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Protein secretion systems of *Pseudomonas aeruginosa* and *P. fluorescens*

Qinhong Ma[a], Yufeng Zhai[a,1], Jane C. Schneider[b], Tom M. Ramseier[b], Milton H. Saier Jr.[a,*]

[a] *Division of Biological Sciences, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA*
[b] *The Dow Chemical Company, 5501 Oberlin Drive, San Diego, CA 92121, USA*

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Abstract

Gram-negative bacteria have evolved numerous systems for the export of proteins across their dual-membrane envelopes. Three of these systems (types I, III and IV) secrete proteins across both membranes in a single energy-coupled step. Four systems (Sec, Tat, MscL and Holins) secrete only across the inner membrane, and four systems [the main terminal branch (MTB), fimbrial usher porin (FUP), autotransporter (AT) and two-partner secretion families (TPS)] secrete only across the outer membrane. We have examined the genome sequences of *Pseudomonas aeruginosa* PAO1 and *Pseudomonas fluorescens* Pf0-1 for these systems. All systems except type IV were found in *P. aeruginosa*, and all except types III and IV were found in *P. fluorescens*. The numbers of each such system were variable depending on the system and species examined. Biochemical and physiological functions were assigned to these systems when possible, and the structural constituents were analyzed. Available information regarding the mechanisms of transport and energy coupling as well as physiological functions is summarized. This report serves to identify and characterize protein secretion systems in two divergent pseudomonads, one an opportunistic human pathogen, the other a plant symbiont.

Keywords: Protein; Secretion; Membrane insertion; Gram-negative bacteria; *Pseudomonas*

1. Introduction

The dual-membrane envelopes of Gram-negative bacteria provide two barriers of unlike nature that pose formidable problems concerning the transport of molecules into and out of these diverse organisms. While nutrients and essential cofactors must be actively transported into the cells, end products of metabolism, toxic substances and secreted macromolecules must be actively extruded. Specific transport systems have evolved to achieve these goals. The diversity of such systems currently recognized in Gram-negative bacteria far exceeds that recognized in Gram-positive bacteria, archaea or eukarya [1].

Protein secretion proves to be illustrative of this fact. Gram-positive bacteria, eukaryotes and archaea exhibit just three known types of functionally characterized protein secretory systems for transport across the cytoplasmic/endoplasmic reticular membranes: first, the so-called general secretory pathway (Sec; GSP) [2–4]; second, the cytochrome oxidase biogenesis (Oxa1/YidC) pathway [5–7]; and third, the twin arginine targeting/translocation (Tat) pathway [8,9]. However, in addition to these systems, Gram-negative bacteria have multiple systems for transport across their outer, lipopolysaccharide-containing membranes (Table 1) [10–13]. Three types of systems are capable of transporting proteins across both membranes of the Gram-negative bacterial cell envelope in a single energy-coupled step. These systems are the ABC (type I), the flagellar/pathogenesis (type III) and the conjugation/virulence (type IV) systems, although several recent publications suggest that type IV systems may catalyze export by a two-step process [14–16]. While ABC peptide exporters are found in Gram-negative and Gram-positive bacteria as well as eukaryotes, ABC protein exporters and types III and IV systems are found only in Gram-negative bacteria [13].

For export across the outer membrane, types I, III and IV secretory pathways use oligomeric outer membrane proteins (OMPs) [17–20]. Four additional protein secreting OMP families are currently recognized in Gram-negative bacteria.

* Corresponding author. Tel.: +1-858-534-4084; fax: 1-858-534-7108.
E-mail address: msaier@ucsd.edu (M.H. Saier).
[1] Present address: IDEC Pharmaceuticals Corporation, 3010 Science Park Road, San Diego, CA 92121, USA.

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Table 1
Comparison of numbers of the protein secretion systems in *P. aeruginosa* PAO1 and *P. fluorescens* Pf0–1[a]

Type of system	TC no.	Membranes transversed		Organisms found				No. of systems	
		IM	OM	G− Bac	G+ Bac	Arch	Euk	Pae	Pfl
Sec secretory pathway	3.A.5	+	−	+	+	+	+	1	1
Twin arginine targeting (Tat) pathway	2.A.64	+	−	+	+	+	+	1	1
Holins	1.E	+	−	+	+	+	−	2	2
Large conductance mechanosensitive ion channel (MscL)	1.A.22	+	−	+	+	+	+	1	1
ATP-binding cassette (ABC) secretory pathway (type I)	3.A.1	+	+	+	+[b]	−	+[b]	4	3
Flagellar/pathogenesis-related secretory pathway (type III)	3.A.6	+	+	+	−	−	−	2	1
Conjugal/virulence-related secretory pathway (type IV)	3.A.7	+	+	+	−	−	−	0	0
MTB secretory pathway (type II)	3.A.14	−	+	+	+[c]	−	−	4	3
Outer membrane FUP	1.B.11	−	+	+	+[c]	−	−	4	2
AT	1.B.12	−	+	+	−	−	−	3	9
TPS system	1.B.20	−	+	+	+[c]	−	+	5	3

[a] Abbreviations: IM, inner membrane; OM, outer membrane; G− Bac, Gram-negative bacteria; G+ Bac, Gram-positive bacteria; Arch, Archaea; Euk, eukaryotes; Pae, *P. aeruginosa*; Pfl, *P. fluorescens*.
[b] ABC-type transporters for peptides and small proteins have been identified in Gram-positive bacteria and eukaryotes, but ABC transporters for large proteins have been identified only in Gram-negative bacteria.
[c] MTB, FUP and TPS are found in only one Gram-positive bacterium, *Deinococcus radiodurans*, which has a dual-membrane envelope [13].

These are the fimbrial usher protein (FUP) family [21–23], the multicomponent main terminal branch (MTB) family, the autotransporter (AT) family [24,25] and the two-partner secretion (TPS) family [26]. Previously, we have examined these outer membrane secretory systems from phylogenetic standpoints [13]. Of these systems, only TPS systems are found outside of Gram-negative bacteria (Table 1).

Many Gram-negative pathogens export virulence factors such as pili, degradative enzymes, adhesins and toxins using a variety of secretory mechanisms [13,27]. However, not all of the secretory pathways are present in a single organism. The human opportunistic pathogen, *Pseudomonas aeruginosa* contains one of the largest genomes among all the bacterial genomes that have been sequenced to date. This bacterium adapts to diverse ecological environments, ranging from water and soil to human bodies, and its large genome size probably reflects its ubiquitous lifestyle. We expect that *P. aeruginosa* possesses more complicated metabolic and protein sorting pathways than Gram-negative bacteria with smaller genome sizes. The availability of the complete genome of *P. aeruginosa* PAO1 [28] allowed us to identify the protein secretory systems and provide insight into the lifestyle and pathogenic capabilities of this organism.

In recent years, several other *Pseudomonas* species have been extensively studied for purposes of (1) secreting enzymes for industrial applications [29], (2) producing novel antibiotics [30,31] and (3) understanding biofilm formation [32,33]. One of these species, the saprophytic plant symbiont, *Pseudomonas fluorescens*, has received considerable attention. Ongoing genome sequencing projects conducted by several institutions have targeted three *P. fluorescens* strains, Pf0-1 [Department of Energy (DOE)–Joint Genome Institute (JGI); http://www.jgi.doe.gov/JGI_microbial/html/pseudomonas/pseudo_homepage.html], SBW25 [The Sanger Center; http://www.sanger.ac.uk/Projects/P_fluorescens/] and Pf-5 [The Institute for Genomic Research (TIGR); http://www.tigr.org/tdb/mdb/mdbinprogress.html]. In this review, we conduct comprehensive analyses of the genomes of two pseudomonads, *P. aeruginosa* PAO1 with a fully sequenced genome [28] and *P. fluorescens* Pf0-1, with a nearly completed genome sequence. We identify all protein constituents of recognized protein secretion systems, analyze them for structural and sequence characteristics, and summarize functional data when available. Comparison of the protein secretory systems found in these two related bacteria, both with unusually large genomes, provides insight into their lifestyles.

2. Computer methods

The complete protein sequence database of *P. aeruginosa* PAO1 was downloaded from http://www.pseudomonas.com (The *Pseudomonas* Genome Project [28]), and the unfinished protein and DNA sequence databases of *P. fluorescens* Pf0-1 (seven-fold coverage, corresponding to an estimated 90% of the genome) were downloaded from http://www.jgi.doe.gov/JGI_microbial/html/pseudomonas/pseudo_homepage.html. One hundred and seventy-two protein sequences of the known protein exporters collected in the Transporter Classification Database (TC-DB, http://www.biology.ucsd.edu/~msaier/transport/) were used as

queries against each *Pseudomonas* protein database using the gapped BLAST program (default parameters: *E* value, <0.01; gap opening, 11; gap extension, 1 [34]). For the ABC transporters involved in protein secretion, the transmembrane (TM) domains but not the ATP-binding domains were used as query sequences to reduce the number of false-positives. For query sequences that yielded no hits, a tBLASTn search was performed to identify putative open reading frames (ORFs) that were not annotated. A total of 92 and 84 proteins in *P. aeruginosa* and *P. fluorescens*, respectively, were found to share statistically significant sequence similarity with the known protein exporters. It should be noted that the absence of a homologue in *P. fluorescens* does not imply that such a protein is absent.

The resultant protein sequences were subsequently searched against the SWISS-PROT database (released on February 26, 2002 and containing 667,446 sequences) for homologues with known biological function using the batch BLAST program in the GCG Wisconsin Package (Version 10, Genetics Computer Group, Madison, WI). Proteins similar to those that are not involved in protein secretion were eliminated. The remaining protein sequences were further analyzed for characteristics of the families to which they belong. First, they were searched against the Pfam domain database (http://www.sanger.ac.uk/Software/Pfam/ [35]) for the presence of recognized domains with conserved motifs. Second, their membrane topologies were determined with the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/ [36]). Finally, we used SignalP 2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/ [37,38]) to determine if the proteins contain putative signal peptides. Multiple sequence alignments were generated using the Clustal X program [39], and the signature sequences of each family were inspected. In addition, the probable secondary structures of the putative AT and TPS systems were predicted with the PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred/ [40]). The proteins belonging to these families tend to share low degrees of sequence similarity but have C-terminal amphipathic β-strands that might form β-barrels in the outer membrane [13]. Amphipathicity of the putative β-strands was plotted with the WHAT program using an angle of 180° as is appropriate for a β-strand (http://saier-144-37.ucsd.edu/biotools/ [41,42]). Finally, we inspected genes that cluster with recognized transporter genes in order to identify the auxiliary proteins for the type I, type II (MTB), type III and FUP secretory pathways as well as the putative substrates for the type I, FUP and TPS pathways.

3. Types of protein secretion systems found in the two *Pseudomonas* species

Table 1 summarizes the protein secretion systems found in the two pseudomonads examined. It also indicates the membranes across which the system secretes proteins, and presents the numbers of systems found in each of the two species. For additional tables and information, please check our web site: http://saier-144-37.ucsd.edu/qma.

The two ubiquitous systems for export across the cytoplasmic membrane are the Sec and Tat systems. While Sec is found in every living organism examined to date [4], Tat is represented in some but not all species within each of the three domains of life [9]. Each of the pseudomonads examined has just one of each such system. They also encode two recognized holins for autolysin secretion [43] and one large conductance mechanosensitive channel protein, MscL, capable of releasing small proteins such as thioredoxin in response to changes in osmotic pressure [44]. While MscL homologues are found in all three domains of life [45,46], holins have been found only in bacteria and archaea [47].

The two pseudomonads examined have three or four ABC-type protein secretory systems that transport proteins across both membranes, but neither has a type IV system. Only *P. aeruginosa* has a type III pathogenicity-related system, but both *Pseudomonas* species have the flagellar secretion system. All four types of outer membrane export systems, MTB, FUP, AT and TPS, were found in multiple copies in the two pseudomonads examined, from two to nine copies each (Table 2). The numbers found for *P. aeruginosa* (Pae) PAO1 were never the same as those found in *P. fluorescens* (Pfl) Pf0-1.

4. The Sec system

The general secretory pathway (Sec) for export of proteins across the cytoplasmic membrane is the dominant mechanism for protein secretion in all living organisms (see Refs. [3,48] for recent reviews). In bacteria, the system includes several ubiquitous proteins (SecY, SecE, SecG, YidC, FtsY, Ffh) and several additional proteins found only in select organisms (SecA, SecB, SecD, SecF, YajC [4]). SecB is an ATP-independent chaperone protein that presents the unfolded preprotein to SecA. SecA is an ATPase that drives export of the unfolded substrate polypeptide chain through the SecYEG channel. The SecDF/YajC complex is an auxiliary system that binds YidC and facilitates protein export but is lacking in many organisms [49]. FtsY, Ffh and YidC function together with the SecYEG complex to promote insertion of signal-peptide-dependent proteins into the membrane [6,50,51]. YidC is also involved in membrane insertion of several Sec-independent membrane proteins [52,53]. All of these proteins were found in single copy in both pseudomonads examined (Table 2). Finally, signal peptidases, SPases I and II, specific for secreted soluble proteins and lipoproteins, respectively, have been identified in both organisms. However, an additional homologue of SPase I (PA1303) is present in Pae but not in Pfl. This is the only major difference we noted for the Sec systems in these

Table 2
Protein constituents of protein secretory pathways in *P. aeruginosa* PAO1[a,b]

System	Prototype	I	II	III	IV	V	VI	VII	Length (aa)	Location
(a) Sec secretory pathway										
Chaperone		SecB							163	cytoplasm
ATPase		SecA							916	cytoplasm
TM complex		SecY							442	IM
		SecE							122	IM
		SecG							129	IM
Auxiliary proteins		SecD							620	IM
		SecF							306	IM
		YajC							112	IM
Membrane insertion		FtsY							455	cytoplasm
proteins		Ffh							457	cytoplasm
		YidC							578	IM
SPase I		LepB	PA1303*						179–284	IM
SPase II		LspA							169	IM
(b) Twin arginine targeting (Tat) secretory pathway										
		TatA							83	IM
		TatB							142	IM
		TatC							268	IM
(c) Holins										
Holin		LrgA	Hol						109–117	IM
Putative substrate		LrgB	Lys						209–228	secreted
(d) Large conductance mechanosensitive ion channel (MscL)										
Channel		MscL							137	IM
(e) Type I (ABC) secretory pathway										
ABC transporter		AprD	HasD*	CvaB*	PA1876**				593–723	IM
MFP		AprE	HasE*	CvaA*	PA1877**				395–443	IM/OM
OMF		AprF	HasF*	OpmK*	PA1875**	OpmH/TolC			425–482	OM
Putative substrate		AprA	HasA*	not found	not found				205–479	extracellular
		AprX*							414	extracellular
(f) Type III (Fla/Path) secretory pathway										
ATPase	(YscN)	PscN*	FliI						440/451	cytoplasm
Cytoplasmic proteins	(YscL)	PscL*	FliH						231/268	cytoplasm
	(YscQ)	PscQ*	FliN						309/157	cytoplasm?
	(YscK)	PscK*	not applicable						206	cytoplasm?
IM components	(YscR)	PscR*	FliP						217/255	IM
	(YscS)	PscS*	FliQ						88/89	IM
	(YscT)	PscT*	FliR						262/258	IM
	(YscU)	PscU*	FlhB						349/378	IM
	(LcrD)	PcrD*	FlhA						706/707	IM
	(YscD)	PscD*	FliG						338/432	IM
Secretin	(YscC)	PscC*	not applicable						600	OM
IM/OM bridge	(YscJ)	PscJ*	FliF						248/598	IM/OM?
Channel gate	(YopN)	PopN*	not applicable						288	extracellular
(g) MTB										
ATPase	(PulE)	XcpR	HxcR	HplR*				PilB PilT PilU	344–594	cytoplasm
Peptidase	(PulO)	XcpA/PilD	XcpA/PilD					XcpA/PilD	290	IM
Major pilin	(PulG)	XcpT	HxcT	HplT*				PilA	144–149	IM/OM
Prepilin	(PulH)	XcpU*	HxcU	HplU*				PilE FimU FimT*	136–172	IM/OM
	(PulI)	XcpV*	HxcV	HplV*				not applicable	124–141	IM/OM
	(PulJ)	XcpW*	HxcW	HplW*				not applicable	196–237	IM/OM
	(PulK)	XcpX*	HxcX	HplX*				not applicable	321–359	IM/OM

Table 2 (*continued*)

System	Prototype	I	II	III	IV	V	VI	VII	Length (aa)	Location
(g) MTB										
IM protein	(PulF)	XcpS	HxcS	HplS*				PilC	374–405	IM
	(PulC)	XcpP*	HxcP	not found	XphA*			not applicable	143–235	IM
	(PulL)	XcpY*	HxcY	not found				not applicable	382/387	IM
	(PulM)	XcpZ*	HxcZ	not found				not applicable	174/197	IM
Secretin	(PulD)	XcpQ	HxcQ	not found	XqhA*	XqhB*	XqhC	PilQ	416–803	OM
(h) FUP										
OM usher		PA0994	PA2130*	PA4084*	PA4652				790–872	OM
Chaperone		PA0993	PA2129*	PA4085*	PA4651				237–262	periplasm
			PA2132*	PA4083*						
Pilin subunit		PA0992	PA2128*	PA4086*					183–381	extracellular
			PA2131*	PA4081*						
Adhesin				PA4082*					1018	extracellular
(i) ATs										
AT		EstA	PA3535	PA0328*					646–995	OM
Putative function		esterase	serine protease?	Amino-peptidase?						
(j) TPS system										
Transporter		PA0040***	PA2463***	PA4540*	PA4624	PA0692			544–568	OM
Putative substrate		PA0041***	PA2462***	PA4541*	PA4625*	PA0690			1417–5627	extracellular
Putative function		hemagglutinin		adhesin	adhesin	adhesin				

Additional protein secretory systems found in *P. fluorescens* Pf0–1 are listed on the web site: http://saier-144-37.ucsd.edu/qma/Pf01.html.

[a] Protein constituents of a single system are shown in the same column, and the homologous transport proteins are presented in the same line.

[b] * Indicates a protein found only in *P. aeruginosa* PAO1, but not in *P. fluorescens* Pf0–1. *P. fluorescens* has two type I systems that are homologous to the PA1875–PA1877 system (**) found in *P. aeruginosa*, but has only one homologous system to the two highly similar TPS systems, PA0040 and PA2463 (***).

two organisms. The protein PA1303 shares 42% sequence identity with SPase I (LepB) in Pae and contains the active site residues (Ser^{90} and Lys^{145}) found in SPase I of *Escherichia coli* [54,55]. However, it lacks the two N-terminal transmembrane segments (TMSs) found in the *E. coli* homologue. Whether or not this *Pseudomonas* protein possesses signal peptidase activity is unknown. The presence of two SPase I homologues in *P. aeruginosa* represents the only example where a constituent of the Sec system has been duplicated.

5. The Tat system

The Tat system specifically translocates proteins with a double-arginine (RR) motif across the inner membrane [8]. In *E. coli*, the Tat system consists of four proteins, TatA, B, C and E, three of which, TatA, B and E, are homologues of each other [9]. Only TatB and C are essential for function; TatA and E are functionally interchangeable [56,57]. In both pseudomonads examined here, only TatA, B and C are present, and they are present in single copy. However, *Pseudomonas stutzeri* has the *tatE* gene [58], showing that the lack of a TatE homologue is not a characteristic of all *Pseudomonas* species. A *tatC* mutant strain of *P. aeruginosa* has been shown to be attenuated for virulence in an animal model, implying that the Tat pathway is involved in pathogenesis [59]. Most Tat substrates are redox proteins located in the periplasm. Nineteen substrates of the *P. aeruginosa* Tat system have been identified, and three of them have been shown to be exported across the outer membrane by the Xcp MTB [59,60]. Evidently, both the Sec and Tat pathways can feed into the MTB.

6. Holin and MscL channels

Holins function primarily in the energy-independent secretion of autolysins in processes relevant to programmed cell death [61]. Two holin-like proteins were found in each of the two *Pseudomonas* species examined. The first (PA3432 and its orthologue in Pfl) are homologous to the LrgA holin of *Staphylococcus aureus* (TC #1.E.14.1.1), which contains four predicted TMSs and exhibits a charge-rich C terminus. Adjacent to these holin-encoding genes are genes encoding potential homologues of autolysins. The second holin-encoding gene in Pae, *hol* (PA0614), is adjacent to an autolysin gene, *lys* (PA0629), in strain PML14 [62]. However, in strain PAO1, close homologues of these two genes are separated by 14 putative genes. Co-expression of *hol* and *lys* induces cell lysis in PAO1 as well as in *E. coli*, suggesting that secretion of the autolysin, Lys, is controlled by Hol [62]. Homologous *hol* and *lys* genes are located in different contigs of the *P. fluorescens* Pf0-1 genome.

The *E. coli*, MscL protein is known to release osmolytes and other small molecules as well as macromolecules

including small proteins following hypoosmotic shock [44]. A single MscL homologue is present in each pseudomonad. Although no functional data are available for these two proteins, their percent identity to the *E. coli* MscL is 64–65%, suggesting an orthologous relationship, and therefore a common function. The structures and many properties of MscL channels have been elucidated [63,64] (for a comprehensive review, see Ref. [65]).

7. ABC exporters (Type I)

Four ABC protein exporters were found in Pae whereas three were found in Pfl (Table 1). In all such systems, three constituents were identified: the ABC transporter itself, a membrane fusion protein (MFP) and an outer membrane factor (OMF) that were encoded by adjacent genes in variable orders. The genes of the Apr systems of several *Pseudomonas* species have been characterized [66–72]. The major substrate of this system is an alkaline protease that contains the conserved Zn^{2+}-binding active-site sequence (TLTHEIGHTL), repeats of the glycine-rich motif (GGXGXD) and an extreme C-terminal motif, D/Ehhh, where h represents hydrophobic residues. Some systems also have been shown to secrete a lipase that exhibits repeats of the glycine-rich motif as well as the extreme C-terminal targeting motifs essential for type I secretion. The nomenclature used for the Apr system has been inconsistent (Apr or Prt versus Tli) and misleading (for instance, AprX refers to two different proteins in various species). Therefore, we propose to unify the designation for all homologous constituents of this system. We suggest using the most commonly used names: AprA is the alkaline protease [73]; AprI is the protease inhibitor; AprD, AprE and AprF are the ABC, MFP and OMF proteins, respectively, and LipA is the substrate lipase. The LipA homologues within an operon are indicated by number. As shown in Fig. 1, the gene orders of *apr* operons vary in different *Pseudomonas* species. However, the central parts that encode AprD, E and F are invariant in that order. *P. fluorescens* and *Pseudomonas brassicacearum* exhibit the gene order, *aprA–aprI–aprD–aprE–aprF*, followed by one or two AT genes and the lipase gene(s).

We identified two homologous lipase genes, *lipA1* and *lipA2*, in Pfl strain Pf0–1. The protein product of *lipA1* is 90% identical to an extracellular lipase of Pfl strain C9 that is known to be secreted by the AprDEF transporter [74]. LipA1 and LipA2 share 52% sequence identity and possess three or two copies of the Ca^{2+}-binding glycine-rich motif as well as the extreme C-terminal motif that is essential for type I secretion [75,76]. Consequently, we speculate that both lipases in Pfl Pf0-1 are secreted by the AprDEF transporter.

The AT genes, designated as PspA and PspB, encode homologues of the two serine proteases, Ssp-h1 and Ssp-h2, of *Serratia marcescens*. One Pfl strain, SIK W1, lacks the AT genes that follow *aprF* in the other strains, but it does have a lipase gene immediately following *aprF*. Pae differs from Pfl in having two putative substrate protease genes, *aprA* and *aprX*, but no AT or lipase gene. *aprA* and *aprI* are present after the *aprD*, *aprE* and *aprF* genes, whereas *aprX* precedes *aprD* (Fig. 1). AprX lacks the conserved Zn^{2+}-binding sequence and the glycine-rich motif found in AprA, but it has an extreme C-terminal motif and was shown to be transported by the AprDEF transporter [77].

The HasDEF system exports the heme acquisition protein, HasA [96]. HasA may function in the acquisition of iron from heme and hemoglobin. This system was not found in Pfl Pf0-1 although it has been identified in one Pfl strain, no. 33 [78]. Similarly, the CvaBA–OmpK system, with no known substrate, is lacking in Pfl. Finally, the PA1876–1877–OmpL system (substrate unknown) is found in dual copy in Pfl. The OmpL homologues in Pfl are 52–73% identical to *aggA* of *Pseudomonas putida* that is involved in

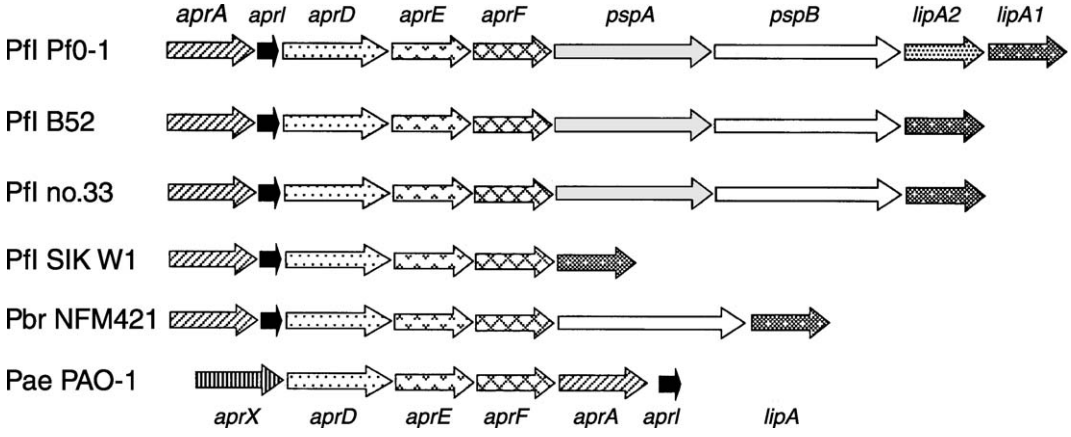

Fig. 1. Comparison of the gene organization of the *apr* operons of various *Pseudomonas* species. Names of the genes are indicated at the top and bottom of the diagram. Homologous genes are drawn with the same shading. Organism and strain are indicated on the left. Abbreviations: Pfl, *P. fluorescens*; Pbr, *P. brassicacearum*; Pae, *P. aeruginosa*. Gene sizes are drawn approximately to scale.

agglutination to plant roots [79]. TolC, an OMF homologue, functions in *E. coli* with multiple transporters and has a homologue (OmpH) in each of the pseudomonads examined.

8. Type III (Fla and Path) secretory pathways

We have previously published phylogenetic analyses of Type III flagellar and pathogenicity-related protein export systems [19]. The pathogenicity-related multicomponent systems transfer proteins directly from the bacterial cytoplasm to the host cell cytoplasm in a single energy-coupled step [80] while the corresponding flagellar systems export flagellins [81]. These complexes, consisting of over a dozen protein constituents each, form needle-like structures. The common protein constituents of the flagellar and pathogenicity-related systems segregate into distinct phylogenetic clusters [19]. Because of this fact, the flagellar-related constituents can easily be distinguished from their pathogenicity-related homologues.

Both pseudomonads have complete flagellar export systems, and Pae has additionally a homologous pathogenicity-related type III system, which secretes virulence factors such as exoenzymes S, T, U and Y directly into the cytoplasm of the host cells [82–84]. Other pseudomonads, including *Pseudomonas syringea* and one strain of *P. fluorescens* (SBW25 [85]), similarly exhibit type III secretion systems. The absence of such a system in most strains of Pfl correlates with the nonpathogenic condition of this species.

9. The main terminal branch

The MTB consists of at least 10 protein constituents that form a large complex in the envelope of a Gram-negative bacterial cell. This system exports folded proteins that are translocated by the Sec or Tat system across the inner membrane. A targeting sequence has not been identified for the MTB. Two complete MTB systems (Xcp and Hxc) are present in Pae (Table 2). In Pfl, a complete Hxc system and a partial Xcp system with homologues of five out of the 12 constituents of the Pae Xcp system were found (Table 2). Since the Pfl genome is not fully sequenced, we could not determine if a complete Xcp system is present. In Pae, the Xcp system secretes most external proteins, including lipases, elastase, alkaline phosphatase, phospholipases and exotoxin A [86]. The expression of this system is controlled by quorum sensing [87]. The Hxc system is specifically designed to secrete alkaline phosphatase and is under *pho* gene regulation [88].

A third MTB system, Hpl, is found in incomplete form in Pae, but no such system was found in Pfl. Its function is unknown. This system lacks an identifiable outer membrane pore-forming oligomeric secretin, but it might form a complex with a secretin encoded by a gene present elsewhere on the chromosome. In fact, three additional secretin genes encoding proteins that are homologous to the secretins of the MTBs were found in the Pae genome. The *xqhA* gene is adjacent to *xphA* that encodes a homologue of an essential constituent of the MTB, XcpP. XqhA has been shown to substitute for XcpQ in type II secretion across the outer membrane in an *xcpQ* mutant strain [89]. The other two secretin genes, XqhB and XqhC, are not associated with other constituents of an MTB, and their functions and expression levels remain to be studied. Finally, a homologous type IV pilus secretory system is present in Pae (Table 2). As can be seen, its protein composition differs considerably from that of the well-characterized Xcp and Hxc systems.

10. Fimbrial ushers

The FUP system is responsible for the biogenesis of numerous fimbriae (pili) in Gram-negative proteobacteria and cyanobacteria. The operon encoding the structural proteins of each fimbrium also encodes a fimbrium-specific periplasmic chaperone and an outer membrane usher protein. Following translocation across the inner membrane by the Sec system, the pilus subunits are bound to the chaperone protein, which prevents the self-assembly of pili in the periplasm. Interaction between the chaperone and usher proteins releases the pilus subunits that subsequently interact with each other and are secreted through the usher protein across the outer membrane. The mechanism by which the assembled fimbrial structure is exported through the usher protein is not well understood [21].

Pae and Pfl have four and two outer membrane FUPs, respectively (Table 2). The corresponding periplasmic chaperones and putative fimbrial subunits were identified by sequence similarity searches of the gene clusters. In Pae, probable substrates were found for three of the four systems identified. Two FUP ushers probably function in conjunction with two fimbrial chaperones [90], and in both of these systems, multiple fimbrial subunits were identified. Of the remaining two FUP systems, a periplasmic chaperone protein was found in only one of the gene clusters encoding the protein. One FUP system (PA2130) has been reported to mediate biofilm formation [90].

11. Autotransporters

ATs were identified by their sequence similarities to known ATs, the presence of an N-terminal signal peptide and a predicted C-terminal 14 or 15 β-strand domain (AT domain [25,91,92]) as revealed using SignalP 2.0 and PSIPRED, respectively (see the Computer methods section). ATs export these protein substrates by passage through an oligomeric ring-shaped structure consisting of 8–10 AT

domains [93]. Pae has three recognizable AT homologues (Table 2) whereas Pfl has nine. PA3535 has a putative serine protease tethered to the AT domain. PA0328 probably exports an aminopeptidase, and EstA (PA5112) exports an esterase that is relevant to the pathogenecity of *P. aeruginosa* [94]. Pfl has five homologues that are closely related to PA3535 and one close homologue of EstA, but no homologue of PA0328. However, two serine protease homologues, PspA and PspB, located in the *apr* operon of Pfl (Fig. 1) may not be proteases because their close homologues could not be shown to exhibit protease activity even though they possess the characteristic active-site motifs [68,70]. Instead, these proteins have been implicated in adhesion or autoaggregation [68].

12. TPS systems

A TPS system consists of two proteins, an outer membrane transporter protein and an exoprotein that is secreted by the transporter. Genes encoding the transporter and substrate proteins are usually found in an operon or within the same locus (see Ref. [26] for a review). Both proteins are translocated across the inner membrane by the Sec system, and the transporter protein probably forms an outer membrane porin with its C-terminal β strands through which the exoprotein is exported. The TPS transporter recognizes a conserved secretion domain in the N terminus of the exoprotein. Exoproteins secreted via the TPS pathway are large proteins with masses ranging between 100 and 500 kDa, and many of them are involved in virulence.

Pae and Pfl have five and three TPS homologues, respectively (Table 2). One of the TPS homologues in Pfl lacks a substrate protein, possibly because of incomplete sequencing. Two of the TPS transporters in Pae (PA0040 and PA2463), only one of which was found in Pfl, are probably specific for hemagglutinins. These systems are 98% identical in amino acid sequence and must have resulted from a recent gene duplication event. The remaining systems in both organisms are possibly transporters for proteins involved in adhesion.

Three additional proteins in Pae, PA2543, PA3648 and PA3339, were retrieved by a PSI-BLAST search with the sequences of known TPS transporters [9]. Although they have several features of TPS channels, such as the presence of N-terminal signal peptides and C-terminal 18 or 19 β-strand domains that might form porins in the outer membrane, their overall sequence similarities to any of the known TPS proteins are less than 30%, and the positions of putative β-strands are inconsistent with those of the TPS proteins in multiple sequence alignments (data not shown). Moreover, we could not identify associated substrate proteins. PA2543 and PA3648 contain a bacterial surface antigen domain (Pfam01103), which is found in a number of OMPs such as the protective antigen, D15 of *Haemophilus influenzae* [95]. PA3339 has part of such a domain as well as a patatin domain (Pfam01734). There is insufficient evidence to allow us to clearly establish these proteins as TPS, although these outer membrane antigens share some features with TPS family members.

13. Conclusion and perspectives

Protein secretion in Gram-negative bacteria has received increasing attention over the past two decades, particularly in the field of bacterial pathogenesis and for production of recombinant proteins of industrial and medical interest. In this review, we identified and characterized all recognizable protein secretory systems in two free-living bacteria, *P. aeruginosa* PAO1 and *P. fluorescens* Pf0-1, using bioinformatics tools. Diverse ecological niches might have driven these bacteria to evolve large genomes (6.3 Mb for Pae and ~ 5.5 Mb for Pfl) and to express different systems under various environmental conditions. Our findings concerning protein secretion, based on genome-wide studies, corroborate this hypothesis. Both pseudomonads not only possess almost all of the recognized protein secretory pathways of Gram-negative bacteria, they also have multiple paralogues of several systems, including type I, MTB, FUP, AT and TPS (Table 2). As noted previously, *P. aeruginosa* contains a high proportion (over 8%) of regulatory genes [28]. Paralogous secretory pathways are undoubtedly controlled by a variety of regulatory proteins, allowing adaptation to a wide range of ecological niches. One example in *P. aeruginosa* is the Hxc system, an MTB exporter that secretes an alkaline phosphatase when environmental phosphate concentrations are low [88].

Being a free-living saprophyte, *P. fluorescens* produces a variety of extracellular proteases and lipases that are presumably required for the utilization of available macromolecular nutrients. It consequently has secretory systems for their export. Some secretory systems, such as the AprDEF transporter, export several substrate proteins, whereas other secretory systems, such as the ATs, export a single substrate protein. Although most proteases produced by Pfl have not been characterized, they may prove to be of industrial values.

Some differences in protein secretion between the two pseudomonads examined shed light on the pathogenic potential of *P. aeruginosa*. First, two FUP systems found in *P. aeruginosa* are missing in *P. fluorescens*. Because *P. fluorescens* Pf0-1 adheres to plant roots rather than animal tissues, these two FUP systems might be involved in *P. aeruginosa* pathogenesis since adherence is a primary infectious step. Second, redundant outer membrane secretins of *P. aeruginosa* might function with a similar MTB system. This has been demonstrated for XqhA, which secretes proteins recognized by the Xcp system when the cognate secretin of the Xcp system, XcpQ, is defective [89]. Such redundant systems may confer upon *P. aeruginosa* survival advantages, allowing it to adapt to a wide range of environ-

mental conditions. Third, the presence of a complete type III secretory system in *P. aeruginosa* but absent in *P. fluorescens* may contribute to its pathogenicity. It should be remembered, however, that our inability to identify these genes could result from incomplete genome sequencing. Characterization of these systems might have implications for vaccine development and treatment of *P. aeruginosa* infections.

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