Spring 2022

Developing an Electroporation Method for Transforming Streptomyces nymphaeiformis

Heather Knott
Bridgewater College, hknott@eagles.bridgewater.edu

Stephen Baron
Bridgewater College, sbaron@bridgewater.edu

Bridgewater College

Developing an Electroporation Method for Transforming *Streptomyces nymphaeiformis*

Heather Knott

Dr. Baron

Spring 2022

Abstract

Streptomyces species are notoriously difficult to transform. *Streptomyces nymphaeiformis* is no different, so a method of electroporation was used to attempt to transform the cells. Multiple growth stages were used in order to alter the degree of development of the cell wall. The procedure did not kill the cells, but the cells were not transformed. Due to the lack of transformation with *S. nymphaeiformis*, transformation was attempted on two other *Streptomyces* strains, *S. lividans* and *S. coelicolor*. Neither was successfully transformed to thiostrepton (*tsr)* resistance, nor did they grow on a plate lacking thiostrepton. One possibility for the lack of transformation and growth was flocculation of the cells in the broth after electroporation. The flocculation may have prevented the electric pulse from shocking all the cells or transferring the sample to the plate for selection was made more difficult. Finding a method to prevent flocculation may allow for successful transformation using an electroporation method.

Introduction

Streptomyces are Gram-positive, filamentous soil bacteria (*1*). Due to their being Gram-positive, *Streptomyces* are not naturally competent for transformation. Recombinant *Streptomyces* are typically formed by polyethylene glycol mediated transformation of protoplasts, cells without the cell wall. The cell wall is stripped away enzymatically by treatment with lysozyme or by mechanical means. However, many strains of *Streptomyces* are poorly transformable by protoplast transformation (*2*).

Streptomyces nymphaeiformis is a strain that is particularly difficult to transform. *S. nymphaeiformis* can degrade polyhydroxybutyrate (PHB), a type of biodegradable plastic. Since this plastic is biodegradable, it has potential environmental and industrial benefits. PHB is degraded to its monomer form, 3-hydroxybutyrate, by PHB depolymerase. PHB depolymerase synthesis in *S. nymphaeiformis* cultures is induced by PHB and repressed by glucose. The long-term goal is to determine how transcription of the PHB depolymerase gene (*phaz*) is regulated under these conditions.

One way to determine how transcription of the PHB depolymerase gene is regulated is to insert the promoter region of *phaZ* into plasmid pIJ4083, a multicopy plasmid designed for detecting promoter activity in *Streptomyces* that contains a selectable thiostrepton (*tsr*) resistance marker and a promoterless reporter gene, *xylE*, which encodes catechol dioxygenase (Fig. 1). The *phaZ* promotor can be inserted into the multiple cloning site (MCS) immediately upstream from *xylE*, thus placing *xylE* expression under the control of the *phaZ* promotor. Ideally, plasmid constructs would be introduced into *S. nymphaeiformis* cells, which would be grown in agar or broth media containing PHB and/or glucose, plus *tsr* for selection. Promoter activity would be evaluated by monitoring levels of catechol dioxygenase activity. However, introduction of DNA into *S. nymphaeiformis* has been unsuccessful with the most commonly used method, transformation of protoplasts.

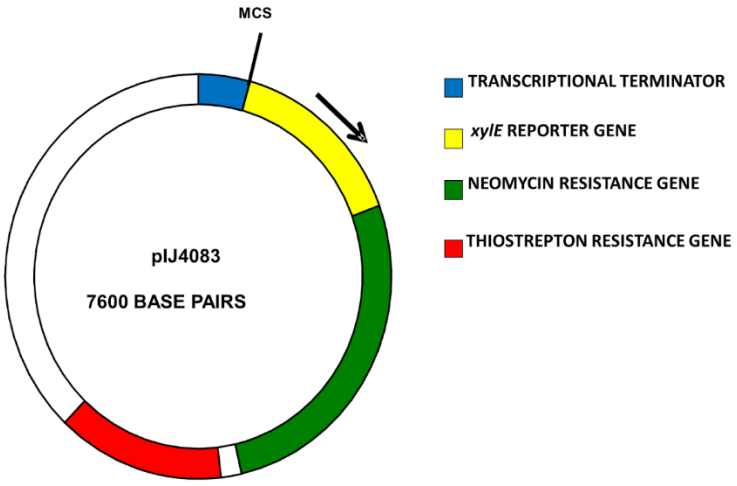

Figure 1. Visual representation of pIJ4083 and the location of important genes. The thiostrepton (*tsr*) resistance gene was used for selectivity. MCS stands for the multiple cloning site. In future research, the *phaZ* promoter will be inserted at that site to place the *xylE* reporter gene under the control of the *phaZ* promotor. Modified from reference 3.

Electroporation is a process in which cells are subjected to a high voltage pulse, which electrically modifies cell membrane permeability (*4*). The electric field both causes pore formation in the cell membrane and provides a driving force for the transport of DNA through the pores (*5*). Electroporation has been examined as a method of transforming *Streptomyces* (*2*). Some authors have had success with the transformation of *Streptomyces* strains by electroporation, but the strains were either pretreated with lysozyme to weaken the cell wall or the work was completed with fully protoplasted cells (*1*) to make the transformation easier. The conditions for electroporation must be optimized for each strain of *Streptomyces* (*2*). The optimal field strength has been found to be between 7 and 12.5 kV/cm depending on the bacterial species being studied (*1*). However, for *Streptomyces parvulus and Streptomyces vinaceus* electric field strengths between 5 and 7.5 kV/cm were optimal for transformation (*1*). In order to optimize the electroporation for *S. nymphaeiformis*, different stages of mycelia growth were examined, using pIJ4083 without the *phaZ* promoter as the incoming DNA.

The different stages of growth to be examined were germinating spores, mid-log phase mycelia, and early stationary phase mycelia. The stage of growth can be monitored by following the absorbance of a culture, which is proportional to the cell density. The above cells were treated with lysozyme to further weaken the cell membrane, potentially allowing for transformation to occur more efficiently. Using these processes, an electroporation method for transforming *S. nymphaeiformis* was explored.

Materials and Methods

S. nymphaeiformis was taken from a mineral salts-PHB agar plate dated 11/27/21. Three colonies of *S. nymphaeiformis* were suspended in about 2 mL of tryptic soy broth containing 0.5% glycine (TSB-G) and incubated at 30℃ at 200 rpm for about 24 hours. The culture was transferred into 40 mL TSB-G and incubated at 30℃, 200 rpm for about 16 hours. This culture was spun down at 6,000 x g, 4℃ for 10 minutes, then the supernatant was removed. The cell pellet was resuspended in 40 mL of ice-cold 10% sucrose. The culture was spun down (same conditions as before) then the supernatant was removed. The cell pellet was resuspended in 20 mL of ice-cold 15% glycerol, then the culture was spun down (same conditions), and the supernatant was removed. The cell pellet was resuspended in 4 mL of 15% glycerol containing lysozyme (100 µg/mL) and incubated at 37°C for 30 minutes. The cell pellet was washed twice with ice-cold 15% glycerol. The final pellet was resuspended in 1.2 mL of 30% PEG 1000 – 10% glycerol – 6.5% sucrose and placed on ice. Then 0.5 µL of pIJ4083 (650 ng/mL) or dI water was added to 50 µL of resuspended final pellet and the mixture was transferred to a 1 mm gapped electrocuvette. A 1200 V electric pulse (12 kV/cm) was applied with an ECM 399 electroporator (BTX, San Diego, CA), and the pulsed mycelium was diluted with 0.75 mL of ice-cold CRM (2.5 g Glucose, 25.75 g Sucrose, 2.53 g $MgCl_2 \cdot 6\ H_2O$, 3.75 g TSB, 1.25 g YE in 250 mL of dI water). The solution was transferred to small vials and was shaken (200 rpm) for 3 hours at 30℃. Then, 0.25 mL of CRM was added to the vial. The cells treated with pIJ4083 were plated (200 µL) on a Tryptic Soy Agar (TSA) plate containing thiostrepton (15 µg/mL). The cells treated with dI water were used for the viability control and selectivity control, both of which were plated with 200 µL of culture. The viability control was plated on TSA while the selectivity control was plated on TSA + thiostrepton (15 µg/mL). The remaining culture was dispensed in 50 µL aliquots and frozen at -80°C for storage. The plates were examined after 4 days. The same procedure was attempted twice more, the first time using three frozen aliquots, but one aliquot was subjected to a higher voltage, 1540 V (15.4 kV/cm), and the second time using one aliquot and *prt*-flip plasmid rather than pIJ4083 at 1050 V (10.5 kV/cm).

Electroporation of mid-log phase cells was attempted next. Three colonies of *S. nymphaeiformis* from the PHB overlay plate dated 11/27/21 were suspended in a starter culture of about 2 mL of TSB + 0.5% glycine and incubated at 30℃, 200 rpm for about 24 hours. The culture was transferred into 40 mL of TSB + 0.5% glycine and incubated at 30℃, 200 rpm until

the absorbance was measured to be 0.654 which is the mid-log phase (about 4 hours). The culture was spun down at 6,000 x g, 4℃ for 10 minutes to pellet the cells. The supernatant was removed, and the cell pellet was resuspended in 40 mL of ice-cold 10% sucrose. The culture was spun down (same conditions as before), and the supernatant was removed. The cell pellet was resuspended in 20 mL of ice-cold 15% glycerol, then spun down (same conditions). The supernatant was removed, and the cell pellet was resuspended in 4 mL of 15% glycerol containing lysozyme (100 μg/mL). The culture was incubated at 37℃ for 30 minutes and then washed twice with ice-cold 15% glycerol. The final pellet was resuspended in 1.2 mL of 30% PEG 1000 - 10% glycerol - 6.5% sucrose and frozen at -80℃. A couple days later, two aliquots were thawed and underwent the previous electroporation process using a 1010 V (10.1 kV/cm) electric pulse.

Next, germinating spores were used for electroporation. To prepare a spore suspension, one aliquot from 2/3/22 (stationary phase) was streaked onto two *ISP2* plates and incubated at 30℃ for 7 days. Sterile water (4.5 mL) was added to each plate then the plates were scraped with a sterile cotton swab to suspend the spores. A sterile pipette was used to transfer the crude suspension to a sterile vial then the liquid was at the maximum speed setting on a vortex mixer for about a minute to break up the spore chains. The suspension was filtered through a sterile cotton using a filter tube. The filtered suspension was poured into a sterile centrifuge tube and spun for 10 minutes (1000 rpm) to pellet the spores. The supernatant was poured off soon after the centrifuge stopped to prevent the spores from detaching from the wall. The tube was agitated on the vortex mixer for a few seconds to disperse the pellet in the drop of water left in the centrifuge tube. Then, 1.5 mL of sterile 20% glycerol was added. Serial dilutions were made (-2, -4, -5, -6, -7, -8, -9) and 10 μL of each dilution was plated onto a plate and incubated at 30℃ for 24 hours. The undiluted spores were frozen at -20℃ for future use. After the 24 hours, the colony-forming units (cfu) were determined. Two weeks later, the spore suspension was thawed and then mixed into 10 mL of TSB + 0.5% glycine. The spore suspension was cultivated for 5 hours at 30℃. The germinated spores were then collected by centrifugation (6000 rpm, 4℃, 10 min.) and then washed twice with chilled sterile water. The spores were suspended in 0.1 mL of 10% glycerol then 50 μL of the suspension was mixed with 1 μL of pIJ4083. The mixture was pipetted into a 1 mm chilled electroporation cuvette and subjected to a 1500 V (15 kV/cm) electric pulse. Immediately after the pulse, 250 μL of TSB medium was added. The sample was

incubated at 30℃ for 3 hours on the shaker (200 rpm). Then, 125 µL of the sample was plated on TSA + thiostrepton (15 µg/mL) and was incubated at 30℃. The viability control (125 µL) was plated on a TSA plate and the selectivity control (125 µL) was plated on a TSA + thiostrepton plate and both were incubated at 30℃. The plates were examined three days later.

Finally, other strains of *Streptomyces* were used for electroporation with pIJ4083. Frozen glycerol *S. lividans* and *S. coelicolor* cultures were replated and incubated at 30℃ for two days. The plates were then refrigerated at 4℃ for storage overnight. Each culture was inoculated in 100 mL of CRM at 30℃ on a rotary shaker for 24 hours. The *S. lividans* culture was collected by centrifugation at 4℃ (10,000 rpm) and the *S. coelicolor* culture was frozen at -80℃. The *S. lividans* culture was resuspended in 100 mL of ice-cold 10% sucrose and centrifuged (same conditions as before). The culture was resuspended in 50 mL of 15% ice-cold glycerol then were frozen at -80℃. After four days, the *S. lividans* mycelia were thawed and centrifuged (same conditions as before). The culture was resuspended in 10 mL of 15% glycerol containing lysozyme (100 µg/mL) and was incubated at 37℃ for 30 minutes. The mycelia was then washed twice with ice-cold 15% glycerol. The pellet was resuspended in 3 mL of 30% PEG 1000 - 10% glycerol - 6.5% sucrose dissolved in dI water and was separated into 50 µL aliquots. The aliquots were frozen at -80℃. The *S. coelicolor* solution was then thawed and the culture was collected by centrifugation at 4℃ (10,000 rpm). The culture was resuspended in 100 mL of ice-cold 10% sucrose and centrifuged (same conditions as before). The culture was resuspended in 50 mL of 15% ice-cold glycerol and was frozen at -80℃ for three days. The culture was then thawed and centrifuged (same conditions as before). The culture was resuspended in 10 mL of 15% glycerol containing lysozyme (100 µg/mL) and was incubated at 37℃ for 30 minutes. The culture was then washed twice with ice-cold 15% glycerol. The pellet was resuspended in 3 mL of 30% PEG 1000 - 10% glycerol - 6.5% sucrose dissolved in dI water and was separated into 50 µL aliquots. The aliquots were frozen at -80℃. Then, two aliquots of the *S. lividans* were thawed at room temperature then placed on ice. pIJ4083 (1 µL) was added to one aliquot while 1 µL of dI water was added to the other aliquot. Each mixture was transferred into a 1 mm - gapped electrocuvette and a 1000 V (10 kV/cm) electric pulse was used. The pulsed mycelium was diluted with 0.75 mL of ice-cold CRM and was shaken in small vials for 3 hours at 30℃. Then, 0.25 mL of CRM was added to each vial and 200 µL was plated. The plates were examined two days later. At that time, two aliquots of *S. coelicolor* were thawed at room temperature then placed on ice. pIJ4083

(1 µL) was added to one aliquot while 1 µL of dI water was added to the other aliquot. Each mixture was transferred into a 1 mm - gapped electrocuvette and a 1000 V (10 kV/cm) electric pulse was used. The pulsed mycelium was diluted with 0.75 mL of ice-cold CRM and was shaken in small vials for 3 hours at 30℃. Then, 0.25 mL of CRM was added to each vial and 200 µL was plated. The plates were examined three days later.

Results and Discussion

Transformation of *S. nymphaeiformis* with pIJ4083 was attempted with an electroporation method at different growth stages. Cells from stationary phase, mid-log phase, as well as germinating spores were electroporated.

With the stationary phase, the viability control (Fig. 2A) had almost a full lawn of growth. This signified the electric shock was not too strong that the cells were killed, and that the many centrifugation steps did not alter the viability of the cells either. The selectivity control (Fig. 2B) had no growth of *S. nymphaeiformis*. The white spot was most likely a contaminant. The lack of growth signified the thiostrepton selected for cells with the plasmid. The *tsr* was shown to prevent the growth of untransformed cells in each trial for every growth stage. The TSB + *tsr* plate (experimental plate) (Fig. 2C) had no growth with a 1200 V (12 kV/cm) electric pulse, thus the stationary phase cells were not transformed with pIJ4083. The cells may not have been transformed due to the voltage not being high enough. Freezing the cells before the electroporation stage may also have been necessary for transformation to occur.

The frozen aliquots were used to determine if freezing altered the cell wall enough for transformation to occur. A higher voltage, 1540 V was also used to help the electroporation process. After repeating the electroporation process on thawed aliquots, the viability control (Fig. 3A) had a full lawn of growth signifying the cells survived being frozen at -80℃ and the cells were able to withstand a higher voltage (1540 V or 15.4 kV/cm). However, the stationary phase *S. nymphaeiformis* were not transformed with a 1200 V (12 kV/cm) pulse, even after the cells were frozen (Fig. 3B). Therefore, freezing the cells did not seem to weaken the cell wall enough for the electroporation to succeed. The stationary phase *S. nymphaeiformis* was also subjected to a higher voltage (1540 V or 15.4 kV/cm) as well as being frozen, yet still was not transformed (Fig. 3C).

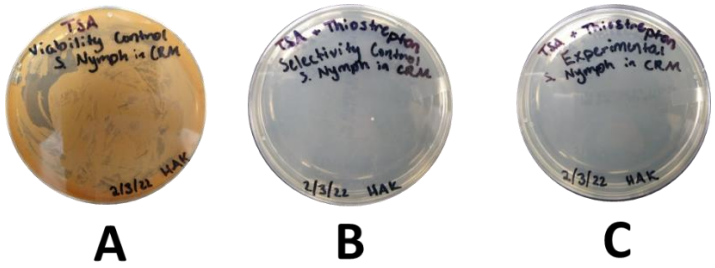

Figure 2. Results of electroporation of unfrozen stationary phase cells of *S. nymphaeiformis* with a 1200 V pulse. The cells were prepared as in Materials and Methods and used immediately for electroporation. **A,** cells treated with deionized water, plated onto TSA (viability control); **B,** cells treated with deionized water, plated onto TSA + tsr (selectivity control); **C,** cells treated with pIJ4083, plated onto TSA + tsr (experimental).

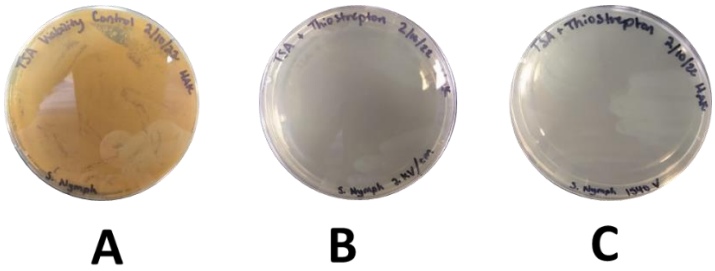

Figure 3. Results of electroporation of frozen stationary phase cells of *S. nymphaeiformis.* The cells were prepared as in Materials and Methods, frozen at -80°C, and then thawed before electroporation. **A,** Cells treated with dI water, pulsed with 1540 V, and plated onto TSA (Viability Control); **B,** cells treated with pIJ4083, pulsed at 1200 V, and plated onto TSA + *tsr* (experimental); **C,** cells treated with pIJ4083, pulsed at 1540 V, and plated onto TSA + *tsr* (experimental).

One possibility for the lack of transformation with stationary cells could be that plasmid pIJ4083 was not compatible with the *S. nymphaeiformis.* Therefore, electroporation was attempted with a different plasmid, pRT-flip, using frozen and thawed stationary phase cell. However, the electroporation was not successful as there was still no growth on the TSB + *tsr* plate (Fig. 4).

Figure 4. Attempted transformation of *S. nymphaeiformis* stationary phase cells with pRT-flip. The cells were prepared as in Material and Methods, frozen at -80°C, and thawed before electroporation with a 1050 V electric pulse. The cells were then plated onto TSA + *tsr*.

The cell wall in stationary phase cells may have been too impermeable for the electroporation to be successful. The mid-log phase was examined next because the cell wall

might be more permeable. The mid-log phase had some interesting results after electroporation. Cultures at mid log phase showed considerable clumping and flocculation after treatment that was not observed with the stationary phase cells. No growth was obtained after electroporation of these cells, even on a viability control plated with water-treated cells (Fig. 5). Either the cells were killed by the high voltage pulse or more likely, the considerable flocculation may have resulted in cells getting stuck in the pipettor and thus not being plated at all. Electroporation and plating of the mid-log phase cells was attempted three times with no success.

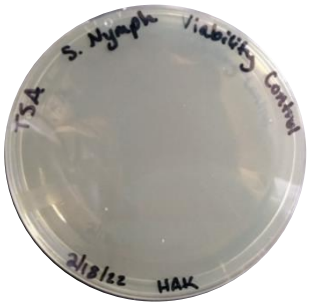

Figure 5. Transformation of mid-log phase cells. The cells were prepared as in Materials and Methods, frozen at -80°C, thawed, treated with deionized water, then subjected to a 1010 V (10.1 kV/cm) electric pulse. The cells were then plated on TSA.

Germinating spores would have a relatively weak cell wall compared to cells at mid-log or stationary phase. Due to time constraints, experimentation progressed to use of germinating spores. The germinated spores were not killed during electroporation as there was a full lawn of growth on the viability control plate (Fig. 6A). However, transformation of *S. nymphaeiformis* germinating spores with pIJ4083 was unsuccessful (Fig. 6B). The lack of success may have been due to the flocculation of the germinating spores, similar to the case with mid-log phase cells. The electric pulse (1500 V or 15 kV/cm) may not have been able to penetrate the clumps of spores, or clumping made accurate pipetting during plating difficult, as previously discussed.

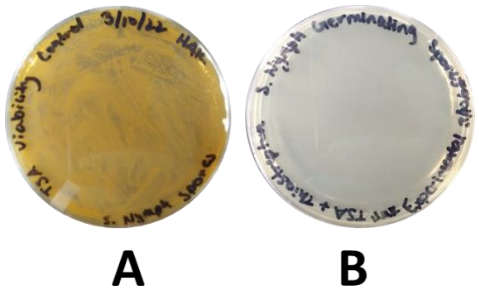

Figure 6. Transformation of germinating spores of *S. nymphaeiformis*. The germinating spores were prepared as in Materials and Methods, frozen at -80°C, and thawed. Electroporation was done with a 1500 V pulse. **A,** cells treated with deionized water, plated on TSA (viability control); **B,** cells treated with pIJ4083, plated on TSA + *tsr* (experimental).

Time constraints prevented research into preventing flocculation, but two different strains of *Streptomyces* were examined to see if transformation with pIJ4083 was possible. Previous studies have shown that both *S. lividans* (*2*) and *S. coelicolor* (*2*) have successfully been

transformed by electroporation. We attempted to transform *S. lividans* and *S. coelicolor* by electroporation with a 1000 V (10 kV/cm) electric pulse. Neither strain showed growth either on the viability control plates (Fig. 7) or experimental plates (not shown). Again, the flocculation of the cells was believed to have interfered with the plating process as well as the electroporation process.

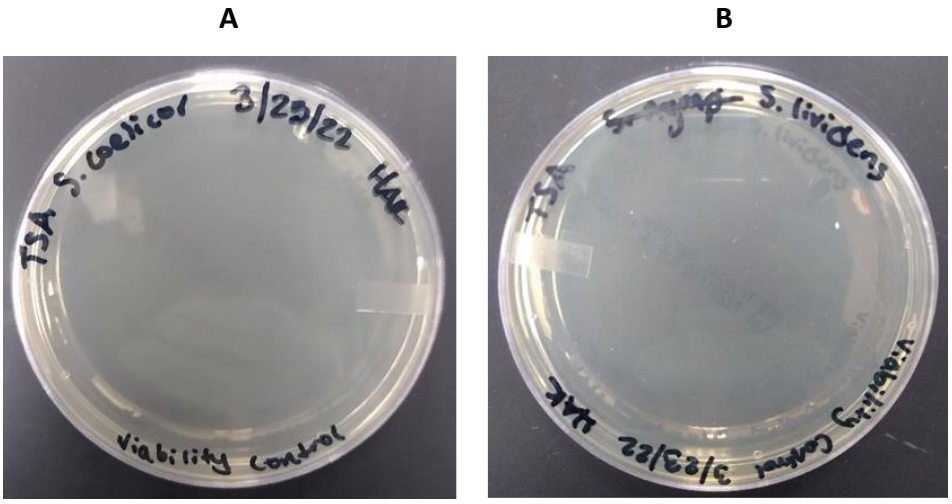

Figure 7. Results of electroporation of *S. coelicolor* **(A)** and *S. lividans* **(B)**. Cells of both strains were prepared as in Materials and Methods, frozen at -80°C, thawed, treated with deionized water, then subjected to a 1000 V (10 kV/cm) electric pulse. The cells were plated on TSA (viability controls). Results of cells treated with pIJ4083 (experimentals) are not shown.

Conclusion

Transformation of germinating spores, mid-log phase cells, or stationary phase cells using an electroporation method was not successful. However, the process may be possible after some more troubleshooting. Although the growth phase of the cells may be important for electroporation, the biggest problem we encountered was flocculation of cells. This occurred during preparation of the germinating spores and mid-log phase cells but not stationary phase cells. Therefore, a method for reducing flocculation needs to be developed in order to increase the efficiency of the electroporation and pipetting the culture onto the plate. Flocculation may be discouraged by using stainless steel springs or other types of baffles to break up clumps during culturing. Another possibility for reducing flocculation is culturing in a complete medium with a high sucrose content, which has been shown to discourage clumping (*2*).

Previous research has shown two genes, designated *matA* and *matB* (for mycelial aggregation), are required for pellet formation (*6*). These genes were discovered through reverse

engineering in a non-pelleting *S. lividans* 66 that was created by selection in a chemostat for over 100 generations (*6*). The researchers went on to analyze a number of *Streptomyces* and found the *mat* genes and corresponding flanking regions were conserved in around two-thirds of all *Streptomyces* genomes (*6*). Therefore, by mutating these genes using point-mutation, flocculation may be reduced. However, this process would be more difficult than just altering the media composition so may not be worth the effort. It may be possible to purchase a culture that has already mutated to be non-pelleting, but this culture may be expensive whereas altering the media composition is not.

Finally, the possibility remains that *S. nymphaeiformis* simply cannot harbor foreign DNA due to an unknown restriction-modification system. If this is the case, transformation will not be possible until the restriction-modification system is understood in more detail.

References

1. Mazy-Servais C, Baczkowski D, Dusart J. Electroporation of intact cells of streptomyces parvulus and streptomyces vinaceus. FEMS Microbiology Letters. 1997 [accessed 2021 Nov 29];151(2):135–138. https://academic.oup.com/femsle/article/151/2/135/487064?login=true. doi:10.1111/j.1574-6968.1997.tb12561.x.
2. Pigac J, Schrempf H. A simple and rapid method of transformation of Streptomyces rimosus R6 and other streptomycetes by electroporation. Applied and Environmental Microbiology. 1995 [accessed 2021 Nov 29];61(1):352–356. https://journals.asm.org/doi/epdf/10.1128/aem.61.1.352-356.1995. doi:10.1128/aem.61.1.352-356.1995.
3. Clayton TM, Bibb MJ. *Streptomyces* promoter-probe plasmids that utilize the *xylE* gene of *Pseudomonas putida*. Nucleic Acids Res. 1990 Feb 25;18(4):1077. doi: 10.1093/nar/18.4.1077. PMID: 2315034; PMCID: PMC330394.
4. Sowers AE, Jordan CA, Neumann E, Tsong TY. Electroporation of Cell Membranes. In: Electroporation and Electrofusion in Cell Biology. Boston, MA: Springer; 1989. p. 149–163. https://link.springer.com/chapter/10.1007%2F978-1-4899-2528-2_9. doi:10.1007/978-1-4899-2528-2.
5. Weaver JC. Electroporation Theory. In: Nickoloff JA (eds) Electroporation Protocols for Microorganisms. Methods in Molecular Biology™. Vol. 47. Totowa, NJ: Humana Press; 1995. p. 1–26. https://link.springer.com/protocol/10.1385%2F0-89603-310-4%3A1. doi:10.1385/0896033104.
6. van Dissel, D., Claessen, D., Roth, M., & van Wezel, G. P. (2015). A novel locus for mycelial aggregation forms a gateway to improved Streptomyces cell factories. *Microbial Cell Factories*, *14*, 44. https://doi.org/10.1186/s12934-015-0224-6.