

## SUPPLEMENTARY INFORMATION

### Structural and functional diversity of type IV secretion systems

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**Supplementary Table 1.** Structures of the T4SS protein components and their subcomplexes solved by electron-microscopy, x-ray crystallography and NMR.

**Supplementary Text Box 1.** Spatiotemporal studies of T4SSs

**Supplementary Text Box 2.** Translocation Signals

**Supplementary Text Box 3.** Diversification of pili or cell surface adhesins for establishment of target cell contacts

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**Supplementary Table 1.** Structures of the T4SS protein components and their subcomplexes solved by electron-microscopy, x-ray crystallography and NMR.

Bacteria	Plasmid Genome Encoded	Subcomplex	Protein nomenclature			Method	Resolution	Entries		Ref
			Original	VirB homologue	Subunits			EMDB	PDB	
<i>Escherichia coli</i>	R388	OMCC (O-layer)	TrwH	VirB7	14	cryo-EM	2.6 Å	12707	703J	1
			TrwF	VirB9	14					
			TrwE	VirB10	14					
<i>Escherichia coli</i>	R388	OMCC (I-layer)	TrwF	VirB9	16	cryo-EM	3.1 Å	12708	703T	1
			TrwE	VirB10	16					
<i>Escherichia coli</i>	R388	Stalk	TrwJ	VirB5	5	cryo-EM	3.7 Å	12709	703V	1
			TrwI	VirB6	5					
<i>Escherichia coli</i>	R388	Arches	TrwG	VirB8	3x6	cryo-EM	6.2 Å	13767	7Q1V	1
<i>Escherichia coli</i>	R388	IMC	TrwM	VirB3	1x6	cryo-EM	3.8 Å	12933	70IU	1
			TrwK	VirB4	2x6					
			TrwG	VirB8	4x6					
<i>Escherichia coli</i>	R388	ATPases	TrwK	VirB4	6	cryo-EM	4.2 Å	12716	7O42	1
<i>Escherichia coli</i>	R388	OMCC (O-layer)	TrwH	VirB7	14	cryo-EM	8.5 Å	2233	3ZBI	1
			TrwF	VirB9	14					
			TrwE	VirB10	14					
<i>Escherichia coli</i>	R388	OMCC (I-layer)	TrwF	VirB9	14	cryo-EM	12 Å	2232	2YPW	1
			TrwE	VirB10	14					
<i>Escherichia coli</i>	R388	ATPase	TrwB	VirD4	6	X-ray	3.0 Å	-	1GKI 1E9R 1E9S 1GL6 1GL7	2
<i>Escherichia coli</i>	R388	Relaxosome	TrwC	Tral	1	X-ray	2.0 Å	-	1OMH	2
<i>Escherichia coli</i>	R388	Relaxosome	TrwC	Tral	1	X-ray	2.5 Å	-	4PCB	3
<i>Escherichia coli</i>	R388	Other	ArdC	-	1	X-ray	2.7 Å	-	6SNA	4
<i>Escherichia coli</i>	R388	IMC	-	-	-	NS	28 Å	3585	-	5
<i>Escherichia coli</i>	R388	IMC Stalk Arches OMCC	-	-	-	NS	20 Å	2567	-	6
<i>Escherichia coli</i>	R388	ATPase	TrwK	VirB4	6	NS	20 Å	5505	-	7
<i>Escherichia coli</i>	R388	Relaxosome	TrwC	VirD2	1	X-ray	2.5 Å	-	4PCB	3
<i>Escherichia coli</i>	pKM101	OMCC (O-layer)	TraN	VirB7	14	X-ray	2.6 Å	-	3JQO	8
			TrwO	VirB9	14					
			TrwF	VirB10	14					
<i>Escherichia coli</i>	pKM101	Pilus	TraM	VirB2	multi	Cryo-EM	3.0 Å	27023	8CW4	9
<i>Escherichia coli</i>	pKM101	Stalk OMCC	-	-	-	Cryo-ET	38 Å	24098	-	10
<i>Escherichia coli</i>	pKM101	IMC Arches	-	-	-	Cryo-ET	43 Å	24100	-	10
<i>Escherichia coli</i>	pKM101	OMCC	-	-	-	NS	20 Å	2136 2137	-	11

<i>Escherichia coli</i>	pKM101	OMCC	-	-	-	Cryo-EM	15 Å	5031 5032 5033 5034 5035	-	12
<i>Escherichia coli</i>	pKM101	Arches	TraE	VirB8	2	X-ray	2.4 Å	-	5I97	13
<i>Escherichia coli</i>	pKM101	Arches	TraE	VirB8	2	X-ray	2.6 Å	-	5WIC 5WII 5WIO 5WIP	14
<i>Escherichia coli</i>	pKM101	Stalk-Pilus	TraC	VirB5	1	X-ray	3.0 Å	-	1R8I	15
<i>Escherichia coli</i>	pKM101	Relaxosome	Tral	VirD2	1	X-ray	2.1 Å	-	8A1B 8A1C	16
<i>Escherichia coli</i>	pKM101	OMCC	TraO	VirB9	1	NMR	-	-	2OFQ	17
			TraN	VirB7	1					
<i>Escherichia coli</i>	F-plasmid	Pilus	TraA	VirB2	multi	Cryo-EM	5.0 Å	4044 4046	5LER 5LFB	18
<i>Escherichia coli</i>	F-plasmid	Stalk OMCC	-	-	-	Cryo-ET	23 Å	9344	-	19
<i>Escherichia coli</i>	F-plasmid	IMC Arches	-	-	-	Cryo-ET	24 Å	9347	-	19
<i>Escherichia coli</i>	F-plasmid	Pilus MS2 phage	TraA	VirB2	multi	Cryo-EM	6.2 Å	9397	6NM5	20
		MS2 maturation protein	-	-	1					
<i>Escherichia coli</i>	F-plasmid	Regulation	FinO	-	1	X-ray	2.0 Å	-	1DVO	21
<i>Escherichia coli</i>	F-plasmid	Regulation	TraJ	-	2	X-ray	1.6 Å	-	4KQD	22
<i>Escherichia coli</i>	F-plasmid	Relaxosome	Tral	VirD2	2	X-ray	2.4 Å	-	3FLD	23
<i>Escherichia coli</i>	F-plasmid	Relaxosome	Tral	VirD2	1	X-ray	2.6 Å	-	1P4D	24
<i>Escherichia coli</i>	F-plasmid	Relaxosome	Tral	VirD2	1	X-ray	2.7 Å	-	2A0I	25
<i>Escherichia coli</i>	F-plasmid	Relaxosome	Tral	VirD2	1	X-ray	2.4 Å	-	2Q7T- 2Q7U	26
<i>Escherichia coli</i>	F-plasmid	Relaxosome	TraM	-	4	X-ray	2.6 Å	-	3D8A	27
		ATPase	TraD	VirD4	4					
<i>Escherichia coli</i>	F-plasmid	Relaxosome	TraM	-	8	X-ray	3.1 Å	-	4QPQ 4QPO	28
<i>Escherichia coli</i>	F-plasmid	Relaxosome	Tral	VirD2	1	NMR	-	BMRB 16971	2L8B	29
<i>Escherichia coli</i>	F-plasmid	Relaxosome	TraM	-	1	NMR	-	BMRB 4584	1DP3	30
<i>Escherichia coli</i>	pED208	OMCC (I-ring)	TraB	VirB10	17	Cryo-EM	3.3 Å	12962	7OKN	31
			TraV	VirB7	17					
<i>Escherichia coli</i>	pED208	OMCC (O-layer)	TraB	VirB10	17	Cryo-EM	3.0 Å	24772 24770 24768	7SPJ 7SPC	32
			TraV	VirB7	17					
<i>Escherichia coli</i>	pED208	OMCC (O-ring)	TraB	VirB10	13	Cryo-EM	3.4 Å	12963	7OKO	31
			TraK	VirB9	26					
			TraV	VirB7	26					
<i>Escherichia coli</i>	pED208	OMCC (I-layer)	TraB	VirB10	26	Cryo-EM	3.0 Å	24769 24771	7SPB 7SPI	32
			TraK	VirB9	26					

			TraV	VirB7	26					
<i>Escherichia coli</i>	pED208	Pilus	TraA	VirB2	multi	Cryo-EM	3.6 Å	4042	5LEG	<sup>18</sup>
<i>Escherichia coli</i>	pED208	Relaxosome	TraM	-	8	X-ray	2.9 Å	-	3ON0 3OMY	<sup>33</sup>
<i>Escherichia coli</i>	R64	ARCHES	TraM	VirB8	1	X-ray	1.5 Å	-	3WZ3	<sup>34</sup>
<i>Escherichia coli</i>	R1	Relaxosome	Tral	VirD2	1	Cryo-EM	3.9 Å	3601	5N8O	<sup>35</sup>
<i>Escherichia coli</i>	R1	Relaxosome	Tral	VirD2	1	X-ray	1.9 Å	-	4L0J	<sup>36</sup>
<i>Escherichia coli</i>	pCU1	Relaxosome	Tral	VirD2?	1	X-ray	2.3 Å	-	3L57 3L6T	<sup>37</sup>
<i>Escherichia coli</i>	R1162	Relaxosome	Tral	VirD2	1	X-ray	2.1 Å	-	2NS6	<sup>3</sup>
<i>Agrobacterium tumefaciens</i>	Ti-Plasmid	Pilus	VirB2	VirB2	multi	Cryo-EM	3.5 Å	28657	8EXH	<sup>9</sup>
<i>Agrobacterium tumefaciens</i>	Ti-Plasmid	Pilus	VirB2	VirB2	multi	Cryo-EM	3.5 Å	28957	8FAI	<sup>38</sup>
<i>Agrobacterium tumefaciens</i>	Ti-Plasmid	OMCC	-	-	-	NS	21 Å	3725	-	<sup>39</sup>
<i>Agrobacterium tumefaciens</i>	Ti-Plasmid	Arches	VirB8	VirB8	2	X-ray	2.2 Å	-	2CC3	<sup>40</sup>
<i>Klebsiella pneumoniae</i>	pKpQIL	Pilus	TraA	VirB2	multi	Cryo-EM	3.9 Å	22460	7JSV	<sup>41</sup>
<i>Klebsiella pneumoniae</i>	pKpQIL	Mating pair	TraN OmpK36	- -	1 3	cryo-EM	2.7 Å	25567	7SZI	<sup>42</sup>
<i>Salmonella enterica serovar Typhimurium</i>	pSLT	Regulation	TraJ	-	2	X-ray	1.7 Å	-	4EW7	<sup>22</sup>
<i>Legionella pneumophila</i>	Genome	ATPase Effector recruitment	DotL	VirD4	1	cryo-EM	3.7 Å	10350	6SZ9	<sup>43</sup>
			DotM	-	1					
			DotN	-	1					
			DotY	-	1					
			DotZ	-	1					
<i>Legionella pneumophila</i>	Genome	ATPase Effector recruitment	DotL	VirD4	1	cryo-EM	3.6 Å	13083	7OVB	<sup>44</sup>
			DotM	-	1					
			DotN	-	1					
			DotY	-	1					
			DotZ	-	1					
<i>Legionella pneumophila</i>	Genome	Effector recruitment	DotY	-	1	X-ray	2.4 Å	-	7UPS	<sup>45</sup>
<i>Legionella pneumophila</i>	Genome	Effector recruitment	DotM	-	1	X-ray	1.8 Å	-	6EXB	<sup>46</sup>
<i>Legionella pneumophila</i>	Genome	Effector recruitment	DotM	-	1	X-ray	1.8 Å	-	5X1U	<sup>47</sup>
<i>Legionella pneumophila</i>	Genome	Effector recruitment	DotN	-	1	X-ray	3.0 Å	-	5X1H	<sup>47</sup>
<i>Legionella pneumophila</i>	Genome	ATPase Effector recruitment	DotL	VirD4	1	X-ray	1.8 Å	-	5X42	<sup>47</sup>
			DotN	-	1					
<i>Legionella pneumophila</i>	Genome	ATPase Effector recruitment	DotL	VirD4	1	X-ray	2.0 Å	-	5X1E	<sup>47</sup>
			IcmS	-	1					
<i>Legionella pneumophila</i>	Genome	ATPase Effector recruitment	IcmW	-	1	X-ray	2.8 Å	-	5X90	<sup>47</sup>
			DotL	VirD4	1					

<i>pneumophila</i>	Genome	ATPase Effector recruitment	IcmS	-	1					
			IcmW	-	1					
			LvgA	-	1					
<i>Legionella pneumophila</i>	Genome	ATPase Effector recruitment	DotL	VirD4	1	X-ray	2.6 Å	-	5XNB	48
			IcmS	-	1					
			IcmW	-	1					
<i>Legionella pneumophila</i>	Genome	ATPase Effector recruitment	DotL	VirD4	1	X-ray	2.8 Å	-	7BWK	49
			IcmS	-	1					
			IcmW	-	1					
			LvgA	-	1					
			VpdB	-	1					
<i>Legionella pneumophila</i>	Genome	OMCC	DotG	VirB10	16					
					18					
			DotH	VirB9	13					
					18					
			DotD	VirB7	26					
			DotF	-	31					
			DotC	-	13	cryo-EM	4.6 Å	24018	7MUQ	50
			DotK	-	13					
			Dis1	-	13					
			Dis2	-	13					
			Dis3	-	13					
<i>Legionella pneumophila</i>	Genome	Other	IcmR	-	1	X-ray	2.4 Å	-	4EYY	51
			IcmQ	-	1					
<i>Legionella pneumophila</i>	Genome	OMCC	-	-	-	Cryo-ET	40 Å	0566	-	52
<i>Legionella pneumophila</i>	Genome	OMCC	-	-	-	Cryo-ET	30 Å	8566 8568 8569	-	53
<i>Legionella pneumophila</i>	Genome	Arches	DotI	VirB8	1	X-ray	3.5 Å	-	3WZ4 3WZ5	34
<i>Legionella pneumophila</i>	Genome	ATPase	DotB	VirB11	6	X-ray	3.2 Å	-	6GEB	54
<i>Coxiella burnetii</i>	Genome	OMCC	-	-	-	Cryo-ET	11 Å	27105	-	55
<i>Brucella suis</i>	Genome	ATPase	VirB11	VirB11	6	X-ray	2.6 Å	-	2GZA	56
<i>Brucella suis</i>	Genome	Arches	VirB8	VirB8	2	X-ray	2.4 Å	-	2BHM	57
<i>Brucella suis</i>	Genome	Arches	VirB8	VirB8	1	X-ray	2.6 Å	-	4AKY	58
<i>Brucella suis</i>	Genome	Arches	VirB8	VirB8	1	X-ray	2.3 Å	-	4AKZ	58
<i>Bartonella tribocorum</i>	Genome	Arches	VirB8	VirB8	2	X-ray	2.9 Å	-	4MEI	59
<i>Bartonella birtlesii</i>	Genome	Arches	VirB8	VirB8	2	X-ray	1.4 Å	-	4JF8	59
<i>Bartonella grahamii</i>	Genome	Arches	VirB8	VirB8	2	X-ray	2.6 Å	-	4KZ1	59
<i>Bartonella grahamii</i>	Genome	Arches	VirB8	VirB8	2	X-ray	2.0 Å	-	4NHF	59
<i>Bartonella quintana Toulouse</i>	Genome	Arches	VirB8	VirB8	1	X-ray	1.7 Å	-	4LSO	59
<i>Rickettsia typhi</i>	Genome	Arches	VirB8	VirB8	2	X-ray	2.0 Å	-	4O3V	59
<i>Helicobacter pylori</i>	Genome	OMCC (O-layer)	Cag3	-	70					
			CagM	-	28	cryo-EM	3.4 Å	22081	6X6S	60
			CagY	VirB10	14					

			CagX	VirB9	14					
			CagT	VirB7	28					
<i>Helicobacter pylori</i>	Genome	OMCC (I-layer)	CagY	VirB10	17	cryo-EM	3.5 Å	20021	6X6J	60
			CagX	VirB9	17					
<i>Helicobacter pylori</i>	Genome	ATPase	Cagα	VirB11	2	X-ray	2.5 Å	-	1G6O	61
<i>Helicobacter pylori</i>	Genome	ATPase	Cagα	VirB11	2	X-ray	2.8 Å	-	1NLY	62
<i>Helicobacter pylori</i>	Genome	ATPase	Cagα	VirB11	6	X-ray	2.4 Å	-	2PT7	63
			HP1451	-	2					
<i>Helicobacter pylori</i>	Genome	ATPase	Cagβ	VirD4	1	X-ray	2.1 Å	-	6JHO	64
			CagZ	-	1					
<i>Helicobacter pylori</i>	Genome	OMCC	CagY	VirB10	2	X-ray	3.0 Å	-	2BHV	57
<i>Helicobacter pylori</i>	Genome	OTHER	CagD	-	2	X-ray	2.8 Å	-	3CWY	65
<i>Helicobacter pylori</i>	Genome	OTHER	CagS	-	1	X-ray	2.3 Å	-	2G3V	66
<i>Helicobacter pylori</i>	Genome	Stalk-Pilus	CagL	VirB5	1	X-ray	2.2 Å	-	3ZCI	67
<i>Helicobacter pylori</i>	Genome	Stalk-Pilus	CagL	VirB5	1	X-ray	3.3 Å	-	3ZCJ	67
<i>Helicobacter pylori</i>	Genome	Stalk-Pilus	CagL	VirB5	1	X-ray	2.2 Å	-	4CII	68
<i>Helicobacter pylori</i>	Genome	Stalk-Pilus	CagL	VirB5	1	X-ray	2.3 Å	-	4X5U	69
<i>Helicobacter pylori</i>	Genome	Arches	CagV	VirB8	2	X-ray	1.9 Å	-	6IQT	70
<i>Helicobacter pylori</i>	Genome	IMC	-	-	-	Cryo-ET	48 Å	0634	-	19
<i>Helicobacter pylori</i>	Genome	OMCC-Stalk-Arches	-	-	-	Cryo-ET	40 Å	0635	-	19
<i>Helicobacter pylori</i>	Genome	OMCC-Stalk-Arches-IMC	-	-	-	Cryo-ET	68 Å	7475	-	71
<i>Helicobacter pylori</i>	Genome	OMCC-Stalk-Arches	-	-	-	Cryo-ET	39 Å	7474	-	71
<i>Xanthomonas citri</i>	Genome	OMCC	VirB7	VirB7	14	Cryo-EM	3.3 Å	0089	6GYB	72
			VirB9	VirB9	14					
			VirB10	VirB10	14					
<i>Xanthomonas citri</i>	Genome	OMCC (O-layer)	VirB7	VirB7	1	NMR	-	BMRB-25512	2N01	73
			VirB9	VirB9	1					
<i>Xanthomonas citri</i>	Genome	OMCC (O-layer)	VirB7	VirB7	1	NMR	-	BMRB-17257	2L4W	74
<i>Yersinia pseudotuberculosis</i>	Genome	ATPase	DotB	VirB11	6	X-ray	2.8 Å	-	6GEF	54
<i>Bacillus subtilis</i>	pLS20	Relaxosome	Aux2	TraM	4	X-ray	1.9 Å	-	7QNQ 7NUV	75
<i>Clostridium perfringens</i>	pCW3	Relaxosome	TcpK	-	2	X-ray	2.5 Å	-	5VFX 5VFX	76
<i>Enterococcus faecalis</i>	pCF10	Arches	VirB8	VirB8	2	X-ray	1.8 Å	-	7AED	77
<i>Enterococcus faecalis</i>	pIP501	Arches	VirB8	VirB8	2	X-ray	2.5 Å	-	4EC6	78
<i>Enterococcus faecalis</i>	pIP501	OMCC (O-layer)	TraK	VirB7	1	X-ray	3.0 Å	-	4HIC	79
<i>Enterococcus faecalis</i>	pIP501	Regulator	TraN	-	1	X-ray	2.0 Å	-	6G1T	80

<i>Enterococcus faecalis</i>	pCF10	Relaxosome	PcfF	-	2	X-ray	1.9 Å	-	6QEQ	<sup>81</sup>
<i>Enterococcus faecalis</i>	Genome	Arches	TraH	VirB8	1	NMR	-	BMRB-26518	5AIW	<sup>82</sup>
<i>Streptococcus thermophilus</i>	ICEst3	Arches	OrfG	VirB8	1	X-ray	1.8 Å	-	6ZGN	<sup>83</sup>
<i>Thermoanaerobacter pseudethanolicus</i>	Genome	ATPase	VirB4	VirB4	1	X-ray	2.4 Å	-	4AG5 4AG6	<sup>11</sup>
<i>Pyrobaculum calidifontis</i>	Genome	Pilus	TedC	VirB2	multi	Cryo-EM	4.0 Å	27413	8DFT	<sup>84</sup>
<i>Aeropyrum pernix</i>	Genome	Pilus	CedA1	VirB2	multi	Cryo-EM	3.3 Å	27414	8DFU	<sup>84</sup>

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54 ‘-’ indicates not identified or not available. OMCC, outer-membrane core complex; IMC, inner-membrane  
55 complex

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### 57 **Supplementary Text Box 1. Spatiotemporal studies of T4SSs**

58 In conjunction with the recent advances in structural definition of T4SSs, fluorescence microscopy approaches  
59 are supplying exciting new insights into dynamic processes associated with T4SS assembly, spatial  
60 organization, and substrate translocation in living cells. This is perhaps best exemplified by recent studies of  
61 the *L. pneumophila* Dot/Icm system, which has been shown to localize at bacterial cell poles<sup>85</sup>. Remarkably, by  
62 introducing an *mreB* mutation to alter *L. pneumophila* cell morphologies from rod to round, the Dot/Icm  
63 machine was delocalized and also no longer translocated T4SS substrates. Consistently, in *Legionella*-infected  
64 mouse macrophages, the secreted substrate SdeC localizes in the host cytoplasm immediately adjacent to the  
65 polar-localized T4SS<sub>Dot/Icm</sub>. These and other findings established that polar secretion of T4SS<sub>Dot/Icm</sub> substrates is  
66 absolutely required for successful *L. pneumophila* replication and infection of host cells<sup>85</sup>.

67  
68 A recent study using cryo-focused ion beam (cryo-FIB) milling, CryoET, and confocal laser scanning  
69 fluorescence microscopy analyses confirmed and extended these findings by showing that, within the *L.*  
70 *pneumophila*-containing vacuole (LCV) which is the replicative niche for the infecting bacteria, *L. pneumophila*  
71 cells are tethered to the LCV membrane by polar-localized and actively-secreting machines<sup>86</sup>. Moreover, two  
72 proteins, DotU and IcmF, were determined to be responsible for T4SS<sub>Dot/Icm</sub> polar localization<sup>52</sup>. Interestingly,  
73 these proteins are not components of other T4SSs, but instead are TssL and TssM homologs associated with  
74 Type 6 Secretion Systems (T6SSs), which play central roles in early steps of machine assembly. While TssL and  
75 TssM are critical for T6SS assembly, they do not localize at cell poles, suggesting that the T4SS<sub>Dot/Icm</sub>  
76 appropriated these components and further adapted them for spatial positioning of the T4SS<sub>Dot/Icm</sub>.

77  
78 The obligate intracellular pathogen *C. burnetii* also deploys a Dot/Icm system for translocation of effectors into  
79 host cells during infection<sup>87,88</sup>. *C. burnetii* undergoes a biphasic life cycle between a metabolically inactive,  
80 environmentally stable small cell variant (SCV) and a large replicative cell variant (LCV) form within the host.  
81 During its infectious cycle, *C. burnetii* lives within the *Coxiella*-containing vacuole (CCV) that has attributes of a  
82 mature phagolysosome. Recently, *C. burnetii* cells and their polar-localized Dot/Icm machines were visualized  
83 at different developmental states *in situ* by cryo-FIB milling and CryoET imaging<sup>55</sup>. These images showed that  
84 the Dot/Icm complex assembles as the bacteria transition from the infectious SCV to the replicative LCV form,  
85 likely in response to environmental cues within the CCV. Interestingly, besides harboring LCVs and SCVs, the  
86 CCV also contains a population of intermediate-sized cells. On detailed examination of the three morphological  
87 variants, the authors postulated that LCV - SCV - LCV transitions occur rapidly and are characterized by stacking  
88 and unstacking of the IM at specific sites within the cell. These structural rearrangements in the IM are  
89 envisioned to accommodate changes in cell sizes accompanying the LCV to SCV developmental transitions<sup>55</sup>.

90  
91 The R388-encoded T4SS similarly localizes at the poles of *E. coli* cells, but, intriguingly, only upon contact of  
92 donor with recipient cells<sup>89</sup>. In the absence of recipient cell contact, fluorescently-tagged versions of TrwB  
93 T4CP, VirB4-like TrwK ATPase, and TrwC relaxase, are distributed around the cell, but upon donor-recipient  
94 contact, all three proteins relocate to cell poles in areas of close juxtaposition to recipient cells<sup>89</sup>. These  
95 findings indicate that T4SS<sub>R388</sub> components must sense a contact-dependent signal and respond by  
96 redistributing to build the intact T4SS<sub>R388</sub> machine at the mating junction for plasmid transfer.

97  
98 In contrast to the above, the F-encoded T4SS preferentially localizes at the quarter positions of rod-shaped  
99 cells and are excluded from cell poles<sup>90</sup>. Visualization of T4SS<sub>F</sub> spatial organization was achieved with  
100 fluorescently-tagged single-stranded-binding protein Ssb, which binds the T-strand transfer intermediate.  
101 When produced in donors or recipients, tagged Ssb was shown to form fluorescent foci at the mating junction,  
102 reflecting the transient accumulation of the processed T-strand in donors and its delivery to recipient cells.  
103 Interestingly, while T4SS<sub>Fs</sub> localize at the quarter positions of donor cells, T-strands preferentially enter  
104 through the poles of recipient cells, suggestive of specific receptor - ligand interactions that dictate the  
105 formation of mating junctions between distinct sites within donor and recipient cells. These investigators  
106 further defined the time course of protein production by F-encoded genes in new transconjugants, showing  
107 that proteins encoded by genes located in the leading region of the plasmid (the first region to enter new  
108 transconjugants) are produced soon after T-strand acquisition from single-stranded promoters. However,  
109 genes encoding the T4SS machinery (the last region to enter new transconjugants) are produced only from  
110 double-stranded promoters once the T-strand is converted to the double-stranded plasmid. Together, these  
111 elegant studies spatiotemporally choreographed the sequence of events associated with the entire  
112 conjugation process and provided new insights into the interplay between plasmid reestablishment and  
113 plasmid gene expression in new transconjugants<sup>90</sup>.



114  
115 Fluorescent tags also have been used to monitor translocation of protein substrates, such as the VirE2 effector  
116 through the T4SS<sub>Agro</sub> during *A. tumefaciens* infection of plant cells or the CagA oncoprotein through the T4SS<sub>Cag</sub>  
117 into gastric epithelial cells<sup>91-93</sup>. Other types of tags, including adenylate cyclase, TEM  $\beta$ -lactamase, or the Cre  
118 recombinase are also widely deployed in screens aimed at identifying new effectors delivered into bacterial or  
119 eukaryotic cells during infection<sup>94-96</sup>. Besides monitoring substrate flow, tags such as dihydrofolate reductase  
120 (DHFR), ubiquitin or GFP that fold rapidly and are resistant to unfolding have been used to characterize the  
121 substrate processing requirements for transfer through the *L. pneumophila* Dot/Icm system, the T4SS<sub>R388</sub>  
122 conjugation machine, and the *H. pylori* Cag systems<sup>93,97</sup>. Invariably, these studies have shown that unfolding  
123 resistant moieties block transit of the tagged secretion substrates, supporting a conclusion that secretion  
124 substrates are obligatorily unfolded prior to export through T4SSs.

### 125 126 **Supplementary Text Box 2. Translocation signals**

127 T4SS secretion substrates carry sequence or structural signatures marking them for recruitment to the VirD4-  
128 like T4CP for translocation. These translocation signals (TSs) vary considerably and for many substrates are not  
129 yet defined. For conjugative transfer, components of the relaxosome assembled at the origin-of-transfer (*oriT*)  
130 sequence of mobile elements mediate the T4CP interaction. Relaxases responsible for *oriT* nicking to initiate  
131 transfer carry internal or C-terminal TSs. Examples of the former include relaxases associated with the F, R388,  
132 RSF1010 plasmids, which carry conserved TSs in the middle of the protein<sup>98-101</sup>. By contrast, the VirD2 relaxase  
133 associated with the *A. tumefaciens* VirB/VirD4 system possesses a C-terminal TS with an overall hydrophilic  
134 character and enrichment of positively-charged residues<sup>102</sup>. Relaxases also can have a combination of internal  
135 and C-terminal TSs that endow promiscuous transfer of the associated mobile element through different  
136 T4SSs. R388-encoded TrwC, for example, relies on distinct TSs to engage with the R388-encoded TrwB T4CP for  
137 translocation through the T4SS<sub>R388</sub> T4CP or the VirD4 T4CP for translocation through a *Bartonella henselae*  
138 VirB/VirD4 effector translocator system<sup>103</sup>. Other components of the relaxosome also can promote  
139 relaxosome - T4CP docking, as exemplified by the accessory factors TraM, TrwA, and TraK associated  
140 respectively with the F, R388, and pKM101 transfer systems<sup>27,104-106</sup>.

141  
142 Protein substrates of T4SSs also carry distinct types of TSs. These can consist of internal motifs, short C-  
143 terminal charged or hydrophobic motifs, or larger C-proximal domains. Examples of the latter include a  
144 conserved XVIPCD motif associated with toxic effectors translocated through the *Xanthomonas spp.*  
145 interbacterial killing systems<sup>107</sup>. The XVIPCD motif recently was shown to bind the AAD of the VirD4 T4CP<sup>108</sup>. In  
146 *Bartonella spp.*, *Bartonella* effector proteins (Beps) are delivered to eukaryotic cells via C-proximal BID (Bep  
147 intracellular delivery) domains. These domains adopt a conserved structural fold of an extended 4-helix  
148 bundle, which mediates T4CP binding and is structurally similar to C-terminal  $\alpha$ -helical domains of TraM  
149 homotetramers that bind TraD T4CPs in F systems<sup>104,109-111</sup>. Some effectors contain several BID domains, and  
150 some BID domains can function dually by mediating the effector - T4CP interaction, and then upon  
151 translocation, binding protein targets to disrupt cellular functions and aid in infection<sup>112</sup>.

152  
153 In the Dot/Icm system, which to date is the most prolific of the T4SS effector translocators<sup>113,114</sup>, effectors  
154 carry at least three types of TSs. First, effectors such as RalF can harbor C-terminal TSs enriched in hydrophobic  
155 residues. These TSs are thought to mediate contacts with the DotL T4CC independently of the  
156 IcmS/IcmW/LvgA adaptor complexes<sup>115</sup>. Second, effectors such as SidM can harbor TSs consisting of a  
157 negatively charged C-terminal glutamate-rich E-block (EExxE) motif required for docking with the T4CC,  
158 potentially through contacts with the positively charged cytoplasmic domain of DotM<sup>43,116</sup>. Lastly, effectors  
159 such as VpdB, SidH, SetA and PieA, carry a characteristic FxxxLxxxK motif that mediates binding with the  
160 IcmSW-associated LvgA adaptor complex<sup>49,104</sup>. In view of the large numbers of Dot/Icm effectors and the  
161 importance of temporal control in regulating effector translocation, other TSs clearly exist whose  
162 characterization awaits further study.

### 163 164 165 **Supplementary Text Box 3. Diversification of pili or cell surface adhesins for establishment of target cell 166 contacts**

167 There is growing evidence for widespread diversification of T4SS-associated pili or pilins during the evolution  
168 of pathogen-host relationships<sup>104</sup>. *Bartonella spp.* carry two general types of T4SS loci, VirB/VirD4-like systems  
169 responsible for effector translocation and Trw systems that lack VirD4-like proteins<sup>111</sup>. The Trw systems have  
170 the coding capacity for multiple allelic variants of pilin subunits suggestive of production of alternative forms

171 of surface-exposed pilins or pili (Figure 4). It is postulated that the variant pili/ pilins facilitate interactions with  
172 different receptors displayed by erythrocytes, either within the reservoir host population (e.g., different blood  
173 group antigens) or among different reservoir hosts<sup>111</sup>.

174

175 The *H. pylori* T4SS<sub>Cag</sub> also elaborates extracellular appendages, but their assembly and production require  
176 different T4SS components than established for the conjugative pili (Figure 4). Production of Cag pili requires  
177 core VirB-like subunits and Cag-specific proteins, but strikingly does not require the VirB2 pilin-like subunit  
178 CagC or the VirB10-like OMCC subunit CagY<sup>117-119</sup>. Also, in contrast to conjugative pili, several Cag subunits  
179 including a domain of CagY, other Cag proteins (Cagl, CagH, CagL), and even the CagA substrate, have been  
180 shown to localize with these extracellular appendages<sup>71,120-125</sup>. The surface display of CagL, Cagl, CagY, and  
181 CagA appears to be biologically relevant in view of evidence that these subunits bind integrin receptors on  
182 host gastric cell surfaces<sup>122-124,126</sup>. Nevertheless, the high-resolution structural determination of the pilus-like  
183 structures of the Cag system is still lacking and these appendages were not observed to be physically attached  
184 to the Cag machinery<sup>71</sup>. In addition to these pilus-like structures, it is interesting to note that *H. pylori* cells  
185 also produce larger appendages on their cell surfaces called sheathed tubes, which have a size of  
186 approximately 37 nm. However, it remains uncertain whether the assembly of these structures is mediated by  
187 the T4SS<sub>Cag</sub>. A recent study suggests that there may not be a direct correlation between the formation of  
188 membrane tubes and the presence of the Cag system<sup>123</sup>.

189

190 Besides pili or other appendages, many T4SSs alternatively rely on surface adhesins to promote target cell  
191 contacts. In F systems, for example, TraN subunits are not core VirB/VirD4 components but nevertheless  
192 contribute to efficient plasmid transfer and production of F pili. TraN subunits have long been known to  
193 contribute to stabilization of mating pairs<sup>16,127,128</sup>, an activity attributed to a central extracellular domain that  
194 binds outer membrane proteins (OMPs) displayed by recipient cells<sup>42,127,128</sup>. Recently, extracellular domains of  
195 TraN subunits from different F plasmids were shown to adopt distinct structures that confer binding to  
196 different target cell OMPs (Figure 4). In a large-scale analysis of TraN proteins, TraN subunits were assigned  
197 into 4 distinct classes,  $\alpha$ ,  $\beta$ ,  $\gamma$ , or  $\delta$  based on predicted structures of the extracellular domains. Experimental  
198 studies further established that TraN subunits of the  $\alpha$ ,  $\beta$ ,  $\gamma$ , or  $\delta$  classes respectively bind OmpW, OmpK36,  
199 OmpA, or OmpW. Enterobacterial species encode different OMP receptors, leading the authors to propose  
200 that distinct TraN - OMP interactions play important roles in determining the dissemination and, ultimately,  
201 the host ranges of F plasmids among different members of the Enterobacteriaceae<sup>42</sup>.

202

203 In the pKM101 system, the VirB5-like subunit TraC is predicted to form part of the central stalk as shown for its  
204 counterpart in the closely related T4SS<sub>R388</sub> machine (Figure 4)<sup>1</sup>. TraC also is exported to the cell surface  
205 independently of the T4SS<sub>pKM101</sub> machine, where it interacts with another pKM101-encoded protein termed  
206 Pep, resulting in assembly of higher order complexes appearing as distinct foci or patches around the cell<sup>129</sup>.  
207 These cell surface complexes function as adhesins to promote efficient pKM101 transfer through the  
208 T4SS<sub>pKM101</sub>. Thus, a pilus-tip adhesin can be alternatively routed to the cell surface to promote interbacterial  
209 adherence independently of the conjugative pilus.

210

211 Other surface proteins functionally interact with but are not part of the T4SS. In *H. pylori*, at least two OMPs  
212 function as adhesins to facilitate delivery of the CagA substrate through the T4SS<sub>Cag</sub> into gastric cells (Figure  
213 4)<sup>130</sup>. One termed BabA promotes CagA delivery to target cells through binding of blood group antigens<sup>131</sup>.  
214 Another, HopQ, binds a group of host cell receptors designated as carcinoembryonic antigen-related cell  
215 adhesion molecule family (CEACAMs)<sup>132</sup>. Through their capacities to promote *H. pylori* binding and T4SS<sub>Cag</sub>-  
216 mediated CagA translocation to specific host cell types, these and other OMPs are implicated as important  
217 virulence factors related to gastroduodenal diseases.

218

219

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