Adjuvant Pluronic F68 is compatible with a plant root-colonizing probiotic, Pseudomonas chlororaphis O6

Amanda R Streeter , Anthony Cartwright , Mohammad Zargaran , Anagha Wankhade , Anne J. Anderson * , David W Britt *

Posted Date: 22 November 2023

doi: 10.20944/preprints202311.1416.v1

Keywords: Colonization; Cryopreservation; Fluorescent labelling; Plant probiotic; Pluronic; Surfactant

Article

Adjuvant Pluronic F68 is Compatible with a Plant Root-Colonizing Probiotic, *Pseudomonas chlororaphis* O6

Amanda R. Streeter [1], Anthony Cartright [2], Mohammad Zargaran [2], Anagha Wankade [2], Anne J. Anderson [2]* and David W. Britt [2,]*

[1] Department of Biological Sciences and Department of Chemistry, State University of New York at Oswego, Oswego, NY 13126. USA
[2] Department of Biological Engineering, Utah State University, Logan, UT 84322. USA
* Correspondence: annejanderson33@gmail.com; David.Britt@usu.edu

Abstract: Plant probiotic bacteria are being increasingly used to maximize productivity and quality of field crops. *Pseudomonas chlororaphis* O6 (*Pc*O6) is a plant root colonizer with probiotic activities that produces metabolites such as phenazines functional in plant protection. This work reports responses of *Pc*O6 to a non-ionic, triblock copolymer surfactant, Pluronic F68. This Pluronic exhibits membrane "healing" activity and improves cryopreservation recovery in eukaryotic cells. The product is FDA approved and is applied as an adjuvant in formulations used in agriculture, medicine, and biotechnology. Growth of *Pc*O6 on Luria broth at 25°C was unhindered by 0.1 and 1.0 g/L F68, reduced at 10 g/L, with more inhibition at 100 g/L F68; micelle formation could account for inhibited growth at higher doses. Phenazine production was not changed by F68, whereas an F68 dose-dependent, surfactant-induced spread of bacterial colonization on 0.5% agar was observed. Exposure of cells to fluorescently labelled F68 resulted in intense fluorescence, stable to washing, showing a direct association of the Pluronic with the bacterium. However, neither protection nor harm was found for *PcO6* cells undergoing repetitive freeze (-20 °C)/thaw cycles with 0.1% or 1% F68. These findings suggest F68 could be compatible for use in agricultural formulations with little effect on probiotics such as *Pc*O6.

Keywords: colonization; cryopreservation; fluorescent labelling; plant probiotic; pluronic; surfactant

1. Introduction

Polymers are present in numerous agroformulations as wetting agents and adjuvants to improve spreading and bioactivity of active ingredients [1]. These surface-active polymer adjuvants may have direct benefits to plant health, particularly when the crop is under stress. Climate change is imposing a greater frequency of nonpredictable drought, temperature extremes, and increased soil salinity on crops. Novel methods to protect plants against abiotic stresses are needed [2-3].

The use of Pluronic F68 may provide adjuvant functionality while introducing membrane-protective activity. Pluronic F68 protects eukaryotic cell membranes from shear stress and bubble-facilitated ruptures in bioreactors and inserts into damaged cell membranes to "heal" permeabilized bilayer limiting electrolyte loss from the cells [4-9]. Pluronic 68 is an 8,400 D triblock copolymer which is 80% hydrophilic and a molecular structure of HO-$[PEO]_{76}$-$[PPO]_{29}$-$[PEO]_{76}$-OH, where PEO is polyethylene oxide and PPO is polypropylene oxide (Figure 1). Of the numerous Pluronics that differ in MW and PEO/PPO ratios, F68 already has FDA approval for human use [10]. Field studies with F68 demonstrate enhanced shoot regeneration in some plants and cryoprotection of frozen plant tissues in others [11-16]. Initial studies from our group confirm that F68 at concentrations up to 10 g/L has no phytotoxicity on wheat seedlings [17].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 22 November 2023 doi:10.20944/preprints202311.1416.v1

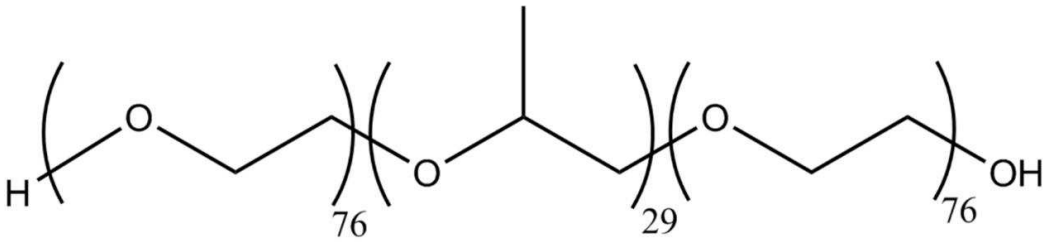

Figure 1. The structure of F68.

Any agricultural formulation application should not harm the association of plants with beneficial microbiomes. The plant's microbiome is essential for plant's welfare, including greater tolerance to both abiotic and biotic stress [18-19]. The Gram-negative strain, *Pseudomonas chlororaphis* O6 (*Pc*O6) isolated from field-grown wheat roots has probiotic effects promoting protection from pathogenic challenge and stresses due to drought and salinity [20-21]. The ability of *Pc*O6 to directly inhibit pathogen growth and induce systemic resistance to pathogens is in part due to production of the metabolites, the phenazines. Phenazines are antimicrobial and are one of the triggers of induced plant resistance [22-24]. Pluronics exhibit a range of MWs and PEO/PPO ratios (Supplemental Table 1). Previous work found that certain Pluronics enhanced, and others decreased phenazine production in isolate *Pc*O6, although they all promoted pseudomonad swarming motility, consistent with their surfactant activity [25]. Phenazines and other surfactants interact positively in the inhibition of pythium species as pathogens [26]. Indeed, a nonionic detergent caused lysis of plant pathogenic *Pythium* and *Phytophthora* species zoospores but were without activity on their mycelia [27]. This background information stimulated our studies directed at understanding the potential interactions between the probiotic pseudomonad, *Pc*O6, and F68.

The studies reported in this paper examined effects on growth and production of phenazines by *Pc*O6 to examine for inhibition or stimulation. The ability of the polymer to alter swarming motility of the bacterium on the surface of 0.5% nutrient agar was studied. F68 was fluorescently labelled to determine whether F68 was associated with bacterial cells and whether labelling was sensitive to washing. Potential cryoprotectant effects of F68 for the prokaryote were determined by assessment of culturability after freeze/thaw cycles at -20 °C in cell suspensions amended with F68.

2. Materials and Methods

2.1. Effects of F68 on growth of PcO6 in liquid culture

Stocks of *Pc*O6 were maintained frozen at -80 °C in 15% sterile glycerol. Stocks were thawed and inoculated onto Lysogeny Broth (LB) or minimal medium (MM) 2% agar plates with growth at 22 C for 30 h. To determine whether F68 showed dose-dependent toxicity for *Pc*O6, cultures were prepared with a starting inoculum of 10^5 colony forming units per mL (CFU/mL) in LB broth containing defined doses of F68 (0 to 100 g/L) with shaking at 125 rpm at 22 °C. Aliquots were withdrawn from the culture and optical density measured at 600 nm using a BioTek Synergy HTX Multimode Reader (Agilent Technologies; Santa Clara, CA) as a measure of cell density. Images of the color of the cultured cells were taken at late stationary phase to record the production of the orange pigmented phenazines typical of this strain when grown on LB medium [36].

To show whether F68 was utilized as a carbon source by *Pc*O6, cultures were established in a defined minimal medium (MM) containing phosphate salts as a pH 7.1 buffer (10.5 g/L dibasic; 4.5 g/L monobasic K phosphates), ammonium ions as a N source from ammonium sulfate (0.125 g/L), and magnesium sulfate heptahydrate (0.125 g/L) as both a S source and Mg ion source. This medium was used without C sources (the no-C control) or with the addition of F68 (1.72 g/L). Additional comparative treatments were included with amendments of an osmolyte glycine betaine (GB) (1.88 g/L), or the mix of 0.86 g/L F68 with 0.94 g/L GB. Three flasks for each treatment were prepared.

 doi:10.20944/preprints202311.1416.v1

Growth was measured after shaking at 125 rpm for 120 h at 22 °C when cultures were into late stationary phase. Images of the cultures were taken to record whether the phenazine pigments were produced. Serial dilutions were prepared in sterile distilled water and aliquots plated onto LB agar. Colonies were counted after 48 h and CFU/ml for the original cultures calculated.

2.2. F68 Effects on PcO6 swarming mobility

The effect of F68 as a surfactant on the swarming motility of *Pc*O6 cells was determined on MM containing 20 g sucrose/L as the C source and 0.5% agar [28]. The agar was amended with defined concentrations of F68 (0, 0.0001 g/L, 0.001 g/L, 0.01 g/L, 0.1 g/L, 1 g/L, and 10 g/L). Above 10 g/L, the F68 formed micelles and the agar would not set. The agar plate was inoculated in the center with 2 µL applications of *Pc*O6 cells at 104 CFU/mL. The *Pc*O6 cells were previously cultured for 48 h on unamended MM broth. The diameters of the colonies were measured after 48 h incubation at 22 °C and their morphologies were noted in images. All chemicals were from Fischer Scientific.

2.3. Labelling of PcO6 cells with fF68

Fluorescein-labelled F68 (fF68) was prepared by the method described in Cartwright et al (2022). Late logarithmic phase *Pc*O6 cells grown in MM broth were exposed in 1 ml aliquots (1-2 x10^8 CFU/mL) to 0.1 mM fF68 or 0.1 mM unlabeled F68 as a control for 30 min. Another control used cells without any treatment. The cells were observed for fluorescence directly or after washing. For washing, cells were pelleted by centrifugation at 10,000 g for 10 minutes and suspended twice in 1 ml MM before final suspension in 1 ml MM. The fluorescence of the cell was observed using a Nikon TE-2000 microscope with a FITC filter set for excitation at 488 nm and emission at 516 nm. The integration time was 1 sec. Cells from three replicates for each treatment were observed with five fields of view for each sample.

2.4. Cryoprotection by F68 for PcO6 cell suspensions

Cells of *Pc*O6, grown to the late logarithmic phase in a 22 °C incubator with shaking at 125 rpm, were pelleted by centrifugation at 10,000 g for 10 minutes in four sterile 50 mL tubes. The cell pellet was resuspended in sterile double distilled water before centrifugation and removal of the wash solutions. The cells in each tube were suspended in either sterile 15% glycerol, 0.1% w/v F68, 1% w/v F68, or double distilled water. The mixtures were shaken to generate homogenous suspensions before 1 ml aliquots were transferred to sterile Eppendorf tubes. The initial concentrations were 3.6 ± 0.5 x 10^8 culturable cells per ml, based on culturability on LB agar medium. All tubes were placed into a temperature-cycling freezer that cycled between -20°C for 48 hours and 20°C for 15 minutes. At defined times one tube for each of the four suspensions was removed and allowed to thaw at room temperature. These samples were serially diluted in sterile water and samples plated onto LB solidified with 2% agar plates, in triplicate. Colonies were counted after 2 d incubation to determine culturable cell densities in the samples.

3. Results

3.1. Growth of PcO6 in liquid cultures, and its phenazine production, are not altered by the presence of F68

On rich LB medium, *Pc*O6 growth was not altered with concentrations of F68 at 0.1 and 1 g/L from the growth rate without F68 (Figure 2 A). Growth rate was decreased by 10 g/L F68 and more extensively with 100 g/L (Figure 2 A). In the LB medium, the presence of F68 did not alter the potential of *Pc*O6 to produce orange - colored phenazines (Figure 2 B).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 22 November 2023 doi:10.20944/preprints202311.1416.v1

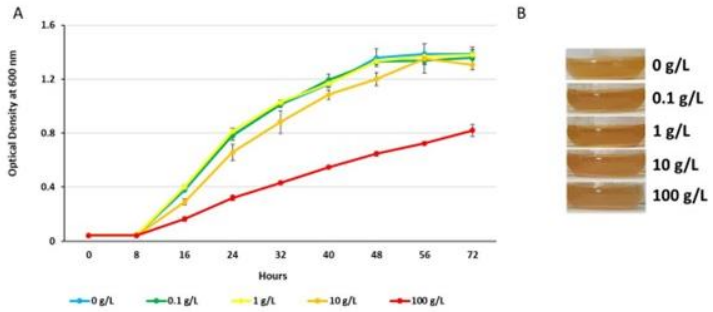

Figure 2. Effect of F68 at defined doses (0 to 100 g/L) on growth rate of *Pc*O6 in shake culture on rich LB medium at 22 °C and 125 rpm. A) The OD 600 nm for the cultures with and without F68 amendments at defined times of incubation. The means of the readings and standard errors for three replicates for each treatment are shown. Cell densities were statistically different (p=0.050 between the cultures without F68 and those with 100 g/L F68 at all time points from 16 h of culture and up to 56 h of culture for the 10 g/L amendments. B) Orange coloration of late stationary – phase 72 h cultures, denoting phenazine formation, is independent of F68 concentration.

When F68 was added to a defined medium lacking a C source, no growth of *Pc*O6 was observed (Figure 3). The CFU/ml at 120 h after inoculation for the medium with only F68 as C source was equal to that of medium inoculated with *Pc*O6 but lacking any C source. This assessment shows that the F68 was not toxic to the pseudomonad cells. Cell density increases were observed when an osmoprotectant, glycine betaine (GB) [29-30], was added into the defined medium, and co-addition of F68 to the GB cultures did not affect the final CFU/ml values. No additions to this defined medium caused the *Pc*O6 cells to generate orange - colored phenazines.

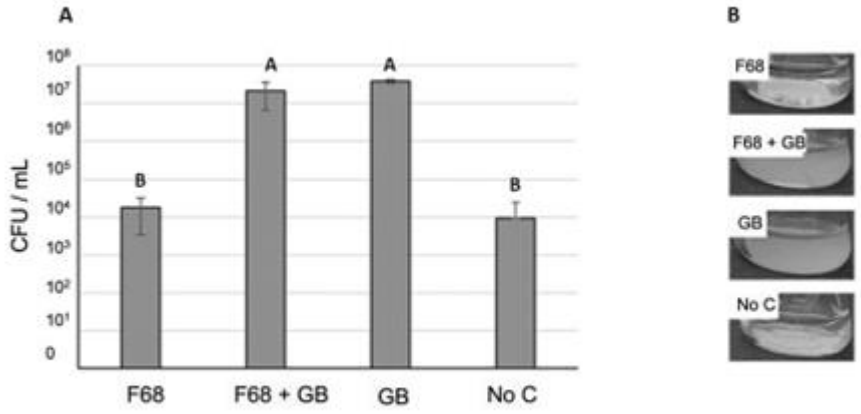

Figure 3. Use of F68 (1.72 g/L) or GB (1.88 g/L) as sole carbon sources for growth of *Pc*O6 in a defined minimal medium. Medium containing no carbon sources (No C) also was inoculated. The inoculum was added at the level of 104 CFU/mL. (A): CFU /ml for 120 h cultures as determined by serial dilutions of the shaken suspensions and growth on LB agar. (B) Images of the 120 h cultures. The data are means and standard errors of CFU/ml with three replicates/treatment. Letters above the bars indicate significant differences between treatments as determined by ANOVA with Tukey's HSD adjustment, at $p < 0.05$.

3.2. F68 enhances swarming behavior of PcO6

A positive effect of F68 was observed on swarming for *Pc*O6. Inocula (20 µl aliquots) added to MM 0.5 % agar plates with sucrose (20 g/L) as the C source grew in 48 h to colonies of approximately 0.8 cm diameter. The diameter of the colony and its tight edged morphology was not affected by

 doi:10.20944/preprints202311.1416.v1

addition of 0.0001 g/L F68 (Figure 4 A). With 0.1 g/L F68 the colony diameter increased (Figure 4 B) but retained a tight-edge morphology so that the colony was still circular in shape. However, with 1 g/L F68 the colony diameter increased, and a dendritic growth morphology was observed (Figure 4 C). This change in morphology occurs after the transition point in the relationship between surfactant activity and concentration of F68 (Supplemental Figure 1). At concentrations of F68 lower than 0.001% there is a rapid change in surface activity but between 0.01% and 0.1% the change in surface activity with concentration levels (Supplemental Figure 1).

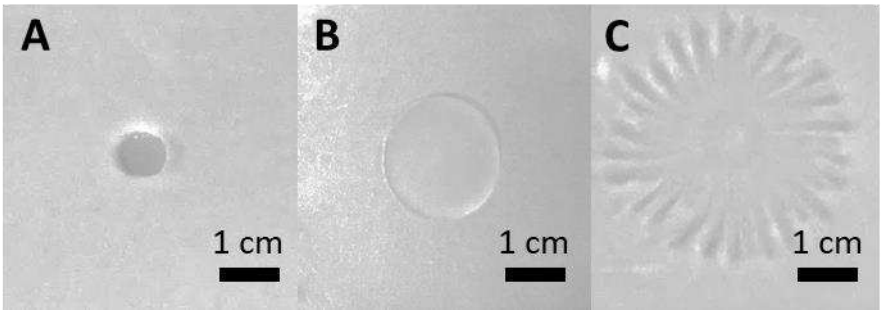

Figure 4. F68 increases colony spread and changes in colony morphology on minimal medium solidified with 0.5% agar to allow swarming. The medium contained sucrose as the carbon source. The images shown are typical of six different replicates for each concentration. A 0.0001 g/L, B 0.1 g/L and C 1 g/L F68.

3.3. PcO6 cells become fluorescent when exposed to fF68

To explore further how F68 influences *Pc*O6 cells, the Pluronic was labelled with fluorescein, producing a fluorescent complex, fF68. Exposure of *Pc*O6, grown to early stationary phase in rich LB in shake culture, to fF68 for 3 h resulted in cells with outlines that were brightly fluorescent as shown in Figure 5. Two control treatments were examined; first the cells alone did not show fluorescence (Supplemental Figure 2) and second of *Pc*O6 cells with nonlabelled F68 for 3 h (Supplemental Figure 2) did not induce fluorescence. The fluorescence of *Pc*O6 caused by treatment with fF68 was stable to wash in minimal medium.

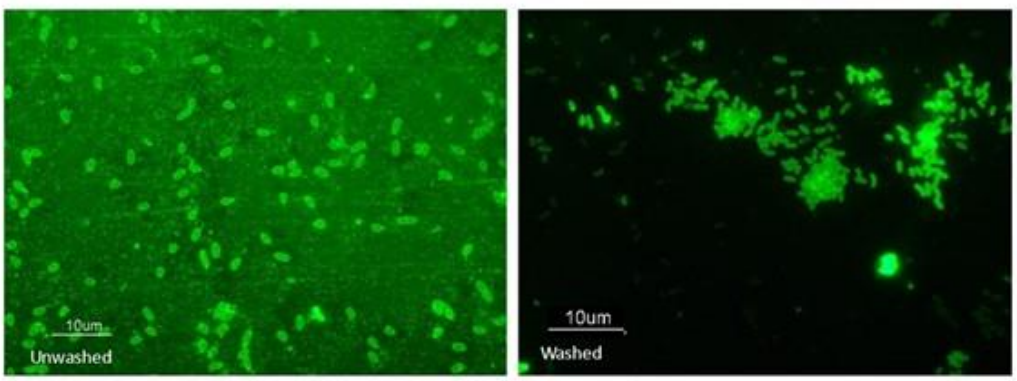

Figure 5. Exposure of *Pc*O6 cells to fF68 results in fluorescent labelling. Fluorescence of *Pc*O6 cells exposed to fF68 for 3 h without washing (A) or after washing (B) with noninoculated minimal medium. The size bar is 10 μm. The images were taken using a FITC filter with 488 nm and emission at 516 nm with an integration time of 1 sec. Images are typical of five fields of view.

3.4. Suspension in F68 does not provide cryoprotection to PcO6

Suspensions of *Pc*O6 cells, with starting CFU/mL of 3.2 ± 0.5 x10^8, showed decreased culturability with the increasing number of freeze thaw cycles from the storage temperature of -20 °C (Figure. 6). Loss in culturability was least for the cell suspensions in glycerol. The suspensions in sterile water

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 22 November 2023 doi:10.20944/preprints202311.1416.v1

showed a greater loss in culturability, losses that were not improved by suspensions in 0.1 or 1% F68 when sampled after one, two and three thaw periods. Suspension in 1% F68 gave statistically higher (p=0.05) culturable cells for the first two cycles but not the third cycle. By the third freeze thaw cycle, the survival of culturable cells was statistically less in 0.1% F68 than in the suspensions in water and 1% F68. During thaw after this third cycle period, the glycerol suspensions thawed faster than samples in water or F68 as shown in Figure 7 where ice is still visible for the suspensions in F68 and water but not for the glycerol suspension. Additionally, a pellet was formed with the glycerol suspension (Figure. 7) whereas thawing of the water- and F68- samples gave optically dense suspensions typical of suspended cells. This pellet in the glycerol sample, when resuspended before the plating of the sample, produced the suspensions with the culturable cell number shown in Figure 7. In some of the published cryoprotection studies (Supplemental Table 2), F68 was active when added during the thawing process [15]. However, when 1% F68 was added to suspensions of cells frozen at -20 °C and without any previous thaw in either 15% glycerol or water, no consistent effect, either protective or detrimental, was observed in thrice repeated studies (data not shown).

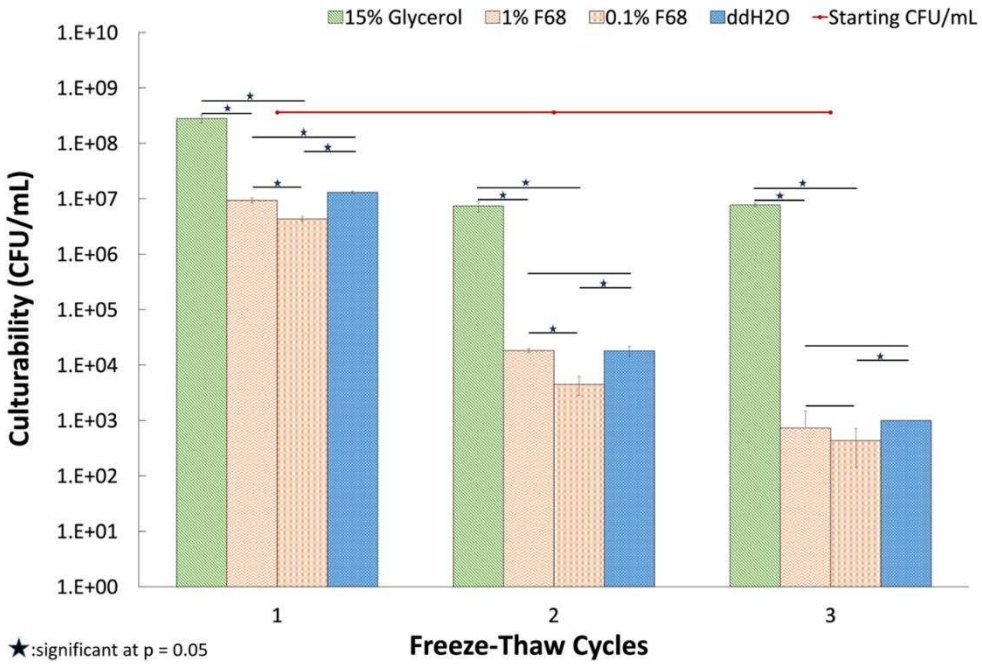

Figure 6. Effect of freeze /thaw cycles on culturability of *Pc*O6. The data show the mean of culturable cells/ ml, obtained from samples completely thawed for 12 min at 22 °C, on LB with 2% agar based on samples of three different tubes for each cycle and treatment. Standard errors are shown, and the asterisks denote a significant difference between the designated treatments at p=0.05 determined using the student's t-test.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 22 November 2023 doi:10.20944/preprints202311.1416.v1

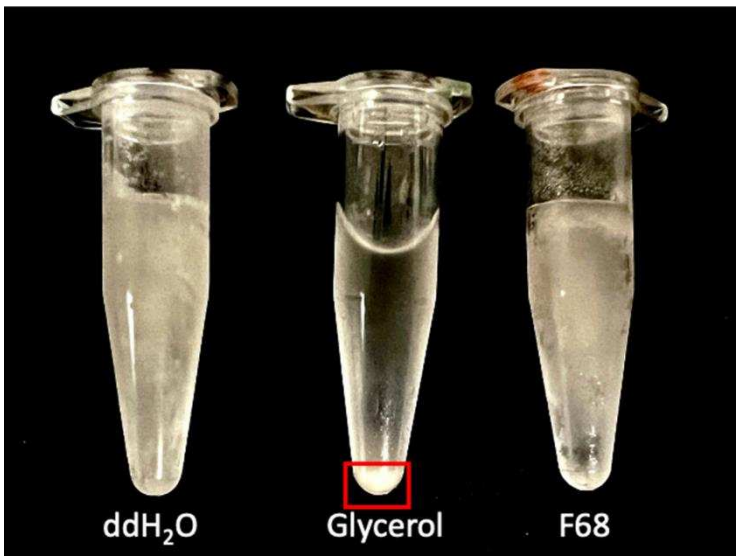

Figure 7. Appearance of tubes of *Pc*O6 cells suspended in water, glycerol and F68 after three freeze (-20 °C) thaw cycles. Thawing proceeded at room temperature (22 °C) for 7 minutes. This image showed the faster thaw for the glycerol suspensions versus the cells suspended in water or water amended with 1% F68. The red box highlights the pellet in the thawed glycerol sample. Ice crystals are apparent at the same time of thawing for *Pc*O6 cells suspended in sterile water or 1% F68 when transferred into the freezer.

4. Discussion

F68 was compatible with the growth of the plant probiotic *Pc*O6 on both rich and minimal defined media confirming its classification as an FDA-approved adjuvant. These findings agreed with a previous publication where cell densities of *Pc*O6 grown in LB or MM liquid shake cultures were not affected by 0.5% (v/v) Pluronics P104, P108, P123 or the reverse Pluronic 25R2 [25]. Each of these Pluronics have different proportions of PPE and PPO as shown in Supplemental Table 1 although all are surfactant active.

F68 also did not alter the production of phenazines from *Pc*O6 unlike the increases during growth on LB medium with 0.5% amendments seen with 25R2 and the decreases with P104 and P123; the null effect of F68 paralleled that of F108 [25]. These responses suggest that the presence of F68 in formulations would not alter the production of phenazines, that are important for biocontrol-active pseudomonads [23]. Like all the Pluronics examined in previous studies by Housley et al (2009), the surface activity of F68 promoted spreading of colonies of *Pc*O6 on a soft agar surface. Such activity may enhance colonization of the root surface, a factor essential for plant protection by biocontrol pseudomonads [31]. The finding that *Pc*O6 does not utilize F68 as a C source for growth would add to its longevity in the rhizosphere if it were present in applied agricultural formulations.

*Pc*O6 cells appeared to sorb a coating of F68, as determined by fluorescence imaging of cells exposed to fF68. This label was stable by washing but currently it is not resolved whether any of the Pluronic is internalized by the bacterial cells. Labelling of organelles of fF68 within root cells has been observed [17]. It is possible internalization is restricted by the complexity of the bacterial surface layers. For the pseudomonad there exists an extracellular polymeric layer, the outer lipopolysaccharide layer that surrounds the periplasmic space and then the plasma membrane. The bacterial plasma membrane is full of active components, including porins, transporters, the housing complexes and signal transduction sites for flagella, environmental sensors, as well as all the structures of the electron transport chain. It is possible this layered complexity in the bacterial membranes limits membrane healing effects proposed for F68 action with eukaryotic membranes [7-9].

We were surprised by the finding that F68 did not act as a cryoprotectant for the *Pc*O6 cells. As explained above, this result may be due to the complexity of the multiple layers that enclose the cytoplasm of the Gram-negative cell preventing healing of ice-crystal damaged membranes. It is

unlikely that these results are due to the concentrations of F68 examined being too low; the values were within the range found in published literature (Supplemental Table 2). Indeed, 1% (w/v) F68 contains 7 x 10^{17} molecules F68/ml. Consequently 2 x 10^{9} molecules of F68 would be available for each *Pc*O6 cell in our studies using suspensions at 3.2 x 10^{8} CFU/ml. Further calculations, based on the size of *Pc*O6 cells and an F68 molecule, propose that 4 x 10^{3} molecules of F68 would be needed to fully cover a single cell of *Pc*O6. The concentration of F68 in the 1% treatments thus exceeds the amount required for complete *Pc*O6 cell coverage; these calculations support the observation of overall cell fluorescence after exposure to fF68. Supplemental Table 2 provides several examples where cryoprotection for eukaryotic cells is observed. Agricultural formulations with a cryogenic-protective role would be valuable under the pressure of climate instability where field soil temperatures fluctuate into freezing conditions. For instance, In Cache Valley Utah USA, where colonized field-grown, winter wheat roots were the source of *Pc*O6 [20], the winter conditions can result in the soil being frozen to depths of 20 cm multiple times creating several freeze/thaw cycles [32]. This freezing depth could subject *Pc*O6 cells to freezing in the soil or when attached to the roots of winter wheat. Studies with another biocontrol-active pseudomonad, *P. fluorescens* Pf5, indicate that the RpoS regulon provides protection against freeze damage and a comparative RpoS regulon active in stress response is present in *Pc*O6 [33-34]. The RpoS regulon could account for the survival through the freeze/thaw cycles of the *Pc*O6 cells suspended only in water. The studies also support the cryoprotection offered by suspending the cells in 15% glycerol, where loss in culturability was restricted to loss of 1 log unit after three free/thaw cycles.

5. Conclusions

In summary, the findings support the adjuvant classification of F68. This Pluronic did not limit growth of a plant probiotic *Pc*O6 or alter its production of the important biocontrol trait of phenazine biosynthesis. Altered swarming might boost colonization of the bacterium on plant surfaces. The surfactant activity of F68 could be valuable for its inclusion as an adjuvant in formulations for crop applications.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. The materials are Supplementary Tables 1 and 2; Supplementary Figures 1 and 2. .

Author Contributions: The following statements should be used "Conceptualization, DWB and AJA .; methodology, ARS,AC,MZ,AG,AJA,DWB; formal analysis, ARS, AC,MZ,AG.; investigation, ARS, AC, MZ, AG.; resources, AJA, DWB.; data curation, ARS,AC,MZ,AG,AJA,DWB.; writing—original draft preparation, ARS,AJA, DWB.; writing—review and editing, ARS,AC,MZ,AG,AJA,DWB.; visualization, ARS, AC, MZ, AJA, DWB; supervision, AJA, DWB; project administration, AJA,DWB; funding acquisition, AJA,DWB. All authors have read and agreed to the published version of the manuscript.".

Funding: This work was supported by grants from the Utah Agricultural Station Projects 1581 and 1746 and the National Science Foundation Research Experience for Undergraduate Program, 1950299.

Data Availability Statement: All of the data from these studies are available upon request to the authors of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hazen, J.L. Adjuvants—Terminology, Classification, and Chemistry1. *wete* **2000**, *14*, 773–784, doi:10.1614/0890-037X(2000)014[0773:ATCAC]2.0.CO;2.
2. Bertrand, A.; Castonguay, Y. Plant Adaptations to Overwintering Stresses and Implications of Climate Change. *Can. J. Bot.* **2003**, *81*, 1145–1152, doi:10.1139/b03-129.
3. Willick, I.R.; Tanino, K.K.; Gusta, L.V. The Impact of Global Climate Change on the Freezing Tolerance of Winter Cereals in Western Canada. *Journal of Agronomy and Crop Science* **2021**, *207*, 88–99, doi:10.1111/jac.12447.
4. Murhammer, D.W.; Goochee, C.F. Sparged Animal Cell Bioreactors: Mechanism of Cell Damage and Pluronic F-68 Protection. *Biotechnol Prog* **1990**, *6*, 391–397, doi:10.1021/bp00005a012.

5. Hua, J.; Erickson, L.E.; Yiin, T.-Y.; Glasgow, L.A. A Review of the Effects of Shear and Interfacial Phenomena on Cell Viability. *Critical Reviews in Biotechnology* **1993**, *13*, 305–328, doi:10.3109/07388559309075700.
6. Palomares, L.A.; González, M.; Ramírez, O.T. Evidence of Pluronic F-68 Direct Interaction with Insect Cells: Impact on Shear Protection, Recombinant Protein, and Baculovirus Production*. *Enzyme Microb Technol* **2000**, *26*, 324–331, doi:10.1016/s0141-0229(99)00176-3.
7. Lee, R.C.; River, L.P.; Pan, F.S.; Ji, L.; Wollmann, R.L. Surfactant-Induced Sealing of Electropermeabilized Skeletal Muscle Membranes in Vivo. *Proc Natl Acad Sci U S A* **1992**, *89*, 4524–4528.
8. Moloughney, J.G.; Weisleder, N. Poloxamer 188 (P188) as a Membrane Resealing Reagent in Biomedical Applications. *Recent Pat Biotechnol* **2012**, *6*, 200–211, doi:10.2174/1872208311206030200.
9. Houang, E.M.; Bates, F.S.; Sham, Y.Y.; Metzger, J.M. All-Atom Molecular Dynamics-Based Analysis of Membrane-Stabilizing Copolymer Interactions with Lipid Bilayers Probed under Constant Surface Tensions. *J Phys Chem B* **2017**, *121*, 10657–10664, doi:10.1021/acs.jpcb.7b08938.
10. Khaliq, N.U.; Lee, J.; Kim, S.; Sung, D.; Kim, H. Pluronic F-68 and F-127 Based Nanomedicines for Advancing Combination Cancer Therapy. *Pharmaceutics* **2023**, *15*, 2102, doi:10.3390/pharmaceutics15082102.
11. Gill, M.I.S.; Cancino, G.O.; Anthony, P.; Davey, M.R.; Power, J.B.; Lowe, K.C. Pluronic F-68 Enhanced Shoot Regeneration in Micropropagated Citrus Rootstock and Passiflora Species. *Acta Biotechnologica* **2003**, *23*, 349–358, doi:10.1002/abio.200390045.
12. Kumar, V.; Laouar, L.; Davey, M.R.; Mulligan, B.J.; Lowe, K.C. Pluronic F-68 Stimulates Growth of *Solanum Dulcamara* in Culture. *Journal of Experimental Botany* **1992**, *43*, 487–493, doi:10.1093/jxb/43.4.487.
13. Kok, A.D.-X.; Wan Abdullah, W.M.A.N.; Tan, N.-P.; Ong-Abdullah, J.; Sekeli, R.; Wee, C.-Y.; Lai, K.-S. Growth Promoting Effects of Pluronic F-68 on Callus Proliferation of Recalcitrant Rice Cultivar. *3 Biotech* **2020**, *10*, 116, doi:10.1007/s13205-020-2118-5.
14. Kok, A.D.-X.; Mohd Yusoff, N.F.; Sekeli, R.; Wee, C.-Y.; Lamasudin, D.U.; Ong-Abdullah, J.; Lai, K.-S. Pluronic F-68 Improves Callus Proliferation of Recalcitrant Rice Cultivar via Enhanced Carbon and Nitrogen Metabolism and Nutrients Uptake. *Frontiers in Plant Science* **2021**, *12*.
15. Anthony, P.; Jelodar, N.B.; Lowe, K.C.; Power, J.B.; Davey, M.R. Pluronic F-68 Increases the Post-Thaw Growth of Cryopreserved Plant Cells. *Cryobiology* **1996**, *33*, 508–514, doi:10.1006/cryo.1996.0054.
16. Lowe, K.C.; Anthony, P.; Davey, M.R.; Power, J.B. Beneficial Effects of Pluronic F-68 and Artificial Oxygen Carriers on the Post-Thaw Recovery of Cryopreserved Plant Cells. *Artificial Cells, Blood Substitutes, and Biotechnology* **2001**, *29*, 297–316, doi:10.1081/BIO-100104232.
17. Cartwright, A.; Anderson, A.J.; Britt, D.W. Pluronic F68-Capped SiO2 Nanoparticles Are Compatible as Delivery Vehicles to Roots and Shoots. *MRS Advances* **2022**, *7*, 327–332, doi:10.1557/s43580-022-00266-7.
18. Pieterse, C.M.J.; Berendsen, R.L.; de Jonge, R.; Stringlis, I.A.; Van Dijken, A.J.H.; Van Pelt, J.A.; Van Wees, S.C.M.; Yu, K.; Zamioudis, C.; Bakker, P.A.H.M. Pseudomonas Simiae WCS417: Star Track of a Model Beneficial Rhizobacterium. *Plant Soil* **2021**, *461*, 245–263, doi:10.1007/s11104-020-04786-9.
19. Gamalero, E.; Glick, B.R. Recent Advances in Bacterial Amelioration of Plant Drought and Salt Stress. *Biology (Basel)* **2022**, *11*, 437, doi:10.3390/biology11030437.
20. Radtke, C.; Cook, W.S.; Anderson, A. Factors Affecting Antagonism of the Growth of Phanerochaete Chrysosporium by Bacteria Isolated from Soils. *Appl Microbiol Biotechnol* **1994**, *41*, 274–280, doi:10.1007/BF00186972.
21. Cho, S.M.; Kang, B.R.; Han, S.H.; Anderson, A.J.; Park, J.-Y.; Lee, Y.-H.; Cho, B.H.; Yang, K.-Y.; Ryu, C.-M.; Kim, Y.C. 2R,3R-Butanediol, a Bacterial Volatile Produced by *Pseudomonas chlororaphis* O6, Is Involved in Induction of Systemic Tolerance to Drought in Arabidopsis Thaliana. *Mol Plant Microbe Interact* **2008**, *21*, 1067–1075, doi:10.1094/MPMI-21-8-1067.
22. Pierson, L.S., III; Pierson, E.A. Phenazine Antibiotic Production in Pseudomonas Aureofaciens: Role in Rhizosphere Ecology and Pathogen Suppression. *FEMS Microbiology Letters* **1996**, *136*, 101–108, doi:10.1111/j.1574-6968.1996.tb08034.x.
23. Biessy, A.; Filion, M. Phenazines in Plant-Beneficial Pseudomonas Spp.: Biosynthesis, Regulation, Function and Genomics. *Environ Microbiol* **2018**, *20*, 3905–3917, doi:10.1111/1462-2920.14395.
24. Kang, B.R.; Han, S.H.; Zdor, R.E.; Anderson, A.J.; Spencer, M.; Yang, K.Y.; Kim, Y.H.; Lee, M.C.; Cho, B.H.; Kim, Y.C. Inhibition of Seed Germination and Induction of Systemic Disease Resistance by *Pseudomonas chlororaphis* O6 Requires Phenazine Production Regulated by the Global Regulator, gacS. *J Microbiol Biotechnol* **2007**, *17*, 586–593.
25. Housley, L.; Anderson, T.; Sontag, N.; Han, S.-H.; Britt, D.W.; Anderson, A.J. Pluronics' Influence on Pseudomonad Biofilm and Phenazine Production. *FEMS Microbiology Letters* **2009**, *293*, 148–153, doi:10.1111/j.1574-6968.2009.01528.x.
26. Perneel, M.; D'hondt, L.; De Maeyer, K.; Adiobo, A.; Rabaey, K.; Höfte, M. Phenazines and Biosurfactants Interact in the Biological Control of Soil-Borne Diseases Caused by Pythium Spp. *Environ Microbiol* **2008**, *10*, 778–788, doi:10.1111/j.1462-2920.2007.01501.x.

27. Stanghellini, M.E. Inhibitory and Lytic Effects of a Nonionic Surfactant on Various Asexual Stages in the Life Cycle of *Pythium* and *Phytophthora* Species. *Phytopathology* **1987**, *77*, 112, doi:10.1094/Phyto-77-112.
28. Kearns, D.B. A Field Guide to Bacterial Swarming Motility. *Nat Rev Microbiol* **2010**, *8*, 634–644, doi:10.1038/nrmicro2405.
29. Smith, L.T.; Pocard, J.A.; Bernard, T.; Le Rudulier, D. Osmotic Control of Glycine Betaine Biosynthesis and Degradation in *Rhizobium meliloti*. *J Bacteriol* **1988**, *170*, 3142–3149, doi:10.1128/jb.170.7.3142-3149.1988.
30. Annunziata, M.G.; Ciarmiello, L.F.; Woodrow, P.; Dell'Aversana, E.; Carillo, P. Spatial and Temporal Profile of Glycine Betaine Accumulation in Plants Under Abiotic Stresses. *Frontiers in Plant Science* **2019**, *10*.
31. de Weger, L.A.; van der Bij, A.J.; Dekkers, L.C.; Simons, M.; Wijffelman, C.A.; Lugtenberg, B.J.J. Colonization of the Rhizosphere of Crop Plants by Plant-Beneficial Pseudomonads. *FEMS Microbiology Ecology* **1995**, *17*, 221–227, doi:10.1111/j.1574-6941.1995.tb00146.x.
32. Cane, J. Global Warming, Advancing Bloom and Evidence for Pollinator Plasticity from Long-Term Bee Emergence Monitoring. *Insects* **2021**, *12*, 457, doi:10.3390/insects12050457.
33. Stockwell, V.O.; Loper, J.E. The Sigma Factor RpoS Is Required for Stress Tolerance and Environmental Fitness of *Pseudomonas fluorescens* Pf-5. *Microbiology (Reading)* **2005**, *151*, 3001–3009, doi:10.1099/mic.0.28077-0.
34. Oh, S.A.; Kim, J.S.; Han, S.H.; Park, J.Y.; Dimkpa, C.; Edlund, C.; Anderson, A.J.; Kim, Y.C. The GacS-Regulated Sigma Factor RpoS Governs Production of Several Factors Involved in Biocontrol Activity of the Rhizobacterium *Pseudomonas chlororaphis* O6. *Can J Microbiol* **2013**, *59*, 556–562, doi:10.1139/cjm-2013-0062.
35. Glauser, S.C.; Talbot, T.R. Some Studies on Freezing and Thawing Human Erythrocytes. *Am J Med Sci* **1956**, *231*, 75–81, doi:10.1097/00000441-195601000-00010.
36. Ashwood-Smith, M.J.; Voss, W.A.G.; Warby, C. Cryoprotection of Mammalian Cells in Tissue Culture with Pluronic Polyols. *Cryobiology* **1973**, *10*, 502–504, doi:10.1016/S0011-2240(73)80004-5.
37. Liu, C.; Lei, J.; Zhang, M.; Wu, F.; Ren, M.; Yang, J.; Wu, Q.; Shi, X. Optimization of Preservation Methods Provides Insights into Photosynthetic Picoeukaryotes in Lakes. *Microbiol Spectr* **2022**, *10*, e0255721, doi:10.1128/spectrum.02557-21.
38. Doğan, A.; Yalvaç, M.E.; Yılmaz, A.; Rizvanov, A.; Şahin, F. Effect of F68 on Cryopreservation of Mesenchymal Stem Cells Derived from Human Tooth Germ. *Appl Biochem Biotechnol* **2013**, *171*, 1819–1831, doi:10.1007/s12010-013-0472-z.
39. González-Hernández, Y.; Fischer, R.W. Serum-Free Culturing of Mammalian Cells - Adaptation to and Cryopreservation in Fully Defined Media. *ALTEX - Alternatives to animal experimentation* **2007**, *24*, 110–116, doi:10.14573/altex.2007.2.110.