Characterization of KfrA proteins encoded by a plasmid of *Paenibacillus popilliae* ATCC 14706[T]

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ABSTRACT

A scaffold obtained from whole-genome shotgun sequencing of *Paenibacillus popilliae* ATCC 14706[T] shares partial homology with plasmids found in other strains of *P. popilliae*. PCR and sequencing for gap enclosure indicated that the scaffold originated from a 15,929-bp circular DNA. The restriction patterns of a plasmid isolated from *P. popilliae* ATCC 14706[T] were identical to those expected from the sequence; thus, this circular DNA was identified as a plasmid of ATCC 14706[T] and designated pPOP15.9. The plasmid encodes 17 putative open reading frames. Orfs 1, 5, 7, 8, and 9 are homologous to Orfs 11, 12, 15, 16, and 17, respectively. Orf1 and Orf11 are annotated as replication initiation proteins. Orf8 and Orf16 are homologs of KfrA, a plasmid-stabilizing protein in Gram-negative bacteria. Recombinant Orf8 and Orf16 proteins were assessed for the properties of KfrA. Indeed, they formed multimers and bound to inverted repeat sequences in upstream regions of both *orf8* and *orf16*. A phylogenetic tree based on amino acid sequences of Orf8, Orf16 and Kfr proteins did not correlate with species lineage.

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Introduction

Paenibacillus popilliae (formerly *Bacillus popilliae*) is a rod-shaped, endospore-forming bacterium with variable Gram stain reactivity (Pettersson et al., 1999; Tanada and Kaya, 1992). The bacterium infects the Japanese beetle *Popillia japonica* and related species, causing milky disease. The name "milky disease" refers to the clinical condition in which the larval hemolymph becomes so clouded with bacterial cells or spores that it appears milky (Steinkraus and Tashiro, 1967). Like other bacteria, *P. popilliae* carry plasmids, some of which have been sequenced (Dingman, 1994; Faust et al., 1979; Longley et al., 1997). Dingman (1994) isolated three plasmids from *P. popilliae*: pBP68, pBP88, and pBP94. Hybridization analysis suggested that pBP88 may be a chimera of pBP68 and pBP94 (Dingman, 1994). A plasmid isolated from *P. popilliae* strain 17, pBP614, encodes homologs of replication proteins for rolling-circle plasmids, suggesting that pBP614 also replicates by a rolling-circle mechanism (Longley et al., 1997).

Plasmids mediate important biological and pathogenic functions such as antibiotic resistance in *Salmonella* spp. (Miriagou et al., 2006), toluene degradation in *Pseudomonas putida* (Jutkina et al., 2013), EPEC adherence factor (EAF) in enteropathogenic *Escherichia coli* (Levine et al., 1985), and the toxins of *Bacillus thuringiensis* subsp. *israelensis* (Berry et al., 2002). The role of the plasmid in *P. popilliae* remains unknown. We previously published a draft genome analysis (Iiyama et al., 2013), but found it difficult to characterize the plasmid sequence because of the number of contigs (583 contigs). Subsequent sequencing analysis revealed the scaffold originated from a plasmid encoding 17 putative proteins, including two KfrA homologs.

The first *kfrA* was described in broad host range IncP plasmid RK2, where the gene product mediates plasmid maintenance (Thomas et al., 1990). Transcription of *kfrA* is repressed by the products of *korA* and *korF*, also encoded on RK2. KfrA is a DNA-binding protein with a long, alpha-helical, coiled-coil tail. Although most KfrA homologs have been found in Gram-negative bacteria, some have been identified in Gram-positive bacteria such as *Virgibacillus halodenitrificans* (CDQ36163) and *Mycobacterium abscessus* (YP_006316029). The role of KfrA in Gram-positive bacteria, however, has not been defined. The present study focused on the multimer formation and specific DNA-binding capacity of recombinant KfrA from the *P. popilliae* plasmid.

Materials and methods

Bacterial strain, culture, DNA extraction, and primers

P. popilliae ATCC 14706[T] was cultured in MYPGP (Costilow and Coulter, 1971), and genomic DNA was extracted (Iiyama et al., 2013). Plasmid DNA was extracted with a LaboPass Mini kit (Hokkaido System Science Co., Ltd, Sapporo, Japan) according to manufacturer instructions. Primers are described in Table 1.

Genome sequence, assembly, BLAST search, gap filling, and annotation

Genomic DNA of *P. popilliae* ATCC 14706[T] was sheared into 3-kb fragments using HydroShear (Digilab Inc., MA, USA) to produce fragments for a paired-end library. Whole genome sequencing was performed on a 454 GS FLX system with a GS FLX Titanium sequencing kit (Roche Diagnostics, Tokyo, Japan). Reads were de novo assembled with 454 Newbler (version 2.7).

In order to identify the putative plasmid sequence, we used NCBI BLAST (version 2.2.25 +) to search for sequences with homology to pBP68 (accession number DQ925488, Dingman, 1994) and pBP614 (U78608, Longley et al., 1997) harbored by *P. popilliae* strains KLN 4 and strain 7, respectively.

To ascertain whether the scaffold (designated scaffold 8) is comprised of circular DNA, PCR was performed with PrimeSTAR GXL DNA polymerase (TAKARA BIO INC., Shiga, Japan) and appropriate primers (652R/14243F, 5036F/7489R) specific to the ends of scaffold 8 and the neighboring gap region. Genomic and plasmid DNA were used as templates. After electrophoresis, PCR products were gel-purified (Wizard SV Gel and PCR Clean-Up system, Promega KK, Tokyo, Japan) and the DNA sequence was determined without cloning by Sanger sequencing. In order to procure highly accurate data, the reads obtained by sequencing on a HiSeq 2000 (Iiyama et al., 2013) were mapped to the circular DNA originated from scaffold 8 with Bowtie2 (version 2.1.0). The sequence was annotated with the Microbial Genome Annotation Pipeline under default settings (MiGAP; Sugawara et al., 2009). The plasmid map was created with PlasmaDNA (Angers-Loustau et al., 2007) and graphically modified.

Table 1
Plasmids and primers used in this study.

Plasmid or oligonucleotide	Relevant features or sequence	Source, reference or purpose
Plasmids		
pET20b	Expression vector, *pelB* leader sequence for potential periplasmic localization, Amp^R	Laboratory stock
pOrf8H	*orf8* in pET20b, expression for Orf8 (8×His-tagged) fused to *pelB* leader sequence, Amp^R	This study
pOrf8F	*orf8* in pET20b, expression for Orf8 (FLAG-tagged) fused to *pelB* leader sequence, Amp^R	This study
pOrf16H	*orf8* in pET20b, expression for Orf8 (8×His-tagged) fused to *pelB* leader sequence, Amp^R	This study
pOrf16F	*orf8* in pET20b, expression for Orf8 (FLAG-tagged) fused to *pelB* leader sequence, Amp^R	This study
pOrf8H2	Plasmid which removed *pelB* leader sequence from pOrf8H, Amp^R	This study
pOrf8F2	Plasmid which removed *pelB* leader sequence from pOrf8F, Amp^R	This study
pOrf16H2	Plasmid which removed *pelB* leader sequence from pOrf16H, Amp^R	This study
pOrf16F2	Plasmid which removed *pelB* leader sequence from pOrf16F, Amp^R	This study
pBBR1MCS2	Cloning vector, Km^R	Kovach et al. (1995)
pIR1	IR1 in pBBR1MCS2 at *Sac*I and *Asp*718 sites, Km^R	This study
pIR2	IR2 in pBBR1MCS2 at *Sac*I and *Asp*718 sites, Km^R	This study
pIR3	IR3 in pBBR1MCS2 at *Sac*I and *Asp*718 sites, Km^R	This study
pIR4	IR4 in pBBR1MCS2 at *Sac*I and *Asp*718 sites, Km^R	This study
Oligonucleotides		
652R	CGCTAAAGTTGAAAGGCAGA	Gap closure
5036F	ATATCCCTCCTTTGGTTTGG	Gap closure
7489R	TGGTTACTCTCGGTTTGCTC	Gap closure
14243F	GGGACCCCTATAAATCCTCA	Gap closure
20bf	CATGGCCATCGCCGGCTGGG	Linearization of pET20b
20br-8His	CACCACCACCACCACCACCACCACTGAGAT	Linearization of pET20b
20br-FLAG	GATTACAAGGATGACGACGATAAGTGAGATCCGGCTGCTAACAAA	Linearization of pET20b
orf8f(-327)	CATCCGTCTGTGGGAGGTAG	Amplification of *orf8*
orf8r1692	GCTGACATCGTTATAGACAACACA	Amplification of *orf8*
orf16f(-233)	ATTGCTGCTCGTCTCCAGTT	Amplification of *orf16*
orf16r1549	CGGGACCCCTATAAATCCTC	Amplification of *orf16*
orf8F	CCGGCGATGGCCATGGCAGGAGTAGCAAAAATCACG	Amplification for In-Fusion substrate
orf8-16RHis	GTGGTGGTGGTGGTGTGACTTTTCCTCACCCTTATTAATAGACTT	Amplification for In-Fusion substrate
orf16F	CCGGCGATGGCCATGGCAGGAGTAGCACGGATTAC	Amplification for In-Fusion substrate
orf8-16RFlag	GTCATCCTTGTAATCTGACTTTTCCTCACCCTTATTAATAGACTT	Amplification for In-Fusion substrate
orf8Fpeldel	ATGGCAGGAGTAGCAAAAATCAC	Removal of *pelB* leader sequence
orf16Fpeldel	ATGGCAGGAGTAGCACGG	Removal of *pelB* leader sequence
orf8-16Rpeldel	ATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTC	Removal of *pelB* leader sequence
orf8(-476)f	CGCGCTGGAGAGATTAAGG	Amplification of *orf8* upstream region
orf16(-570)f	GGGGTGCTATATGTCGAGGA	Amplification of *orf16* upstream region
orf8-16(71)r	TTACCCTCTTGTAACAACTTTTCTG	Amplification of *orf8* and *orf16* upstream regions
IR1f	CATGAAAAAATCATG	Creation of IR1
IR1r	GTACCATGATTTTTTCATGAGCT	Creation of IR1
IR2f	CATATTGTAGTATG	Creation of IR2
IR2r	GTACCATACTACAATATGAGCT	Creation of IR2
IR3f	CATACTACATATTACATATTACATACTACATACTACAATATGGAGGGTG	Creation of IR3
IR3r	GTACCACCCTCCATATTGTAGTATGTAGTATGTAATATGTAATAT GTAGTATGAGCT	Creation of IR3
IR4f	CATACTACATACAATATACAACATACTACAATATGGAGGGTG	Creation of IR4
IR4r	GTACCACCCTCCATATTGTAGTATGTTGTATATTGTATGTAGTATGAGCT	Creation of IR4
FITC-UnivF	(FITC)-CGCCAGGGTTTTCCCAGTCACGAC	Preparation of FITC-labeled fragment
UnivR	TCACACAGGAAACAGCTATGAC	Preparation of FITC-labeled fragment

Secondary structure prediction

The secondary structure of Orf8 and Orf16 was predicted with PSIPRED v3.3 (Jones, 1999). KfrA proteins in *Thiothrix nivea*, *Methylophaga frappieri*, pMCBF1 of *P. putida* and broad-host-range plasmid RK2 were analyzed for comparison. Amino acid sequences were aligned with ClustalX (Thompson et al., 1997). The secondary structural information was overlaid on the alignment.

Construction of recombinant protein expression vectors

pET20b was linearized by PCR with primer pairs 20bf/20br-8His and 20bf/20br-FLAG to construct plasmids for His- and FLAG-tagged protein expression. The *orf8* fragment was amplified from plasmid DNA extracted from *P. popilliae* ATCC 14706^T with primers orf8f(-327)/orf8r1692 and PrimeSTAR GXL DNA polymerase. Nested PCR was performed with primers orf8F/orf8-16RHis and orf8F/orf8-16RFlag for His- and FLAG-tagged protein expression. Similarly, *orf16* was amplified with orf16f(-233)/orf16r1549, orf16F/orf8-16RHis and orf16F/orf8-16RFlag primer pairs. Four expression plasmids were constructed with In-Fusion reagents (TAKARA BIO, Shiga, Japan): pOrf8H (His-tagged Orf8), pOrf8F (FLAG-tagged Orf8), pOrf16H (His-tagged Orf16), and pOrf16F (FLAG-tagged Orf16).

To eliminate the *pelB* leader sequence for potential periplasmic localization from pOrf8H and pOrf8F, PCR products generated with primers orf8Fpeldel/orf8-16Rpeldel were circularized by intramolecular ligation with T4 DNA ligase to yield pOrf8H2 and pOrf8F2, respectively. The same procedure was used to construct pOrf16H2 and pOrf16F2 with orf16Fpeldel/orf8-16Rpeldel from pOrf16H and pOrf16F.

Expression and purification of recombinant Orf8 and Orf16

E. coli BL21 DE3 (pLysS) harboring an expression vector including pOrf8H, pOrf8F, pOrf16H, pOrf16F, pOrf8H2, pOrf8F2, pOrf16H2, or pOrf16F2 was cultured in LB broth containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37 °C overnight. A 30-μL aliquot of seed culture was transferred to 50 mL fresh broth and cultured at 37 °C. After 3.5 h, gene expression was induced with 0.4 mM IPTG at 37 °C for 3 h. Cells were harvested by centrifugation (2000 ×g, 4 °C, 30 min) and frozen at −80 °C.

To investigate production and cellular localization of recombinant proteins, cells were lysed with complete Lysis-B (Roche Diagnostics K.K., Tokyo, Japan) and the lysate was centrifuged (20, 630 ×g, 4 °C, 5 min). An equal volume of 2× Laemmli sample buffer was added to the supernatant (soluble fraction) and the pellet was solubilized with 1× Laemmli sample buffer (insoluble fraction). Whole cell lysis was performed with 1× Laemmli sample buffer (total cell fraction). A 10-μL culture equivalent of each sample was analyzed by 10% SDS-PAGE.

His-tagged proteins in the insoluble fraction were dissolved with a small volume of binding buffer containing 8 M urea, 0.5 M NaCl, 5 mM imidazole, and 20 mM NaH_2PO_4–Na_2HPO_4 (pH 7.4). Target proteins were purified by standard affinity chromatography using HisTrap HP (GE Healthcare Japan Corporation, Tokyo, Japan). After washing with binding buffer, the target proteins were eluted with a gradient of 5 mM to 250 mM imidazole. Fractions containing the target proteins were pooled and dialyzed against 2 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 7.4) supplemented with 137 mM NaCl. Aliquots of the retentate were stored at −20 °C until use. FLAG-tagged proteins in the soluble fraction were dialyzed without purification and the aliquots were stored at −20 °C.

Homomultimer formation of recombinant Orf8 and Orf16

Homomultimer formation of recombinant protein under various pH conditions was assayed by cross-linking and ultrafiltration methods.

Cross-linking method

Recombinant proteins (5 μg) were incubated at 30 °C for 2 h in 50-μL reactions containing 137 mM NaCl and 20 mM buffer. Buffers were citric acid–sodium citrate (pH 2.5–3.5), acetic acid–sodium acetate (pH 4.0–5.5), NaH_2PO_4–Na_2HPO_4 (pH 6.0–8.0), boric acid–NaOH (pH 8.5–9.0), and $NaOCO_3$–NaOH (pH 9.5–10.0). Glutaraldehyde (2.5 μL of 2.5%) was added and the samples were incubated at 30 °C for 1 h. The cross-

linking reaction was quenched by adding 5 µL of 1 M Tris–HCl (pH 8.0). The samples were added to an equal volume of 2× Laemmli sample buffer and analyzed by 7.5% SDS-PAGE.

Ultrafiltration method

Recombinant proteins (20 µg) were incubated in 200-µL reactions containing 137 mM NaCl and 20 mM buffer at 30 °C for 2 h. Buffers were citric acid–sodium citrate (pH 2.5) and NaH_2PO_4–Na_2HPO_4 (pH 7.5). After ultrafiltration on a 100 K Nanosep device (20 °C, 14,000 ×g, 5 min, Pall Life Sciences, MI, USA), the permeate (< ca. 100 kDa) was collected and added to an equal volume of 2× Laemmli sample buffer. The retentate (> ca. 100 kDa) was diluted with 200 µL of the same buffer and centrifuged to eliminate the remaining low-molecular-weight protein. This washing step was repeated five times. The final retentate was collected and brought to a 200-µL volume with water. The samples were added 200 µL of 2× Laemmli sample buffer. Permeate and retentate fractions were analyzed by 10% SDS-PAGE.

In order to investigate the pH stability of homomultimer formation, recombinant proteins (1 µg/10 µL) were incubated under various pH conditions as described above. The samples were added to 90 µL of 137 mM NaCl and 22.2 mM secondary buffer. NaH_2PO_4–Na_2HPO_4 buffers of pH 8.0 and pH 7.5 were used for the samples previously incubated at pH 2.5–3.5 and pH 4.0–10.0, respectively. In a preliminary experiment, we confirmed that addition of the secondary buffer restored the reaction pH to 7–8, a suitable range for cross-linking by glutaraldehyde. After incubation at 30 °C for 2 h, 5 µL of 2.5% glutaraldehyde was added for cross-linking. The reaction was terminated and analyzed by 7.5% SDS-PAGE. Since protein concentrations were low in this pH stability test, the proteins were visualized by silver staining.

Molecular interaction between recombinant Orf8 and Orf16

Preliminary testing revealed that the recombinant proteins formed monomers in 8 M urea, 20 mM NaH_2PO_4–Na_2HPO_4, and 137 mM NaCl. FLAG-tagged Orf8 and His-tagged Orf16 (6 µg each) were co-incubated in 100 µL buffer at room temperature for 2 h to promote monomer formation. FLAG-tagged Orf8 alone was used as a control. Multimer formation was initiated by transferring to 20 mM NaH_2PO_4–Na_2HPO_4 (pH 7.4), and 137 mM NaCl using a 10 K Nanosep device. The retentate was incubated at 30 °C for 2 h, added to 20 µL of 50% slurry of equilibrated COSMOGEL His-Accept (Nacalai Tesque, Kyoto, Japan), and mixed with mild rotation at 4 °C overnight. The mixtures were transferred to a 0.45-µm Nanosep MF device. After centrifugation (20 °C, 1 min, 3000 ×g), the flow-through fraction was collected and washed five times with 500 µL of 20 mM NaH_2PO_4–Na_2HPO_4 buffer (pH7.4), 0.5 M NaCl, and 5 mM imidazole. The bound proteins were eluted with the same buffer containing 250 mM imidazole. The proteins in the flow-through and eluent fractions were separated by 10% SDS-PAGE. For immunoblot analysis, the proteins were electrotransferred to nitrocellulose membranes (BioTrace NT, Pall Corporation, NY, USA) by using a semidry transfer system (2 mA/cm^2, 90 min). The blot was blocked with 0.5% Alkali-soluble Casein (Merck Millipore, MA, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h. Antibody to FLAG tag (1:1000 dilution, Anti-DDDDK-tag, MBL, Aichi, Japan) and Goat Anti-Rabbit IgG (Fc), AP conjugate (1:7500 dilution, Promega KK, Tokyo, Japan) were used as primary and secondary antibodies. At each step, unbound antibody was washed away with three 10-min washes of TBS-T. The blot was developed using Western Blue™ Stabilized Substrate for Alkaline Phosphatase (Promega KK, Tokyo, Japan).

Electrophoresis mobility shift assays

Electrophoresis mobility shift assays (EMSAs) were performed to investigate the DNA-binding capacities of Orf8 and Orf16. DNA substrates were prepared as described below (Fig. 6A). A 547-bp fragment containing a 476-bp upstream segment of *orf8* (US$_8$) was amplified from the plasmid with primers orf8(-476)f and orf8-16(71)r and Phusion DNA polymerase (New England Biolabs Japan Inc., Tokyo, Japan). Similarly, a 641-bp fragment containing a 570-bp upstream segment of *orf16* (US$_{16}$) was amplified with primers orf16(-570)f and orf8-16(71)r. The purified PCR products (US$_8$ and US$_{16}$) and their *Tas*I digests were used as substrates for EMSAs. For further EMSA, synthetic double stranded DNA, IR1, IR2, IR3 and IR4 were created with appropriate oligonucleotides, IR1f–IR4r (Table 1). These dsDNA, IR1, IR2, IR3 and IR4 were ligated into *Sac*I–*Asp*718 sites of pBBR1MCS2 to construct pIR1, pIR2, pIR3 and pIR4, respectively. PCR was carried out using universal primers, FITC-UnivF and UnivR to label the insert DNA of the plasmids with FITC. The PCR products were

A

Similarity to (■ > 90%, ▬ 80-90%, ▬ 70-80%)

pBP68
pBP614

◁ 652R   5036F ▷   ◁ 7489R   14243F ▷

Scaffold 8

1.9   3.5   3.1   0.8   3.5   0.1

X   gap   X   P   P

0.5 0.8   0.5 1.4

P   P

(1 kb)

B

Template

Total genome | Plasmid

lambda/HindIII | 14243F, 652R | 5036F, 7489R | 14243F, 652R | 5036F, 7489R

(kb)

◀ 2.6
◀ 2.2

C

100-bp ladder | lambda/HindIII | uncut | PstI | XbaI | PstI/XbaI

(kb)

◀ 8.4
◀ 7.6
◀ 6.7
◀ 5.2   ◀ 4.4, 3.9,
◀ 3.5   ◀ 3.5
◀ 2.7

◀ 0.8
◀ 0.6   ◀ 0.6

D

orf16 orf17 orf1
orf15   orf2
orf3
orf14
orf13   orf4
orf12
pPOP15.9
(15,929 bp)
orf11   orf5

orf10 orf9 orf8 orf7 orf6

P P
P
X   X
P

purified to remove unreacted primers. Since the PCR products contained vector sequences, the labeled DNA fragments were termed IR1V, IR2V, IR3V, and IR4V to avoid confusion with IR1–IR4.

DNA substrates (US_8, US_{16}, their *Tas*I digests, and IR1V–IR4V) were incubated with appropriate amounts of His-tagged Orf8 or His-tagged Orf16 in binding buffer (10 mM Tris–Cl, 1 mM EDTA, 100 mM KCl, 0.1 mM DTT, 5% glycerol, 10 µg/mL BSA, pH 8.0) at 30 °C for 30 min. When required to clarify binding-specificity, IR1–IR4 were added as competitors. The reactions were analyzed by 1.5% agarose or 12% polyacrylamide gel electro-phoreses. Signal of FITC-labeled DNA was detected using a molecular imager FX (Bio-Rad Laboratories, Inc., CA, USA). Non-labeled DNA was stained with ethidium bromide and detected by visualization under UV light.

Phylogenetic analysis of KfrA proteins

The amino acid sequences of KfrA proteins from various bacteria were aligned in ClustalX (Thompson et al., 1997). The phylogenetic tree was visualized with an unrooted NJ-plot (Perrière and Gouy, 1996).

Results and discussion

Sequencing and identification of open reading frames in pPOP15.9

A total of 407,478 reads was assembled and 16 scaffolds of 3,973,017 nucleotides with an N50 of 681.8 kb were generated. The mean coverage was 25.2-fold. BLAST search indicated that pBP68 and pBP614 shared partial homology with scaffold 8 (Fig. 1A). The length of scaffold 8 is 14.5 kb of linear DNA with a 1.7-kb assembly gap. PCR products were amplified using appropriate primers from genomic and plasmid DNA templates (Fig. 1B). The results indicated that scaffold 8 is a circular DNA. After sequencing, the length of the circular DNA was determined to be 15,929 bp. Mapping of HiSeq 2000 reads to the sequence yielded a map ratio and read depth of 3.286% and 7760.6. The plasmid extracted from *P. popilliae* was digested with *Pst*I and/or *Xba*I. The digestion profiles were consistent with expectations for the circular DNA originated from scaffold 8 (Fig. 1C), indicating that this circular DNA is a plasmid, designated pPOP15.9.

MiGAP predicted 17 open reading frames (orfs) on pPOP15.9 (Fig. 1D; Table 2). Amino acid sequence alignment indicated that Orf1, Orf5, Orf7, Orf8, and Orf9 are homologous with Orf11, Orf12, Orf15, Orf16, and Orf17, respectively, with ≥ 40% similarity. One or two domains were identified in Orf1, Orf6–8, Orf11, and Orf15–16 (Table 2). Putative domains in Orf1, Orf11 and Orf6, Orf15 are found in replication initiator pro-teins and transcription regulators, respectively. Interestingly Orf8 and Orf16 are closely related to proteins found in Gram-negative bacteria (KfrA proteins). Some KfrA-like protein sequences in Gram-positive bacteria are listed in the NCBI database. The phylogenetic analysis based on the amino acid sequences of KfrA proteins is described in Section 3.7.

In most strains of *B. thuringiensis*, the delta endotoxin-encoding *cry* gene is located on a plasmid (Dean, 1984). Although *P. popilliae* also carries a *cry* gene (Zhang et al., 1997), it is not encoded on pPOP15.9, nor are other virulence factors such as toxins; therefore, this plasmid does not contribute to *P. popilliae* pathogenesis. Since the significance of the *P. popilliae* plasmid remains unknown, pPOP15.9 is considered a cryptic plasmid.

Prediction of secondary structure of Orf8 and Orf16

The secondary structures of Orf8 and Orf16 were compared to those of KfrA proteins of *T. nivea*, *Methylophaga* sp., *P. putida*, and broad-host-range plasmid RK2 (Fig. 2). Orf8, Orf16, and other KfrA proteins are highly α-helical and share nearly identical predicted secondary structures, with an N-terminal globular domain and a long, coiled-coil α-helical tail (Adamczyk et al., 2006; Jagura-Burdzy and Thomas, 1992). Addition of FLAG- or His tags to the carboxyl termini of Orf8 and Orf16 had no effect on the secondary structure (data not shown).

Fig. 1. Plasmid pPOP15.9 found in *P. popilliae* ATCC 14706[T]. Similarity of scaffold 8 to pBP68 and pBP614 (A). Open triangles indicate primers for gap enclosure. Restriction nuclease sites are shown as P (*Pst*I) and X (*Xba*I). The numbers on the bold line for scaffold 8 represent DNA fragment length (kb). PCR was performed for gap closure of scaffold 8 (B). Plasmid was extracted and digested with *Pst*I, *Xba*I, or both (C). Seventeen open reading frames were predicted. The inner circle represents the restriction map.

Table 2
Putative open reading frames (*orf*) found in pPOP15.9 and their homologs.

Orf	Length (aa)	Mass (kDa)	Putative domain [a]		Homolog				
					pPOP15.9 [b]		Other organisms [c]		
			Domain (ID)	E-value	Orf	Identity/similarity	Protein (bacterial species)	Locus	E-value
Orf1	461	55.5	Replication initiation factor (pfam02486)	2.14e−04	Orf11	90.5/95.9	Hypothetical protein (Ppol)	WP_016818384	3e−180
							Replication initiation protein (Bthu)	WP_001135316	1e−123
							Replication initiation protein (Bcer)	WP_001141237	5e−122
Orf2	72	8.19	No hit	–	No hit	–	No hit	–	–
Orf3	143	16.7	No hit	–	No hit	–	No hit	–	–
Orf4	168	18.2	No hit	–	No hit	–	Hypothetical protein (Palv)	WP_005543610	3e−04
Orf5	167	18.5	No hit	–	Orf12	29.9/43.4	Hypothetical protein (Psp.)	WP_017687016	3e−29
							Hypothetical protein (Pgin)	WP_019535067	4e−25
							Hypothetical protein (Psp.)	WP_016818383	3e−22
Orf6	184	22.0	MerR HTH family regulatory protein (pfam13411)	1.01e−05	No hit	–	Hypothetical protein (Blat)	WP_003338843	5e−31
							Hypothetical protein (Blat)	WP_003335127	2e−20
							DNA-binding protein (Bcer)	WP_002167222	3e−20
Orf7	111	12.6	Helix-turn-helix XRE-family like proteins (cd00093)	5.30e−15	Orf15	45.0/63.3	Hypothetical protein (Pbar)	WP_016311381	1e−21
							Transcriptional regulator (Ppeo)	WP_010349619	2e−21
							Transcriptional regulator (Pter)	YP_005073849	2e−21
Orf8	327	36.0	Plasmid replication region DNA binding N-term (pfam11740)	1.44e−15	Orf16	95.1/97.9	KfrA protein (Pput, pMCBF1)	AAY97967	2e−29
			5′ nucleotidase family (cl17687)	7.11e−03			KfrA protein (Tniv)	WP_002706521	2e−27
							KfrA protein (Mfra)	YP_006297578	3e−27
Orf9	61	7.08	No hit	–	Orf17	87.1/90.3	No hit	–	–
Orf10	70	8.22	No hit	–	No hit	–	Hypothetical protein (Ppol)	WP_016822061	1e−06
							Hypothetical protein (Bsp.)	WP_019123583	2e−04
							Hypothetical protein (Psp.)	WP_009673026	0.001
Orf11	462	55.7	Replication initiation factor (pfam02486)	1.28e−5	Orf1	90.5/95.9	Hypothetical protein (Ppol)	WP_016818384	1e−179
							Replication initiation protein (Bthu)	WP_001135316	7e−124
							Replication initiation protein (Bcer)	WP_001141237	9e−122
Orf12	206	22.9	No hit	–	Orf5	29.9/43.4	Hypothetical protein (Palv)	WP_021254164	5e−13
Orf13	63	7.39	No hit	–	No hit	–	No hit	–	–
Orf14	89	9.77	No hit	–	No hit	–	No hit	–	–
Orf15	110	12.8	Helix-turn-helix XRE-family like proteins (cd00093)	7.42e−13	Orf7	45.0/63.3	Hypothetical protein (Bmas)	WP_019156383	8e−41
			Predicted transcriptional regulators (COG1396)	8.01e−06			Transcriptional regulator (Pter)	YP_005073849	6e−33
							Transcriptional regulator (Ppol)	YP_003872652	2e−30
Orf16	326	35.8	Plasmid replication region DNA binding N-term (pfam11740)	7.77e−17	Orf8	95.1/97.9	KfrA protein (Pput, pMCBF1)	AAY97967	2e−29
							Hypothetical protein (Tter)	WP_021249357	5e−28
							KfrA protein (un-b)	NP_598100	1e−27
Orf17	61	7.27	No hit	–	Orf9	87.1/90.3	No hit	–	–

Expression of recombinant Orf8 and Orf16 proteins

pOrf8H, pOrf8F, pOrf16H, and pOrf16F yielded small amounts of expressed protein that mainly localized to the cytoplasm (data not shown), suggesting failure of periplasmic export; the *pelB* leader peptide remained in almost all of the proteins. Extraction of recombinant protein from the periplasmic space was difficult because the contaminating cytoplasm could not be eliminated. The *pelB* leader sequence was deleted to create pOrf8H2, pOrf8F2, pOrf16H2, and pOrf16F2, all of which expressed abundant recombinant proteins (Fig. 3). FLAG-tagged proteins localized in the cytoplasm as soluble proteins and His-tagged proteins were detected in the insoluble fraction. The His-tagged proteins were purified as described in Materials and methods section (data not shown).

Homomultimer formation of recombinant proteins

Broad bands of 180 kDa were detected when His-tagged proteins were incubated at pH 6.5 or higher, indicating multimer formation (Fig. 4A). A previous report of RK2-encoded KfrA cross-linking yielded a clear dimer band and a faint tetramer band that was difficult to reproduce (Jagura-Burdzy and Thomas, 1992). We did not observe the dimer band in this study. Although the detected bands may suggest tetramer formation, low resolution in the high molecular mass range and the observed broad band could not rule out other levels of multimerization; thus, we adopted the term "multimer" in this manuscript.

No signals were detected in the pH 4.5–6.0 samples, in which His-tagged proteins likely aggregated and formed macromolecules with glutaraldehyde, which could not enter the polyacrylamide gel. A monomer band was detected at pH 2.5–4.0, with clearer signals at lower pH. Glutaraldehyde reacts with the amino group over a wide pH range and the reaction increases with increasing pH (Okuda et al., 1991). Therefore, the monomer band detected at low pH may have been due to the absence of cross-linking. To verify this, His-tagged proteins incubated at pH 2.5 and 7.5 were subjected to ultrafiltration (Fig. 4B). Recombinant proteins were detected only in the retentate fractions of samples treated at pH 7.5 and pH 2.5. It is unlikely that the proteins could fold properly at low pH; they likely aggregated under these conditions. Homomultimer formation was restored to proteins treated at pH 2.5–7.0 when they were shifted to pH 7–8 (Fig. 4C). Thus, His-tagged proteins formed homomultimers at pH 6.5–10.0; multimerization was retained over a pH range of 2.5–10.0 at 30 °C for 2 h. Similar results were obtained for the FLAG-tagged proteins (Supplemental Fig. S1), which differed from the His-tagged proteins only in homomultimer formation at pH 6.0. The hydrophilic nature of the FLAG tag prevents aggregation. The difference in tag hydrophilicity may also have affected cellular localization in *E. coli* (Fig. 3).

Orf8 and Orf16 multimerized at pH 6.5 or higher. Although the proteins seemed to aggregate at low pH, the ability to form multimers was not completely lost and was reversible. In *E. coli*, the cytoplasmic pH fell within 10 to 20 s to pH 5.6 to 6.5 when the external pH was lowered from pH 7.5 to 5.5. Rapid recovery, however, occurred until about 30 s after HCl addition and was followed by slower recovery over the next 5 min (Wilks and Slonczewski, 2007). If it is hypothesized that *P. popilliae* has a similar pH homeostasis, and that multimerization is indispensable to the function of Orf8 and Orf16, thus, pH stability enables multimerization after a shift from acidic to neutral pH.

Notes to Table 2:

[a] Putative domains were searched against NCBI's Conserved domain database (Marchler-Bauer et al., 2011).

[b] An end-to-end pairwise alignment of nucleotide and amino acid sequences was performed using the EMBOSS Needle provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/psa/). Similarity less than 40% is indicated as "no hit".

[c] Each amino acid sequence was analyzed using NCBI's BLASTP with E-value cutoff at 0.1. When four or more hits were obtained, the top three are indicated. Abbreviations: Bcer, *Bacillus cereus*; Blat, *Bacillus laterosporus*; Bmas, *Bacillus massiliosenegalensis*; Bsp., *Brevibacillus* sp.; Bthu, *Bacillus thuringiensis*; Mfra, *Methylophaga frappieri*; Palv, *Paenibacillus alvei*; Pbar, *Paenibacillus barengoltzii*; Pgin, *Paenibacillus ginsengihumi*; Ppeo, *Paenibacillus peoriae*; Ppol, *Paenibacillus polymyxa*; Pput, *Pseudomonas putida*; Psp., *Paenibacillus* sp.; Pter, *Paenibacillus terrae*; Tniv, *Thiothrix nivea*; Tter, *Thauera terpenica*; un-b, uncultured bacterium

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Orf8    MAGVAKITKEQIWAAAEKLLQEGKS---PTLAAVRGVVGGGSYTTISEAMSEFRAVQEKTDAPIKEQPLPPVLDEAAARMMAEVWLVATGLANDRLKAER
Orf16   MAGVARITKEQIWAAAEKLLQEGKS---PTLAAVRGVVGGGSYTTISEAMSEFRAVQEKSDAPIKE-PLPPVLDEAAARMMAEVWLIATGLANERLKAER
Tniv    ----MALTKEQIFHAADQLAESGES---PTLANVRKALGGGSFTTISEAMNEWKAKQQATTTPMRE-PAPEAITSRLDEIGAEIWAVALDKANARLSSER
Mfra    ----MAISKEQIFEVADELDAAGQN---PTLAAVRKALDGGSYTTISEAMKEWRAAKAADATPIQE-PPPPGVMDKLSEVGAEIWGVALELANGRLASER
Pput    ----MAITKEQIWAAADELDASGQN---PTLASVRKIIGGGSFTTISEAMNEWRSRKTAQAAPMRE-PAPQAVSDRLAELGADLWAVALEMANSRLMAER
RK2     -MELNKETRDRIFAAADELFEQGDRENFPTVDAVRKAARVN-MNDASAGMREWRRQRTAQAAPLAV-QVPDAVQQAGNQAVAALWQAAQALANESLQAAQ
            ::::*:  .*::*   *.   **: **     . .* *::    :*:    *   : .   *  :*  *   ** *  * ::

Orf8    EALDAARLEHERVQAETAEMADQLADEIEALRLENESLKDTLDASMKDTMDAQEELQNERLKVTQLQARLDVLEESMKELRADKERLGRVN-EEYAKEMR
Orf16   DALDVARAEHERVQSETAEMADQLADEIEALRLASDSLNEALDASMKDTMHAQEELQNERLKVTQLQARLDVLEESMKELRADKERLGRVN-EEYAKEMR
Tniv    EALEATRVQLETAQQEEATELADQLSVELETLQAQHLQATQDLQAASTTIETLRQENASKSRQLATTEARAEETTKRADDLKAELQHAHAENTAQRQRHTQ
Mfra    EGLEAARLEMEASRLEAVELADQLSAELDDSRNQVASLEGVAQELRAEVDDLKAKLTTASERATTAEARSSEMELRASDLRSELDRAHREADKNRQALDR
Pput    EALEAARLDMEAARQEAAELADQLTQELDETKSRLASIEAAEAAARGEAGDLRESLTAMKERAATAEARAAELRTELDHAHQEGRQMRGER-DQAQGVAL
RK2     AGWDRERNELEAVRGELADAFEAQARELEEAQARVTLLEQQAAEEAAELAARQRQALAEARTALAGAEQRAALATQRADEVERRAQELRAELDYAHQDARA
           . :   * : *   : .* .:   :   : *::  :                :              :  : :   *         .. .        .

Orf8    ELDKSNGRLLAEVERLNGVVDDKENWIQGLTTEVEQLRAAVQDKEKLAAEVKQQQIVVA---AQETEAAKLSAVAAAEKEAAIMAAKVEQLEAHAKR-Q
Orf16   ELDKSNGRLLAEVERLNGVVDDKENWIQGLTTEVEQLRAAVQDKEKLAAEVKQQQIVVA---AQETEAAKLSAVAAAEKEAAIMAAKVEQLEAHAKR-Q
Tniv    ETQALQERHTSEVQALQTNLSHTSDTLKTQTAEAVRLQAQLDQAQTQLASLHQQQQVDQERHAREIQQLGERLDKAQAEKEQVLQAASTSREDAAGLRGE
Mfra    VQDAAEKKAAKDHKAELAQAQGEAKQAAKEHQKEVEALRAQHSKALENAQEAKAAAQSEVER--LTAATSSLEYKLAQAEEKLAVLAGEVEQAAKERDS-A
Pput    ERAEVIEKLRDELATARTTAEQSERRAAELRLELDRAHQDADQARQALAEQQQASQTKAA--ELDQLKGELVKVQAKAEADQAAYQEQRKQMAQEAARQA
RK2     FKEESRKTLDAANQEMQALRGERD----ATRREAESLRTELAAVKAKAQADAEAHQEQRKLAAQEAAARQAERFTTVQAEDREARQEAKAAQEEAAMLRGR
           . :.    :           *     :             : :  *         . .                     . ** .. .   . :

Orf8    EEVFKATIIDYAATHGAAMSGKKPTKSINKGEEKS---------
Orf16   EEVFKATIIDYAATHGAAMSGKKPTKSINKGEEKS---------
Tniv    LEALRVQNAALLATLTPAAP-DTPKSSKAKPTNKQ---------
Mfra    LEAAGTAREQAAALRGRVEGLEIALSRGEKDAGSQK--------
Pput    ERYTKAQADRDAARHDAAQAREDAAALRGRLEALESRGVSGKYG
RK2     LDAETSGRKHKAGGK----------------------------
           .
```

Fig. 2. Secondary structure of Orf8, Orf16, and other KfrA proteins. Tniv, Mfr, Pput, and RK2 represent the KfrA proteins of *Thiothrix nivea*, *Methylophaga frappieri*, pMCBF1 of *Pseudomonas putida*, and RK2 plasmid. The gray regions indicate alpha helix structures predicted by PSIPRED v3.3.
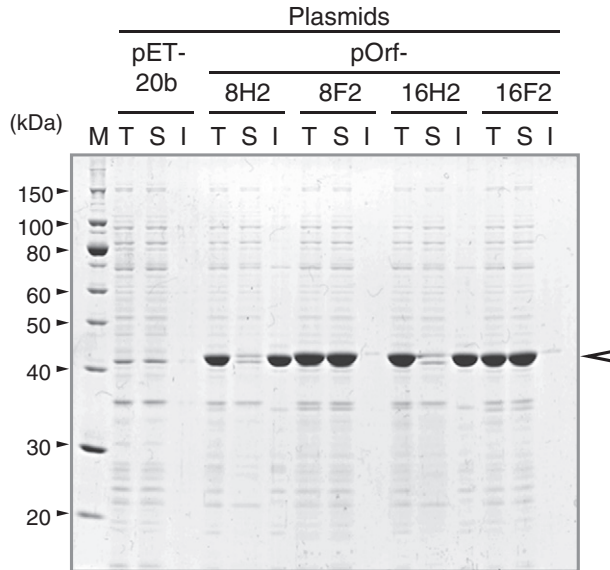
Fig. 3. Cellular localization of recombinant Orf8 and Orf16 proteins. M, T, S, and I indicate molecular weight marker, total cell, soluble, and insoluble fractions. An equivalent of 10-μL culture per lane was analyzed. Open triangle indicates the recombinant protein band.

Molecular interaction between recombinant Orf8 and Orf16

When FLAG-tagged Orf8 alone was applied to the pull-down assay as a control, it was detected in the flow-through fraction but not in the eluent (Fig. 5). Co-incubation with His-tagged Orf16, however, brought the FLAG-tagged Orf8 into the flow-through and elution fractions. Western blot analysis indicated the FLAG-tagged Orf8 interacted with His-tagged Orf16, suggesting heteromultimer formation.

In this study, the number of subunits per multimer and the function of the proteins were not determined. If the proteins form a tetramer, three heterotetramer combinations (1:3, 2:2, 3:1 in Orf8:Orf16) are possible. After having solved these remaining problems, it is necessary to determine whether the heteromultimers are functional.

Electrophoresis mobility shift assays (EMSAs)

EMSAs were performed to evaluate the interaction between recombinant proteins and DNA substrates. In all EMSAs, it was difficult to detect the protein–DNA complex as anything but a smeared band; otherwise, the complex remained in the sample well because of charge, molecular mass, or some unexplained reason. Therefore, we evaluated binding capacity based on the decrease or disappearance of free DNA.

In the first EMSA with upstream regions of *orf8*, US_8, and *orf16*, US_{16} (Fig. 6A), DNA bands were not detected after co-incubation of 40 pmol His-tagged proteins with 0.025 pmol DNA substrates (Fig. 6B). The decrease in DNA signal intensity correlated with an increase in protein dose. Although His-tagged Orf8 did not cause a loss of signal intensity at 10 pmol, His-tagged Orf16 caused a clear reduction at this dose, suggesting that Orf16 has a greater DNA-binding affinity than Orf8. Next, *Tas*I-digested US8 and US16 (Fig. 6A) were used as substrates to more closely identify the binding site. The second EMSA indicated that Orf8 bound to a 156-bp fragment of US8 and a 149-bp fragment of US16 (Fig. 6C). On the other hand, Orf16 bound to these DNA fragments and to 49-bp fragments of both US8 and US16. This result suggested that Orf8 and Orf16 could bind to sequence(s) 205 bp upstream of *orf8* ($205US_8$) and 198 bp upstream of *orf16* ($198US_{16}$).

KfrA, encoded on the broad-host-range RK2 plasmid, specifically binds to an inverted repeat sequence upstream of *kfrA* (Jagura-Burdzy and Thomas, 1992). Three putative inverted repeat sequences were found in $205US_8$ (designated as IR1, IR2 and IR3) and $198US_{16}$ (IR1, IR2 and IR4). IR1 and IR2 were common to
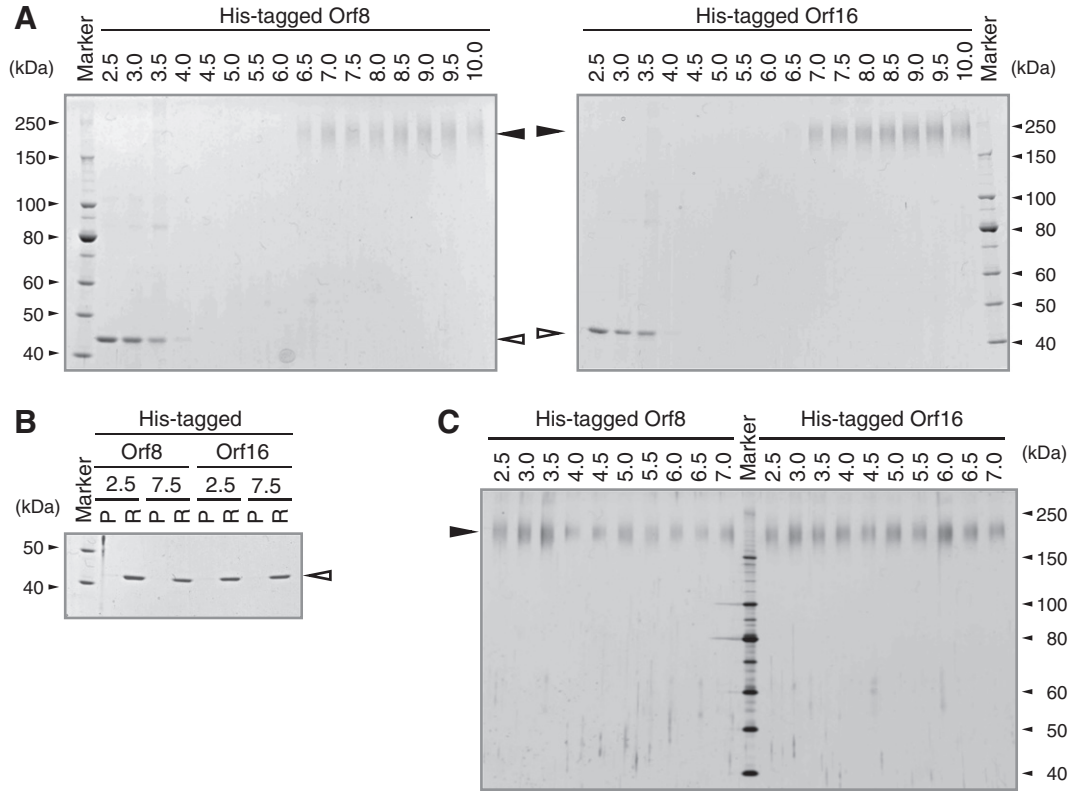
Fig. 4. Effect of pH on homomultimer formation of His-tagged Orf8 and Orf16 proteins. The recombinant proteins were incubated at various pH values and cross-linked with glutaraldehyde. The samples (250 ng/lane) were electrophoresed and CBB stained (A). The recombinant proteins were incubated at pH 2.5 and pH 7.5. After ultrafiltration (100-kDa cutoff), permeate (P) and retentate (R) fractions were electrophoresed and CBB stained (B). To ascertain pH stability, the recombinant proteins were incubated at various pH values, diluted, and shifted to pH 7–8. After cross-linking, the samples (25 ng/lane) were electrophoresed and silver stained (C). In all panels, large closed and open triangles indicate multimers and monomers, respectively.
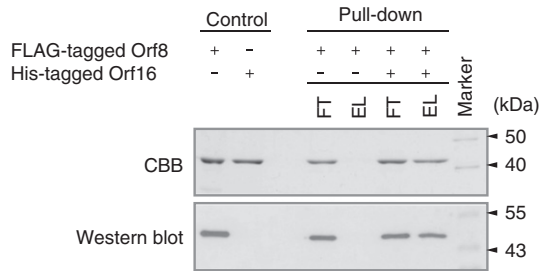
Fig. 5. Pull-down assay to test interaction between recombinant Orf8 and Orf16 proteins. Total proteins were detected by CBB staining (upper panel). FLAG-tagged Orf8 was detected with a FLAG antibody.

both regions (Fig. 6A). These fragments were labeled with FITC (IR1V, IR2V, IR3V and IR4V) and used as EMSA substrates. Free IR3V and IR4V were reduced by co-incubation with Orf8. Other free substrates were not reduced by Orf8 (Fig. 6D). On the other hand, Orf16 induced loss of free IR1V and IR2V, as well as IR3V and IR4V. However, a decrease of free insert-less DNA fragment (EV) was also induced by Orf16 (Fig. 6D). This unexpected result suggests that Orf16 could also bind to the vector sequence. Therefore, to clarify the sequence-specific binding capacity, EMSA was performed with non-labeled competitors (IR1, IR2, IR3, IR4). The competitors did not contain vector sequences (Fig. 6A). Binding of Orf8 to IR3V or IR4V was completely inhibited by excess IR3 or IR4 (Fig. 6E). Interactions of Orf16 with EV and IR1V were inhibited by IR2, IR3, and IR4, but not completely by IR1. This result suggests that Orf16 interacts with the vector sequence in IR1V. The Orf16–IR2V complex was dissociated by IR2, IR3, and IR4. IR1 did not cause this dissociation. Binding of Orf16 to IR3V and IR4V was completely inhibited by IR3 and IR4. IR2 partially inhibited this binding. IR1 did not affect this interaction.

The EMSA results demonstrate binding of His-tagged Orf8 and Orf16 to IR3 (upstream of *orf8*) and IR4 (upstream of *orf16*). Orf16 also weakly bound to IR2 (upstream of both genes).

Phylogenetic analysis of KfrA proteins

The KfrA phylogenetic tree clearly differed from the species tree (Fig. 7). For example, KfrA proteins of Gram-positive bacteria are scattered and do not form a phylogenetic clade in the tree, suggesting that the *kfrA* gene was horizontally transferred from other bacteria. The *kfrA* gene was first described in an IncP-1 plasmid RK2 (Thomas et al., 1990). The IncP-1 plasmids can replicate and are stably maintained in almost all Gram-negative bacteria and may be transferred by conjugation to Gram-positive bacteria, yeasts, and eukaryotic cell lines (Adamczyk and Jagura-Burdzy, 2003). The *kfrA* genes in pPOP15.9 may have been obtained from a broad-host-range plasmid like RK2. The host range of pPOP15.9 remains unresolved.

In this study, Orf8 and Orf16 encoded on pPOP15.9 formed a multimer and bound to upstream regions of both *orf8* and *orf16*. However, the function of these proteins and the contribution of pPOP15.9 to bacterial host behavior were not investigated and remain to be clarified in the future.
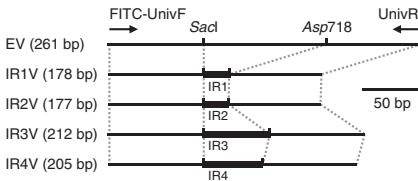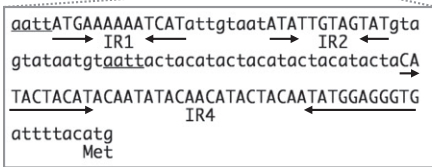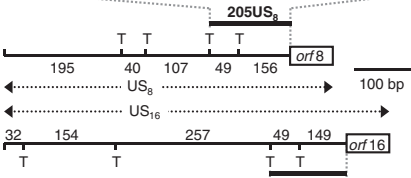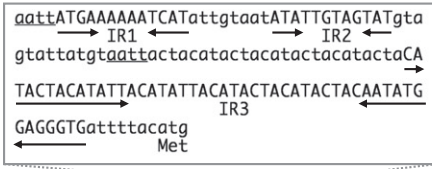
Accession number

The nucleotide sequence of pPOP15.9 has been deposited in DDBJ/EMBL/GenBank under accession number AB931111.

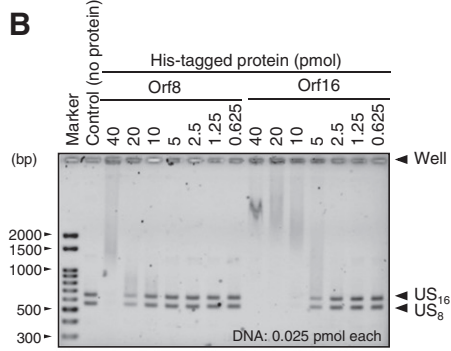Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2015.03.001.

A

aattATGAAAAAATCATattgtaatATATTGTAGTATgta
→ IR1 ←   → IR2 ←
gtattatgtaattactacatactacatactacatactaCA

TACTACATATTACATATTACATACTACATACTACAATATG
              IR3   ←

GAGGGTGattttacatg
        ←
        Met

205US₈

195   40 107   49 156   orf8

US₈

US₁₆

32   154   257   49 149   orf16
T   T   T T

198US₁₆

aattATGAAAAAATCATattgtaatATATTGTAGTATgta
→ IR1 ←   → IR2 ←
gtataatgtaattactacatactacatactacatactaCA
                                    →
TACTACATACAATATACAACATACTACAATATGGAGGGTG
              IR4   ←

attttacatg
Met

FITC-UnivF SacI   Asp718   UnivR
→
EV (261 bp)
IR1V (178 bp) IR1
IR2V (177 bp) IR2
IR3V (212 bp) IR3   50 bp
IR4V (205 bp) IR4

B

His-tagged protein (pmol)

Marker | Control (no protein) | Orf8 | Orf16

Orf8: 40 20 10 5 2.5 1.25 0.625
Orf16: 40 20 10 5 2.5 1.25 0.625

(bp)
2000
1500
1000

500
300

◄ Well
◄ US₁₆
◄ US₈

DNA: 0.025 pmol each

C

TasI-digestions (0.2 pmol)

Marker | US₈ | US₁₆

His-tagged Orf8 (pmol)   - 80 -   - 80 -
His-tagged Orf16 (pmol) - - 80   - - 80

(bp)
300
200
100

◄ Well
(US₈) (US₁₆)
◄156 ◄149
◄ 49 ◄ 49

D

FITC-labeled DNA (probe, 0.25 pmol)

EV | IR1V | IR2V | IR3V | IR4V

no Orf8 Orf16 | no Orf8 Orf16 | no Orf8 Orf16 | no Orf8 Orf16 | no Orf8 Orf16

His-tagged protein (50 pmol)

◄ Well
◄ Free probe
◄ Residual primer

E

His-tagged protein (50 pmol)

Orf8 | Orf16

IR3V | IR4V | EV | IR1V | IR2V | IR3V | IR4V

FITC-labeled DNA (probe, 0.25 pmol)

no IR3 IR4 | no IR3 IR4 | no IR1 IR2 IR3 IR4 | no IR1 IR2 IR3 IR4 | no IR1 IR2 IR3 IR4 | no IR1 IR2 IR3 IR4 | no IR1 IR2 IR3 IR4
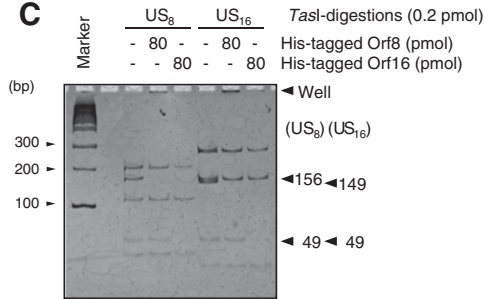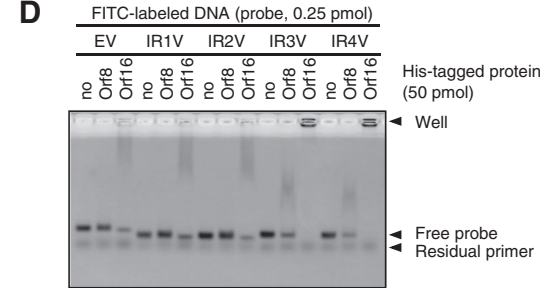
Competitor (100 pmol)
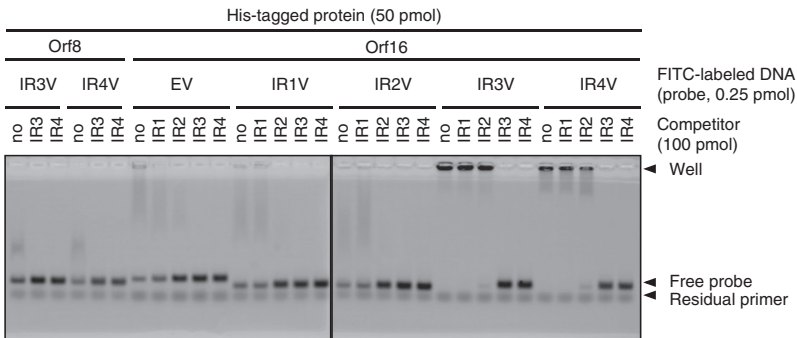
◄ Well
◄ Free probe
◄ Residual primer

Fig. 7. Unrooted phylogenetic tree based on amino acid sequences of Orf8, Orf16, and other KfrA proteins. Abbreviations of class names (AC, Actinobacteria; BA, Bacilli; AP, Alphaproteobacteria; BP, Betaproteobacteria; GP, Gammaproteobacteria; DP, Deltaproteobacteria; CY, Cyanophyceae; NI, Nitrospira) are shown in bold letters with the exception of RK2. Gram-positive bacteria are indicated in larger letters.

Fig. 6. DNA binding capacity of Orf8 and Orf16 by electrophoretic mobility shift assays (EMSAs). Preparation of DNA substrate is graphically shown in panel A. US_8 and US_{16} indicate upstream regions of *orf8* and *orf16* genes. T indicates *Tas*I site, and the numbers beside *Tas*I map mean length of fragment in bp. $205US_8$ and $198US_{16}$ indicate 205-bp upstream region of *orf8* and 195-bp upstream region of *orf16*, respectively. Sequences of $205US_8$ and $198US_{16}$ are boxed. In the sequences, underlined letters and arrows indicate *Tas*I sites and inverted repeat sequences, respectively. FITC-UnivF and UnivR show primers for FITC labeling. US_8, US_{16} (panel B), *Tas*I digested US_8, US_{16} (panel C) and FITC-labeled EV and IR1V–IR4V (panels D and E) were used as DNA substrates. Panel E shows the EMSA result for non-labeled IR1–IR4 competitors. The ethidium bromide and FITC signals were detected in panels A, B and C, D, respectively.

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