Towards an Understanding of *Psychrophlexus mondsii*: Knowledge of the Unknowable

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Abstract

In this study we have isolated a bacterium belonging to the genus *Psychroflexus* from a marine tidal marsh in Woods Hole, MA and present initial characterization of this organism with respect to its close relatives *P. torquis* and *P. tropicus*. This analysis has led as to propose a new species designation of *mondii*, within the genus *Psychroflexus*. We also present initial physiological characterization of a unique aggregation phenotype referred to as cushballness. We demonstrate environmental and genetic regulation of cushball formation by *P. mondii* and identify putative extracellular structures that may be required for intracellular aggregation. *P. mondii*, like other Flavobacteria is capable of gliding motility. We present preliminary data supporting the presence of cushball formation pathways that are both independent and dependent of pathways required for gliding motility, suggesting that the decision to pursue different lifestyles, motile vs sessile, may be integrated at a genetic level. In general we demonstrate that *P. mondii* offers a genetically tractable system to address cushball formation, gliding motility as well as many other interesting questions about the biology of the *Flavobacteria* in general.

Introduction

Bacterial genetics has tended to center around the study of a few so called ‘model’ organisms such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* to name a few. And surely we have learnt a lot about these bacteria and their biology. Monod’s famous quote, that “what is true for *E. coli* is true of Elephants” relates the idea that life, at the heart of it, is built around common themes, such that detailed study of the few can be translated to information about the many. In the context of the genetic code and fundamental principles of gene regulation, Monod was largely vindicated. However, in the context of general microbiology the concept of the model system has fallen well short, in both practical and theoretical terms. Currently we are undergoing a renaissance in microbiology. The microbial universe is seemingly infinitely diverse in its metabolic capacity and biogeographical plasticity. The challenge for modern day microbiologists is not just to understand what is there, but to also understand what they are doing there and how they are interacting with their environment to and other organisms. A formidable task within the general chaos that is biological systems, but a challenge worth rising to. How does one begin to address these issues in microbiology? How does one begin to understand the biology of diverse microbes? Genetics is clearly a powerful tool, but currently there is an extreme lack of bacterial systems that developed for genetic manipulation. This task is not easy, as even culturing bacteria is not trivial. I believe though that the challenge must be undertaken. The genetic potential relates the biological potential and with recourse to thoroughly understanding the environment of the organisms in question I believe we can start to understand the roles organisms play in different biological niches.

With this concept in mind, this paper presents preliminary work to characterize a novel species of *Psychroflexus* within the context of providing a genetic platform to understand the general biology of members of the genus *Psychroflexus* and more broadly the family Flavobacteriaceae.
Methods

Growth and media

*P. mondsii* was routinely cultured on both SS (5 mM MOPS pH 7.2, 0.05% tryptone, 0.05% yeast extract, 1 mM succinate, 1 mM maltose and 1x SW base salts) and SWC media (0.5% tryptone, 0.1% yeast extract, 3 mL glycerol and 0.75x SW base salts. Media was solidified by addition of agar to a percentage of 1.5% unless otherwise stated. *P. mondsii* was routinely grown at 30°C unless otherwise stated. *E. coli* was routinely cultured on lysogeny broth (LB) also at 30°C.

Antibiotics used were erythromycin (Em; 200 µg/mL), tetracycline (Tc; 15 µg/mL) ampicillin (Ap; 100 µg/mL) and kanamycin (Km; 50 µg/mL).

PCR and Sequence Analysis

Universal bacterial primers were used to amplify the 16s rDNA from *P. mondsii*. Three primers were used to sequence the full 16s rDNA fragment. Sequence was analyzed and aligned into a single contig using Vector NTi (Invitrogen). Closest cultured relatives were determined using ARB software, and identity to known sequences was determined with Blast (NCBI).

Pigment Analysis

Pigments were extracted from whole cells with ethyl acetate. Cells were concentrated from an o/n culture in SS medium by pelleting 4 mLs of cells and resuspending in 500 µL of fresh SS medium. To this, 2 mLs of ethyl acetate was added and mixed by vortexing. The aqueous and organic layers were allowed to separate and then an absorbance spectra was obtained from the organic phase relative to an ethyl acetate blank.

Gliding analysis

Gliding was visualized predominantly using wet mounts and phase contrast microscopy. Visualization of single cell gliding was facilitated by growth of cells in 5% salt as this prevents aggregation of cells. Gliding was also visualized using SS medium solidified with 1% agar.

SEM preparation

To prepare samples of *P. mondsii* cushballs for SEM, 8 µL of o/n culture grown in SS medium was spotted on to a glass cover slip that had been treated with poly-L-Lysine to promote cell adherence. After 20 -30 min adsorption time, the excess media was wicked away using a paper towel. Then the slide was flooded with a 3% solution of glutaraldehyde in 100 mM Tris pH 8.0. Cells were allowed to fix for two hours before removal of glutaraldehyde by wicking and washing with 100 mM Tris pH 8.0. At this stage samples were either left in buffer overnight or directly put through an ethanol dehydration series. Coverslips were passaged through an ethanol series containing 50%, 70%, 95% and 100% EtOH. Each slide was left for 10 min in each ethanol solution before transferring to the next. Two washes were done at 100% EtOH before critical point drying using standard procedures. Coverslips were cut to size and mounted in SEM stubs before sputter coating in gold/ paladium. At this point sampled were ready to be viewed by SEM.

TEM preparation

Cells from overnight cultures were negatively stained for TEM. This was done by adsorbing cells to a formvar TEM grid followed by staining with uranyl acetate. Cells were adsorbed for 30 sec to a minute and stained with uranyl acetate for no more than one minute. Grids were allowed to dry, before carbon coating. At this time grids were able to be imaged my TEM.
**Tn4531 mutagenesis**

*E. coli* BW19851 harboring pEP4531 was washed twice with LB and mixed with *P. mondsii* in a 1:4 donor/host ratio, spotted on SWC agar plates and incubated at 30°C o/n. Cells were then recovered and plated on SWC media containing erythromycin. *E. coli* does not express Em resistance due to promoter incompatibility. Some background resistance is observed in *E. coli* donor controls, so to combat this, transformations were plated on media with 5% salt to further select for growth of *P. mondsii*. Lastly pigmentation of *P. mondsii* distinguishes it from *E. coli* on selective plates. *P. mondsii* was never seen to develop resistance to Em within the course of these assays.

**Table 1. Comparison of phenotypes within genus *Psychroflexus***

<table>
<thead>
<tr>
<th></th>
<th><em>P. torquis</em></th>
<th><em>P. tropicus</em></th>
<th><em>P. mondsii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolism</strong></td>
<td>chemoheterotroph</td>
<td>chemoheterotroph</td>
<td>chemoheterotroph</td>
</tr>
<tr>
<td><strong>Aerobe/anaerobe</strong></td>
<td>aerobe</td>
<td>aerobe</td>
<td>aerobe</td>
</tr>
<tr>
<td><strong>Cell size (µM)</strong></td>
<td>0.4 - 1.5 wide 0.5 - 50 long</td>
<td>0.18 – 0.25 wide 2.0 – 2.5 long</td>
<td>0.35 – 0.5 wide 1.5 – 2.5 long</td>
</tr>
<tr>
<td><strong>Coccoid bodies</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Flagellar Motility</strong></td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Pigment</strong></td>
<td>Orange</td>
<td>Not Known</td>
<td>Orange/Pink</td>
</tr>
<tr>
<td><strong>Temp Growth °C</strong></td>
<td>-16 to 20</td>
<td>4 to 43</td>
<td>4 to 30</td>
</tr>
<tr>
<td><strong>NaCl dependence</strong></td>
<td>req. sea water</td>
<td>optimum @ 5-7%</td>
<td>optimum @ 5%</td>
</tr>
<tr>
<td><strong>Gliding</strong></td>
<td>Yes</td>
<td>Not known</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Cushball</strong></td>
<td>Not reported</td>
<td>Not reported</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Results

Isolation and identification of *P. mondsii*

We carried out enrichment for thin bacteria, such as spirilla and spirochetes, by using a 0.22 µM filter as physical barrier for inoculation of SS medium plates. The source of inoculum for this enrichment was a sample from the bacterial mat located at Sippewisset tidal marsh, Woods Hole, MA. We were successful in recovering several spirilla isolates, but in addition an orange-pigmented colony was observed growing within the zone where the filter paper had been placed. This colony was restreaked to purity on SS medium and used as template for amplification of the 16s rDNA. The total 16sDNA sequence was obtained and compared to reference databases to identify the nearest cultured relative (Appendix 1).

This analysis indicated *Psychroflexus torquis* to be the closest relative with 97% identity over all nucleotides. *Psychroflexus torquis* was isolated from sea ice in Antarctica as an aerobic chemoheterotroph capable of growth at low temperatures. Another closely related species was that of *Psychroflexus tropicus*, which is seemingly a mesophillic species within the genus *Psychroflexus*. The major difference being a shift in the temperature range that supports growth. Where as *P. torquis* cannot grow above 20 °C, *P. tropicus* can grow up to 43 °C. This is consistent with the fact that *P. tropicus* was isolated off the shores of Hawaii. Identity over aligned sequence was 98% with *P. tropicus*, however a gap giving no significant alignment was observed between nucleotide 93 and 116 of the submitted sequence (appendix 1).

Below we outline phenotypic analysis supporting inclusion of our isolate in the genus *Psychroflexus*. However, based on polyphasic evidence, one of which being ego size, we were directed to create the new species *mondsii*, within the genus *Psychroflexus*. For a summary of phenotypes compared to *P. torquis* and *P. tropicus* refer to table 1.

Phenotypic characterization of *P. mondsii*

**Cell size and morphology**

Cell dimensions were measured via two sources: 1) using length measurement available on Axiovision software (Zeiss), and 2) measuring dimensions directly from TEM micrographs. Both sources provided comparable results. It should be noted that *P. mondsii* cell size depended on the medium and the phase of growth.

![Figure 1](image)

*Figure 1.* A) TEM micrograph of *P. mondsii*. Scale is 500 nM. B) & C) Phase contrast images of *P. mondsii* demonstrating coccoid bodies developing at the point of cell division.
Cells earlier in the growth phase are shorter than those in stationary phase. In fact, filaments are commonly observed in SWC media late in stationary phase. Typically though, cells range between 0.35 and 0.5 µM in width and between 1.5 and 2.5 µM in length.

Cells are Gram-negative rods, but were observed to form coccoid bodies when grown in SWC media. Coccoid bodies are spherical nonrefractile cell masses that grow and originate at the mid point of a dividing cell. They can be observed to bud from the cell and released into the culture medium. The biological fate or indeed function of these coccoid bodies is unknown.

*P. mondsii* appears to prefer lower nutrient conditions, as cells failed to grow on LB or nutrient broth even if media was adjusted to sea water levels of NaCl.

**Temperature range supporting growth**

*P. mondsii* was streaked on to both SS and SWC medium before incubating at 4°C, 30°C, and 37°C. *P. mondsii* was capable of growth at both 4°C and 30°C, but not 37°C, on both SS and SWC mediums. Growth was qualitatively slower at 4°C that at 30°C, but yield was roughly equivalent.

**Salt requirements for growth**

*P. mondsii* was grown for 48 hours in SWC media containing a range of NaCl concentrations (0-25% w/v). In these experiments 2% NaCl was the lower limit observed to support growth. Yield was maximal in 5% NaCl, but growth was not observed in salt concentrations above 10%.

These experiments were repeated in SS media, to gauge how nutrient conditions affected salt tolerance. In general yield is lower in SS media relative to SWC, however SS was observed to support growth at higher salt concentrations. *P. mondsii* grew in SS media supplemented with up to 15% NaCl.

![Figure 2](image)

**Figure 2.** NaCl dependence for growth by *P. mondsii* in either SWC medium of SS medium. Cell density was quantified after 48 hr growth at OD 600.

We further tested the specific requirement for sodium chloride as opposed to other salts. SWC media was supplemented with either 5% NaCl, 5% KCl or 2.5% MgSO₄ and tested for their ability to support growth. Only SWC supplemented with NaCl supported growth, suggesting that sodium ions are specifically required for growth of *P. mondsii*. 
Pigment Analysis

*P. mondsii* colonies are pigmented, showing pink/orange coloration on SS medium and a more orange coloration on SWC medium. These colorations are suggestive of carotenoid pigments, however to confirm this, absorbance profiles were obtained for ethyl acetate cell extracts. This analysis clearly showed three peaks closely group between 400 nM and 500 nM, which is a signal indicative of carotenoid pigments. Interestingly, there was one other major peak at around 920 nM. This wavelength does not correspond to any known pigments involved in photoenergetic reactions.

![Figure 3](image)

**Figure 3.** A) *P. mondsii* streaked on SWC medium producing an orange pigment. B) Absorbance Spectra for *P. mondsii* pigment extracted in ethyl acetate.

Gliding Motility

Cells from the genus *Flavobacterium* commonly demonstrate the ability to glide across surfaces. We tested the ability of *P. mondsii* to glide in two ways. First, we examined the colony morphology of *P. mondsii* growing on plates. Typically, cells that glide show diffuse colony margins. On close inspection we could see evidence for this when *P. mondsii* was grown on SS plates. Evidence for gliding was more prominent on plates that were moister and had an agar percentage around 1% (w/v). Second, we examined the movement of *P. mondsii* along a glass surface using phase contrast microscopy. Cells grown in either SS media or SWC media with 5% salt were grown o/n and used to prepare a wet mount for microscopy.

![Figure 4](image)

**Figure 4.** Diffuse colony margins of *P. mondsii* growing on SS medium.

We could visualize gliding motility of *P. mondsii* in both media conditions. However, cells did not glide for more than a body length before flipping over 180 degrees, back to where they started. In this manner cells seemed to glide on the spot. Gliding is known to be affected by surface and nutrient conditions. Possibly *P. mondsii* is capable of more prolific gliding given a more appropriate
environment. Irrespective, the motion exhibited by *P. mondsii* is characteristic of gliding, the cell body visibly constricting as it contacts the surface to generate force.

*Intracellular aggregation (Cushballness)*

*P. mondsii* was initially isolated on SS medium. When cells from colonies were observed under the microscope they were seen to be part of multicellular aggregates. Aggregates were structured in that cells seemed to be attached to each other via one of the two poles. This phenomenon was even more obvious when cells were grown shaking o/n in SS liquid medium. In this context the majority of cells formed discrete aggregates. In contrast to other bacteria, very few *P. mondsii* cells were free swimming. Cells tethered by a single pole showed dynamic movement around that pole. This aggregate structure was commonly referred to as a cushball to reflect the polar arrangement of cells around a central focal point.

![Figure 5](image)

**Figure 5.** A) Low magnification picture of cell aggregates that form in an overnight culture of *P. mondsii* in SS medium B) 630x Phase image of an individual cluster.

*Further Exploration of Cushballness*

*Environmental modulation of Cushball formation by salt concentration*

We tested the affect of increasing NaCl concentrations on the ability of *P. mondsii* to form cushballs in SS medium during shaking overnight growth. We observed that cushballs formed robustly in 2% NaCl (w/v) but concentrations equaling 5% and higher strongly inhibited cushball formation. We further dissected this phenomenon by assessing whether this effect was mediated by osmotic stress or increases in ionic strength. To do this we tested the ability of SS media with 2% NaCl and 6% sucrose to support cushball formation. Sucrose is a non-polar osmolyte so should not increase the ionic strength of the media. *P. mondsii* was observed to form cushballs with the addition of sucrose suggesting it is the ionic properties of NaCl that are more likely to be responsible for inhibition of cushball formation.

If NaCl is interfering with surface interactions in a nonspecific way we hypothesized that increased levels of NaCl would also inhibit surface attachment and gliding motility. We tested this hypothesis and found the opposite to be true. Cells grown in SS + 5% NaCl (w/v) could attach to the glass substratum and where capable of gliding. Potentially the affect of NaCl on gliding is more specific and could represent an environmental cue for regulation of cushball formation. This concept is discussed further later.
Analysis of Cushball structure with Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

Cushball aggregates were examined by SEM to more accurately determine the three-dimensional structure, as well as detect the presence of surface structures required for intracellular attachment. Representative pictures are shown in figure 7 & 8. Interestingly, high magnification SEM images showed that cells were connected by a network of fibers, and that these fibers commonly emanated from the polar region of cells (Fig. 8A). It is unclear whether these structures constitute a polysaccharide matrix or more formal cellular structures such as pili or fimbrae. But their presence is consistent with the polar nature of attachment observed under phase contrast.

Figure 6. Effect of Salt concentration on *P. mondsii* cushball formation.

Figure 7. SEM images of *P. mondsii* cushballs attached to a glass coverslip.
We further assessed the presence of putative surface structures using TEM. This analysis allowed us to look under higher magnification at single cells, but precluded visualization of clusters. TEM imaging gave some support to the presence of more classical surface structures such as pili or fimbrae. Cells could be regularly found with lightly stained protrusions from the polar region of the cell (Fig. 8B). How these putative structures relate to the network of fibers seen in SEM is unknown. It should be noted that TEM pictures showing the presence of pili like structures were from cells grown in SS + 5% salt conditions where cells do not form cellular clusters.

![A) TEM micrograph of P. mondsii showing putative pili/fibril structures present at the pole. B) High mag SEM image looking down on a P. mondsii cushion ball. Shown are connecting fibers emanating from the poles of attached cells.](image)

**Figure 8.** A) High mag SEM image looking down on a *P. mondsii* cushion ball. Shown are connecting fibers emanating from the poles of attached cells. B) TEM micrograph of *P. mondsii* showing putative pili/fibril structures present at the pole.

**Genetic Analysis of Cushball formation**

Previous experiments suggested that cushion ball formation could be regulated or modulated by environmental conditions, namely salt concentration. Interestingly conditions that affected cushion ball formation did not impact on gliding. We next sort to understand whether cushion ball formation and gliding constituted separate genetic pathways in the cell by performing a genetic screen to isolate mutants of *P. mondsii* that were unable to form cushion balls in SS medium. To do this we utilized a bulk enrichment procedure using spontaneous mutation as our source of genetic variation. Selection for non-cushball formers was imposed by taking advantage of the fact that cells that form cushion balls sink to the bottom of the test tube if left sitting on the bench. Simply, media was transferred from the top of the tube to fresh media once cells had settled to the bottom. The culture was then allowed to grow up again and this procedure repeated. After four passages cells were noticeably seen to remain suspended in the media rather than sinking to the bottom. At this stage cells where streaked to isolation and single colonies were tested for cushion ball formation using phase microscopy and the same sedimentation assay used to isolate them originally. A range of mutants were isolated showing complete loss of the ability to form cushion balls.

We also assessed these mutants for their ability to glide on glass surfaces in an attempt to understand if there was coupling between these phenotypes at the genetic level. Interestingly we obtained mutants that were able to glide in a similar fashion to the wild type, and we also isolated a mutant that could attach to surfaces but was unable to glide on that surface. These results may suggest that at some point gliding and cushion ball formation are on the same genetic pathway but that a bifurcation occurs at some point in that pathway.
Development of genetic tools for use with \textit{P. mondsii}

The mutants obtained in the above genetic screen are unmarked and more likely to be single point mutations than large insertions deletions. It would be nice to be able to carry out marked insertion deletions using a transposon. The main advantage here is that it is much easier to locate and characterize the nature of the mutation responsible for the observed phenotype. Also of much use would be \textit{E. coli}/\textit{P. mondsii} shuttle plasmids. Such constructs would greatly facilitate complementation studies, which are of paramount importance in demonstrating a causal link between a mutation and a given phenotype. To this end we tested the usability of several transposons and plasmids described for use with \textit{Flavobacterium johnsonae} (3,4).

In summary, we were able to transform \textit{P. mondsii} with Tn4531 using the erythromycin resistance marker. however, we were unable to obtain transformants with the plasmid pCP29. Given the success of conjugation for transformation by Tn4531, it seems likely that the origin of pCP29 does not replicate in \textit{P. mondsii}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{A) Aggregation phenotypes of Wt (\textit{P. mondsii}) and three mutants isolated from genetic enrichments. B) Phase contrast micrographs showing loss of aggregation phenotype by mutants relative to the wild type when grown in SS medium.}
\end{figure}
Discussion

We have isolated a bacterium belonging to the genus *Psychroflexus* and present initial characterization of this organism with respect to its close relatives *P. torquis* and *P. tropicus*. This analysis has led us to propose a new species designation of *mondsii*, within the genus *Psychroflexus*. We also present initial physiological characterization of a unique aggregation phenotype referred to as cushballness. As part of this characterization we have begun to develop genetic tools and protocols for use with *P. mondsii*. We present preliminary data supporting the presence of cushball formation pathways that are both independent and dependent of pathways required for gliding motility.

We initially isolated *P. mondsii* due to an interest in the cellular aggregates that it formed when grown in SS medium. Subsequent phylogenetic analysis using the 16s rDNA sequence indicated that *P. mondsii* was closely related to the Flavobacterium *Psychroflexus torquis* as well as *Psychroflexus tropicus*. Our initial characterization of *P. mondsii* indicated that it does indeed display many attributes consistent with its inclusion within the phylum Flavobacterium and more specifically the genus *Psychroflexus*. For instance, similar to *P. torquis* and/or *P. tropicus*, *P. mondsii* is an obligate aerobic chemoheterotroph. It does not display flagellar mediated motility but is capable of gliding motility across surfaces. Size and morphology are also roughly consistent. A few points are worth noting in more detail.

We commonly observed the development of a spherical body at the point of cell division. Coccoid body formation is a common trait of this genus, however very little is known about the biology associated with this. Other researchers suggest that this is dependent on the “age” of the culture and indicative of a starvation response. However contrary to this generalization we observed that cells cultured in low nutrient media such as SS medium form spherical bodies at a much lower frequency than cells cultured in SWC media irrespective of the age of the culture per se. Potentially growth dynamics in general play a role, or even density dependant mechanisms since the overall cell yield is much greater in SWC media.

The different climates of isolation seem to reflect the temperature range that supports growth of *P. torquis*, *P. tropicus* and *P. mondsii*. *P. torquis* was isolated from Antarctica and is able to grow at temperatures below zero degrees Celsius. Conversely, *P. tropicus* was isolated off the shores of Hawaii, so predictably is more mesophilic, growing from 4 to 43 degrees. In this case, *P. mondsii* was isolated off the shores of Woods Hole in Massachusetts, USA. This environment is certainly intermediate in temperature range to that of Antarctica and Hawaii, which is consistent with its ability to grow down to at least 4 degrees but not much above 30 degrees. The different temperature dependence of *P. tropicus* was the major evidence provided in support of its designation as a new species, so in that respect, a similar argument applies for *P. mondsii*. Whether this is a sufficient argument shall be left at the mercy of the taxonomists.

Our primary interest was in understanding more of the biology behind the ability of *P. mondsii* to form the structured multi-cellular aggregates we termed cushballs. We first asked whether the ability to form cushballs was subject to modulation by growth conditions. As a result we found two conditions that inhibit cushball formation. That being, growth in SWC medium as opposed to SS medium, or growth in SS media amended to contain 5% NaCl. The underlying cause of this may well be different for the two conditions as SWC medium contains only 1.5% salt, which is sufficiently low to allow cushball formation in SS medium. Currently we do not know exactly the component of growth in SWC that prevents cushball formation. The ability of salt to inhibit cushball formation could seemingly be for three reasons 1) increase in osmolarity of the medium 2) increase in ionic strength of the medium 3) increase and specific sensing of sodium or chloride ions.
To begin to discern between these two models we analyzed the ability of sucrose to inhibit cushball formation. Sucrose is non-polar so would not adjust the ionic strength of the medium. We observed that cushball formation still occurred, ruling out increases in osmotic stress as the reason for the ability of salt to inhibit cushball formation. It may be that increases in NaCl lead to general interference with electrostatic interactions between surface structures. However, one piece of evidence against this is the observation that inhibition of cushball formation by salt does not inhibit surface attachment or gliding. *P. mondsii* is a marine bacterium isolated in a tidal marsh. In this environment the concentration of NaCl is likely to fluctuate considerably due to natural cycles, suggesting NaCl concentration is an environmentally relevant signal. One possibility is that *P. mondsii* regulates cushball formation specifically in response to the level of NaCl. To further address the specificity of this response to NaCl, other ionic salts such as KCl and NaSO4 should be tested for their ability to interfere with cushball formation.

Another question of interest was whether *P. mondsii* possessed surface structures that may be involved in cushball formation and or gliding. This is of interest because Flavobacteria are not commonly found to have pili or fimbriae. To investigate this we carried out TEM imaging of single cells and SEM imaging of cushball clusters. This analysis gave support for two potentially different types of extracellular structures. First, TEM analysis indicates the potential presence of fimbriae and or pili at one of the poles. Not all cells could be seen to have these structures so it will be important to confirm this with multiple cell preps in different growth conditions. Also it would be of value to carry out embedding and sectioning of *P. mondsii* cells, rather than simply using negative staining techniques. SEM of cushballs indicated the presence of connecting fibers spanning cells. Interestingly, these fibers were seen to commonly emanate from the pole of cells within the cushball, which is consistent with phase contrast imaging showing that cells are generally tethered by a single pole to a surface and/ or each other. Currently it is not possible to determine the biological nature of these fibers. Are they the same structures seen in the TEM analysis? Further analysis of fiber structure and composition will be required to answer this and other questions. In any respect cushball formation by Flavobacteria is a novel phenotype, and it would be of interest to understand more about its structural basis and regulation.

The final undertaking in this project was to understand more about the genetic relationship between pathways for cushball formation and those for gliding. We wondered whether there would be common regulation of these differential lifestyle decisions, motility vs sessility. To address this question we isolated mutants of *P. mondsii* that were unable to form cushballs, and then asked whether they were still able to glide similar to the wild type. In fact we found both classes of mutant, suggesting that some parts of the cushball pathway are common to gliding pathways, but at some point they diverge and are independent. The nature of these mutations is currently unknown preventing further speculation. Due to time constraints mutants were obtained by enrichment for spontaneous mutants, as such they are difficult to map and characterize. To address this we began optimizing a transposon mutagenesis system for use with *P. mondsii*. This would allow the acquisition of marked insertion mutants, which would facilitate faster characterization of the genes involved in cushball biosynthesis and regulation.

In summary, we demonstrated environmental and genetic regulation of cell aggregation by *P. mondsii* and identified putative extracellular structures that may be required for intracellular aggregation. *P. mondsii* offers a genetically tractable system to address cushball formation, gliding motility as well as many other interesting questions about the biology of the Flavobacteria in general.
Appendix 1: P. mondsii 16s rDNA sequence

GCTGACGACCGGCACGGGTGCTAAGCGGTATACAAATCTACCTATTACTAGAGGATAGCAGCCAGGAAATTT
GGATTAATATTTTATGTTATTAACGATTTGGCCTAACAGTGTAAATTAGGTTCAGGATAGTAGGATAGGCA
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TCGCTAATGCTAGCATACGGCCATGATGCGGTGAATACGCTGGGCGCTTATGACACCGCCCGCTCAAGCC
ATGGAAGCTGGGGGTACCTGAAG
References


Acknowledgements

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