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The Twin Arginine Transport System Appears To Be Essential for Viability in *Sinorhizobium meliloti*▽

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The twin arginine transport (Tat) system is responsible for transporting prefolded proteins to the periplasmic space. The Tat pathway has been implicated in many bacterial cellular functions, including motility, biofilm formation, and pathogenesis and symbiosis. Since the annotation of *Sinorhizobium meliloti* Rm1021 genome suggests that there may be up to 94 putative Tat substrates, we hypothesized that characterizing the twin arginine transport system in this organism might yield unique data that could help in the understanding of twin arginine transport. To initiate this work we attempted a targeted mutagenesis of the *tat* locus. Despite repeated attempts using a number of different types of media, the attempts at mutation construction were unsuccessful unless the experiment was carried out in a strain that was merodiploid for *tatABC*. In addition, it was shown that a plasmid carrying *tatABC* was stable in the absence of antibiotic selection in a *tat* deletion background. Finally, fluorescence microscopy and live/dead assays of these cultures show a high proportion of dead and irregularly shaped cells, suggesting that the loss of *tatABC* is inversely correlated with viability. Taken together, the results of this work provide evidence that the twin arginine transport system of *S. meliloti* appears to be essential for viability under all the conditions that we had tested.

Sinorhizobium meliloti is a Gram-negative alphaproteobacterium capable of entering into a symbiotic relationship with leguminous plants such as alfalfa. Within the rhizosphere, rhizobia are capable of sensing flavones or isoflavones secreted by the host plant (4, 46, 57). In response, a cascade of events ensues that leads to the eventual attachment of the bacteria to the plant root, infection thread development, and finally release of the bacteria within the differentiated plant cells of the developing nodule structure (34, 45). It is within this tightly regulated environment that the rhizobia express the genes that encode the proteins required for nitrogen fixation and that result in the reduction of atmospheric N_2 to NH_4. In exchange for the production of nitrogen, the plant provides nutrients for the bacteria to grow and to establish the symbiotic relationship (33, 50).

Protein targeting and translocation are important processes for correct cellular function within all living organisms. It is predicted in *Escherichia coli* that more than 450 proteins are transported across the cytoplasmic membrane (43). The vast majority of these proteins are transported through the general secretory (Sec) system, with a minority being transported by the more recently discovered twin arginine transport (Tat) pathway (43). Proteins that are targeted to the cytoplasmic membrane in Gram-negative bacteria via the Sec system rely on a core set of proteins that include SecA, a protein that has ATPase function, SecYEG, which define the minimum membrane transport apparatus, and in some cases a chaperone protein, SecB (18, 54). The translated protein is carried toward the membrane with help from the chaperone SecB and relayed to the SecYEG apparatus that threads the proteins through the membrane in a linear fashion, with the energy for transport being derived from the hydrolysis of ATP, which is provided by SecA (18).

In contrast, the Tat system is believed to transport proteins that have already undergone folding and, in many cases, cofactor insertion (41, 60). In brief, following protein translation, a chaperone may be involved to help transfer the substrate to the TatBC complex, where the TatC component recognizes the twin arginine signal motif, (S/T)RRXFLK (1, 42). The TatBC complex subsequently recruits TatA oligomers that coordinately make up the membrane pore required for transport (8, 29, 31). Using the pH gradient (ΔpH), the Tat substrate protein is transported through the TatA pore in its folded state and integrated into the membrane or transported further to the periplasmic space (3, 39).

Approximately 30 proteins are predicted to be transported through the Tat system in *E. coli* (43). The majority of these appear to be expressed or function anaerobically (43). Interestingly, bioinformatic analysis of *S. meliloti* and *Rhizobium leguminosarum* suggests that a much larger number of proteins may use the Tat system in these organisms (36). In addition, these organisms are classified as obligate aerobic organisms (12, 28, 55).

Since *tat* mutations have been shown to affect many bacteria-host interactions (17, 25, 36, 49, 62), we set out to construct a *tat* mutation in *S. meliloti* to elucidate the role that *tat* may have in determining the bacteria's ability to interact with its host plant and affect nodule development. Moreover, we reasoned that a *tat* mutation in *S. meliloti* might help characterize putative Tat substrates in a different model organism. Surprisingly, we were able to construct a *tat* mutation only in a merodiploid strain that contained the *tatABC* genes on a plasmid *in trans*. Using plasmid stability, transduction experiments, and live/dead assays, we show that the *tat* region in *S. meliloti* appears

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
S. meliloti		
Rm1021	SU47 *str-21* Sm^r	35
Rm2011	SU47 *str-3* Sm^r	11
Rm5000	SU47 *rif-5* Rif^r	19
SRmA363	Rm1021 $expR^+$	38
SRmA938	Rm1021 pKnock-Gm containing 5′ *nptII*::3′ flanking *tatABC*	This work
SRmA946	Rm1021 pRK7813*tatABC*::pKnock-Gm	This work
SRmA947	Rm1021 *tatABC*Δ/pRK7813*tatABC*	This work
S. medicae		
SmD100	WSM419 Sm^r	This work
SmD104	SmD100(pBP92)	This work
SmD107	SmD107(pBP93)	This work
E. coli		
DH5α	λ^- ϕ*lacZ*DM15 D(*lacZYA-argF*)*U169 recA1 endA hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	24
S17-1	*recA* derivative of MM294A with integrated RP4-2 (Tc::Mu::Km::Tn*7*)	58
MM294A	*pro-82 thi-1 hsdR17 supE44*	20
MT607	MM294A *recA56*	20
MT616	MT607(pRK600)	20
Plasmids		
pEX18Tc	Broad-host-range gene replacement vector; *sacB* Tc^r	26
pBlueScript II SK	Cloning vector; ColE1 *oriV* Ap^r	Stratagene
pRK600	pRK2013 *npt*::Tn*9* Cm^r	20
pRK7813	RK2 derivative carrying pUC9 polylinker and lambda *cos* site; Tc^r	27
pKnock-Gm	Suicide vector for insertional mutagenesis; R6K *ori* RP4 *oriT* Gm^r	2
pPH1JI	IncP plasmid; Gm^r	7
pBP59	*sacB* from pEX18Tc inserted into pRK7813	This work
pBP79	pBP59 with *tatABC* cloned into polylinker	This work
pBP91	pRK7813 *tatC*::*nptII*; oriented in the same direction as P_{lac}	This work
pBP92	pRK7813 *tatC*::*nptII*; oriented in the opposite direction as P_{lac}	This work
pBP103	pKnock-Gm containing upstream 5′ *tatABC* fragment	This work
pBP105	pBP103containing 3′ downstream *tatABC* fragment	This work
pBP121	pBP105 containing *nptII* cassette	This work
pMM4	*tatC* fragment cloned into pBS	This work
pMM13	Kan^r fragment from pMM22 cloned into pMM4	This work
pMM22	Kan^r fragment cloned as a SmaI fragment into pBlueScript	This work

[a] Cm, chloramphenicol; Gm, gentamicin; Kan, kanamycin; Nm, neomycin; Ap, ampicillin; Sm, streptomycin.

to be required for viability and is an essential region of the chromosome. This is the first work to show that Tat is required for viability in a bacterial species.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains, plasmids, and primers are listed in Tables 1 and 2. *E. coli* strains were routinely grown at 30°C or 37°C in Luria-Bertani (LB) medium. *S. meliloti* strains where routinely grown at 30°C in either LB medium, LB medium containing 2.5 mM magnesium and calcium (LB^{MC}), or Vincent's minimal medium (VMM), as previously described (47, 51, 56, 61). For defined medium, carbon sources were filter sterilized and added to medium at a final concentration of 15 mM. When required, final concentrations of antibiotics were as follows: tetracycline (Tc), 5 μg ml^{-1}; gentamicin (Gm), 20 μg ml^{-1} or 60 μg ml^{-1}; neomycin (Nm), 100 μg ml^{-1}; streptomycin (Sm), 200 μg ml^{-1}; kanamycin (Km), 40 μg ml^{-1}; ampicillin (Amp), 200 μg ml^{-1}; and chloramphenicol (Cm), 60 μg ml^{-1}.

Genetic manipulations. Triparental matings for plasmid transfer between strains of *S. meliloti* and *E. coli* were carried out as previously described using MT616 as a helper strain (51). Transductions were carried out essentially as previously described (19). Constructed mutants were recombined essentially as previously described (23, 53).

DNA manipulations and plasmid constructions. All DNA isolation, restriction digestion, gel electrophoresis, ligations, transformations and PCRs, and Southern blot analyses were performed essentially as previously described using standard protocols (56).

Construction of pRK7813*sac*. Construction of pRK7813*sac* was carried out as follows. The *sacB* gene of pEX18Tc was PCR amplified using primers 1 and 2 and cloned into a unique NcoI site found in pRK7813. Transformants were screened onto LB medium supplemented with Tc (LB^{Tc}) and LB^{Tc} medium plus 5% sucrose. Sucrose-sensitive plasmids were purified, restriction mapped, and retested for sucrose sensitivity.

Construction of pBP79. pBP79 was constructed to harbor *tatABC* as well as the entire 5′ upstream intergenic region such that the transcription of *tatABC* could be controlled by its native promoter. In addition, the orientation of *tatABC* was such that it was in the opposite orientation to the P_{lac} promoter found in pRK7813*sac*. To generate this construct, a 2,674-bp PCR amplification product was generated using primers 9 and 10 and Rm1021 DNA as a template; the product was isolated, restricted, and ligated into pRK7813*sac*. A transformant that appeared to be correct on the basis of restriction was sequenced to confirm that the entire insert was identical to the wild type. The construct was designated pBP79.

Construction of pBP121. The construction of pBP121 was carried out in three steps. First, the region immediately flanking *tatA* was PCR amplified using primers 5 and 6 (Table 2 and Fig. 1). This 601-bp product was ligated into pKnock-Gm as a NotI/SmaI fragment and designated pBP103. Second, the downstream region flanking *tatC* was amplified using primers 7 and 8 (Table 2 and Fig. 1). The 486-bp PCR product was restricted with SmaI/KpnI and ligated

TABLE 2. Primers used in this work

No.	Primer name	Sequence (5′ → 3′)
1	sacB Fwd	ATATCCCGGGCCATGGCCATCACATATACCTGCCGTTC
2	sacB Rvs	ATATCCCGGGCCATGGTTATTTGTTAACTGTTAATTGTCCTTG
3	tatABC Fwd	ATATGAATTCTGCGCGAGACGCGCGCGA
4	tatABC Rvs	ATATAAGCTTCTCCTTCGACGCAGCATTGCG
5	5′ upstream tat Fwd	ATATGCGGCCGCGGTTTCAGATGCGCGTAACG
6	5′ upstream tat Rvs	ATATCCCGGGGTATGGCCAGCGTGTCGGC
7	3′ downstream tat Fwd	ATATCCCGGGACCGCGGAGTTCTTGCGCC
8	3′ downstream tat Rvs	ATATGGTACCCGACATTTGCGCTTTCGTCCG
9	tatC Fwd	ATATGAATTCCGCCGGCTGCGAATAAG
10	tatC Rvs	ATATGAATTCGCTGCATGACATCGGAG
11	tat confirmation Fwd	CACCGAAGCCTGAAGAAGAC
12	Tat confirmation Rvs	GCTCCTTCGAAGTCCATCAG
13	*nptII* out Left	TTCGGAATCGTTTTCCGGGAG
14	*nptII* out Right	TTAGCAGCCCTTGCGCCCTG

into pBP103 that was cut with the same restriction enzymes. This construct was named pBP105. The plasmid pBP121 was ultimately constructed by cutting pBP105 with SmaI and ligating a gel-isolated 1.4-kb SmaI fragment containing the Kan^r cassette from pMM22 into this site.

Construction of pBP91 and pBP92. To construct pRK7813*tatC*::*nptII*, primers 3 and 4 were used to PCR amplify *tatC*. The 950-bp fragment was then cloned into pBluescript SK to yield pMM4. A gel-isolated *nptII* cassette from pMM22 was cloned into pMM4 as a SmaI fragment to produce pMM13. The *tatC*::*nptII* construct was subsequently cut out of pMM13 as an EcoRI fragment and ligated into pRK7813. Transformants were isolated, and the plasmids were screened for the orientation of the EcoRI fragment relative to P_{lac} by restriction digest. The insert in pBP91 was oriented such that *tatC*::*nptII* was in the same direction as P_{lac}, whereas the insert in pBP92 *tatC*::*nptII* was in the opposite orientation to P_{lac}.

Plasmid stability experiments. Cultures of *Rhizobium* strains containing either pRK7813 or pBP79 were grown in LB^{Tc} medium (2.5 μg ml^{-1}) to ensure that all bacteria contained the appropriate plasmid. These cultures were then subcultured into LB broth that did not contain tetracycline. Cultures were then allowed to grow overnight at 30°C; they were subcultured the next day into fresh LB medium and again incubated overnight. The process was repeated until the experiment was completed. At each subculture, cells were serially diluted and plated onto LB agar, and 100 of the resultant colonies were screened for the presence of the plasmid on the basis of resistance to tetracycline.

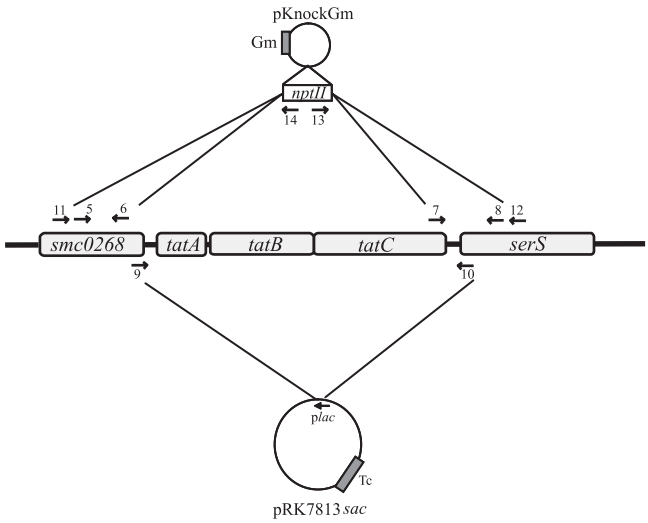


FIG. 1. Genetic region containing *tatABC* of *S. meliloti*. A schematic diagram showing the relative positions of the relevant primers used to construct and verify constructs in the *tat* region. Numbered arrows correspond to the primers listed in Table 2. The plasmid above corresponds to pBP121, whereas the plasmid below corresponds to pBP79. Details of the construction and verification are in the text. The arrow above the P_{lac} promoter indicates the direction of transcription *nptII*, neomycin phosphotransferase cassette.

Microscopy and plate reader analysis. Cultures of *Rhizobium* strains were grown in LB medium to mid-log phase. Cells were pelleted, washed three times, resuspended in saline, and incubated for 1 h at room temperature without shaking. When required, killed cells were incubated in 70% ethanol for 1 h in the place of saline. Cells were then pelleted, resuspended, and incubated with an appropriate amount of stain from a Molecular Probes BacLight detection kit. Cells were then incubated for 15 min in the dark. For microscopy, cells were added to an agarose-covered slide and analyzed using a Zeiss fluorescent microscope; for plate reader assays, cells were aliquoted into a 96-well plate and read using a fluorescent plate reader. In order to set up a calibration curve to assess the percentage of live cells, different proportions of live cells were mixed with killed cells. Stained cultures were directly compared to this curve. A log-phase culture of Rm1021 was arbitrarily set as a theoretical maximum (100%).

RESULTS

Plasmid promoter activity is required for chromosomal recombination. To characterize the twin arginine transport pathway in *S. meliloti*, we set out to create a gene disruption using a Kan^r cassette targeted to the *tatC* gene. The *tatC* gene was PCR amplified, cloned into pBluescript, disrupted with an antibiotic cassette, recloned into pRK7813, and used for an allelic exchange experiment, as previously described (47). Although putative recombinants were isolated, none of these was a bona fide double-crossover event; they appeared to be single-crossover events in which the vector was also integrated into the genome. As a result, in addition to having a mutant copy of *tatC*, the recombinants also contained a wild-type copy of the *tat* gene under the control of the vector promoter.

Since these experiments were carried out using LB medium as a complex medium, we hypothesized that the medium could be limiting the growth of a *tat* mutant, potentially providing a false-negative result. To address this hypothesis, the selection was attempted on various medium types. Defined media that were used included VMM (61), rhizobial minimal medium (RMM) (9), and M9A (13). For each of these, medium trials were carried out with glucose, mannitol, or succinate as a carbon source, and ammonia, nitrate, or glutamate were used as a nitrogen source. Complex media that were tried included tryptone-yeast extract (TY) (6), yeast extract-mannitol (YEM) (61), and LB media (37). In every case we were unable to isolate *tat* mutants that had lost the wild-type copy of the *tat* gene.

TABLE 3. Frequency of single-crossover recombinants is dependent upon the orientation of P_{lac}[a]

Construct	Orientation relative to P_{lac}	No. of recombinants	
		S. meliloti	*S. medicae*
pBP91	Same direction	1.8×10^{-4}	1.7×10^{-4}
pBP92	Opposite direction	$<10^{-8}$	$<10^{-8}$

[a] Plasmid pBP91 or pBP92 were conjugated from *E. coli* into either *S. meliloti* or *S. medicae* and recombinants were selected on the basis of antibiotic resistance. Data presented are the mean of one experiment with three independent replicates where the standard deviation is less than 10%. The experiment was also replicated on three separate occasions with comparable data.

Since Rm1021 is known to be compromised with respect to phosphate utilization (64, 65) and since it is known that the strain carries an insertion element within a major regulator (44), we reasoned that perhaps our inability to isolate a *tatC* mutant was because of the Rm1021 background. To address this, we attempted a similar strategy using the closely related wild-type strains Rm2011 and Rm5000, which are independently derived from the parental strain SU47 and not compromised with respect to phosphate utilization (65), as well as SRmA363, which was constructed to be *expR*$^+$ (38). The results of these experiments were consistent with our previous findings. Taken together, these data suggested that the *tat* genes may play an essential role under laboratory growth conditions and that *S. meliloti* could not grow in the absence of *tatC*.

To address the hypothesis that we were preferentially selecting for single-crossover recombinants that reconstructed the wild-type *tatC* gene *in vivo*, we reoriented the fragment in pBP92 such that *tatC* was in an opposite orientation to the P_{lac} promoter on the vector. We reasoned that if this, indeed, was the case, there should be a marked difference in the frequencies with which we could isolate single-crossover recombinants based on which construct was used in the allelic exchange experiment.

Consistent with this hypothesis, we were able to easily isolate single-crossover mutants using pBP91 (Table 3). When pBP92 was used, even after repeated attempts, we were unable to isolate any colonies. When the single-crossover events were expressed as a frequency, this represents a difference that is greater than 4 orders of magnitude (Table 3).

To determine if our inability to generate single-crossover recombinants was a function of using *S. meliloti*, we decided to attempt a similar experiment in the sequenced *Sinorhizobium medicae* strain WSM419. Since WSM419 is a wild-type strain that is sensitive to many commonly used laboratory antibiotics, we first isolated a streptomycin-resistant variant. This variant was designated SmD100 (Table 1). The plasmids pBP91 and pBP92 were subsequently conjugated into SmD100 and used for allelic exchange experiments. The results were comparable to the *S. meliloti* data (Table 3). Our ability to generate recombinants in these two closely related species correlated with the orientation of the construct with respect to the P_{lac} promoter in the vector (Table 3).

A *tat* deletion can be generated in a strain that is merodiploid for *tatABC*. The inabilities to construct a mutation in the *tat* region led us to hypothesize that the Tat system may be essential for either the growth or viability of *S. meliloti*. Alternately, the region may represent a region of the chromosome that is poorly recombinogenic. However, since we were able to isolate single-crossover events in this region, the latter possibility seemed to be unlikely.

A classic method for demonstrating that a gene is essential utilizes the isolation of temperature-sensitive alleles of the gene in question (15, 32). The isolation and characterization of temperature alleles are often time-consuming and difficult strategies to pursue. To address the possibility that *tatABC* could be essential for growth or viability in *S. meliloti*, a strategy was devised utilizing a host that would be merodiploid for *tatABC* (SRmA938, containing *tatABC* on a replicating plasmid) and a suicide vector (pBP121) that carried the regions immediately flanking this region (Fig. 1). The suicide vector was chosen such that the vector could easily be conjugated into *S. meliloti*, would not replicate in Rm1021, and did not contain any region of homology with pBP79. Due to problems generating a PCR product, we note that pBP79 and pBP121 do have approximately 200 bp of common DNA sequence (Fig. 1).

To generate a chromosomal deletion, pBP121 was mobilized into SRmA938 using pRK600 as a helper plasmid. Sm^r Nm^r transconjugants were selected, and approximately 200 were screened for Gm^r and Tc^r. Two colonies were found to be Gm^s, Tc^r, and Nm^r. These colonies putatively represented strains in which a double-crossover event had occurred where the chromosomal *tatABC* region was replaced with the insert carried on pBP121, and the plasmid pBP79 was retained. These were designated SRmA946 and SRmA947.

Subsequent Southern analysis of SRmA946 and SRmA947 using pKnock-Gm as a probe revealed that SRmA946 contained vector sequences, whereas SRmA947 did not contain sequences derived from this plasmid. SRmA946 was subsequently shown to be an extrachromosomal cointegration of pBP79 and pB121.

To ensure that SRmA947 contained a *tatABC* chromosomal deletion, PCR products were generated using primers (the primer pair 11 and 14 and the primer pair 12 13) outside the region in pBP121 and primers specific to the Nm resistance cassette. These PCR products were sequenced to confirm integration into the chromosome. The sequence data clearly show that SRmA947 contains a chromosomal deletion of *tatABC*.

Transduction of the *tatABC* deletion is host dependent. To ensure that the ability to delete *tatABC* was not due to a second site mutation that might have occurred in SRmA947 and allowed this genetic event to occur, a lysate of SRmA947 was prepared and used to transduce Rm1021. Since it was possible to have very few or no transductants on the selection plates, if the incorporation of a *tat* deletion was deleterious to the wild type, a control transduction in which the transduction of plasmid pBP79 into Rm1021 from SRmA947 was also carried out.

The results clearly show that after repeated independent attempts, we were unable to isolate Rm1021 transductants with the *tat* deletion ($<10^{-8}$ recombinants/recipient). In contrast, Rm1021 transductants carrying pBP79 were readily isolated at frequencies similar to those previously published (2.2×10^{-7} recombinants/recipient [19]). To ensure that these were real transductants, five were single-colony purified, and the Tc^r plasmid was conjugated into *E. coli* DH5α, isolated, and ana-

lyzed by restriction analysis. Each of these plasmids was identical to pBP79.

Since SRmA938 was merodiploid for *tatABC*, we wished to determine if the transduction of the *tat* deletion was dependent upon the presence of an additional copy of *tatABC*. To carry out this experiment a lysate of SRmA947 was used to transduce SRmA938. Utilizing SRmA938, it was possible to generate Nm^r transductants at frequencies similar those found for other markers (3.0×10^{-6} recombinants/recipient [19]).

pBP79 is completely stable in an *S. meliloti* strain containing a *tat* deletion. Through routine growth and maintenance of SRmA947, the strain was observed to grow more slowly than the wild type. Since SRmA947 contains a plasmid and was routinely grown in broth cultures in the presence of tetracycline, it was thought that the slow growth might be a consequence of growing in the presence of the antibiotic. To address this, growth rates of Rm1021 containing pBP79 were directly compared to the growth of SRmA947. It was found that SRmA947 had a growth rate that was lower than that of the wild type. Whereas a typical doubling time for *S. meliloti* grown on complex is medium is about 3 h when it is incubated at 30°C (38), the doubling time for SRmA947 was found to be 5 h (data not shown).

Initially, it was unclear why SRmA947 would have a lower growth rate. One possible reason for the slow growth could be the presence of *tatABC* in multicopy. An alternate explanation might be that cells that lose pBP79 become nonviable or cease to grow. Since the plasmid vector pRK7813 is inherently unstable (27), it seemed plausible that a portion of a population of SRmA947 could be losing the plasmid and thus ceasing to grow.

To address the latter possibility, it was hypothesized that all cells carrying a chromosomal *tat* mutation must retain the *tatABC* genes expressed *in trans*. To assess this, we performed an assay to measure stability of pBP79 in either a wild-type background (SRmA938) or a *tat* deletion background (SRmA947). In addition we included SRmA946, which contains a cointegrate of pBP79 and pBP121.

Strains were first grown to an equivalent optical density in LB medium containing tetracycline and then subcultured into LB broth without tetracycline. Cells were removed after each subculture and plated for viability on LB medium. Colonies that grew on LB plates were subsequently screened for tetracycline resistance. It was reasoned that this assay could help determine whether the slow growth that was observed for SRmA947 was due to a cessation of growth or a loss of viability since plasmid portioning is not associated with any putative or known *tat* substrates.

The results show that under our growth conditions, approximately 50% of the population of Rm1021 that harbored pBP79 would lose the plasmid at each subculture step. After 5 days there was a complete loss of the pBP79 in SRmA938 (Fig. 2). Similarly, SRmA946, which contains a cointegrate of pBP79 and pBP121, was also lost at a similar rate (data not shown). In addition, Rm1021 lost the plasmid pRK7813 at a rate similar to that of pBP79, suggesting that the loss of the plasmid was not due to overexpression of *tatABC* (data not shown). In contrast, pBP79 was completely stable in SRmA947 even after more than a week of subcultures (Fig. 2 and data not shown).

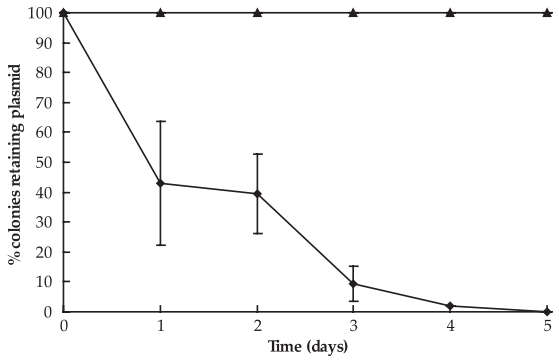


FIG. 2. Stability of pBP79 containing the *tat*ABC operon. Presence of pBP79 was measured by the proportion of Tc-resistant colonies found at each subculture. Error bars represent standard deviations of three independent experiments. Where not shown, error bars are smaller than the symbol. ▲, SRmA947; ◆, SRmA938.

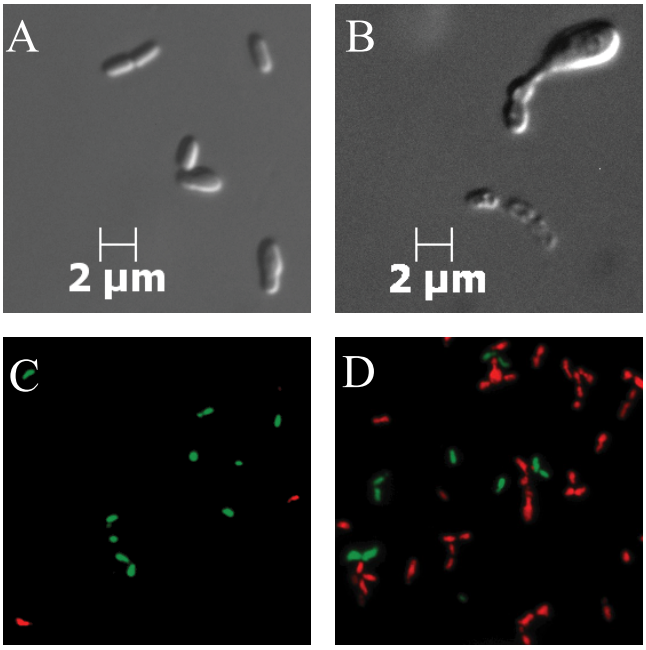
A proportion of SRmA947 cells display aberrant morphologies. Since the plasmid stability experiments showed that pBP79 was unstable in *S. meliloti*, it would be unlikely that every cell of SRmA947 contained a plasmid. We predicted that a proportion of cells in a growing culture of SRmA947 would lose pBP79, in essence yielding a genotypic *S. meliloti tat* mutant. Although these cells would be *tat* deficient, TatABC would deplete as the cells continued to grow. Therefore, observing the cells microscopically might give some insight into the role that TatABC might play in the overall physiology of *S. meliloti*.

It is known that in *E. coli* a twin arginine transport mutant undergoes improper cell division, resulting in elongated cells (59). Cell morphology is an important developmental indicator of a cell's adaptation to its environment. Rapid cell division during logarithmic growth indicates a healthy population attuned to its environment. Delay of cell division or changes to cell appearance can indicate that there may be one problem or many problems occurring within the cell.

Using both phase-contrast and differential interference contrast (DIC) microscopy, we were able to see that, like *E. coli*, a proportion of the *S. meliloti* mutants exhibited aberrant cell morphologies. Whereas the wild-type cells appeared predominantly as short rods, a higher proportion of SRmA947 cells appeared to be elongated, reaching lengths approximately two to three times that of wild-type cells. In addition, the cells appeared to be typically swollen or branched or were found joined together with cells of heterogeneous size, possibly indicating an inability to undergo proper cell separation or perhaps defects in cell wall assembly (Fig. 3).

A growing culture of SRmA947 contains a significantly higher proportion of dead cells than the wild type. Bacterial populations typically contain a number of live and dead cells. Microscopic observation suggested that SRmA947 contained a large number of misshapen cells. From these observations, we are unable to ascertain whether these cells are viable. We wished to determine whether a culture of SRmA947 contained a higher number of dead cells and if these cells are correlated with the altered cell morphology observed.

To determine cell viability, we utilized a BacLight LIVE/DEAD bacterial viability kit (Molecular Probes, Invitrogen).



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Strain	Relevant genotype	% wild type
Rm1021	-	100.0 (10)
SRmA938	p*tatABC*	107.5 (21)
SRmA947	*tatABC*; pRK7813*tatABC*	66.8 (8)

FIG. 3. Effect of *tatABC* mutation on cell morphology and viability in *S. meliloti*. (A and C) Rm1021. (B and D) SRmA947. Panels A and B show DIC images of *S. meliloti* cells. Panels C and D show cells that were stained using a BacLight LIVE/DEAD bacterial viability kit (Molecular Probes, Invitrogen) and viewed using a fluorescent microscope. Live cells fluoresce green, whereas dead cells fluoresce red. (E) Percentage of live cells in cultures of Rm1021, SRmA938, and SRmA947, stained using a BacLight LIVE/DEAD kit and quantitated using fluorescence. Data are presented as the mean of three independent replicates. Values in parentheses represent standard deviations.

When visualized, Rm1021 cultures were predominantly seen as small rod-shaped cells. The majority of these stained green, indicating that they were live cells (Fig. 3C). In contrast, cultures of SRmA947 had a marked increase in the number of dead cells that were observed microscopically (Fig. 3D). It was not uncommon to find many of the large cells associated in chains or abnormally shaped cells stained red, suggesting that these were cells with compromised membranes that were either dead or dying (Fig. 3D). We note that similar shapes were also described for *Agrobacterium tumefaciens* cells with *tat* mutations (17).

Direct microscopic analyses to determine the proportion of viable cells within each culture were complicated by the fact that with SRmA947 it was possible to find many areas where there were high proportions of dead cells. In an effort to remove possible sources of bias in determining the proportion of dead cells in each culture, viability was assayed using fluorescence measurements. Consistent with what was observed microscopically, it was found that the proportion of dead cells in a culture of SRmA947 was significantly higher than that of Rm1021 (Fig. 3). To ensure that the plasmid pBP79 was not skewing our quantitation, SRmA938 was also assayed in a similar manner. The data show that the proportion of dead cells is not significantly different from that of the wild type, suggesting that pBP79 expressed in Rm1021 did not account for the differences that were observed between SRmA947 and Rm1021 (Fig. 3E).

DISCUSSION

Protein targeting and translocation play important roles within the bacterial cell. Most bacterial species contain a number of different secretion systems including everything from type I ABC-type transporters through to the recently discovered type VI secretion system in *Pseudomonas aeruginosa* (40). Not all species contain each of these systems. The twin arginine transport pathway, however, is widely distributed across a large number of bacterial species (52, 63). In spite of this, the Tat pathway is often viewed as an accessory system, with the general secretory system being of primary importance and essential for viability within many bacteria (48).

Our data show that it is possible to construct a *tat* mutation in *S. meliloti* but that cell viability is dependent upon the presence of a second copy of *tatABC* within the cell (Table 3). Moreover a slow-growth phenotype associated with this strain is correlated with an inability to isolate a viable strain not containing the plasmid carrying *tatABC* and a significant proportion of dead cells with aberrant shapes in the culture medium (Fig. 2 and 3). Taken together, these data strongly suggest that *tatABC* is essential for *S. meliloti* viability under standard growth conditions.

In an attempt to determine what twin arginine transport-dependent gene products are required for viability, a list of putative Tat substrates was compared with genes annotated as essential. As determined from the database of essential genes (DEG), smc04024 and smc04449 were homologous with two genes annotated to be essential in *Haemophilus influenzae*. smc04024 is a membrane-bound lytic murein transglycosylase required for correct cell wall assembly and recycling. *tat* mutants typically have increased membrane sensitivity to detergents and chemicals. We note that in *R. leguminosarum*, it has been suggested that increased amounts of extracellular proteins are found in the culture supernatant of a *tatC* mutant, suggesting defects in cell permeability (30). A simple search of the *S. meliloti* genome, however, shows that there are at least seven annotated murein transglycosylases. The second gene, smc04449, is a 2′,3′-cyclic-nucleotide 2′-phosphodiesterase, which is used for salvaging nucleosides and nucleotides (5, 10, 21, 22). We note that the *S. meliloti* genome contains at least one other gene, smc04018, which is annotated to have the same putative function. Moreover, since *S. meliloti* can also live as a formate-dependent autotroph, it seems unlikely that a salvage pathway gene would be essential in *S. meliloti* (47). However, these prospective candidates will need to be functionally tested to determine whether they may, indeed, be essential for *S. meliloti* viability.

To our knowledge, the Tat system has been shown to be essential for survival only in the halophilic archaeon *Haloferax volcanii* (16). More recently, the use of the term "essential gene" has been questioned (15). Although our data strongly

support the supposition that *tatABC* are necessary for cell viability, our data can be interpreted only in light of the growth conditions that we have used. In addition, gene essentiality can be genome specific. A bacterium that is capable of utilizing a diverse set of compounds as carbon and nitrogen sources will have very different requirements than an organism with fastidious growth requirements.

Although well over a thousand bacterial genomes have been sequenced, *tat* mutations have been characterized in relatively few species. Our work to characterize the twin arginine transport system in *S. meliloti* provides the first evidence that the Tat pathway is essential in any eubacterial species. Interestingly, *tat* mutations have been published for a number of closely related species, including *R. leguminosarum* (30, 36) and *A. tumefaciens* (17). Although these mutants had pleiotropic phenotypes, they were viable using standard laboratory media. Preliminary comparative searches have not been successful in identifying why *S. meliloti* is different from these species. As more bacterial species are characterized, it will be interesting to determine if other species require the Tat system for viability.

Tat has been associated with many functions, such as anaerobic respiration, biofilm formation, pathogenesis, and symbiosis (14, 17, 36, 41). Although Tat has been implicated in these processes, the Tat substrate(s) that is directly affected often has not been identified. Since *S. meliloti* is predicted to contain up to 94 putative Tat substrates (36, 52), future studies focusing on identifying and characterizing these Tat substrates and elucidating their physiological roles may provide insight into how *S. meliloti* uses the Tat system, both as a saprophytic free-living organism and as a symbiont.

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