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Filamentous phage assembly: variation on a protein export theme[1]

Marjorie Russel *, Nora A. Linderoth, Andrej Šali

Box 302, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

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Abstract

Biogenesis of both filamentous phage and type-IV pili involves the assembly of many copies of a small, integral inner membrane protein (the phage major coat protein or pilin) into a helical, tubular array that passes through the outer membrane. The occurrence of related proteins required for assembly and export in both systems suggests that there may be similarities at the mechanistic level as well. This report summarizes the properties of filamentous phage and the proteins required for their assembly, with particular emphasis on features they may share with bacterial protein export and pilus biogenesis systems, and it presents evidence that supports the hypothesis that one of the phage proteins functions as an outer membrane export channel.

Keywords: f1; M13; IKe; Extracellular secretion; type-IV (type-4) pili; Outer membrane channel; Multimeric protein; Vancomycin sensitivity

1. Introduction

The filamentous phage constitute a large family of bacterial viruses that infect a variety of Gram-negative bacteria, using pili as receptors. The best characterized are the very similar phages M13, fd and f1, that infect *Escherichia coli* (*Ec*) via F pili and, to a lesser extent, IKe, which uses N or P pili. The phage particles are rods about 65 Å in diameter and 9000 Å in length, and they contain a circular ss DNA which is extended along the particle axis with an imperfectly base-paired segment that constitutes the packaging signal at one end. The particle is a tube formed by many copies of the major coat protein, with a few copies each of several minor coat proteins located at the ends. The packaging signal end, which is first to emerge from the cell (Webster et al., 1981), contains two proteins (pVII, pIX) necessary for efficient particle assembly, while the other end contains two proteins (pVI, pIII) required for particle stability and phage infectivity. pIII mediates phage attachment to pili and is also necessary for virus uncoating and penetration of phage DNA into the host cell cytoplasm, a process that requires the action of the host TolQ, R and A proteins (Levengood and Webster, 1989; Sun and Webster, 1987; Webster, 1991).

Upon entry into the cytoplasm, host enzymes convert the phage ssDNA to a super-coiled ds form which is the template for rolling-circle replication and for phage gene expression (reviewed in Model and Russel, 1988). Replication is mediated by pII, the phage-encoded site-specific nicking enzyme and host enzymes. Initially, newly synthesized ss are converted to ds form. However, when pV, the phage-encoded ssDNA-binding protein, reaches a sufficient concentration, it binds the ssDNA, thereby sequestering them from the replication proteins. pV forms a dimer in solution at physiological salt concentrations (Bulsink et al., 1988). Thus when it covers the ssDNA it collapses the circular DNA into a flexible rod, leaving the packaging signal exposed at one end (Bauer and Smith, 1988). This complex is the substrate for particle assembly.

The phage genome encodes eleven proteins, of which five are part of the virion, three are required for phage DNA synthesis and three serve assembly functions

* Corresponding author. Tel. +1 212 3278659; Fax +1 212 3277850; e-mail: russelm@rockvax.rockefeller.edu

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Abbreviations: aa, amino acid(s); Ap, ampicillin; Ap^R, Ap resistance marker (gene); DOC, deoxycholate; ds, double strand(ed); *Ec*, *Escherichia coli*; eop, efficiency of plaque formation; *Hi*, *Haemophilus influenzae*; *Ko*, *Klebsiella oxytoca*; LB, Luria broth; Orf (*orf*), open reading frame; p, gene product (protein); *Pa*, *Pseudomonas aeruginosa*; SDS, sodium dodecyl sulfate; ss, single strand(ed); *Vc*, *Vibrio cholerae*; wt, wild type.

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(Model and Russel, 1988). Two of the proteins (pX and pXI) are products of internal translational initiation (within gene *II* and gene *I*, respectively); the smaller proteins, which encompass the C-terminal third of the larger proteins, are required in their own right for efficient particle production (Fulford and Model, 1984; Rapoza and Webster, 1995). An intergenic region that does not code for proteins contains the signals for initiation of synthesis of both the (+) and (−) strands of DNA, for termination of RNA synthesis and the packaging signal.

Filamentous phages do not kill their host, and infected cells continue to grow and divide indefinitely while producing phage. Phage particles do not form in the cytoplasm; rather they are continually extruded or secreted across the bacterial membranes as they are assembled, without causing cell lysis. These properties – and their filamentous morphology – distinguish them from most other bacterial viruses which are icosahedral in shape, accumulate in the cell cytoplasm and accomplish their release from the host cell by lysing it. A consequence of this life style is that all steps subsequent to the formation of the pV-ssDNA complex occur at or in a membrane. All five structural proteins of the virus particle are anchored in the inner membrane prior to their incorporation into phage particles (Endemann and Model, 1995; Ohkawa and Webster, 1981), as are the phage-encoded morphogenetic proteins pI, pXI and pIV. Similarities between these morphogenetic proteins and certain bacterial proteins (d'Enfert et al., 1989, and see below) – and potential mechanistic similarities between filamentous phage assembly, type-IV pilus biogenesis and specialized forms of extracellular protein secretion in bacteria (Russel, 1994b) – account for inclusion of this work in this volume.

1.1. Relationship to bacterial export pathways

Although most proteins that are translocated across the inner membrane of Gram$^-$ bacteria remain in the aqueous compartment between the inner and outer membrane, the periplasm, many bacteria are capable of exporting certain proteins to the external milieu, as they do filamentous phage. Multiple genes, thought to encode a multiprotein export apparatus, are required for this process (reviewed in Hobbs and Mattick, 1993; Pugsley, 1993; Van Gijsegem et al., 1993). In the first pathway to be discovered, the pullulanase export pathway of *Klebsiella oxytoca* (*Ko*), approx. 14 proteins are required for the externalization of a single enzyme (Pugsley, 1993). Homologous systems (called type II) found in other species export different proteins or mediate assembly of type-IV pili. The exported proteins use the standard *sec* system for translocation across the inner membrane and must fold correctly to be translocated to the external milieu (Hardie et al., 1995; Pugsley, 1992; Py et al., 1993). An equally complex secretion system (called type III) translocates at least some proteins directly from the bacterial cytoplasm to the cytoplasm of a eukaryotic cytoplasm (Rosqvist et al., 1994, 1995; Sory and Cornelis, 1994). The proteins that constitute the type-III secretion system are unrelated to the type-II system, with two exceptions: both include a protein with a nucleotide-binding motif, and both require a protein homologous to filamentous phage pIV (Fig. 1). This suggests that the pIV homolog plays a key role that is common to both. As the only integral outer membrane components, the pIV homologs are good candidates for mediating passage of exported proteins across the outer membrane (Pugsley, 1993; Russel, 1991).

1.2. Proteins required for filamentous phage assembly

Thioredoxin is the only host protein known to be required for filamentous phage assembly. Although known as a potent reductant of disulfide bonds in proteins (Lim et al., 1985; Russel and Model, 1985), its redox activity is not necessary for phage assembly. Some *trxA* mutants defective in assembly retain full redox activity, while others lack redox activity yet remain competent for phage assembly (Russel and Model, 1986). The DNA polymerase of phage T7 contains thioredoxin, which functions as a processivity factor (Tabor et al., 1987). Thioredoxin may function similarly in the iterative process of filamentous phage assembly – removing pV from the DNA and/or replacing it by coat protein(s) to promote elongation of the particle (Huber et al., 1986). It remains to be seen whether additional host proteins participate in phage assembly; of particular interest are GenBank sequences from the *Ec* chromosome that bear striking similarity to type-II export and pilus biogenesis genes (including *two* gene *IV* homologs).

The phage-encoded proteins pI, pXI and pIV are required for phage assembly but are not present in the virus particle nor involved in phage DNA synthesis or gene expression (Russel, 1991). pI spans the inner membrane once, leaving an N-terminal domain of approx. 250 aa in the cytoplasm and a C-terminal domain of 75 aa in the periplasm; pXI retains approx. 10 aa of the cytoplasmic domain, which may form an amphipathic helix, plus the membrane spanning and periplasmic domains of pI (Guy-Caffey et al., 1992; Rapoza and Webster, 1995). The individual roles of pI and pXI have not yet been dissected. Genetic analysis suggests that pI acts to initiate assembly by recognizing the packaging signal in phage DNA (Russel and Model, 1989), to bind thioredoxin (Russel and Model, 1983, 1985) and to interact with the major phage coat protein, possibly to promote its incorporation into nascent phage (Russel, 1993). In addition, pI and/or pXI interact with pIV (Russel, 1993). pI/pXI have also been implicated in the formation of phage-specific adhesion zones,

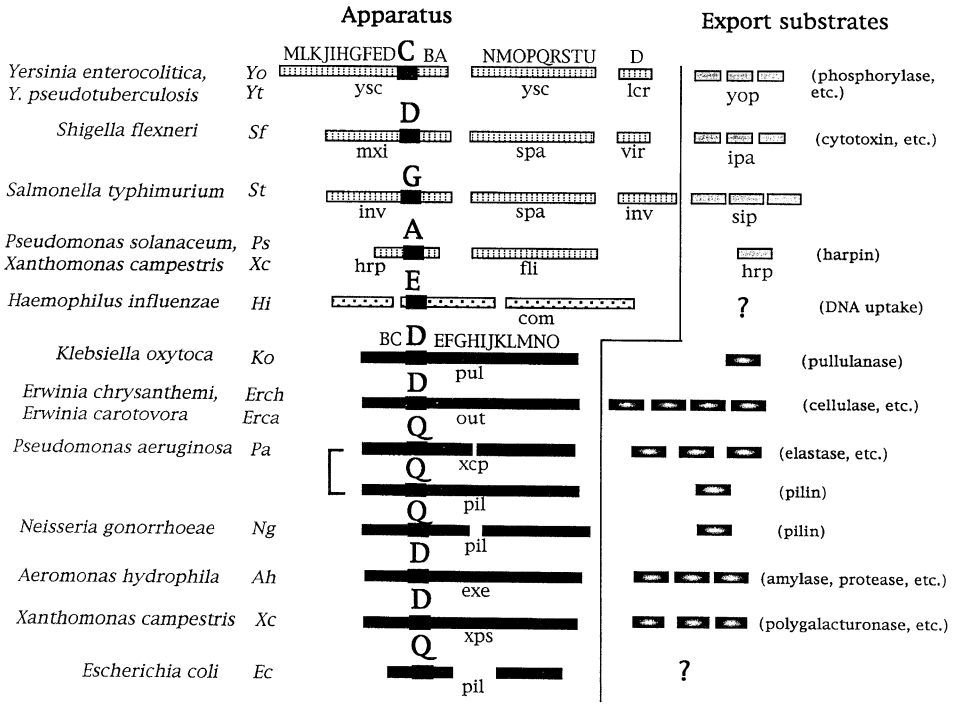

Fig. 1. Diversity of type-II and type-III export pathways. Upper left (stippled segments): type-III genes are organized similarly in different animal and plant pathogens. Lower left (shaded): the type-II genes in some species are clustered in a single operon, and in others they are dispersed (indicated by gaps). *Xc* contains a type-II and a type-III system; *Pa* contains two type-II systems. Genetic competence in *Hi* appears to be distinct from both the type-II and type-III systems. Export substrates (right): The nature and number of proteins exported varies widely, from a single protein (e.g., pilin, the structural protein of type-IV pili) to ≥11 (e.g., Yop, Ipa proteins). The genes (shown by enlarged letters) with homology to gene *IV* are indicated by black bars. The functions of the other export genes, largely unknown, are described in other parts of this volume.

regions in which the inner and outer membranes of *E. coli* are in close contact (Lopez and Webster, 1985). These contact sites, visualized by electron microscopy, may reflect the existence of special assembly sites or exit complexes necessary for phage assembly and extrusion. pI contains a nucleotide-binding motif located in the cytoplasmic domain that is conserved amongst divergent filamentous phages (Russel, 1991). The role of this motif has not yet been determined, but mutation of either of two consensus aa in the motif in f1 gene *I* abolishes pI function (J.-N. Feng, P. Model and M.R., unpublished). When expressed at even moderate levels from a plasmid, pI kills the host; membrane potential is lost and protein synthesis ceases before appreciable pI is synthesized (Horabin and Webster, 1988).

pIV is an outer membrane protein, although its behavior in cell fractionation is somewhat problematic (Russel and Kazmierczak, 1993). Following cleavage of its signal sequence and translocation across the inner membrane, the 43-kDa mature protein is transiently soluble in the periplasm, then integrates into the membrane (Brissette and Russel, 1990). Like outer membrane porins (Sen and Nikaido, 1990), it is secreted when synthesized in spheroplasts, which lack an outer membrane (Russel and Kazmierczak, 1993). Like outer membrane proteins of known structure (Cowan et al., 1992), its C-terminal half is predicted to consist of many short segments of β-sheet. Deletion analysis indicates that this portion of pIV mediates membrane integration (Russel and Kazmierczak, 1993).

An alignment of filamentous phage pIVs, type-II and type-III homologs was generated by the MACAW program (Schuler et al., 1991) (Fig. 2). Although some subsets of these proteins are homologous along their entire length (not shown), a 60-aa block near their C termini is common to all, with shorter blocks scattered over an approx. 200-aa region. The similarities consist largely of short patches (3–4 aa) of hydrophobic aa and conserved Gly and Pro, which suggests structural conservation. Porins consist of many short β-sheet strands that lie perpendicular to the plane of the outer membrane, with 3–4 hydrophobic aa in each strand forming the 'belt' that interacts with the membrane (Cowan et al., 1992). pIV and the homologs – indeed all outer membrane proteins – may be anchored similarly.

With the exception of the relatively small *Haemophilus influenzae* (*Hi*) homolog (required for DNA uptake), size differences between the bacterial proteins (70–80 kDa) and pIVs (35–44 kDa) reflect N-terminal length differences. Interestingly, pIV from the *Ec* phage f1 is more similar to the *Ko* homolog PulD (31.8% identity over 217 aa) than it is to pIV from the *Pseudomonas aeruginosa* (*Pa*) phage pf3 (25.3% identity over 175 aa). Furthermore, although the gene order is

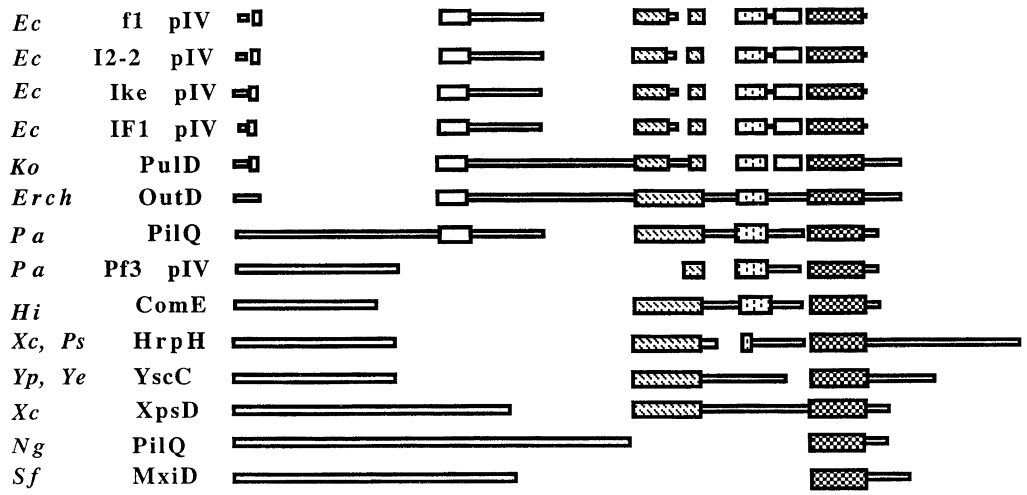

Fig. 2. Multiple alignment of filamentous phage pIV protein and bacterial proteins. Sequences were obtained from GenBank using f1 pIV as a query sequence. Those from other filamentous phage, those known to be required for extracellular secretion, biogenesis of type-IV pili, or genetic competence were aligned using the MACAW program (Schuler et al., 1991). Wide bars indicate homologous regions, narrow bars indicate non-conserved regions and spaces indicate gaps in the alignment.

largely preserved between f1 and pf3, their gene *IV*s are located in different positions (Fig. 3). This suggests that filamentous phages may have obtained their gene *IV*s from an ancestral bacterial host on separate occasions.

2. Results and discussion

2.1. Extreme stability of the pIV multimer

At least some bacterial homologs share certain structural and functional properties with pIV suggesting that this family of proteins may play a common role in distinct export pathways. Like pIV, some type-II homologs (Akrim et al., 1993; Possot et al., 1992; Russel and Kazmierczak, 1993) and a type-III homolog (Wengelnik et al., 1996) induce expression of the *Ec psp* operon (Brissette et al., 1990, 1991), although the mechanism by which they do so is unclear. When extracted from membranes with non-ionic detergents and analyzed by sedimentation or cross-linking, pIV can be detected as a homo-multimer composed of ten to twelve monomers (Kazmierczak et al., 1994). When synthesized in the same cell as pIV, several type-II bacterial homologs form mixed multimers with f1 pIV, suggesting that they also normally exist as homo-multimers (Kazmierczak et al., 1994). Multimeric forms of two homologs analyzed by size chromatography are consistent with a

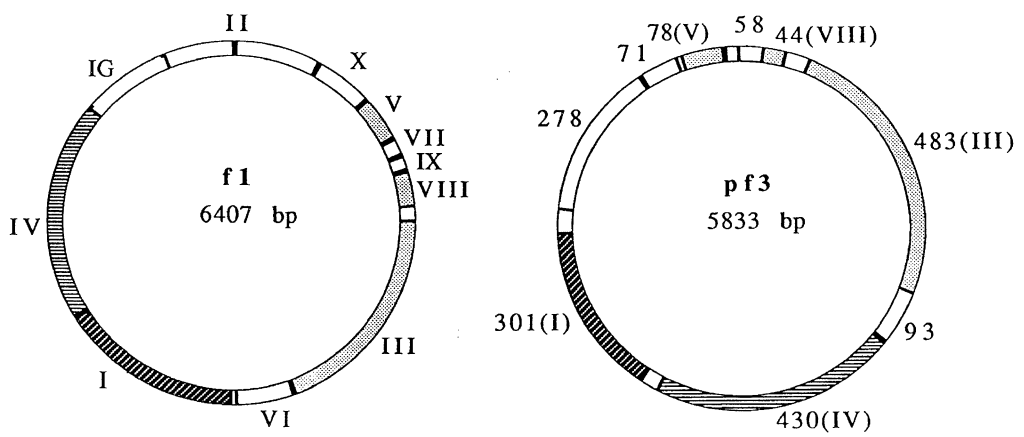

Fig. 3. Comparison of the gene order of divergent filamentous phage. Genes of *Ec* filamentous phage f1 (Hill and Petersen, 1982) and deduced open reading frames of *Pa* filamentous phage pf3 (Luiten et al., 1985) are shown. With two exceptions noted below, these phages have no detectable aa sequence similarity. Putterman et al. (1984) identified the major coat protein (Orf44, pVIII) and ssDNA-binding protein (Orf78, pV) of pf3, and Luiten et al. (1985) suggest that Orf483 is equivalent to pIII. Our identification of *orf430* as gene *IV* is based on homology at its 3′ end (approx. 200-codon span) to gene *IV* of other phage; identification of *orf301* as a gene *I* counterpart is based on the presence of a nucleotide-binding motif (Walker et al., 1982) at its 5′ end, a feature conserved amongst gene *I*s of the *Ec* filamentous phages.

twelve-subunit complex (Chen et al., 1996; Newhall et al., 1980). Recently, a more unusual means of diagnosing multimers has been reported; several of these proteins, including pIV (Linderoth et al., 1996), type-II (Drake and Koomey, 1995; Hardie et al., 1996) and type-III (Plano and Straley, 1995) homologs fail to migrate at their expected monomer position in denaturing (SDS-containing) polyacrylamide gels. Instead, they collect in the well or the stacking gel. This material is not due to aggregation, and its formation requires export from the cytoplasm (Hardie et al., 1996; Linderoth et al., 1996). Extreme treatments (heating to 100°C in 4% SDS, treatment with phenol, or reduction and alkylation) are necessary to dissociate it to the monomer species. Although most wt pIV remains multimeric (i.e., in the stacking gel) after heating to 100°C in protein sample buffer (which contains 4% SDS), pIV with a single aa substitution (P375A) is predominately monomeric and at lower temperatures also migrates as an approx. 90-kDa dimeric species (Fig. 4). Perhaps dimers are a normal intermediate in the assembly pathway of pIV and its homologs. The P^{375} in pIV is within the 60-aa homology block (see above) and is one of two aa conserved in every homolog identified to date. P^{375}-mutant pIV was previously shown to be nonfunctional in phage assembly and defective in multimerization by its inability to be cross-linked (Russel, 1994a). A different, fully functional mutation (S318I) rendered the protein totally resistant to dissociation under all conditions tested (Linderoth et al., 1996).

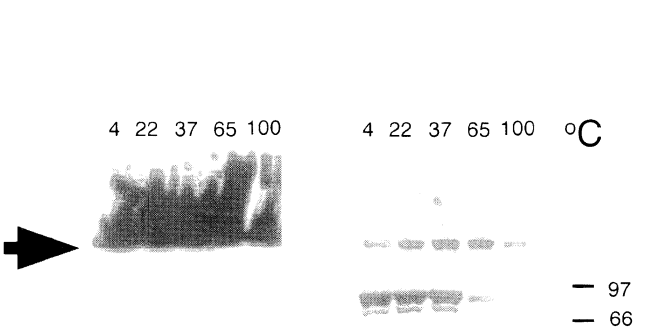

Fig. 4. Stability of wt and P375A mutant pIV multimers to dissociation. The large arrow indicates the boundary between the stacking and running gels; the small arrow indicates the position of pIV monomer. Molecular weight standards (in kDa) are shown on the right margin. **Methods:** Total membranes prepared by osmotic shock of lysozyme-treated (Russel and Kazmierczak, 1993) K38 (HfrC) cells infected by R643 (pIV^+) or R616 (P375A pIV) were solubilized in protein gel sample buffer (4% SDS/20% glycerol/10% β-mercaptoethanol/125 mM Tris · HCl, pH 6.8) and heated at the indicated temperatures for 5 min. Following electrophoresis, proteins were transferred to a nitrocellulose membrane, probed with polyclonal anti-pIV serum, and detected using enhanced chemiluminescence.

2.2. *Size prediction of a putative export channel*

The outer membrane location, multimeric nature and critical role in the export of large macromolecules of members of this protein family suggest that they may form a channel through which proteins or filamentous phage exit the cell. The channels formed by trimeric porins, the only outer membrane proteins whose structures have been determined (Cowan et al., 1992), are too small to permit egress of folded proteins or phage. Because proteins with channel diameters sufficient to accommodate phage or large, folded proteins have not been described and because no structural information about pIV or its homologs is available, an attempt has been made to determine whether a pIV multimer *could* form a channel of the requisite size.

Fig. 5a shows the combined range of minimal and maximal dimensions (in Å) of a set of unrelated proteins from the Brookhaven Protein Structure Database as a function of their length. The pIV monomer is 405 aa long, but only the C-terminal half is involved in membrane association and multimerization. Thus boundary values for a protein of approx. 220 aa were used (32 Å, minimal; 75 Å, maximal). Two arrangements of subunits were considered, one in which monomers contact one another at their smallest dimension, thereby minimizing the channel diameter (shown in Fig. 5b for a 12-mer), and another where contact via the largest dimension maximizes the channel diameter (Fig. 5c). Then channel diameters were calculated using either 32 Å subunits arrayed to minimize the diameter (minimal estimate) or 75 Å subunits arranged to maximize the diameter (maximal estimate). The minimal and maximal channel diameters are plotted as a function of the number of subunits in the multimer (Fig. 5d). This analysis suggests that even if only ten subunits contact one another along their smallest dimension, they could generate a channel large enough for filamentous phage to pass through.

The estimate of pIV size could have used only the dimensions of proteins with a secondary structure content thought to resemble that of the pIV monomer, although this would have involved making an additional assumption. This more restricted subset of proteins would only have increased the estimate of the minimal subunit diameter and thus increased the estimate of the minimal pore diameter.

2.3. *pIV mutants that make* E. coli *sensitive to vancomycin*

The pIV oligomer may function as an exit channel (Kazmierczak et al., 1994), but several observations suggest that its role must be more complex. First, pIV from the filamentous phages f1 and IKe cannot substitute for one another (Russel, 1992), despite the fact that their particles have indistinguishable diameters and

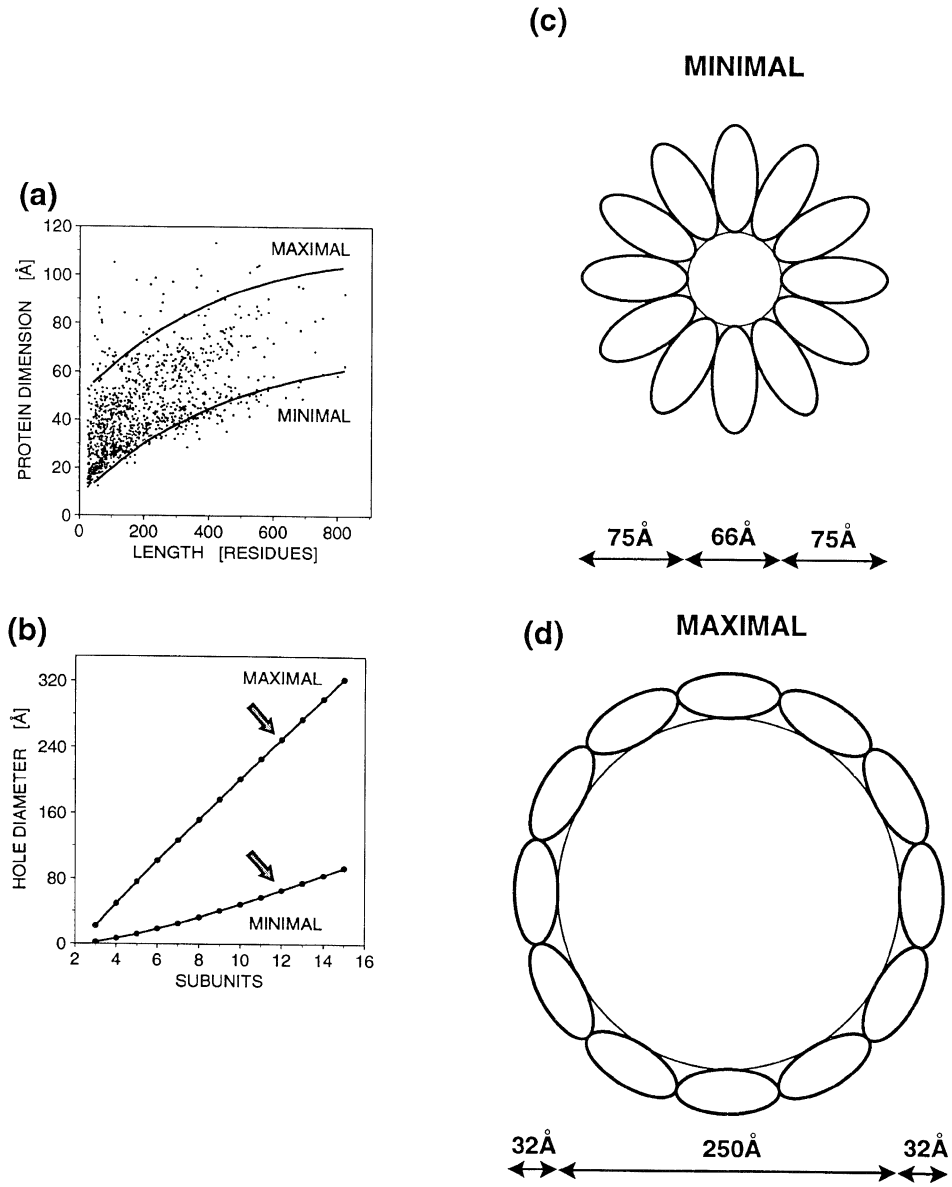

Fig. 5. Modeling the putative pIV channel. (**a**) Minimal and maximal dimensions of protein 3D structures as a function of their chain length. A representative set of 594 unrelated or weakly related proteins (Šali et al., 1995) was obtained from the December 1994 release of the Brookhaven Protein Databank (Abola et al., 1987). The maximal and minimal dimensions were defined as the maximal inter-atomic distances along the longest and shortest inertia axes, respectively. Each protein contributes two points to this plot, corresponding to the minimal and maximal dimensions. The two lines indicate a likely range of protein dimensions as a function of chain length. (**b**) The minimal and maximal diameters of a centro-symmetric pore as a function of the number of identical subunits forming the pore. In this calculation and in panels **c** and **d**, the assumed minimal and maximal diameters of the subunits were 32 and 75 Å, respectively (from panel **a**). These minimal and maximal dimensions were used because the smallest (**c**) and largest pores (**d**) are formed by subunits that are most elongated. The two arrows correspond to the two cases illustrated in panels **c** and **d**. (**c**) The smallest pore consisting of twelve identical maximally elongated subunits. (**d**) The largest pore consisting of twelve identical maximally elongated subunits.

symmetry properties (Makowski, 1983). Second, in the absence of pIV, not even cell-associated (i.e., periplasmic) particles can be detected (unpublished results). In addition, a passive hole in the outer membrane large enough to permit passage of the phage (65 Å in diameter) would almost certainly render *Ec* sensitive to compounds too large or too hydrophobic to cross the outer membrane, and expression of wt pIV does not. For example, the FepA protein forms a gated channel in the outer membrane; synthesis of a mutant FepA (channel diameter approx. 20 Å) that lacks the gating loop (but not wt FepA) makes *Ec* highly sensitive to detergents and other normally impermeant compounds (Liu et al., 1993). Thus if the pIV multimer is an exit channel, its opening must be regulated. Interestingly, expression of plasmid-borne gene *IV* with point mutations affecting

Gly^{355}, an aa conserved in all known bacterial homologs, does make *Ec* sensitive to vancomycin and deoxycholate (Russel, 1994a). Expression of wt pIV does not have this effect. Vancomycin is too large (1449 Da) to penetrate through porin channels (which can accommodate molecules up to approx. 650 Å) and too hydrophilic to diffuse through the outer membrane of normal cells. Perhaps it enters through pIV channels defective in the gating function.

The Gly^{355} mutants are completely non-functional for phage assembly, but more recently isolated mutations at four additional sites in gene *IV* retain the ability to assemble phage (Table 1). In addition to rendering cells vancomycin- and DOC-sensitive, production of some mutant proteins (from a plasmid that also specifies β-lactamase) prevents colony formation on plates containing 100 μg Ap/ml; addition of 20% sucrose to the plates or reduction of the Ap concentration eliminates this effect (not shown). These phenotypes suggest a 'leaky' outer membrane and are consistent with the possibility of a defective pIV gate. Mutants at two positions (N^{295}, S^{324}) lie in a poorly conserved region of pIV; the others (N^{335}, G^{367}) are within the 60-aa conserved block. Most homologs contain Asn (N) at the position equivalent to N^{335} in pIV, while positively charged aa predominate at the position equivalent to G^{367}.

Although pIV from f1 and IKe are not functionally interchangeable, when *both* pI and pIV are exchanged, some heterologous phage are assembled (Russel, 1993). The region of lowest similarity between f1 pIV and IKe pIV is located within the periplasmic portion of these proteins, which forms a distinct, protease-resistant domain (Brissette and Russel, 1990). Furthermore, a mutation in the periplasmic region of pI compensates for a mutation in the periplasmic domain of pIV (Russel, 1993). These observations suggest that pI and pIV interact in the periplasm to promote assembly. This, in turn, suggests a possible mechanism for coupling opening of the putative pIV channel to initiation of phage assembly; binding of the packaging signal (the hairpin that protrudes from the ss DNA-pV complex) to the cytoplasmic domain of pI could cause a conformational change in the periplasmic domain of pI that would enable it to interact with and open the pIV channel (Kazmierczak et al., 1994), as diagramed in Fig. 6. A similar idea has been proposed for the bacterial export systems (Sandkvist et al., 1995). Although there is no

Table 1
Some properties of DOC^S gene *IV* mutants[a]

	IV function[b]	eoc		Radius of vancomycin inhibition (mm)[e]	
		on Ap[c]	on DOC[d]	3 μg Vm/disc	30 μg Vm/disc
		38/30°C	38/30°C		
no pIV	−	1.1	0.9	0	1.5
pIV wt	+	1.0	0.9	0	1.5
N295S	+	$\approx 10^{-3}$	$< 10^{-4}$	5	6
N295I	+	$\approx 10^{-3}$	$< 10^{-4}$	6	8
S324G	+	$\approx 10^{-3}$	$< 10^{-4}$	4	6
S324C	+	1.0	10^{-3}	4	7
S324R	+	$\approx 10^{-3}$	$< 10^{-4}$	4	6
N335S	+ (sm)	$\approx 10^{-3}$	$< 10^{-4}$	4	5
N335I	+ (v. sm)	10^{-3}	$< 10^{-4}$	5	6
G367S	+	0.6	0.01	0	5
G367C	+	0.6	$< 10^{-4}$	0	2

[a]*E. coli* cells (A527, F^+ ($\lambda cI857$)) containing a mutagenized population of gene *IV* were originally isolated by cloning (into the phage λ p_L expression vector pPMR60) gene *IV* that had been amplified under conditions that enhance the error rate. Transformants were screened for sensitivity to DOC. Nucleotide sequence analysis of gene *IV* from twelve sensitive isolates (of 135 screened) showed that each had incurred several different mutations, but that some isolates shared the same mutation (shown in bold); clones containing a single mutation (those that had arisen twice) were constructed in a wt gene *IV* background by mutagenesis with oligodeoxynucleotides that had a degeneracy at a single position (so that more than a single allele could be obtained). Each single mutant retained the DOC sensitivity of the multiply mutant parental gene *IV*-containing strain.

[b]Dilutions of R484 phage (deleted for gene *IV*) were spotted onto 30°C overnight lawns of A527 cells containing the vector (pPMR60, Ap^R), its derivative pPMR112 (gene IV^+ under control of the phage λ p_L promoter), or pPMR112 with the indicated single mutations in gene *IV*, and the plates were incubated overnight at 38°C. +, eop approx. 1; −, eop $< 10^{-5}$; sm, small plaques; v. sm, very small plaques.

[c]Culture dilutions were plated on LB plates with no additions or containing 100 μg Ap/ml, and incubated overnight at 30°C or 38°C. Little, if any, pIV is produced at 30°C, and at 38°C the pIV level is approximately the same as that produced in f1-infected cells. The efficiency of colony formation (eoc) at 38°C versus 30°C was approx. 1 on plates lacking Ap; the cells recovered from these colonies plated at close to unit efficiency on Ap-containing plates at 30°C, indicating that the failure to form a colony at 38°C on Ap-containing plates was not due to plasmid loss.

[d]Culture dilutions were plated on plates containing 1% DOC and incubated overnight at 30°C or 38°C.

[e]Overnight cultures were plated, discs containing the indicated amount of vancomycin were added, and the plates were incubated overnight at 38°C.

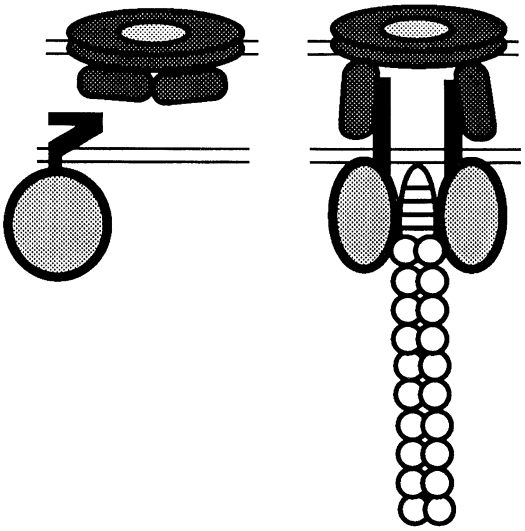

Fig. 6. Model of phage assembly through the exit complex. Binding of the packaging signal, which protrudes from the ssDNA-pV complex, may induce a conformational change in pI that enables its periplasmic domain to bind to the periplasmic domain of the pIV multimer. This, in turn, might stimulate opening of the pIV channel, providing a specific conduit through which the assembling phage could be extruded.

protein with extended sequence homology to pI/pXI in these systems, there is a protein with a nucleotide-binding motif (Hobbs and Mattick, 1993; Pugsley, 1993; Van Gijsegem et al., 1993). These bacterial proteins lack a membrane-spanning segment but are membrane-associated. It has been shown in *Vc* that the protein with this motif is capable of autophosphorylation, and that its membrane association is due to its interaction with an integral inner membrane component (Sandkvist et al., 1995). Thus, these two proteins together could play a similar role to pI. No enzymatic activity has yet been demonstrated for pI (J.-N. Feng and P. Model, unpublished), but it is attractive to think that nucleotide binding and hydrolysis facilitate removal of pV from the DNA and/or addition of coat protein.

3. Conclusions

The ability of pIV multimers (and, implicitly, of a pI/pXI complex) to actually form channels and the ability of pI to interact with the packaging signal, with thioredoxin and with pIV all remain to be tested directly. The biochemistry of this system is rudimentary because essentially all its components are membrane proteins and thus difficult to work with. Nonetheless, the promise that a deeper understanding of the mechanism of filamentous phage assembly and export will be useful for the investigation of assembly and export of proteins that function as virulence factors in so many bacterial species should be a spur to further work.

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References

Abola, E.E., Bernstein, F.C., Bryant, S.H., Koetzle, T.F. and Weng, J. (1987) Protein Data Bank. In: Allen, F.H., Bergerhoff, G. and Sievers, R. (Eds.), Crystallographic Databases — Information, Content, Software Systems, Scientific Applications. Data Commission of the International Union of Crystallography, Bonn/Cambridge/Chester, pp. 107–132.

Akrim, M., Bally, M., Ball, G., Tommassen, J., Teerink, H., Filloux, A. and Lazdunski, A. (1993) Xcp-mediated protein secretion in *Pseudomonas aeruginosa*: identification of two additional genes and evidence for regulation of *xcp* gene expression. Mol. Microbiol. 10, 431–443.

Bauer, M. and Smith, G.P. (1988) Filamentous phage morphogenetic signal sequence and orientation of DNA in the virion and gene V protein complex. Virology 167, 166–175.

Brissette, J.L. and Russel, M. (1990) Secretion and membrane integration of a filamentous phage-encoded morphogenetic protein. J. Mol. Biol. 211, 565–580.

Brissette, J.L., Russel, M., Weiner, L. and Model, P. (1990) Phage shock protein, a stress protein of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 87, 862–866.

Brissette, J.L., Weiner, L., Ripmaster, T.L. and Model, P. (1991) Characterization and sequence of the *Escherichia coli* stress-induced *psp* operon. J. Mol. Biol. 220, 35–48.

Bulsink, H., Harmsen, B. and Hilbers, C.W. (1988) DNA-binding properties of gene V protein encoded by bacteriophage M13. The kinetics of the dissociation of gene V protein, polynucleotide complexes upon addition of salt. Eur. J. Biochem. 176, 589–596.

Chen, L.-Y., Chen, D.-Y., Miaw, J. and Hu, N.-T. (1996) XpsD, an outer membrane protein required for protein secretion by *Xanthomonas campestris* pv. *campestris*, forms a multimer. J. Biol. Chem. 271, 2703–2708.

Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., Jansonius, J.N. and Rosenbusch, J.P. (1992) Crystal structures explain functional properties of two *E. coli* porins. Nature 358, 727–733.

d'Enfert, C., Reyss, I., Wandersman, C. and Pugsley, A.P. (1989) Protein secretion by Gram-negative bacteria. Characterization of two membrane proteins required for pullulanase secretion by *Escherichia coli* K-12. J. Biol. Chem. 264, 17462–17468.

Drake, S.L. and Koomey, M. (1995) The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. Mol. Microbiol. 18, 975–986.

Endemann, H. and Model, P. (1995) Location of filamentous phage minor coat proteins in phage and in infected cells. J. Mol. Biol. 250, 496–506.

Fulford, W. and Model, P. (1984) Gene X of bacteriophage f1 is required for phage DNA synthesis – mutagenesis of in-frame overlapping genes. J. Mol. Biol. 178, 137–153.

Guy-Caffey, J.K., Rapoza, M.P., Jolley, K.A. and Webster, R.E. (1992) Membrane localization and topology of a viral assembly protein. J. Bacteriol. 174, 2460–2465.

Hardie, K.R., Schulze, A., Parker, M.W. and Buckley, J.T. (1995)

Vibrio spp. secrete proaerolysin as a folded dimer without the need for disulphide bond formation. Mol. Microbiol. 17, 1035–1044.

Hardie, K.R., Lory, S. and Pugsley, A.P. (1996) Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein. EMBO J. 15, 978–988.

Hill, D.F. and Petersen, G.B. (1982) Nucleotide sequence of bacteriophage f1 DNA. J. Virol. 44, 32–46.

Hobbs, M. and Mattick, J.S. (1993) Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. 10, 233–243.

Horabin, J.I. and Webster, R.E. (1988) An amino acid sequence which directs membrane insertion causes loss of membrane potential. J. Biol. Chem. 263, 11575–11583.

Huber, H.E., Russel, M., Model, P. and Richardson, C.C. (1986) Interaction of mutant thioredoxins of *Escherichia coli* with the gene 5 protein of phage T7; the redox capacity of thioredoxin is not required for stimulation of DNA polymerase activity. J. Biol. Chem. 261, 5006–5012.

Kazmierczak, B.I., Mielke, D.L., Russel, M. and Model, P. (1994) pIV, a filamentous phage protein that mediates phage export across the bacterial cell envelope, forms a multimer. J. Mol. Biol. 238, 187–198.

Levengood, S.K. and Webster, R.E. (1989) Nucleotide sequences of the *tolA* and *tolB* genes and localization of their products, components of a multistep translocation system in *Escherichia coli*. J. Bacteriol. 171, 6600–6609.

Lim, C.J., Haller, B. and Fuchs, J.A. (1985) Thioredoxin is the bacterial protein encoded by fip that is required for filamentous bacteriophage f1 assembly. J. Bacteriol. 161, 799–802.

Linderoth, N.L., Model, P. and Russel, M. (1996) Essential role of an SDS-resistant protein IV multimer in assembly-export of filamentous phage. J. Bacteriol. 178, 1962–1970.

Liu, J., Rutz, J.M., Feix, J.B. and Klebba, P.E. (1993) Permeability properties of a large gated channel within the ferric enterobactin receptor, FepA. Proc. Natl. Acad. Sci. USA 90, 10653–10657.

Lopez, J. and Webster, R.E. (1985) Assembly site of bacteriophage f1 corresponds to adhesion zones between the inner and outer membranes of the host cell. J. Bacteriol. 163, 1270–1274.

Luiten, R.G.M., Putterman, D.G., Schoenmakers, J.G.G., Konings, R.N.H. and Day, L.A. (1985) Nucleotide sequence of the genome of Pf3, an IncP-1 plasmid-specific filamentous bacteriophage of *Pseudomonas aeruginosa*. J. Virol. 56, 268–276.

Makowski, L. (1983) Structural diversity in filamentous bacteriophages. In: McPherson, A. (Eds.), The Viruses, Vol. 1. Wiley, New York, pp. 204–253.

Model, P. and Russel, M. (1988) Filamentous bacteriophage. In: Calendar, R. (Eds.), The Bacteriophages, Vol. 2. Plenum, New York, pp. 375–456.

Newhall V, W.J., Wilde III, C.E., Sawyer, W.D. and Haak, R.A. (1980) High-molecular-weight antigenic protein complex in the outer membrane of *Neisseria gonorrhoeae*. Infect. Immun. 27, 475–482.

Ohkawa, I. and Webster, R.E. (1981) The orientation of the major coat protein of bacteriophage f1 in the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. 256, 9951–9958.

Plano, G.V. and Straley, S.C. (1995) Mutations in *yscC*, *yscD*, and *yscG* prevent high-level expression and secretion of V antigen and Yops in *Yersinia pestis*. J. Bacteriol. 177, 3843–3854.

Possot, O., d'Enfert, C., Reyss, I. and Pugsley, A.P. (1992) Pullulanase secretion in *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. Mol. Microbiol. 6, 95–105.

Pugsley, A.P. (1992) Translocation of a folded protein across the outer membrane in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 89, 12058–12062.

Pugsley, A.P. (1993) The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. 57, 50–108.

Putterman, D.G., Casadevall, A., Boyle, P.D., Yang, H., Frangione, B. and Day, L.A. (1984) Major coat protein and single-stranded DNA-binding protein of filamentous virus Pf3. Proc. Natl. Acad. Sci. USA 81, 699–703.

Py, B., Chippaux, M. and Barras, F. (1993) Mutagenesis of cellulase EGZ for studying the general protein secretory pathway in *Erwinia chrysanthemi*. Mol. Microbiol. 7, 785–793.

Rapoza, M.P. and Webster, R.E. (1995) The products of gene I and the overlapping in-frame gene XI are required for filamentous phage assembly. J. Mol. Biol. 248, 627–638.

Rosqvist, R., Magnusson, K.-E. and Wolf-Watz, H. (1994) Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. 13, 964–972.

Rosqvist, R., Hakansson, S., Forsberg, A. and Wolf-Watz, H. (1995) Functional conservation of the secretion and translocation machinery for virulence proteins of Yersiniae, Salmonellae and Shigellae. EMBO J. 14, 4187–4195.

Russel, M. (1991) Filamentous phage assembly. Mol. Microbiol. 5, 1607–1613.

Russel, M. (1992) Interchangeability of related proteins and autonomy of function. The morphogenetic proteins of filamentous phage f1 and IKe cannot replace one another. J. Mol. Biol. 227, 453–462.

Russel, M. (1993) Protein-protein interactions during filamentous phage assembly. J. Mol. Biol. 231, 689–697.

Russel, M. (1994a) Mutants at conserved positions in gene IV, a gene required for assembly and secretion of filamentous phage. Mol. Microbiol. 14, 357–369.

Russel, M. (1994b) Phage assembly: a paradigm for bacterial virulence factor export? Science 265, 612–614.

Russel, M. and Kazmierczak, B. (1993) Analysis of the structure and subcellular location of filamentous phage pIV. J. Bacteriol. 175, 3998–4007.

Russel, M. and Model, P. (1983) A bacterial gene, *fip*, required for filamentous bacteriophage f1 assembly. J. Bacteriol. 154, 1064–1076.

Russel, M. and Model, P. (1985) Thioredoxin is required for filamentous phage assembly. Proc. Natl. Acad. Sci. USA 82, 29–33.

Russel, M. and Model, P. (1986) The role of thioredoxin in filamentous phage assembly. Construction, isolation, and characterization of mutant thioredoxins. J. Biol. Chem. 261, 14997–5005.

Russel, M. and Model, P. (1989) Genetic analysis of the filamentous bacteriophage packaging signal and of the proteins that interact with it. J. Virol. 63, 3284–3295.

Šali, A., Potterton, L., Yuan, F., Van-Vlijmen, H. and Karplus, M. (1995) Evaluation of comparative protein modeling by MODELLER. Proteins 23, 318–326.

Sandkvist, M., Bagdasarian, M., Howard, S.P. and DiRita, V.J. (1995) Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. EMBO J. 14, 1664–1673.

Schuler, G.D., Altschul, S.F. and Lipman, D.J. (1991) A workbench for multiple alignment construction and analysis. Proteins Struct. Funct. Genet. 9, 180–190.

Sen, K. and Nikaido, H. (1990) In vitro trimerization of OmpF porin secreted by spheroplasts of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 87, 743–747.

Sory, M.-P. and Cornelis, G.R. (1994) Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol. Microbiol. 14, 583–594.

Sun, T. and Webster, R.E. (1987) Nucleotide sequence of a gene cluster involved in the entry of the E colicins and the single-stranded DNA of infecting filamentous phage into *Escherichia coli*. J. Bacteriol. 169, 2667–2674.

Tabor, S., Huber, H.E. and Richardson, C.C. (1987) *Escherichia coli* thioredoxin confers processivity on the DNA polymerase activity of

the gene 5 protein of bacteriophage T7. J. Biol. Chem. 262, 16212–16233.

Van Gijsegem, F., Genin, S. and Boucher, C. (1993) Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol. 1, 175–180.

Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1, 945–951.

Webster, R.E. (1991) The tol gene products and the import of macromolecules into *Escherichia coli*. Mol. Microbiol. 5, 1005–1011.

Webster, R.E., Grant, R.A. and Hamilton, L.A.W. (1981) Orientation of the DNA in the filamentous bacteriophage f1. J. Mol. Biol. 152, 357–374.

Wengelnik, K., Marie, C., Russel, M. and Bonas, U. (1996) Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. J. Bacteriol. 178, 1061–1069.