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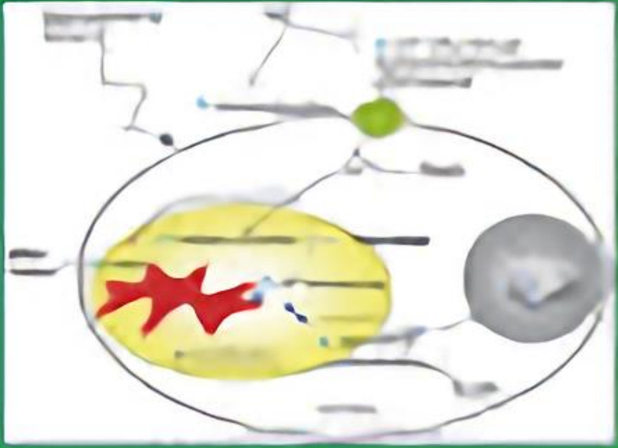

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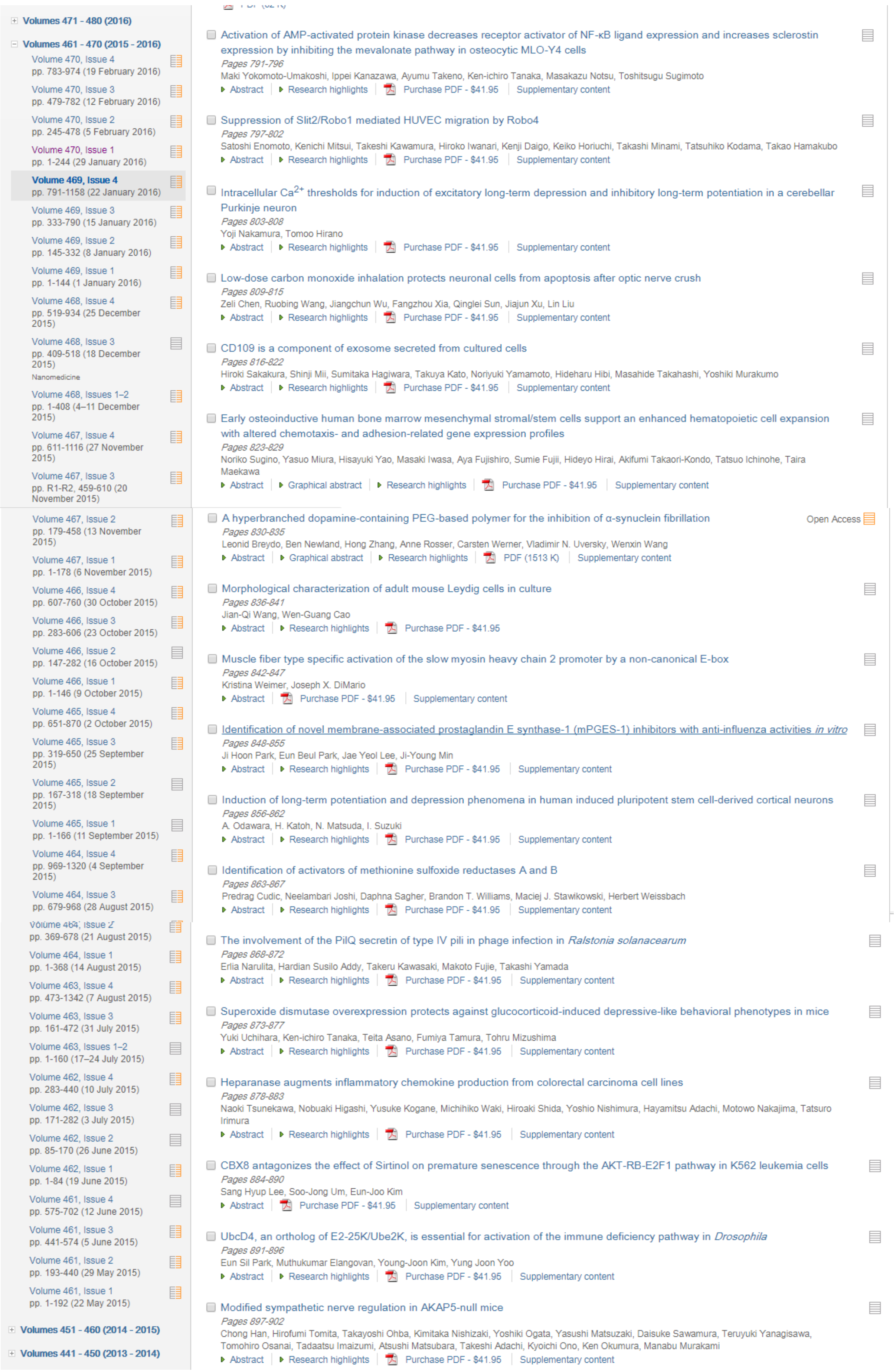
Cover photo. Hypothetical model of the mechanisms involved in AIF processing and release. Exposure of NSCLC cells to the protein kinase C inhibitors, staurosporine or PKC412, results in a hyperpolarization of the plasma membrane. As a consequence, the hyperpolarization-activated HCN2 channel opens and permits Ca^{2+} to enter the cell. Both plasma membrane hyperpolarization and the activation of HCN2 channel are inhibited by Cs^{+}. The resulting Ca^{2+} elevation in the cytosol also translocates to the intermembrane space of the mitochondria and results in the activation of calpain as well as enhanced ROS formation. The calcium chelator, BAPTA is able to inhibit both calpain activation and ROS accumulation, whereas only the latter is inhibited by NAC and Trolox. AIF is cleaved by mitochondrial calpain-I. This cleavage is prevented by PD150606, a selective calpain inhibitor. Cleaved AIF is released into the cytosol and translocates to the nucleus, where it contributes to chromatin condensation and highmolecular weight DNA fragmentation. Nuclear translocation of AIF can be inhibited by binding to Hsp70 in the cytosol. (BBRC Volume 396, pages 95–100). It is reproduced by kind permission of the authors – Sten Orrenius, et al.



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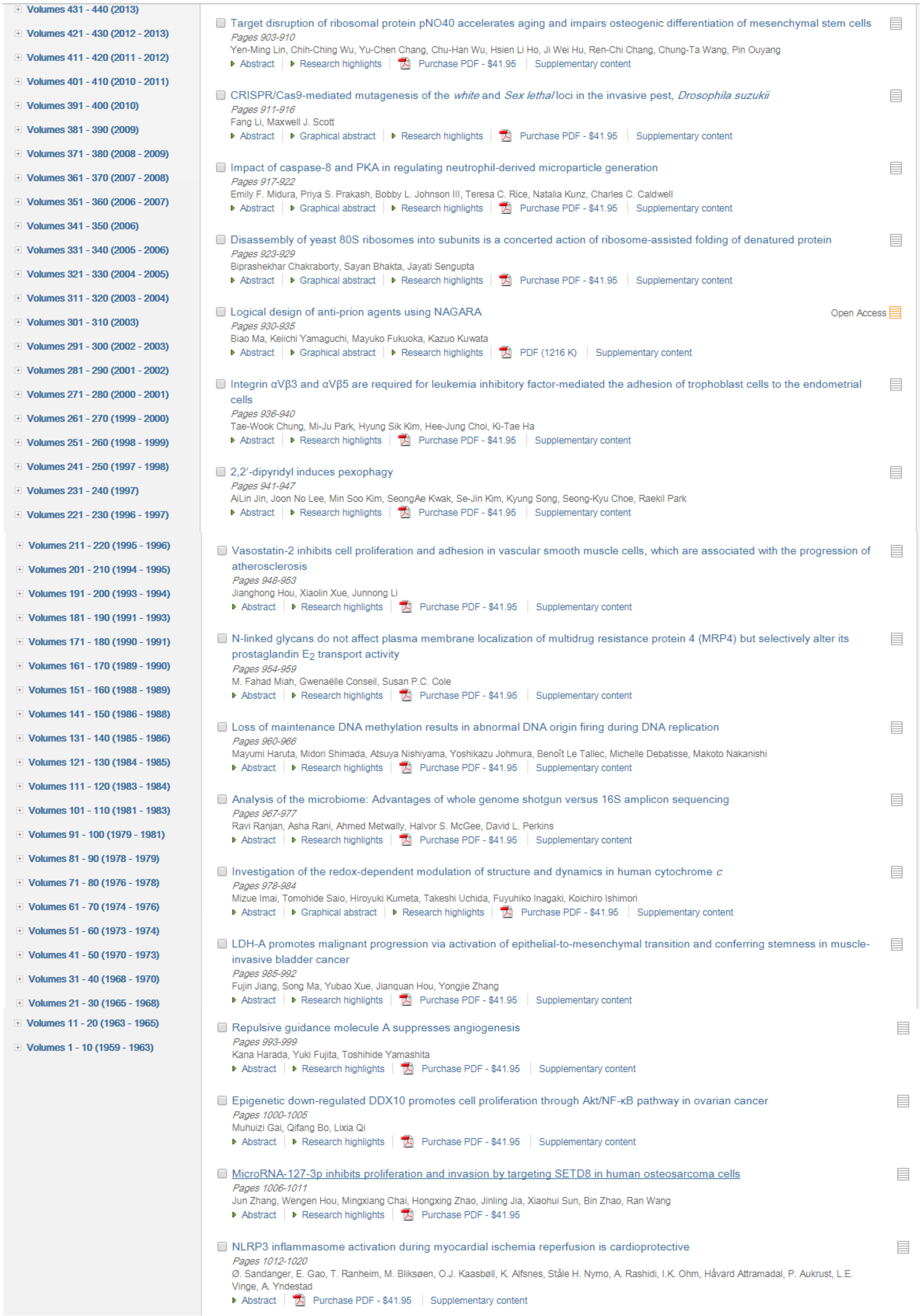

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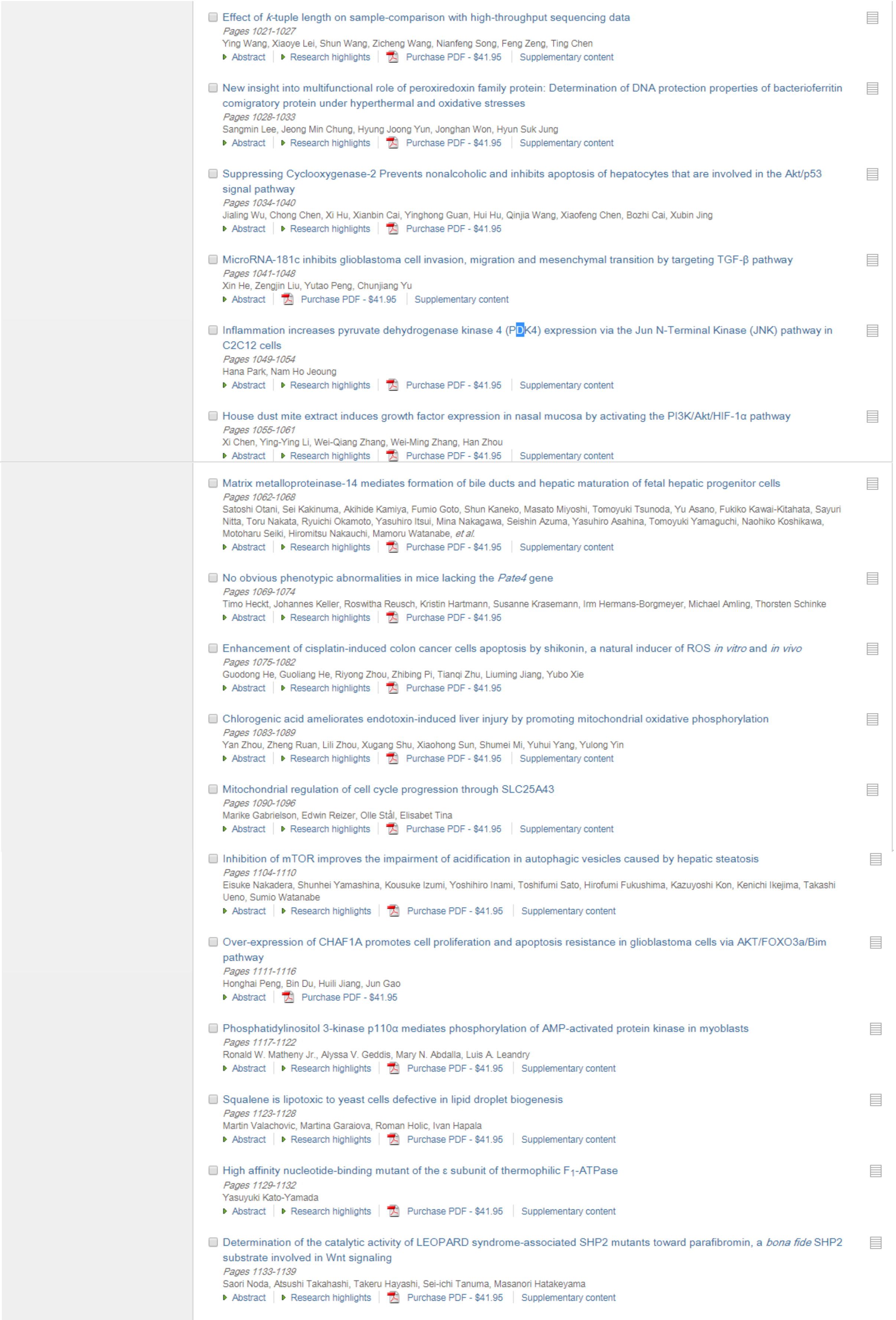

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The involvement of the PilQ secretin of type IV pili in phage infection in *Ralstonia solanacearum*

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ABSTRACT

PilQ is a member of the secretin family of outer membrane proteins and specifically involved in type IV secretion. Here we report the effects of *pilQ* mutation in *Ralstonia solanacearum* on the host physiology including susceptibility to several phage types (*Inoviridae, Podoviridae* and *Myoviridae*). With three lines of cells, namely wild type, Δ*pilQ* and *pilQ*-complemented cells, the cell surface proteins, twitching motility and sensitivity to phages were compared. SDS-PAGE analysis revealed that the major TFP pilin (PilA) was specifically lost in *pilQ* mutants and was recovered in the complemented cells. Drastically inactivated twitching motility in *pilQ* mutants was recovered to the wild type level in the complemented cells. Several phages of different types including those of *Inoviridae*, *Podoviridae*, and *Myoviridae* that infect wild type cells could not form plaques on *pilQ* mutants but showed infectivity to *pilQ*-complemented cells. These results indicate that *PilQ* function is generally required for phage infection in *R. solanacearum*.

1. Introduction

Ralstonia solanacearum is a gram-negative plant pathogen that forms 3–6 nm (in diameter) filaments on the cell surface, called type IV pili (Tfp) [1–3]. Tfp is important as a virulence factor in pathogenic bacteria for cell adhesion, aggregation, biofilm formation, horizontal gene transfer, multicellular development, pathogenesis, and twitching motility [4,5]. In gram-negative bacteria, the Tfp system requires at least 35 *pil* genes for the synthesis, display, and function of polar and retractable Tfp, including *pilA*, *pilB*, *pilC*, *pilQ*, and *pilT* [6,7]. The *pilA* gene encodes a 17 kDa monomer of major pilin protein [8]. PilB is required for pilus extension while *pilC* is an inner membrane protein that might facilitate pilin translocation [5,9]. PilT encoded by the *pilT* gene is required for pilus retraction [3]. PilB and PilT are ATPases acting antagonistically [10]. We addition, *pilQ*, encoded by *pilQ*, is a 50–58 kDa secretin [11,12] located in the outer membrane and acts as a gated-channel for which the pilin subunits (PilA) extrude into extracellular milieu [3].

On the other hand, Tfp is also important as a receptor site for some bacteriophages [13] such as ϕRSM of *R. solanacearum* [14], VGJϕ and CTXϕ of *Vibrio cholera* [15,16], phage IF1 of *Escherichia coli* [17], and XacF1 of *Xanthomonas axonopodis* pv. *citri* [18]. In the case of *R. solanacearum*, strains were generally separated into two groups based on the TFP type that was differentially recognized by different phages such as ϕRSS and ϕRSM [14]. All of these phages are filamentous and belong to the family *Inoviridae*. However, other types of phage recognize and bind to different molecules on the host cell surface as receptors such as outer membrane proteins, lipopolysaccharides (LPS), teichoic acids, etc. For example, T4 phage (*Myoviridae*) binds to LPS as its receptor [19] and λ phage (*Siphoviridae*) binds to an outer membrane protein lamB of *E. coli* [20]. R phage and related 7 phages (*Podoviridae*) bind to different parts of the LPS core on *Yersinia pestis* [21]. LPS was also suggested as receptors for *Ralstonia* phages such as ϕRSA1 (*Myoviridae*) [22], ϕRSB1 (*Podoviridae*) [23], and ϕRSL1 (*Myoviridae*) [24].

In this study, we reported the effects of disruption of Tfp porin (PilQ) in *R. solanacearum* on the host susceptibility to various phage types (*Inoviridae*, *Podoviridae* and *Myoviridae*).

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2. Materials and methods

2.1. Bacterial strains, phages, media, and growth conditions

R. solanacearum strains were obtained from the National Institute of Agrobiological Sciences, Japan and several types of bacteriophage were from the collection of Laboratory of Biomolecular Technology, Grad. Schl. of ADSM, Hiroshima University, Japan (Table S1). The bacterial cells were cultured in casamino acid-peptone-glucose (CPG) medium [25] at 28 °C with shaking at 200–300 rpm. All phages were routinely propagated with appropriate host strains. An overnight culture of bacterial cells grown in CPG medium was diluted 100-fold with 100 ml fresh CPG medium in a 500 ml flask. To collect sufficient amounts of phage particles, a 500-mL bacterial culture was grown. When the culture reached 0.1 units at OD_{600}, the phage was added at a multiplicity of infection (moi) of 0.01–0.05. After further growth for 16–18 h, the cells were removed by centrifugation at 8000 × g for 15 min at 4 °C (R12A2 rotor, Hitachi Himac CR21E centrifuge). The supernatant was passed through a 0.45-μm membrane filter, and then phage particles were precipitated by addition of 0.5 M NaCl and 5% polyethylene glycol 6000. Phage preparations were stored at 4 °C until use.

2.2. Isolation and characterization of nucleic acids from bacteria

Standard molecular biological techniques for DNA isolation, digestion with restriction enzymes and other nucleases, and construction of recombinant DNAs were performed according to [26]. Genomic DNA was isolated from the purified phage particles by phenol extraction. In some cases, extrachromosomal DNA was isolated from phage-infected *R. solanacearum* host cells by the mini-preparation method [27].

2.3. Construction of a disruption mutant of pilQ::Kan and complementation test

A 2.2-kbp fragment of *pilQ* was PCR amplified from the MAFF 106603 genomic DNA using primers: forward 5′-TACCTCTAGAGACCCTGAAAGTTCAGGAGGGCGG-3′, and reverse 5′-TACCTCTAGACTTCAGCGACAGCTGGTCGGACAG-3’. The amplimers were ligated into the *Eco*RV site of pBlueScript II-SK+ (Toyobo Biochemicals, Tokyo, Japan) to generate pSKP. To a unique *Stu*I site within the *pilQ* coding region of pSKP, a 1.3-kbp Kan resistance cassette (cleaved from pUC4-KIXX by digestion with *Sma*I [28], was inserted to create pSKP-Kan. The plasmid was introduced into strains MAFF 106603 and MAFF 730138 by electroporation to disrupt *pilQ* by homologous recombination. pSKP-Kan cannot replicate in *R. solanacearum*, so that disruptants containing pilQ:-Kan could be selected on CPG plates containing kanamycin (50 μg/ml). Inactivation of *pilQ* in strains MAFF 106603 and MAFF 730138 was checked by the criterion of forming colonies that had lost twitching motility and also by SDS-PAGE of cell surface proteins for lacking PilA protein. For complementation test, we transformed a pRSSTG-PilQ plasmid into the electrocompetent pilQ:Kan strains by electroporation technique as described by Ref. [22]. The pRSSTG-PilQ plasmid was constructed from pRSS-TG (carrying both tetracycline resistant and Green Fluorescent Protein genes) [28]. A full-length *pilQ* fragment described above was inserted into the *Sma*I site of pRSSTG. Purified-plasmid was introduced into cells of PilQ:Km strains by electroporation with a Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA) with a 2-mm cell at 2.5 kV in accordance with the manufacturer's instructions. Transformants that produce Green Fluorescent Protein (GFP) were selected on CPG plates containing 50 μg/ml kanamycin and 12 μg/ml tetracycline.

2.4. Twitching motility and SDS-PAGE analysis

To investigate the twitching motility, bacterial cells (1×10^4 CFU/ml) were spotted (initial spot diameter, 4 mm) on the surface of minimal medium (MM) [0.175% K_2HPO_4, 0.075% KH_2PO_4, 0.015% sodium citrate, 0.025% $MgSO_4.7H_2O$, 0.125% $(NH_4)_2SO_4$, 0.5% glucose, and 1.5% agar] plates that were air dried prior to the spotting. Petri dishes were then incubated at 28 °C and the diameter of the spots were measured daily for 2 days. The twitching activity was examined by placing a petri dish without its lid on the stage of an upright light microscope (Olympus CKX41) equipped with 4 × and 10 × objectives.

Extracellular structure proteins were isolated from 24-h-old bacterial cells grown on solid MM [29]. Cells were suspended in 10 mM Tris–HCl (pH 8.0) buffer, and surface structures were shaved from the cells by passing the cell suspension through a 25-gauge needle. Bacterial cells were removed by centrifugation at 6000 × g for 20 min at 4 °C (R12A2 rotor, Hitachi Himac CR21E centrifuge). The bacterial surface proteins were collected by ultracentrifugation at 136,000 × g for 60 min (P50S2 rotor, Hitachi Himac CP80WX centrifuge). The precipitates were separated by Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) according to [30]. For total bacterial proteins, cells were lysed by using sonication and subjected to SDS-PAGE.

2.5. Phage susceptibility and absorption assays

Phage susceptibility was assayed by spotting onto bacterial lawn on CPG agar with the phage suspension adjusted to contain 10^8 PFU per spot. Clear zone formation for susceptibility was observed after incubation at 28 °C for 24–48 h.

Phage adsorption was assayed as described by Ref. [18]. The exponentially growing cells (OD_{600} 0.1) of the test strains were mixed with bacteriophage at multiplicity of infection (moi) of 0.01, and the mixture was incubated for 0 min (no adsorption) and 30 min at 28 °C. To collect non-adsorbed phages, mixture was centrifuged at 15,000 × g for 5 min at 4 °C in a Sakuma M150-IV microcentrifuge (Sakuma Seisakusho, Tokyo, Japan) followed by filtration using 0.45 μm membrane filter. The phage titer in the supernatant was determined by a standard plaque assay with MAFF 106603 and MAFF 730138 as indicator strains.

3. Results

3.1. Characteristics of pilQ mutants of R. solanacearum strains MAFF 106603 and MAFF 730138

We constructed ΔpilQ mutants in two strains of *R. solanacearum*, MAFF 106603 and MAFF 730138 (showing different phage host ranges), to examine roles of TFP in the interaction between phages and the host. The growth rates of both mutants in CPG liquid culture measured by optical density at OD_{600} were almost similar to those of the wild-type strains (data not shown), indicating the *pilQ* deficiency did not affect the bacterial growth under the experimental conditions. Compared with wild-type strains, these mutants formed smaller and less viscous colonies on CPG plates.

R. solanacearum cells show twitching motility by function of Tfp [3]. When the bacterial cell suspensions ($OD_{600} = 0.1$, 2 μl) of wild types and pilQ:Kan mutants were dropped onto the surface of minimum agar medium and incubated for 48 h, the wild-type strains formed colonies with thin edges and irregularly shaped spearheads, showing active twitching motility, whereas the both mutants pilQ:Kan MAFF 106603 and pilQ:Kan MAFF 730138 formed colonies with smooth-colony edge and lacking spearheads (Fig. 1). These morphological aspects observed for the mutant colonies corresponded to those of *pilQ* mutant of K60, which lacked Tfp and did not twitch [3]. This deficiency of twitching motility in the mutant cells was recovered to the wild-type in both pilQ:Kan MAFF 106603 and pilQ:Kan MAFF 730138 when the complementary plasmid pRSSTG-PilQ was introduced into the mutant cells (Fig. 1).

In the next step, we confirmed changes on the cell surface structural components caused by the *pilQ* mutation. Cell surface structural proteins were prepared as described in Material and methods. As presented in Fig. S1, SDS-PAGE protein separation patterns showed that the pilQ mutant (pilQ:Kan MAFF 106603) lacked major component of type IV pili (pilA), compared with the wild type [31]. Almost the same result was obtained in the experiments where MAFF 730138 and pilQ:Kan MAFF 730138 were compared. These results indicated that the cells of *pilQ* mutants could not form Tfp on the cell surface.

3.2. Infection of filamentous phages on pilQ mutants of R. solanacearum

Two groups of filamentous phages (ϕRSS and ϕRSM) are known to infect strains of *R. solanacearum* [23,32,33]. In each group, phages are separated into two types based on the host range represented by ϕRSM1-type and ϕRSM3-type that contain a different pIII receptor binding protein and differentially recognize host strains [14]. Therefore, “two different types of Tfp” were suggested for receptors of these phages. Strains MAFF 106603 and MAFF 730138 serves as the host for ϕRSM3 and ϕRSM1, respectively but not vice versa, and are expected to have different types of Tfp. When Δ*pilQ* mutants of these strains were tested for infection by ϕRSM1 and ϕRSM3, either mutant could not be infected by these phages (Fig. 2). However, complemented mutants with pRSSTG-PilQ showed the wild-type host range. Same results were obtained in the case of ϕRSS1 (infective to MAFF 106603 and not infective to MAFF 730138) and ϕRSS2 (infective to MAFF 730138 and not infective to MAFF 106603) infection. These results indicated that “two different types of Tfp” are dependent of the PilQ function. Most likely ϕRSM1 and ϕRSM3 selectively recognize minor components of pilins.

3.3. Infection of several types of phage on pilQ mutants of R. solanacearum

The *pilQ* mutants provided an opportunity to examine changes in physiological states of the cells, especially interaction with various phages. Wild-type, *PilQ* mutants and *PilQ*-complemented strains of MAFF 106603 and MAFF 730138 were subjected to phage susceptibility tests against ϕRSA1 (myovirus), ϕRSB1 (podovirus), ϕRSB3 (podovirus), ϕRSJ2 (podovirus), ϕRSJ5 (podovirus), and ϕRSL1 (myovirus). In the case of MAFF 106603, wild-type cells were infected by 7 of 10 phages, but unexpectedly the *pilQ* mutant showed resistance to all the phages (Fig. 2A). *pilQ*-complemented mutant cells recovered to the wild-type host range. Also in strain MAFF 730138, 8 of 10 phages infected to the wild-type cells, but no phage infected to the *pilQ* mutant (Fig. 2B). The *pilQ*-complemented mutant showed the wild-type host range. These results indicated that pilQ was also important for various kinds of phages to infect *R. solanacearum* cells. In the case of filamentous phages such as ϕRSS and ϕRSM, Tfp serves as a receptor so that deficiency of Tfp results in no attachment of phage particles to the cells. To see the effects of *pilQ* mutation on the attachment of these different kinds of phage, phage adsorption rates were determined by a standard method described in Materials and methods. The results shown in Table 1 indicated that no significant changes occurred in adsorption rates for each phage between the wild type and *pilQ* mutant cells in either MAFF 106603 or MAFF 730138.

4. Discussion

4.1. Tfp as a phage receptor

In addition to various phages of *Inoviridae*, several phages of

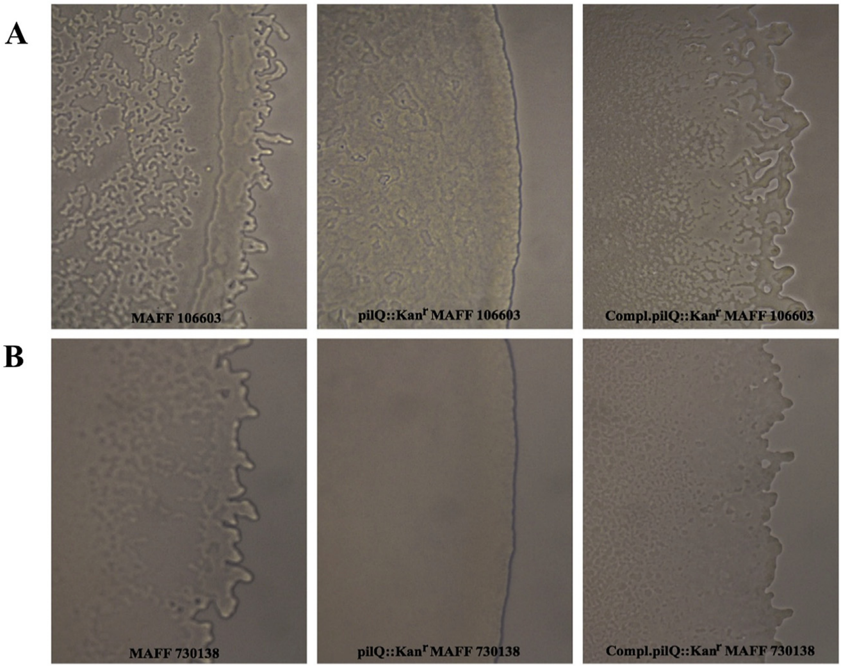

Fig. 1. Twitching motility of *R. solanacerum* cells. **(A)** Cells of strain MAFF 106603 (wild type, Δ*pilQ* mutant and Δ*pilQ* mutant complemented with a *pilQ* plasmid) and **(B)** strain MAFF 730138 (wild type, Δ*pilQ* mutant and Δ*pilQ* mutant complemented with a *pilQ* plasmid. Twitching motility was observed under a microscope 2 days post-inoculation (dpi) on the twitching plates.

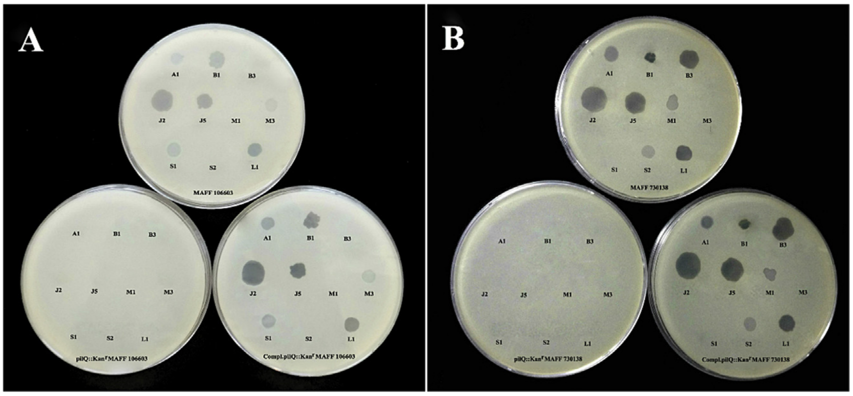

Fig. 2. Infectivity of various phages to *R. solanacearum* cells. Cells of wild type, Δ*pilQ* mutant and Δ*pilQ* mutant complemented with a *pilQ* plasmid in two strains MAFF 106603 **(A)** and MAFF 730138 **(B)** were tested for susceptibility to phages (Table 1). None of the phages tested were able to infect Δ*pil*Q mutants (either in MAFF 106603 **or** MAFF 730138).

Table 1
Phage adsorption to cells of wild-type and ΔpilQ mutant.

Phage	MAFF 106603	pilQ::Kan MAFF 106603	MAFF 730138	pilQ::Kan MAFF 730138
ΦRSA1	16.99 ± 1.10	16.77 ± 1.36	18.16 ± 1.74	18.06 ± 1.49
ΦRSB1	12.97 ± 1.90	12.82 ± 1.13	14.37 ± 2.93	14.22 ± 1.31
ΦRSJ2	63.24 ± 1.52	62.05 ± 1.67	65.15 ± 2.63	65.30 ± 2.39
ΦRSJ5	52.79 ± 3.01	52.64 ± 2.16	54.41 ± 3.20	54.26 ± 1.32
ΦRSL1	22.43 ± 1.78	22.85 ± 1.55	25.59 ± 1.19	25.70 ± 2.49

*Values are mean ± SD of three independent experiments.

other families are known to use Tfp as a phage receptor, including *Pseudomonas aeruginosa* phages such as PP7 (levivirus) [34], PO4 (siphovirus) [35], F116 (siphovirus) [36], and D3112 (siphovirus) [37], and *Xylella fastidosa* and *Xanthomonas* spp. phages Sano (siphovirus), Salvo (siphovirus), Prado (podovirus), and Paz (podovirus) [38]. In all of those cases, phages attached to the host cells via Tfp. Therefore, if a *pilQ* mutation leading to deficiency of Tfp formation like in this study occurs in the host cells, those phages are expected to lose infectivity. Contrasting to those cases, most phages used in this study (except for inoviruses) recognize LPS as a primary receptor [39]. For all of these phages, host adsorption rates were not significantly changed when compared between the wild-type cells and *pilQ* mutant cells (Table 1). Therefore, involvement of PilQ (Tfp) suggested in the infection process of these phages should be in a different way.

4.2. *PilQ secretin involved in DNA uptake*

Besides various biological functions of Tfp, its role in the transport of DNA from the extracellular milieu into cytoplasm is well known [40]. Especially, the secretin PilQ functioning in Tfp biogenesis is characterized to be involved in DNA uptake. The central cavity in the PilQ 12-mer, with a diameter of 6.5 nm could easily accomodate the DNA double helix (~2.4 nm), either by itself or in a nucleoprotein complex [41]. Recent electron microscopic observation revealed that the PilQ complex of *Thermus thermophilus* HB27 is 15 nm wide and 34 nm long and consists of an extraordinary stable "core" and "cup" structure and five ring structures with a large central channel [42]. The PilQ complex was found to span the entire cell periphery. Therefore, PilQ can mediate DNA transport across the outer membrane and periplasmic space in a single-step process. It was also suggested in *Neisseria meningitidis* that transforming DNA is introduced into the cell through the outer-membrane channel formed by the PilQ complex, and that DNA uptake occurs by non-specific induction of DNA coupled to pilus retraction, followed by presentation to DNA-binding component(s), including PilQ [43].

4.3. *Possible roles of PilQ in phage infection*

In this study, it was shown that Δ*pilQ* mutants of two different strains were converted to be resistant to phages of different families including *Myoviridae*, *Podoviridae* as well as *Inoviridae* (Fig. 2). Most of such myoviruses and podoviruses recognize LPS on the cell surface as a receptor and adsorbed normally to the cells of Δ*pilQ* mutants (Table 1). Therefore, infection processes after cell adsorption were somehow blocked in these pilQ mutants. Phages inject their DNA into the host cytoplasm. The classical "syringe model" is not enough to explain the mechanism and several sources of energy are suggested [44]. Many phages lack a tail long enough to span the cell envelope, and simple injection by a syringe model would result in the phage genome being deposited in the extracellular medium or in the cell periplasm. As described above, PilQ can mediate DNA transport across the outer membrane and periplasmic space in a single-step process. To our knowledge, this is the first clear demonstration of involvement of PilQ in phage infection. It is reasonably understood that many phages use Tfp (associated with pilQ) as a receptor on the host cells.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.12.071.

Transparency document

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