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ORIGINAL PAPER

Role of VcrD1 protein in expression and secretion of flagellar components in *Vibrio parahaemolyticus*

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Abstract VcrD1 protein is a component of type III secretion system (T3SS) 1 in *Vibrio parahaemolyticus*. A comparative analysis of secretomes of wild-type and *ΔvcrD1* strains revealed that the mutant was defective in secretion of diverse proteins including several flagellar components. Western blot analyses using specific antibodies confirmed that the secretion of at least four flagellar components, such as FlaA, FlgL, FlgE, and FlgM, was affected by the *vcrD1* mutation, which was consistent with decreased motility on soft agar plates and the non-flagellated morphology of the mutant. The *ΔexsA* mutant, another T3SS1 mutant, did not showed reduced motility, but became non-motile phenotype with the additional *ΔvcrD1* mutation. Complementation of wild-type *vcrD1* gene into *ΔvcrD1* mutant resulted in restored motility. Fractionation of bacterial cytoplasm from the periplasm and membrane revealed lower levels of FlaA and FlgM in the cytoplasm of the *ΔvcrD1* mutant, indicating that VcrD1 might regulate the expression of flagellar genes in addition to the secretion of flagellar components in *V. parahaemolyticus*. Quantitative RT-PCR assays of seven representative flagellar genes in the wild-type and Δ*vcrD1* mutant strains demonstrated that transcript levels of two early flagellar genes, *flaK* and *flaL*, were not reduced by the *vcrD1* mutation, whereas the middle and late flagellar genes were expressed at a lower level in the *vcrD1* mutant. This study raises a possibility that VcrD1 plays a role in flagellar morphogenesis in *V. parahaemolyticus* by regulating the expression and secretion of flagellar components.

Keywords *Vibrio parahaemolyticus* · VcrD1 protein · Flagellar proteins

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Introduction

Vibrio parahaemolyticus, a common resident in marine and estuarine environments (Daniels et al. 2000), infects humans and typically results in gastroenteritis (Nair et al. 2007). In addition to a well-known virulence factor of *V. parahaemolyticus*, thermostable direct hemolysin (TDH) (Honda and Iida 1993), the type III secretion system (T3SS), is considered to be an important virulence factor for delivering effectors into host cells (Park et al. 2004). Genome sequencing of *V. parahaemolyticus* RIMD2210633 has revealed the presence of two T3SS, T3SS1 and T3SS2 (Makino et al. 2003). Roles of these T3SSs were examined using T3SS1- or T3SS2-deficient *V. parahaemolyticus* strains, in which the *vcrD1* or *vcrD2* gene, respectively, was mutated. The phenotypes of these mutants indicated that T3SS1 plays a role in cytotoxicity toward tissue culture cells, whereas T3SS2 is associated with bacterial enterotoxicity in the rabbit ileal loop model (Makino et al. 2003; Park et al. 2004). A T3SS1-deficient *V. parahaemolyticus* strain was also defective in inducing the death of HEp-2 cells, which occurs via a MAPK-dependent (p38 and ERK1/2) and caspase-independent mechanism

(Yang et al. 2011). Expression of T3SS1 genes is induced when *V. parahaemolyticus* is cultivated in tissue culture medium in which ExsA and ExsD play roles as a positive and negative regulators, respectively (Zhou et al. 2008).

In this study, several polar flagellar proteins were secreted at lower levels in the media grown by the *vcrD1* mutant *V. parahaemolyticus* than the culture media of the wild-type strain. The secretion system for bacterial flagellar components belongs to the T3SS superfamily (Macnab 2004). While the T3SSs responsible for secreting effectors are defined as non-flagellar (NF) T3SSs, they are thought to have evolved from flagellar T3SSs (Abby and Rocha 2012). Interestingly, *V. parahaemolyticus* has two flagellar systems, i.e., a polar sheathed flagellum and lateral unsheathed flagella (reviewed by McCarter 1999; reviewed by Duan et al. 2013). The polar flagellum functions constitutively and is responsible for bacterial swimming. On the other hand, synthesis of lateral flagella is induced under a specific condition such as growth on solid surfaces (Belas et al. 1986).

Approximately sixty genes are involved in formation of the polar flagellum in *V. parahaemolyticus* (Kim and McCarter 2000). Most of the polar flagellar genes are found in two locations of the larger chromosome of *V. parahaemolyticus* with exceptions of several motor genes, *motAB*, *motX*, and *motY* (McCarter 2001). Transcription of these flagellar genes is temporally regulated in a tight coordination with the orders of flagella assembly (Kim and McCarter 2000). These flagellar genes are grouped into three hierarchies according to their temporal orders of transcription during flagella formation. The early genes, *flaK* and *flaLM*, are master regulators, which interact with σ54 and play a role in transcription of the middle genes. Interestingly, *flaK* mutant *V. parahaemolyticus* was found to retain the swimming motility until the bacteria had an additional defect in formation of lateral flagella, indicating the presence of cross-regulation between these two flagellar systems of *V. parahaemolyticus* (Kim and McCarter 2004). The middle flagellar genes transcribed by σ54 encode proteins involved in assembly of the hook-basal-body structure, HAP1, HAP3, MotY, some chemotaxis proteins, and an alternative sigma factor, σ28. Sigma factor 28 then transcribes the other set of flagellar genes, the late flagellar genes, which encode additional motor proteins, additional chemotaxis proteins, 5 flagellins, a distal capping protein HAP2, and the anti-σ28 FlgM.

Besides flagellar T3SSs (Kim and McCarter 2000), *V. parahaemolyticus* possesses two NF T3SSs (T3SS1 and T3SS2) to convey its virulence factors into the host cells (Park et al. 2004). In this study, we further characterized the role of NF-T3SS1 in polar flagella formation by examining the altered expression and secretion of flagellar components in a *ΔvcrD1* mutant.

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used to prepare plasmid DNA and transfer of the plasmid by conjugation were grown in Luria–Bertani (LB) broth (1 % bacto-tryptone, 0.5 % yeast extract, and 1 % NaCl) or on LB plate containing 1.5 % agar. *V. parahaemolyticus* RIMD2210633 (ATCCBAA-238; American Type Culture Collection, Manassas, VA, USA) was used as the wild-type strain in this study and was cultured in LB medium supplemented with an additional 2 % NaCl (LBS). Ampicillin was added to the medium at 100 μg/ml to maintain the plasmids in *E. coli*. Chloramphenicol (2 μg/ml) was used to select *V. parahaemolyticus* exconjugants. For *V. parahaemolyticus* carrying pRK415 or pRK415-derived plasmid, tetracycline was added into the medium at a concentration of 3 μg/ml. All medium components were purchased from Difco (Lawrence, KS, USA), and the chemicals and antibiotics were from Sigma (St. Louis, MO, USA).

Preparation of secreted proteins from *V. parahaemolyticus*

Secreted proteins were prepared from wild-type and *ΔvcrD1 V. parahaemolyticus* strains cultivated in LBS broth at 37 °C. In addition, secreted proteins were harvested from these strains grown in Dulbecco's modified eagle medium (DMEM) as an inducing condition for T3SS1 as reported (Zhou et al. 2008). Once bacterial cells grew to an optical density at 600 nm (OD_{600}) of 0.8, they were centrifuged at 8,000 rpm for 15 min at 4 °C and the resultant supernatants were passed through a 0.2-μm pore membrane filter. Proteins were concentrated using a Centricon YM-10 (Millipore, Bedford, MA, USA) at 4 °C. Filtered supernatants were mixed with an equal volume of prechilled 10 % trichloroacetic acid (TCA) and incubated on ice for 30 min. After a 30-min centrifugation at 9,000 rpm and 4 °C, the pellets were washed with acetone three times, dried, and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0). The protein content in the secretome preparations was determined with a Protein Assay kit (Bio-Rad, Hercules, CA, USA).

2D gel electrophoresis of secreted proteins

Secreted proteins (wild-type and *ΔvcrD1* mutant *V. parahaemolyticus*) were subjected to isoelectric focusing with the IPGphor system and Immobiline Drystrip gel strips (Life Sciences, New York, USA). Briefly, 200-μl aliquots of sample protein in rehydration solution were applied to the strips (pH 4–7, 18 cm) and rehydrated for 16 h at room temperature. After rehydration, the proteins were focused for 36 kV/h at 20 °C (100 V

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source, reference
E. coli		
DH5α	*supE44 ΔlacU169* (Φ80 *lacZ ΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10 λ*pir*	*thi thr leu tonA lacy supE recA*::RP4-2-Tc::Mu λ*pir*, OriT of RP4 Km^r; conjugational donor	Simon et al. (1983)
BL21 (DE3)	*E. coli* B F^- *dcm ompT hsdS* (rB^- mB^-) *gal* λ (DE3)	Invitrogen
V. parahaemolyticus		
RIMD2210633	Kanagawa phenotype-positive, serotype O3:K6	ATCC
NK1	*ΔvcrD1* mutant derived from RIMD2210633	Yang et al. (2011)
MJ21	*ΔexsA* mutant derived from RIMD2210633	This study
MJ22	*ΔexsA* mutant derived from NK1	This study
MJ23	*ΔflaK* mutant derived from RIMD2210633	This study
MJ24	*ΔlafK* mutant derived from RIMD2210633	This study
MJ25	*ΔlafK* mutant derived from MJ23	This study
Plasmids		
pET21b	Expression vector, Ap^r	Novagen
pETompV	pET21b, 754-bp $ompV^+$ (VPA0318)	This study
pETflgL	pET21b, 1,191-bp $flgL^+$ (VP0786)	This study
pETflgM	pET21b, 315-bp $flgM^+$ (VP0771)	This study
pBluescript (II) SK (+)	Cloning vector, Ap^r	Stratagene
pSKexsAD	pBluescript (II) SK (+), 792-bp downstream region of *exsA*	This study
pSKexsAUD	pSKexsAD, 747-bp upstream region of *exsA*	This study
pSKflaKU	pBluescript (II) SK (+), 766-bp upstream region of *flaK*	This study
pSKflaKUD	pSKflaKU, 647-bp downstream region of *flaK*	This study
pSKlafKU	pBluescript (II) SK (+), 750-bp upstream region of *lafK*	This study
pSKlafKUD	pSKlafKU, 640-bp downstream region of *lafK*	This study
pDM4	Suicide vector, Cm^r	Miller and Mekalonos (1988)
pDMexsAUD	pDM4, 1,539-bp *Apa*I–*Sac*I fragment of pSKexsAUD	This study
pDMflaKUD	pDM4, 1,413-bp *Xho*I–*Spe*I fragment of pSKflaKUD	This study
pDMlafKUD	pDM4, 1,390-bp *Apa*I–*Sac*I fragment of pSKlafKUD	This study
pRK415	Broad-host-range plasmid, Tc^r	Keen et al. (1998)
pRKvcrD1	pRK415, 1,035-bp $vcrD1^+$	Yang et al. (2011)

for 2 h, 500 V for 1 h, 1,000 V for 1 h, 2,000 V for 1 h, 4,000 V for 1 h, 6,000 V for 2 h, and 8,000 V for 2 h). The strips were then soaked in sodium dodecyl sulfate (SDS) equilibrium buffer containing 6 M urea, 2 % SDS, 5× gel buffer (pH 8.8), 50 % glycerol, and 2.5 % acrylamide for 15 min. The strips were transferred onto 10 % acrylamide gels and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie brilliant blue G-250 containing 17 % ammonium sulfate, 3 % phosphoric acid, 34 % methanol, and 0.1 % Coomassie blue G-250 in distilled water.

In-gel trypsin digestion and MALDI-TOF mass spectrophotometry

Protein spots were selected, which were at decreased levels in the secretome of *ΔvcrD1* mutant (below to 33 % of the wild type), but present in considerable amounts pertinent to further analysis. Protein spots of interest were excised and washed three times with distilled water. The excised spots were incubated in destaining solution (15 mM potassium ferricyanide, 50 mM sodium thiosulfate) and washed with 25 mM ammonium bicarbonate/50 % acetonitrile until the Coomassie blue G-250 dye disappeared. After drying in a vacuum concentrator, the gel was rehydrated with 5 μl trypsin (12.5 ng/μl in 50 mM NH_4HCO_3) at 4 °C for 45 min and incubated overnight at 37 °C. The tryptic peptides were extracted with 60 % acetonitrile and 0.1 % trifluoroacetic acid (TFA), and dried with a vacuum concentrator. The resultant peptide mixtures were dissolved in 0.5 % TFA. Using a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1 % TFA/50 % acetonitrile as a matrix, mass spectrometry was performed on a MALDI-TOF/TOF mass

Table 2 Primers used in this study

Protein/gene	Primer name	Nucleotide sequence (5′–3′)[a]
For recombinant proteins		
OmpV	VPA0318-F	GATCGGATCCGATGAACAAGACACTTATCG
	VPA0318-R	GATCCTCGAGGGAAGTGGTAAGACACGCTTG
FlgL	VP0786-F	GATCGGATCCGGTGTTAACCCGAATTTCTAG
	VP0786-R	GATCCTCGAGAATGTAGTTAAACAATGTCAG
FlgM	VP0771-F	ATGCGAATTCCATGGCAGGTATAGATAATATA
	VP0771-R	ATGCCTCGAGGCTTTTGCCTTGCAATTCGTT
Construction of mutant strains		
ΔexsA	exsAupF	ATGCGGGCCCAGTGATAGGGAAACTCTATTC
	exsAupR	ATGCTGCAGTTTCTACCCTTCATAATTTTT
	exsAdownF	ATGCCTGCAGAATGACTTATGACTAAAATTT
	exsAdownR	ATGCGAGCTCGTGTATTTAGCTGCGCTTTTA
ΔflaK	flaKupF	GATCCTCGAGTCACAAATTCCGTTATGGCCA
	flaKupR	GATCCTGCAGAAGTAAGAATGATTGCCTTTA
	flaKdownF	GATCCTGCAGATCGAGCTTCAGTTCAATCAC
	flaKdownR	GATCACTAGTTCGTCGCTGGCGGAAAATTAG
ΔlafK	lafKupF	ATGCGGGCCCGGAACCAAATAATAAGATTTC
	lafKupR	ATGCCTGCAGTGAACCTTAACCTTCTTCTGT
	lafKdownF	ATGCCTGCAGCCTAATAGGAGAAAAATATAG
	lafKdownR	ATGCGAGCTCCAATCTCGCCAATATCGAATC
For real-time PCR		
flgE	flgE-F	TGGGGTGAATCAAACAAAGGC
	flgE-R	TTATCTAATCTGCAGGATATT
fliF	fliF-F	GAAACCAGCCTAATTGGTAGC
	fliF-R	TTAGCCATTTTCGTTCATCCA
fliD	fliD-F	ATTCGTACGCGAGAGAAAAGT
	fliD-R	TTAACCTAACGCACTCATAAG
flaA	flaA-F	ATCAGCAACCTAGACAACATA
	flaA-R	TTAGCCCAACAAGCTTAGCGC
flgM	flgM-F	AGCCAACAAAGTAAAGCCGTC
	flgM-R	TTAGCTTTTGCCTTGCAATTC
gap	gap-F	TTTGAAGAGCGTCCGTTGGTG
	gap-R	TTAAGCCAACACAACGTTACG

[a] Restriction enzyme sites are underlined

spectrometer (Applied Biosystems, Foster City, CA, USA). The Mascot program (http://www.mtrixsceince.com) was used to search the Swiss-Prot and NCBInr databases.

Preparation of polyclonal antibodies

The DNA encompassing the open reading frame (ORF) of each selected protein (FlgL, FlgM, and OmpV) was amplified using a pair of specific primers listed in Table 2 and then cloned into a pET21b (+) expression vector (Novagen, Darmstadt, Germany). The recombinant candidate protein was overexpressed in *E. coli* BL21 (DE3) by adding 1 mM isopropyl thio-β-galactosidase and then separated by 12 % SDS-PAGE. The induced protein band was excised and resuspended with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). This protein was then injected intraperitoneally into pathogen-free rats (CrjBgi: CD[S.D.]IGS, 6-week-old, female) as an antigen to produce polyclonal antibodies. After three injections at 2-week intervals, serum was obtained from the immunized rats, and the resultant antibody titers were tested.

Western blot analysis

Bacterial extracts were prepared by sonicating bacterial cells in TNT buffer (10 mM Tris–HCl, pH 8.0, 150 mM

NaCl, and 0.05 % Tween 20). Protein in the culture supernatants was concentrated 200-fold by precipitation with TCA or filtration through Amicon/Centricon YM-10 columns (Millipore, Billerica, MA, USA). Cell lysates and concentrated supernatants in sample buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2 % SDS, 0.1 % bromophenol blue, and 20 % glycerol) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). For each extract, two different amounts of proteins were analyzed by Western blot. Membranes were blocked with 5 % skim milk in Tris-buffered saline with Tween 20 (150 mM NaCl, 50 mM Tris–HCl, and 0.1 % Tween 20) and incubated overnight at 4 °C with polyclonal antibodies (1:5,000 dilution). Horseradish peroxidase-conjugated secondary antibodies were used. Immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

In addition to antibodies against candidate proteins, secretion of FlgE and FlaA was monitored using antibodies specific to homologous proteins from *V. vulnificus* (Lee et al. 2004; Lee and Kim unpublished result, respectively). Antibodies specific to TDH (Yang et al. 2011) were included in Western blot analyses as a loading control for each secretome.

Construction of deletion mutants of *V. parahaemolyticus*

For construction of *ΔexsA* mutant, the downstream region of the *exsA* gene was amplified from the genomic DNA of *V. parahaemolyticus* RIMD2210633 with the primers, exsAdownF and exsAdownR (Table 2). The resultant 792-bp DNA fragment was then digested with *Pst*I and *Sac*I, and ligated into pBlueScript (II) SK (+) (Stratagene, La Jolla, CA, USA) to produce pSKexsAD. The upstream region of the *exsA* gene was amplified using the primers, exsAupF and exsAupR (Table 2). The resultant DNA fragment of 747 bp was treated with *Apa*I and *Pst*I and ligated into pSKexsAD to yield pSKexsAUD. The 1,539-bp *Apa*I–*Sac*I DNA fragment of pSKexsAUD was transferred into a suicide vector pDM4 (Miller and Mekalonos 1988), resulting in formation of pDMexsAUD. The plasmid pDMexsAUD in SM10 λ*pir* (Simon et al. 1983) was mobilized to *V. parahaemolyticus* RIMD2210633 or *ΔvcrD1* mutant, and the conjugants were selected by plating the conjugation mixture of *E. coli* and *V. parahaemolyticus* on LBS plates supplemented with 2 μg/ml chloramphenicol. A colony with characteristics indicating a double homologous recombination event (resistance to 5 % sucrose and sensitivity to chloramphenicol) was further confirmed by PCR using the primers, exsAupF and exsAdownR, and then named MJ21 or MJ22 for the *ΔexsA* or *ΔexsAΔvcrD1* mutant strain, respectively.

A mutant *V. parahaemolyticus* losing both FlaK and LafK, key regulators for polar flagellum and lateral flagella, respectively (Kim and McCarter 2004), was constructed and included for motility assays as a non-motile control. For construction of the *ΔflaK* mutant, the upstream (766-bp) and downstream (647-bp) regions of the *flaK* gene were amplified using the primer set of flaKupF/flaKupR and flaKdownF/flaKdownR, respectively (Table 2). The *Xho*I–*Spe*I DNA fragment of pSK-flaKUD was transferred into pDM4 to produce in pDM-flaKUD, which was then used to generate *ΔflaK* mutant as described above. A plasmid (pSKlafKUD) includes the upstream (750 bp) and downstream (640 bp) regions of the *lafK* gene, which have been amplified by the following primer sets, lafKupF/lafKupR and lafKdownF/lafKdownR (Table 2). The *Apa*I–*Sac*I DNA fragment of the resultant plasmid was ligated into pDM4 to produce pDMlafKUD, which was used to make the *ΔflaKΔlafK* mutant, as described above.

Motility assay

Wild-type and mutant *V. parahaemolyticus* strains (*ΔvcrD1*, *ΔexsA*, *ΔexsAΔvcrD1*, and *ΔflaKΔlafK* mutants) were freshly grown in LBS at 37 °C with aeration to an OD_{600} of 0.8, washed, and resuspended in PBS to a final concentration of 10^6 colony forming units/ml. The cell suspension was inoculated into LBS medium containing 0.3 % agar and incubated for 4 h at 37 °C. Motility of the *vcrD1* mutant *V. parahaemolyticus* carrying the complementation plasmid, pRKvcrD1 (Yang et al. 2011) was also examined in the same manner along with two control strains (wild type carrying pRK415 and *vcrD1* mutant carrying pRK415). The degree of bacterial motility was quantified by measuring the diameters (mm) of the motility halos made by these *V. parahaemolyticus* strains. Data are presented as the means ± standard deviation from three independent experiments.

Transmission and scanning electron microscopy

Wild-type and *ΔvcrD1* mutant strains were examined for the presence of a flagellum. Bacterial cells were negatively stained with 2 % uranyl acetate (pH 7.4) on a Formvar carbon-coated grid and observed with a transmission electron microscope (CM-10; Philips, Amsterdam, Netherlands) at 75 kV.

Vibrio parahaemolyticus strains for the complementation study (wild type carrying pRK415, *vcrD1* mutant carrying pRK415, and *vcrD1* mutant carrying pRKvcrD1) were also examined for the presence of flagellum using a scanning electron microscope (S-3000 N, Hitachi, Tokyo, Japan) at 30 kV. Before examination, the specimens were

coated with an electro-conductive layer of gold, which was evaporated by an ion sputter coater (E101, Hitachi).

Fractionation of bacterial cells

Wild-type and *ΔvcrD1 V. parahaemolyticus* strains were grown to $OD_{600} = 0.8$ at 37 °C in LBS broth. Through centrifugation, the culture supernatant was separated from the bacterial cells. The harvested cells were divided into cytoplasmic and periplasmic fractions as described (Manoil and Beckwith 1986). Harvested cells were resuspended in a spheroplast buffer (100 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, 1 mM $MgCl_2$, and 20 μg/ml phenylmethylsulfonyl fluoride) and subjected to centrifugation (3 min, 13,000 rpm, 4 °C). The pellets were warmed, resuspended in ice-cold water, and treated with 20 mM $MgCl_2$. The osmotic shocked-cells were centrifuged (5 min, 13,000 rpm, 4 °C). The supernatants were saved as periplasmic fractions, and the pellets were further resuspended in PBS with 1 % Triton X-100. The cells in the pellet were lysed by sonication. After centrifugation, the resultant supernatant was saved as the cytoplasmic fraction.

The culture supernatant, periplasmic fraction, membrane fraction, and cytoplasmic extract of each strain were analyzed by Western blot using anti-FlaA antibodies or anti-FlgM antibodies. Antibodies against glucose-specific enzyme IIA^{Glc} (Lee and Lee unpublished result) and anti-OmpV antibodies were used as a loading control for cytoplasmic and membrane fractions, respectively. Anti-TDH antibodies were used as a loading control for the secretome.

Quantitation of flagellar mRNAs

The degree of expression of seven flagellar mRNAs was evaluated in wild-type and *ΔvcrD1* mutant *V. parahaemolyticus* by real-time PCR (RT-PCR). *V. parahaemolyticus* was cultured in LBS broth at 37 °C with shaking. At $OD_{600} = 0.8$, the bacteria cells were centrifuged at 8,000 rpm for 5 min at 4 °C. Total RNAs were isolated from harvested cells using TRizol (BioRad) and then treated with the RNase-free DNase (Qiagen, Hilden, Germany). After amount of RNA was measured using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), cDNA was synthesized from 4 μg of RNA using PrimeScript RT reagent kit (TaKaRa, Shiga, Japan) as directed by the manufacturer. The cDNAs were analyzed by quantitative RT-PCR (qRT-PCR) on a Light Cycler 480 II real-time PCR system (Roche Applied Science, Mannheim, Germany) using Light Cycler 480 DNA SYBR green I master kit (Roche Applied Science). The qRT-PCR was carried out in a 96-well plate. The amount of each transcript was estimated using the specific pair of primers (Table 2). Each experiment was performed in triplicate for each sample and repeated three times with independent RNA samples. The *gap* gene of *V. parahaemolyticus* was used as an endogenous control to normalize each transcript in wild-type and *ΔvcrD1* mutant cDNAs. Data analysis was based on the relative quantification method by determining the crossing point (C_p) value using the Light Cycler 480 II real-time PCR system software program (Roche Applied Science, version LSC480 1.5.0.39).

Statistical analysis

Data are presented as the means ± standard deviation from three independent experiments. Statistical analyses were performed using Student's *t* test (SYSTAT, SigmaPlot version 11; Systat Software Inc. Chicago, IL, USA) to evaluate the statistical significance of the results. Differences were considered significant when $P < 0.05$. Data with $P < 0.01$ are indicated with two asterisks, whereas data with *P* values between 0.01 and 0.05 are indicated with a single asterisk.

Results

Identification of extracellular proteins via T3SS1 in culture supernatants of *V. parahaemolyticus*

To identify the proteins secreted via the T3SS1 apparatus, 2D-gel electrophoresis (2DGE) was performed with bacterial culture supernatants of the wild-type and the *ΔvcrD1* mutant *V. parahaemolyticus* strains, and their proteomic patterns were compared. Culture supernatants were prepared from bacteria grown in two different media, LBS (Fig. 1a) and DMEM (Fig. 1b). Among proteins that were present at lower levels in the mutant supernatant (at least threefold less), protein spots in amount pertinent to further analysis were selected. Nineteen protein spots were identified and are listed in Table 3 in case of LBS-grown cells. Fifteen protein spots were chosen for further analysis in the supernatants derived from the DMEM-grown bacteria (Table 4).

In case of secretome prepared from bacterial cells grown in LBS, a majority of proteins secreted less in the *ΔvcrD1* mutant were associated with parts of the polar flagellum: FliK, FlaH, FlgK, and FlgL, which are in the flagellar hook; FlgD, which is a cap protein of a flagellar hook; and FlaA, FlaD, and FlaG, which are flagellins. This result suggests that VcrD1 plays a significant role in the assembly and development of a polar flagellum of *V. parahaemolyticus*. Earlier studies reported that T3SS is evolutionarily related to the secretion system for bacterial flagella, flagellar T3SS, through sharing structural and functional features (Jorges 2008). The other proteins are enzymes, including a chitodextrinase, chitinases, and an alkaline serine protease.

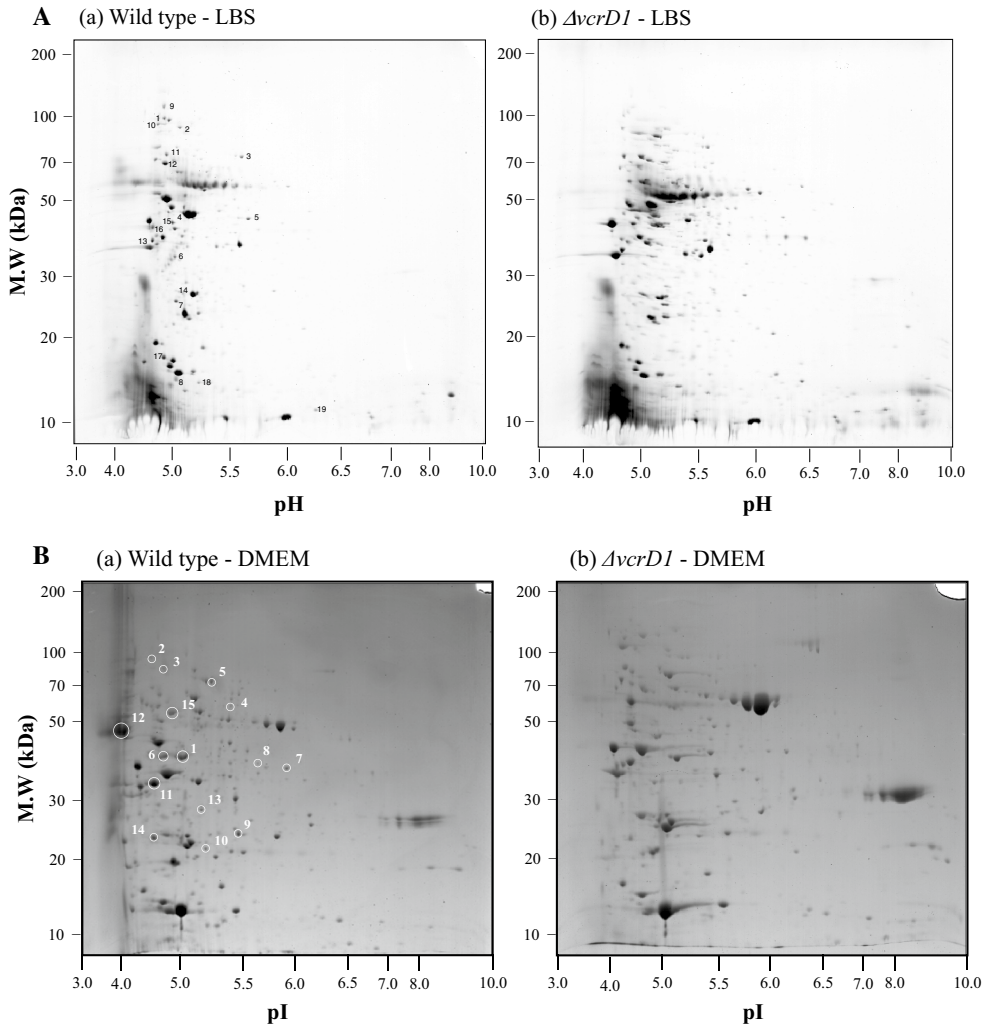

Fig. 1 Comparative analysis of secreted proteins using 2D gel electrophoresis of culture supernatants of wild-type and *ΔvcrD1 V. parahaemolyticus* strains. Bacteria were incubated in LBS broth (**a**) or DMEM (**b**) at 37 °C. Secreted proteins were analyzed by 2D-gel electrophoresis, including isoelectric focusing (pH 4–8) and SDS-PAGE. The resultant gels were stained with Coomassie brilliant blue G-250. Numbered proteins were identified by MALDI-TOF mass spectrometry. Identity of these proteins is listed in Tables 3 and 4 for LBS-secretome and DMEM-secretome, respectively

Two outermembrane proteins, OmpA and OmpV, were also secreted at lower levels in the *ΔvcrD1* mutant. Finally, six hypothetical proteins without any known function were present at lower levels in the secretome of the mutant.

Comparative proteomic analysis on the secretomes derived from *V. parahaemolyticus* grown in DMEM also demonstrated that secretion of the polar flagellin FlaA protein was affected in *ΔvcrD1* mutant (Table 4). In addition, an outermembrane protein OmpA and chitinase were found at lower levels in secretome of *ΔvcrD1* mutant than that in wild type. Unlike secretomes derived from LBS-grown cells, several metabolic enzymes for amino acids were secreted less from the *ΔvcrD1* mutant than wild type. Interestingly, some transport proteins were found at lower levels in the mutant secretome.

Confirmation of lower secretion of the flagellar proteins from the *ΔvcrD1* mutant by Western blot

Two of the eight flagellar proteins identified by 2DGE (Tables 3, 4), FlaA and FlgL, were confirmed for their low level in LBS-secretome of the *ΔvcrD1* mutant using their specific polyclonal antibodies. A 40-kDa immunoreactive band was present at a lower level in secretome of the *vcrD1* mutant than in the wild type (Fig. 2a). The *ΔvcrD1* mutant secretome also contained less FlgL than did the wild-type secretome (Fig. 2b). We also examined secretion of an additional flagellar protein, FlgE, in the culture supernatants of wild type and the *ΔvcrD1* mutant by Western blot using antibodies against *V. vulnificus* FlgE (Lee et al. 2004; Fig. 2c). Similar to FlaA and FlgL, the *vcrD1* mutant secreted less FlgE than did the wild type.

Table 3 Proteins with diminished secretion in the *vcrD1* mutant compared with wild type (cultivated in LBS)

Spot	Protein (ORF#)	MW (kDa)	pI	Score[a] (valid score)
1	Polar flagellar hook-length control protein FliK (VP2244)	68	4.5	126 (77)
2	Flagellar capping protein (VP2256)	73	4.7	125 (58)
3	Flagellar hook-associated protein FlgK (VP0785)	71	5.5	274 (58)
4	Polar flagellin FlaA (VP2258)	40	4.9	86 (58)
5	Flagellar hook-associated protein FlgL (VP0786)	45	5.5	201 (58)
6	Flagellin (VP0790)	40	4.9	61 (58)
7	Flagellar basal body rod modification protein (VP0777)	25	4.8	132 (58)
8	Flagellar protein FlaG (VP2257)	16	4.7	115 (58)
9	Chitodextrinase (VPA0832)	112	4.6	92 (58)
10	Chitinase (VPA0055)	90	4.4	102 (58)
11	Putative chitinase (VP0619)	63	4.6	48 (58)
12	Alkaline serine protease (VPA0227)	72	4.8	56 (58)
13	Outer membrane protein OmpA (VPA1186)	36	4.6	122 (58)
14	Putative outer membrane protein OmpV (VPA0318)	28	5.1	60 (58)
15	Hypothetical protein (VPA0917)	41	4.8	115 (58)
16	Hypothetical protein (VPA1077)	43	4.3	84 (58)
17	Hypothetical protein (VPA0242)	20	4.8	84 (58)
18	Hypothetical protein (VPA0934)	23	5.6	52 (58)
19	Hypothetical protein (VPA0669)	11	5.9	46 (58)

[a] Cutoff values for validation are in parentheses

Table 4 Proteins with diminished secretion in the *vcrD1* mutant compared with wild type (cultivated in DMEM)

Spot	Protein (ORF#)	MW (kDa)	pI	Score (valid score)
1	Polar flagellin FlaA (VP2258)	40	4.9	80 (71)
2	Chitinase (VPA0055)	90	4.4	128 (71)
3	Aminopeptidase (VP1604)	98	4.6	117 (71)
4	Urocanate hydratase (VP1274)	62	5.4	96 (71)
5	Lysine decarboxylase (VP2890)	80	5.2	211 (71)
6	Dihydrolipoamide dehydrogenase (VP2517)	51	5.7	80 (71)
7	Alanine racemase (VPA1001)	44	6.4	147 (71)
8	Arginine/ornithine succinyltransferase (VP2796)	38	5.5	75 (71)
9	Uridine phosphorylase (VP0960)	27	5.4	109 (71)
10	Purine nucleoside phosphorylase (VPA1475)	26	5.2	91 (71)
11	Outermembrane protein OmpA (VP0764)	34	4.4	76 (71)
12	Peptide ABC transporter periplasmic peptide-binding protein (VP0048)	57	6.0	172 (71)
13	ABC transporter substrate-binding protein (VP2080)	33	6.0	133 (71)
14	Spermidine/putrescine ABC transporter periplasmic spermidine/putrescine-binding protein (VP1525)	39	4.6	156 (71)
15	Molecular chaperone GroEL (VP2851)	58	4.7	94 (71)

[a] Cutoff values for validation are in parentheses

Lower levels of three flagellar proteins in the *vcrD1* mutant suggest that overall expression of flagellar proteins is decreased in the *ΔvcrD1* mutant. In the next experiment, secretion of FlgM was examined because it is an anti-σ28 controlling the temporal expression of flagellar genes, especially late genes (Kutsukake and Iino 1994). Thus, accumulation of FlgM in the cytoplasm of the *vcrD1* mutant affects the expression of downstream flagellar genes. However, Western blot analysis of LBS-culture supernatants demonstrated that less FlgM was present in the culture supernatant of the *ΔvcrD1* mutant (Fig. 2d). The TDH levels in wild-type and *vcrD1* mutant secretomes were evaluated by Western blot with anti-TDH antibodies as a loading control.

Both wild-type and mutant secretomes were prepared from *V. parahaemolyticus* incubated in DMEM and also analyzed by Western blot using these anti-flagellar protein

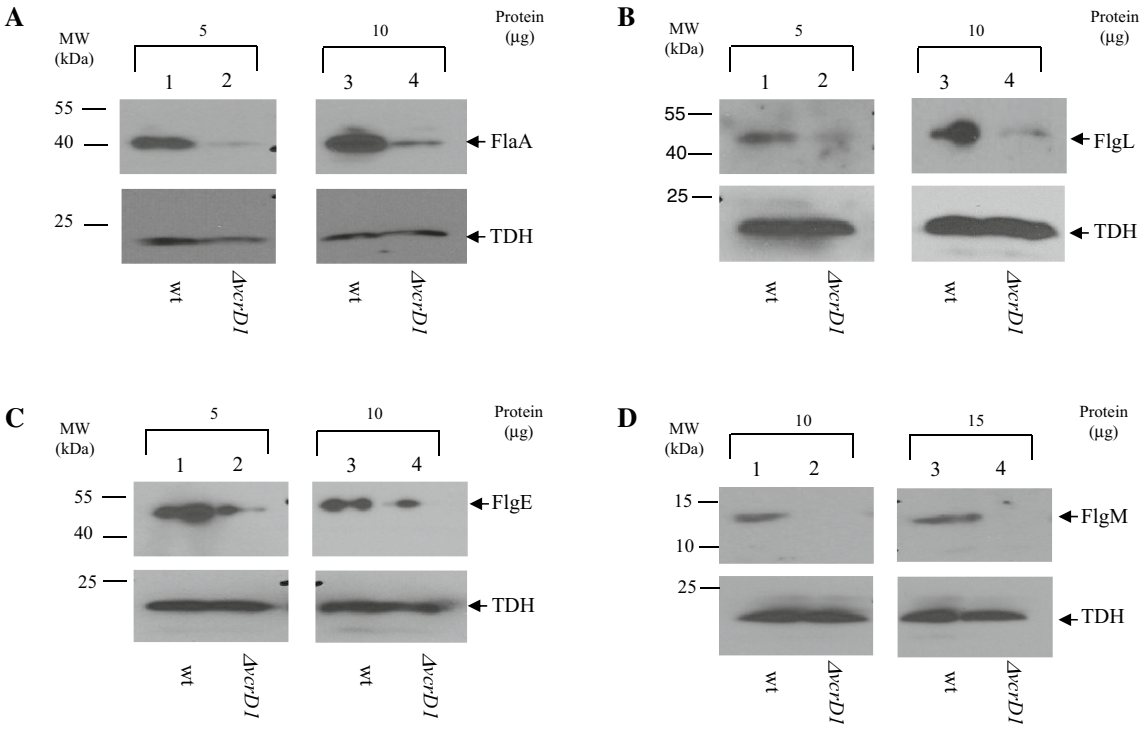

Fig. 2 Western blot analysis of LBS-secretomes of wild-type and *ΔvcrD1* mutant strains using polyclonal antibodies against four selected flagellar proteins. Two different amounts of proteins (5–15 μg) were used for Western blot analysis. Western blots using anti-FlaA antibodies (**a**), anti-FlgL antibodies (**b**), anti-FlgE antibodies (**c**), anti-FlgM antibodies (**d**), and anti-TDH antibodies as a loading control (**e**). *Lanes 1* and *3*, wild-type secretomes; *lanes 2* and *4*, *ΔvcrD1* secretomes

antibodies (Fig. 3). All of the four flagellar proteins, FlaA, FlgE, FlgL, and FlgM, were present at lower levels in the mutant secretome than wild-type secretome indicating that secretion of flagellar proteins was also affected by the *vcrD1* mutation in DMEM like the LBS-cultivated cells.

Role of *vcrD1* in bacterial flagella/motility of *V. parahaemolyticus*

Inoculation of the bacterial suspensions into LBS medium containing 0.3 % agar showed that the *ΔvcrD1* mutant strain did not have any motility, whereas wild-type *V. parahaemolyticus* had a distinct motile phenotype with a large diffuse spreading halo (Fig. 4a, b). Reduced diameter of the motility halo of the *ΔvcrD1* mutant was not caused from its decreased growth as shown in the similar growth curves of wild type and *vcrD1* mutant (Fig. S1).

The next experiment was to examine a possibility whether the loss of VcrD1 protein causes defects in various secretion system including flagellar components due to toxic accumulation of unexported T3SS1 components. An additional T3SS1 mutant with a defect in the master T3SS1 regulator, ExsA, was constructed and examined for its motility in combination with the *ΔvcrD1* mutant (Fig. 4a, b). The *ΔexsA* mutant showed intact motility similar with the wild type. Interestingly, *ΔvcrD1ΔexsA* double mutant demonstrated non-motile phenotype as did the *ΔvcrD1* mutant. A mutant *V. parahaemolyticus* with defects in LafK and FlaK was also included in the motility assays as a non-motile control. No significant difference in diameter of motility halos was detected among the *ΔvcrD1*, *ΔvcrD1ΔexsA*, and *ΔflaKΔlafK* strains when they were incubated up to 7 h in the motility plates (Fig. S2).

Transmission electron micrographs clearly showed that wild-type *V. parahaemolyticus* retained a polar flagellum but that the *ΔvcrD1* mutant had completely lost its flagellum (Fig. 4c). Less than 1 % of the *ΔvcrD1* mutant had a flagellum.

To confirm that loss of bacterial motility in the *ΔvcrD1* mutant is derived from absence of the *vcrD1* gene, a complementation strain, *ΔvcrD1* mutant carrying pRKvcrD1, was examined for its motility along with two control strains, wild type with pRK415 and *ΔvcrD1* strain with pRK415 (Fig. 5a, b). As expected, wild-type *V. parahaemolyticus* with pRK415 had motility, whereas *ΔvcrD1* mutant carrying pRK415 did not show any motility. The *ΔvcrD1* mutant with pRKvcrD1 had a comparable motility with wild type carrying pRK415.

Secretomes of these *V. parahaemolyticus* strains were analyzed by Western blot using anti-flagellar proteins,

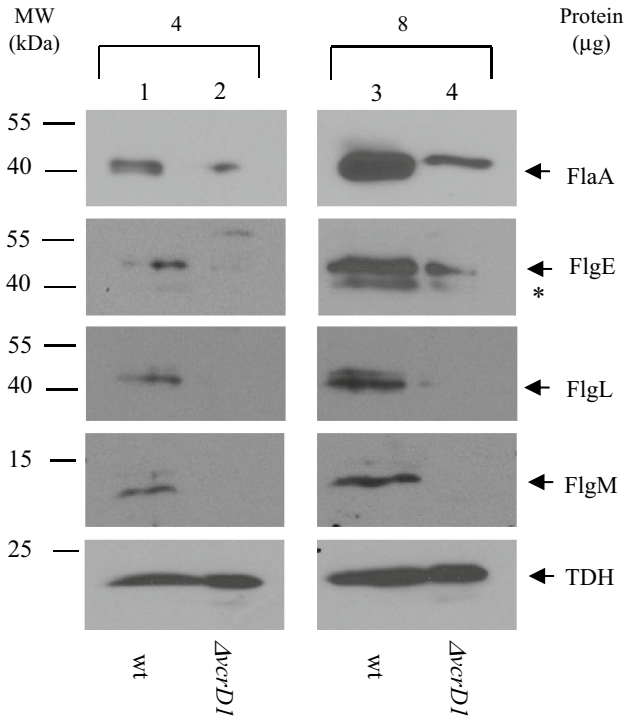

Fig. 3 Western blot analysis of DMEM-secretomes of wild-type and *ΔvcrD1* mutant strains using polyclonal antibodies against four selected flagellar proteins. Two different amounts of proteins (4 and 8 μg) were used for Western blot analysis using anti-FlaA antibodies, anti-FlgE antibodies, anti-FlgL antibodies, anti-FlgM antibodies, and anti-TDH antibodies. *Lanes 1* and *3*, wild-type secretomes; *lanes 2* and *4*, *ΔvcrD1* secretomes. Nonspecific protein is indicated with an *asterisk*

FlaA, FlgL, FlgE, and FlgM (Fig. S3A). Unexpectedly, FlaA protein was present at a similar level in these strains (wild type carrying pRK415, *ΔvcrD1* mutant with pRK415, and *ΔvcrD1* mutant with pRKvcrD1). The rest three proteins were found less in the secretome of the complemented strain, *ΔvcrD1* mutant with pRKvcrD1. Scanning electron micrographs of these strains revealed that wild type carrying pRK415 and *ΔvcrD1* mutant with pRKvcrD1 had a polar flagellum, whereas *ΔvcrD1* mutant with pRK415 did not have (Fig. S3B).

Determination of the expression level of flagellar components in wild-type and *ΔvcrD1* mutant *V. parahaemolyticus* strains

Since four flagellar proteins were present at lower levels in culture supernatant of the *ΔvcrD1* mutant, we examined the cellular fractions containing these proteins in *ΔvcrD1* mutant *V. parahaemolyticus*. Specifically, we determined whether FlaA or FlgM in the cytoplasm of the *ΔvcrD1* mutant was not successfully secreted through the outer membrane. Extracts of wild-type and *ΔvcrD1 V. parahaemolyticus* strains were divided into four fractions (cytoplasmic, periplasmic, membrane, and culture supernatant) that were analyzed by Western blot using anti-FlaA or anti-FlgM antibodies (Fig. 6a, b, respectively). FlaA was detected in all four fractions from the wild-type strain, but was barely detected in any fraction from the *ΔvcrD1* strain, indicating that decreased secretion of FlaA in the mutant resulted from decreased FlaA synthesis.

FlgM was found in all of the four fractions of wild-type cells. The amount of FlgM in the cytoplasmic and secreted fractions from the *ΔvcrD1* mutant was decreased, whereas it did not show any difference in the periplasmic and membrane fractions of the mutant compared with those of wild type. Western blot analysis of these fractions indicated that IIA^{Glc} was found in the cytoplasm, whereas TDH was only detected in the secreted proteins. In all of the fractions, OmpV was found, but mainly presented in the membrane fractions. Decreased levels of both FlgM and FlaA in the cytoplasmic fraction of the *ΔvcrD1* mutant raised a possibility that transcription of flagellar genes is decreased in this mutant.

Determination of transcript level of flagellar genes in wild-type and *ΔvcrD1* mutant *V. parahaemolyticus*

Using primers designed to amplify specific flagellar genes (Table 2), the levels of flagellar gene transcripts were monitored in the wild-type and *ΔvcrD1* mutant strains (Fig. 7). The *gap* gene encoding glyceraldehyde 3-phosphate dehydrogenase was constitutively expressed. Therefore, all transcript levels were normalized to the amount of *gap* transcript in the same RNA sample. Expression of two early flagellar genes, *flaK* and *flaL*, was not decreased in the *ΔvcrD1* mutant. Rather that, *flaK* transcription was significantly increased by the *vcrD1* mutation. Transcripts of the middle (*flgE*, and *fliF*) and late (*fliD*, and *flaA*) flagellar genes were decreased significantly in the *ΔvcrD1* mutant (from 28 to 3 % of wild-type). In addition, expression of the *flgM* gene was also reduced in the *ΔvcrD1* mutant, which is an anti-σ factor and one of middle/late genes. This result demonstrated that deficiency of *vcrD1* resulted in decreased transcription of flagellar genes, especially middle and late flagellar genes.

Discussion

Vibrio parahaemolyticus has two distinct NF-T3SSs, T3SS1 and T3SS2 (Park et al. 2004). VcrD protein is postulated to be a conserved member of the NF-T3SS secretion apparatus (Buttner 2012). Knocking out this conserved inner membrane proteins of these NF-T3SSs, VcrD1 or VcrD2, resulted in significant attenuation in bacterial

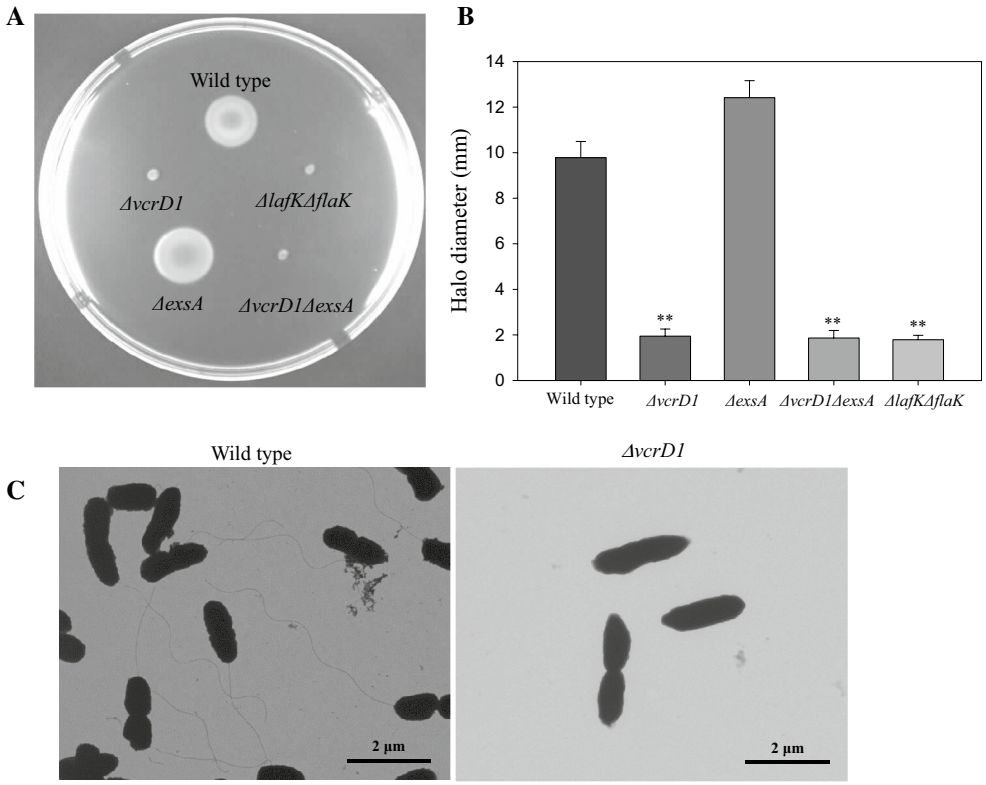

Fig. 4 Bacterial motility and polar flagellum of wild-type and *ΔvcrD1* mutant strains of *V. parahaemolyticus*. **a** Swimming motility of various *V. parahaemolyticus* strains (wild type, *ΔvcrD1* mutant, *ΔexsA* mutant, and *ΔvcrD1ΔexsA* mutant) was measured in LBS/0.3 % agar plates for 4 h. A double *ΔlafKΔflaK* mutant was also included in this motility assay as a non-motile control. **b** Quantification of bacterial motility by measuring the diameters (mm) of the motility halos made by these *V. parahaemolyticus* strains. Data are presented as the means ± standard deviation from three independent experiments. Statistical analyses were performed using Student's *t* test to evaluate the statistical significance of the results. Differences were considered significant when $P < 0.05$. Data with $P < 0.01$ are indicated with *two asterisks*. **c** Transmission electron micrographs of wild-type and *ΔvcrD1* mutant strains negatively stained with uranyl acetate. The *bar* represents 2 μm

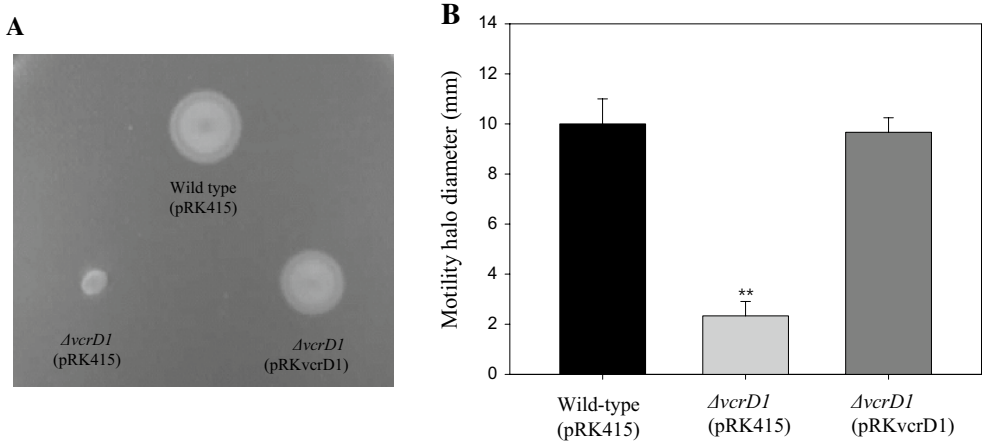

Fig. 5 Complementation of bacterial motility with an addition of wild-type *vcrD1* gene. **a** Swimming motility of various *V. parahaemolyticus* strains (wild-type carrying pRK415, *ΔvcrD1* mutant strain carrying pRK415, and *ΔvcrD1* mutant strain carrying pRKvcrD1) was measured in LBS/3 % agar plates for 4 h. **b** Quantification of bacterial motility by measuring the diameters (mm) of the motility halos made by these *V. parahaemolyticus* strains. Data are presented as the means ± standard deviation from three independent experiments. Statistical analyses were performed using Student's *t* test to evaluate the statistical significance of the results. Differences were considered significant when $P < 0.05$. Data with $P < 0.01$ are indicated with *two asterisks*

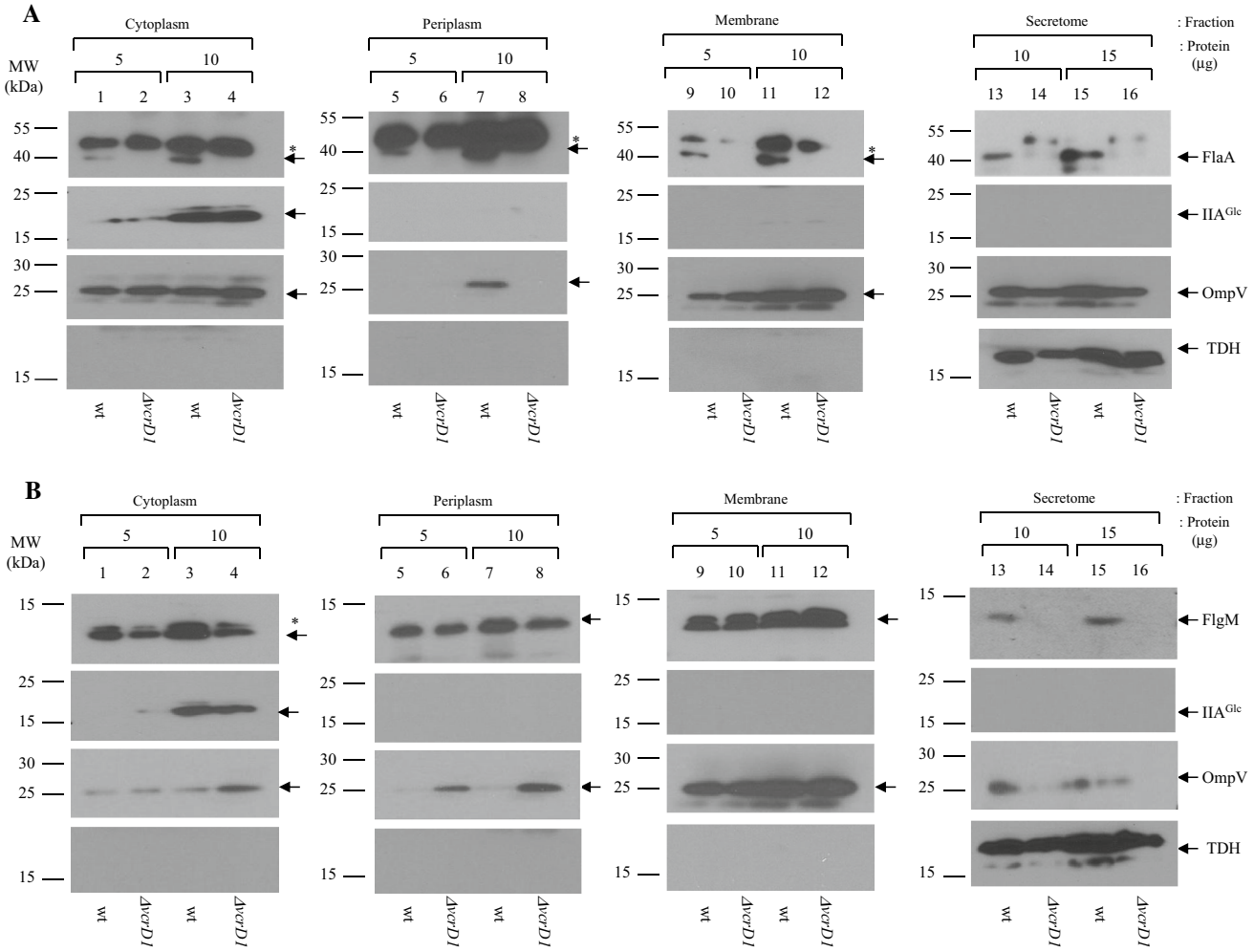

Fig. 6 Fractionation of wild-type and *ΔvcrD1* mutant *V. parahaemolyticus* into cytoplasmic, periplasmic, membrane and secretome proteins, and Western blot analysis of these fractions using anti-FlaA antibodies (**a**) or anti-FlgM antibodies (**b**). Two different amounts of each fraction (5–15 μg) were used for Western blot analysis. *Lanes 1* and *3*, cytoplasmic proteins of wild type; *lanes 2* and *4*, cytoplasmic proteins of *ΔvcrD1* mutant; *lanes 5* and *7*, periplasmic proteins of wild type; *lanes 6* and *8*, periplasmic proteins of *ΔvcrD1* mutant; *lanes 9* and *11*, membrane proteins of wild type; *lanes 10* and *12*, membrane proteins of *ΔvcrD1* mutant; *lanes 13* and *15*, secretomes of wild type; *lanes 14* and *16*, secretomes of *ΔvcrD1* mutant. Intracellular level of glucose-specific enzyme IIA^{Glc} was monitored as a control for the cytoplasmic proteins, whereas OmpV serves as a loading control for membrane proteins. TDH is a loading control for secretome proteins. Nonspecific proteins are indicated with *asterisks*, whereas specific proteins (FlaA, FlgM, glucose-specific enzyme IIA^{Glc}, OmpV, and TDH) are denoted with *arrows*

cytotoxicity and enterotoxicity, respectively (Park et al. 2004; Yang et al. 2011). This microorganism has dual flagellar systems: (1) a single polar flagellum that is continuously produced and (2) numerous lateral flagella that are synthesized under certain conditions, such as surface conditions (McCarter 1999). Further characterization of the *ΔvcrD1* mutant *V. parahaemolyticus* showed decreased secretion of several flagellar components (Figs. 1, 2, 3; Tables 3, 4) and raised a possibility that VcrD1, a component of NF-T3SS1, also plays a role in formation of the polar flagellum.

As expected, the *ΔvcrD1* mutant did not have motility and the polar flagellum (Fig. 4). Absence of a polar flagellum on the *ΔvcrD1* mutant of *V. parahaemolyticus* indicates that VcrD1-containing T3SS1 system might function in flagellum secretion. Non-motile phenotype of the double *ΔexsAΔvcrD1* mutant clearly indicated that the *exsA* mutation could not alleviate the defect caused by the *vcrD1* mutation (Fig. 4a, b). This data excluded a possibility that deficiency of VcrD1 causes malfunction of general secretion systems including the flagellar secretion. *V parahaemolyticus* has 60 potential flagellar genes, and their roles in flagellum assembly and transcriptional organization have been reported (Kim and McCarter 2000). Therefore, further experiments are needed to reveal how VcrD1 is involved in flagellum formation. For

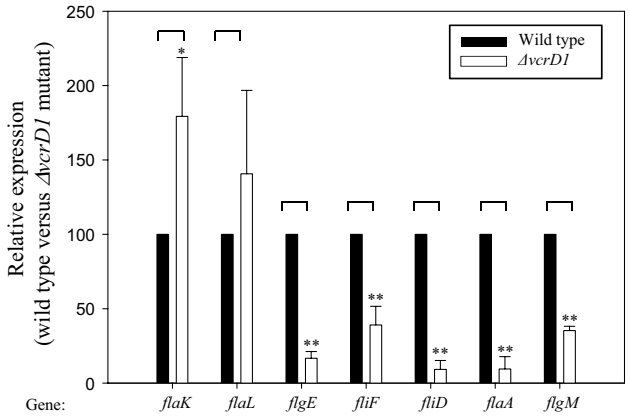

Fig. 7 Quantitative analysis of transcripts of seven flagellar genes. The wild-type and *ΔvcrD1* mutant *V. parahaemolyticus* cultured in LBS broth up to $OD_{600} = 0.8$, were harvested to isolate RNAs using TRizol (BioRad), which were treated with the RNase-free DNase (Qiagen). Four micrograms of RNAs were used to make cDNAs using PrimeScript RT reagent kit (TaKaRa). cDNA was analyzed by qRT-PCR on a Light Cycler 480 II real-time PCR system (Roche Applied Science) using Light Cycler 480 DNA SYBR green I master kit. The amount of each transcript was estimated using the specific pair of primers (Table 2). The experiments were performed in triplicate for each sample. The *gap* gene of *V. parahaemolyticus* was used as an endogenous control to normalize each transcript in wild-type and *ΔvcrD1* mutant cDNAs. Data analysis was based on the relative quantification method by determining the crossing point (C_p) value using the Light Cycler 480 II real-time PCR system software program (Roche Applied Science, version LSC480 1.5.0.39). Data are presented as the means ± standard deviation from three independent experiments. Statistical analyses were performed using Student's *t* test to evaluate the statistical significance of the results. Differences were considered significant when $P < 0.05$. Data with $P < 0.01$ are indicated with *two asterisks*, whereas data with *P* values between 0.01 and 0.05 are indicated with a *single asterisk*

example, physical association of VcrD1 with other flagellar components is an interesting question needed to be verified.

Motility was restored when the *ΔvcrD1* mutant was complemented with the wild-type *vcrD1* gene (Fig. 5a, b). In contrast with the bacterial motility, flagellar components were found in the secretomes of all the strains (wild type carrying pRK415, mutant carrying pRK415, and the complemented *vcrD1* mutant). A hypothesis was made to explain this discrepancy between motility and secretion of flagellar proteins that *V. parahaemolyticus* carrying pRK415 or pRKvcrD1 may have dramatic change in its membrane topology resulting in secretion of these proteins, but as inappropriate forms to assemble flagella. That is, the *ΔvcrD1* mutant carrying pRK415 may secrete flagellar components, but fail to assemble the flagellum in a correct form as demonstrated in Fig. S3B.

Lower cytoplasmic level of FlaA protein in the *ΔvcrD1* mutant suggests that decreased FlaA secretion could be caused by decreased expression of FlaA (Fig. 6a). Flagellum morphogenesis is a complex cascade of events, which requires coordinate expression 60 genes encoding structural subunits, regulatory proteins, and chemosensor machinery. These genes have been categorized into three groups in relation to their temporal expression during assembly of the polar flagellum: early, middle, and late genes (Aldridge and Hughes 2002).

Early genes encode FlaK and FlaLM, transcriptional regulators, required for expression of the middle genes. The middle genes transcribed by σ54 include ORFs encoding assembly proteins, σ28, hook proteins, and basal body proteins. Sigma 54 also transcribes the late genes encoding flagellins, motor proteins, and the anti-σ factor, FlgM (McCarter 2001). FlgM is an important regulatory factor for temporal coordination of flagella gene expression, which blocks premature expression of late filament genes by binding to FliA. Secretion of FlgM through a complete hook-basal-body results in expression of the late filament genes (Ohnishi et al. 1992). Therefore, we examined whether decreased expression of flagellar proteins is caused by the altered level of cytoplasmic FlgM (Fig. 6b). Western blot analysis indicated that FlgM expression is also decreased in the *ΔvcrD1* mutant *V. parahaemolyticus*. This result suggests that the altered FlaA level is not due to increased amount of cytoplasmic FlgM caused by unsuccessful FlgM secretion.

Quantitative measurement of flagellar transcripts demonstrated that the *ΔvcrD1* mutation affected early genes and middle/late genes differently (Fig. 7). Failed secretion of flagellar components has been reported to result in abortion of transcription of downstream flagellar genes (Chilcott and Hughes 2000). Malfunctioning flagellar secretion caused by VcrD1 deficiency does not interfere with the early steps of flagellum assembly, but prevents the transcriptional cascade in the middle and late stages of flagellum morphogenesis.

This observation suggests that the role of T3SS1 in cytotoxicity of *V. parahaemolyticus* should be re-evaluated. The loss of polar flagellum/motility in the *ΔvcrD1* mutant (Fig. 4) may be a factor responsible for the attenuated cytotoxicity of the mutant. In *V. vulnificus*, flagellum/motility has been reported as an important virulence factor (Lee et al. 2004). So far, three effector proteins delivered by T3SS1 have been identified: VopQ (VepA) (Burdette et al. 2009), VopS (Yarbrough et al. 2009), and VPA0450 (Broberg et al. 2010). To define the exact role of T3SS1 as an NF-T3SS, additional mutants that are defective in secretion of these effectors but retain polar flagellum/motility should be analyzed. Another NF-T3SS of *V. parahaemolyticus*, T3SS2, has been shown to be involved in delivering several effectors, such as VopA/P, VopC, VopV, VopT, and VopL (Zhang and Orth 2013; Ham and Orth 2012). A T3SS2-deficient *V. parahaemolyticus* was constructed by deleting

the *vcrD2* gene that has an amino acid sequence 25 % identical to VcrD1 (Park et al. 2004; Lee and Park unpublished result). This *ΔvcrD2* mutant demonstrated intact motility (Noh and Park unpublished result), suggesting that T3SS1 and T3SS2 of *V. parahaemolyticus* function differentially with respect to flagellum biogenesis.

Concluding remarks

Absence of the VcrD1 protein, which has been considered to be a NF-T3SS component, resulted in a loss of bacterial flagellum/motility in *V. parahaemolyticus* due to decreased secretion of flagellar components and decreased expression of middle and late flagellar genes. The regulatory mechanism for VcrD1 involvement in flagella formation needs to be elucidated in future studies.

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Conflict of interest The authors have no conflict of interest.

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