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A Preliminary Foray into Domesticating *Pirellula*, the little pears, as a model *Planctomycetes*

Jeff Holmes
Warren Wilson College

jholmes@warren-wilson.edu

Background

The *Planctomycetes* constitute a distinct phylogenetic branch of the Bacteria that has been recognized for some time because of their distinctive morphology, which has been subsequently confirmed and refined through molecular phylogenetics. There most provocative feature is the presence of characteristic internal membrane organelles that divide the cytoplasm into two or three major compartments, which has fuelled speculation as to their evolutionary relationship to eukaryotes. They are also remarkable for being completely devoid of peptidoglycan, which is almost universally present in the cell walls of bacteria (Fuerst 2011). However, because no planctomycete is known to cause any disease of plants or animals, and until recently they were not known to be responsible for any economically or ecologically important chemical transformations, they have been relatively unstudied, except as curiosities.

At least two developments have renewed interest in the *Planctomycetes*. One was the discovery that a distinct clade of the *Planctomycetes* is able to perform the anaerobic oxidation of ammonia to nitrogen gas. This previously unknown step in the nitrogen cycle has global ecological implications, and appears to be performed only by these "annamox" planctomycetes. Understanding the physiology and lifestyle of these organisms has been hampered by their inability to be grown in pure culture and their slow growth under difficult to maintain enrichment conditions. What is clear now is that they are probably quite widespread and need to be considered in any potentially anaerobic environment. Which leads to the second development that has spurred interest in the *Planctomycetes*, their frequent occurrence in metagenomic datasets from almost any soil or aquatic environments. They rarely represent more than 1 to 2 percent of the total sequences present, but they seem to be almost everywhere, provoking the question of what are they doing there. Our knowledge of "garden variety" (or non-annamox) planctomycetes is derived almost exclusively from cultured representatives, which in general all seem to be standard aerobic organoheterotrophs with slow doubling times. Without any obvious metabolic tricks to suggest a unique ecological niche for them, it is something of a mystery as to how these garden variety planctomycetes are keeping from being outcompeted by the *Proteobacteria*. Thus the mystery of the planctomycetes lies in both their uniqueness and their seeming banality.

This project was motivated, in part, by the wish to develop methods to begin to better understand the *Planctomycetes*, and in particular the genus *Pirellula*, which seems to have to most promise for laboratory cultivation. The absence of a model planctomycete that could be easily manipulated in the lab and was genetically tractable seemed a major impediment to further understanding the basic biology of these organisms. The need for such a model system was endorsed by the on-line publication soon after this mini-project was initiated of methods for transformation of *Planctomyces limnophilus*, with exactly this justification (Jogler, et al. 2011). *Planctomyces* and *Pirellula* are the two genera of the *Planctomycetes* that have been recognized for the longest time and have the most cultured and named species. The

genus *Planctomyces* is characterized by long stalked cells reminiscent of the prosthecate *Caulobacter* that anchor themselves to substrates or one another by their fibrous stalks. *Pirellula* cells are more ovoid or pear shaped (*Pirellula* = "little pear") and have numerous small fibers capable of attachment at one end that can be seen in the electron microscope, but lack the long thick stalk of *Planctomyces* that is evident by phase microscopy. *Planctomyces limnophilus* was chosen for it's rapid doubling time for a planctomycete, which they report as being 6 to 14 days to obtain visible single colonies. For this project I studied *Pirellula staleyi*, which consistently produced visible colonies in about two days, and was specifically chosen because of this "weedy" growth. Why the Kolter group didn't chose this well characterized species that also has a full published genome sequence is not clear (the supplementary material was not available with the on-line publication), but for the purposes of a three week MBL Microbial Diversity project, *Pirellula staleyi* would seem the more logical choice, and it is the hope of this author that these results might stimulate the choice of this species for planctomycete projects in the future.

Methods

For the enrichment of *Pirellula* species from environmental samples, modifications of published methods (Ward, et al. 2006) based on the use of N-acetyl glucosamine as the sole source of carbon and nitrogen were used. Two carbon and nitrogen free media bases were used, one for freshwater and the other for saltwater samples. FW-PST contained 1X Fresh Water Base salts; 1 mM sodium sulfate; 10 mM potassium phosphate buffer (pH 7); 1X trace salts solution. SW-PST was identical except for the substitution of Salt Water Base salts for fresh. For solid media, agar was added at 15 grams/liter. Media was autoclave-sterilized and then filter sterilized N-acetyl glucosamine was added from a 100mg/stock to a final concentration of 2mg/ml, unless stated otherwise. All agar media contained 1x vitamin stock solution added after autoclaving, while it was generally left out of the liquid media unless noted otherwise.

Enrichments or selections for *Pirellula* were performed both by streaking small samples of liquid or sediment on agar plates containing N-acetyl glucosamine (NAG) as the carbon and nitrogen source, or by placing approximately one gram samples into 10 mls of liquid NAG enrichment media in 16mm test tubes. Incubation was done on the bench at room temperature. Turbidity in the liquid cultures was detected between 1 to 4 days later, checked on the microscope and then samples from these enrichments were streaked for single colonies on NAG plates. Single colonies were screened microscopically using the 40X phase objective or the 100X oil immersion lens, looking for the characteristic *Pirellula* or *Planctomyces* morphology.

Tests of varying media composition and antibiotic sensitivity were done by making serial dilutions in 24-well sterile polystyrene plates (Corning). *Pirellula staleyi* cells were grown on Nutrient Agar plates (Difco), resuspended in liquid to the point of just being visibly turbid, and then aliquoted into the wells to be tested. Serial dilution and plating of some of these suspensions indicated that the cell density was

approximately 1-2 X 10^7 cells/ml. The final volume in each well was always one milliliter or very close to that. The extent of turbidity was judged visually, which proved at least as accurate at using a Spect20 spectrophotometer in a few early comparisons. The use of a plate reader was not tried.

Results and Discussion

A variety of innocula were used to try to enrich for *Pirellula* species on media that contained N-acetyl glucosamine (NAG) as the only carbon and nitrogen source. These included water samples from Eel Pond, the School Street Marsh, Little Sippiwissett Marsh, Cedar Swamp, and Trunk River. In most cases a small amount of sediment was included in the sample, given the primarily sedentary nature of *Pirellula*. Because previous work suggested that the numbers of Pirellula often increase with storage of sample water, samples up to 20 days of age were used whenever possible. Sediment samples from the seawater table were used as well for the same reason, including a Thiovulum culture, several old Sippiwissett mat samples, a bucket of clams and whatever else looked like it had maybe been sitting around for a while. All incubations were at room temperature either on the bench (liquid) or in a dark drawer (plates). In most cases samples were both enriched in liquid NAG media and plated directly on NAG agar media. In every sample tested growth was observed to occur by either colony formation or turbidity. Growth was detectable in most samples by day 2, and in all samples by day 5. Some of the fast and slow growing liquid enrichments were streaked out onto NAG plates for the isolation of pure cultures.

Individual colonies were picked with toothpicks into 4 microliter drops of PBS, three per slide, and covered with 18mm coverslips. Cells were observed with the 40X phase objective, and if necessary the 100X oil immersion lens, to look for the ovoid or pear shape characteristic of *Pirellula*, the presence of a budding type of cell division, or a stalked or tethered cell morphology characteristic of *Planctomyces*. Fast growing isolates were screed first and then only small colonies that appeared after several days were looked at, considering the relatively slow doubling times expected of planctomycetes. In all, over 100 single cell isolates were screened in this way. No candidate *Pirellula* or *Planctomyces* were identified in this screen. Instead the vast majority of the isolates obtained were long thin rods that were vigorous swimmers, looking much like typical *Pseudomonas* sp. Less than 5% of the isolates appeared to lack flagellar motility, even the ones the ones that had not be enriched in liquid. There were lots of differences in the isolates in terms both of cell morphology, colony morphology and pigmentation, suggesting that the NAG selection is not particularly selective for *Pirellula*, and that lots of different bacteria can use this compound as a sole carbon and nitrogen source. I have been unable to determine what the rationale for using this NAG enrichment technique was, but from my limited experience it would seem to be of limited value. Some recent papers on planctomycete enrichment have apparently successfully isolated strains both with and without NAG in the media, but do not discuss its relative merits.

Given these results, the addition beta-lactam antibiotics to the enrichment media was considered. This is frequently mentioned as a method for enriching for planctomycetes, and with an apparently sound biochemical basis, as these antibiotics inhibits the cross-linking of peptidoglycan peptides and planctomycetes lack peptidoglycan in their cell walls. The concentration of Ampicillin or Penicillin suggested for enrichment media by Staley, et al. is 2 milligram/ml. This seemed like a high concentration, given that ampicillin levels used to select for ampicillin-resistant *E. coli* transformants are generally in the range of 40-50 microgram/ml. For this reason it was decided to test a type strain of *Pirellula* to determine what concentrations of ampicillin it was resistant to, as a guide to what concentrations to use in my enrichments. This turned out to be a turning point in my project and consumed the remainder of my time working on *Pirellula*. No further work was done on enrichment, and from the few works that have recently described enrichment procedures for planctomycetes, I think it is fair to conclude that a method that can enrich for the apparent 1% of planctomycetes in most soil and water samples does not yet exist.

Antibiotic sensitivity of Pirellula staleyi

Pirellula staleyi (strain ATCC 27377 / DSM 6068) was isolated by James Staley from the freshwater Lake Lansing, in Lansing, Michigan in the early seventies. It was originally identified as a different species, and then renamed later. Little has been published on its biology since its original isolation, but it has been repeatedly used in phylogenetic trees to orient the relationships between newly isolated and reclassified planctomycete species. It was later selected by the Joint Genome Institute as one of the GEBA genomes to be sequenced, based solely on it's phylogenetic position in a part of the tree that was underrepresented in sequenced genomes. In 2009 its complete genome sequence, with an automated annotation, was made publically available. It was the availability of this complete sequence, and the JGI's promotion of its use in undergraduate education, coupled with its apparent ease of cultivation and relatively rapid growth, that originally attracted me to it as a potential model system for studying planctomycete biology in the context of a small liberal arts college. Therefore, last year I contacted Jim Staley about the possibility of doing this and he thought it ought to be possible, though he was unaware of anyone who had ever tried to genetically manipulate this species, or who was working on it in any capacity. He generously supplied me with a couple lyophilized capillaries from his freezer that were probably ten to twenty years old. I resuscitated one of these cultures on CM media (DSM culture collection recipe) with is essentially a one-tenth dilution of Luria-Bertani media. I had success growing it on full strength LB plates, Nutrient agar plates, and Tryptic Soy agar plates. I froze down both glycerol and DMSO cultures and have done nothing with the strain until bringing it to Woods Hole this summer. In Woods Hole, the *P. staleyi* grew well on the Nutrient Agar, LB agar, and Salt Water complete agar. The later was somewhat surprising, as according to published accounts *Pirellula* species are supposed to exhibit a narrow salt tolerance range. The colonies on SWC were smaller than those on NA, and distinctly more yellow than beige, but there seemed to be no significant decrease in plating efficiency.

The ampicillin sensitivity of *P. staleyi* was first tested by streaking it out on stock LB-Amp plates made for generating *E. coli* clone libraries. No single colonies were obtained, even after incubation for 7 days at room temperature. Growth of *P. staleyi* on LB-Amp plates only occurred when a particularly heavy visible innoculum was used, and then only in the region of the plate that contains the thin cell paste. This result suggested that *P.staleyi* might be resistant to ampicillin, but only in a cell density dependent manner, as might be expected if cells can protect each other, as through the production of an inactivating beta-lactamase enzyme. But at low cell densities *P. staleyi* appears to be sensitive to ampicillin, contrary to the published behavior of members of the *Planctomycetes* (Fuerst, 2011, Cayrou 2010).

To further explore the drug sensitivity of *P. staleyi*, serial dilutions of various antibiotics, by either $1/5^{th}$ or ½ in Nutrient broth were added to approximately 10^5 cells in final volumes of one milliliter. The minimum inhibitory concentrations for these antibiotics are given in Table 1.

Table 1
Minimum inhibitory concentrations for various antibiotics against *P. staleyi* in nutrient broth.

Antibiotic	Minimum inhibitory concentration (microgram/ml)	
Ampicillin	200-100	
Penicillin G	400-200	
Amoxicillin	200-40	
Cefotaxime sulfate	1-0.5	
Vancomycin	2-1	
Kanamycin	1.6-0.32	
Streptomycin	8-1.6	
Fosfomycin	64-32	No clear cutoff conc.

These results were surprising in a number of ways. Ampicillin, penicillin, and amoxicillin are all so-called first generation beta-lactam antibiotics that supposed to have the same mechanism of action: binding to the active site of the enzyme that cross-links the short peptides of peptidoglycan and thereby halts cell wall growth, though is some cases this may lead to eventual cell lysis. It is therefore not

surprising that these three antibiotics have similar MIC values. What is more surprising is that I had previously shown that 40 microgram/ml ampicillin completely inhibited *P. staleyi* on plates, but here in liquid nutrient broth it was unaffected by concentrations up to 100 microgram/ml. I repeated the plate assay by making LB plates that contained 400, 100, 40, 4, or no ampicillin. A dilution of *P. staley*i was applied to each of these plates that produced several thousand single colonies on the no ampicillin control. No colonies were present on any of the other plates, even after 5 days. Similar results were obtained be streaking cells directly on these plates without dilution. This seems to indicate a MIC value for ampicillin below 4 microgram/ml. on plates, but above 100 microgram/ml in liquid. The one glaring caveat to this comparison is that significantly more cells were used to inoculate the wells than the plates, so the difference may have more to do with the initial cell density when the antibiotic is applied, rather than the difference between liquid and agar media. Further evidence for the importance of cell density was that while these MIC values were pretty clear after 24 to 36 hours growth, with additional time growth was evident as the higher concentrations of antibiotic. Again, this seemed reminiscent of late satellite colony formation on *E. co*li transformation plates, due to the inactivation of ampicillin by the beta lactamase produced by the resistance gene.

But perhaps the most significant surprise was that *P. staleyi* was sensitive to beta-lactams at all. This contradicted the general consensus about planctomycetes. For this reason additional antibiotics were tested for their effectiveness on *P. staleyi*. Significantly, both cefotaxime and vancomycin proved to be very effective at inhibiting the growth of *P. staleyi* in nutrient broth. The MIC values for these two antibiotics were in the 1 microgram/ml range, which is usually considered sensitive for species that they are used against clinically. Cefotaxime is a so-called third-generation beta lactam that has been designed to be resistant to the most common forms of beta lactamase. The results for this antibiotic add further support to the hypothesis that *P. staleyi* is sensitive to beta lactam antibiotics, but achieves a level of resistance to them through the production of beta lactamase enzymes that can hydrolyze the penicillin types, but not the later cephalosporins.

The sensitivity of *P. staleyi* to vancomycin is perhaps the most surprising, unless this Pirellula does produce standard peptidoglycan and needs to crosslink the peptides in order for the cells to either grow or divide. Vancomycin is a large hydrophilic aminoglycoside that also inhibits the crosslinking of peptidoglycan, but not by binding to the crosslinking enzyme, but rather to the D-alanine containing peptides, making them unavailable for crosslinking. In general, vancomycin is not a widely useful antibiotic because nearly all Gram-negative organisms are resistant to it because it can't cross the outer membrane. The sensitivity of *P. staleyi* to both beta-lactams and vancomycin would seem strong evidence that it must produce and crosslink peptidoglycan, most likely as part of its cell wall. The likelihood that it might be sensitive to both kinds of antibiotics due to cross-reacting to targets in other cellular pathways just by chance seems vanishingly small. As surprising as this finding is, it is notable, and probably not a coincidence, that a similar phenomenon

appears to be in play in *Chlamydia*. The *Chlamydia, Planct*omycetes, and *Verrucomicrobia* are generally considered to be all members of a single clade, or super-phylum sometimes called the VBC's. At least some members of the Verrucomicrobia have peptidoglycan in their cell walls, but the *Chlamydia* have long been known to lack a peptidoglycan cell wall. Nonetheless, until relatively recently the drug most commonly prescribed for a *Chlamydia* infection was amoxicillin. The contradiction of successfully using a beta-lactam on an infection by a bacteria that lacks peptidoglycan was always recognized, but successfully ignored by referring to it as the "chlamydial anomaly". More recent work has suggested that beta-lactams specifically block the multiple fission event that occurs in the reticulate body of the chlamydial lifecycle. The one intriguing, but unreplicated observation made on *P. staleyi* that may relate to this is that in one dilution series of penicillin G, cells that were right at the inhibitory concentration of penicillin were able to grow slowly, but formed large clumps and aggregates, suggesting they were experiencing difficulty separating the newly budded cells.

According to the BioCyc database, the *Chlamydia trachomonas* genome contains 9 of the thirteen genes required for peptidoglycan synthesis, and *Pirellula staleyi* contains 5 of them. It is hard to know whether the missing genes are actually gone because the organisms have lost the ability to complete normal peptidoglycan synthesis, or whether their phylogenetic divergence from other bacteria is masking the functional homologs that are there. Certainly *P. staleyi* contains a high percentage of hypothetical genes of unknown function, but it is also possible that these organisms synthesize some sort of molecule that is not a true peptidoglycan, but still contains D-alanine peptides that need to be crosslinked.

In this mini-project I was limited to the antibiotics available in the freezer from past year's courses. There is obviously a huge armamentarium of well-characterized antibiotics that interfere with cell wall biosynthesis that could be tested on *P. staleyi* now. Synergistic interactions might also be looked at, though this might be best done in overlapping disc diffusion assays. One preliminary test I did suggested that sub-inhibitory doses of penicillin might actually suppress the inhibition of vancomycin, but I was unable to repeat this result.

These simple and quick experiments, if nothing else, would seem to support the contention that *P. staleyi* is worthy experimental organism to address simple questions about the biology of PVC organisms that are difficult to do in organisms requiring anaerobic mixed cultures or growth inside eukaryotic host cells.

A defined media for *P. staleyi*

My experience with the purported ampicillin resistance of Pirellula species made me test the ability of *P. staleyi* to grow on my defined media containing N-acetyl glucosamine as the carbon and nitrogen source. It didn't grow on either the salt water or freshwater version of NAG plates that contained the vitamin supplement. This was frustrating not only because this was the media I had been trying to enrich for *Pirellula* species, but also because the annotated *P. s*taleyi genome indicated the

presence for all three genes needed for NAG catabolism. These results led to a number of preliminary experiments into determining what was missing from this medium necessary for the growth of *P. staleyi*. These experiments were somewhat inconclusive and difficult to summarize, but I was unable to develop a defined media that would consistently work, and the need for such a media became increasingly obvious for trying to dissect the physiology and metabolism of this organism in the way I hoped to in the future.

Yeast extract was the most successful undefined media component, which could support growth at a concentration of 0.5 mg/ml all by itself when dissolved in milliQ water. Supplementation of this low yeast extract concentration with various carbon and nitrogen sources, or vitamin or trace metal supplements did not seem to have any effect on this initial growth. However there was some indication that if the broth dilutions were left for several days that some of these supplements did support increased growth at a much slower level than initially seen. These results were not really evident until the day of lab cleanup, when my dishes were being tossed out, but I would say there was pretty good evidence that *P. staleyi* can use NAG, glucose and succinate as carbon sources, and does respond mildly to vitamin supplementation. There is need for more work here, but with new optimism that a reasonably good defined media can be developed for *P. staleyi* in the future under less chaotic and time limited conditions.

The bottle effect

Finally, I feel obligated to acknowledge that I also spent either too much or too little time messing around with trying to develop a definitive experiment to explore the biological basis for what has come to be known as the bottle effect. This is the phenomenon that is used to explain the changes in microbial composition that occur over time after an environmental sample is left inside its sampling container. I encountered it during the first weeks of class when I discovered that the number of colony forming units (CFU's) detected in our group's Eel Pond water sample had increased by about 5-fold after a weeks storage on our bench. I was then led into the arcane historical literature on the bottle effect, and ultimately seduced by the simplicity of the plating assay as a way of trying to address basic questions about microbial succession, adaptation, specialist vs. generalist lifestyles, r's vs. K's, and on and on.

To anyone foolhardy enough to try to engage this phenomenon again, I have just a few suggestions: prepare large batches of plates so that all your before and after counts are made on the same media. Having plates that are flat and very dry is critical. Beware that some water samples contain significant numbers of organisms that form huge spreading colonies that make CFU counts impossible. Autoclave your media well; I was ultimately undone by a batch of plates that had just enough viable cells in the agar to kill the whole thing. Some questions I think still need to be addressed: are marine water samples more likely to exhibit an increase in CFU number with storage than freshwater/still water ones? I was never able to repeat my original observation of an increase in CFU count with time. All of my samples

either stayed unchanged or decreased in total CFU count, including two new Eel Pond samples. Possibly marine plankton samples are more prone to increases than the pond and swamp samples I was focusing on. Also I was often including a little sediment in my bottles because they were doing double duty for *Pirellula* enrichments. That was a mistake in hindsight. Also, consider testing whether your plate counts at high dilution are consistent with your plate counts at low dilution. Others (T. Schmidt, pers. comm.) have seen consistent and pronounced effects of this type. I did not see any dramatic effects in the eleven samples I looked at, but these were again mostly planktonic microbial communities and there are reasons you might expect to see more of a dilution effect with soil or sediment samples.

This last question seems simple and straightforward enough that I am considering trying to develop it into a freshman lab exercise. I also re-learned in my investigations how aesthetically appealing and imagination stirring it is to scrutinize a multispecies plates of diverse colony morphologies under the dissecting microscope, and I would like to try to leverage that in my introductory biology courses in the future.

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