

## The Twin Arginine Translocation System Is Essential for Virulence of *Yersinia pseudotuberculosis*

Moa Lavander,<sup>1,2</sup> Solveig K. Ericsson,<sup>1</sup> Jeanette E. Bröms,<sup>1,2</sup> and Åke Forsberg<sup>1,2\*</sup>

Department of Medical Countermeasures, Division of NBC Defense, Swedish Defense Research Agency, SE-901 82 Umeå, Sweden,<sup>1</sup> and Department of Molecular Biology, Umeå University, SE-901 87 Umeå, Sweden<sup>2</sup>

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***Yersinia* species pathogenic to humans have been extensively characterized with respect to type III secretion and its essential role in virulence. This study concerns the twin arginine translocation (Tat) pathway utilized by gram-negative bacteria to secrete folded proteins across the bacterial inner membrane into the periplasmic compartment. We have shown that the *Yersinia* Tat system is functional and required for motility and contributes to acid resistance. A *Yersinia pseudotuberculosis* mutant strain with a disrupted Tat system (*tatC*) was, however, not affected in *in vitro* growth or more susceptible to high osmolarity, oxidative stress, or high temperature, nor was it impaired in type III secretion. Interestingly, the *tatC* mutant was severely attenuated via both the oral and intraperitoneal routes in the systemic mouse infection model and highly impaired in colonization of lymphoid organs like Peyer's patches and the spleen. Our work highlights that Tat secretion plays a key role in the virulence of *Y. pseudotuberculosis*.**

As the integrity of the bacterial cytoplasm is protected by membranes, the necessity of means for transport of molecules between the bacterial compartments, periplasm and cytoplasm, and the surrounding milieu is obvious. Not only do bacteria require the ability to take up nutrients and get rid of waste, transport across the membranes is also highly involved in the ability of bacteria to cause disease. A number of specialized protein secretion systems are essential for delivery of virulence factors across the two membranes of gram-negative pathogens (39). The importance of these systems is reflected in the multitude of mechanisms that have evolved not only for protein translocation across the bacterial membranes but also for targeting of anti-host factors across the host cell membrane. The means of translocation across the bacterial outer membrane have been grouped into five different pathways (I to V) (66). In pathways I, III, and IV, the substrate appears to be targeted directly across the two membranes without any periplasmic intermediates (13, 18, 20, 24, 42, 53), whereas in pathways II (58) and V (32), the process is divided into discrete steps where translocation across the inner membrane occurs as the substrate is targeted to the periplasm either by the general secretory pathway (sec system) (23) or via the more recently discovered twin arginine translocation (Tat) pathway (6).

The most extensively studied secretion and virulence mechanism of the pathogenic *Yersinia* species *Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* is the plasmid-encoded type III secretion system (T3SS). Utilizing this mechanism, the pathogens deliver Yop effectors across the bacterial envelope and the host cell membrane in a bacterium-cell contact-dependent process (48, 55). A chromosomal T3SS is also present in all three species and has been shown to be important for

virulence in *Y. enterocolitica* (30). In contrast, a role in virulence for the chromosomal systems encoded by *Y. pseudotuberculosis* and *Y. pestis* has not been established (51). The plasmid-encoded T3SS has, on the other hand, been shown to be a powerful tool for subversion of the host immune defense and is essential for virulence in all three human-pathogenic *Yersinia* species. Hence, T3SS mutants are highly attenuated in mouse infection models. Although *Y. pseudotuberculosis*, in contrast to *Y. pestis*, rarely causes systemic infections in humans, it inflicts a systemic plague-like disease in mice (28), making it a relevant and convenient model to study the mechanisms of severe infections.

The rapid progress in genome sequencing of bacterial pathogens has extended the possibilities to identify novel putative virulence determinants, as well as secretion systems (45). The genomes of the closely related species *Y. pseudotuberculosis* and *Y. pestis* both contain homologues to genes encoding the components required to assemble a functional Tat system in *Escherichia coli* (14, 47). The Tat pathway targets folded proteins, identified via an N-terminal signal peptide with an S/T/R-R-x-F-L-K consensus motif, for secretion across the inner membrane of gram-negative bacteria. A majority of the *E. coli* Tat substrates bind cofactors in the cytoplasm (5, 6), therefore demanding complete folding prior to their export. Tat substrates with no cofactor have, however, also been identified. Halophilic archaea require a way to rapidly stabilize their proteins in protection against the nearly saturated salt conditions in their cytoplasm. Thus, rapid folding of newly synthesized proteins is performed in the cytoplasm, facilitated by the ability of the majority of the exported proteins in these archaea to take the Tat route (34, 54). In *E. coli*, where Tat has been paradigmatically researched, the minimal components of the Tat protein translocation system are the membrane proteins TatA, TatB, and TatC (8, 59, 70). In many bacteria, a wide range of substrates are secreted by the Tat pathway and as a result, *tat* mutants often show pleiotropic phenotypes with defects in bio-

\* Corresponding author. Mailing address: Department of Medical Countermeasures, Division of NBC Defense, Swedish Defense Research Agency, SE-901 82 Umeå, Sweden. Phone: 46 90 10 66 60. Fax: 46 90 10 68 03. E-mail: ake.forsberg@foi.se.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<b>Strains</b>		
<i>Y. pseudotuberculosis</i>		
IP32953	Wild type, serotype I	E. Carniel <sup>a</sup>
IP32953c	IP32953, cured of pYV	S. Garbom, <sup>b</sup> unpublished
IP32953tatC	IP32953, <i>tatC</i> insertion mutation; Cml <sup>r</sup>	This study
<i>Y. pestis</i>		
KIM5	Wild type	R. R. Brubaker <sup>c,d</sup>
KIM5tatC	KIM5, <i>tatC</i> insertion mutation; Cml <sup>r</sup>	This study
<i>E. coli</i> S17-1λpir	RP4-2 Tc::Mu-Km::Tn7 (λpir)	19
<b>Plasmids</b>		
pNQ705	Suicide integration vector; Cml <sup>r</sup>	41
pML30	pNQ705 with bases 19–329 of <i>tatC</i> ; Cml <sup>r</sup>	This study
pMMB66HE	P <sub>tac</sub> expression vector; Amp <sup>r</sup>	26
pML31	pMMB66EH with <i>tatC</i> ; Amp <sup>r</sup>	This study
pZD51	pMMB22 with <i>torA::GFP</i> ; Amp <sup>r</sup>	21

<sup>a</sup> Pasteur Institute, Paris, France.

<sup>b</sup> Umeå University, Umeå, Sweden.

<sup>c</sup> Michigan State University, East Lansing.

<sup>d</sup> KIM5 is also called KIM10 or substrain D27 (49).

genesis of the cell envelope (36), biofilm formation (35), motility (21, 43), and resistance to various environmental stress conditions (62). However, the Tat system has also been shown to be involved in secretion of virulence determinants like phospholipase toxins in *Pseudomonas aeruginosa* (43, 69), *P. syringae* pv. tomato DC3000 (11), and *Legionella pneumophila* (56), as well as Shiga toxin 1 in enterohemorrhagic *E. coli* (50).

In this work, we have established that *Y. pseudotuberculosis* and *Y. pestis* both express functional Tat translocation systems and further investigated the phenotypes of a *Y. pseudotuberculosis* *tatC* mutant strain. Somewhat surprisingly, disruption of the Tat system did not influence in vitro growth under low-nutrient conditions or stress conditions like high osmolarity, high temperature, and oxidative stress. Nor was the *tat* mutant defective for phenotypes coupled to functional T3S, including secretion of Yop effectors under low-Ca<sup>2+</sup> conditions, their translocation into target cells, and resistance against phagocytosis by macrophages. The only in vitro defects we could confirm for the *tatC* mutant were loss of motility at environmental temperatures and a slight decrease in acid resistance. Interestingly, Tat was demonstrated to have an essential role in *Yersinia* pathogenicity, as the *tatC* mutant was severely attenuated for virulence in the systemic mouse infection model. Thus, our work highlights that twin arginine translocation plays a key role in the ability of *Y. pseudotuberculosis* to cause disease.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Routine culturing of bacteria occurred in Luria-Bertani (LB) broth (17) or on blood agar base (Merck) plates at 26°C (*Y. pseudotuberculosis* and *Y. pestis*) or at 37°C (*E. coli*) with aeration. For analysis of in vitro growth at 26°C or 37°C, defined *Yersinia* medium (TMH; 63) (nutrient-rich medium) or *Yersinia* defined minimal medium (YDM; 1× M9 minimal salts, 0.4% glucose, 0.4% Casamino Acids, 10 mM MgCl<sub>2</sub>, 5 mM K<sub>2</sub>SO<sub>4</sub>, 10-μg/ml thiamine) (nutrient-poor medium) was used. When growth was investigated at 37°C, the cultures were pregrown for 1 h at 26°C and then shifted to 37°C. To induce or repress T3S, media were supplemented with 5 mM EGTA

and 20 mM MgCl<sub>2</sub> (inducing medium) or 2.5 mM CaCl<sub>2</sub> (noninducing medium), respectively. Where appropriate, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml. For induction of the *tac* promoter, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the growth medium at a final concentration of 0.4 mM.

**DNA methods.** Preparation of plasmid DNA, restriction enzyme digests, ligation, and transformation of *E. coli* strains were performed according to standard methods (57).

**Generation of *tatC* insertion mutants in *Yersinia*.** An internal fragment of the *tatC* gene was amplified by PCR with IP32953 as the template and primers 5'-GCACTCGAGCTCCAACCCCTTATCTCTCATCTGA-3' (SacI site underlined) and 5'-GCTCACTCTAGATAACGGCACCATCAAACGGCG-3' (XbaI site underlined). Nucleotides in italics correspond to bases 306379 to 306400 and 306690 to 306670, respectively, in the IP32953 sequence (GenBank accession number BX936398). The resulting 311-bp fragment was introduced into XbaI/SacI-digested pNQ705 (41), resulting in the suicide vector pML30. Conjugal mating experiments with S17-1λpir as the donor strain allowed the integration of pML30 within regions of complementary sequences on the chromosomes of *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM5 (41), thereby generating mutants IP32953tatC and KIM5tatC, respectively.

**Construction of a vector for *trans* complementation of *tatC* mutants.** The expression vector pML31, encoding *tatC* under the control of the *tac* promoter, was generated as follows. By PCR with primers 5'-GCACTCGGATCCCGGCTAAATATCAACGCATCGA-3' (BamHI site underlined) and 5'-GCACTCGAATTCATCTCGTATTCACCAACCTGGC-3' (EcoRI site underlined), an 814-bp fragment encoding *tatC* was obtained. Italicized letters correspond to nucleotides 306325 to 306346 and 307160 to 307139, respectively, in the IP32953 sequence (GenBank accession number BX936398). The fragment was introduced into BamHI/EcoRI-digested pMMB66HE (26), resulting in plasmid pML31. Plasmids were transferred into recipient *Yersinia* by conjugation.

**Yop expression and secretion assay.** Induction of type III substrate synthesis and secretion from *Y. pseudotuberculosis* were performed as previously described (4). Total protein levels were assessed by sampling directly from the bacterial culture suspension containing both proteins secreted into the culture medium and bacteria associated. Sampling of the cleared supernatant allowed assessment of secreted protein levels. All protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to immunoblotting. Detection of specific proteins on membrane support was achieved by the use of rabbit polyclonal antiserum raised against all secreted Yops (AgriSera AB), followed by ECL detection (Amersham Pharmacia Biotech).

**Use of the *torA::GFP* reporter system to analyze Tat functionality in *Yersinia*.** The reporter plasmid pZD51 encoding the Tat signal sequence of trimethylamine *N*-oxide reductase (TorA) of *E. coli* fused to green fluorescent protein

(GFP), under control of the IPTG-inducible *tac* promoter (21), was introduced by electroporation into wild-type or *tatC* mutant *Y. pseudotuberculosis* and *Y. pestis*. Due to the leakiness of the *tac* promoter, no IPTG addition was required to achieve detectable levels of the GFP reporter. The cellular location of GFP was determined by fluorescence microscopy on bacteria grown in liquid culture.

**Motility assay.** Motility assays were conducted on 0.3% minimal motility agar containing (wt/vol) 1% peptone and 0.5% NaCl. Where appropriate, antibiotics and IPTG were added.

Cell cultures grown in LB broth overnight were normalized to an optical density at 600 nm of 2.5, and 1  $\mu$ l of each strain was inoculated onto the surface of the motility plates. Motility was examined at 24, 48, 60, and 96 h of incubation at 22 and 37°C. At least four independent motility assays were carried out for each strain and condition.

**Environmental stress experiments.** The effects of high osmolarity, oxidative stress, low pH, and high temperature were determined for strains of *Y. pseudotuberculosis* grown overnight in LB broth at 26°C and followed established protocols (12, 22, 44, 72, 73). For high-temperature stress experiments, stationary-phase cells were transferred to prewarmed tubes and incubated at 55°C for 5 min. Control bacteria were incubated at ambient temperature (22°C). In the oxidative-stress experiments, cells were incubated for 1 h at 26°C in 40 mM H<sub>2</sub>O<sub>2</sub> in deionized water. For high-osmolarity stress, cells were incubated in 2.4 M NaCl in deionized water for 1 h at 26°C. To evaluate survival at low pH, cells were incubated in LB broth adjusted to pH 3 with HCl for 5 min at ambient temperature. Control bacteria were incubated in deionized water or LB broth. After exposure, the bacteria were pelleted at 12,000  $\times$  g for 5 min at 22°C and resuspended in sterile phosphate-buffered saline (PBS) (pH 7.4), and appropriate dilutions were plated on LA agar, LB broth (17) with 1.5% agar, for the wild type and on LA agar containing 20  $\mu$ g/ml chloramphenicol for *tatC* mutant cells. Plates were incubated at 26°C for 2 days for CFU counting. All survival experiments were repeated at least three times with duplicate samples of each strain in each individual experiment.

**Cultivation and infection of HeLa cells.** Culture maintenance and infections with *Yersinia* followed established protocols (65). The cytotoxic effect induced in the infected HeLa cells was monitored by light microscopy, and images were collected at successive time points.

**Survival in J774 macrophage cell cultures.** Culture maintenance and infections of macrophages with *Yersinia* were performed essentially as described previously (44), with the exception that the number of viable bacterial cells at different time points after infection was determined after growth on LA agar supplemented with appropriate antibiotics. Duplicate samples were taken at all time points, and the assay was repeated twice.

**Phagocytosis inhibition assay.** The phagocytosis inhibition assay followed established protocols (64), with subtle modifications. *Y. pseudotuberculosis* cultures were adjusted to an optical density at 600 nm of 0.2 in Dulbecco's modified Eagle's medium and subsequently grown at 26°C for 30 min before a shift to 37°C for an additional 2 h. J774 macrophages seeded at a density of  $5 \times 10^4$  cells onto an eight-chamber slide were infected with bacteria to a final  $A_{600}$  of 0.1 or 0.01. After infection, macrophages were washed and fixed in 2% formalin. Cells were further washed, blocked for 10 min in 0.1 M glycine, washed, and then incubated with anti-*Yersinia* rabbit antiserum, followed by Alexa Fluor 568 red-conjugated goat anti-rabbit antibodies (Molecular Probes). Macrophages were permeabilized (0.15% saponin), washed, and incubated with anti-*Yersinia* antiserum (containing 0.15% saponin, 2% bovine serum albumin, and 0.1 M glycine). Diluted in the same solution, Alexa Fluor 488 green-conjugated antibodies (Molecular Probes) were added. This staining procedure allowed us to distinguish between external (red) and internal (green) bacteria by fluorescence microscopy.

**Infection of mice.** This study was approved by the Local Ethical Committee on Laboratory Animals in Umeå, Sweden. Mice were housed under conventional conditions, given food and water ad libitum, and allowed to acclimatize for at least 7 days before infection. For oral infection of mice, the parental strain IP32953, the *tatC* mutant, and as a negative control, an avirulent, plasmid-cured strain (IP32953c) were used. Bacteria grown overnight in LB broth at 26°C were harvested and resuspended in 50 ml of 0.9% NaCl in sterilized tap water to concentrations of  $10^9$ ,  $10^8$ , and  $10^7$  CFU/ml. Each suspension was given to groups of five C57BL/6 (Scanbur BK) female mice deprived of food and water overnight (18 h), after which the mice were allowed to drink the bacterium-containing water ad libitum for 6 h. Each mouse consumed approximately 3 ml of the bacterial suspension. To ensure that the strains tested did not succumb under these conditions during the feeding period, their ability to survive in the drinking water was monitored for 8 h. For intraperitoneal infection, bacteria grown overnight in LB broth at 26°C were harvested and resuspended in PBS at concentrations ranging from approximately  $10^4$  to  $10^8$  bacteria/ml. For each concentration used, 0.1 ml was injected intraperitoneally into five mice. Aliquots

of the diluted cultures were also plated on blood agar base plates with appropriate antibiotics to determine the number of CFU injected. An in vivo segregation assay for the strains in Peyer's patches and the spleen was also performed. The mice were infected orally with a concentration of  $10^9$  CFU/ml as described above. Two mice from each group were sacrificed daily, and six Peyer's patches and the spleen were removed by dissection. The organs were homogenized in PBS, and serial dilutions were spread on *Yersinia* selective agar base (Difco) plates for viable counting. In all of the infection experiments, the mice were monitored at least two times daily for 14 days. Mice that showed severe symptoms like hunched carriage and tousled fur, no longer moved around, and had stopped feeding and drinking were sacrificed according to the instructions of the Local Ethical Committee on Laboratory Animals. Fifty percent infective doses were calculated based on the number of animals that had to be sacrificed as a result of severe symptoms by the Reed-Muench method for estimation of 50% endpoints (52). Based on previous experience, the calculated infective doses are comparable to the 50% lethal doses of earlier studies.

**Data mining.** By using the protein and nucleotide BLAST resources at the National Center for Biotechnology Information homepage (<http://www.ncbi.nlm.nih.gov/BLAST>), genes encoding Tat components (*tatA*, *-B*, *-C*, and *-E*) and a gene colocalizing with the *tat* operon (*tatD*) were identified in the genome sequences of *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM5. The accession numbers retrieved for *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM5 gene products were YP\_068804/NP\_667790 (*TatA*), YP\_068805/NP\_667791 (*TatB*), YP\_068806/NP\_667792 (*TatC*), YP\_068807/NP\_667793 (*TatD*), and YP\_069628/NP\_668496 (*TatE*). By using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi>), the amino acid identities between the *Yersinia* proteins and their *E. coli* homologues *TatA* (NP\_418280), *TatB* (YP\_026270), *TatC* (NP\_418282), *TatD* (YP\_026271), and *TatE* (NP\_415160) were determined. For identification of potential Tat substrates, pattern searches of the KIM5 and IP32953 genomes were performed with the Pedant genome database (<http://pedant.gsf.de>). The search pattern used was <X(2,32)-RR-X-[VFLIMA](2)-X(0,10)-{RDEK}(13), i.e., a range of 2 to 32 amino acids after the N terminus prior to the twin arginine, one open residue, two amino acids each being either Val, Phe, Leu, Ile, Met, or Ala, and finally the restraint that the following 23 residues must contain an uncharged stretch of at least 13 amino acids (54). The results from the pattern search were then put through the TatP program (<http://www.cbs.dtu.dk/services/TatP>) (3). To reduce the number of false positives, we excluded all proteins that had negative results for all the parameters measured by the program.

## RESULTS

**The Tat system is functional in *Y. pseudotuberculosis* strain IP32953 and *Y. pestis* strain KIM5.** Investigation of the genome sequences of *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM5 revealed the presence of a chromosomal region encoding four genes with high homology to *E. coli* genes *tatA*, *tatB*, *tatC*, and *tatD* and with identities ranging from 52 to 77% on the amino acid level. The genetic organization of the *tatABCD* operon is similar to that of *E. coli* (7), and the genes are most likely transcribed from a common promoter. In addition, the *hemB* gene was found to localize downstream of the *tatABCD* operon and is possibly also part of the same transcriptional unit. In *Y. pestis* KIM5, *tatD* is represented by a pseudogene. This is, however, not likely to affect the function of Tat since *tatD*, given its name due to colocalization with the *tatABC* operon, encodes a DNase and is not required for Tat translocation in *E. coli* (71). Further, the *Yersinia* genomes also contain the monocistronic gene encoding *TatE*, which has functions overlapping those of *TatA* and is believed to be the result of gene duplication (59).

For investigation of Tat functionality, plasmid pZD51 (Table 1) (21), encoding a GFP reporter fused to the *E. coli* Tat signal peptide from *TorA*, was introduced into *Yersinia*. Similar to *E. coli*, specific targeting of GFP to the periplasm could be observed for wild-type strains of both *Y. pseudotuberculosis* and *Y. pestis* (Fig. 1 and data not shown). In contrast, when pZD51 was introduced into *tatC* mutant strains, the GFP fluorescence

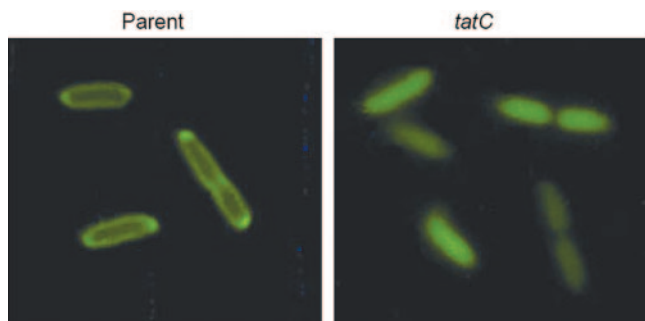


FIG. 1. TatC-dependent export of TorA::GFP in *Yersinia*. Fluorescence images of the parental IP32953 and mutant IP32953*tatC* strains carrying plasmid pZD51, encoding the signal peptide from the *E. coli* Tat substrate TorA fused to GFP. Note the periplasmic location of GFP in the parental strain.

was evenly distributed in the bacterial cytoplasm, demonstrating that Tat is disrupted in these strains. From this, we conclude that *Yersinia* indeed encodes a functional Tat system and that, similar to what has been shown in other gram-negative bacteria, *tatC* is essential for the function of the system.

**Disruption of the Tat system does not affect *Yersinia* growth in liquid culture.** We first wanted to determine the importance of Tat under in vitro conditions. Growth in both rich (TMH) and minimal (YDM) media at 26°C or 37°C was compared for the *Y. pseudotuberculosis* wild-type strain and the *tatC* mutant. Irrespective of culture conditions, the mutant did not show any growth deficiency compared to the parental strain (Fig. 2). Therefore, the Tat protein translocation system is unlikely to have a major role in cell growth and division in *Yersinia*.

**A functional Tat system is required for motility under environmental conditions.** Tat secretion has been shown to be important for the motility of the gram-negative pathogens *P. aeruginosa*, *P. syringae* pv. tomato DC3000, *E. coli* O157:H7, and *Agrobacterium tumefaciens* (11, 21, 43, 50). In the latter two cases, the Tat-defective strains have been shown not to express flagellin, indicating that components of the flagellar secretion assembly and/or secretion system are Tat substrates. No role for motility per se in connection with the ability of human-pathogenic *Yersinia* to cause disease has been defined. In fact, both *Y. enterocolitica* and *Y. pseudotuberculosis* are motile only at environmental temperatures (2, 37, 38) and the genes encoding flagella are nonfunctional in *Y. pestis* (47). Cultivation of the *tatC* mutant and wild-type strains of *Y. pseudotuberculosis* on semisolid minimal agar revealed that the mutant strain was impaired in motility at 22°C. As expected, neither of the strains was motile at 37°C (Fig. 3). The motility deficiency of the mutant was fully restored by introduction of the *tatC* gene in *trans* under the control of the inducible *tac* promoter from pML31 (Fig. 3). Thus, the phenotype of the mutant could be confirmed to be the result of lack of a functional *tatC* gene and not due to effects on downstream genes. Our results also indicate that there might be a link between flagellar assembly and/or secretion and Tat secretion also in *Yersinia*.

**Contribution of Tat to survival under environmental stresses.** Since Tat previously has been implicated in the resistance to a range of environmental stress conditions (62), we wanted to determine whether Tat could play a similar role in *Yersinia*. Hence, the survival rates of the *Y. pseudotuberculosis* wild-type and *tatC*

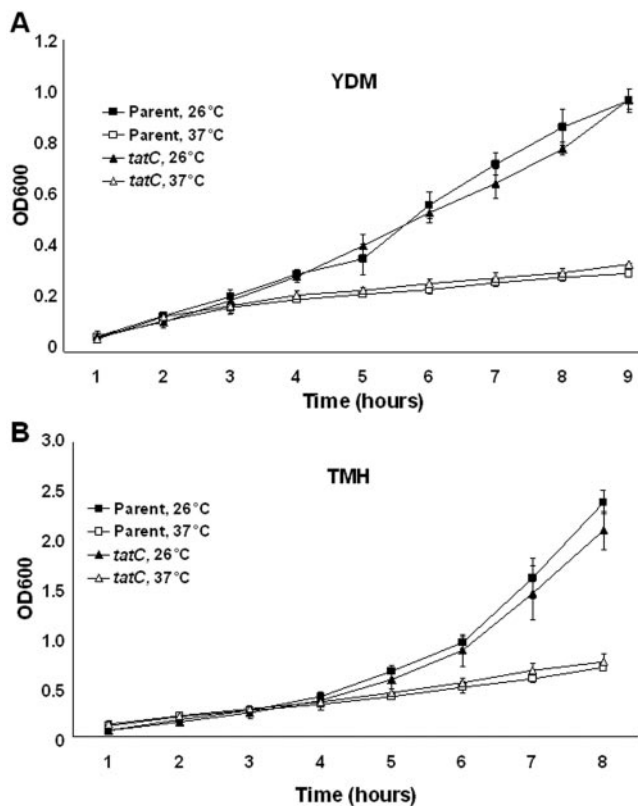


FIG. 2. In vitro growth of Tat mutant *Yersinia*. Growth of the parental IP32953 and mutant IP32953*tatC* strains was determined in poor (YDM) (A) and rich (TMH) (B) media at 26°C and 37°C under low-calcium conditions. Growth assays were performed in duplicate, with the error bars representing the standard deviation. For details, see Materials and Methods. OD600, optical density at 600 nm.

mutant strains after exposure to various stresses were examined. No effect on viability as a result of high temperature, oxidative stress, or high osmolarity could be detected for the mutant, indicating that Tat has no role in protection against these stresses. Under all of these stress conditions, the survival rates of both the wild-type and mutant strains were on the order of 85% or higher. The *tatC* mutant did, however, display a slightly reduced survival rate (50.4%  $\pm$  28.9%) compared to that of the wild type (88.5%  $\pm$  8.2%) when exposed to a low pH. The reduced survival of the *tatC* mutant strain was statistically significant ( $P < 0.05$ , Student's two-tailed *t* test with unequal variance). Introduction of plasmid pML31, carrying *tatC* under control of the *tac* promoter, restored the survival rate of the *tatC* mutant strain to wild-type levels (90.3%  $\pm$  9.3%), suggesting that there is indeed a small decrease in acid resistance associated with disruption of the Tat system.

**Tat is not coupled to *Yersinia* T3S.** T3S is a virulence mechanism common to human-pathogenic *Yersinia* species. The T3SS of *Yersinia* and that of the opportunistic pathogen *P. aeruginosa* are closely related, and some of the key components are also functionally interchangeable (9, 10, 25). Interestingly, Tat secretion is clearly important for *P. aeruginosa* virulence (43, 69). In addition to mediating secretion of phospholipases and iron uptake systems, a functional Tat system has been shown to be required for T3S (Michael Vasil, personal communication). PscO,

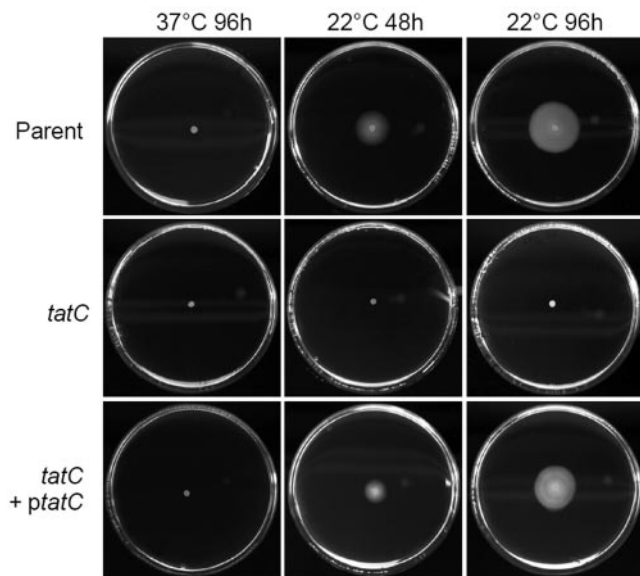


FIG. 3. Analysis of motility of Tat mutant *Yersinia*. The motility of the parental IP32953, mutant IP32953*tatC*, and *trans*-complemented IP32953*tatC*(pML31) strains was assayed by growth on semisolid minimal medium plates. Results after growth at 22°C or 37°C for 48 and 96 h are shown. For details, see Materials and Methods.

one of the core components of the T3SS, has an N-terminal motif fitting the consensus for Tat substrates, although it remains to be shown that PscO can be secreted via the Tat pathway (M. Vasil, personal communication). To explore whether there is a link between Tat and T3S also in *Yersinia*, we assayed the *tatC* mutant for phenotypes coupled to T3S. Initially, we investigated the ability of the *tatC* mutant to produce and secrete Yop proteins under T3S-inducing conditions *in vitro* (growth in calcium-depleted medium at 37°C). The mutant strain was found to express and secrete wild-type levels of Yops into the culture medium (Fig. 4A). Accordingly, the *tatC* mutant was fully capable of delivering Yop effectors into host cells, as visualized by induction of a YopE-mediated cytotoxic effect on HeLa cells (Fig. 4B), as well as of resisting phagocytosis by macrophages (Fig. 4C). Thus, in contrast to the highly related T3SS of *P. aeruginosa*, the T3SS of *Yersinia* is not dependent on a functional Tat translocon for function, and importantly, no putative Tat secretion signal can be identified in the N terminus of YscO, the PscO homologue of *Yersinia* (data not shown).

**Tat is essential for *Yersinia* virulence.** Finally, we wanted to determine if the Tat secretion system plays a role in *Yersinia* virulence. To test this, we used the mouse infection model to study the virulence of *Y. pseudotuberculosis* strain IP32953. This strain is very closely related to *Y. pestis* and causes a systemic plague-like disease in mice. When mice were infected via the oral route or intraperitoneally, the *tatC* mutant strain was revealed to be highly attenuated. The kinetics after oral infection is shown in Fig. 5. For the intraperitoneal route, the 50% infective doses (see Materials and Methods) were calculated to be  $2.2 \times 10^3$  bacteria for wild-type strain IP32953 and  $3.6 \times 10^7$  bacteria for the *tatC* mutant. This is comparable to the 50% infective dose of the virulence plasmid-cured strain, which was  $2.2 \times 10^7$  bacteria. Thus, the level of attenuation of

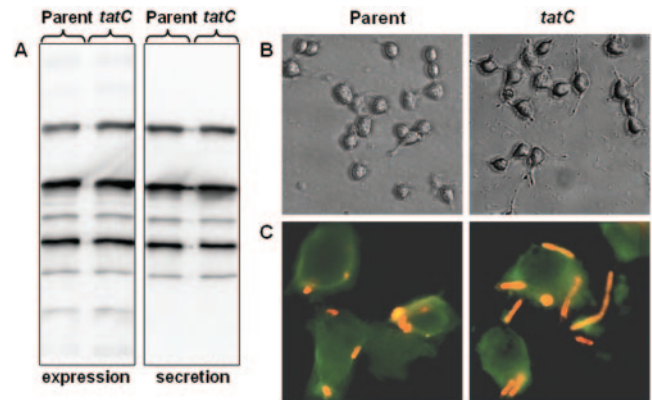


FIG. 4. Analysis of T3S-related phenotypes. Parental IP32953 and mutant IP32953*tatC* were compared for phenotypes related to T3S. (A) Yops in the total protein fraction (left) or secreted into the extracellular medium (right) under T3S-inducing conditions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and identified by immunoblot analysis with anti-Yop antiserum. (B) *Yersinia* strains were allowed to infect a monolayer of growing HeLa cells. After 3 h of infection, the effect on the HeLa cells was recorded by phase-contrast microscopy. Note the extensive rounding of the HeLa cells induced by translocated YopE. (C) Fluorescence microscopy of *Yersinia*-infected J774 macrophages, where extracellular bacteria, resistant to phagocytosis, are labeled in red. For details, see Materials and Methods.

the *tatC* mutant for both the oral and intraperitoneal routes was similar to that of a virulence plasmid-cured strain lacking the T3SS. We have previously shown that in mice infected via the oral route, the Peyer's patches are rapidly colonized where virulent strains overcome the primary host defense and further disseminate to colonize the spleen and liver (33). In contrast, mutants affected in the T3SS are highly attenuated in the mouse model, and while fully capable of initially colonizing the Peyer's patches, these mutants are unable to disseminate from this locus to cause a systemic infection (27, 33). To get a better understanding of the virulence defect of Tat secretion-negative strain IP32953*tatC*, we compared the infection kinetics in lym-

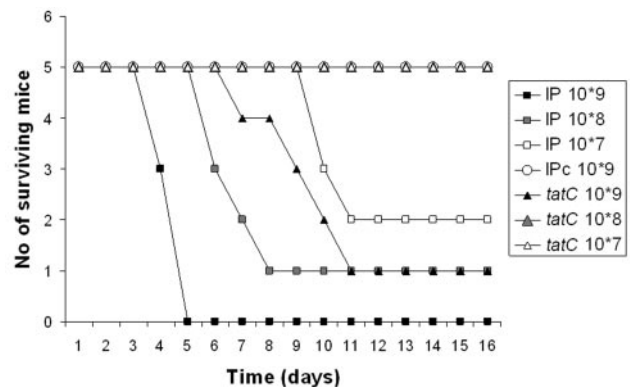


FIG. 5. Oral infection of mice. Survival curves were determined for mice infected with parental IP32953, mutant IP32953*tatC*, and virulence plasmid-cured strain IP32953*c* over a 14-day period. Mice were infected orally with successive 10-fold dilutions ( $10^7$  to  $10^9$ ) of a bacterial suspension and monitored at least twice daily for 14 days. For details, see Materials and Methods.

TABLE 2. Number of colony-forming bacteria in Peyer's patches and spleens of mice infected orally with strains of *Y. pseudotuberculosis*

Day and site	No. of CFU after infection with:					
	Parent strain IP32953		IP32953 <i>tatC</i>		IP32953 <i>c</i>	
	Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 1	Mouse 2
1						
Peyer's patches	$9.4 \times 10^5$	$5.2 \times 10^5$	30	10	<10	<10
Spleen	$1.2 \times 10^2$	$4.2 \times 10^3$	<10	10	<10	<10
2						
Peyer's patches	$2.0 \times 10^4$	$2.7 \times 10^4$	60	20	$1.4 \times 10^2$	20
Spleen	$2.7 \times 10^3$	$9.8 \times 10^2$	80	<10	<10	<10
3						
Peyer's patches	$1.6 \times 10^5$	$8.5 \times 10^5$	30	$5.0 \times 10^2$	80	$3.8 \times 10^2$
Spleen	$2.4 \times 10^4$	$1.6 \times 10^4$	30	50	<10	<10
4						
Peyer's patches	$2.7 \times 10^6$	$1.5 \times 10^4$	$1.3 \times 10^2$	80	50	40
Spleen	$1.9 \times 10^5$	$1.9 \times 10^4$	70	<10	10	$4.9 \times 10^2$
5						
Peyer's patches	NA <sup>a</sup>	NA	$3.0 \times 10^2$	70	40	10
Spleen	NA	NA	<10	<10	$2.3 \times 10^2$	20

<sup>a</sup> NA, not applicable. On day 5, all mice infected with parent strain IP32953 had been sacrificed due to severe symptoms (see Materials and Methods).

phoid organs of orally infected mice with that of isogenic wild-type strain IP32953, as well as a virulence plasmid-cured derivative, IP32953*c*, lacking the T3SS. We found that the *tatC* mutant strain was severely impaired in the colonization of both Peyer's patches and the spleen, with bacterial numbers several log units lower than for wild-type *Yersinia* and comparable to those of the avirulent, plasmid-cured strain (Table 2). This indicates either that the *tatC* mutant does not enter these organs or that it is rapidly cleared by the primary immune system. In conclusion, our work has established that the Tat system has an important and unique role in the virulence of human-pathogenic *Yersinia*.

## DISCUSSION

Specialized protein secretion systems comprise essential virulence mechanisms in many bacterial pathogens. For human-pathogenic *Yersinia* species, the T3SS, which targets the primary host defense by delivery of effectors into host cells, is the most extensively studied virulence strategy. The focus of this work was the Tat mechanism, which we here verify to be functional in both *Y. pseudotuberculosis* and *Y. pestis*. Importantly, we also confirm that a functional Tat system is essential for the ability of *Y. pseudotuberculosis* to cause a systemic infection in mice via both the oral and intraperitoneal routes. Our findings indicate that *Yersinia* encodes specific virulence factors that are secreted by the Tat system.

In pathogens where Tat-mediated protein translocation has been studied so far, the system has been found to be involved in several important processes like biogenesis of the cell envelope, biofilm formation, assembly of the electron transport chain, survival under various environmental stress conditions, and motility (11, 21, 35, 36, 43, 62). The facts that Tat-negative bacteria display pleiotropic defects and are often impaired for in vitro growth make it difficult to establish a more direct contribution of Tat

substrates to virulence. The situation seems to be different in *Yersinia*, where the *tatC* mutant strain displayed no growth defects during in vitro growth at either environmental or host temperature in rich and poor media alike. We also investigated the importance of the Tat system for survival under different environmental stress conditions and found that the *tatC* mutant was not impaired in the ability to survive after exposure to high temperature, oxidative stress, or high osmolarity. In line with this is also our finding that only 11 of the 27 predicted or verified Tat substrates in *E. coli* (46) can be identified as potential Tat substrates in *Yersinia* (Table 3 and data not shown).

As in many other pathogens, we did observe a motility defect when the *Yersinia* Tat system was disrupted (21, 43, 50). We do not believe that this defect per se is of high significance for *Yersinia* virulence, as *Y. pseudotuberculosis* is only motile at environmental temperatures (2) and the most virulent species, *Y. pestis*, is nonmotile due to several mutations in operons encoding gene products involved in flagellar secretion and assembly (47). However, if the deficiency in motility is the result of impaired secretion of flagellar components, we cannot exclude the possibility that other proteins secreted by the flagellar T3SS contribute to virulence, similar to what has been found in *Y. enterocolitica* (74).

Upon disruption of the Tat system, some bacterial species display impaired cell division, leading to chain formation. In the case of a *Yersinia* *tatC* mutant, elongated cell morphology was occasionally observed (Fig. 4C). This was, however, most likely not due to defects in cell division but rather an effect of chloramphenicol selection, a phenomenon previously observed for *Yersinia* (Fabienne Deleuil, unpublished observation).

The T3SS of *P. aeruginosa* is closely related to the plasmid-encoded system of *Yersinia*. Interestingly, in *P. aeruginosa* there appears to be a link between the Tat system and T3S. Introduction of mutations that disrupt the Tat system causes defects

TABLE 3. Putative Tat substrates of *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM5<sup>a</sup>

Physiological role	Cellular localization	<i>Y. pseudotuberculosis</i>		<i>Y. pestis</i>		<i>E. coli</i> Tat substrate
		Accession no.	Protein	Accession no.	Protein	
<b>Respiration</b>						
Nitrate reduction	Periplasmic	YP_071269	NapF	NP_668763	NapF	NapG
Nitrate reduction	Periplasmic	YP_071267	NapA			NapA
DMSO <sup>b</sup> reduction	Inner membrane	YP_069346	DmsA	NP_668198	DmsA	DmsA
DMSO reduction	Inner membrane	YP_071198	DmsA	NP_668839	DmsA	
Formate oxidation	Periplasmic?	YP_072403	FdoG			FdoG
<b>Cell cycle</b>						
Cell division	Periplasmic	YP_071879	SufI	NP_670801	SufI	SufI
Cell wall amidase	Periplasmic	YP_071528	AmiC	NP_670460	AmiC	AmiC
<b>Iron acquisition</b>						
Ferrichrome binding	Periplasmic			NP_668133	FhuD	FhuD
Transport protein	Inner membrane	YP_070126	YbtP	NP_669704	YbtP	
Transport protein	Inner membrane	YP_070127	YbtQ	NP_669703	YbtQ	
Transport lipoprotein	Periplasm	YP_070252		NP_669757		YcdO
<b>Transport</b>						
HlyD family	Inner membrane/ extracellular?	YP_068640				
HlyD family	Inner membrane/ extracellular?	YP_069921		NP_670114		
Transport	Periplasmic	YP_069763		NP_670302		
Sugar transport	Periplasmic	YP_069857		NP_670191		
Sugar transport	Periplasmic			NP_668205		
Phosphate transport	Inner membrane			NP_668720		
Aliphatic sulfonate transport	Inner membrane			NP_667585		
Sulfate transport	Membrane	YP_071223	CysZ	NP_668810		
<b>Others</b>						
CA	?	YP_071573		NP_670506		
Proline-specific aminopeptidase	?	YP_071686	PepP	NP_670569	PepP	
Copper homeostasis	Periplasmic	YP_069263		NP_668113		CueO
Sulfatase	?	YP_071579		NP_670514		
<b>Unknown</b>						
	Membrane	YP_069840				
	Membrane			NP_668871		
	?	YP_070253		NP_669756		YcdB
	Exported/outer membrane	YP_069963		NP_670063		YcbK
	?			NP_669146		
	Extracellular			NP_670676		
	Extracellular	YP_071753		NP_670675		

<sup>a</sup> Putative substrates were retrieved with the TatP software (see Materials and Methods).

<sup>b</sup> DMSO, dimethyl sulfoxide.

in T3S, possibly due to PscO, one of the core T3SS components, which has a putative Tat signal peptide and thus may be secreted via the Tat system (M. Vasil, personal communication). Recently, a *P. syringae* *tatC* mutant strain was also shown to display decreased T3S-dependent translocation of anti-host factors into plant cells, indicating that functional Tat is required for efficient type III translocation in this pathogen (11). When we examined the *Yersinia* *tatC* mutant, we could, however, not see any defects in phenotypes coupled to functional T3S. The mutant was indistinguishable from the isogenic wild-type strain with respect to Yop expression and secretion, the ability to induce a YopE-mediated cytotoxic effect on infected HeLa cells, and the ability to block phagocytosis by J774 macrophages. Thus, the virulence defect of the *tatC* mutant is not an indirect effect caused by impaired function of the T3SS.

When investigating the susceptibility of the *tatC* mutant to various environmental stresses, we detected a small decrease in acid resistance. One critical phase of infection where the pathogen faces a low pH is inside macrophages, where *Yersinia* is believed to reside at early stages of infection. We found that the *tatC* mutant was not impaired for survival in the macrophage-like cell line J774.1. Even if J774 cells are less efficient in bacterial killing compared to activated macrophages, we still believe that these results are relevant, as J774 cells have been shown to cause impaired intracellular survival of *phoP* mutants in *Y. pestis*, as well as in *Y. pseudotuberculosis* (29, 44). In both cases, the impaired intracellular survival was coupled to a moderate decrease in virulence. Impaired acid resistance could impact the ability to cause disease via the oral route, as the pathogen is exposed to a low pH during its entry into the gastrointestinal tract. Importantly, how-

ever, the *tatC* mutant strain was highly attenuated not only via the oral route but also in intraperitoneal infection of mice, when the gastrointestinal tract is circumvented.

When in silico prediction methods were used to identify putative Tat substrates in the genomes of *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM5, most of the potential substrates were shared by the two strains (Table 3). Among the putative Tat substrates, we identified a few which could explain the phenotypes in which the *tatC* mutant differs from the wild-type strain. One of the substrates encodes a potential carbonic anhydrase (CA) (accession number YP\_071573; Table 3). This is interesting since CAs have been shown to play a role in pH regulation and have been proposed to be important for survival of intracellular pathogens within their hosts (60). Prokaryotic CAs have, in *Helicobacter pylori* and cyanobacteria, been shown to localize to all bacterial compartments, the cytoplasm, the inner and outer membranes, and the periplasm, and also extracellularly (15, 61). A recent publication shows a periplasmically located CA of *H. pylori* to be required for in vitro acid resistance (40). Other interesting potential substrates, with respect to virulence, include proteins involved in iron acquisition, which could have a significant impact on the ability of *Yersinia* to establish an infection. In addition, several putative substrate genes encode homologues of systems potentially involved in the transport of sugar, amino acids, phosphate, and sulfur sources (Table 3), and there are also a number of potential Tat substrates of unknown function.

Our findings that the Tat pathway is critical for the ability of *Y. pseudotuberculosis* to cause a systemic infection in mice has implications also for *Y. pestis* as the two species are very closely related (1) and cause similar systemic infections in mice. We have verified that the Tat pathway is functional also in *Y. pestis* KIM5 and that most of the putative secretion targets are shared between the two genomes (Table 3), suggesting that there is a Tat substrate(s) that is involved in virulence also in *Y. pestis*.

The identification of Tat as a key virulence mechanism could be important for future development of novel component vaccines against *Y. pestis*. A subunit vaccine is currently under development that is based on two virulence proteins, LcrV of the T3SS and capsule-like antigen fraction 1 (F1). The new vaccine is very promising and induces high levels of protection even against an aerosol challenge with *Y. pestis* (31, 67, 68). This shows that the principle of using secreted virulence factors as vaccine works well for a pathogen like *Y. pestis*, which is believed to reside mainly extracellularly during infection. One potential pitfall of the new vaccine is the occurrence of natural isolates of *Y. pestis* that lack F1 with fully retained virulence (16). Therefore, there is an urgent need to identify additional surface-located or secreted proteins that are required for *Yersinia* virulence to evaluate for protection. The essential role played by the Tat pathway in virulence suggests that candidate proteins that are promising as vaccine components could be found among the Tat substrates of *Yersinia*. Work to identify the Tat substrates of *Yersinia* and to verify the role of the Tat system in *Y. pestis* is under way and is likely to uncover a novel major virulence mechanism of pathogenic *Yersinia*.

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