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Analysis of the *Brucella suis* twin arginine translocation system and its substrates show that it is essential for viability

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Running Title: *Brucella suis* Tat is essential

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Abstract

Bacteria use the Twin Arginine Translocator (Tat) system to export folded proteins from the cytosol to the bacterial envelope or to the extracellular environment. As with most Gram-negative bacteria, the Tat system of the zoonotic pathogen *Brucella* is encoded by a three gene operon; *tatABC.* Our attempts, using several different strategies, to create a *Brucella suis* 1330 *tat* mutant were all unsuccessful. This suggests that, for *B. suis*, Tat is essential and is in contrast to a recent report for *B. melitensis.* This was supported by our findings that two molecules that inhibit the *Pseudomonas aeruginosa* Tat system also inhibit *B. suis, B. melitensis* and *B. abortus* growth *in vitro*. In a bioinformatic screen of the *B. suis* 1330 proteome, we identified 28 proteins with putative Tat signal sequences. We used a heterologous reporter assay based on export of the Tat dependent amidase AmiA using the Tat signal sequences from the *Brucella* proteins to confirm that 20 of the 28 candidates can engage the Tat pathway.

Introduction

Translocation of proteins across membranes is essential for all aspects of the physiology of bacteria, ranging from basic metabolic processes to pathogenic/symbiotic interactions with a host. Gram negative bacteria use two pathways to translocate proteins across the cytoplasmic membrane; the Sec system, which exports unfolded proteins, and the Tat system, which exports folded proteins. The Tat pathway is found in many bacteria, including most animal and plant pathogens. It has the unique ability to transport fully-folded proteins, including a subset that binds redox cofactors, across the cytoplasmic membrane [1]. Tat substrates are involved in a large array of bacterial processes such as respiratory metabolism, cellular division, motility, adaptation of bacteria to particular environments, or biofilm formation [2–5].

Proteins that are targeted to the Sec or Tat pathways have specific N-terminal signal peptides that allow their recognition by their respective machineries. Sec and Tat signal peptides have the same overall tripartite organization (n-, h- and c-region), but Tat signal peptides are longer than their Sec counterparts (27 to 35 aa for Tat signal peptides versus 17 to 24 aa for Sec signal peptides). One of the main features of Tat signal peptides is the presence of a distinctive twin arginine motif S/TRRxFLK, in the n-region, where the arginine pair is almost invariant and other amino acids are present at a frequency >50%. Another characteristic of Tat signal peptides is that their h-region tends to be less hydrophobic than the corresponding region of Sec signal peptides [6]. These characteristics have allowed the development of three prediction programs that recognize the features of Tat targeting sequences [7–9].

While in most bacteria, *tat* mutants are viable, in a few organisms a functional Tat system appears to be essential under standard laboratory conditions. These include *Haloferax volcanii* [10], *Sinorhizobium meliloti* [11] and *Mycobacterium tuberculosis* [12]. In other bacteria,

including *Burkholderia thailandensis* [13], *Myxococcus xanthus* [14], *Mycobacterium smegmatis* [15] and *Shewanella oneidensis* [16], a functional Tat system is needed for optimal growth in aerobic conditions. Conversely, for *E. coli O157:H7* [17], *Pseudomonas aeruginosa* [5], *Agrobacterium tumefaciens* [18], and *Ralstonia solanacearum* [19] the Tat system is required for anaerobic growth.

Brucella are facultative intracellular pathogens. Intracellular survival and multiplication of *Brucella* are essential to the establishment, development and chronicity of the disease. *Brucella* enters cells via lipid rafts, a route that ensures their intracellular survival by avoiding fusion of their membrane-bound compartment with lysosomes. *Brucella* then uses its VirB type IV secretion system [20] to secrete effector proteins into the infected cells which direct the maturation of the vacuole into a replication-permissive organelle derived from the endoplasmic reticulum [21].

The genome sequences of all *Brucella* species contain genes that encode a Tat system and proteins have been annotated as 'Tat dependent'. A recent report describes the construction and characterization of a *B. melitensis tat* mutant , suggesting that the Tat system is required for stress resistance and virulence [22]. Here we report that we are unable to create a *Brucella suis tat* mutant and we demonstrate that two inhibitors of the *P. aeruginosa* Tat system inhibit the *in vitro* growth of several strains of *Brucella* in a dose dependent manner, suggesting that Tat is essential for viability. A bioinformatic screen for proteins with Tat signal sequences allowed us to identify a set of potential Tat substrates for *B. suis*, and we were able to confirm that the signal peptides of 20 of these engage the Tat pathway using a heterologous translocation reporter system.

Materials and Methods

Bacterial strains, growth conditions, plasmids.

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in Lysogeny broth (LB) or on LB plates and *Brucella* on Trypticase Soy (TS). When required, the medium was supplemented with ampicillin (Amp; 100 µg/mL), Kanamycin (Kan; 25µg/mL) or Chloramphenicol (Cm; 30µg/mL). N-Phenyl maleimide and Bay 11-7082 were from Sigma. Plasmid constructions were generated using standard methods or synthesised commercially (Genwiz).

In silico prediction of Tat substrates and signal sequence peptide sites.

A BLASTP search with the conserved SRRXFLK motif was used to identify putative Tat substrates encoded by the *B. suis* 1330 genome. Candidate substrates were analysed using the three existing programs for Tat signal prediction: TatP (http://www.cbs.dtu.dk/services/TatP/ [8]), TATFIND (http://signalfind. org/tatfind.html [9]) and PRED-TAT (http://www.compgen.org/ tools/PRED-TAT/ [7]).

For signal peptide domain prediction the SignalP 3.0 (HMM) and Phobius servers were used. Most likely signal peptide cleavage sites were inferred from Sec, Tat and lipoprotein signal peptide predictions algorithms (SignalP 3.0, LipoP 1.0, TatP, TATFIND and PRED-TAT).

Plasmid constructions for the different strategies to obtain a *tat* mutant in *Brucella*

Clean deletion of the *tatABC* operon The genomic sequences immediately upstream (535bp) and downstream (512bp) of the *tatABC* operon were amplified from *B. suis* 1330 genomic DNA as template with *Pfu* DNA polymerase (Life technologies, USA) using primer pairs tatA-F1FW/tatA-F1REV and tatC-F2FWD/tatC-F2REV. Primers used for the constructions in this

study are described in Supplemental Table S1. The PCR products were cloned into pUC29 and, after sequence verification (MWG Operon Eurofins, Germany), excised using the restriction introduced in the primers and ligated together in the SacB dependent sucide vector pIN60, generating pIN365.

Non polar deletion and marked *tatA* mutants. The same strategy was used to create a non polar deletion of *tatA,* with the same DNA fragment downstream of *tatA* and a 472 bp region upstream of *tatA* designed so that the ATG of *tatA* is followed by the second codon of *tatB.* This was amplified with the tatA2UPstream-F1FWD/tatA2UPstream-F1FWD primer pair. Again, the two fragments were ligated into pIN60 to give pIN367. To allow positive and visual selection of an insertion, we introduced replaced the *tatA* sequence with a chloramphenicol resistance cassette and *gfp,* amplified from pIN310 with primers eGFPXbaI/CmRMfeI giving a 1583bp. After sequence verification this was cloned into pIN367 as a *Xba*I and *Mfe*I fragment to give pIN368.

Conditional mutant in the Tat system An anydrotetracycline-inducible *tatABC* operon was created by inserting the *tetR* repressor sequence and the Tn*10 tetA* promoter/operator (tet-p/o) to drive expression of the *tat* operon. This was used to replace the native promoter of the *tat* operon, allowing us to control its expression by adding or removing anhydrotetracycline. We designed two constructs, the first contains 400bp upstream of the *tatABC* operon (F1) and the kanamycin resistance gene (810bp). The second construct had the *tetR* repressor gene and the promoter of the divergent *tetA* resistance gene followed by a 499bp sequence containing *tatA* and part of *tatB.* These two sequences were synthetized commercially (Genewiz) and cloned together in a SacB dependent suicide vector (pIN11) to generate plasmid pIN371.

N-Phenyl maleimide and Bay 11-7082 inhibition assay

Brucella strains were growth overnight in TS broth. Stock solutions of N-Phenyl maleimide or Bay 11-7082 at 100mM were made in ethanol and DMSO respectively. Serial 2 fold dilutions of each inhibitor were made in TS broth starting at 200 µM. One mL of each dilution was inoculated with *B. suis*, *B. abortus* and *B. melitensis* at an OD_{600} of 0.05 and incubated at 37C with or without shaking (200rpm). After 24h (shaking) or 48h (static), the OD_{600} was measured in a spectrophotometer. Results are expressed as the % of the OD of a culture with no inhibitor, experiments were repeated three times.

Heterologous system to detect predicted *Brucella* Tat substrates

DNA sequences where the sequence encoding the signal sequence of the *E. coli* AmiA protein was replaced by the signal sequences of the 28 predicted *Brucella* Tat substrates were synthesised commercially (Genwiz) and cloned into plasmid pssAmiA-AmiAH [23]. A total of 32 plasmids were constructed to account for alternative cleavage sites (Table S2). *E. coli* strains (MC4100, MC4100 Δ*ssamiAC,* and MC4100 Δ*tatC*) were transformed with these plasmids. Strains with no plasmid or transformed with a plasmid encoding wild type AmiA were used as controls.

Results

Characterisation of the Tat system in *Brucella*

All *Brucella* have the typical *tatABC* operon structure [24]. Alignment of the *tatABC* genes from different *Brucella* species showed a high degree of homology: *tatA* shows 99.54% identity, *tatB* at least 94.53 % and *tatC* 99.88% across the genus (data not shown). At the amino acid level,the annotated TatA sequences in Genbank have protein sequences of either 72 or 80 residues, however this is probably just an annotation error with an incorrect start codon used in the longer versions. The 72 amino acid sequences show100% identity to each other. TatC also shows 100% sequence identity. TatB sequences vary in length from 184 to 203 aa. While this is partly due to different predicted start sites, TatB displays a greater degree of heterogenicity than TatA and C; there are numerous deletions in a proline rich region in the TatB proteins of *B. ovis*, *B. vulpis* and several atypical isolates, as well as two shorter deletions at the C-terminus of *B. ovis* TatB (Fig. 1).

The Twin arginine system is essential for *Brucella*

To investigate the role of the *Brucella* Tat system in virulence, we tried several strategies to construct a *B. suis tat* mutant.

***tatABC* deletion.** We first attempted to create a mutant with an unmarked deletion of the whole region encoding the *tat* operon. The genomic flanks (aproximately 500bp) immediately upstream and downstream of the *tat* operon were amplified using *B. suis* 1330 genomic DNA and cloned together in a SacB dependent suicide vector to give pIN365. Plasmid pIN365 was then electroporated into *B. suis* 1330 and Kanamycin-resistant (Kan^R) clones were selected; PCR analysis confirmed that the plasmid had integrated into the *tat* locus. A second homologous recombination to get the allelic replacement with excision of the *tat* operon and

integrated plasmid, was selected using sucrose sensitivity. We performed PCR on 200 sucrose resistant, kanamycin sensitive colonies and were unable to detect a colony where the *tatABC* operon had been deleted; all had reverted to wild type. This suggests that, for *B. suis*, the Tat system is essential.

Inframe deletion of *tatA*. Data from a Tn-seq screen performed on *B. abortus* [25] showed a very low number of transposon insertions in *tatABC* (Fig 2). Analysis of the Tnseq profile in the *tatABC* operon region shows that there are no insertions in the *tatC* region, however some insertions could be seen in the *tatA* region. Therefore, we reasoned that a deletion of *tatA* may be easier than deletion of the entire operon. Primers were designed to amplify upstream and downstream sequences which, when ligated together, replaced the ATG start codon of *tatA* with that of *tatB.* Here again we used a SacB dependent suicide vector to select a first integration but were again unable to identify a clone where the *tatA* gene had been deleted by the second recombination event (300 colonies tested).

Disruption of *tatA* with a chloramphenicol cassette and GFP marker. Due to the unsuccessful attempts in identifying an unmarked, inframe deletion mutant of the whole operon or the *tatA* gene, we decided to use a strategy that would allow us to positively select mutants by inserting two selectable markers, chloramphenicol resistance and GFP, into the *tatA* sequence. Here, attempts to isolate clones with even a single-crossover event were unsuccessful. No kanamycin resistant colonies were isolated after several attempts at electroporation.

Construction of conditional mutant in *tat* system. As a final strategy to generate a *tat* mutant, we attempted to construct a conditional mutant where *tatABC* expression was inducible. We used the anhydrotetracycline (ATC) inducible tetracycline resistance promoter from Tn*10*,

which has already been used with success in *Brucella*, [26] to control expression of the *tatABC* operon.

To determine the opitmal ATC concentrations to induce expression of the *tatABC* genes from the *tetA* promoter, we used a plasmid carrying the *gfp* gene under control of the *tetA* promoter (pJC45). We establish by flow cytometry (FACS) that the minimal concentration for induction was 0.2 µM, GFP expression was maximal at 5 µM, and there was a reduction of fluorescence at 10 µM (data not shown). After electroporation with pIN371, colonies were grown on plates with different concentrations of ATC (0.2, 0.5, 1, 1.5, 2.0, 2.5, 5 and 10 µM) and the appropriate antibiotics. From the many conditions tested, colonies resulting from the first crossover were only obtained when 0.2 µM ATC was present in both the liquid medium used during the 4-hour recovery post electroporation and in the plates. The colonies obtained were smaller than usual. This suggests that the integration of the plasmid affects expression of the Tat system which has a negative effect on the bacterium. Again, attempts to isolate second crossover events, where the wild type promoter was replaced by the *tet* promoter were not successful and the first crossover colonies were lost after a few passages on agar.

Taken together, the results of this work provide evidence that the twin arginine transport system of *B. suis* is essential for viability under all the conditions that we tested.

Inhibitors of the *Pseudomonas aeruginosa* Tat system inhibit *Brucella* growth *in vitro*

As we could not genetically inactivate the Tat system, we tested two compounds previously reported to inhibit some bacterial Tat systems: N-Phenyl maleimide and Bay 11-7082. Both compounds have been proposed as potent Tat inhibitors and have been shown to inhibit the

secretion of Phospholipase C, a Tat dependent substrate, in *P. aeruginosa* [27]. We tested the effects of the two compounds on the growth *B. suis, B. abortus* and *B. melitensis.* In initial disc diffusion experiments with *B. suis* on plates, no growth inhibition was seen (not shown). However, when tested in liquid culture both compounds appeared to inhibit bacterial multiplication for all three *Brucella* strains. Similar effects were seen in shaking and non shaking conditions with both inhibitors. Bay 11-708 showed a stronger capacity to inhibit *Brucella* growth than N-Phenyl maleimide. We sought to test the effects of these compounds on *Brucella* in cell infection assays. However, we found that both were highly cytotoxic at concentrations below 50 µM (not shown) meaning that they could not be used.

In silico prediction of Tat substrates in the *Brucella* proteome

Proteins are targeted to the Tat machinery through specific N-terminal signal peptides that have a distinctive twin arginine motif. A BlastP search with the conserved motif (SRRXFLK) was used to identify potential Tat substrates in *B. suis* 1330 proteome and then analysed with three programs for Tat signal prediction: TatP, TATFIND and PRED-TAT. We considered that a protein contained a putative Tat signal if it was predicted by at least two of the programs: a total of 28 proteins were identified (Table 2 and Table S2).

Heterologous system to confirm predicted *Brucella* substrates

To confirm the Tat-dependence of our 28 putative substrates, we used a heterologous reporter assay in *E. coli*. This assay is based on the Tat dependent export of the AmiA amidase which allows the identification of Tat signal peptide. When fused to a Tat signal peptide, AmiA is exported into the periplasm which rescues the SDS sensitive phenotype of *E. coli* MC400 Δ*ssamiAC* that lacks periplasmic AmiA and AmiC activities. AmiA does not rescue SDS sensitivity

when fused to a Sec signal peptide or when expressed in a *tat* mutant [3]. DNA sequences encoding signal sequence-AmiA fusions were synthesised and cloned into pssAmiA-AmiAH comercially [3,23]. If a putative Tat substrate had more than one predicted cleavage site, all were tested; we thus tested 33 putative signal peptides. We found that 20 of the 33 putative signal sequences restored SDS resistance of the Δ*ssamiAC* but not of the Δ*tatC* strain (Fig. 4 and Fig S1). These data show that the *B. suis* genome encodes at least 20 *bona fide* Tat substrates.

Discussion

There is a high degree of conservation in genome organization between *tatA*, *tatB* and *tatC* genes across the *Brucella* genus and groups of highly related bacteria such as *Ochrobactrum* spp. and the well characterised *tatABC* operon of *E. coli*. While TatA and TatC are highly conserved (other than a two possible start sites for TatA), TatB shows the greatest degree of variability with deletions in a proline rich region and at the C-terminus. The NMR structure of the *E. coli* TatB protein suggests that this region is non structured [28]. These regions do not interact with TatC or the signal sequence of the transported substrate, but may interact with the substrates [29].

We attempted to create a *tat* mutant by deleting or interrupting the chromosomally encoded *tat* genes using several different strategies. None were sucessful, strongly suggesting that the Tat system is essential. The essentiality of the system is further suggested by Tn-seq analysis that was performed in *B. abortus* [25]. It could be possible that our attempts to disrupt the *tat* operon had also disrupted expression of *serS* which is also essential (Fig 2). However, the inhibitory effects of N-phenyl maleimide and Bay 11-7082, reported to inhibit Tat function in

P. aeruginosa [27] again suggest the essentiality of the Tat system. Unfortunately, these molecules were cytotoxic for both HeLa and J774 cells, which will limit their use as antimicrobial agents or for use as experimental Tat inhibitors in cell culture based infection models. Yan et al described the construction and characterisation of a *B. melitensis* mutant with a deletion of the *tatA* gene [22]. The reasons why construction of a *tat* mutant was possible in *B. melitensis*, while it appears essential in both *B. suis* and *B. abortus* and both inhibitors had an effect *B*. *melitensis* 16M are not clear. However, it may be possible that the *B. melitensis* strain used in that study has acquired unknown compensatory mutations that make inactivation of Tat possible (as recently reported for the central carbon metabolism in *B. suis* [30]). While many of the Tat substrates we identified are important to *Brucella* physiology, and some contribute to *Brucella* virulence, there are none that could explain the essentiality of the *B. suis* Tat system; however it is possible that this is due to a cumulative effect of loss of more than one Tat substrate.

Our bioinformatic screen identified 28 putative Tat substrates in *B. suis* 1330, two more than in the study by Nunez et al [24]. Using the amidase heterologous reporter screen we were able to confirm that 20 were true Tat substrates. Four of the remaining candidates were able to export AmiA in a *tat* mutant, indicating that the fusions are at least partially routed to the Sec pathway. There have been reports that some Tat and Sec signal sequences can show a degree of promiscuity [31] and that a small number of AmiA fusions can restore SDS resistance to a *tatC* mutant [32].

Yan *et al* described a similar bioinformatic screen in *B. melitensis* [22]. This study also tested translocation using the amidase reporter assay and constructed and analysed mutants in the

predicted Tat substrates, suggesting that 6 contribute to virulence. As expected, given the high degree of sequence identity between the two strains, there were many similarities, however there were some differences in the list of predicted Tat substrates and the results from the amidase reporter assay they presented were very different to those of our study. While some so the differences are due to annotation anomalies, many cannot be explained. A comprehensive comparison of the two data sets is shown in supplemental data (Table S3). Several of the identified proteins in our study, including a multicopper oxidase, the Rieske protein, MsrP, NosX, NosZ and NirK are are well known Tat substrates in other organisms. Others, however, are not 'classical' Tat substrates; these include the two DsbA proteins, the L,D transpeptidases, the periplasmic binding proteins plus several proteins with unknown function. We identified homologues of all the confirmed substrates encoded by the genomes of closely related alpha proteobacteria (including *Ochrobactrum*, Rhizobiales and *Agrobacterium*) using BlastP and bioinformatically confirmed the presence of Tat signal sequences for all except the homologues of BR_RS10515 (data not shown). This suggests that the alphaproteobacterial have a repertoire of Tat substrates that differs from those in the enterobacteria and *Pseudomonas.* As seen with *P. aeruginosa* [3], a large number of the Tat substrates are predicted to contain co-factors (metal ions, iron-sulphur clusters..) and are predicted to be involved in electron transport, respiration and other oxidoreduction reactions. These include the Rieske Fe/S protein, an essential subunit of mitochondrial and bacterial cytochrome *bc1* complexes, NosX, NosZ, and NirK. Several of the Tat substrates we identified are involved in resistance to oxidative stress and survival at low oxygen concentrations (*Brucella* is generally classed as a strict aerobe, but can use alternative terminal electron acceptors to survive anaerobiosis), characteristics that have been associated with *Brucella* virulence [33,34], although they are not essential as *nosX* is a pseudogene in *B. ovis* [35].

Transport of a wide range of substances across bacterial envelopes, including the uptake of many nutrients, is performed by ABC transporters. *Brucella* has an exceptionally large number of predicted ABC transporters [36]. The uptake systems generally work with periplasmic binding proteins that concentrate the substrate in the periplasm and deliver it to the inner membrane transport components. We identified 7 Tat dependent periplasmic binding proteins. This seems to be common in the α-proteobacteria since 10, 14 and 23 Tat dependent periplasmic binding proteins were predicted for *Ochrobactrum anthropi*, *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*, respectively [18,24,37].

Many of the Tat substrates are important for the formation and maintenance of the bacterial envelope. The L,D transpeptidases are involved in peptidoglycan remodeling and biosynthesis. The *Brucella* genome encodes over 80 predicted lipoproteins, which play an essential role in envelope integrity and, in some cases, bacterial virulence [38]. Two lipoproteins were identified as Tat substrates. It has been suggested that Tat dependent lipoproteins, often fast folding hydrolases and oxido-reductases, have evolved in extremophiles, environemental bacteria such as *Streptomyces coelicolor, L. pneumophila, and Haloferax volcanii* [39]. This fits with our observations that *Brucella* have evolved from soil bacteria by a process of genome reduction [40]. Formation of disulphide bridges in the periplasm by the Dsb system is essential for the correct folding of envelope proteins. We identified two DsbA proteins encoded by *B. suis* as Tat substrates. Transposon mutagenesis studies have shown that DsbA proteins are both required for full virulence [41]. While it is possible to mutate both genes individually, a double mutation has not been tested, and may be lethal. Their role in virulence may be due to reduced fitness or due to incorrect folding of virulence proteins, as reported for *Legionella pneumophilia*, where a DbsA protein is required

for folding of Dot/Icm proteins [42]. Disulphide bond formation systems vary in prokaryotes probably reflecting specific requirements for survival in diverse ecological niches [43]. In *E. coli*, the DsbA signal sequence directs it to an unusual pathway depending on SecY, SecA and SRP, possibly because it folds rapidly in the cytoplasm [44,45]. Analysis of the genomes of other alpha proteobacteria closely related to *Brucella* suggests that their DsbA homologues are also Tat substrates (not shown), suggesting the DsbA proteins fold too rapidly in the cytoplasm to engage the SecY, SecA and SRP pathway.

All together our data show that the Tat system is essential for *B. suis* survival and the originality of its substrates might account for this essentiality. More work will be needed to understand why Tat is essential and if, as for most pathogens, it has a role in virulence.

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Table 1 Bacterial strains and Plasmids

Bacterial Strains	Description	Source/Reference
Brucella suis 1330		ATCC 23444^{T}
Brucella abortus 2308		Laboratory collection
Brucella melitensis 16M		ATCC 23456^{T}
E. coli DH5α	Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17 (rk–, mk +) *phoA supE44 thi-1 gyrA*96 *relA1* λ–	Invitrogen
E. coli MC4100	F-Δl*acU*169 *araD*139 *rpsL*150 r*elA*1 *ptsF* rbs *flbB*5301	Laboratory collection
E. coli MCDSSAC	MC4100 *amiA*Δ2–33, *amiC*Δ2–32	[4]
E. coli B1LKO	MC4100 Δ*tatC*	[46]
Plasmids		
pIN60	SacB dependent suicide vector. (KanR)	[47]
pIN11	SacB dependent suicide vector. (CmR)	[47]
pJC45	pBBR1 based, expresses GFP under control of plasmid tetR/tetA promoter. (KanR)	[26]
pIN310	pUC29 expressing eGFP and CmR	Laboratory strain

pIN365	pIN60 with upstream and downstream regions flanking the *tat* operon. To create a clean deletion of the operon.	This study
pIN367	pIN60 with sequences encoding eGFP and CmR between upstream and downstream regions flanking *tatA*. To create a GFP CmR marked *tatA* deletion.	This study
pIN371	pIN11 derivative carrying 400bp upstream of the *tatABC* operon, KmR, *tetR* repressor, *tatA* under control of the *tetA* promoter..To replace the *tat* operon promoter with an inducible promoter.	This study
pUNI-PROM	pT7.5-derived vector allowing constitutive expression under the control of the *E. coli tat* promoter or inducible expression from the upstream T7 promoter (ApR)	[48]
pssAmiA-AmiAH	ssAmiA fused to mature AmiA carrying a C-terminal hexa-histidine tag in pUNI-PROM (ApR)	[23]

Table 2 *B. suis* 1330 proteins predicted to be Tat substrates

B. suis locus	Annotation[1]	Tat dependent[2]
BR_RS00235	L,D-transpeptidase	Yes
BR_RS01455	CAP domain-containing protein/alkaline phosphatase	Yes
BR_RS01605	ABC transporter substrate-binding protein	Yes
BR_RS01705	DsbA family protein	Yes
BR_RS02290	DsbA family protein	Yes
BR_RS03515	L,D-transpeptidase	Yes
BR_RS07125	Rieske Fe-S protein.	Yes
BR_RS10155	Molybdopterin-dependent oxidoreductase	Yes
BR_RS10515	ABC transporter periplasmic substrate-binding protein UF1007	Yes
BR_RS11255	NirK:Putative multicopper oxidases	Yes
BR_RS11325	NosZ : Nitrous-oxide reductase	Yes
BR_RS11350	NosX protein/FAD:protein FMN transferase	Yes
BR_RS12255	ABC transporter periplasmic substrate-binding protein	Yes
BR_RS13015	Amidohydrolase family protein	Yes
BR_RS13330	CueO : Multicopper oxidase	Yes
BR_RS13635	FrpC	Yes
BR_RS13715	ABC transporter periplasmic substrate-binding protein	Yes
BR_RS13835	ABC transporter periplasmic substrate-binding protein	Yes
BR_RS14675	MsrP : TMAO/DMSO reductase	Yes

BR_RS15485	XylF - ABC transporter periplasmic substrate-binding protein	NT[3]
BR_RS15655	SsuA: ABC transporter substrate-binding protein	yes
BR_RS10115	ABC transporter periplasmic substrate binding protein	No
BR_RS11370	ABC transporter periplasmic Substrate binding protein	No
BR_RS14540	MetQ/NlpA family	No
BR_RS11250	Hypothetical protein	No
BR_RS11985	Bme12	No
BR_RS12600	Hypothetical protein	No
BR_RS02595	L,D-transpeptidase	No

[1] Full details of annotation, Tat dependent homologues in other bacteria, predicted signal peptidase cleavage sites and sequences cloned into pssAmiA-AmiAH are given in Supplemental Table S2

[2] Results of the amidase SDS resistance assay. See supplemental Table S2 and Figure S1 for full data sets

[3] Not tested

Figure legends

Fig 1 TatB alignment

Alignment of *Brucella* TatB proteins with CLUSTALΩ

Fig 2 TnSeq analysis of the *B. abortus tat* region.

The red line represents sliding R200 values across the genome, the thin gray line represents the mean R200 per chromosome. Data from Sternon et al

Fig 3. N-Phenyl maleimide and Bay 11-7082 are inhibit *Brucella* replication.

Trypticase soy broth (1 mL) containing the inhibitors at the indicated concentrations was inoculated with *B. suis*, *B. abortus* and *B. melitensis* at an OD600 of 0.05. After 24h (shaking) or 48h (static) culture, the OD600 were measured. Data is expressed as % of the OD600 of a culture with no inhibitor. Data represents the mean ± SD of three independent experiments. Statistical analysis was performed using a One-way ANOVA followed by the Sidak's test. Statistical differences are denoted with ∗∗∗∗ P ≤ 0.0001;*** = P ≤ 0.001; ** = P ≤ 0.01;* = P ≤ 0.03).

Fig 4. Analysis of predicted *Brucella* Tat signal sequences in the amidase reporter assay.

SDS viability assay of *E. coli* Δ*ssamiAC* mutant (MCDSSAC) strain carrying derivatives of pssAmiA-AmiAH where the AmiA signal sequence has been replaced by candidate *Brucella* Tat signal peptides identified *in silico*. Examples show positive negative (wrong cleavage site) and negative constructions. The full data set is shown in Fig S1.

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Fig S1. Analysis of all predicted *Brucella* Tat signal sequences in the amidase reporter assay.

SDS viability assay of *E. coli* Δ*ssamiAC* mutant (MCDSSAC) strain carrying derivatives of pssAmiA-AmiAH where the AmiA signal sequence has been replaced by candidate *Brucella* Tat signal peptides identified *in silico*.

```
B._ovis                     MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._vulpis                   ----------MFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
Brucella_sp._PacMan_Frog    ----------MFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._melitensis_bv2           MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._microti                  MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._neotomae                 MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._suis_bv5                 MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._abortus                  MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._melitensis_bv1           ----------MFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._suis_bv1                 MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._pinnipedialis            MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
B._ceti                     MCAQSARYQIMFDIAWSELLIIAIVMIVVVGPKDLPKMLRAFGKATARMRTTANEFRHQF
                                ************************************************

B._ovis                     NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._vulpis                   NEALKEAELEDVKTIIDEARSLDPRSRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
Brucella_sp._PacMan_Frog    NEALKEAELEDVKTIIDEARSLDPRSRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._melitensis_bv2           NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._microti                  NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._neotomae                 NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._suis_bv5                 NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._abortus                  NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._melitensis_bv1           NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._suis_bv1                 NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._pinnipedialis            NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
B._ceti                     NEALKEAELEDVKTIIDEARSLDPRTRLTQVFDPIRSAGEDLRSGLQSATSMSPVTENKV
                            *************************:**********************************

B._ovis                     GEVTTPVEPGGTPVP-----VPMITAPEEPAKPRKTSPRPAAKAGLKPTTTKTAKKNRSY
B._vulpis                   GEVTAPVEPDGTPVPA----------PEEPVKPRKTSPRPAAKAGPKPTTAKTAAKKTGA
Brucella_sp._PacMan_Frog    GEVTTPVEPDGTPVPA-----PTISAPEEPAKRRKTSPRPAAKAGPKPTTAKAAAKKTGA
B._melitensis_bv2           GEVTTPVEPGGTPV-----PVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._microti                  GEVTTPVGPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._neotomae                 GEVTTPVEPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._suis_bv5                 GEVTTPVEPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._abortus                  GEVTTPIEPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._melitensis_bv1           GEVTTPVEPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._suis_bv1                 GEVTTPVEPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._pinnipedialis            GEVTTPVEPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
B._ceti                     GEVTTPVEPGGTPVPAPPVPVPMITAPEEPAKPRKTSPRPAAKAGPKPTTTKT-AKKTGA
                            ****:*: *.****            ****.* ************ ****:*:  *: .

B._ovis                     PQ-ADGRQ--------DRNAG---
B._vulpis                   TPKPMADKTATPVKKTTKKTGTKA
Brucella_sp._PacMan_Frog    TPKPMADKTATPVKKTTKKTGTKA
B._melitensis_bv2           TPKPTADKTATPVKKTTKKTGTKA
B._microti                  TPKPTADKTATPVKKTTKKTGTKA
B._neotomae                 TSKPTADKTATPVKKTTKKTGTKA
B._suis_bv5                 TPKPTADKTATPVKKTTKKAGTKA
B._abortus                  TPKPTADKTATPVKKTTKKTGTKA
B._melitensis_bv1           TPKPTADKTATPVKKTTKKTGTKA
B._suis_bv1                 TPKPTADKTATPVKKTTKKTGTKA
B._pinnipedialis            TPKPTADKTATPVKKTTKKTGTKA
B._ceti                     TPKPTADKTATPVKKTTKKTGTKA
                                 . :         :::*
```

Fig 1 TatB alignment

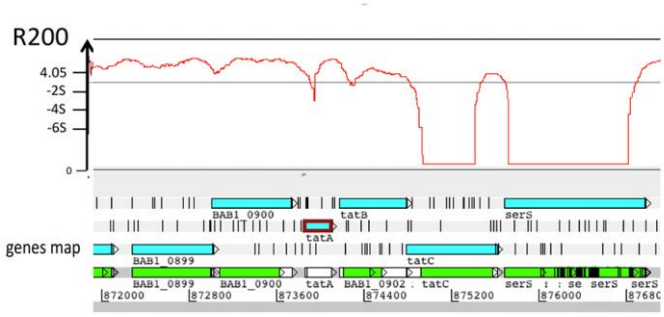

Fig 2 TnSeq analysis of the *B. abortus tat* region.

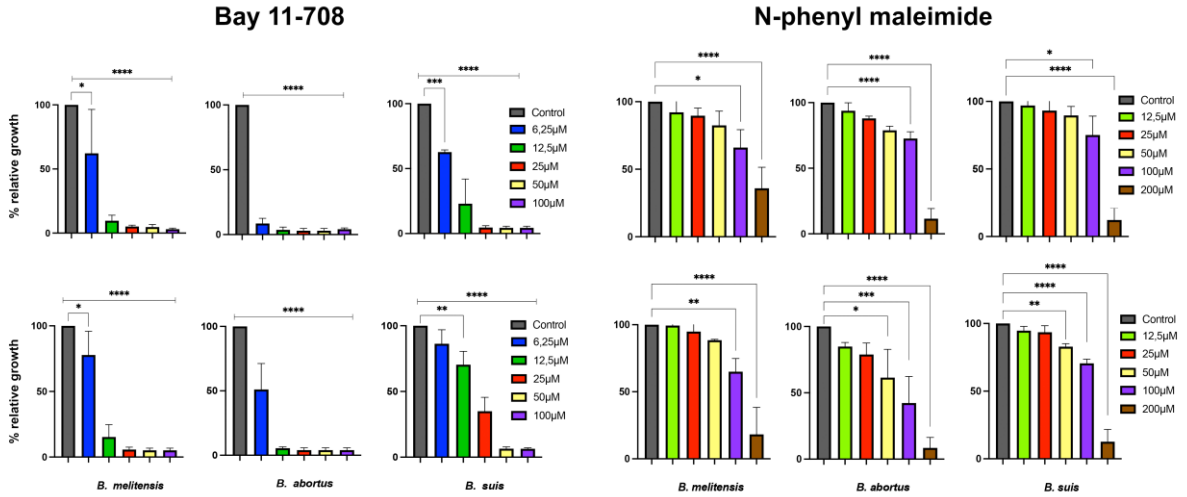

Fig 3. N-Phenyl maleimide and Bay 11-7082 are inhibit *Brucella* replication.

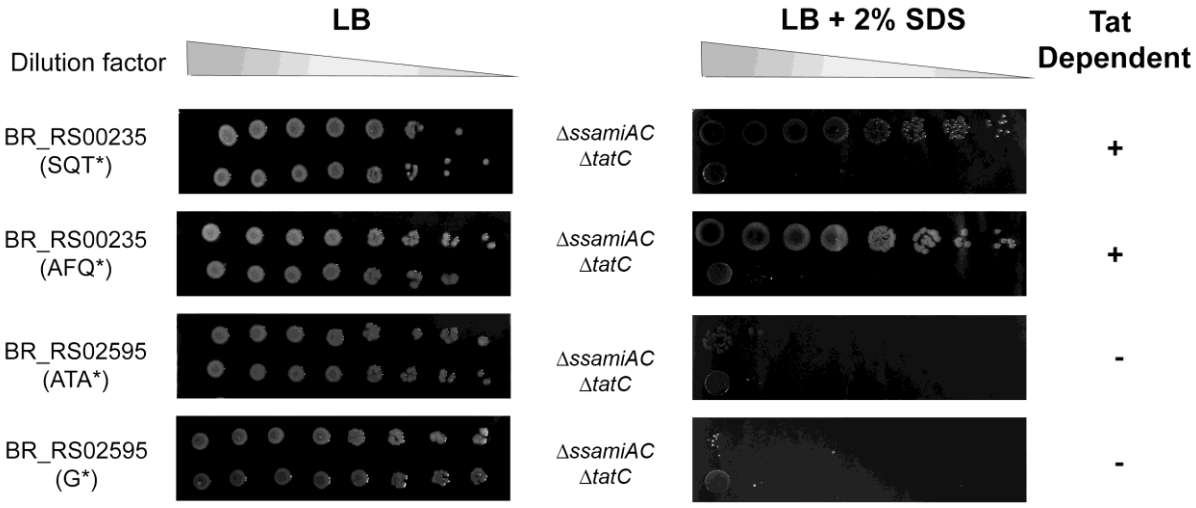

Fig 4. Analysis of predicted *Brucella* Tat signal sequences in the amidase reporter assay.