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Deletion of *degQ* gene enhances outer membrane vesicle production of *Shewanella oneidensis* cells

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Abstract

Shewanella oneidensis is a Gram-negative facultative anaerobe that can use a wide variety of terminal electron acceptors for anaerobic respiration. In this study, *S. oneidensis degQ* gene, encoding a putative periplasmic serine protease was cloned and expressed. The activity of purified DegQ was inhibited by diisopropyl fluorophosphate, a typical serine protease-specific inhibitor, indicating that DegQ is a serine protease. In-frame deletion and subsequent complementation of the *degQ* was carried out to examine the effect of envelope stress on the production of outer membrane vesicles (OMVs). Analysis of periplasmic proteins from the resulting *S. oneidensis* strain showed that deletion of *degQ* induced protein accumulation, and resulted in a significant decrease in protease activity within the periplasmic space. OMVs from the wild-type and mutant strains were purified and observed by transmission electron microscopy. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the OMVs showed a prominent band at ~37 kDa. Nano liquid chromatography-tandem mass spectrometry analysis identified three outer membrane porins (SO3896, SO1821 and SO3545) as dominant components of the band, suggesting that these proteins could be used as indices for comparing OMV production by *S. oneidensis* strains. Quantitative evaluation showed that *degQ*-deficient cells had a five-fold increase in OMV production compared with wild-type cells. Thus, the increased OMV production following the deletion of DegQ in *S. oneidensis* may be responsible for the increase in envelope stress.

Introduction

Many Gram-negative bacteria form outer membrane vesicles (OMVs) as part of their natural growth cycle (Rothfield and Pearlman-Kothencz 1969; Wensink and Witholt 1981; Nowotny et al. 1982; Beveridge 1999). OMVs are spherical bilayered proteolipids with a diameter of 20–250 nm, and are compositionally similar to the outer membranes of bacteria, which contain outer membrane proteins, lipids, periplasmic proteins, lipopolysaccharides, RNA, and DNA (Knox et al. 1966; Kadurugamuwa and Beveridge 1996). Gram-negative bacteria living in fresh water environments and as pathogens in animal hosts produce OMVs (Schwechheimer et al. 2013). Although OMV production appears to be a ubiquitous physiological process, the factors triggering OMV formation have not been conclusively identified. Previous research suggested that disturbances in growth, exposure to antibiotics, or simply turnover in cell wall components initiates vesicle formation (Knox et al. 1966; Kadurugamuwa and Beveridge 1995; Zhou et al. 1998).

Recent work has also suggested that OMV production is closely related to envelope stress (McBroom and Kuehn 2007; Schwechheimer and Kuehn 2015). The hypothesis that vesiculation plays an important role in disposing envelope ‘‘garbage’’ was first proposed with respect to proteinaceous waste accumulation in an *Escherichia coli* *degP*-deficient strain. DegP is a periplasmic chaperone and protease in *E. coli* that manages envelope stress caused by unfolded and misfolded periplasmic proteins (Lipinska et al. 1990; Schwechheimer and Kuehn 2013). Random mutagenesis of *E. coli* determined that a transposon insertion immediately following the start codon of *degP* resulted in hypervesiculation (McBroom et al. 2006). Misfolded proteins were not digested in the *degP*-deficient strain, and such undigested substrates can cause lethality

under conditions of relatively high temperature, at which protein misfolding is more likely to occur (Strauch et al. 1989). Notably, the lumen of OMVs produced by the *degP*-deficient strain contained misfolded outer-membrane proteins, which can be substrates for DegP (Schwechheimer and Kuehn 2013). Thus, *E. coli* strains lacking DegP likely increase OMV production as a survival strategy to eliminate these undesired proteins from the cell envelope.

Shewanella oneidensis is a Gram-negative facultative anaerobe that can use a wide variety of terminal electron acceptors for anaerobic respiration (Myers and Nealson 1988; Myers and Nealson 1990; Myers and Myers 1994; Myers and Myers 2000). It plays an important role in many environmental and biotechnological processes, including removing toxic metal contaminants, such as uranium and chromium, by reductive reactions (Fredrickson et al. 2008; Belchik et al. 2011). The genome sequence of *S. oneidensis* suggested the presence of a putative periplasmic serine protease, DegQ (Dai et al. 2015). Furthermore, deletion of *degQ* resulted in severe growth defects at higher temperatures, indicating that DegQ might act as a major protease for protein quality control in the periplasm (Dai et al. 2015). It is therefore important to clarify the relationship between OMV production and periplasmic protease DegQ in *S. oneidensis*.

In this study, we investigated the relationship between DegQ and OMV production in *S. oneidensis* from the perspective of envelope stress. DegQ was confirmed as a functional serine protease by using purified DegQ. In addition, the *degQ*-deficient *S. oneidensis* mutant was compared with the wild type to identify differences in amount and level of protease activity in the periplasmic proteins. OMV production was examined, and the proteins associated with the isolated OMVs from *S. oneidensis* were

identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses.

Materials and methods

Bacterial strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. For genetic manipulations, *E. coli* and *S. oneidensis* strains were cultured in lysogeny broth (LB) medium (10 g L^{-1} Hipolypepton (Wako Pure Chemical Industries), 5 g L^{-1} Bacto-yeast extract, and 10 g L^{-1} NaCl) at 37 and 30°C, respectively. The culture media of strains harboring the plasmid were supplemented with 25 mg L^{-1} chloramphenicol and 1 mM isopropyl thiogalactoside (IPTG) when necessary.

All *S. oneidensis* strains were precultured in LB medium for 18 h at 30°C, and then inoculated into 80 mL of fresh LB medium in a flask to give an optical density at 660 nm (OD_{660}) = 0.01. The cultures were placed on a rotary shaker (NR-20, Taitec) with shaking at 120 strokes per minute. Cell growth was recorded on a basis of dry cell weight (DCW) by measuring changes in OD_{660}.

In-frame deletion mutagenesis

The *degQ* (SO3942) gene was deleted in-frame from the *S. oneidensis* genome as described previously (Fennessey et al. 2010; Gao et al. 2010; Sundararajan et al. 2011). Briefly, a 592-bp fragment of the region upstream of the open reading frame (ORF), a 364-bp fragment of *degQ* (from 989 - 1353 bp), and a 292 bp fragment of the region downstream of the ORF were amplified by PCR, generating fragments F1 and F2, which were fused by overlap extension PCR to generate fragment F3 (see

Supplementary Fig. S1). The primers used for construction are listed in Supplementary Table S1. Fragment F3 was cloned into suicide plasmid pRE112 (Edwards et al. 1998) using *SacI* and *XbaI* restriction endonucleases, and then transformed into JM109 λpir (Penfold and Pemberton 1992) by the calcium chloride method. The resulting plasmid, pRE112-F3, was transferred into recipient *S. oneidensis* MR-1 cells by conjugation from *E. coli* SM10 λpir (Miller and Mekalanos 1988). Integration of the mutagenesis construct into the chromosome was induced to generate the final deletion strain. Gene deletion was verified by PCR using the F1-F and F2-R primers. The resulting mutation was confirmed as an in-frame deletion that removed approximately 1000 bp of the *degQ* gene. The mutant strain was named Δ*degQ*.

Knock-in complementation analysis

The *degQ* region (SO3942) was amplified from *S. oneidensis* MR-1 genomic DNA using primer pair *degQ*-F and R (see Supplementary Table S1). The amplicon was ligated into pHSG399 at the *Xho*I and *Bam*HI restriction sites to generate recombinant plasmid pHSG399-*degQ*. The purified pHSG399-*degQ* (approximately 2 μg) was transformed into Δ*degQ* cells by electroporation using 0.2-cm Gene Pulser/MicroPulser cuvettes (Bio-Rad) and a Bio-Rad XPulser. Immediately after transformation, 0.5 mL of super optimal broth with catabolite repression (SOC) medium devoid of antibiotics was added to the cell suspension. Cells were transferred into a sterile culture tube and incubated at 30°C for 1 h with continuous shaking. Following incubation, the cells were spread onto LB agar plates supplemented with 25 μg/mL chloramphenicol and incubated at 30°C for 2–3 days. The complementation strain was named Δ*degQ*+*degQ*.

Characterization of DegQ

Plasmid pHSG399-*degQ*-His was also constructed to express DegQ with a His6-tag sequence at the C-terminus. The plasmid was transformed into wild-type *S. oneidensis* MR-1 cells by electroporation, and the resulting strain was named WT+*degQ*-His. The cells were pre-cultured and then inoculated into 80 mL of LB medium containing 25 μg/mL chloramphenicol and incubated with shaking at 30°C. When OD_{660} = 0.5, IPTG was added to the cultures at a final concentration of 1 mM, and the cultures were further incubated for 20 h. The cells were harvested by centrifugation at 4,000 ×*g* for 10 min at 4 °C. The cell pellets were resuspended in phosphate-buffered saline (PBS; pH7.4) and disrupted by ultrasonication. The supernatant was recovered by centrifugation at 2,800 ×*g* for 10 min at 4 °C, followed by additional centrifugation at 10,000 ×*g* for 10 min at 4 °C. The supernatant was purified by using a Bio-Scale Mini Profinity IMAC cartridge as well as a Profinia protein purification system (Bio-Rad Laboratories Inc.) based on the affinity of the His6-tag, according to the manufacturer's protocol. The concentration of purified DegQ was determined by a Pierce BCA Protein Assay kit (Thermo Fisher Scientific Inc.) using bovine serum albumin as a standard. A 5-μL aliquot of purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie Blue staining. For western bloting, protein was transferred from the gel to a membrane sheet of Hybond P (GE Healthcare Ltd.) using the semi dry transfer method. Hybridization was conducted using an anti-His6 primary antibody and an ECL Western Blotting Starter kit (GE Healthcare Ltd.) according to the manufacturer's protocol. Hybridization signals were detected using a ChemiDoc imaging system (Bio-Rad Laboratories Inc.).

Protease activity was determined as described previously (Secades and Guijarro

1999) using azocasein as a substrate, with slight modifications. A 120-µL solution of purified DegQ containing 0.12 mg protein was mixed with 480 µL of 0.1% (w/v) azocasein in 25 mM Tris-HCl buffer (pH 7.6) with 5 mM $MgCl_2$, and the mixture was incubated for 30 min at 30°C. When necessary, diisopropyl fluorophosphate (DFP) was added as a serine protease-specific inhibitor. A 600-µL volume of 10% (v/w) trichloroacetic acid was added to terminate the reaction, and samples were incubated for 30 min on ice, followed by centrifugation at 15,000 ×*g* for 10 min at 4°C. An aliquot of supernatant (800 µL) was mixed with 200 µL of 1.8 M NaOH, and the absorbance at 440 nm (A_{440}) was measured using an ultraviolet-visible (UV-Vis) spectrometer (UV-2600 PC, Shimadzu Corp.). One unit of enzyme activity was defined as the amount of enzyme that yielded an increase in A_{440} of 0.01 per minute at 30°C.

Gene expression analysis

To examine gene expression, each strain was cultured in 80 mL of LB medium at 30°C and then harvested at 4 h post-inoculation by centrifugation at 4°C for 10 min at 5 000 ×*g*. Total RNA was extracted from the collected cells as described elsewhere (Nguyen et al. 2014), and then reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio Inc.) with random hexamer primers. Gene expression was analyzed by real-time PCR, as described in our previous study (Nguyen et al. 2014). The gene expression level was normalized against that of *rrsA* (16S rRNA). The specific primer pairs are listed in Table S1.

Isolation of periplasmic proteins and assay for proteolytic activity

Periplasmic proteins from *S. oneidensis* were extracted according to the previously

reported method (Quan et al. 2013) with some modifications. *S. oneidensis* cells were harvested from 80 ml of culture broth at 6 h post-inoculation by centrifugation at 3,000 ×*g* for 20 min at 4°C. Precipitant was gently resuspended in 2 mL of Tris-sucrose-EDTA (TSE) buffer (200 mM Tris-HCl buffer containing 500 mM sucrose and 1 mM EDTA, pH 8.0) using a wire loop. The cells were then incubated on ice for 30 min, and the supernatant containing envelope extract was collected by centrifugation at 16,000 ×*g* for 30 min at 4°C. Finally, the periplasmic fraction was isolated as a supernatant by ultracentrifugation at 100,000 ×*g* (CS100FNX, Hitachi) for 1 h at 4°C. Protein concentrations were determined by the Bradford assay, with bovine serum albumin as a standard.

Protease activity of periplasmic proteins was also determined using azocasein, as described above. The periplasmic fraction from the sample (120 μL) was mixed with 480 μL of 0.1% (w/v) azocasein in 25 mM Tris-HCl buffer (pH 7.6) containing 5 mM $MgCl_2$.

Isolation and transmission electron microscope (TEM) observation of OMVs

OMVs were isolated as previously described (Gujrati et al. 2014) with some modifications. Following incubation for 24 h, *S. oneidensis* culture broth (80 mL) was centrifuged at 3,970 ×*g* for 10 min at 4°C. The supernatant was then passed through a 0.45 μm pore–size filter. The contents were precipitated using ammonium sulfate (final concentration, 400 g L^{-1}) at room temperature for 1 h. The crude OMVs obtained by centrifugation at 12,450 ×*g* for 30 min at 4°C were dissolved in 500 μL of 15% (v/v) glycerol, and then concentrated by ultracentrifugation (CS100FNX, Hitachi) at 109,000 ×*g* for 1 h. The OMV pellets were resuspended in 100 μL of 15% (v/v) glycerol solution.

As a result, the OMV samples were concentrated 800–fold as compared with the original culture broth. For observation of flocs using a TEM (H6000, Hitachi), the OMV samples were dropped onto a mesh copper grid and negatively stained with 4% uranyl acetate.

SDS-PAGE and nano LC-MS/MS analyses of OMVs

A 5-μL sample of isolated OMVs from each strain was analyzed by SDS-PAGE with Coomassie Blue staining. OMV production was quantified according to the previously described method (Schwechheimer and Kuehn 2013) with some modifications. The SDS-PAGE band at ~ 37 kDa was analyzed by densitometry (NIH Image J software) as an index of OMV concentration. The OMV density value was divided by dry cell weight (DCW) in each culture to calculate cell-based OMV production, which was then normalized against OMV production of the wild-type strain. The 37-kDa band was cut from the gel and subjected to in-gel digestion with trypsin. The proteins were analyzed using nano-flow liquid chromatography with online tandem mass spectrometry (nanoLC/ESI MS/MS system) composed of an LTQ Orbitrap Velos (Thermo Fisher Scientific Inc.) coupled with a nanoLC (Advance, Michrom BioResources) and an HTC–PAL autosampler (CTC Analytics), at the Core Instrumentation Facility of the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan). Tandem mass spectra were acquired automatically, and then searched against an *S. oneidensis* database from the National Center for Biotechnology Information using the Mascot Server (Matrix Science).

Results and Discussion

Characterization of DegQ

The His6-tagged DegQ protein expressed in the *S. oneidensis* WT+*degQ*-His strain was purified and then analyzed by SDS-PAGE. DegQ was detected as a major band at a molecular weight of 45 kDa (Fig. 1A), corresponding to the expected size of DegQ protein. This indicated that the His6-tagged DegQ was successfully expressed in the form of soluble molecules in the WT+*degQ*-His cells. In addition, western blot analysis using anti-His primary antibody was conducted to further confirm the presence of DegQ. A clear signal at ~ 45 kDa was observed (Fig. 1B), confirming that the purified protein is DegQ.

The protease activity of purified DegQ was confirmed by the azocasein hydrolysis assay. As shown in Fig. 1C, the activity of DegQ was 5.4 U mg-protein^{-1}. As DegQ is thought to be a putative periplasmic serine protease, the effect of DFP, a serine protease-specific inhibitor (Swamy et al. 1983), on the activity of DegQ was examined. As expected, 20 mM DFP inhibited protease activity of DegQ by 85%, suggesting that the purified protein was a serine protease. Thus, these results confirmed that the *S. oneidensis degQ* gene product is a serine protease.

Deletion and complementation of *degQ* in *S. oneidensis*

To confirm the in-frame deletion of *degQ*, genomic DNA was extracted from wild type *S. oneidensis* and the Δ*degQ* mutant. The *degQ* gene (1353 bp) plus 592 bp and 292 bp of the upstream and downstream regions, respectively, were amplified by PCR. Electrophoresis results are shown in Supplementary Fig. S1. An approximately 2.2–kbp fragment was amplified from the wild type cells, corresponding to the combined size of *degQ* plus the surrounding regions. In contrast, an ~ 900 bp band was amplified from

the putative Δ*degQ* mutant. Furthermore, real–time PCR analysis did not detect *degQ* mRNA expression from the mutant cells (Table 2). These results indicate the successful deletion of *degQ* from the chromosome of *S. oneidensis*.

The complete *degQ* gene was expressed and re-introduced into the Δ*degQ* mutant using the pHSG399–*degQ* plasmid. The relative expression of *degQ* (normalized against that of *rrsA*) in the resultant Δ*degQ*+*degQ* cells at 4 h post-inoculation was 1.5×10^{-2}, which was much higher than the expression in wild type cells (6.3×10^{-5}) (Table 2). This increased expression compared with the wild type can be attributed to the high copy number of pHSG399 plasmid (Takeshita et al. 1987).

Periplasmic protease activity

Figure 2 shows representative growth curves of the wild type *S. oneidensis*, deletion mutant Δ*degQ*, and complemented mutant Δ*degQ*+*degQ* strains. The wild type and Δ*degQ* strains showed similar growth profiles (average growth rate of about 0.6 g–DCW h^{-1} during 6-12 h). The cell densities of the wild type and Δ*degQ* cultures at 24 h post-inoculation reached DCW = 6.9 and 7.8 g L^{-1}, respectively. It can be mentioned that the deletion of *degQ* did not repress cell growth at 30°C. Meanwhile, the growth of Δ*degQ*+*degQ* cells appeared to be slightly slower at 12 h than the other two strains, probably owing to the load of the high-copy number plasmid. The cell density of Δ*degQ*+*degQ* reached DCW = 6.7 g L^{-1} at 24 h. On the whole, these results suggest that the deletion and complementation of *degQ* did not drastically influence the growth of *S. oneidensis*.

Next, cells of each strain were harvested at exponential growth phase (culture time of 6 h post-inoculation) for isolation of periplasmic proteins and analysis of

protease activity. Table 2 shows protease activity on a DCW basis, as well as the concentration of periplasmic proteins obtained from each strain. The specific protease activity of the periplasmic proteins from the wild type was 19.4 U g–DCW^{-1}, while the activity of the Δ*degQ* cells was 7.0 U g–DCW^{-1}, corresponding to only 30% of that of the wild type. Thus, the periplasmic protease activity was significantly lowered by deletion of *degQ*. In contrast, the complemented mutant, Δ*degQ*+*degQ*, had the highest protease activity of 61.3 U g–DCW^{-1}. This corresponded with the increased *degQ* mRNA expression observed in Δ*degQ*+*degQ* cells compared with the wild type.

Interestingly, the concentration of periplasmic proteins from the Δ*degQ* cells was 2.56 mg g–DCW^{-1}, which was approximately 2.5 times greater than that from the wild type cells (1.03 mg g–DCW^{-1}). This result strongly suggests that deletion of *degQ* induced protein accumulation in the periplasmic space of *S. oneidensis* cells. In contrast, the complemented mutant Δ*degQ*+*degQ* cells produced the lowest concentration of periplasmic proteins (0.44 mg g–DCW^{-1}), which corresponds with the increased protease activity of this strain. Thus, our results strongly suggest that DegQ is a dominant periplasmic protease of *S. oneidensis*, and that lack of DegQ induces protein accumulation in the periplasmic space.

Comparison of OMV production among strains

As in the case of *E. coli* (McBroom and Kuehn 2007; Schwechheimer and Kuehn 2013), it is likely that accumulation of periplasmic proteins in *S. oneidensis* cells would induce OMV production, owing to envelope stress. Therefore, the insoluble fraction of the supernatant containing OMVs was obtained from wild type, Δ*degQ*, and Δ*degQ*+*degQ* culture broths at a culture time of 24 h by ultracentrifugation. The

extracted OMVs from each strain were observed by TEM with negative staining (Fig. 3). All strains produced OMVs, and while OMVs from the wild type culture tended to aggregate, there was no significant difference in the size of OMVs between the wild type and Δ*degQ* strains. On the other hand, fewer OMVs were obtained from the Δ*degQ*+*degQ* cells compared with the other strains, indicating reduced OMV production. In addition, co-precipitating appendages such as fimbriae and flagella were confirmed in the sample from the Δ*degQ*+*degQ* cells.

To compare OMV production, the fractions containing OMVs from each *S. oneidensis* strain were analyzed by SDS-PAGE and qualitatively compared with the wild type. The protein bands in the sedimented fractions from the Δ*degQ* cells were much more intense (Fig. 4A), while the bands from the Δ*degQ*+*degQ* cells were much weaker. These results suggested that deletion of *degQ* enhanced OMV production, as was observed in *E. coli* (McBroom et al. 2006). However, it has been reported that a portion of the periplasmic proteins is included inside the OMV structure when OMVs are generated (Lee et al. 2007). Therefore, we cannot exclude the possibility that the more intense bands from the Δ*degQ* sample is a consequence of increased amounts of periplasmic proteins inside the OMVs, rather than being indicative of enhanced OMV production.

From this perspective, in the case of *E. coli*, the bands observed in the sample at ~37 kDa (OmpF, OmpC, and OmpA) could be used as an index of OMV concentration, as these membrane proteins are expressed specifically and abundantly within the outer membrane of cells, and thus, are also found in abundance in OMVs (Schwechheimer and Kuehn 2013). Therefore, the same type of index could be used for evaluation of OMV production by *S. oneidensis*. As shown in Fig. 4A, a prominent band with a

molecular mass of ~37 kDa was confirmed from all strains by SDS-PAGE analysis. The proteins contained within this band were then examined by nano LC-MS/MS analysis. Table 3 summarizes the properties of 10 proteins in descending order according to matching score from the first analysis. Among these proteins, OmpS38 (SO3896) had the highest score. Omp35 is a putative porin on the outer membrane of *S. oneidensis* cells that was confirmed by subcellular fraction analysis (Maier and Myers 2004). OmpS38 is upregulated under anaerobic conditions and is involved in respiration of non-oxygen electron acceptors (Gao et al. 2015). The protein with the next highest score was also identified as a putative outer membrane porin (SO1821) (Heidelberg et al. 2002). These two outer membrane porins were confirmed to have the highest scores during the second analysis run (Table 3). MotA, a flagellar motor transmembrane channel protein, had the third highest score, while, the fourth protein (SO3545) was OmpA, which is also outer membrane porin (Gao et al. 2015). Thus, outer membrane proteins dominated the OMV samples, indicating that the bands at ~37 kDa can be used as an index of OMV production in *S. oneidensis*. Taking these findings into account, the OMV production was quantitatively analyzed based on the densitometry of the band at ~37 kDa from each strain. As a result, we determined that OMV production by the Δ*degQ* strain was about five times greater than that of the wild type strain (Fig. 4B). In contrast, the complemented Δ*degQ*+*degQ* strain showed about a 60% reduction in OMV production compared with the wild type strain. Together with the data from periplasmic protease activity analysis and periplasmic protein concentration, our results suggest that OMV production was promoted by the deletion of *degQ* in *S. oneidensis* cells as a result of the accumulation of excessive amounts of periplasmic proteins.

In conclusion, our results show that *degQ* encodes a serine protease in *S. oneidensis*. Deletion of *degQ* resulted in the accumulation of periplasmic proteins, and led to a significant decrease in protease activity within the periplasmic space. SDS-PAGE analysis of isolated OMVs showed the band corresponding to outer membrane porins was more intense in the Δ*degQ* cells. Thus, OMV production by *S. oneidensis* was promoted by the lack of DegQ, most likely as a result of an increase in envelope stress.

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FIGURE LEGENDS

Fig. 1 Purification and characterization of DegQ protein. (A) SDS-PAGE analysis of purified DegQ protein. DegQ was expressed in the *S. oneidensis* WT+*degQ*-His strain. (B) Western blot analysis of purified DegQ protein. Hybridization was conducted using anti-His6 primary antibody. (C) Protease activity of purified DegQ protein with or without serine protease inhibitor, diisopropyl fluorophosphates (DFP). Data was determined from three independent experiments. Vertical bars indicate standard deviation. An asterisk indicates statistical significance as determined by a Student's t-test (p<0.05).

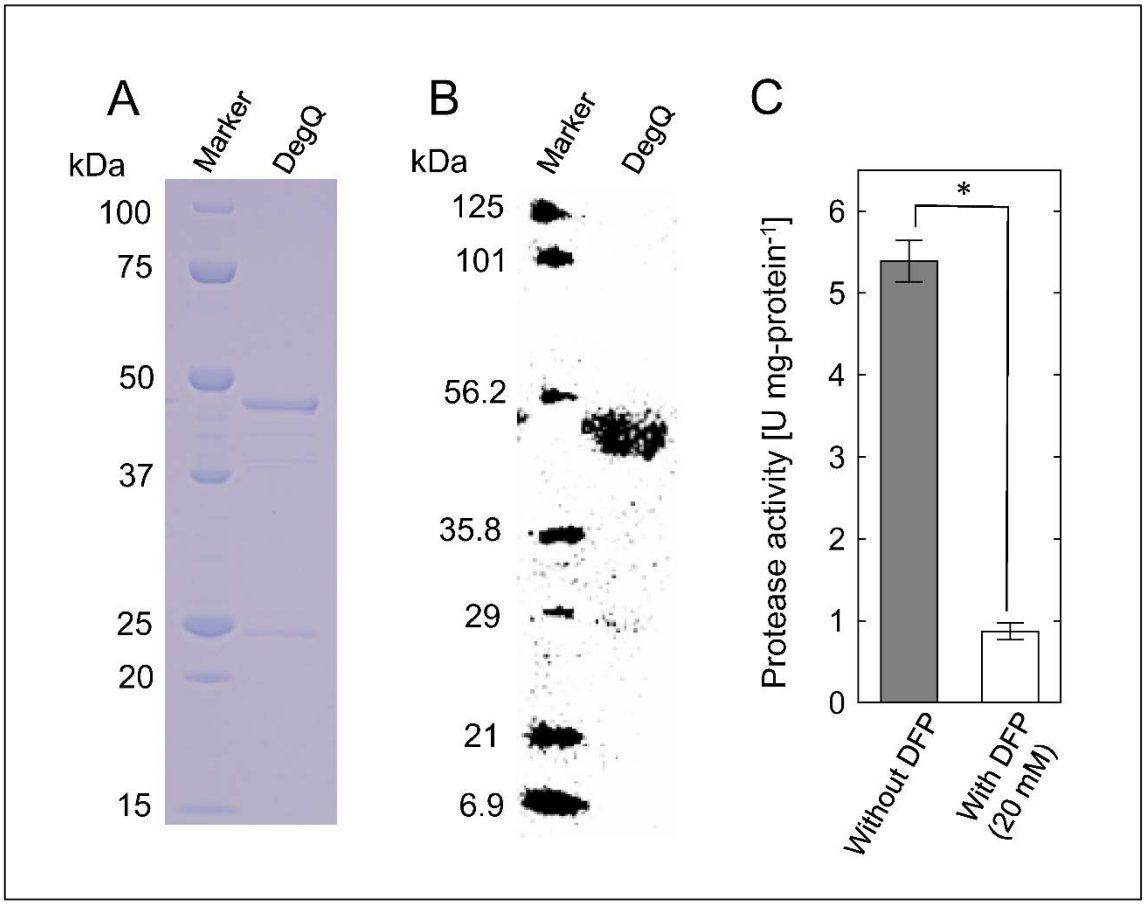


Fig. 2 Growth profiles of *S. oneidensis* wild type, Δ*degQ*, and Δ*degQ*+*degQ* strains in LB medium at 30°C. Data were determined from three independent experiments. Vertical bars indicate standard deviation. DCW was calculated by DCW = α × OD_{660} when α = 0.87 (wild type), 0.96 (Δ*degQ*), and 0.93 (Δ*degQ*+*degQ*) g L^{-1}, respectively.

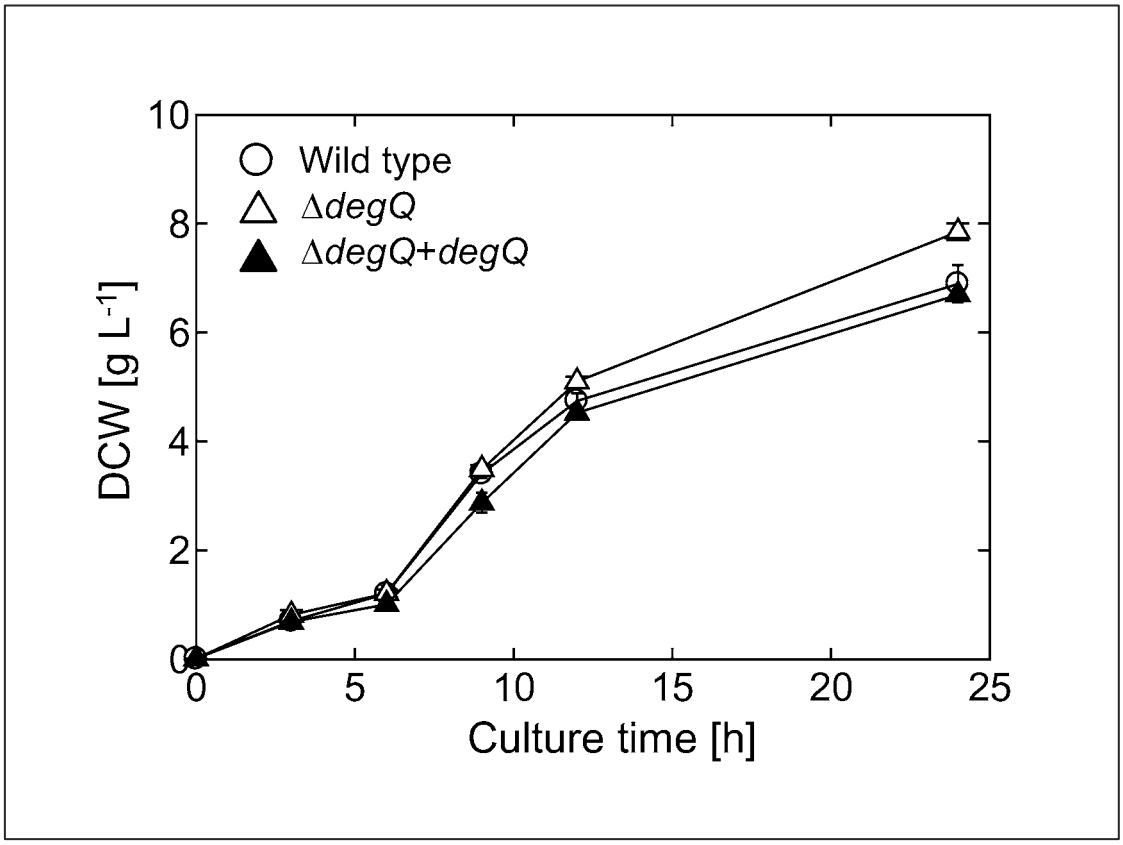


Fig. 3 TEM images of OMVs isolated from *S. oneidensis* wild type, Δ*degQ*, and Δ*degQ*+*degQ* strains. The OMVs were stained with uranyl acetate. The scale bars indicate 200 nm.

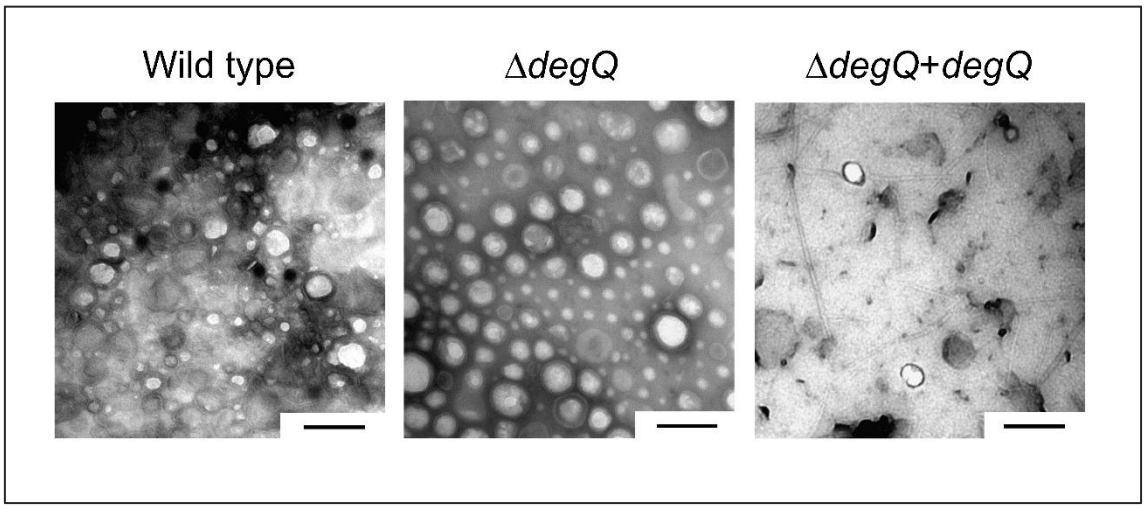
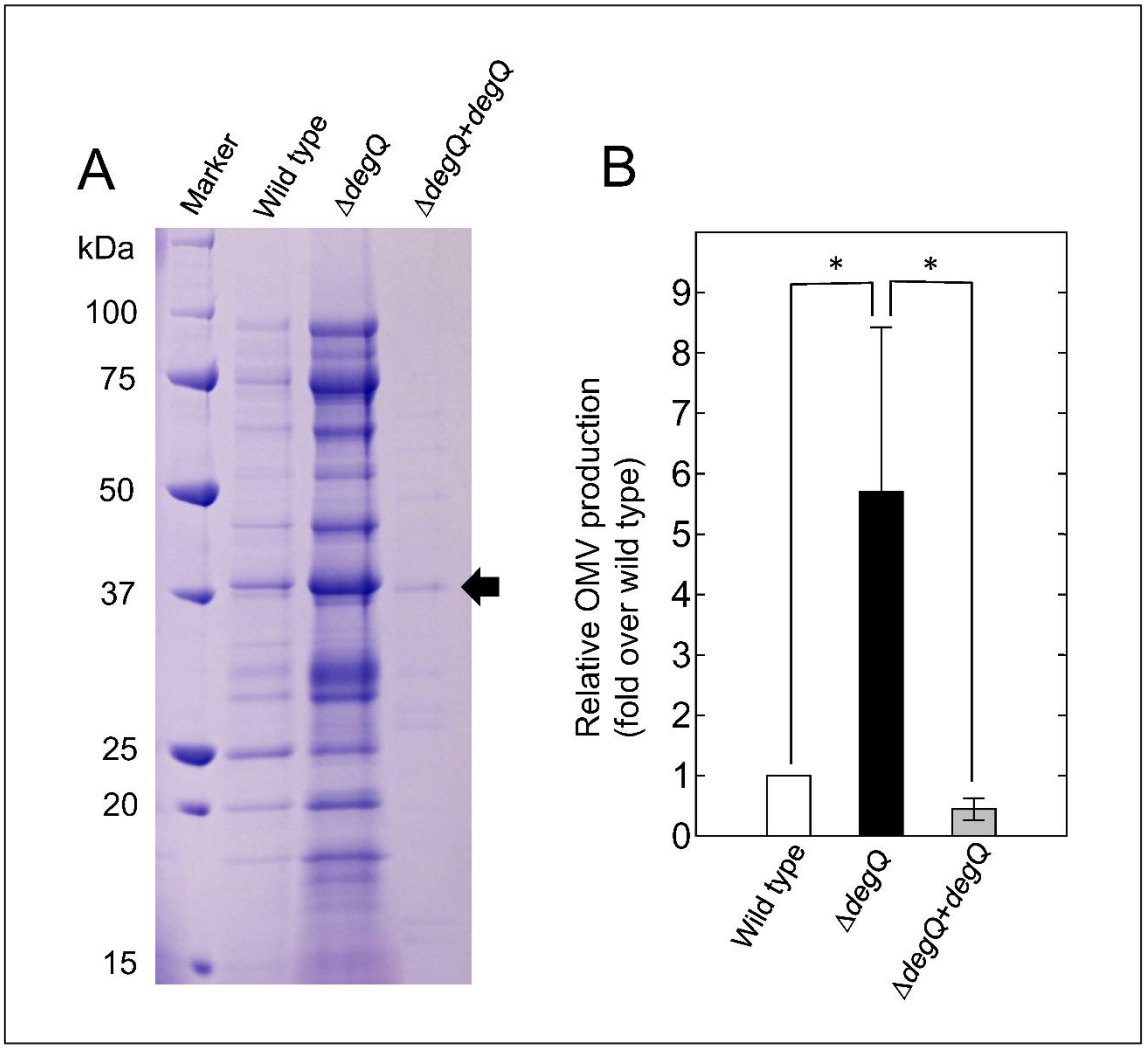


Fig. 4 Correlation between *degQ* and OMV production. (A) SDS-PAGE analysis of OMVs isolated from the *S. oneidensis* wild type, Δ*degQ*, and Δ*degQ*+*degQ* strains. The closed arrow indicates the protein bands of interest that were analyzed by nano LC-MS/MS. (B) Comparison of OMV production among *S. oneidensis* wild type, Δ*degQ*, and Δ*degQ*+*degQ* strains. OMV production was determined as relative values by normalizing against the value of the wild type strain. Data was determined from three independent experiments. Vertical bars indicate standard deviation. An asterisk indicates the statistical significance as determined by ANOVA with Tukey's test ($p<0.05$).



SUPPORTING INFORMATION

Table S1 Primers used in this study.

Fig. S1 Electrophoresis of the *degQ* fragment amplified from genomic DNA isolated from *S. oneidensis* wild type (lane 1) and Δ*degQ* (lane 2) strains. Lane M; *HindIII*-digested λ DNA marker.

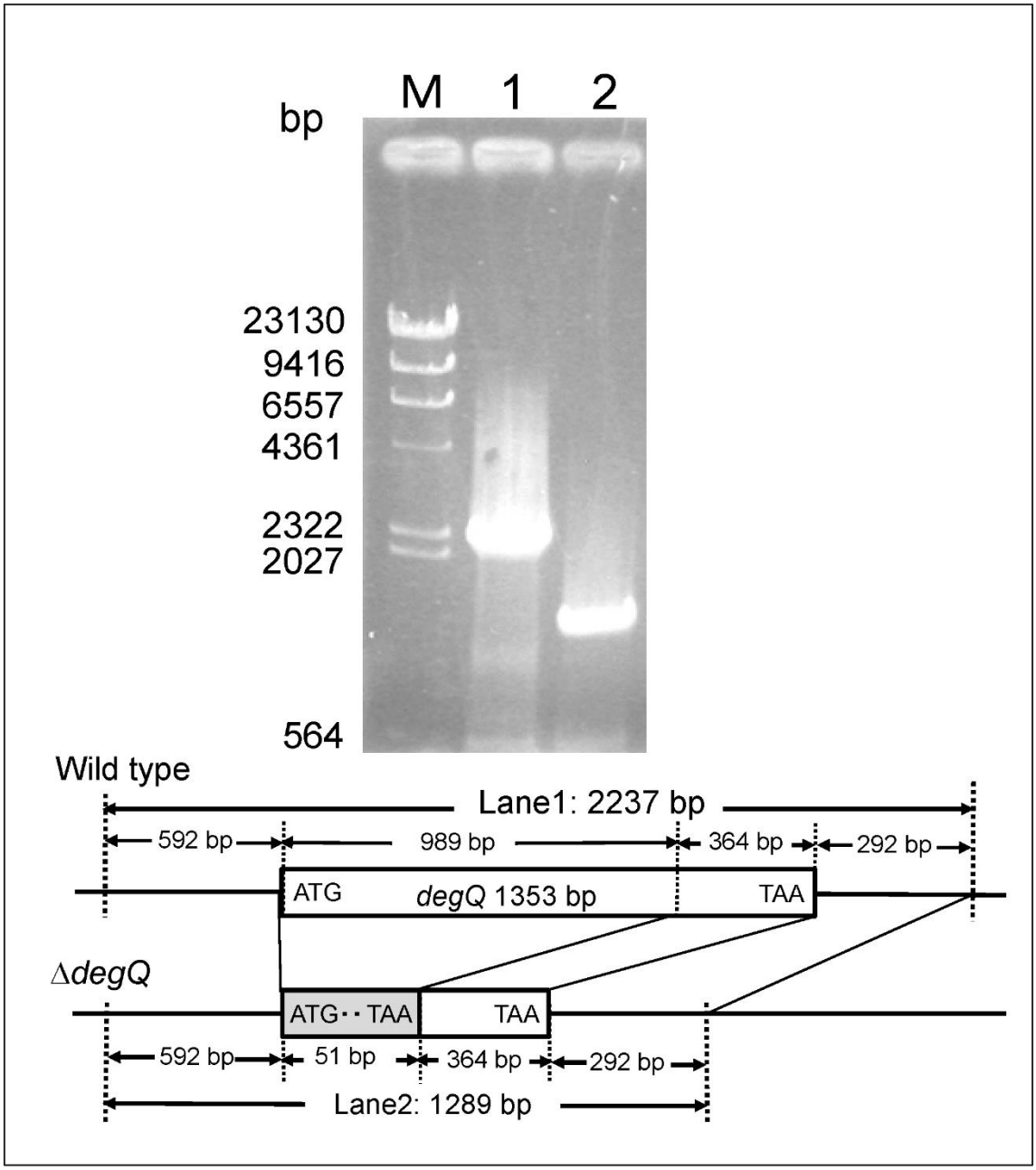


Table 1

Table 1 Strains and vectors used in this study.

Strain and vector	Description	Reference
Strains		
S. oneidensis MR-1	Wild type strain	(Myers and Nealson 1988)
WT+*degQ*-His	Wild type strain with pHSG399-*degQ*-His	This study
Δ*degQ*	In-flame deletion of *degQ*	This study
Δ*degQ*+*degQ*	Cm^r, complemented strain with pHSG399-*degQ*	This study
Escherichia coli		
SM10 λpir	Mating strain	(Miller and Mekalanos 1988)
JM109 λpir	Cloning strain	(Penfold and Pemberton 1992)
Plasmids		
pRE112	Cm^r, pir-dependent suicide vector	(Edwards et al. 1998)
pRE112-F3	Cm^r, pRE112 caring F3 fragment	This study
pHSG399	Cm^r, expression vector	(Takeshita et al. 1987)
pHSG399-*degQ*	Cm^r, expression vector carrying *degQ*	This study
pHSG399-*degQ*-His	Cm^r, expression vector carrying *degQ* with the His6-Tag sequence at C-terminus	This study

Table 2

Table 2 mRNA expressions of *degQ* gene, protease activities of DegQ protein, and amounts of periplasmic proteins from *S. oneidensis* wild type, Δ*degQ*, and *degQ*+*degQ* strains.

Strain	mRNA expression [−]	Protease activity [U g–DCW^{-1}]	Periplasmic proteins [mg g–DCW^{-1}]
Wild type	$(6.3 \pm 4.2)\times10^{-5}$	19.4 ± 2.9	1.03 ± 0.12
Δ*degQ*	N.D.	7.0 ± 2.3	2.56 ± 0.37
Δ*degQ*+*degQ*	$(1.5 \pm 1.2)\times10^{-2}$	61.3 ± 14.6	0.44 ± 0.03

The data were determined using the cells harvested at culture times of 4 h for mRNA expression analysis and 6 h for protease activity assay. The data were determined from three independent experiments.
N.D.: Not detected

Table 3

Table 3 Proteins identified in OMVs from *S. oneidensis* wild type strain.

Protein	Gene	Mass	Score	Peptide no.	Accession	Note
First analysis						
Outer membrane porin	*ompS38*	39874	34463	1166	gi|499386175	SO3896
Outer membrane porin	-	39654	6912	327	gi|499385907	SO1821
Flagellar motor protein MotA	*motA*	48740	1097	36	gi|499384378	SO4287
Outer membrane porin	*ompA*	43970	1033	28	gi|499384375	SO3545
iron ABC transporter substrate-binding protein	*fbpA*	37394	953	30	gi|499383481	SO0744
TonB-dependent ferric putrebactin siderophore receptor PutA	*putA*	81373	877	20	gi|499385445	SO3033
ATP synthase subunit beta	*atpD*	49856	784	21	gi|499386863	SO4747
alcohol dehydrogenase	*adhB*	40387	783	23	gi|499384108	SO1490
cytochrome C	*omcA*	79992	725	27	gi|499384335	SO1779
5'-nucleotidase	*ushA*	61353	671	29	gi|499384520	SO2001
Second analysis						
Outer membrane porin	*ompS38*	39874	11280	488	gi|499386175	SO3896
Outer membrane porin	-	39654	1960	109	gi|499385907	SO1821
alcohol dehydrogenase	*adhB*	40387	709	28	gi|499384108	SO1490
TonB-dependent receptor	*hmuA*	81373	527	22	gi|499385445	SO3669
Outer membrane porin	*ompA*	43970	384	22	gi|499384375	SO3545
Isocitrate dehydrogenase	-	36173	370	13	gi|499384148	SO1538
ABC transporter substrate-binding protein	*potF*	40834	368	16	gi|499383922	SO1270
Phage capsid protein	-	43497	319	18	gi|499385376	SO2963
Cytochrome C	*omcA*	79992	316	12	gi|499384335	SO1779
Iron ABC transporter substrate-binding protein	*fbpA*	37394	299	16	gi|499383481	SO0744

Supplementary Table S1

Supplementary Table S1 Primers used in this study

Primers	Sequence
Mutagenesis	
F1-F	5’- ATCGGAGCTCCA AAGAGTTAGGCTCTTCGGCT -3’
F1-R	5’- GACTGGCTTAGGTCGTCTCTATTTCGTTTTCATCTATTCATA -3’
F2-F	5’- AGAGACGACCTAAGCCAGTCCCAAGAGCTGCGTGCTAAAGTC -3’
F2-R	5’- ATCGTCTAGAGCCTTGGCAAAGGAGAGTTCA -3’
Complementation	
degQ-F	5’- ATCGAAGCTTGAAAACGAAATTATCT -3’
degQ-R	5’- ATCGGAATTCTTAACGAAGCACTAA -3’
Purification	
degQ-His-R	5’- ATCGGAATTCTCAGTGGTGGTGGTGGTGGTGACGAAGCACTAAGTAAAG -3’
Realtime PCR	
rrsA(house keeping)-RT-F	5’- GCAACGCGAAGAACCTTACC -3’
rrsA (house keeping)-RT-R	5’- CAGCACCTGTCTCACGGTTC-3’
degQ(SO3942)-RT-F	5’- AACAAGTGCAAGAGCGTCCA -3’
degQ(SO3942)-RT-R	5’- CTTCACGGCCATCGTGTAAA -3’