



Procedia

Environmental Sciences

The 4th International Conference on Sustainable Future for Human Security
SUSTAIN 2013

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4th International Conference on Sustainable Future for Human Security, Sustain 2013

Editorial



The 4th International Conference on a Sustainable Future for Human Security (SUSTAIN 2013) was held at Kyoto University (Japan) on 19-21 October, 2013. The conference was organized by Sustain Society and the Indonesian Students Associations of Kyoto, with the support of the Organization for the Promotion of International Relations (OPIR) Kyoto University, Research Institute for Sustainable Humanosphere (RISH), Global Center for Education and Research on Human Security Engineering (HSE), Global COE Program for Sustainability / Survivability Science for a Resilient Society Adaptable to Extreme Weather Conditions (GCOE-ARS), and Inter-Graduate School Program for Sustainable Development and Survivable Societies (GSS).

The conference originated from the need to provide an inter-disciplinary forum where the most serious problems affecting a sustainable future for human security could be discussed, in recognition of the fact that many future problems cannot be solved by a “siloeed” approach. The emphasis on sustainable futures is in response to the general awareness of the need to solve numerous human-related problems resulting from the rapid growth of modern society. The topic of sustainable futures for human security needs to be discussed in an integrated way, in accordance with the principles of sustainability, considering energy and materials supply, economics and trade, technology, cities, agriculture, social and environmental aspects.

To continue providing adequate technology to cope with the demands of human quality of life requires intensive research and development with multidisciplinary perspectives. Research and development towards achieving future human security should embrace sustainability perspectives, to avoid negatively impacting the environment and necessitating or exacerbating inefficient use of natural reserves, increasing emissions and hazardous wastes and jeopardizing human health and society.

The conference covered a wide range of issues with the aim of highlighting potential issues and paths towards a sustainable future. It attracted a high level of attendance from countries of the global North and South, with a wide geographical coverage. Overall, 160 participants were involved, with 120 presentations over the course of the conference. The quality of papers received was a testament to the reputation that the conference has been building over the past 3 years.

Papers presented at SUSTAIN 2013 were divided into five thematic areas: (1) Energy and Environment (EnE); (2) Sustainable Forestry and Agriculture (FA); (3) Sustainable Built Environment in Tropical Hemisphere Countries (BE); (4) River Basin and Disaster Management (RnD); (5) Social Science and Economics (SE). Under these broad areas, a wide-ranging series of presentations was given, which elaborated on current research across Asia and the world. Being held in Kyoto, a city of great cultural heritage, the participants also took part in a tour of some of the main sights and experiences that link modern and ancient Japan.

The two programmed days of the conference each commenced with keynote presentations which, like the conference itself, were wide-ranging. In the first session on day one, Dr. Ir. Edi Effendi Tedjakusuma, delivered an address on issues of a sustainable future for human security in the context of Indonesia. Dr. Puppim de Oliveira, Assistant Director and Senior Research Fellow at the United Nations University Institute of Advanced Studies (UNU-IAS), then discussed the future sustainability of cities in Asian nations. In the last keynote, Professor Satoshi Fujii, a Japanese cabinet adviser on Disaster Prevention and Reduction, introduced Japanese policy towards a more resilient country.

More than 230 participants attended the conference from 23 countries in Asia, North America and Europe. Around 161 papers were presented in the two days of conference. Only selected papers will be published in the *Procedia Environmental Science* and a special issue of the *International Journal for Sustainable Futures for Human Security (J-SUSTAIN)*.

The organizers appreciate the support and assistance of the co-operating organizations, the participants, presenters and staff. The next SUSTAIN conference is highly anticipated by all the attendees of SUSTAIN 2013 and the committee expect to further build on the success of this year's event.

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Selection and peer-review under responsibility of the SustaiN conference committee and supported by Kyoto University; (RISH), (OPIR), (GCOE-ARS) and (GSS) as co-hosts



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4th International Conference on Sustainable Future for Human Security, Sustain 2013

Disruption of *gspD* and its effects on endoglucanase and filamentous phage secretion in *Ralstonia solanacearum*

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Abstract

Ralstonia solanacearum is a phytopathogenic bacterium that causes bacterial wilt disease in many agricultural, horticultural and ornamental crops. During pathogenesis, *R. solanacearum* synthesizes and secretes cell wall degrading enzyme (CWDE) such as endoglucanase through type II secretion system. On the other hand, the virulence of *R. solanacearum* is also affected by filamentous phage infection and is secreted through cell-wall membrane via secretin-like channel. *R. solanacearum* is known to have a secretin channel to secrete its virulence factors, such as GspD, a type II secretion system secretin. To confirm that GspD have a role on the secretin of endoglucanase and ϕ RSS1 phage, we constructed a *gspD* mutant that lacks secretin formation on the cell membrane of *R. solanacearum*. The results showed that a *gspD* mutant only abolished to secrete endoglucanase enzyme, but not ϕ RSS1 secretin. According to the ϕ RSS1 genomic organization, we found that ϕ RSS1 phage has a gene responsible for secretin-like protein due to phage secretion through cell membrane.

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Selection and peer-review under responsibility of the Sustain conference committee and supported by Kyoto University; (RISH), (OPIR), (GCOE-ARS) and (GSS) as co-hosts

Keywords: *Ralstonia solanacearum*; secretin; filamentous phage; endoglucanase

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1. Introduction

Ralstonia solanacearum is an important bacterium that causes bacterial wilt disease in many crop plants for more than 200 species of plants [1]. Once the bacteria enter a tissue of susceptible host, they colonize the intercellular spaces of the root cortex and vascular parenchyma. The bacteria eventually enter the xylem vessels and spread into the upper parts of the plant, causing wilt symptoms [2]. The development of bacterial wilt disease depends on the virulence and pathogenicity of its pathogen [3]. During pathogenesis, *R. solanacearum* secretes enzymes that degrade the plant cell wall through the type II secretion system (T2SS), also known as the general secretory pathway (GSP) [4]. The type II protein secretion systems (T2SS) are membrane protein complexes that transport folded proteins from the periplasm to the extracellular medium through the outer membrane channel formed by the protein secretin [5].

The components of the T2SS and their functions have been well characterized in some phytopathogenic bacteria such as *Pseudomonas*, *Xanthomonas*, and *Ralstonia* [6]. The T2SS contains a secretion ATPase, pseudopilus, and major outer membrane protein, are called “secretin”. The T2SS secretin is a member of a family of outer membrane secretin transporters. The role of this secretin is similar to other secretins including the type III secretion system (T3SS), toxin co-regulated pili, type IV pilus, type IV bundle-forming pili, and filamentous phage (Strozen *et al.*, 2012). The secretin is encoded by *gspD* that assembles in the outer membrane of bacteria and act as channel for extrusion of enzyme or toxin [7]. The GspD in *Pseudomonas* species, also called as XcpQ, EpsD in *Vibrio* species [8], OutD in *Erwinia*, and XpsD in *Xanthomonas*, are used to secrete a protein and exoenzyme [9]. In addition, secretion of protein, including enzyme and toxin, has been largely studied from Cholera cell that extrude-out through outer membrane channel (secretins) [10]. On the other hand, some researches have shown that the secretin is also used by bacteriophage for extrusion phage particles [11].

In general, the genome of Ff-like phage is organized in a modular arrangement, in which functionally related genes are grouped [12] and always have three functional modules [13]. One among the modules is the assembly and secretion module containing the genes (*gI* and *gIV*) for morphogenesis and extrusion of phage particles [14]. Gene *gIV* encodes protein pIV, an aqueous channel (secretin) in the outer membrane through which phage particles exit from the host cell. However not all of filamentous phages have the pIV protein that correspond for phage extrusion like in the case of CTX ϕ [10], ϕ RSM1 [13], ϕ RSM3 [15].

The aim of this research is to construct the mutant of *R. solanacearum* that lack *gspD*-like gene and test the hypothesis, that endoglucanase and filamentous phage are also secreted via GspD secretin.

2. Materials and Methods

2.1. Bacterial strains, media, and growth condition.

R. solanacearum strains MAFF 106603 were obtained from the National Institute of Agro biological Sciences, Japan. The bacterial cells were cultured in casamino acid peptone glucose (CPG) medium [16] at 28 °C with shaking at 200 to 300 rpm. *Escherichia coli* strain XL10GOLD was obtained from Laboratory of Biotechnology Molecular, Hiroshima University and was routinely cultured on yeast tryptone broth medium. In some cases, antibiotics such as kanamycin and ampicillin, was added into medium.

2.2. Isolation and characterization of nucleic acids.

Standard molecular biological techniques for DNA and plasmid isolation, digestion with restriction enzymes and other nucleases, and construction of recombinant DNAs were performed according to Sambrook and Russell [17]. Genomic DNA was isolated from the bacterial strain by phenol extraction [16].

2.3. Isolation of kanamycin cassette.

The kanamycin (Kan) cassette was isolated from pUC4-KIXX plasmid. The plasmid was transformed into *E. coli* XL10GOLD and harvested by mini preparation plasmid DNA [17]. Then, the plasmid was cut with *EcoRI* to

digest and cut out the fragment of kanamycin cassette from the plasmid. The resulting fragment of 1.5 kbp was then isolated and used for generating the pSKG::Kan vector .

2.4. Construction of disruption mutant of *gspD*::Kan^r.

A 1,837-bp internal fragment of *gspD* was PCR amplified from MAFF 106603 genomic DNA using a pair-primer of *gspD*(1837)-F: ATGAAGTCTCGTTGAACTTCGTCAA and *gspD*(1837)-R: GCTCGCTGTCCTGGTAA TTGTCTTCC and resulting a blunt-end fragment (Manual of PCR mix). The amplicon was ligated into blunt-end restriction site of Multiple Cloning Site (MCS) of pBlueScript-SK+ to generate pSKG. The allele of the gene was inactivated by inserting a 1.3-kbp Km resistance cassette to create pSKG::Km (Fig. 1). The plasmid was introduced into MAFF 106603 by electro poration to disrupt *gspD* as described below. pSKG::Km cannot replicate in *R. solanacearum*, so selection for Km-resistance was achieved by screening for specific physiological changes. Inactivation of *gspD* in MAFF 106603 was determined by picking colonies that had lost Egl production in the CMC plate assay [18]. Briefly, a single colony on CMC plate was removed and washed off with sterile water. The medium was covered with Congo Red 0.1% (w/v) for 20 min, washed with water and several times with NaCl 1 M. Endoglucanase-negative transformant loss a yellow halo against a red background on this cellulase plate assay.

2.5. Electroporation.

All strains were made electro-competent as described by Sambrook and Russell [17]. Briefly, an aliquot of 100 μ l of the competent cell was mixed with 1 μ l of the recombinant plasmid (pSK-*gspD*::Kan^r). The mixture was placed in a pre-chilled sterile electro poration cuvette (1 mm electrode gap, Bio-Rad) and immediately pulsed by use of a Bio-Rad Gene Pulser (2.5 kV, 200 W, and 25 μ F). The mixture was incubated at 28°C for 1 h with 1 ml of CPG broth. Cells were spread on CPG agar containing the kanamycin and ampicillin and incubated at 28°C.

2.6. Assay on bacteriophage.

To confirm the infection and the production of bacteriophage on bacterial strain, we performed infection and plaque assays. Briefly to infection assay, we spotted a one microliter of bacteriophage (suspension on SM buffer at 10⁸ PFU/mL) on a lawn of *R. solanacearum* culture in CPG agar. The plaque forming was recorded after 24 hours of incubation at 28°C. To confirm the phage production, we infected bacterial cell with bacteriophage (m.o.i of 0.1) in CPG broth. After 24 hours post infection, we collected the supernatant by centrifugation and filtered the liquid culture through 0.45 μ m membrane filter. Five microliter of filtrate the dropped on to the lawn of *R. solanacearum* as infection assay.

3. Results

3.1. Construction of *GspD* mutant of *R. solanacearum*

To create a mutant of *GspD*, we amplified a fragment of *gspD* sequence from MAFF106603 strain using a pair-primer of *gspD* (1837) (Fig 1). The resulting fragment, about 1.8 kbp (Fig. 2a), was connected into pSK plasmid resulting a pSKG plasmid (4,87 kbp) and had some restriction site including *EcoRI* and *Clal* (Fig 1). The construct of pSKG was confirmed by its restriction site showed responding sizes of restriction fragments while digested with using *EcoRI* and/or *Clal* that was two fragments when digested with *Clal* and one fragment when digested with *EcoRI* (Fig 2a). In addition, the pSKG was separated into three fragments when digested with both enzymes (Fig 2a). In order to create a *gspD*::kan mutan, we removed about 1.5 kbp of internal sequence of *gspD* fragment on the pSKG using *SphI* and *BglII* restriction enzymes (Fig 1). A resulting plasmid, 3.3 kbp (Fig. 2b), was inserted with a kanamycine cassette with size of 1.3 kbp (Fig 2b). A generated plasmid, pSKG::Kan (4.6 kbp), a suicide vector for *R. solanacearum*, was then transformed into the *R. solanacearum* competent cell to generate a *gspD*::Kan mutant through homologous recombination.

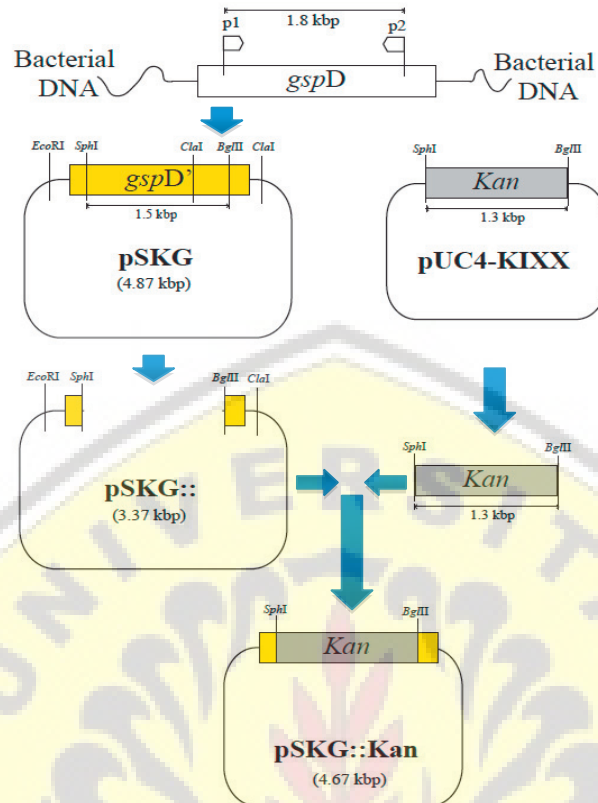


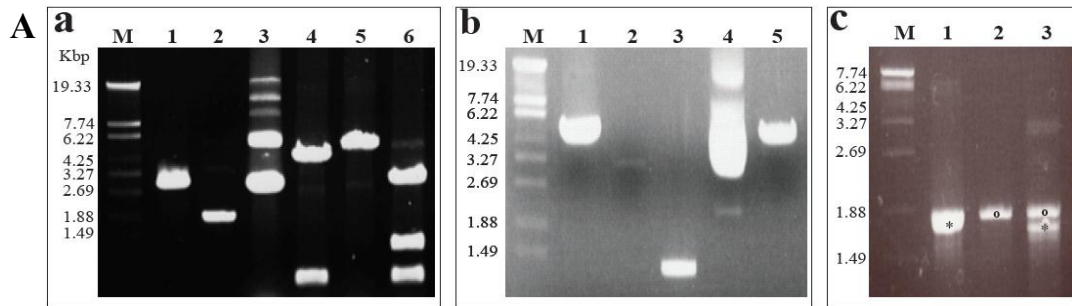
Fig. 1. Scheme for the construction of suicide vector pSKG::Kanr for *R. solanacearum*. Plasmid pSKG was created by inserting an internal part of GspD sequence (called *gspD'* with length of 1.8 kbp) from *R. solanacearum* and was amplified in *E. coli* XL10Gold. Then the pSKG was digested with SphI and BglII and was predicted to result in the loss of an internal 1.5-kbp fragment of *gspD'* part. A Kanamycin cartridge was obtained by extracted 1.5 kbp fragment from digestion product from pUC4-KIXXX [13] by SphI and BglII. The suicide vector pSKG::Kanr was obtained by joining after digestion of both with SphI and BglII and the resulting plasmid was selected with ampicillin and kanamycin in XL10Gold.

In addition, the pSKG was separated into three fragments when digested with both enzymes (Fig 2a). In order to create a *gspD*::kan mutan, we removed about 1.5 kbp of internal sequence of *gspD* fragment on the pSKG using *SphI* and *BglII* restriction enzymes (Fig 1). A resulting plasmid, 3.3 kbp (Fig. 2b), was then inserted with kanamycine cassette with the size of 1.3 kbp (Fig 2b). A generated plasmid, pSKG::Kan (4.6 kbp), a suicide vector for *R. solanacearum*, was transformed into the *R. solanacearum* competent cell to generate a *gspD*::Kan mutant through homologous recombination.

The selection of *gspD*::Kan mutant was chosen by collecting a single colony that grow on the kanamycine-contained medium and was confirmed the transformant through PCR. The result showed that all transformants were single crossovers by showing two fragments separated on the agarose gel, that were 1.6 kbp responsible for *gspD*::Kan fragment and 1,8 kbp responsible for a part of *gspD* fragment from original sequence (Fig 2c).

3.2. *GspD* mutant abolished to secrete endoglucanase but still produces ϕ RSS1 particles

To confirm the successful transformant that was *GspD*::Kan mutant we also checked and confirmed the enzyme secretion, such as endoglucanase. Interestingly, although the transformant was a single-crossover mutant, it showed no enzyme activity was detected on the CMC medium (Fig 3). This result also confirmed that the transformant was a mutant that lacked to form *GspD* secretion and no endoglucanase was secreted through a membrane channel (Fig 3).



B

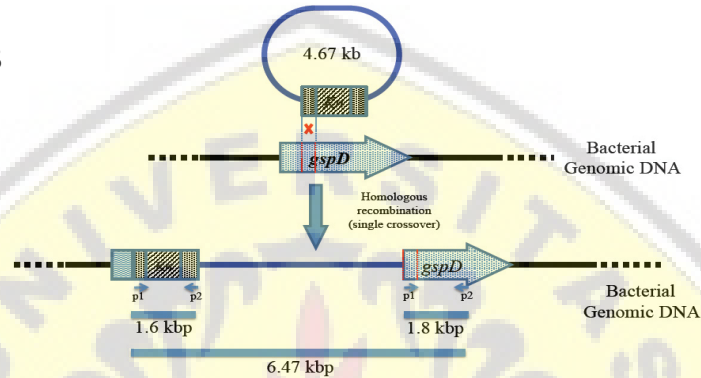


Fig. 2. DNA pattern on agarose gel electrophoresis 1 % stained with ethidium bromide (A); DNA pattern of plasmid pSK (lane a1), *gspD* fragment (lane a2), replicative-form of ligated pSK+*gspD* (pSKG) in *E. coli* XL10GOLD (lane a3), pSKG digested with *Cla*I (lane a4), pSKG digested with *Eco*RI (lane a5), and pSKG digested with *Cla*I and *Eco*RI (lane a6). The pSKG plasmid (lane b1) digested with *Sph*I and *Bgl*III to remove a 1.5 kbp internal sequence of *gspD* fragment (lane b2), then connected with kanamycin cassette (lane b3) resulting a replicative form of constructed plasmid pSKG::Kan^r (lane b4), and was confirmed by digesting pSKG::Kan^r on single restriction site to give single linear fragment (lane b5). For further confirmation, a pSKG::Kan^r (lane b5) then checked by PCR using specific pair primer for to amplify *gspD* (lane c1, asteric) or *gspD*::Kan (lane c2, circle), or both sequences (lane c3). Probable schematic of the mutant of *gspD*::Kan with single cross-over (B).

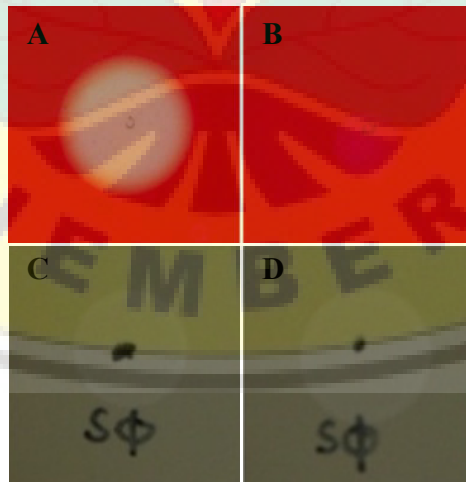


Fig. 3. Endoglucanase production by *R. solanacearum*. Wild-type strain still showed the ability to form clear zone on CMC medium (A) while not on the *gspD* mutant (B). Plaque forming (C and D) on MAFF 106603 strain using culture-free supernatant of MAFF 106603-infected-φRSS1 (C) or with culture-free supernatant of Δ*gspD*::Kan-infected-φRSS1 (D).

Table 1. Characteristic of *R. solanacearum* strain and its *gspD* mutant

No	Strains	Features	Secreted-phage
1.	MAFF 106603	WT, Kan ^r	-
2.	MAFF 106603 ϕ RSS1	WT, Kan ^r , ϕ RSS1 infected	+
3.	MAFF 106603 Δ <i>gspD</i> ::Kan	WT, Kan ^r , single cross-over mutant	-
4.	MAFF 106603 ϕ RSS1 Δ <i>gspD</i> ::Kan	WT, Kan ^r , single cross-over mutant, ϕ RSS1 infected	+

WT: Wild-type strain; Kn^r: kanamycin susceptible; Kn^r: kanamycin resistant; Δ *gspD*: disruption *gspD* gene by single crossover;

4. Discussion

Secretion of hydrolytic enzymes and toxins are transported via membrane channel that occur through T2SS channel [18]. In *R. solanacearum*, the secretion of these enzyme and toxin as well as β -1,3 endoglucanase has been well studied by Liu *et al.* [20] using deletion mutant, GMI-D, through homologous recombination. The GMI-D mutant, that abolished to form secretin channel (*sdpD* or *gspD*) on the cell membrane, is unable to secrete some enzymes such as β -1,4-endoglucanase (Egl), endopolygalacturonase (PehA), exopolygalacturonases (PehB and PehC), β -1,4-cellobiohydrolase (CbhA), and a pectin methylesterase (Pme) [20]. In the other bacteria like *E. carotovora* and *V. cholerae*, the secretin OutD and EspD, respectively have the same role for translocating exoproteins in a folded form across the outer membrane including enzyme and toxin [9]. This secretin protein is specific to a bacterial species and could not be replaced with secretin protein from other related-bacterial species [21].

Obtaining mutant through homologous recombination is usually give two possibilities of recombination, single crossover and double crossover [22]. Although, a double crossover mutant is more stable than single crossover mutant [23], the use of single crossover mutant is widely used in some studies with some treatments [24, 25, 26]. In this study we always used antibiotic-contained medium in all experiment, especially during enzyme and bacteriophage assay (Fig 3), using single crossover mutant to avoid polar effects. The presence of antibiotic on the medium will kill the curing cell due to loss of inserted-antibiotic resistant gene on the chromosome.

On the other hand, The filamentous phage is a group of bacteriophage, infects host cell, but do not lyses the bacterial host and establishes a persistent association, releasing phage particles from the growing host cells [15]. Releasing phage particles from the bacterial host is also determined through a secretin channel that encoded by bacterial host or phages itself [10, 27]. Although, ϕ RSS1 phage has assembly-secretion module, pI and pIV [13], no similarity of known secretin-like protein like in filamentous ϕ 1 or M13 phages that infect *E. coli* [7, 28].

A filamentous phage that has of pIV protein like Filamentous phage ϕ 1, is extruded from its *E. coli* host through phage-encoded pIV protein [27]. In this type of phage, the role of pIV is collaborated with other protein, pI and pXI, with specific function for phage assembly or extrusion [7]. In the other hand, another type of filamentous phage, the non-phage-encoded pIV protein like CTX ϕ , the phage particles are extruded through host-encoded secretin. The similar secretin on *V. cholerae*, EspD, is occupied for secretion Cholera toxin and phage particles [10].

In this study, we confirmed that, ϕ RSS1 is not secreted via its host secretin, but probable using their own secretin, encoded by *gl* and *gIV* on the assembly and secretion module. Although, the no homologous to any secretin-like protein, *gl* and *gIV* are seem to be specific secretin to each filamentous phage type [29, 30, 31].

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