Looking at the Genetics and Biochemical Components

The road to demonstrating the causes of WS emergence is complicated, but the conclusions can be relatively easily summarized. There has been a fairly clear map made of the genetic mutations that lead to biochemical responses that ultimately lead to WS phenotypes. For our purposes, the important thing is that with proper explanations, this map is accessible to a High School student. It is possible to gloss over the extreme details and instead focus on the broader regulatory pathways without losing much depth of understanding. In fact, because this material is fairly accessible, the biochemical pathways that lead to WS phenotypes offer a wonderful jumping off point to a broader unit on the relationship between genes, proteins, signaling and phenotypes. Similarly, the studies that led to our current understanding of the mutational causes of WS emergence offer a comprehensible template for how genetic research is done. Thus, contained within this unit is a study of modern evolutionary theory, contemporary research practices and a working example of the central dogma.

Phenotypes: SR and WS

The ancestral, wild-type strain of *P. Fluorescens* SBW25 forms smooth, round colonies when plated. These smooth (SM) genotypes form the majority of bacteria within a microcosm and are free-floating within a liquid (planktonic). With regular consistency, whenever a liquid containing *P.Flu* is left static, a specific phenotype emerges that has been named "Wrinkly Spreader" (WS). WS phenotypes are characterized by their ability to create a biomat that allows them to colonize the surface of the liquid, commonly referred to in the literature as the "Air-Liquid" (A-L) interface. The Wrinkly Spreaders produce a biofilm that allows cells to stick to a surface (such as test tube glass or a plastic bead) and also causes the cells to stick together after dividing. Although it is energetically costly to devote nutrients to creating this biofilm, the WS receive the benefit of having greater access to oxygen. The WS bacteria can thus be considered a niche colonizer.

Chemically, WS overproduce an acetylated cellulose polymer. Research has demonstrated that overproduction of this polymer is sufficient for the WS phenotype to be presented. However, there are various genetic changes that can lead to this overproduction. The coding for the production of the polymer itself is located within a 10-gene operon known as *wss* (**WS s**tructural). However, no mutations in the *wss* operon were found to lead to overexpression of the enzymes, leading researchers to conclude that gain-of-function mutations within *wss* were an unlikely source of the WS phenotype. Researchers then looked to upstream causes of polymer overproduction.

The Wsp Operon

Cellulose-synthesizing enzymes can be activated by cyclic-dimeric-guanosine monophosphate (c-di-GMP). Therefore, it was thought possible that overproduction of c-di-GMP would lead to overexpression of *wss* and thus increased biofilm production. One source of c-di-GMP within *P.Flu* was discovered and labeled *wspR* (**WS p**henotype **r**egulator). *wspR* is made of two domains, one of which contains a region that is specifically known to produce c-di-GMP. However, an analysis of the *wspR* gene could not find any mutations that would lead to overproduction of c-di-GMP. Further analysis of the *wspR* region indicated an entire *wsp* operon that ultimately led to regulation of *wspR* (and thus c-di-GMP) expression.

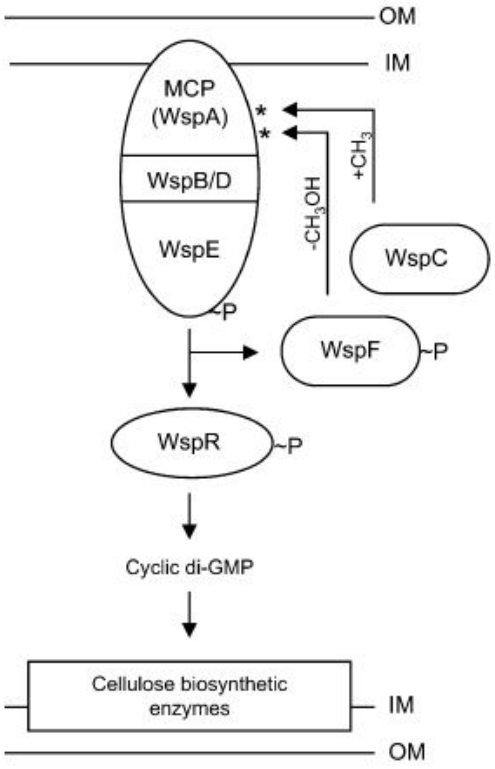

Figure : The Wsp Operon

It turns out that *wsp* is a 7-gene operon, with genes *wspABCDEFR*. With the exception of *wspR*, the start codon of each gene overlaps the stop codon of the previous one, meaning the genes are likely all transcribed at once (*wspR* was also demonstrated to be part of the same transcriptional unit). The *wsp*ABDE proteins form a complex that is embedded in the inner membrane of the bacterial wall. *wspC* and *wspF* turn the complex on and off, respectively, by adding (*wspC*) and removing (*wspF*) a methyl group. When the complex is turned on, *wspE* works as a kinase that phosphorylates *wspR*, which thus activates *wspR*, causing it to produce c-di-GMP.

In essence, the wild-type *wsp* operon works to both create c-di-GMP and to self-regulate against the overproduction of c-di-GMP by a negative feedback loop. When the kinase *wspE* is activated, it activates *wspF*, which then turns off the kinase. Once the kinase is deactivated, *wspC* is ready to come in and reactivate it. The normal state seems to be one of random fluctuation between activation and deactivation, which allows only a small amount of c-di-GMP (and thus the cellulose polymer) to be synthesized.

There are multiple places within the *wsp* operon that could harbor mutations that would lead to overproduction of c-di-GMP. Upstream of *wspR* there are three different proteins, *wspACE*, that must be activated for the complex to produce c-di-GMP. These proteins thus all act as "on switches." Only one protein, however, acts as an "off switch" that shuts down the complex. The important thing to keep in mind is that for a mutation in the "on switch" regions to cause excess c-di-GMP, it must be a gain-of-function mutation. That is, it must not only fail to damage the operation of the protein but actually make it do its job better. These types of mutations are much more rare than loss of function mutations, and the rate and consistency with which WS phenotypes emerge indicates that the mutation is instead the more common loss-of-function. The only gene that would have a loss of function associated with overexpression of the polymer is the *wspF* that acts as the "off switch."

Consider what happens when you deactivate (or at least limit) the protein that turns off this complex. Without a properly functioning *wspF*, the kinase would not be deactivated and would therefore be constantly activating the c-di-GMP-producing proteins, which would finally cause an overproduction of acetylated cellulose polymer, leading to the WS phenotype. When researchers looked at gene sequences from 26 WS colonies, 13 of them

had mutations in *wspF* that led to either reduced activity or loss of function of the protein. Clearly some answers had been found, but the final picture was not yet in sight.

Aws and Mws Operons

Because mutations in the *wsp* operon could only account for some of the WS phenotypes (13 out of 26), researchers had to look for other genes that may also code for biofilm production. To determine alternate pathways to WS, researchers deleted the *wsp* operon from the ancestral SM genome. This new strain of *P.Flu* was known as Δ*wspABCDEFR* and was phenotypically indistinguishable from the ancestral SM type. When this new strain was cultured as usual, new WS phenotypes were seen to emerge after only 5 days of growth. Clearly those new strains could not have been caused by mutations in the *wsp* operon, because that operon had been deleted from the genome.

Researchers chose a single WS genotype to analyze, calling it *Aws* (**a**lternative **w**rinkly **s**preader). They found a specific operon composed of three parts: *awsX, awsR, and awsO.* As might be expected, *awsX* was found to be a negative regulator of c-di-GMP, and it was demonstrated that a loss-of-function mutation in *awsX* (from a 39-bp deletion) was sufficient for the creation of the WS phenotype.

When researchers analyzed the remaining WS phenotypes—i.e. those not accounted for by *wsp* mutations—they found that some of them had *Aws* mutations, but not all. Therefore there was yet another gene that could cause WS emergence.

Repeating the same process that pointed towards *Aws*, scientists deleted both the *wsp* and *Aws* operons from the SM ancestor genotype (Δ*wsp* Δ*aws*) and again cultured this new strain. Again, WS phenotypes emerged, now dubbed *Mws* (**M**ike's **w**rinkly **s**preader). Researchers found another gene, *mswR*, which once again negatively regulates c-di-GMP production, so that when this gene loses function biofilm will be overproduced.

Following all of the above processes, scientists were able to find a final genotype, with deleted *wsp, Aws,* and *Mws*. This other genotype took an additional 3 days for any WS phenotypes to emerge, and was therefore named *SWS* (**s**low **w**rinkly **s**preader).

Conclusion

The biochemical and genetic details involved with studying WS evolution can be very complex. But by presenting a gist of the above studies, advanced students can gain significant insight into how geneticists think about and research mutations and evolution. The salient details can be presented in simplified form:

- WS phenotypes emerge when bacteria overproduce biofilms.
- *P.Flu* are constantly producing biofilm in small amounts, but when mutations damage the negative regulator (the "off switch"), the new strain will overproduce biofilm and become WS phenotypes.
- More than one type of gene triggers the biochemical pathways that cause biofilm production.
    - As of 2009, the four known genes that result in biofilm production are known as *wsp*, *Aws*, *Mws*, and *SWS*.
    - Once researchers find a gene will cause WS emergence when mutated, they delete that gene from the original SM strain, grow the *P.Flu* until WS emerges again, and then tries to find a new gene that has mutated to cause this phenotype change.

These details should be generally understandable at the high school level. Teachers can then decide how much of the bigger picture would be appropriate for their class.