Genetic and Molecular Characterization of Flagellar Assembly in *Shewanella oneidensis*

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Abstract

*Shewanella oneidensis* is a highly motile organism by virtue of a polar flagellum. Unlike most flagellated bacteria, it contains only one major chromosome segment encoding the components of the flagellum with the exception of the motor proteins. In this region, three genes encode flagellins according to the original genome annotation. However, we find that only *flaA* and *flaB* encode functional filament subunits. Although these two genes are under the control of different promoters, they are actively transcribed and subsequently translated, producing a considerable number of flagellin proteins. Additionally, both flagellins are able to interact with their chaperon FliS and are subjected to feedback regulation. Furthermore, *FlaA* and *FlaB* are glycosylated by a pathway involving a major glycosylating enzyme, PseB, in spite of the lack of the majority of the consensuses glycosylation sites. In conclusion, flagellar assembly in *S. oneidensis* has novel features despite the conservation of homologous genes across taxa.

Introduction

In environments where living conditions change constantly, microorganisms must respond to those changes rapidly. One of the common approaches is that cells move away from detrimental environments and reach relatively favorable niches. Bacteria use a wide variety of cellular structures to facilitate motility, of which the flagellum is the most important and thus the best studied [1]. More than 80% of known bacterial species are motile by means of flagella [2]. In addition, flagella are indispensable in adhesion to substrates, biofilm formation, pathogenicity, and reduction of insoluble metal minerals [3–6]. In general, bacteria have either polar (i.e. *Vibrio cholera*) or lateral (i.e. *Escherichia coli*) flagella, which are alike structurally. Exceptions which have both are found [7–8]. Although the bacterial flagellum is one of the most complex of all prokaryotic organelles, the structure of the conventional flagellum is relatively well understood and excellent reviews are available [1,9–10].

A flagellar system is tightly regulated because its synthesis and functioning is highly costly for the cell (about 2% of biosynthetic energy expenditure in *E. coli*) [10–11]. Genes encoding proteins involved in flagellum synthesis are organized into an ordered cascade in which the expression of genes at a given level is required for the expression of other genes at a lower level of assembly [10]. In Gram-negative bacteria, the regulatory cascades for lateral and polar flagella are dramatically different [12]. In bacteria with lateral flagella, the top of the hierarchy is the *flhDC* operon, encoding the FlhD and FlhC proteins, which are essential for expression of downstream flagellar genes [12]. In the case of polar flagella, genes are transcribed in a four tiered hierarchy [13].

It is well established that a σ^{34}-associated transcription activator is the master regulator at the top level among microorganisms in which such a master regulator has been identified [14]. This master regulator controls transcription of genes in the second tier, which encode components of the MS ring-switch complex as well as the regulatory factors FlrB, FlrC and *FlaA* (σ^{34}). FlrB and FlrC are responsible for transcription of genes in the third tier, most of which encode the basal body-hook, cap, and some of flagellins. The rest of flagellar genes encoding flagellins, the anti-sigma factor *flgM*, and the motor components, which make up the fourth tier, are σ^{28}-dependent.

Owing to countless scientific efforts along with advances in glycoprotein detection and identification, it is now established that flagellins are heavily glycosylated. Studies on *C. jejuni* and *H. pylori* have illustrated two O-linked-glycosylation pathways, Pse and Leg [15–16]. Such modification via the O-glycan pathway is essential for flagellum assembly and bacterial motility [17]. However, *C. jejuni* 81–176 is the only strain for which the sites of glycosylation are known [18]. Although 19 serine or threonine residues, of which 18 reside in the D2–D3 domains, can be glycosylated only 8 are required for motility and flagellar assembly [19–20]. The D2–D3 domains are heavily glycosylated because they form the projections on the filament surface [21].

The *Shewanella* species have expanded during the last two decades as an important family of facultative Gram-negative anaerobes [21]. Initially, studies on this group of microorganisms were mostly aimed at exploring the ability for the bioremediation of metal/radionuclide contaminants in the environment. In recent years, *S. oneidensis*, the representative species of *Shewanella*, has
become a research model for respiration diversity, metabolic network, and biofilm formation [21]. For motility, all *Shewanella* species produce a polar flagellum. Since flagellar structures and gene organization are highly conserved, it is reasonable to assume that the synthesis of the flagella in *S. oneidensis* is similar to that in *V. cholerae*, the paradigm for the polar flagellum and the only organism sharing extensive regions of similar gene order [9,22]. Nevertheless, extensive diversity among flagellated bacteria exists in the content and organization of these flagellar genes and their regulation [23–25]. This may be especially true in the case of *S. oneidensis*, which has two sets of stator systems to drive flagellar rotation [26].

*Shewanella* species are extremely diverse in phenotypic or ecological features, making it difficult to accurately define the core characteristics of the genus [21,27]. As one of the most genetically conserved organelles composed of a large number of components, flagella may perfectly serve that purpose [24]. Additionally, flagella of *Shewanella* species have been reported to be involved in formation of biofilms and pellicles [28–29]. In spite of the increasing importance placed on the organelle, it is surprising that flagella of *Shewanella* species have not been investigated in detail. In the present study we first elucidated the content and organization of flagellar genes in *S. oneidensis* and compared these features to all sequenced *Shewanella* strains. We then systematically examined components with uncertain functions. We found that of the two major flagellin genes major one was under the direct control of σ^II^ and the minor one was also actively transcribed. A bacterial two-hybrid assay revealed that FliD was unable to interact with SO3234, the predicted chaperon of FlID, suggesting the lack of such a chaperon in the polarly flagellated bacteria. Furthermore, PseB was identified to be essential for flagellating glycosylation although the entire glycosylation pathway remains elusive in *S. oneidensis*.

**Results**

*In silico* analysis of flagellar genesin *Shewanellae*  
22 *Shewanella* genomes at Integrated Microbial Genomes at DOE Joint Genome Institute (http://img.jgi.doe.gov) were used for sequence comparison analyses. A preliminary analysis of flagellar genes revealed a high level of similarity, enabling us to use the best studied *S. oneidensis* as the representative species. Differences in other *Shewanella* genomes are discussed in the text to provide a more comprehensive view of diversity.

The *S. oneidensis* genome contains up to 70 genes encoding the flagellar and chemotaxis proteins that are homologous to those of other polar flagellar and chemotaxis systems [22]. These include the three sets of chemotaxis genes at different locations, of which only the third is likely to be functional since a mutation in cheA-3(SO3207) eliminated bacterial chemotactic response [30]. To elucidate organization of flagellar genes in *S. oneidensis*, flagellar gene systems from *E. coli* and *V. cholerae* were chosen as general references, representing the best understood and the closest in phylogeny. Unlike *E. coli* and *V. cholerae*, *S. oneidensis* allocates only one major location (SO3200-58) on the chromosome for flagellar genes and the third set of chemotaxis genes. This 59- gene fragment (approximately 155KB in length) can be divided into three clusters, resembling three regions of the polar flagellar genes of *V. cholerae* (Fig. 1). The structure of operons was derived from *in silico* analyses (http://www.microbesonline.org, http://biocyc.org/SONE211586/) with slight adjustments in reference to the well-defined counterparts of *V. cholerae* [13,31–33]. In this 59-gene fragment, thremisidentified genes in the original annotation (SO3246, SO3224, and SO3201 between flaE and flaF, flaB and flaA, and cheW3 and SO3290, respectively) were removed when the genome was re-annotated, as presented at http://img.jgi.doe.gov.

The first cluster (A-I) includes *flgT*, *flgOP*, *flgNM*, *flaA* (SO3255), *cheV3-R3*, and *flgBCDEFGHIJK* (SO3246L). While *flaM* and *cheVR* respectively encode an anti-σ^II^ factor and achemotaxis protein, the rest of the genes are annotated as structural genes for a sodium-driven motor ring (FlgT), a gene encoding a protein of unknown function (FlgOP), one for a basal body rod, others for rings, and hook protein (FlgBCDEFGHIJK), a chaperon (FlgN) and an assembly protein (FlgA) [34–35]. The *flaA* gene was not assigned in the original genome sequencing but it shows sufficient homology with *flaA* of other bacteria to be included so after validating its requirement for motility [29], we designated it *flaA*. Another unassigned gene in the first annotation is SO3240 (encoding an ortholog of FlgK), which was proposed to be non-functional due to two frameshifts in its predicted gene sequence. In contrast, all other sequenced *Shewanella* strains contain a full-length *flgK*, suggesting that there may be sequencing errors in the *flgK* gene of *S. oneidensis*.

The second cluster (A-II) of *S. oneidensis* flagellar genes consists of *flad* (SO3228, *flaR* (SO3237), *flgA*, *flgD* (SO3234-SO3235), *flhA*, *flhBC*, and *flEGFHIJKLMNOPQR*). Except for *flaBC* encoding regulatory proteins, products of all these genes are components of the *S. oneidensis* flagellum such as filament, basal body, switch, and export proteins. Among genes in this cluster, SO3237, SO3228 and SO3234 were not named in the original annotation. Sequence analysis of SO3237 and SO3238 by BLAST revealed that the best fits were to flagellins, especially those of less than 300 a.a. in length. We therefore named SO3237 and SO3238 as *flaB* and *flaA*, respectively. Previously, *flaA*, *flaB*, and *flaC* were proposed to encode flagellar subunits and removal of all three genes or *flaA* and *flaB* together resulted in a complete loss of motility [26,36]. However, several lines of evidence suggest that FlgA is unlikely to be a flagellin subunit. These include that 1) the protein is too small (119 a.a.) to include all necessary domains of a functional flagellin [37], 2) the low sequence similarity to FlaA or FlaB (9%), 3) a 119 a.a. fragment appears to have all necessary domains of a functional flagellin [37], 4) the *flaA* is the first gene of the four-gene “non-flagellin” operon *flaG-flaD-flaA-flB* in *V. cholerae* [13].

A great diversity in flagellar filament genes among sequenced *Shewanella* strains was observed. Like *S. oneidensis*, 12 other strains possess two genes encoding flagellins of 265-275 a.a., implying that flagellar filament subunits of this length represent the ancestral set of flagellins in *Shewanellae*. Intriguingly, *S. baltica* OS185 and OS195 possess four genes encoding flagellin subunits while two other sequenced *S. baltica* strains (OS155 and 222) have only two (Fig. S1). The additional two flagellar filament genes reside on a fragment of 5.4 kb between analogs of *S. oneidensis* flaA and *flad*.* These fragments appear to have resulted from transposition events perhaps due to the presence of IS4 family transposase genes. The second largest group consists of 5 strains including *Shewanella* sp. MR-4, MR-7, *S. benthica*, *S. violacea*, and *S. frigidimarina*, whose genes encode the flagellin subunits of 465-482 a.a. It is worth noting that *S. benthica* KT99 not only carries transposase gene between the flagellin gene and *flaB* but also has two additional genes encoding 104 a.a. and 344 a.a. proteins in place of the counterpart of *S. oneidensis* flaB. Given that both proteins exhibit high levels of sequence similarity to known flagellins, we speculate that this unusual structure may be the result of sequencing errors. The other two strains *S. pealeana* and *S. piezotolerans* contain genes encoding flagellins of 393–394 a.a. and 434–463 a.a., respectively. It is interesting to note that *S. piezotolerans* is the only strain whose two flagellins differ from each other by more than 5 a.a.
The only ORF in the A-II cluster that could not be functionally assigned is SO3234. The gene encodes a small protein (106 a.a.) of unknown function. Although the proteins are highly conserved among sequenced Shewanella strains, its counterparts in other organisms have not been confidently identified. In V. cholerae and Vibrio parahaemolyticus, the gene at the same location is designated flaI but its product shares sequence identity of less than 20% with SO3234 [13].

The third cluster (A-III) contains the chemotaxis genes cheY3Z3A3B3W3, the export gene flhA, the regulatory genes flIA and flhFG, and the four ORFs encoding proteins of unknown function. The flhA gene product (S28) is a sigma factor specific for expression of some late flagellar genes encoding the filament proteins, motor proteins and other flagellar-related secreted proteins [1]. In polarly flagellated bacteria, flhF and flhG encode proteins controlling the number and location of the flagella.

The flagellar genes that are not on this 59-gene fragment include two sets of motor genes: pomAB (SO1529-30), motAB (SO4287-6), motX (SO3936), and motY (SO2754). Region A, divided into three clusters I, II, III, encompasses genes SO3200-58. Thick arrows denote ORFs (not drawn to scale) with corresponding genes below. Genes with an asterisk above were subjected to the physiological analyses in this study. Putative promoters are designated by thin arrows based on operon structures predicted at two sites (http://www.microbesonline.org; http://biocyc.org/SONE211586/). Solid and dash lines represent ones identified at both sites and ones found at only either site, respectively. Pseudogenes removed from the genome are in light grey.

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Development of suitable complementation plasmids for flagellar genes

The bacterial flagellar system is relatively stable in evolution and up to 24 core genes are found in every microorganism possessing flagella [24]. While genomic analyses allow for the direct detection of genes encoding most flagellar components, it is still necessary to carry out mutational analyses on uncertain ones for functional validation.

In our previous studies, broad host range vectors pBBR1MCS-2 and pBBR1MCS-5 were used for complementation by introduction of the intact gene and/or its promoter region into the multiple cloning site (MCS) of either plasmid [40–42]. However, the majority of the flagellar gene operons are unusually large (the predicted largest is 13 kb in S. oneidensis), consisting of multiple genes with different function. As a result, a vectors such as pBBR1MCS is not suitable because: 1) mutation(s) may be introduced during amplification of the targeted gene and its promoter; 2) the other genes within the same operon may interfere with complementation, especially those encoding regulatory proteins. To circumvent such an obstacle, we constructed two plasmids: pHG101, a promoter-less plasmid for genes next to their promoter and pHG102, hosting the S. oneidensis arcA promoter for genes some distance from their promoter. The arcA promoter was chosen because the gene has been found to be expressed at the considerable level under both aerobic and anaerobic
conditions [40,43]. Th is promoter with an MCS was generated by PCR [primers available upon request unless otherwise noted].

In this study, all mutants that displayed phenotypes distinguishable from their parental strains were subjected to complementation. Although motility of each strain on both swimming and swarming plates was examined in comparison with the wild type only swimming results are presented for there was no statistically significant difference between swimming and swarming unless otherwise noted. The results are summarized in Table 1 and discussed in the text when necessary.

**S. oneidensis** contains an intact and functional FlgK

According to the genome sequence, two ORFs encoding proteins of 396 and 141 a.a. reside at the flgK locus. flgK encodes a hook-filament junction protein, which is essential for flagellar assembly and motility [11]. Given the synteny and the indispensable role of FlgK, it is unlikely that this crucial protein could be missing. To verify the sequence, an S. oneidensis culture with flagellated cells (confirmed by a microscope) was used as the chromosomal DNA source for gene amplification. Three independent sequencing results unambiguously indicated that SO3240 in the original annotation missed out A and C immediately after nt1134 and verified the sequence, an FlgK filament junction protein, which is essential for flagellar assembly.

**flaA and motility** [11]. Given the synteny and the indispensible role of FlgK is functional in S. oneidensis (Table 1) (Fig. 2).

**FlaB is the major flagellinsubunit for motility**

In *Shewanella*, genes encoding the flagellar filament subunits display the largest diversity asmentioned above, which warrants an in-depth analysis. Two flagellin genes are found in the S. oneidensis genome: flaA(SO3238) and flaB(SO3237). FlaA and FlaB share a sequence identity of 89% although they are rather short, only 272-3 a.a. in length compared to 376 a.a. for V. cholerae, 490 a.a. for E. coli, and 572 a.a. for Campylobacter jejuni [44]. Structural analyses on the 495 a.a. filament protein of *S. typhimurium* reveal that the protein consists of seven domains: D1-D2-D3-D5-D6-D7-D8, of which D5-D7-D8 are not required for formation of full-length flagella for motility [37,45-47]. We therefore speculate that the S. oneidensis subunits may retain only necessary domains. To test this hypothesis, alignments of the S. oneidensis FlaA and FlaB amino acid sequences with *S. typhimurium* LT2 FlIC were performed. Indeed, the D5-D7-D8 domains were not found in either S. oneidensis flagellar filament subunit (Table 2).

In *V. cholerae*, mutation of flaA completely abolished motility whereas the other four flagellin genes were dispensable [48]. C. jejuni flaA was essential for motility but a flaB deletion strain produced full-length functional flagellar filaments [49-50]. Moreover, in *V. parahamolyticus*, loss of one of six flagellar filament genes had little or no effect on motility or flagellar structure [11]. Consequently, in *S. oneidensis* it is possible that FlaA or FlaB may not be essential for formation of full-length flagella despite the high degree of conservation in all of the domains of these two proteins.

To determine the role of these two genes, we constructed S. oneidensis strains containing an in-frame deletion in flaA, flaB, or flaA/flaB. Motility assays demonstrated that compared to the wild type strain, the mutation in flaB had little or no effect on swimming motility but ΔflaB retained only about 40% of its swimming/swarming capability based on the radius of the swimming rings (Table 1, Fig. 2). The double mutantΔflaA/ΔflaB, however, lost motility completely. To confirm the observation, ΔflaB was transformed with the pHG101 containing flaB. Surprisingly, the strain with plasmid-borne flaB showed a stronger capacity for swimming than its parental wild type, which is likely due to multiple copies of the gene in the complemented strain. However, in the case of swimming, the motility increased much less significantly. This suggests that over-expressed flaB may elevate motility of individual cells as swimming motility is a form of movement in multicellular groups rather than as individuals [51]. To test whether over-expressed flaB alone is enough to promote motility, ΔflaAΔflaB was complemented with flaB. A swimming assay showed that the plasmid borne flaB was only able to elevate motility to a level equal to that of the wild type. Examination of the ΔflaB cells by electron microscopy revealed that the flaB mutant produced heavily truncated flagella (data not shown). In contrast, ΔflaA displayed filament morphology similar to the wild type. All of these results indicate that both flagellin subunits are constituents of the filaments with FlaB dominating.

**flaB but not flaA is under the control of σ^28**

Our observation that one of the flagellins is predominant in the *S. oneidensis* flagellum is supported by other examples. In *V. cholerae*, transcription of the essential flaA was directed by σ^28 whereas the other four non-essential flagellin genes were controlled by σ^70 [48]. In contrast, in *C. jejuni* the essential flagellin filament gene flaA was under the control of σ^28 whereas the σ^70-controlled flaB played a minor role.
Given the high degree of sequence identity between *S. oneidensis* FlaA and FlaB, the contrasting phenotypes of these two mutants are likely due to differences in gene expression levels.

To elucidate the underlying mechanism determining the essentiality of FlaB in *S. oneidensis*, we first examined the promoter regions of *flaB* and *flaA*. The σ₂₅-dependent promoters in the *S. oneidensis* genome have been predicted using pattern matching (PM) and iterative position specific score matrix (PSSM)-based approaches [52]. PM and PSSM identified σ₂₅ binding sites in upstream regions of 9 and 12 genes, respectively, and only 6 were in common supporting the use of these programs. We then constructed the σ₂₅ binding weight matrix using the experimentally verified σ₂₅ binding sequences from *E. coli*, *S. typhimurium* and *V. cholerae* to screen for the σ₂₅ binding sites in the *S. oneidensis* genome [13,40,43,53]. In total, 169 putative σ₂₅ binding sites were identified using RSAT with the default setting [54] as given in the supplemental material (Table S1). The σ₂₅ recognition sites were found in the promoter regions of flagellar genes including *motY*, *flaB*, *flgM*, and *pomA*, all of which were among the top 15 most confident. Furthermore, these four sites are the only sites that have been identified by all three approaches (in our method, the top 15 were chosen for comparison). These results strongly suggest that *motY*, *flaB*, *flgM*, and *pomA* are under the control of σ₂₅ in *S. oneidensis* in agreement with similar results in *V. cholerae* [13,31].

To experimentally validate that *flaB* is under the direct control of σ₂₅, we fused each of the *flaA* and *flaB* promoters to the full-length lac operon in a newly developed reporter system [43]. For the promoter activity assay, a *fliA* null mutant was constructed to provide a genetic background lacking σ₂₅. As expected, the mutant produced a truncated flagellum and lost motility completely, matching the phenotype of *fliA* defective mutants from various bacteria (Table 1, Fig. 2 & 3). The reporter plasmids were introduced into the wild type and a Δ*fliA* strain and the activity of the *flaA* and *flaB* promoters was measured. Although

![Figure 2. Motility of *S. oneidensis* wild type and isogenic mutation strains.](image-url)
both flaA and flaB were actively transcribed in the wild type background, the promoter activity of flaB was approximately three times higher than that of flaA (Fig. 4). In contrast, in the fliA defective background, flaB was barely transcribed whereas expression of flaA was not significantly altered. These results verify that flaB but not flaA is under the control of σ28.

Both FlaA and FlaB are under the feedback control

The relative transcription levels of flaA and flaB agree well with the contents of their products in the flagella filament. However, in recent studies it was shown that the relative amounts of flagellins is subject to multiple levels of regulation and modification, including post-transcriptional, posttranslational controls as well as secretion [17,55–57]. To test whether mechanisms at other levels account for the contrasting phenotypes of the flaA and flaB mutants, we raised antibodies against a peptide fragment shared by FlaA and FlaB and used them to detect flagellin subunits in the DflaA, DflaB, DfliA strains and in their parental wild type strains. Western blotting revealed a single band in the wild type but not in the DflaA DflaB double mutation strain. At the same position, a band was visible in both DflaA and DflaB, indicating that the two flagellins migrated identically on sodium dodecyl sulfate-poly-

**Table 2.** Alignment of S. typhimurium FliC with S. oneidensis FlaA and FlaB.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Residues within domain</th>
<th>% identity with FliC</th>
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<tbody>
<tr>
<td>FliC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D0 (N)</td>
<td>1–45 1–45</td>
<td>66 68 (91)%</td>
</tr>
<tr>
<td>D1 (N)</td>
<td>46–180 46–168</td>
<td>43 46 (86)%</td>
</tr>
<tr>
<td>D2 (N)</td>
<td>181–190</td>
<td>– –</td>
</tr>
<tr>
<td>D3</td>
<td>191–284</td>
<td>– –</td>
</tr>
<tr>
<td>D4 (C)</td>
<td>285–407</td>
<td>– –</td>
</tr>
<tr>
<td>D1 (C)</td>
<td>408–455 186–233</td>
<td>53 51 (93)%</td>
</tr>
<tr>
<td>D0 (C)</td>
<td>456–495 234–272</td>
<td>57 58 (94)%</td>
</tr>
</tbody>
</table>

*The domain boundaries within the S. oneidensis FlaA and FlaB proteins were assigned based on alignment with S. typhimurium FliC.

Per cent identity was determined using CLUSTALW with a gap penalty of 10.0 and a gap length penalty of 0.2.

Numbers in bracket represent %identity between S. oneidensis FlaA and FlaB.

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**Figure 3.** Transmission electron micrographs of S. oneidensis wild type and flagellar mutantstrains. Cells were stained with 1% phosphotungstic acid and applied to TEM. 1. The wild type. 2. Δfgk, aflagellated. 3. ΔfliD, truncated filaments. 4. ΔSO3234, flagella indistinguishable from that of the wild type.

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**Figure 4.** Promoter activities of flaA and flaB in S. oneidensis strains. Whole-cell lysates were prepared from S. oneidensis cultures in mid-exponential growth phase and assayed as described in Experimental Procedures. Promoter activities were determined by measuring β-galactosidase levels using flaA-lacZ and flaB-lacZ reporter constructs in the wild type, ΔfliA, and ΔfliD strains. Quantification of the promoter activities was normalized to the total protein in each strain.

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acrylamide gel (SDS-PAGE) (Fig. 5). This single band was at a position approximately 4–5 kDa higher than expected for the deduced molecular masses of each of the flagellins (∼29 kDa), suggesting the possibility of posttranslational modification, mostly likely glycosylation. The band in ΔfliA was much stronger than that in ΔfliB, supporting other evidence that FlaB is produced in substantially greater amount than FlaA. Additionally, results showed that ΔfliA possessed the smallest number of flagellin proteins, all presumably FlaA. The immunoblotting assay also confirmed the successful complementation of mutations in flaA and fliB. It is noteworthy that the plasmid borne flaB promoted overproduction of FlaB flagellin in comparison to the amount of this protein in the wild type.

As a considerable number of FlaA and FlaB proteins were present, a fliD mutant was constructed and assayed by immunoblotting to demonstrate whether these flagellin subunits are subjected to the feedback control. Results showed that the fliD mutant was completely non-motile on swarming or swimming plates and the plasmid borne fliD fully rescued the defect (Table 1, Fig. 2). Consistently, TEM studies demonstrated that the mutant cells possessed a truncated flagellum (Fig. 3), a characteristics of feedback control occurs at the transcriptional level, we performed the same assay on FlaG and FliS. As expected, no interactions were detected between these two proteins, providing additional evidence that FlaG is not a flagellin subunit.

Both FlaA and FlaB are glycosylated

A recent study supported a novel mechanism for flagellar gene expression is dictated by the rate of protein secretion [59]. As a consequence, intracellular levels of flagellins may have inhibitory effects on their own production. The flagellin subunits are synthesized in the cytosol and transported as monomers to the plasma membrane where they are secreted. To investigate whether the feedback control occurs at the transcriptional level, we performed the same assay on FlaA/FliS and FlaB/FliB were able to form colonies on 3-AT in less than 24 h, similar to cells hosting the positive control plasmid pair. These positive interactions were confirmed by growth of these colonies on plates containing both 3-AT and streptomycin (12.5 µg/ml). In contrast, cells with the negative control plasmid pair failed to produce any colonies in 40 h. These results indicate that FliS is able to interact with both FlaA and FlaB. Meanwhile, we performed the same assay on FlaG and FliS. As expected, no interactions were detected between these two proteins, providing additional evidence that FlaG is not a flagellin subunit.

Figure 5. Western blot analysis of FlaA and FlaB. Whole-cell lysates were prepared from S. oneidensis cultures in mid-exponential growth phase, loaded equally, separated on 12% SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride (PVDF). S. oneidensis flagellins were probed with polyclonal antibodies that recognize both FlaA and FlaB and detected by chemiluminescence. In all panels, M represents the protein marker. The samples in each lane are the (A) 1, ΔfliA/ΔfliB strain, 2, ΔfliA/ΔfliB strain, 3, ΔfliA/ΔfliB strain, 4, wild type strain, 5, ΔfliA strain, 6, ΔfliB strain, 7, ΔfliA strain; (B) 1, wild type strain, 2, ΔfliD/ΔfliD strain, 3, ΔfliD strain; (C) 1, wild type strain, 2, ΔfliD/ΔfliD strain, 3, ΔfliD/ΔfliD strain.

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SO3234 is important for motility but may not be the chaperon of FliD

In *E. coli* and *S. typhimurium*, operon *fliDST* encodes the cap *FliD*, the flagellar chaperon *FliS*, and the *FliD* chaperon *FliT* [1]. Since *FliT* is a regulator that inhibits the flagellar master regulatory proteins *FliDC* by direct binding [66], *FliD* can function as an anti-regulator by sequestering *FliT* from *FlhDC* [1]. Unlike the *fliD* defect mutant that possess truncated flagella and lose motility fully, the *fliT* mutants produce flagellar structures indistinguishable from those produced by their parental wild type [67]. Consequently, the mutants are as motile as the wild type [68]. In *S. oneidensis*, the genes downstream of *fliD* are *SO3234* and *fliS*. Intriguingly, although this type of organization is also observed in a variety of bacteria including *V. cholera*, little has been done to elucidate the role of *SO3234* and its analogs in flagellar assembly. The question arose whether *SO3234* (106 a.a.) is functionally equivalent to *FliT*.

Our mutational analysis has validated that the annotated *fliD* indeed encodes the cap of the flagellar filament so we constructed an *S. oneidensis* strain devoid of *SO3234*. The mutant *ΔSO3234* displayed a decrease in motility to approximately 56% relative to its parental strain (Table 1, Fig. 2), in contrast to the findings that *fliT* mutants produced functional flagella in general [38,67–69]. When pHG101 containing *fliD*-*SO3234* fused to the arcA promoter was inserted, both mutant strains had their motility fully restored (Fig. 2, Table 1). Surprisingly, the mutants appeared to produce full-length flagella (Fig. 3), indicating that the reduced motility may not be due to impaired filament formation.

To further dissect the role of *SO3234*, the bacterial two-hybrid system was employed to examine the direct interaction of *SO3234* with *FliS* and *FliD*. The *SO3234* gene was cloned into the bait plasmid while *FliD* was used as the target, and these were co-transformed into the *E. coli* reporter strain. In contrast to the positive control, no colonies were found on plates containing 3-AT after 24 hours of incubation (Table 3). Additional incubation for 16 hours which allows the growth of cells containing weak interactors did not help. These results rule out the possibility that *FliD* interacts with *SO3234* in vivo, thus confirming that *SO3234* is unlikely to be the chaperone for *FliD*.

**Discussion**

Flagellar synthesis in *S. oneidensis* has been presumed to be similar to that in *V. cholera*, the research paradigm for polarly flagellated bacteria, despite apparent permutations in the flagellar gene contents and organization between the two genomes [13,33,34,70]. As a result, only a couple of studies have been done in deciphering the *S. oneidensis* motility system in contrast to extensive investigation on its anaerobic respiration and metal reduction for more than two decades [21,26,36,39]. In this study, we have performed a relatively comprehensive genetic analysis of the polar flagellum *S. oneidensis*, generating three contributions to the current

**Table 3.** Bacterial two-hybrid assay of *FliS*-FlaA, *FliS*-FlaB, and *SO3234*-FliD.

<table>
<thead>
<tr>
<th>Bait</th>
<th>Target</th>
<th>Colonies on nonselective platesa</th>
<th>Colonies on Selective platesb</th>
<th>Confirmationc</th>
<th>Result Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBT</td>
<td>pTRG</td>
<td>201</td>
<td>0</td>
<td>–</td>
<td>no bait + no target – no interaction</td>
</tr>
<tr>
<td>pBT</td>
<td>pTRG-Gal11Tc</td>
<td>177</td>
<td>0</td>
<td>–</td>
<td>no bait + target Gal11T – no interaction</td>
</tr>
<tr>
<td>pBT-LGF2</td>
<td>pTRG-Gal11Tc</td>
<td>188</td>
<td>159</td>
<td>159</td>
<td>bait LGF2 + target Gal11T – strong interaction</td>
</tr>
<tr>
<td>pBT</td>
<td>pTRG-FlaIS</td>
<td>231</td>
<td>0</td>
<td>–</td>
<td>no bait + target PseB – no interaction</td>
</tr>
<tr>
<td>pBT-FlaA</td>
<td>pTRG</td>
<td>143</td>
<td>0</td>
<td>–</td>
<td>bait FlaA + no target – no interaction</td>
</tr>
<tr>
<td>pBT-FlaA</td>
<td>pTRG-FlaIS</td>
<td>150</td>
<td>47</td>
<td>47</td>
<td>Bait FlaA + target FliS – strong interaction</td>
</tr>
<tr>
<td>pBT-FlaB</td>
<td>pTRG</td>
<td>128</td>
<td>0</td>
<td>–</td>
<td>bait FlaB + no target – no interaction</td>
</tr>
<tr>
<td>pBT-FlaB</td>
<td>pTRG-FlaIS</td>
<td>192</td>
<td>104</td>
<td>104</td>
<td>Bait FlaB+ target FliS – strong interaction</td>
</tr>
<tr>
<td>pBT-FlaG</td>
<td>pTRG</td>
<td>222</td>
<td>0</td>
<td>–</td>
<td>Bait Flag+ no target – no interaction</td>
</tr>
<tr>
<td>pBT-FlaG</td>
<td>pTRG-FlaIS</td>
<td>266</td>
<td>0</td>
<td>–</td>
<td>Bait Flag+ target FliS – no interaction</td>
</tr>
<tr>
<td>pBT</td>
<td>pTRG-SO3234</td>
<td>163</td>
<td>0</td>
<td>–</td>
<td>no bait + target SO3234 – no interaction</td>
</tr>
<tr>
<td>pBT-FliD</td>
<td>pTRG</td>
<td>145</td>
<td>0</td>
<td>–</td>
<td>bait FliD + no target – no interaction</td>
</tr>
<tr>
<td>pBT-FliD</td>
<td>pTRG-SO3234</td>
<td>138</td>
<td>0</td>
<td>–</td>
<td>Bait FliD+ target SO3234 – no interaction</td>
</tr>
</tbody>
</table>

*a* M9 agar +25 μg/ml chloramphenicol +12.5 μg/ml tetracycline.

*b* 12.5 mM 3-AT.

*c* +12.5 μg/ml streptomycin.

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understanding of polar flagellar synthesis. First, we provide insights about the two flagellin subunits in respect to their functionality and regulation. Second, we demonstrated that both FlaA and FlaB are glycosylated by anovel pathway utilizing PseB. Third, we present data that SO3234 may not be the chaperon for FliT, arguing that the polar flagella may not require chaperons for their assembly.

Most of polar flagellated bacteria possess multiple flagellins which are similar to one another [11,49,71–73]. These microbes can be readily classified into two groups based on whether functionally predominant flagellins are present. V. parahaemolyticus, Caulobacter crescentus, and Aeromonas hydrophiliae representatives of the group without dominant filament subunits [11,71,73]. For bacteria in the other group, the removal of the predominant flagellin subunits causes a loss in motility [11,49,74–75]. Meanwhile, the minor flagellins are so insignificant that their mRNAs (C. jejuni) may not be above a detectable level [49,74]. Consistently, major flagellins outnumber the minor ones hundreds or thousands of times. This phenomenon has largely been accredited to the fact that flagellin genes are under the control of different promoters, recognized by specialized regulatory proteins and/or sigma factors. In V. cholera, the major flagellin gene is σ +− dependent [11] while C. jejuni and C. coli employs σ 201 to direct expression of the predominant flagellin [49,74]. In S. oneidensis, disruption of either flagellin gene fails to lead to a complete non-motile phenotype whereas σ 201 – dependent FlaB is evidently more important than FlaA in motility. More intriguingly, transcription of both genes is considered as is the amounts of FlaA and FlaB. Collectively, compared to the other studied polarly flagellated bacteria S. oneidensis seems to assemble flagella with ‘weaker’ major and ‘stronger’ minor flagellins, thus representing a novel model.

Flagella production is a metabolically costly endeavor for bacterial cells and therefore is tightly regulated, particularly at the transcriptional level. To prevent production of unnecessary flagellin subunits, additional feedback loops are exploited to ensure that during flagellar assembly every stage is signaled prior to synthesis of the components for the next stage [76]. In a recent study, it was reported that secretion efficiency of flagellins plays an important role [44]. The secretion signal modulating efficiency is located in the amino-terminal sequence, more precisely the N-terminal D0 domain of flagellin [77]. For example, protein-specific conserved residues are identified between C. jejuni FlaA and FlaB in the D0 domain by sequence alignment of both proteins from multiple strains [44]. These protein-specific residues dictate the secretion efficiency. In S. oneidensis, however, this may not be the case. Amino acid sequence alignment of the D0 domains of FlaA and FlaB from 23 sequenced Shewanella strains reveals a similar degree of conservation in N- and C-terminal D0 domains. More importantly, no protein-specific residues are found within either domain, suggesting that secretion of FlaA and FlaB may be similar if it is dependent on this terminal sequence. In addition, the chaperon FliS may not make a difference because the D0 domain binding region of these two flagellins share the highest identity [60–61]. However, there are some features residing in these two flagellins that distinguish them because the greater motility of the S. oneidensis strain with the over-expressed FlaB is only found in the presence of FlaA. This certainly merits further investigation.

Although great efforts have been made to increase our understanding of the polar flagellar system in recent years, the function of FliT is still elusive. In bacteria with lateral flagella such as Salmonella, chaperon FliT of FliD inhibits the transcriptional activator FliD,C2, suggesting that FliT is a regulatory component conserved throughout the flagellar systems [66,78–79]. Interestingly, despite multiple roles of FliT the mutation in this gene did not affect either flagellar structure or motility [67] but export of FliD was significantly reduced in the mutant [68].

Unlike most bacteria with multiple separating segments for flagellar genes, S. oneidensis allocates only one major region, making it an ideal organism for identification of unknown flagellar genes. Among a few unknown flagellar genes, SO3234 appears most likely to be the counterpart of FliT because it shares conserved synteny with fliT in peritrichously flagellated bacteria and is also similar in size. We found that although the SO3234 null mutant showed reduced motility, it produced a flagellum indistinguishable from that in its parental strain. This observation is resonant with the finding by Capdevila et al. [38], indicating that the SO3234 mutation phenotype is different from fliT mutants. Furthermore, SO3234 is unable to interact with FliD, eliminating the possibility of chaperoning FliD. Given that most of the polarly flagellated bacteria also lack the second interaction partner of FliT, FliC, we therefore assume that FliT is unlikely to be present in this group of microorganism. However, whether FliT is only conserved among enteric bacteria as suggested by Imaeda et al. [79] demands further investigation because FliDC has been identified in a polarly flagellated bacterium, Burkholderia glumae [25].

Strikingly, both flagellins of S. oneidensis lack the D2–D3 domains, retaining only one glycosylation site identified in the flagellins of C. jejuni 81–176 (Fig. 6). Assuming that the basic structure of S. oneidensis flagellin is similar to that of Salmonella [20], it is unlikely that the majority of glycosylation sites are located in the D3–D4 domains because they are not exposed. If this holds, there are at most 7 residues suitable for glycosylation. Consequently, it is almost certain that Pse (MW = 316) is not the pathway glycosylating flagellins because it needs at least 15 sites to account for the observed mass increase of 4–5 kDa. However, S. oneidensis apparently employs PseB to initiate glycosylation. This is not surprising because the bacterium has a complete set of enzymes and transporters for GlcNac metabolism to generate abundant substrates [80]. Therefore, flagellar glycosylation in S. oneidensis may be carried out by anovel pathway by components which may evolve independently as promiscuous enzymes that work in multiple pathways. Additional work is in progress to identify other genes in the pathway and to dissect the role of glycosylation in the flagellar assembly in S. oneidensis.

Methods

Bacterial strains, plasmids, and culture conditions

A list of all bacterial strains and plasmids used in this study is given in Table 4 [81]. For genetic manipulation, E. coli and S. oneidensis strains were grown under aerobic conditions in Luria-Bertani (LB) medium at 37 and 30°C, respectively. When needed, the growth medium was supplemented with antibiotics at the following concentrations: ampicillin at 50 μg/ ml, kanamycin at 50 μg/ ml, and gentamycin at 15 μg/ml.

Construction and complementation of in-frame deletion mutants

In this study, in-frame deletion strains were constructed using the Fusion PCR method [82]. Primers used for generating PCR products for mutagenesis are available upon request. In brief, two fragments flanking the targeted gene were amplified independently first and joined together by the second round of PCR. The resulting fusion fragment for each individual gene was introduced into plasmidpDS3.0. The resulting mutagenesis vector was transformed into E. coli WM3064, and then transferred into S. oneidensis by conjugation. Integration of the mutagenesis construct into the chromosome was selected by gentamycin resistance and confirmed
by PCR. Verified transconjugants were grown in LB broth in the absence of NaCl and plated on LB supplemented with 10% of sucrose. Gentamycin-sensitive and sucrose-resistant colonies were screened by PCR for deletion of the targeted gene. The deletion mutation was then verified by sequencing of the mutated region.

To facilitate complementation experiments, two plasmids were constructed in this study. The first plasmid, pHG101, was formed by replacing lacZ and its promoter region on pBBR1MCS with the amplified MCS of the same plasmid [83]. The second plasmid, pHG102, was derived from pGH101 by placing the S. oneidensis arcA promoter in the front of the MCS. For complementation of genes next to their promoter, a fragment containing the targeted gene and its native promoter was generated by PCR and cloned into pHG101. For other genes, the targeted gene was amplified and inserted into MCS of pHG102 under the control of the arcA promoter. Introduction of each verified complementation vector into the corresponding mutant was done by mating with WM3064 containing the vector, and confirmed by plasmid extraction and restriction enzyme mapping.

**Physiological characterization**

Growth of deletion strains in LB was measured by recording cell densities of cultures at 600 nm under aerobic conditions in triplicate with strain MR-1 as the control. To determine the
Table 4. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
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</tr>
<tr>
<td>WM3064</td>
<td>Donor strain for conjugation; ΔdapA</td>
<td>[81]</td>
</tr>
<tr>
<td><strong>S. oneidensis strains</strong></td>
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<tr>
<td>MR-1</td>
<td>Wild-type</td>
<td>ATCC 700550</td>
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<td>HG3210</td>
<td>fliA deletion mutant derived from MR-1; ΔfliA</td>
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</tr>
<tr>
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<td>SO3234 deletion mutant derived from MR-1; ΔSO3234</td>
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<td>HG3235</td>
<td>flaD deletion mutant derived from MR-1; ΔfliD</td>
<td>This study</td>
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<td>HG3237</td>
<td>flaB deletion mutant derived from MR-1; ΔfliB</td>
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<tr>
<td>SO3230-3238</td>
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<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<td>pDS3.0</td>
<td>Ap’, Gm’, derivative from suicide vector pCD442</td>
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<td>pDS3.0 containing the PCR fragment for deleting fliD</td>
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</tr>
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<tr>
<td>pDS-FLGK</td>
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<td>pTP327</td>
<td>Broad host lacZ reporter vector</td>
<td>[43]</td>
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<td>pTP327-FLABp</td>
<td>pTP327 containing the S. oneidensis flaB promoter</td>
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<tr>
<td>pBR1MCS-2</td>
<td>Broad host Km’ vector used for complementation</td>
<td>[83]</td>
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<td>pHG101</td>
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<td>pHG102</td>
<td>pHG101 containing the arcA promoter</td>
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</tr>
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</table>

doi:10.1371/journal.pone.0021479.t004

swarm and swim motility of mutants, one microliter of an overnight culture was spotted in the middle of a swarm LB plate (0.5% agar) or a swarm LB plate (0.25% agar) and allowed to dry for 1 h at room temperature. All plates were incubated at 30°C for 10 h or as noted otherwise. For phase-contrast microscopic analysis, swarm or swim cells were scraped from the leading edges of each swarm and then visualized in NB or saline on a glass slide.

Electron microscopy visualization

For transmission electron microscopy (TEM), cells grown overnight 1% tryptone agar plates were suspended in sterile distilled water, spread onto carbon-Formvar copper grids, and then negatively stained with 1% phosphotungstic acid (pH 7.4). Preparations were viewed under a CM12 Philips TEM.

β-Galactosidase activity assay

A lacZ reporter system for S. oneidensis has been developed [43]. To construct the flaA-lacZ and flaB-lacZ reporters, the flaA and flaB promoter DNA fragments were generated by PCR (primers available upon request), cloned into pTP327, and verified by sequencing. The reporter plasmids were moved into S. oneidensis ΔfliA ΔfliD or the MR-1 strain by conjugation. Cells in the log phase (30°C, OD600 = 0.4) were harvested by centrifugation, washed with PBS (phosphate buffered saline), and treated with lysis buffer (0.25 M Tris/HCl, pH 7.5, 0.5% Trion-X100). The resulting soluble protein was collected after centrifugation and used for enzyme assays employing the High Sensitivity β-Galactosidase Assay Kit (Stratagene) according to manufacturer’s instructions. β-galactosidase activity was determined by monitoring color development at 575 nm every minute for 30 min by using a Synergy 2 Multi-Detection Microplate Reader. The protein concentration of the cell lysates was determined using a Bradford assay with BSA as a standard (Bio-Rad).

Bacterial two-hybrid assay

The BacterioMatch II Two-Hybrid system (Stratagene) was used to investigate protein-protein interaction in vivo in E. coli cells according to manufacturer’s instructions. Briefly, plasmid constructs were created by cloning the target and bait proteins in the pTRG and pBT vectors and verified by sequencing. The resultant plasmids were used to co-transform BacterioMatch II Validation Reporter Competent Cells on M9 salt agar plates containing 25 µg/ml chloramphenicol 12.5 µg/ml tetracycline with or without 3-amino-1,2,4-triazole (3-AT), pBT-LGF2, pTRG-Gal11P, and empty pBT and pTRG constructs were used as positive and negative controls. The plates were incubated at 37°C for 24 h and then moved to room temperature for an additional 16 h (the colonies indicating a positive interaction usually appeared between 18 and 24 h). The positive interactions were confirmed by streaking colonies on plates containing both 3-AT and streptomycin (12.5 µg/ml).
Immunoblotting assay
Rabbit polyclonal antibodies against a fragment of FlaB (CRDLTIQSENGANST) were prepared in accordance with standard protocols provided by the manufacturer (Genscript) and used for immunoblotting analysis. Bacterial cells in log phase (30°C, OD_{600} = 0.4) were used. For these experiments, cell samples were washed once with TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), and resuspended to an optical density at 600 nm (OD_{600}) of 1.0 in lysis buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl). The total protein concentration of the cell lysates was then determined by the bicinchoninic acid assay ( Pierce). Samples were loaded onto SDS-12% polyacrylamide gels and either stained with Coomassie brilliant blue or electrophoretically transferred to polyvinylidene difluoride (PVDF) according to the manufacturer’s instructions (Bio-Rad). The gels were blotted for 1 h at 50 V using a Criterion blotter (Bio-Rad). The blotting membrane was probed with anti-FlaB antibody, followed by a 1:10,000 dilution of goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate, and the alkaline phosphatase reaction was developed.

Supporting Information

**Figure S1** Organization of flagellin genes in *Shewanella*. Among sequenced *Shewanella* strains, 12 including *S. oneidensis* possess two genes encoding flagellins of 265–275 aa. The second largest group consists of 5 strains including *Shewanella* sp. MR-4, MR-7, *S. benthica*, *S. violacea*, and *S. frigidimarina*, whose genes encode flagellin subunits of 463–482 aa. *S. baltica* OS185 and OS195 possess four genes encoding flagellins, which appear to have resulted from transposition events. The other two strains *S. pealeana* and *S. piezotolerans* contain genes encoding flagellins of 393–394 aa and 434–463 aa, respectively.

**Table S1** σ^{28}-dependent genes in *Shewanella oneidensis*. (PDF)

**Acknowledgments**
We thank Arthur Arouson (Purdue U., USA) for taking the time to carefully read and edit this paper, and Yuzhong Zhang (Shangdong U., China) for valuable assistance with the bacterial two-hybrid assay.

**Author Contributions**
Conceived and designed the experiments: HG. Performed the experiments: LW JW PT HCP. Analyzed the data: LW HG. Contributed reagents/materials/analysis tools: HG. Wrote the paper: LW HG.

References

Flagellar System in *Shewanella oneidensis*
Flagellar System in Shewanella oneidensis