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Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events

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Abstract

Type III secretion systems (TTSS) are unique bacterial mechanisms that mediate elaborate interactions with their hosts. The fact that several of the TTSS proteins are closely related to flagellar export proteins has led to the suggestion that TTSS had evolved from flagella. Here we reconstruct the evolutionary history of four conserved type III secretion proteins and their phylogenetic relationships with flagellar paralogs. Our analysis indicates that the TTSS and the flagellar export mechanism share a common ancestor, but have evolved independently from one another. The suggestion that TTSS genes have evolved from genes encoding flagellar proteins is effectively refuted. A comparison of the species tree, as deduced from 16S rDNA sequences, to the protein phylogenetic trees has led to the identification of several major lateral transfer events involving clusters of TTSS genes. It is hypothesized that horizontal gene transfer has occurred much earlier and more frequently than previously inferred for TTSS genes and is, consequently, a major force shaping the evolution of species that harbor type III secretion systems.
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1. Introduction

Many Gram-negative bacteria, pathogens and symbionts of animals and plants, have developed secretion systems, termed type III secretion systems (TTSS), which mediate elaborate interactions with their hosts. These secretion systems translocate proteins that lack a signal sequence and may require specific chaperones for their secretion. TTSS systems are unique in the dependence of secretion on external signals, usually the contact with host cells. The TTSS export mechanism is usually composed of more than 20 different proteins, and includes soluble cytoplasmic proteins, outer membrane proteins, and integral membrane proteins. TTSS enable bacteria to deliver a variety of effectors directly into the host cytosol, allowing them to manipulate host cellular processes and subvert them for their benefit (for reviews, see Hueck, 1998; Galan et al., 1999; Aizawa, 2001). Effects include promoting bacterial internalization by mammalian cells in *Salmonella* and *Shigella* (Zychlinsky and Sansonetti, 1997; Hayward and Koronakis, 1999; Zhou et al., 1999a,b), induction of macrophage apoptosis in *Yersinia* spp. (Mills et al., 1997; Monack et al., 1997), and creation of pores in plant cells (Lee et al., 2001). Though sequences of effectors are often poorly conserved among different bacterial species, a high degree of similarity is observed in many proteins comprising the secretion apparatus required for their delivery.

A high degree of sequence similarity exists between TTSS proteins and flagellar proteins. Bacterial flagella are complex propeller-like molecular machines responsible for motility in both Gram-positive and Gram-negative bacteria. The flagella are prevalent in many types of bacteria, including free living species of diverse ecological niches, pathogens and symbionts. Since many constituents of the type III secretion apparatus have paralogs in the export system required for the assembly of the bacterial flagellum, it has often been suggested that TTSS genes evolved from genes encoding flagellar proteins (Galan and Collmer, 1999; Macnab, 1999; Nguyen et al., 2000). Two issues are worth

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mentioning in this context. First, both systems are complex multi-component structures and, consequently, several components of TTSS have no flagellar homologs and many flagellar components have no homologous counterparts in TTSS. Second, the suggestion that a simpler system (TTSS) is derived from a more complex system (flagella) is quite odd in an evolutionary context since it runs against the progressionist grain that pervades evolutionary thought since the days of Jean-Baptiste Lamarck. As was pointed out by Aizawa (2001): "The flagellum is a beautifully designed architecture almost completed in evolution. Why should those sophisticated skills be abandoned to go back to boring soluble proteins?"

Genes encoding type III secretion systems are predominantly located on unstable genetic elements - plasmids or pathogenicity islands (PAIs). These include PAI-1 and PAI-2 in *Salmonella enterica* serovar *Typhimurium*, LEE on enteropathogenic *Escherichia coli, hrp*-PAI in *Pseudomonas syringae* and plasmids of *Shigella flexneri, Yersinia enterocolitica* and *Ralstonia solanacearum*. Thus, TTSS could have been acquired by one or more horizontal gene transfer events. The study of TTSS evolution is, therefore, complicated by the need to consider the possibility of horizontal transfer events occurring at high frequencies.

The importance of TTSS in a variety of host-bacterium interactions makes the study of its molecular evolution particularly interesting. A few intriguing questions present themselves: Was the emergence of the TTSS ancient or relatively recent on the evolutionary scale? Did TTSS originate from flagella? Did TTSS genes evolve first in plant-pathogens as an adaptation of the flagellar basal body as was recently suggested (Galan and Collmer, 1999) or did they emerge earlier in evolution and facilitated interaction with unicellular hosts as suggested for *Chlamydia* (Kim, 2001)? Did horizontal transfer events play a major role in the molecular evolution of TTSS, and when?

2. Methods

2.1. Protein sequences

The nomenclature of TTSS proteins is difficult to follow, as each protein is known by many different names according to species, first discovered function, etc. Therefore, for clarity, we adopted the unified nomenclature suggested by Hueck (1998), who used the abbreviation Sct (secretion and cellular translocation), followed by a specific suffix, e.g. SctR. Flagellar homologs of Sct proteins have standardized names (e.g. Fli, Flh) that are used consistently in all bacteria.

While there are nine protein families with homologs in both flagella and TTSS, we limited our analysis to only the four families in which the amino-acid identity between the TTSS homolog of Yersinia and its closest flagellar relative is at least 35%. This was done to avoid unsupported internal branches. Moreover, low identity levels may result in nonsensical trees, in which some internal branches may exhibit extravagant, yet misleading bootstrap values.

Protein sequences of the SctN/FliI, SctV/FlhA, SctR/Flip and SctS/FliQ homologs were obtained using NCBI BLAST (Altschul et al., 1997) against the SwissProt, PIR, PRF, and PDB databases, as well as against translations from the annotated coding regions in GenBank. To avoid biasing the phylogenetic tree, when protein sequences of more than one species belonging to the same genus were available, and the sequences were nearly identical to one another, only one protein was included in the analysis. Protein sequences used in this study are listed in Tables 1–4.

2.2. DNA sequences

Bacterial 16S-rDNA sequences were obtained from GenBank. To avoid errors due to the alignment of DNA sequences of varying lengths (Table 5), only the first 1,400 nucleotides in each sequence were used for the phylogenetic analysis.

2.3. Phylogenetic analyses

DNA and amino acid sequences were aligned, and phylogenetic trees were reconstructed by the neighbor-joining method (Saitou and Nei, 1987) as implemented in ClustalX (Thompson et al., 1997). DNA distances were calculated with Kimura's two-parmeter correction (Kimura 1980). Amino-acid distances were corrected by assuming the Poisson distribution. In all cases, positions with gaps in the multiple alignment were excluded from the analysis.

2.4. Gene trees and species trees

The trees constructed from the amino acid sequences will be regarded as gene trees that may or may not be congruent with the species trees due to stochastic errors and horizontal gene transfers distorting the vertical evolutionary history. The trees constructed from the 16S-rDNA sequences were checked for contradictions with established taxonomy of Proteobacteria (e.g. Staley et al., 1989) as well as for violation of the monophylies of the Alpha, Beta, Gamma, Delta, and Epsilon subphyla. In the absence of such discrepancies, the trees were treated as the species trees against which the gene trees were contrasted.

2.5. Detection of horizontal transfer

Putative horizontal gene transfer events were identified through comparisons of inferred TTSS trees with appropriate 16S-rDNA species trees. The comparison was performed with the algorithm of Hallett and Lagergren (2001) as implemented in the LATTRANS program by Dr. Louigi Addario-Berry (http://www.cs.mcgill.ca/~laddar/lattrans/download.html). To use the program, both

Table 1
SctN/FliI sequences used in this study

No.	Abbreviation	Apparatus	Bacterial species	Protein	Length (aa)	Accession no.
		TTSS				
1	Ypes_YscN1		*Yersinia pestis*	YscN	439	NP_395174
2	Yent_YscN		*Yersinia enterocolitica*	YscN	439	NP_052401
3	Yent_YsaN		*Yersinia enterocolitica*	YsaN	465	AAB69192
4	Paer_PscN		*Pseudomonas aeruginosa*	PscN	440	AAB86534
5	Bbro_BscN		*Bordetella bronchiseptica*	BscN	444	AAC38612
6	Xcam_HRB6		*Xanthomonas campestris*	HrpB6	442	AAB08461
7	Rsol_HrcN		*Ralstonia solanacearum*	HrcN	439	NP_522431
8	Mlot_HrcN		*Mesorhizobium loti*	HrcN	452	NP_106866
9	Bjap_RhcN		*Bradyrhizobium japonicum*	RhcN	451	AAG60799
10	Cpne_YopN		*Chlamydophila pneumoniae*	YopN	442	NP_224903
11	Sent_SSAN		*Salmonella enterica* (serovar *Typhimurium*)	SsaN	433	NP_460380
12	Ypes_YscN2		*Yersinia pestis*	YscN2	445	NP_403918
13	Ecol_EivC		*Escherichia coli* (O157:H7)	EivC	439	BAB37153
14	Ecol_EscN		*Escherichia coli* (O157:H7)	EscN	446	BAB37991
15	Bpse_SctN		*Burkholderia pseudomallei*	SctN	449	AAK73233
16	Eamy_HrcN		*Erwinia amylovora*	HrcN	454	AAB06001
17	Pagg_HrcN		*Pantoea agglomerans*	HrcN	454	CAC43015
18	Psyr_HrcN		*Pseudomonas syringae*	HrcN	449	CAD22886
19	Sfle_Spa		*Shigella flexneri*	Spa47	430	C42284
20	Sent_InvC		*Salmonella enterica* (serovar *Typhimurium*)	InvC	431	NP_461815
		Flagella				
21	Tmar_FliI		*Thermotoga maritima*	FliI	438	NP_228033
22	Aaeo_FliI		*Aquifex aeolicus*	FliI	443	NP_214096
23	Vcho_FliI		*Vibrio cholerae*	FliI	439	AAF95275
24	Cace_FliI		*Clostridium acetobutylicum*	FliI	438	NP_348777
25	Bbur_FliI		*Borrelia burgdorferi*	FliI	436	NP_212422
26	Bsub_FliI		*Bacillus subtilis*	FliI	440	NP_38950
27	Hpyl_FliI		*Helicobacter pylori*	FliI	434	NP_208211
28	Cjej_FliI		*Campylobacter jejuni*	FliI	461	CAB72678
29	Cpne_FliI		*Chlamydophila pneumoniae*	FliI	433	NP_225053
30	Paer_FliI		*Pseudomonas aeruginosa*	FliI	451	NP_249795
31	Ypes_FliI1		*Yersinia pestis*	FliI1	446	NP_404349
32	Ypes_FliI2		*Yersinia pestis*	FliI2	484	NP_405393
33	Eco_FliI		*Escherichia coli* (O157:H7)	FliI	457	NP_310707
34	Sent_FliI		*Salmonella enterica* (serovar *Typhimurium*)	FliI	456	NP_460925
35	Tpal_FliI		*Treponema pallidum*	FliI	447	NP_218842
36	Rsol_FliI		*Ralstonia solanacearum*	FliI	481	NP_521954
37	Baph_FliI		*Buchnera aphidicola*	FliI	466	NP_660429
38	Ccre_FliI		*Caulobacter crescentus*	FliI	444	AAC45616
39	Zmob_FliI		*Zymomonas mobilis*	FliI	443	AAG29861
40	Lmon_FliI		*Listeria monocytogenes*	FliI	433	NP_464243
41	Sfle_FliI		*Shigella flexneri*	FliI	457	NP_707826
42	MlotFliI		*Mesorhizobium loti*	FliI	466	NP_104138
43	Rsph_FliI		*Rhodobacter sphaeroides*	FliI	442	JC4733
44	Atum_FliI		*Agrobacterium tumefaciens*	FliI	473	O34171
45	Bjap_FliI		*Bradyrhizobium japonicum*	FliI	441	NP_768841

species trees and protein trees had to be converted to binary trees with identical numbers of branches. Thus, any species having two Sct paralogs had to be represented twice in the species tree. The scenarios generated by the program were compared to the original trees. Inferred horizontal transfer events not involving the 'crossing' of at least one branch associated with a bootstrap value of 95% or higher were discarded. Of the alternative scenarios suggested by the algorithm, the most parsimonious events were selected based on information external to the trees.

3. Results

3.1. Evolution of the type III secretion system from flagella is unlikely

As stated previously, four highly conserved elements in both systems were used to infer evolutionary relationships between flagellar proteins and TTSS components. Among these four proteins, SctN, a cytoplasmic ATPase known to be essential for type III secretion in various bacteria

Table 2
SctV/FlhA sequences used in this study

No.	Abbreviations	Apparatus	Bacterial species	Protein	Length (aa)	Accession no.
		TTSS[a]				
1	Ypes_LcrD1		*Yersinia pestis*	LcrD	704	P31487
2	Yent_LcrD		*Yersinia enterocolitica*	LcrD	704	P21210
3	Yent_YsaV		*Yersinia enterocolitica*	YsaV	690	O30436
4	Paer_PcrD		*Pseudomonas aeruginosa*	PscD	706	Q9I327
6	Xcam_HRPC2		*Xanthomonas campestris*	HRPC2	645	P80150
7	Rsol_HrcV		*Ralstonia solanacearum*	HrcV	690	P35656
8	Mlot_HrcV		*Mesorhizobium loti*	HrcV	681	Q989N4
9	Bjap_RhcV		*Bradyrhizobium japonicum*	RhcV	699	BAC47065
10	Cpne_LcrD		*Chlamydophila pneumoniae*	LcrD	710	Q9Z8L5
11	Sent_SsaV		*Salmonella enterica* (serovar *Typhimurium*)	SsaV	681	P74856
12	Ypes_LcrD2		*Yersinia pestis*	LcrD2	684	AAM84113
13	Ecol_EivA		*Escherichia coli* (O157:H7)	EivA	686	Q8X6E0
14	Ecol_EscV		*Escherichia coli* (O157:H7)	EscV	675	O52139
15	Bpse_SctV		*Burkholderia pseudomallei*	SctV	705	Q93KZ1
16	Eamy_HrpI		*Erwinia amylovora*	HrpI	697	P35654
17	Pagg_HrcV		*Pantoea agglomerans*	HrcV	719	Q937I5
18	Psyr_HrpI		*Pseudomonas syringae*	HrpI	695	P35655
19	Sfle_MxiA		*Shigella flexneri*	MxiA	686	P35533
20	Sent_InvA		*Salmonella enterica* (serovar *Typhimurium*)	InvA	685	P35657
		Flagella[b]				
21	Tmar_FlhA		*Thermotoga maritima*	FlhA	678	Q9X010
22	Aaeo_FlhA		*Aquifex aeolicus*	FlhA	678	O67265
23	Vcho_FlhA		*Vibrio cholerae*	FlhA	697	Q9KQD1
24	Cace_FlhA		*Clostridium acetobutylicum*	FlhA	690	Q97H66
25	Bbur_FlhA		*Borrelia burgdorferi*	FlhA	697	Q44909
26	Bsub_FlhA		*Bacillus subtilis*	FlhA	677	P35620
27	Hpyl_FlhA		*Helicobacter pylori*	FlhA	733	Q9ZM40
28	Cjej_FlhA		*Campylobacter jejuni*	FlhA	724	Q9PP48
29	Cpne_FlhA		*Chlamydophila pneumoniae*	FlhA	582	Q9Z8I0
30	Paer_FlhA		*Pseudomonas aeruginosa*	FlhA	707	Q9I3P9
31	YpesFlhA		*Yersinia pestis*	FlhA	692	Q8ZFC3
32	Eco_FlhA		*Escherichia coli* (O157:H7)	FlhA	692	NP_288316
33	Sent_FlhA		*Salmonella enterica* (serovar *Typhimurium*)	FlhA	692	P40729
34	Tpal_FlhA		*Treponema pallidum*	FlhA	707	Q56338
35	Rsol_FlhA		*Ralstonia solanacearum*	FlhA	696	Q8XQ93
36	Baph_FlhA		*Buchnera aphidicola*	FlhA	702	AAO26954
37	Ccre_FlhA		*Caulobacter crescentus*	FlhA	700	Q03845
38	Zmob_FlhA		*Zymomonas mobilis*	FlhA	707	Q9Z5S8
39	Lmon_FlhA		*Listeria monocytogenes*	FlhA	691	AH1159
40	Bjap_FlhA1		*Bradyrhizobium japonicum*	FlhA1	693	BAC52116
41	Mlot_FlhA		*Mesorhizobium loti*	FlhA	695	Q98HA3
42	Rsph_FlhA		*Rhodobacter sphaeroides*	FlhA	682	ZP_00006082
43	Atum_FlhA		*Agrobacterium tumefaciens*	FlhA	723	Q8UHU8
44	Bjap_FlhA2		*Bradyrhizobium japonicum*	FlhA2	747	NP_768847
45	Xcam_FlhA		*Xanthomonas campestris*	FlhA	697	NP_637274

[a] No ortholog 7gs for SctV of *B. bronchiseptica* were found in databases.
[b] No ortholog 7gs for FlhA in *S. flexneri* were found in the databases.

(Eichelberg et al., 1994; Woestyn et al., 1994) is the most conserved across systems, due mostly to constraints on its ATP binding domains and Mg^{2+} binding site (Hueck, 1998). Paralogs of these ATPases, called FliI, are found in the flagellar export mechanisms, where they energize the translocation of substrates across the membrane (Minamino and Macnab, 2000). The SctN/FliI tree is shown in Fig. 1a. Trees were also reconstructed for three conserved inner membrane proteins SctV, SctR, and SctS whose paralogous flagellar proteins are FlhA, FliP and FliQ, respectively (Fig. 1b–d).

All four protein trees are compatible with the hypothesis that both flagellar and TTSS protein subfamilies are monophyletic (Fig. 2a). We note, however, the division into two monophletic groups has a high bootstrap support in only one tree (Fig. 1b). Branch lengths indicate that the levels of diversity are similar in the TTSS and flagella subtrees implying a similar degree of antiquity for both

Table 3
SctR/FliP sequences used in this study

No.	Abbreviation	Apparatus	Bacterial species	Protein	Length (aa)	Accession no.
		TTSS[a]				
1	Ypes_YscR1[a]		*Yersinia pestis*	YscR	217	P40297
2	Yent_YscR		*Yersinia enterocolitica*	YscR	217	NP_783679
3	Yent_YsaR		*Yersinia enterocolitica*	YsaR	224	AAK84105
4	Paer_PscR		*Pseudomonas aeruginosa*	PscR	217	NP_250384
5	Xcam_HrcR		*Xanthomonas campestris*	HrcR	214	NP_636600
6	Bbro_BscR		*Bordetella bronchiseptica*	BscR	223	AAF25801
6	Rsol_HrcR		*Ralstonia solanacearum*	HrcR	217	Q52488
7	Mlot_HrcR		*Mesorhizobium loti*	HrcR	221	NP_106869
8	Bjap_RhcR		*Bradyrhizobium japonicum*	RhcR	221	NP_768459
9	Cpne_YopR		*Chlamydophila pneumoniae*	YopR	306	NP_225020
10	Sent_SsaR		*Salmonella enterica* (serovar *Typhimurium*)	SsaR	215	CAA68199
11	Ypes_YscR2		*Yersinia pestis*	YscR2	216	NP_403922
12	Ecol_EpaP		*Escherichia coli* (O157:H7)	EpaP	221	A85940
13	Ecol_EscR		*Escherichia coli* (O157:H7)	EscR	217	NP_290283
14	Bpse_SctR		*Burkholderia pseudomallei*	SctR	216	AAD11411
15	Eamy_HrcR		*Erwinia amylovora*	HrcR	217	Q46646
16	Pagg_HrcS		*Pantoea agglomerans*	HrcS	217	CAA68098
17	Psyr_HrcS		*Pseudomonas syringae*	HrcS	208	AAC25069
18	Sfle_SpaP		*Shigella flexneri*	SpaP	216	P35529
19	Sent_SpaP		*Salmonella enterica* (serovar *Typhimurium*)	SpaP	224	P40700
		Flagella				
20	Tmar_FliP		*Thermotoga maritima*	FliP	249	NP_228507
21	Aaeo_FliP		*Aquifex aeolicus*	FliP	239	O67750
22	Vcho_FliP		*Vibrio cholerae*	FliP	299	NP_231754
23	Cace_FliP		*Clostridium acetobutylicum*	FliP	261	Q97H63
24	Bbur_FliP		*Borrelia burgdorferi*	FliP	254	NP_212409
25	Bsub_FliP		*Bacillus subtilis*	FliP	221	P35528
26	Hpyl_FliP		*Helicobacter pylori*	FliP	248	NP_223343
27	Cjej_FliP		*Campylobacter jejuni*	FliP	244	D81354
28	Paer_FliP		*Pseudomonas aeruginosa*	FliP	255	Q51468
29	Ypes_FliP1		*Yersinia pestis*	FliP	246	NP_405386
30	Eco_FliP		*Escherichia coli* (O157:H7)	FliP	245	P33133
31	Sent_FliP		*Salmonella enterica* (serovar *Typhimurium*)	FliP	245	P54700
32	Sfle_FliP		*Shigella flexneri*	FliP	204	NP_707833
32	Tpal_FliP		*Treponema pallidum*	FliP	271	P74930
33	Rsol_FliP		*Ralstonia solanacearum*	FliP	249	NP_521936
34	Baph_FliP		*Buchnera aphidicola*	FliP	360	AAO26812
35	Ccre_FliP		*Caulobacter crescentus*	FliP	266	Q45980
36	Ypes_FliP2		*Yersinia pestis*	FliP	256	AE0087
37	Lmon_FliP		*Listeria monocytogenes*	FliP	255	NP_464203
38	Bjap_FliP1		*Bradyrhizobium japonicum*	FliP	250	NP_772456
39	Mlot_FliP		*Mesorhizobium loti*	FliP	243	NP_104149
40	Rsph_FliP		*Rhodobacter sphaeroides*	FliP	301	O85133
41	Atum_FliP		*Agrobacterium tumefaciens*	FliP	245	Q44344
42	Bjap_FliP2		*Bradyrhizobium japonicum*	FliP	246	NP_773507
43	Xcam_FliP		*Xanthomonas campestris*	FliP	280	NP_637281

[a] No homologs for FliP of *Chlamydophila pneumoniae* and *Zymomonas mobilis* were found in the databases.

groups. Thus, the suggestion that TTSS evolved from flagella (e.g. Galan and Collmer, 1999; Macnab, 1999; Nguyen et al., 2000), by what can only be called ‘reductive evolution,’ receives no topological support from the phylogenetic trees. Let us assume that TTSS are indeed derived from flagella. Then, flagellar proteins are expected to be paraphyletic (Fig. 2b). Alternatively, if the more complex flagellar export system is assumed to be derived from TTSS, then the TTSS proteins are expected to be paraphyletic (Fig. 2c). We note, however, that since our trees are essentially unrooted, the question of monophyly or paraphyly cannot be resolved simultaneously for both TSSS and flagella. However, the vast majority of the possible paraphyletic trees would have been revealed by unrooted trees too. Thus, the phylogenetic reconstruction does not support the claim that type III secretion systems elements

Table 4
SctS/FliQ sequences used in this study

No.	Abbreviation	Apparatus	Bacterial species	Protein	Length (aa)	Accession no.
		TTSS[a]				
1	Ypes_YscS1		*Yersinia pestis*	YscS	88	P40298
2	Yent_YscS		*Yersinia enterocolitica*	YscS	88	AAD16829
3	Yent_YsaS		*Yersinia enterocolitica*	YsaS	89	AAK84110
4	Paer_PscS		*Pseudomonas aeruginosa*	PscS	88	AAG05081
5	Xcam_HrcS		*Xanthomonas campestris*	HrcS	80	NP_636599
6	Bbro_BscS		*Bordetella bronchiseptica*	BscS	88	AAF25802
7	Rsol_HrcS		*Ralstonia solanacearum*	HrcS	86	NP_522420
8	Mlot_HrcS		*Mesorhizobium loti*	HrcS	82	BAB52656
9	Bjap_RhcS		*Bradyrhizobium japonicum*	RhcS	91	NP_768460
10	Cpne_YopS		*Chlamydophila pneumoniae*	YopS	95	B72030
11	Sent_SsaS		*Salmonella enterica* (serovar *Typhimurium*)	SsaS	88	P74891
12	Ypes_YscS2		*Yersinia pestis*	YscS2	93	AAM84119
13	Ecol_EpaQ		*Escherichia coli* (O157:H7)	EpaQ	86	NP_311751
14	Ecol_EscS		*Escherichia coli* (O157:H7)	EscR	89	NP_312609
15	Bpse_SctS		*Burkholderia pseudomallei*	SctS	87	AAD11412
16	Eamy_HrcS		*Erwinia amylovora*	HrcS	86	AAB06006
17	Pagg_HrcS		*Pantoea agglomerans*	HrcS	83	CAA68099
18	Psyr_HrcS		*Pseudomonas syringae pv. Phaseolicola*	HrcS	88	AAG33885
19	Sfle_SpaQ		*Shigella flexneri*	SpaQ	86	P40705
20	Sent_SpaQ		*Salmonella enterica* (serovar *Typhimurium*)	SpaQ	86	P40704
		Flagella				
21	Tmar_FliQ		*Thermotoga maritima*	FliQ	88	NP_228506
22	Aaeo_FliQ		*Aquifex aeolicus*	FliQ	89	O67774
23	Vcho_FliQ		*Vibrio cholerae*	FliQ	89	NP_231753
24	Cace_FliQ		*Clostridium acetobutylicum*	FliQ	89	NP_348767
25	Bbur_FliQ		*Borrelia burgdorferi*	FliQ	87	Q44906
26	Bsub_FliQ		*Bacillus subtilis*	FliQ	89	P35535
27	Hpyl_FliQ		*Helicobacter pylori*	FliQ	88	O25964
28	Cjej_FliQ		*Campylobacter jejuni*	FliQ	89	NP_282802
29	Paer_FliQ		*Pseudomonas aeruginosa*	FliQ	89	AAG04836
30	Ypes_FliQ1		*Yersinia pestis*	FliQ	89	NP_405385
31	Ecol_FliQ		*Escherichia coli* (O157:H7)	FliQ	89	NP_310715
32	Sent_FliQ		*Salmonella enterica* (serovar *Typhimurium*)	FliQ	89	P54701
33	Sfle_FliQ		*Shigella flexneri*	FliQ	89	NP_707834
34	Tpal_FliQ		*Treponema pallidum*	FliQ	94	P74931
35	Rsol_FliQ		*Ralstonia solanacearum*	FliQ	89	NP_521935
36	Baph_FliQ		*Buchnera aphidicola*	FliQ	90	Q8KA36
37	Ccre_FliQ		*Caulobacter crescentus*	FliQ	87	Q45974
38	Ypes_FliQ2		*Yersinia pestis*	FliQ	89	AD0087
39	Lmon_FliQ		*Listeria monocytogenes*	FliQ	90	AE1159
40	Bjap_FliQ1		*Bradyrhizobium japonicum*	FliQ	88	BAC52117
41	Mlot_FliQ		*Mesorhizobium loti*	FliQ	88	NP_104165
42	Rsph_FliQ		*Rhodobacter sphaeroides*	FliQ	88	ZP_00004707
43	Atum_FliQ		*Agrobacterium tumefaciens*	FliQ	88	F97429
44	Bjap_FliQ2		*Bradyrhizobium japonicum*	FliQ	87	BAC51076
45	Xcam_FliQ		*Xanthomonas campestris*	FliQ	89	NP_637280

[a] No homologs for FliQ of *Chlamydophila pneumoniae* and *Zymomonas mobilis* were found in the databases.

originated from components of the flagellar export apparatus.

The particularly high branch lengths of two *C. pneumoniae* flagellar homlogs (Fig. 1a,b) can be attributed to the supposed non-functionalization of flagellar genes in this bacterium, which is immobile and lacks flagellar rod, hook and filament (Kim, 2001), resulting in the accelerated evolution of what may well be 'pseudogenes-in-waiting.'

3.2. Ancient horizontal DNA transfer of TTSS genes

As TTSS genes are located on unstable genetic elements (PAIs or plasmids), which frequently facilitate horizontal gene transfer, we looked for horizontal transfer between different bacterial species. It is now widely accepted that horizontally acquired DNA in bacteria undergoes a 'species-adaptive' process in which it gradually becomes

Table 5
16S-rDNA sequences used in this study

No.	Bacterial species	Accession no.
1	*Yersinia pestis*	AJ232238
2	*Yersinia enterocolitica*	Z75316
3	*Pseudomonas aeruginosa*	15595198
4	*Bordetella bronchiseptica*	X57026
5	*Shigella flexneri*	X96963
6	*Xanthomonas campestris*	X99299
7	*Ralstonia solanacearum*	17544719
8	*Mesorhizobium loti*	AP003001
9	*Bradyrhizobium japonicum*	U69638
10	*Salmonella enterica* (serovar *Typhimurium*)	16763390
11	*Escherichia coli* (O157:H7)	AE005174
12	*Burkholderia pseudomallei*	U91839
13	*Erwinia amylovora*	X83265
14	*Pantoea agglomerans*	AJ506794
15	*Pseudomonas syringae*	Z76669
16	*Aquifex aeolicus*	15282445
17	*Thermotoga maritima*	AE001703
18	*Chlamydophila pneumoniae*	15617929
19	*Clostridium acetobutylicum*	NC_003030
20	*Bacillus subtilis*	Z99104
21	*Borrelia burgdorferi*	AF091367
22	*Vibrio cholerae*	AE004096
23	*Helicobacter pylori*	AE000620
24	*Campylobacter jejuni*	6968128
25	*Caulobacter crescentus*	AE006011
26	*Buchnera aphidicola*	NC_004545
27	*Zymomonas mobilis*	AF117351
28	*Listeria monocytogenes*	16802048
29	*Agrobacterium tumefaciens*	17936711
30	*Rhodobacter sphaeroides*	46451

indistinguishable from the rest of the genome in terms of GC content and codon usage. Therefore, in order to identify relatively ancient horizontal transfer events it is necessary to compare between protein trees and species trees and identify deviations from vertical evolution. In this study, horizontal transfer was inferred from cases where topology was significantly different between the species tree (as inferred from 16S rDNA sequences) and the protein tree for SctN (Fig. 3).

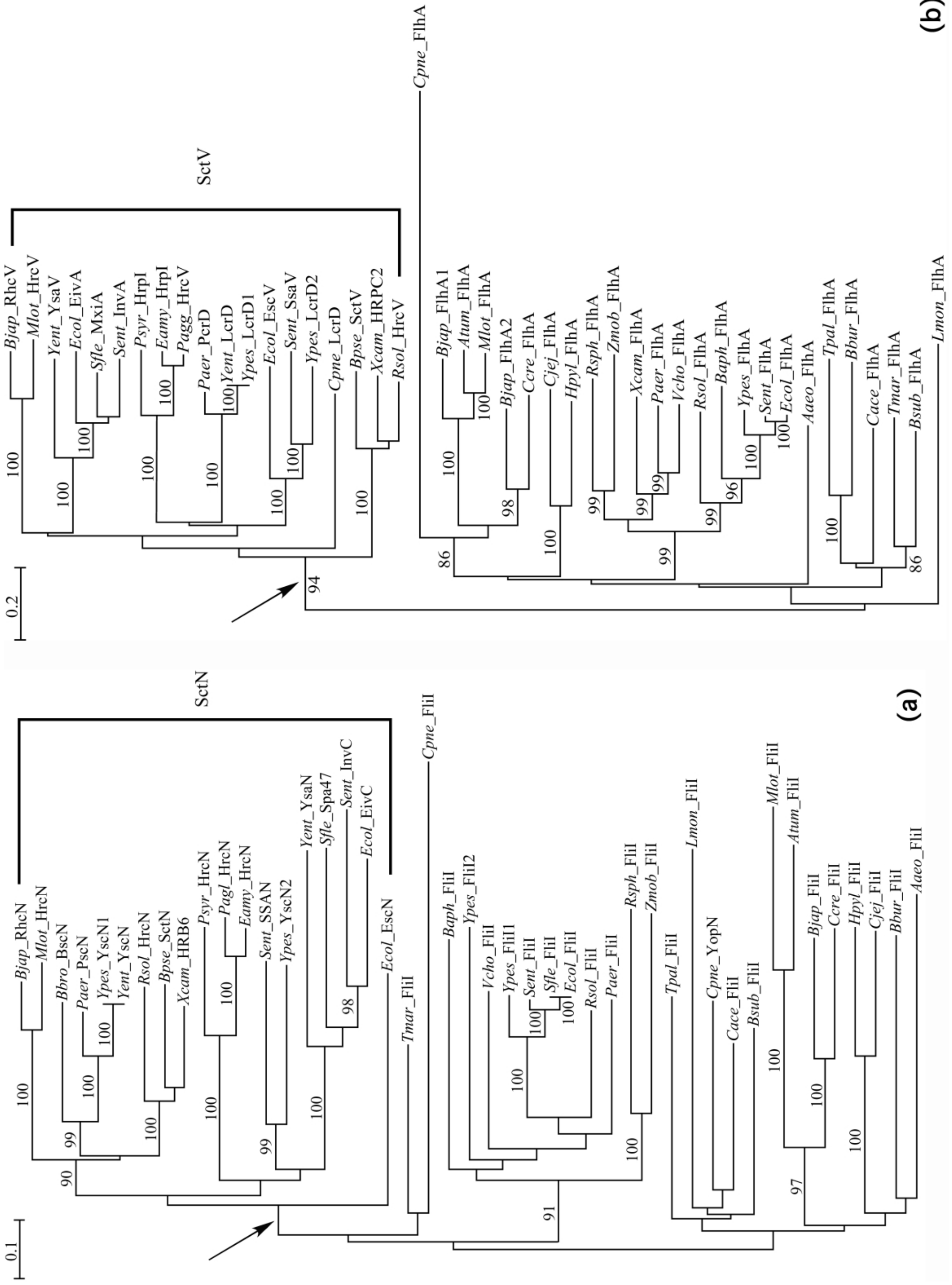
To reach statistically significant congruence between the species tree and the gene tree, one must assume at least six horizontal gene transfer events, of which at least four are inferred to involve internal branches on the tree. Horizontal transfers between bacterial subdivisions (classes) are inferred to have occurred at least twice: (1) between the ancestor of *B. pseudomallei* and *R. solanacearum* (in the Beta subdivision) and the ancestor of *X. campestris* (in the Gamma subdivision), and (2) between the ancestor of *B. bronchispetica* (in the beta subdivision) and the ancestor of the *Yersinia* species (in the Gamma subdivision). Horizontal transfer between families are inferred to have occurred between an ancestral *Yersinia* (Enterobacteriaceae) and *P. aeruginosa* (Pseudomonadaceae), and between the progenitor of *Erwinia* and *Pantoea* (Enterobacteriaceae) and an ancestor of *P. syringae* (Pseudomonadaceae). At least two more horizontal transfer events occurred within family Enterobacteriaceae: between *Y. pestis and S. enterica* and between *S. enterica* and *E. coli*. Most of the transfer events identified in SctN were also detected when we examined the phylogenetic trees of the other three type III proteins: SctV, SctS, and SctR, supporting the hypothesis that many genes in the TTSS loci have been acquired through horizontal transfer of gene clusters. There were however a few exceptions: (1) There is no *B. bronchispetica* SctV homolg in the databases so it is impossible to infer a lateral gene transfer event involving this gene, (2) For the SctS tree, which is based on a protein whose length is less than 100 amino acids, it proved very difficult to show statistically significant lateral transfers involving either *B. bronchispetica* or the internal events within Enterobacteriacea.

In unrooted trees, direction of transfer cannot be determined unequivocally. However, in many cases the most likely direction may be deduced from auxiliary knowledge. Thus, since the two closely related *Pseudomonas* species in our study have distantly related TTSS genes, the most parsimonious explanation is that each acquired its corresponding TTSS gene separately. The alternative explanation, that the ancestral *Pseudomonas* had two separate TTSS systems, and that *P. syringae* and *P. aeruginosa* each lost one cluster during diversification and then became the source of the TTSS cluster of *Erwinia*/Pantoea and *Bordetella*/*Yersinia*, respectively, implies two additional gene loss events and is, therefore, less likely. Our hypotheses is further supported by GC-content considerations. We assume that the TTSS cluster of the ancestral *Bordetella* was transferred to *Yersinia*, and not vice versa since the SctN of *Bordetella* appears to predate the divergence between the two SctNs of *Yersinia*. The SctN from *X. campestris* diverged last from its common ancestor with SctN from *Ralstonia* and SctN from *Burkholderia* and is, therefore, more likely to be the recipient rather than the donor of this TTSS cluster.

The 'evolutionary promiscuity' of these secretion systems is even more conspicuous when contrasted with their flagellar paralogs. Thus, when a similar procedure was applied to detect horizontal gene transfer of FliI, only one case of horizontal transfer was found - between a progenitor of *Rhodobacter sphaeroides* and *Zymomonas mobilis* (in the Alpha subdivision) and a progenitor of Enterobacteriaceae and Vibrionaceae (in the Gamma subdivision).

4. Discussion

Type III secretion systems (TTSS) deliver bacterial proteins important for interactions with the host. So far, they have been identified in pathogens and symbionts of plants and animals. It is often assumed that the TTSS evolved from


(a)
0.1
SctN
Bjap_RhcN
Mlot_HrcN
Bbro_BscN
Paer_PscN
Ypes_YscN1
Yent_YscN
Rsol_HrcN
Bpse_SctN
Xcam_HRB6
Psyr_HrcN
Pagl_HrcN
Eamy_HrcN
Sent_SSAN
Ypes_YscN2
Yent_YsaN
Sfle_Spa47
Sent_InvC
Ecol_EivC
Ecol_EscN
Tmar_FliI
Cpne_FliI
Baph_FliI
Ypes_FliI2
Vcho_FliI
Ypes_FliI1
Sent_FliI
Sfle_FliI
Ecol_FliI
Rsol_FliI
Paer_FliI
Rsph_FliI
Zmob_FliI
Tpal_FliI
Lmon_FliI
Cpne_YopN
Cace_FliI
Bsub_FliI
Mlot_FliI
Atum_FliI
Bjap_FliI
Ccre_FliI
Hpyl_FliI
Cjej_FliI
Bbur_FliI
Aaeo_FliI
(b)
0.2
SctV
Bjap_RhcV
Mlot_HrcV
Yent_YsaV
Ecol_EivA
Sfle_MxiA
Sent_InvA
Psyr_HrpI
Eamy_HrpI
Pagg_HrcV
Paer_PcrD
Yent_LcrD
Ypes_LcrD1
Ecol_EscV
Sent_SsaV
Ypes_LcrD2
Cpne_LcrD
Bpse_SctV
Xcam_HRPC2
Rsol_HrcV
Cpne_FlhA
Bjap_FlhA1
Atum_FlhA
Mlot_FlhA
Bjap_FlhA2
Ccre_FlhA
Cjej_FlhA
Hpyl_FlhA
Rsph_FlhA
Zmob_FlhA
Xcam_FlhA
Paer_FlhA
Vcho_FlhA
Rsol_FlhA
Baph_FlhA
Ypes_FlhA
Sent_FlhA
Ecol_FlhA
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Cace_FlhA
Tmar_FlhA
Bsub_FlhA
Lmon_FlhA

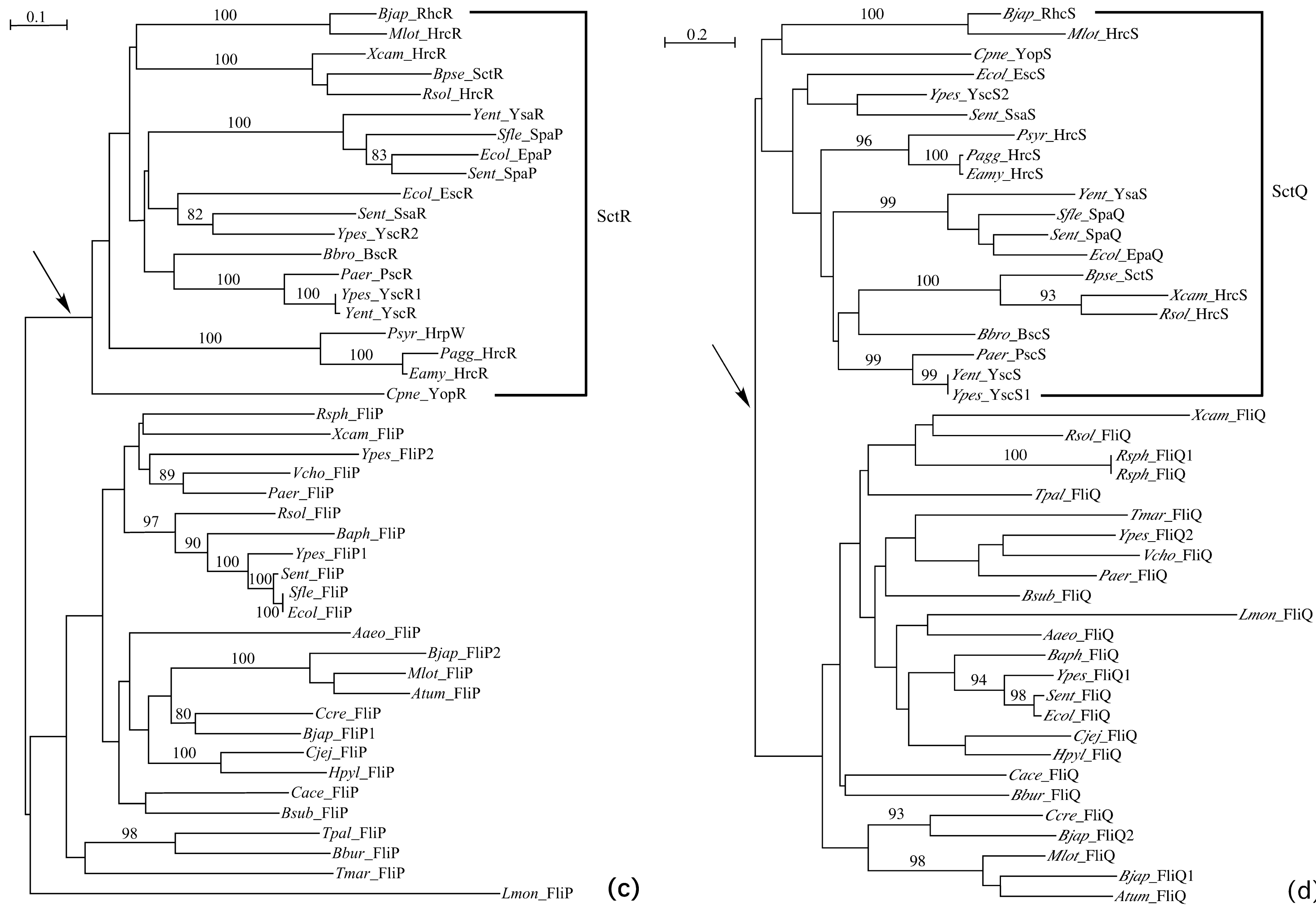

Fig. 1. Unrooted neighbor-joining phylogenetic trees of proteins from the flagellar export and type III secretion systems. Bootstrap percentages out of 1,000 replicates are shown for branches supported by values larger than 80%. Scale bars represent numbers of substitutions per site. Proteins belonging to the type III secretion system subfamily are marked with a bracket. The arrows indicates that it is possible to position the roots so that all trees are compatible with the monophy of the flagellar export subfamily as well as with the monophyly of its paralogous TTSS subfamily. (a) SctN/FliI; (b) SctV/FlhA; (c) SctR/FliP; (d) SctS/FliQ.

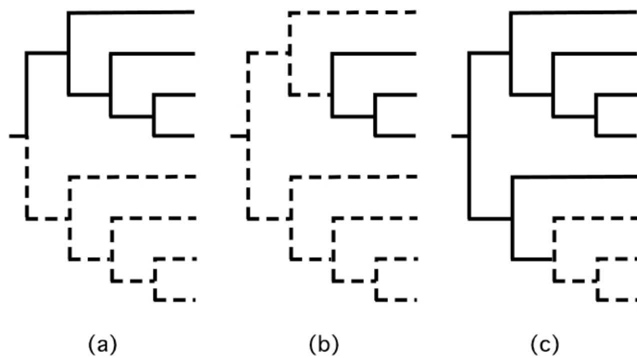

Fig. 2. Schematic representation of three possible phylogenetic relationships between Sct and flagellar-export protein subfamilies. Sct lineages are shown in dashed lines. (a) Independent evolution of Sct and flagellar-export proteins from a common ancestor. (b) Flagellar-export proteins are derived from Sct. (c) Sct proteins are derived from flagellar-export proteins.

flagellar paralogs, since they resemble the flagellar export apparatus in protein components as well as in supramolecular structure (Aizawa, 2001). The results presented in this paper do not support this assumption, as they indicate that TTSS are as ancient and most probably share a common ancestor with the flagellar export apparatus. A similar work published by Nguyen and colleagues (Nguyen et al., 2000) also addressed the same issues by using similar methodologies. However, despite arriving at similar results, the authors chose not to trust their own findings and reverted to the old dogma of the flagellar progenitor of TTSS. In order to justify their conclusions concerning the antiquity of flagella, which was incongruent with their empirical findings, Nguyen and colleagues resorted to four non-phylogentic (or anti-phylogenetic) arguments. First, they claimed that flagella are common in bacteria but absent from eukaryotes and archaea. Second, they claimed that type III secretion systems are limited to Gram negative bacteria. Third, they assumed that TTSS are selected for by interactions with eukaryotes. And finally, they claimed that 'higher eukaryotes' (i.e., multicellular organisms) only appeared within the last billion years, whereas flagellated bacteria most probably existed for 4 billion years.

Each of these arguments can be easily refuted. First, flagella are common in both eukaryotes and archaea, but share no homology with their functional analogs in prokaryotes. These immensely complex yet morphologically similar molecular motors are very different among the kingdoms, indicative of parallel evolution. Had the ancient life forms, which existed before the branching of archeaa and eukarya, been flagellated, then a certain degree of homology between kingdoms would have been evident. Thus, it is far more plausible that functional flagella are not nearly as ancient as implied by Nguyen and colleagues. As for absence of TTSS in Gram positive bacteria, this is easily explainable by the fact that TTSS structures have to span two membranes, such that their presence in one-membrane organisms cannot be beneficial. However TTSS are present in several *Chlamydia* species that belong to the Chlamydiae/-Verrucomicrobia superphylum, which are as distant from the Gram-negative Proteobacteria phylum as are the Gram-positive Firmicutes. The present role of TTSS in virulence towards higher eukaryotes does not preclude other roles, such as interactions with other taxa (see below). Furthermore, the ancestor of both flagella and TTSS may have been a simple but versatile export apparatus that could have secreted a variety of proteins with multiple functions, such as intra-bacterial interactions and assembly of surface appendages.

Our analysis of the most conserved paralogs between TTSS and flagella indicates that the divergence between TTSS and flagella may have been very ancient. Based on an estimate of 120–160 million years for the divergence between *Escherichia* and *Salmonella* (Ochman and Wilson, 1987), and assuming molecular-clock regularity, the divergence between TTSS and flagella may have occurred hundreds of millions of years ago, much earlier that the appearance of the first multicellular eukaryotes on the evolutionary stage.

Several TTSS families evolved independently as plant and animal commensals, pathogens and symbionts long before the appearance of warm-blooded vertebrates. This is in agreement with the finding that the insect pathogen *Sodalis glossinidius* also harbors a TTSS related to the one located on a pathogenicity island (SPI I) of *S. enterica* (Dale et al., 2001). Ancestry of TTSS genes of the PAI I of *S. enterica* has been previously studied at the DNA level. It was suggested that *Yersinia*, *Salmonella* and *Shigella* acquired TTSS genes independently from an external source, whereas the alternative that TTSS was ancestral in the Enterobacteriaceae was considered unlikely (Li et al., 1995). The discovery of a second, chromosomally located, TTSS gene cluster in *Y. enterocolitica* (Fig. 1), which clusters with the TTSS of the other Enterobacteriaceae, renders Li et al.'s interpretation untenable. Furthermore, the recently discovered TTSS of *S. glossinidius* is also in the same cluster. (This sequence was not included in the present analysis since its 16S-DNA has not yet been sequenced.) The ancestral nature of the TTSS does not rule out the possibility that *Salmonella* (or even *Shigella*) acquired at least some of their TTSS genes from either *Yersinia*, as had been suggested previously based on GC content (Altmeyer et al., 1993) or from a common ancestor of the Enterobacteriaceae. The TTSS cluster is not present in all the Enterobacteriaceae, probably due to independent sporadic deletions (Morschhauser et al., 1994; Bach et al., 1999). This assumption is supported by the finding that the TTSS gene clusters are located on unstable DNA regions, such as a plasmids or PAIs. The loss of TTSS could be beneficial, since the synthesis of the large secretion apparatus is energetically costly and would be selected against in environmental isolates or some non-invasive strains, which have no use of the system. Effects of such rapid processes of gene acquisition and gene loss can be observed

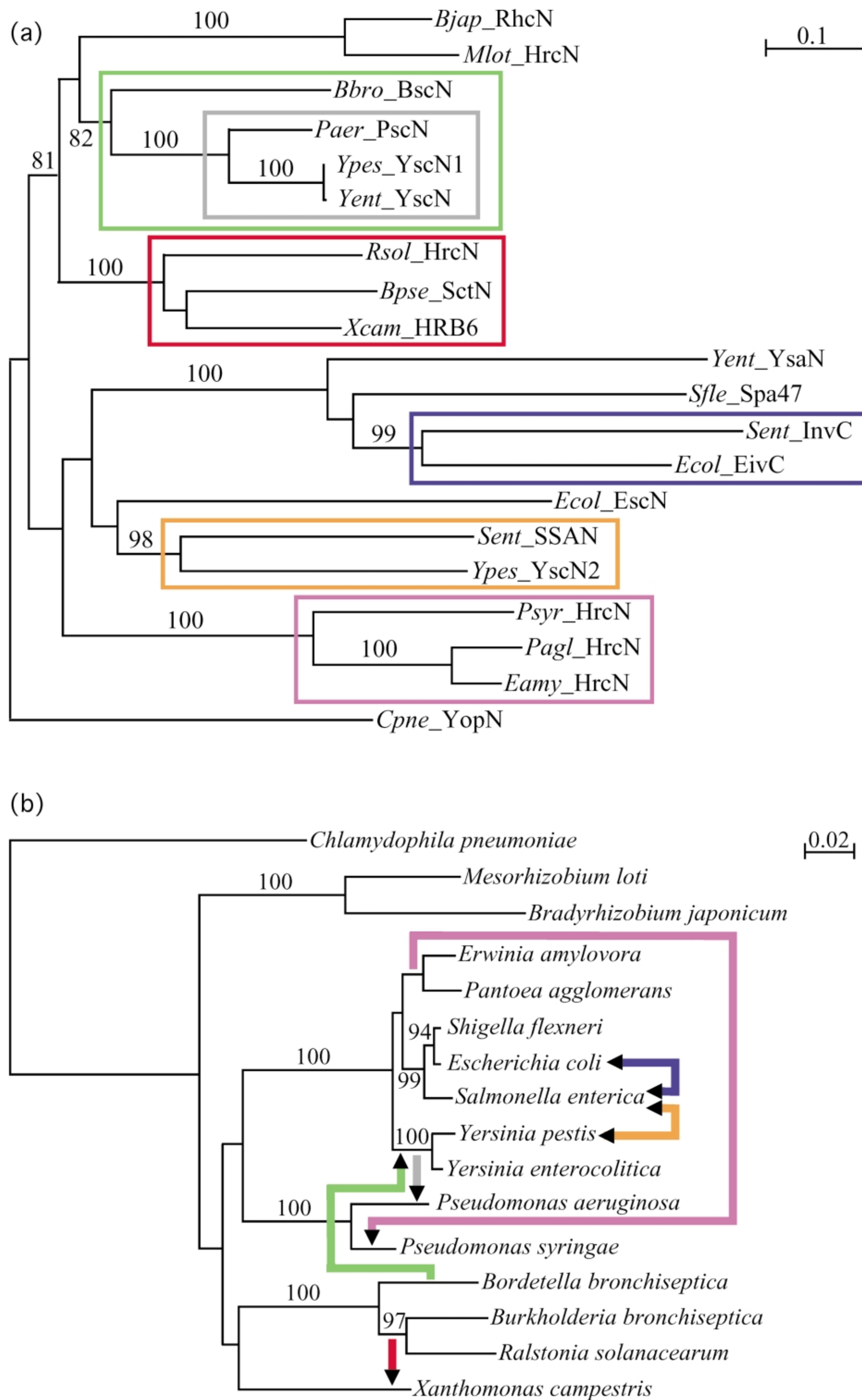

Fig. 3. Horizontal gene transfers of type III ATPase genes. Neighbor-joining trees for SctN protein sequences (a) and 16r-DNA sequences (b) were constructed excluding positions with gaps and correcting for multiple replacements or substitutions, respectively. Bootstrap percentages out of 1,000 replicates are shown for branches supported by values larger than 80%. Scale bars represent numbers of changes per site. Identification of horizontal gene transfer events was achieved with LATTRANS. Only horizontal transfers with high bootstrap support are shown on the species tree (16S rDNA tree). Boxed clades in (a) indicate conflicts with the species tree due to horizontal transfers of TTSS gene clusters. Arrows represent inferred horizontal gene transfers, and have the same color as the phylogenetic incongruences which they resolve. Double-headed arrows indicate transfers for which no direction could be inferred.

in the genus *Yersinia*, where *Y. pestis* and *Y. enterocolitica,* which diverged less than 20,000 years ago (Achtman et al., 1999), share the same plasmid-encoded TTSS but have different chromosomal TTSSs. This finding is compatible with recent genomic data for *Y. pestis* indicating a very rapid rate of evolution and a very high incidence of gene loss, which probably represent an adaptation of this organism to its niche as a systemic pathogen (Parkhill et al., 2001).

Our TTSS phylogenetic trees reveal no clear division between bacteria of mammals and plants, thus, lending no support for the assumption that the TTSS emerged first in plant bacteria. Furthermore, ancient TTSS could have been involved in various interactions with insects (as was recently shown for *S. glossinidius*), nematodes, or even unicellular organisms. This hypothesis is supported by reports on infections of protozoans by bacteria known to possess type III secretion systems (e.g. Amann et al., 1997; Barker et al., 1999). These findings raise the possibility that the historical roles played by ancestral TTSS proteins might have been radically different from the host-bacteria interactions mediated by present type III secretion systems.

In our present study of TTSS protein evolution we identified relatively ancient horizontal gene transfer events, which are mostly undetectable at the DNA level. The vast majority of the inferred horizontal transfer events in our study occurred among species sharing similar host ecologies (e.g. from one plant pathogen to another or from one animal pathogen to another, but not from animal to plant bacteria). This observation strengthens our conclusion concerning the high frequency of horizontal gene-transfer events, which by definition require a modicum of physical proximity between donor and acceptor bacteria. The only exception seems to be SctN from the mammalian pathogen *B. pseudomallei,* which clusters with homologous proteins from phytopathogens. However, this finding can be explained by the fact that the environmental reservoir of *B. pseudomallei* is rice fields. Moreover, a recent report based on the analysis of the incomplete *B. pseudomallei* genome revealed the existence of a second TTSS, related to *Salmonella*, which seems to be derived from a mammalian bacterium (Attree and Attree, 2001).

In *Pseudomonas*, two different species have TTSS clusters that are highly dissimilar, each adapted to its own host. The mammalian pathogen *P. aeuroginosa* and the plant pathogen *P. syringae* have SctN orthologs that are phylogenetically related to those of Enterobateriaceae residing in their respective hosts. These results suggest that the two *Pseudomonas* species probably acquired their TTSS by horizontal gene transfer. This hypotheses is further supported by the fact that the GC content of the *P. syringae* TTSS cluster is lower than the mean for the other *P. syringae* genes (54% *versus* 60%; Jackson et al., 1999). The GC-content value for the *P. syringae* TTSS cluster is closer to that of enterobateriaceal genomes (about 48–50%). In two other cases (*X. campestris* and *B. bronchiseptica*), there is a reasonable factual basis for inferring the direction of transfer, based on the time of divergence and the number of species involved.

The large number of horizontal transfer of TTSS genes among bacteria, which occurred at different time points in evolution, is probably the result of the selective advantage bestowed by these molecular syringes upon the bacterium. What is especially noteworthy is the fact that many horizontal gene transfers occurred far earlier than previously reported. We assume that at later evolutionary stages, horizontal gene transfer becomes more difficult as both the secretion apparatus and its substrates become fine-tuned by the co-evolution of the bacterium and its host.

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