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Prokaryotic Utilization of the Twin-Arginine Translocation Pathway: a Genomic Survey

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The twin-arginine translocation (Tat) pathway, which has been identified in plant chloroplasts and prokaryotes, allows for the secretion of folded proteins. However, the extent to which this pathway is used among the prokaryotes is not known. By using a genomic approach, a comprehensive list of putative Tat substrates for 84 diverse prokaryotes was established. Strikingly, the results indicate that the Tat pathway is utilized to highly varying extents. Furthermore, while many prokaryotes use this pathway predominantly for the secretion of redox proteins, analyses of the predicted substrates suggest that certain bacteria and archaea secrete mainly nonredox proteins via the Tat pathway. While no correlation was observed between the number of Tat machinery components encoded by an organism and the number of predicted Tat substrates, it was noted that the composition of this machinery was specific to phylogenetic taxa.

Prokaryotes have a number of distinct pathways dedicated to the process of protein secretion. In general, these organisms translocate the majority of their secretory proteins in an unfolded conformation via the universally conserved and essential Sec pathway (15, 16, 20). Proteins secreted by this pathway are directed to the membrane-embedded proteinaceous Sec pore by an N-terminal signal peptide (9). While Sec signal peptides are similar structurally, they do not show sequence conservation (33). Once targeted to the membrane, Sec substrates can be translocated through the pore by the energetics of translation and/or ATP hydrolysis (reference 15 and references therein).

An alternate secretion mechanism, the twin-arginine translocation (Tat) pathway, was originally identified in chloroplasts and has recently been found in bacteria and archaea (24, 27, 32, 37). It is distinct from the Sec pathway in that (i) Tat substrates are secreted in a folded conformation (11, 22, 31), (ii) Tat signal peptides contain a highly conserved twin-arginine motif (3, 6, 18), (iii) the energy driving translocation is provided solely by the proton motive force (7, 24), and (iv) the Tat pathway is not a universally conserved secretion mechanism (36, 37).

Previous analyses of *Escherichia coli* Tat mutants and substrates suggested that the major role of this pathway in prokaryotes is to translocate redox proteins that integrate their cofactors in the cytoplasm and therefore possess some degree of tertiary structure prior to secretion (3, 22, 35). However, the recent identification of nonredox Tat substrates (such as virulence factors from *Pseudomonas aeruginosa*) indicates a broader role for the pathway than merely the secretion of redox proteins (19, 34). Furthermore, genomic data suggest that one group of organisms, the halophilic archaea, have routed nearly all of their secretome to the Tat pathway (23).

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While Tat components have been identified in many prokaryotes (36, 37), the extent to which this secretory pathway is utilized in bacteria and archaea is not well characterized. We have identified putative Tat substrates from 84 diverse prokaryotic proteomes available from the National Center for Biotechnology Information (ftp://ftp.ncbi.nih.gov/) using a modified version of the previously described program TATFIND version 1.1 (23). The results of these studies, as well as phylogenetic analyses of Tat components, allowed us to investigate correlations between the number of components of the Tat system and the number of putative Tat substrates.

TATFIND version 1.2. Previous genome-wide identification of Tat signal sequences by using merely the twin-arginine motif followed by multiple hydrophobic amino acids as search criteria has been shown to drastically overestimate the number of proteins secreted via the Tat pathway. Therefore, to identify putative Tat substrates in this study, we used the more stringent TATFIND program (18), which was designed by using mainly the sequences of putative haloarchaeal Tat signal peptides. It defines a Tat substrate as any protein that meets two criteria: (i) the presence of an $(X^{-1})R^{0}R^{+1}(X^{+2})(X^{+3})(X^{+4})$ motif within the first 35 amino acids of the protein, where each position X represents a defined set of permitted residues, and (ii) the presence of an uncharged stretch of at least 13 amino acids downstream of the $R^{0}R^{+1}$ (23). The recent identification of novel Tat substrates in *P. aeruginosa* (19) has led us to extend the rules of TATFIND to allow a methionine at position X^{-1} and a glutamine at position X^{+4}, thus creating the program used in this study, TATFIND version 1.2. Mutational analyses of certain Tat signal sequences suggest that in specific instances the substitution of lysine, asparagine, and glutamine for one of the two conserved arginines does not prevent Tat-dependent export (5, 8, 13, 30). However, only two naturally occurring Tat substrates are known to deviate from the conserved RR motif in their signal sequence (10, 12). Therefore, modifications of the program allowing for a variable RR motif due to these recent reports

TABLE 1. Predicted Tat substrate and component numbers in a diverse group of prokaryotes

Organism	Domain	Phylum	Open reading frames	No. of TATFIND 1.2 positives	Tat component(s)[a]		
					A/E	B	C
Streptomyces coelicolor A3(2)	*Bacteria*	*Actinobacteria*	7899	145	1	—	1
Mesorhizobium loti	*Bacteria*	*Proteobacteria* (alpha)	7279	95	1	—	1
Sinorhizobium meliloti	*Bacteria*	*Proteobacteria* (alpha)	6206	94	1	1	1
Caulobacter crescentus	*Bacteria*	*Proteobacteria* (alpha)	3737	88	1	1	1
Ralstonia solanacearum	*Bacteria*	*Proteobacteria* (beta)	5116	71	1	1	1
Halobacterium sp. strain NRC-1	*Archaea*	*Euryarchaeota*	2446	68	1	—	2
Pseudomonas aeruginosa	*Bacteria*	*Proteobacteria* (gamma)	5567	57	1	1	1
Xanthomonas campestris 3391	*Bacteria*	*Proteobacteria* (gamma)	4181	55	1	1	1
Agrobacterium tumefaciens	*Bacteria*	*Proteobacteria* (alpha)	5299	51	1	1	1
Xanthomonas axonopodis 306	*Bacteria*	*Proteobacteria* (gamma)	4312	50	1	1	1
Escherichia coli K-12	*Bacteria*	*Proteobacteria* (gamma)	4279	34	2	1	1
Escherichia coli O157:H7 EDL933	*Bacteria*	*Proteobacteria* (gamma)	5335	33	2	1	1
Salmonella enterica serovar Typhimurium LT2	*Bacteria*	*Proteobacteria* (gamma)	4559	33	2	1	1
Escherichia coli O157:H7	*Bacteria*	*Proteobacteria* (gamma)	5361	32	2	1	1
Mycobacterium tuberculosis H37Rv	*Bacteria*	*Actinobacteria*	3927	31	1	—	1
Nostoc sp. strain PCC 7120	*Bacteria*	*Cyanobacteria*	6129	31	2	—	1
Mycobacterium tuberculosis CDC1551	*Bacteria*	*Actinobacteria*	4187	29	1	—	1
Salmonella enterica serovar Typhi	*Bacteria*	*Proteobacteria* (gamma)	4768	28	2	1	1
Deinococcus radiodurans	*Bacteria*	*Deinococcus-Thermus*	2997	22	2	—	1
Synechocystis sp. strain PCC 6803	*Bacteria*	*Cyanobacteria*	3167	21	2	—	1
Yersinia pestis	*Bacteria*	*Proteobacteria* (gamma)	4083	19	2	1	1
Brucella melitensis	*Bacteria*	*Proteobacteria* (alpha)	3199	19	1	—	1
Xylella fastidiosa 9a5c	*Bacteria*	*Proteobacteria* (gamma)	2768	17	1	1	1
Aquilex aeolicus	*Bacteria*	*Aquificae*	1529	15	2	—	1
Corynebacterium glutamicum	*Bacteria*	*Actinobacteria*	3041	15	2	—	1
Pyrobaculum aerophilum	*Archaea*	*Euryarchaeota*	2605	14	1	—	1
Neisseria meningitidis Z2491	*Bacteria*	*Proteobacteria* (beta)	2065	12	1	1	1
Pasteurella multocida	*Bacteria*	*Proteobacteria* (gamma)	2015	12	1	1	1
Campylobacter jejuni	*Bacteria*	*Proteobacteria* (epsilon)	1654	11	1	1	1
Neisseria meningitidis MC58	*Bacteria*	*Proteobacteria* (beta)	2079	11	1	1	1
Haemophilus influenzae	*Bacteria*	*Proteobacteria* (gamma)	1714	9	1	1	1
Archaeoglobus fulgidus	*Archaea*	*Euryarchaeota*	2420	9	2	—	2
Mycobacterium leprae	*Bacteria*	*Actinobacteria*	2720	9	1	—	1
Vibrio cholerae	*Bacteria*	*Proteobacteria* (gamma)	3835	7	2	1	1
Bacillus subtilis	*Bacteria*	*Firmicutes*	4112	7	3	—	2
Aeropyrum pernix	*Archaea*	*Crenarchaeota*	1840	7	2	—	1
Methanosarcina mazei Goe1	*Archaea*	*Euryarchaeota*	3371	6	2	—	2
Treponema pallidum	*Bacteria*	*Spirochaetes*	1036	6	—	—	—

Continued on following page

TABLE 1—*Continued*

Organism	Domain	Phylum	Open reading frames	No. of TATFIND 1.2 positives	Tat component(s)[a]		
					A/E	B	C
Bacillus halodurans	*Bacteria*	*Firmicutes*	4066	5	2	—	2
Methanosarcina acetivorans strain C2A	*Archaea*	*Euryarchaeota*	4540	5	2	—	2
Sulfolobus solfataricus	*Archaea*	*Crenarchaeota*	2977	5	3	—	2
Chlorobium tepidum TLS	*Bacteria*	*Chlorobi*	2252	5	2	—	1
Pyrococcus horikoshii	*Archaea*	*Euryarchaeota*	1801	5	—	—	—
Sulfolobus tokodaii	*Archaea*	*Crenarchaeota*	2826	4	2	—	1
Helicobacter pylori 26695	*Bacteria*	*Proteobacteria* (epsilon)	1576	3	1	1	1
Helicobacter pylori J99	*Bacteria*	*Proteobacteria* (epsilon)	1491	3	1	1	1
Clostridium perfringens	*Bacteria*	*Firmicutes*	2723	3	—	—	—
Pyrococcus furiosus DSM3638	*Archaea*	*Euryarchaeota*	2065	3	—	—	—
Thermotoga maritima	*Bacteria*	*Thermotogae*	1858	3	—	—	—
Listeria innocua	*Bacteria*	*Firmicutes*	3043	2	1	—	1
Staphylococcus aureus Mu50	*Bacteria*	*Firmicutes*	2714	2	1	—	1
Staphylococcus aureus MW2	*Bacteria*	*Firmicutes*	2632	2	1	—	1
Staphylococcus aureus N315	*Bacteria*	*Firmicutes*	2625	2	1	—	1
Thermoplasma acidophilum	*Archaea*	*Euryarchaeota*	1482	2	1	—	1
Thermoplasma volcanium	*Archaea*	*Euryarchaeota*	1500	2	1	—	1
Chlamydia trachomatis	*Bacteria*	*Chlamydiae*	895	2	—	—	—
Chlamydophila pneumoniae AR39	*Bacteria*	*Chlamydiae*	1112	2	—	—	—
Chlamydophila pneumoniae CWL029	*Bacteria*	*Chlamydiae*	1054	2	—	—	—
Pyrococcus abyssi	*Archaea*	*Euryarchaeota*	1769	2	—	—	—
Listeria monocytogenes EGD-e	*Bacteria*	*Firmicutes*	2846	1	1	—	1
Ricketisia conorii	*Bacteria*	*Proteobacteria* (alpha)	1374	1	1	—	1
Ricketisia prowazekii	*Bacteria*	*Proteobacteria* (alpha)	835	1	1	—	1
Chlamydia muridarum	*Bacteria*	*Chlamydiae*	909	1	—	—	—
Chlamydophila pneumoniae J138	*Bacteria*	*Chlamydiae*	1069	1	—	—	—
Clostridium acetobutylicum	*Bacteria*	*Firmicutes*	3848	1	—	—	—
Lactococcus lactis subsp. *lactis*	*Bacteria*	*Firmicutes*	2267	1	—	—	—
Methanothermobacter thermautotrophicus	*Archaea*	*Euryarchaeota*	1873	1	—	—	—
Streptococcus pneumoniae R6	*Bacteria*	*Firmicutes*	2043	1	—	—	—
Streptococcus pyogenes	*Bacteria*	*Firmicutes*	1697	1	—	—	—
Streptococcus pyogenes MGAS8232	*Bacteria*	*Firmicutes*	1845	1	—	—	—
Thermoanaerobacter tengcongensis	*Bacteria*	*Firmicutes*	2588	1	—	—	—
Buchnera aphidicola	*Bacteria*	*Proteobacteria* (gamma)	545	—	—	—	—
Buchnera sp. strain APS	*Bacteria*	*Proteobacteria* (gamma)	564	—	—	—	—
Fusobacterium nucleatum 25586	*Bacteria*	*Fusobacteria*	2067	—	—	—	—
Methanococcus jannaschii	*Archaea*	*Euryarchaeota*	1729	—	—	—	—
Methanopyrus kandleri AV19	*Archaea*	*Euryarchaeota*	1687	—	1	—	—
Mycoplasma genitalium	*Bacteria*	*Firmicutes*	484	—	—	—	—
Mycoplasma pneumoniae	*Bacteria*	*Firmicutes*	689	—	—	—	—
Mycoplasma pulmonis	*Bacteria*	*Firmicutes*	782	—	—	—	—
Streptococcus pneumoniae TIGR4	*Bacteria*	*Firmicutes*	2094	—	—	—	—
Ureaplasma urealyticum	*Bacteria*	*Firmicutes*	614	—	—	—	—
Borrelia burgdorferi	*Bacteria*	*Spirochaetes*	1638	—	—	—	—

[a] —, no homologs identified.

were not included, as these are likely to be exceptions and would lead to strong overprediction.

Tat substrates. TATFIND 1.2 predicted that the Tat pathway is utilized to various extents in the 84 different organisms analyzed, based on the number and identity of their putative Tat substrates (Table 1 and www.sas.upenn.edu/~pohlschr/tatprok.html). TATFIND 1.2 identified all previously confirmed Tat substrates containing twin-arginine motifs in *E. coli* and

other prokaryotes (14, 19, 21, 29, 34) and predicted few or no substrates of this pathway in organisms which lack all Tat component homologs or contained only a TatA homolog (e.g., *Chlamydia trachomatis* and *Methanopyrus kandleri* AV19, respectively) (Table 1). Strikingly, in the analyses of the 29 prokaryotic genomes with zero or one identified Tat component(s), we predicted a total of only 37 false positives (including cytoplasmic, membrane, and secreted proteins), of which only 4 were putative secreted Sec substrates. This result strongly suggests that our program is highly efficient in distinguishing Tat signal peptides from Sec signal peptides.

Surprisingly, some organisms (e.g., *Rickettsia prowazekii* and *Staphylococcus aureus*) seem to have maintained the Tat pathway for the secretion of a small number of proteins, as TATFIND 1.2 identified only one and two Tat substrates, respectively, for these bacteria (Table 1). Moreover, it was intriguing that other bacteria and archaea appear to make extensive use of this pathway, which originally was thought to be required for the translocation of only a minor subset of secreted proteins (Table 1). For example, 88, 94, and 145 putative Tat substrates were identified in *Caulobacter crescentus*, *Sinorhizobium meliloti*, and *Streptomyces coelicolor*, respectively (Table 1). While analyses of putative Sec substrates suggest that the haloarchaea remain the only organisms that use this pathway for the translocation of the majority of their secreted proteins, our results predict that some prokaryotes use the Tat pathway for the secretion of as many as 20% of their extracytoplasmic proteins (see below and reference 2).

The diverse utilization of the Tat pathway observed in the 84 prokaryotes was also observed within phylogenetically related groups. For example, we found a range of 9 (*Mycobacterium leprae*) to 145 (*S. coelicolor*) putative Tat substrates in the bacterial phylum *Actinobacteria* (Table 1). Similarly, we observed that the number of predicted Tat substrates varies widely within the phyla *Proteobacteria*, *Cyanobacteria*, *Euryarchaeota*, and *Crenarchaeota* (Table 1). Hence, our results indicate that the degree to which the Tat pathway is used is quite variable, even among related organisms.

To further characterize the utilization of the Tat pathway by the prokaryotes examined in this study, we identified the function and localization of TATFIND 1.2-positive proteins of four organisms by their annotation (or that of their homolog) in the SWISS-PROT database. Since the Tat pathway has been shown to play a role in the transport of only secreted proteins, we excluded known and putative multispanning membrane proteins (predicted by TMHMM [28]) and cytoplasmic proteins from further analyses. Proteins predicted to be secreted in *Pyrobaculum aerophilum* were exclusively redox proteins, while the majority of putative Tat substrates of the gram-positive *Bacillus subtilis*, the gram-negative *C. crescentus*, and the archaeon *Halobacterium* sp. strain NRC-1 were nonredox proteins (Table 2). A relatively large number of the TATFIND 1.2-positive nonredox proteins found in *C. crescentus* and *Halobacterium* sp. strain NRC-1 were identified as substrate-binding proteins, a phenomenon also predicted for the plant pathogen *Agrobacterium tumefaciens* (Christie, personal communication). Thus, while some organisms seem to preferentially use this pathway for the secretion of redox proteins (as does *E. coli*, for example; see Table 2), our results suggest that in certain organisms the Tat pathway is responsible for the secretion of a wider variety of substrates.

TABLE 2. Classification of TATFIND 1.2 positives[a]

Organism	No. of secreted proteins		No. of cytoplasmic/integral membrane proteins	No. of proteins with no annotated function or localization
	Redox	Nonredox		
B. subtilis	1	2	0	4
P. aerophilum	7	0	0	7
E. coli K-12	12	5	6	11
Halobacterium sp. strain NRC-1	5	17	1	45
C. crescentus	6	28	5	49

[a] For comparative purposes, only proteins whose function and putative localization could be determined (see text) were classified as redox, nonredox, and cytoplasmic/integral membrane proteins.

Certain protein homologs were identified as Tat substrates in many prokaryotes. Analyses of some redox proteins, such as the dimethyl sulfoxide reductase chain A (DmsA) that is found in many of the 84 organisms analyzed, revealed that the majority of the DmsA homologs in these prokaryotes contained Tat signal peptides (see www.sas.upenn.edu/~pohlschr/tatprok.html). Surprisingly, we also noted that virtually all homologs of certain nonredox proteins, such as alkaline phosphatase D and phospholipase C, had typical Tat signal sequences (Fig. 1). This suggests the existence of an unknown selective pressure favoring the conserved targeting of these and other nonredox proteins to the Tat pathway.

An exception to the observed Tat motif conservation among phospholipase C homologs was found in *Xanthomonas axonopodis*, as one of its two phospholipase C homologs contained an aspartic acid residue at position X^{+2} (a variation not accepted by the rules of TATFIND 1.2). While this phospholipase C homolog in fact may not be a Tat substrate, this single deviation may reflect the existence of certain organism-specific Tat substrate characteristics. Possible organism specificity is also indicated by the 54 additional putative Tat substrates identified in *S. coelicolor* when alanine is allowed at position X^{+4}, as suggested by Ochsner et al. (19); alanine at this position, however, does not markedly affect predictions for virtually all other organisms (data not shown). Further in vivo and in silico analyses of Tat substrates may lead to the development of different versions of TATFIND tailored to phylogenetically related groups of organisms (similar to the separate analyses performed by SignalP to identify Sec signal sequences [17]).

Tat components in sequenced prokaryotes. At least one copy of a TatA homolog and one copy of a TatC homolog are required for a functional Tat pathway (4, 25, 37). In certain prokaryotes (such as *E. coli*), an additional protein, TatB, is necessary for Tat-dependent secretion (26). Expanding on previous analyses (37), we searched for the presence of these components in all organisms analyzed using PSI-BLAST and its iterations (Table 1) (1). Detailed phylogenetic analyses revealed that all bacteria in the obligate intracellular pathogen phylum *Chlamydiae* completely lacked Tat component homologs. Conversely, the presence of the Tat machinery was conserved in all *Crenarchaeota*, *Actinobacteria*, *Cyanobacteria*, and *Proteobacteria* (with the exception of the obligate symbi-

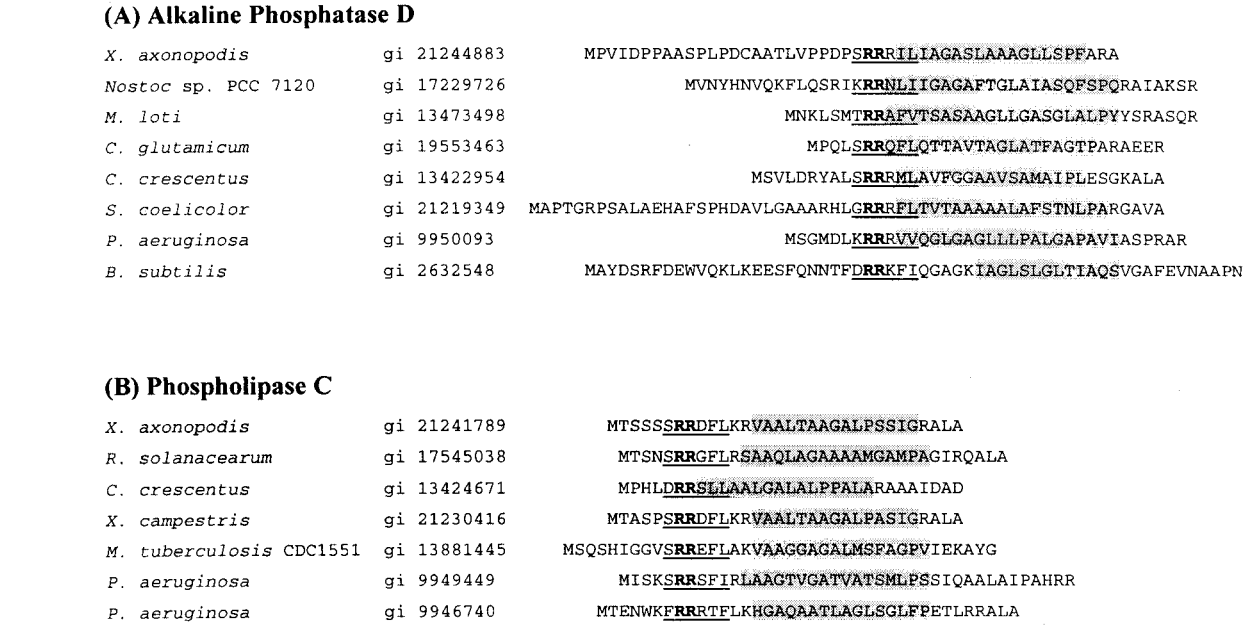

FIG. 1. Signal sequences of alkaline phosphatase D (A) and phospholipase C homologs (B) contain the conserved twin-arginine motif (underlined) and a downstream uncharged stretch (highlighted).

otic genus *Buchnera*) that we examined, even though the extent to which this machinery was utilized varied dramatically within each phylum (Table 1). Interestingly, homologs of TatB were found exclusively in the proteobacteria and were missing from only a few organisms in the α subdivision (Table 1).

In the archaeal phylum *Euryarchaeota* and the bacterial phylum *Firmicutes*, approximately half of the organisms analyzed had homologs of the Tat machinery components. Curiously, a number of organisms from these phyla, unlike the *Proteobacteria* or *Actinobacteria*, were found to contain multiple copies of TatC homologs (Table 1) (37). It is intriguing that the majority of the *Firmicutes* and *Euryarchaeota* organisms were predicted to have a relatively small number of putative Tat substrates, which suggests that in prokaryotes, there is no direct correlation between the number of Tat substrates and TatC homologs. Furthermore, additional examination of the 84 proteomes demonstrated that the number of TatA homologs or the presence of a TatB homolog in any of these organisms is also unrelated to the number of Tat substrates. This notion is underscored by the observation that *B. subtilis* contains three TatA and two TatC homologs, yet has only seven putative Tat substrates (Table 1). As suggested for *B. subtilis*, multiple copies of the Tat translocon components may be present in order to form separate translocons, each responsible for the secretion of a subset of distinct Tat substrates (14).

Concluding remarks. The development of a program that identifies proteins with typical Tat signal sequences provides intriguing insight into the extent to which prokaryotes use this pathway. Furthermore, our results raise a number of questions concerning the mechanism of the poorly understood Tat pathway. While our analyses suggest that this pathway is used to widely varying extents and for diverse substrates, we do not yet understand the selective pressure that favors the use of the Tat pathway. We did not observe a correlation between the number of Tat component homologs and substrates encoded by the 84 organisms, leaving the question as to why some organisms encode several copies of TatA and TatC homologs unanswered. It was, however, intriguing that organisms with only one copy of these homologs possess a variety of predicted substrates, which suggests that similar to the Sec system, the Tat pathway is a general secretory pathway.

The identification of organisms that use this pathway extensively may offer new opportunities for the production of heterologous secretory proteins. Furthermore, the absence of Tat translocon components (TatA/B and TatC) in mammals, along with the observation that the elimination of a functional TatC attenuates virulence of *P. aeruginosa*, suggests that the Tat translocon may represent a novel antibiotic target (19, 34). Our results strongly suggest that it is critical to study this process in a diverse set of microorganisms in order to reveal the importance of this pathway in bacteria and archaea.

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