Biofilm Formation by Purple Nonsulfur Bacteria

By Rachel Whitaker and Jennifer Hughes

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(Fig 1). The strains were initially grown in batch cultures either in anaerobic conditions under lights or in aerobic conditions on a platform shaker in the dark. After 24-36 hours, the cultures were turbid and had a (1:10) OD600 of 0.25-0.3. These cultures were then used for inoculating the flow chamber experiments described below.

Biofilm setup

The flow cells were made of plexiglass on three sides and covered glass microscope cover slip. The interior dimensions of the chamber were 1 x 3 x 40 mm. The chamber was then washed with 95% ETOH and flushed with media before inoculation. We connected the inflow of the flow cell to a media bottle with silicone tubing. Media (see Appendix) was pumped through the chambers by a peristaltic pump and into a waste bottle at a rate of 18 ml/hr. When possible, the media was pumped through a bubble trap immediately before the flow cells.

To inoculate the chambers, we incubated 1 ml of the batch culture cells for 30 minutes before initiating the flow. We covered the aerobic biofilms with foil to keep out any light. The anaerobic biofilms were setup in an anaerobic hood and placed under a 25W incandescent bulb approximately 30 cm above the flow chambers.

Experiments.

To test for biofilm formation and examine the influence of an aerobic versus anaerobic environment, we inoculated the wild type strain of *R. sphaeroides* into 2 flow chambers in aerobic conditions and 2 flow cells in anaerobic conditions. The aerobic strains were examined after 4 days, and the anaerobic strains were examined after 3 days.

To examine the influence of the chemotaxis operons on biofilm structure, we inoculated 2 flow cells with the wild type, delta 2, and delta 3 strains under anaerobic conditions. This experiment was run twice, each time for 3 days.

Microscopy

After we removed the flow chambers from the pump, we immediately stained them with a 1:5 dilution of SYBR green in PNSB media for 45 minutes. Following the staining, we added 10 ul of antifade solution diluted in a 1:1 solution of Glycerol and PBS. We took 3 to 4 pictures of each biofilm along the length of the chamber on a single channel confocal microscope using the FITC filter with a 10X objective. Approximately 50-60 slices were taken per image stack at 2.2 um intervals. Each image was 512 x 512 um. Higher resolution images were also taken with the 40X and 63X objectives.

Quantitative analyses

To quantify structural differences in the biofilms, we measured three statistics using the Zeiss LSM software. First, we measured the highest point of the biofilm in each picture. Second, using the orthogonal function in the image viewer, we counted the number of peaks above 50% of the tallest height along three transects. These transect ran parallel to the y-axis and crossed the x-axis at 128, 256, and 384 um (Fig 2). Finally, we measured the width of the peaks that crossed the 50% mark along the 256 um transect.

Results

Macroscopic observations

Under anaerobic conditions, noticeable purple biofilms of both *R. sphaeroides* and the natural isolate grew up in about 3 days. By eye, it appeared that the positioning of the chambers under the light influenced the thickness of the growth (Fig 3). In all of the experiments, the flow cells directly under the light had less growth than the flow cells flanking it. We reconfirmed this result by growing three replicates of wild type strain. The flow cell in the center, and directly under the light, again had less apparent growth. For this reason, we exclude data from the center position in the remaining analyses. Under aerobic conditions biofilm formation was less obvious to the naked eye, however, biofilms also formed under these conditions.

Microscopic observations

Biofilms were formed in all of the flow chambers. The *R.sphaeroides* wild type strain grew much faster anaerobically than aerobically. After three days, wild type *R.sphaeroides* formed evenly spaced aggregates (mushrooms) on the plexiglass (bottom) surface of the anaerobic flow cell chambers. The average maximum height of these peaks in a 10X frame was 74 um. Channels appeared to separate these peaks. After four days, the aerobic strains formed a monolayer on the bottom surface of the chamber. This layer was patterned, but lacked the well developed mushroom aggregates observed under anaerobic conditions (Fig 4).

There were no obvious macroscopic growth differences between *R. sphaeroides* and the natural isolate (JKH4); however, their biofilm structures were quite distinct. The natural isolate formed aggregates on the coverslip (top) surface and a continuous layer of growth on the plexiglass surface (Fig 5). At higher magnification, the aggregates appeared filamentous.

The presence of different chemotaxis operons affected biofilm structure (Fig 6). Under anaerobic growth conditions, the *R. sphaeroides* delta 3 mutant formed larger aggregates than either the wildtype or delta 2 (Fig 7). Delta 3 formed tall mushroom formations that were not evenly spaced across the surface. The peaks of delta 3 were also fewer and wider than the peaks of wild type or delta 2 (Fig 8 and 9). In contrast, delta 2 and the wild type were not distinguishable by these measures. Both formed small, relatively evenly spaced aggregates on the plexiglass surface.

Discussion

R. sphaeroides is an abundant and widely distributed species in the environment, and thus is an obvious candidate for initial tests of PNSB biofilms. Well-developed biofilms formed under all initial experimental conditions, indicating that biofilm growth may be preferable for growth in these organisms. The results of the natural isolate demonstrate that biofilm formation may be widespread among PNSB species and that there is taxonomic variation in PNSB biofilm structure.

In addition to variation among species in the group, microhabitat variation may greatly affect biofilm structure in the natural environment. We observed striking differences in the macroscopic and microscopic appearance of growth chambers places

only a few centimeters apart (Fig. 3). It appears that the primary difference between these chambers may be growth rate since chambers in the middle chamber had similar, but less developed microscopic structure. We hypothesize that the differences are due to variation in temperature, rather than light intensity. Given that only a few centimeters make a difference in a relatively constant laboratory environment, it would be interesting to examine how much of the variation in biofilm structure in the natural environment is due to microhabitat variation.

It is unclear whether the differences in the development of the aerobic and anaerobic biofilms are due to phototrophic versus chemotrophic growth conditions, growth rate, or effects on other aspects of the strain's physiology. It will be important to tease apart the influence of these variables before it is possible to draw conclusions about how phototrophy influences biofilm structure.

Other studies have shown that motility, physiology, quorum sensing, and cell adhesion mechanisms are important for biofilm formation (Davies et al 1998, Loo et al 2000, Davey and O'Toole, 2000). The importance of chemotaxis on biofilm formation is less resolved. Pratt and Kolter (1998) showed that motility but not chemotaxis affect biofilm formation in *Escherichia coli*. However, their observations only showed that chemotaxis mutants were able to form biofilms, but they did not investigate whether the structure of the biofilms was distinct from the wild type. In contrast, Parkins (2001) showed that the GacA response regulator of *Pseudomonas aeruginasa* was essential for biofilm formation.

From the experiments presented here, it appears that while chemotaxis mutants are able to form biofilms, the structure of the biofilm formed by mutants in operon 3 are distinct from the wild type and delta 2 mutants. Further investigation of chemotaxis mutants using confocal microscopy are necessary before broader conclusions can be drawn about the importance of chemotaxis to biofilm structure. We hypothesize that chemotaxis operon 3 is important for *R. sphaeroides* to sense gradients created by the growth of other cells. The loss of this sensory function may prevent the cells from evenly distributing themselves along the surface to maximize access to resources and results instead in large towering aggregates. It would be interesting to observe the long-term survival of these mutants in biofilms and determine whether this function of chemotaxis contributes to the persistence of the community.

Addendum

On the side of the PNS experiment, we tested two addition setups. First, we wanted to see if one could study the structure of "biofilms" on glass coverslips immersed in batch cultures. Although these experiments did not include any flow or stress across the surface, this technique may be potentially useful to examine whether the interaction of species on surfaces differs in different environments. We placed sterile coverslips in sulfate reducing bacteria enrichment culture and in cultures of *Methanospirillum hungatei*. We took pictures of these slides by creating "mini-aquaria" on a glass microscope slide. A metal washer was glued onto a glass slide, antifade solution was place in the hole of the washer, and the stained coverslip was glued to the top of the washer. Both the mixed SRB cultures and the methanogens attached and formed structures on the coverslips.

We also setup a flow cell chamber in anaerobic conditions for the *M. hungatei* in an anaerobic hood. A concern was providing enough H_2 for the strain. We filled a rubber innertube with H_2/CO_2 and attached that to the air intake of the media bottle. This method appeared to work, as after only 4 days, cells had attached to the surfaces of the flow chamber.

Acknowledgements

We thank the faculty, TAs, and fellow students of the 2001 MBL Microbial Diversity course for constant support and guidance during this project. We would especially thank Dale Pelletier for help with the anaerobic hood and anaerobic media preparation, Alfred Spormann for orientation to the confocal microscope, Kirk Harris for providing his strain for our use and Claudia Lupp for preparing the SYBR green staining reagents. We also thank Judy Armitage and George Wadhams for the use of their strains and much of the inspiration for this project.

References

Dang, Hongyue and Lovell, Charles. 2000 "Bacterial Primary Colonization and Early Succession on Surfaces in Marine Waters as Determined by Amplified rRNA Gene Restriction Analysis and Sequence Analysis of 16S rRNA Genes." *Applied and Environmental Microbiology* 66:467-475.

Davey, Mary Ellen and George O'Toole. 2000. "Microbial Biofilms: From ecology to molecular genetics." *Microbiology and Molecular Biology Reviews* 64:847-867.

Davies, D.G., M.R. Parsek, J.P. Pearson, B.H. Iglewski, J.W. Costerton, and E.P. Greenberg. 1998. "The involvement of cell-to-cell signals in the development of a bacterial biofilm." *Science* 280:295-298.

Loo, CY, DA Corliss and W. Ganeshkumar. 2000. "*Streptococcus gordonii* biofilm formation: Identification of genes that code for biofilm phenotypes." *Journal of Bacteriology* 182:1374-1382.

Parkins, Michael, Howard Ceri and Douglas Storey. 2001. "*Pseudomonas aeriginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation." *Molecular Microbiology* 40:1215-1226

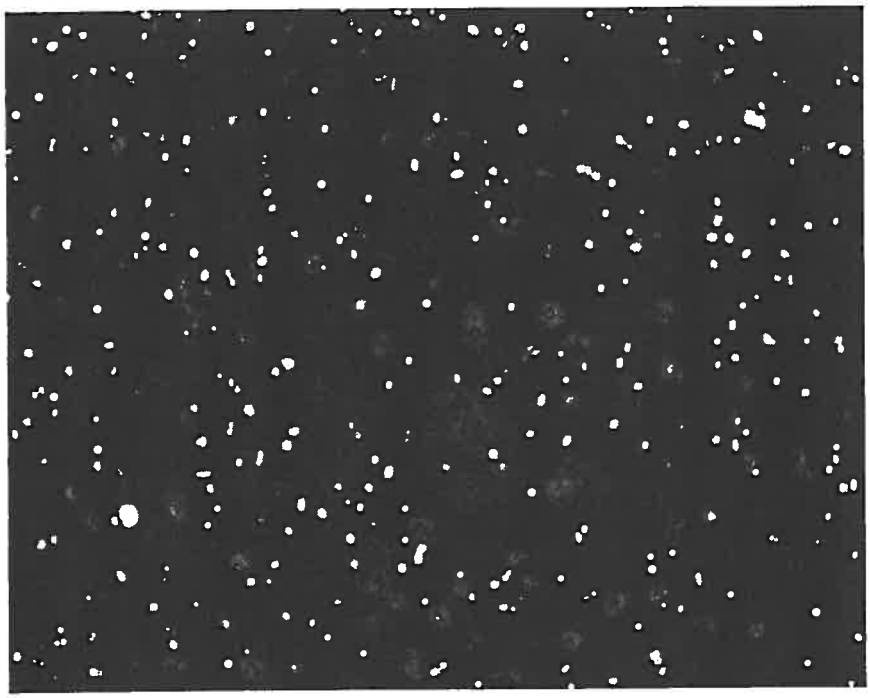
Pratt, L.A. and R. Kolter 1998. "Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili." *Molecular Microbiology* 30:285-293.

Shah, D.S.H., S.L. Porter, A.C. Martin, P.A. Hamblin, and J.P Armitage. 2000. "Fine tuning bacterial chemotaxis: analysis of *Rhodobacter sphaeroides* behaviour under aerobic and anaerobic conditions by mutation of the major chemotaxis operons and CheY genes." *EMBO Journal* 19:4601-13.

Wadhams, G. in prep. "The study of chemotaxis receptor proteins in *Rhodobacter sphaeroides*. PhD thesis, Oxford University.

Figure 1: A 40X dark field image of *Rhodobacter sphaeroides* (a) and the natural isolate (b) from Sippewisset salt marsh (JKH4).

(a)



(b)

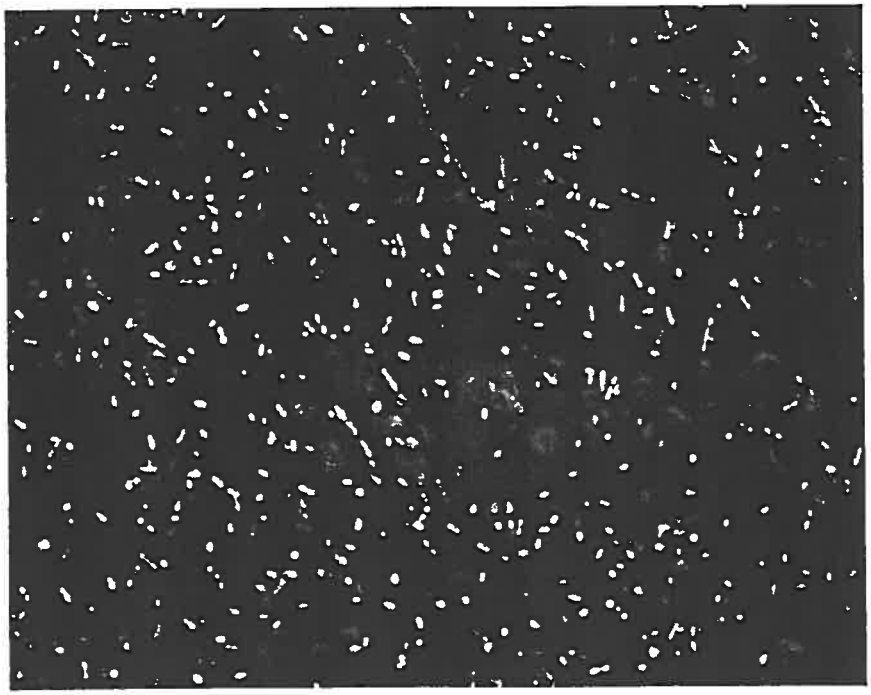


Figure 2: A snapshot of a delta 3 biofilm. The quantitative measures discussed in the text were obtained from the pictures in the Zeiss LSM software. In this particular figure, 3 peaks are above the 50% height line on the middle transect parallel to the y-axis. The widths of the aggregates were determined by measuring the width of the peak above which crosses the 50% height line.

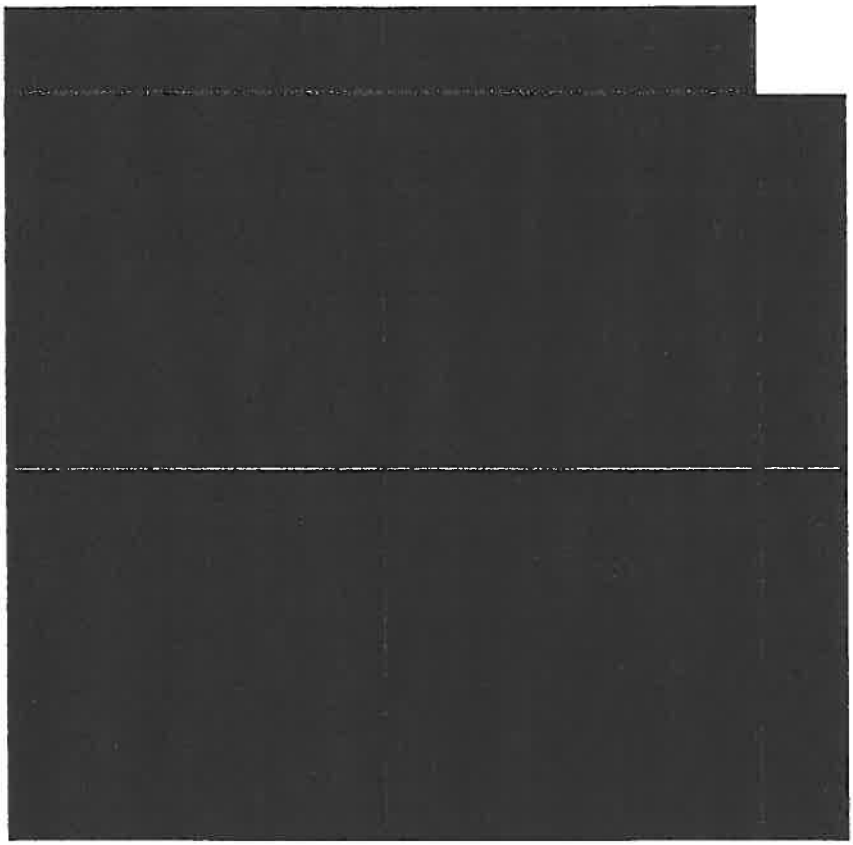


Figure 3: A picture of 3 anaerobic flow chambers. The middle chamber, which was directly under the light bulb, has less growth than the upper and lower chambers.

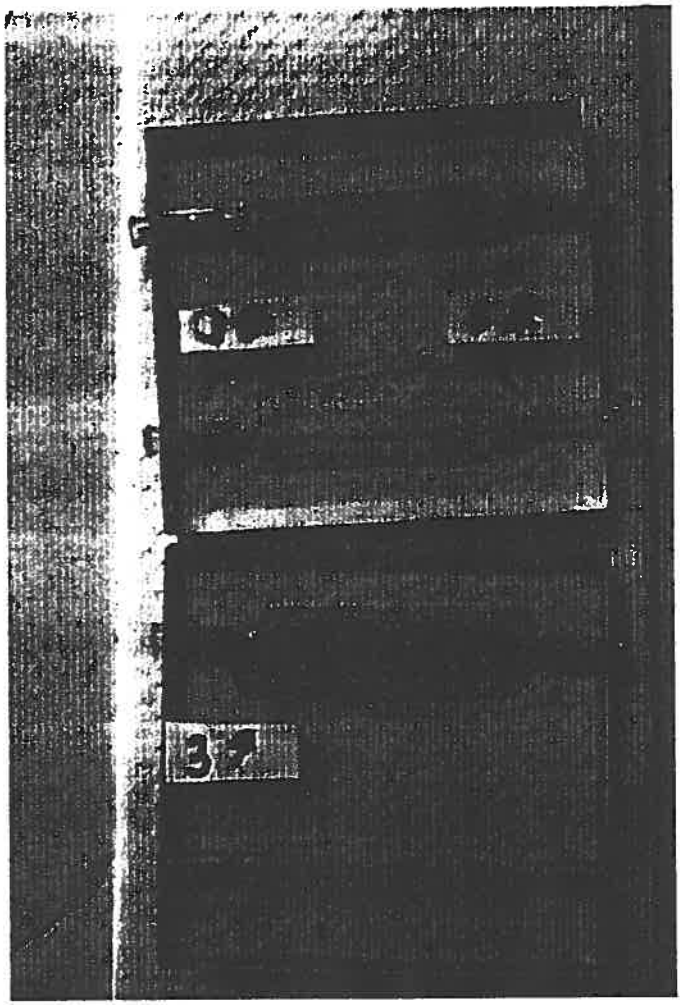
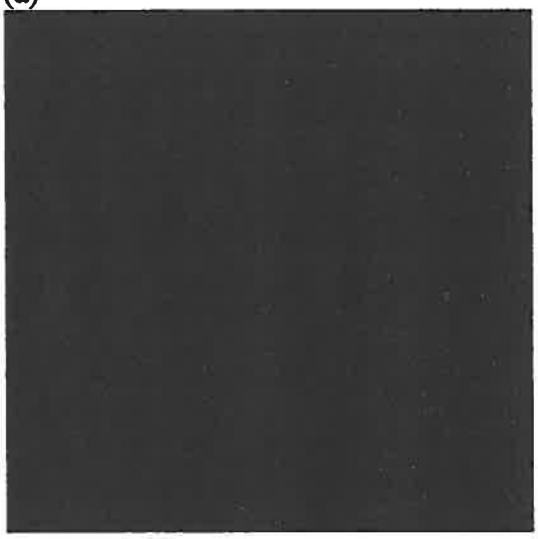


Figure 4: *R. sphaeroides* biofilms grown in aerobic (a) and anaerobic (b) conditions. Magnification is 10X.

(a)



(b)

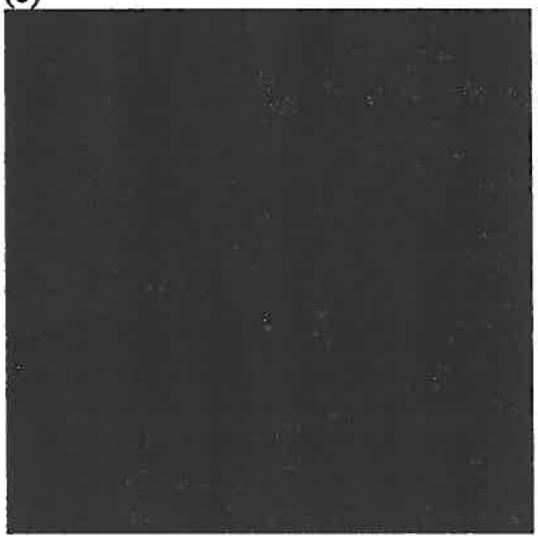
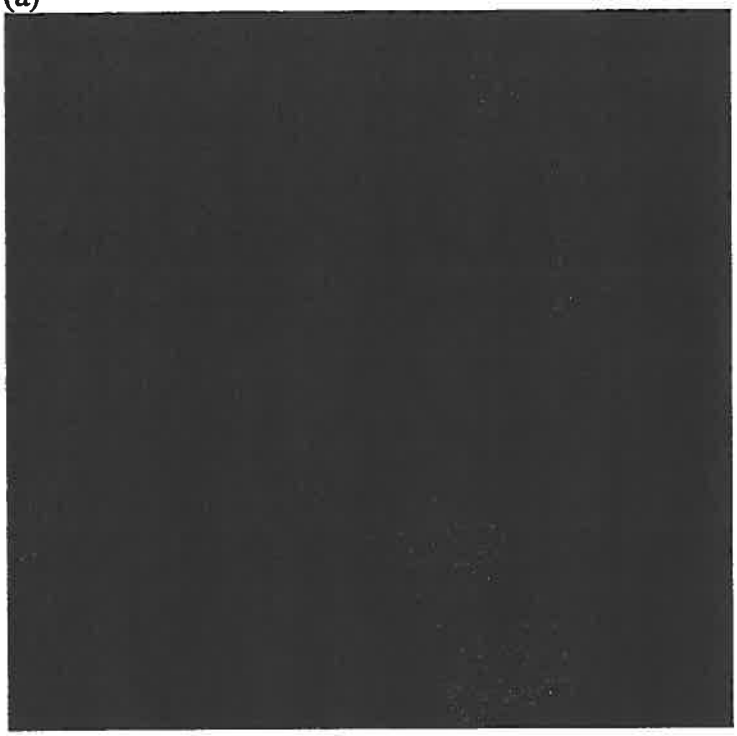


Figure 5: Biofilms of the natural PSNB isolate (JKH4) on the (a) coverslip surface and (b) plexiglass surface.

(a)



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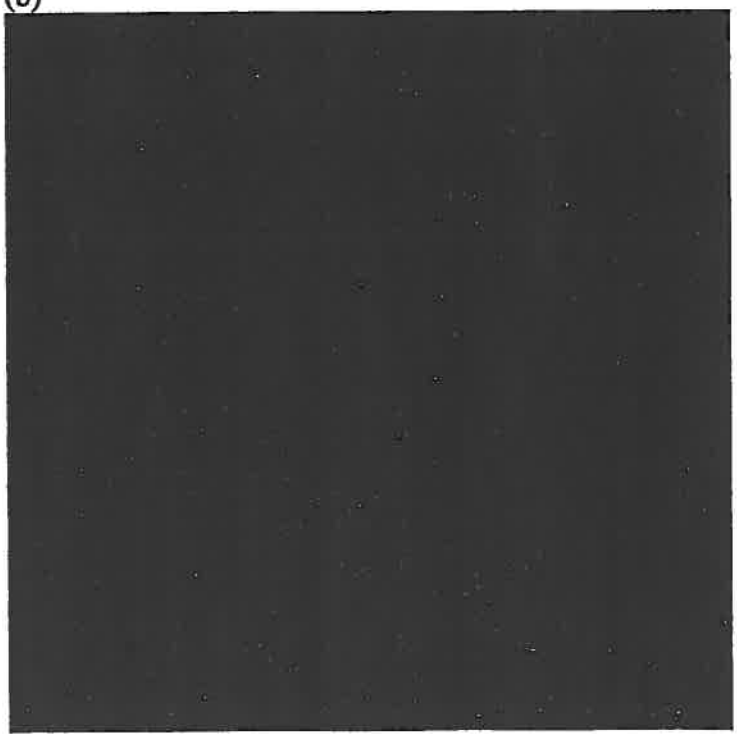
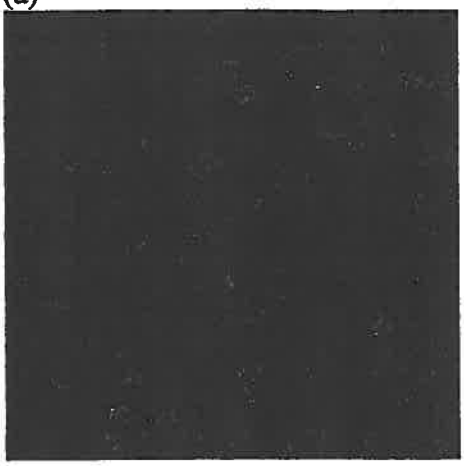


Figure 6: Typical pictures of *R. sphaeroides* biofilms at 10X. The strains are (a) wild type, (b) delta 2, and (c) delta 3.

(a)



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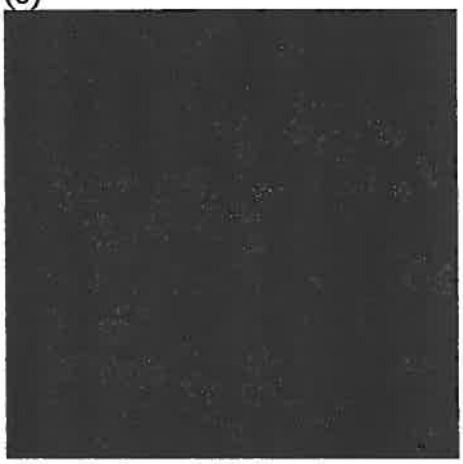


(c)

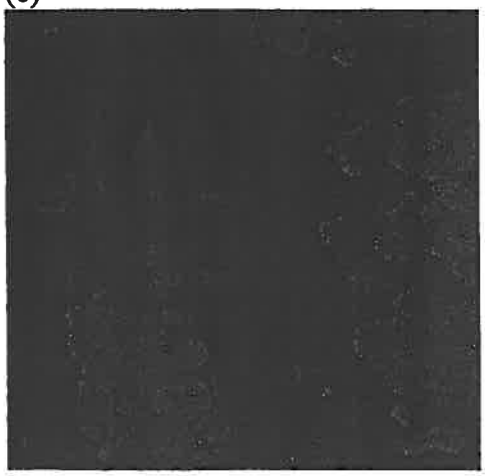


Figure 7: The average maximum height of biofilms across 3 or 4 places in the flow chamber. Error bars are standard deviation.

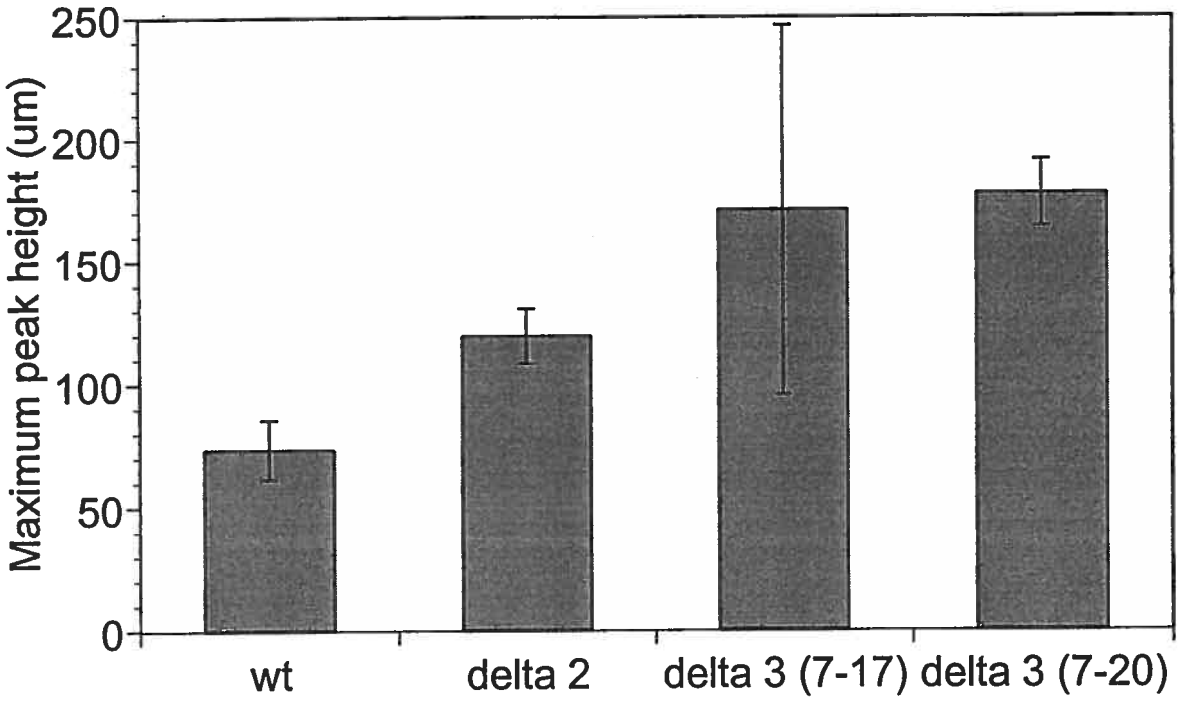


Figure 8: The average number of aggregates across taller than 50% the maximum height in the picture across three linear transects. Error bars are standard deviation.

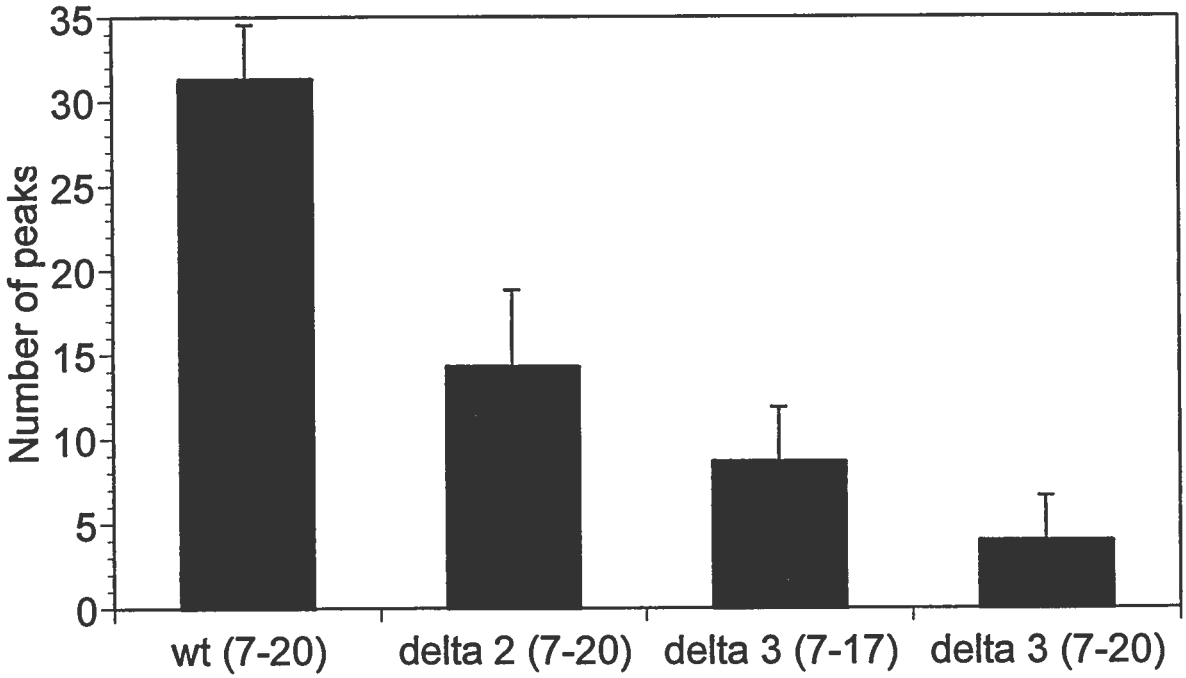
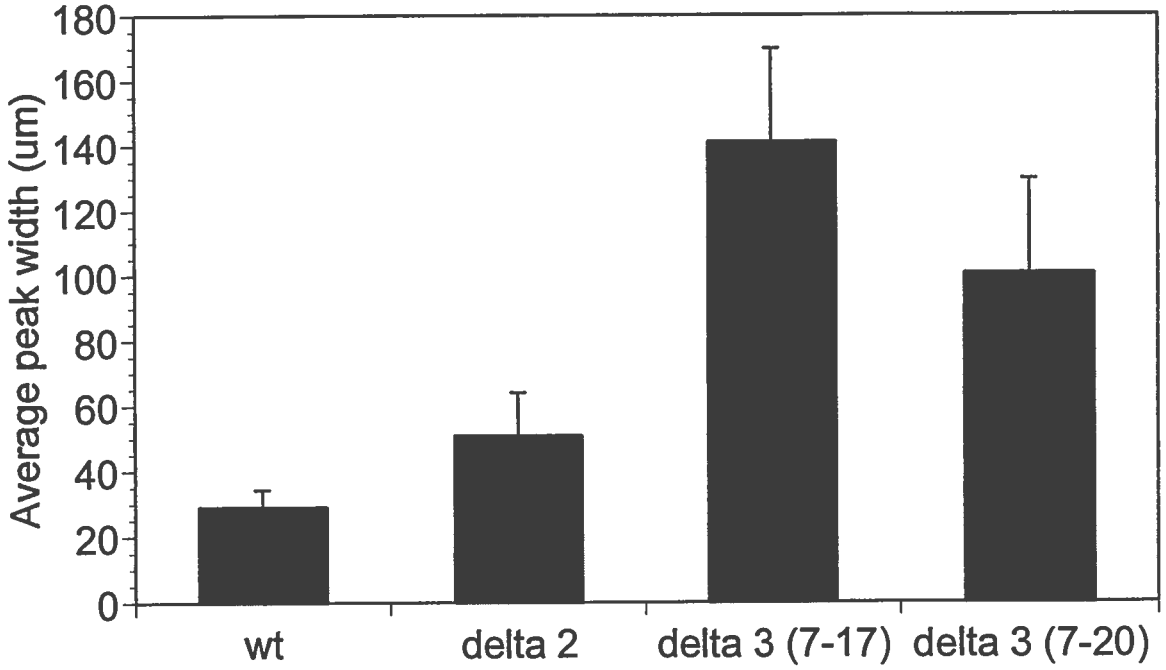


Figure 9: The average width of the aggregates along one transect. Error bars are standard deviation.



Appendix

The purple non-sulfur media is the same for anaerobic and aerobic environments.

Per liter:

NaCl	1 g
$MgCl_2 .6H_2O$	0.4 g
$CaCl_2 .2H_2O$	0.1 g
KH_2PO_4	0.2 g
KCl	0.5 g
Succinic acid	1.18 g
Yeast extract	0.5 g
HEPES	2.38 g
NH_4Cl	0.25 g

After autoclaving add:

SL12	1 ml
12 Vitamin solution (1000x)	1 ml
B_{12} solution (1000x)	1 ml

The anaerobic media was autoclaved and flushed with N_2/CO_2 while it cooled and while the vitamins and trace metals were added.