ATTEMPT TO TRANSFORM *STREPTOMYCES NYMPHAEIFORMIS* USING ELECTROPORATION

Heather Knott and Stephen F. Baron

Department of Biology and Environmental Science: Bridgewater College, Bridgewater, VA

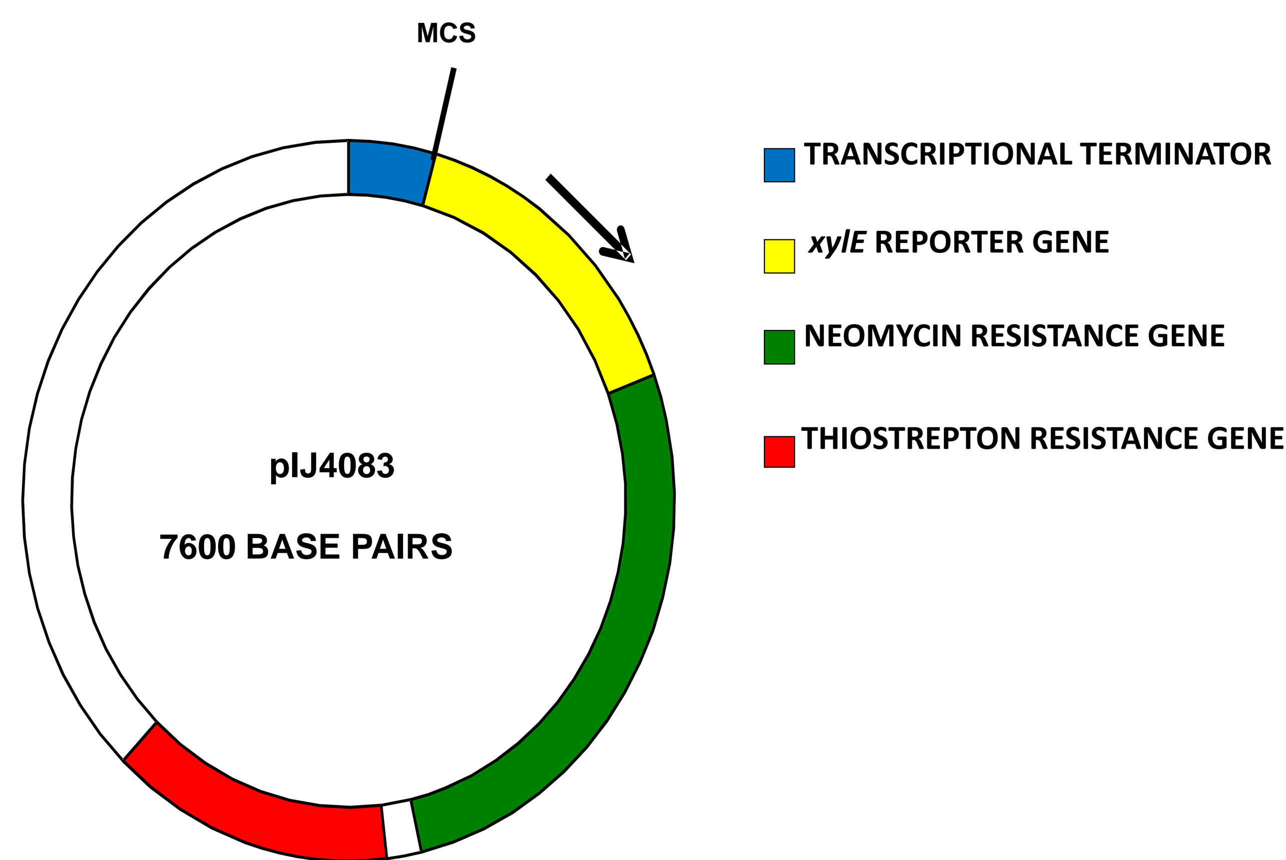
INTRODUCTION

STREPTOMYCES NYMPHAEIFORMIS IS A FILAMENTOUS SOIL BACTERIUM THAT 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. OUR LONG-TERM GOAL IS TO DETERMINE HOW TRANSCRIPTION OF THE PHB DEPOLYMERASE GENE (*PHAZ)* IS REGULATED UNDER THESE CONDITIONS.

ONE WAY TO DO THIS IS TO INSERT THE PROMOTER (CONTROL) REGION OF *PHAZ* INTO PLASMID pIJ4083, WHICH CONTAINS A PROMOTERLESS REPORTER GENE, *XYLE,* ENCODING CATECHOL DIOXYGENASE (FIG. 1). ACTIVITY OF THE INSERTED PROMOTER UNDER DIFFERENT GROWTH CONDITIONS CAN EASILY BE ASSAYED BY MEASURING CATECHOL DIOXYGENASE ENZYME ACTIVITY. HOWEVER, THIS NECESSITATES PLACING THE PLASMID CONSTRUCT INTO *S. NYMPHAEIFORMIS* CELLS. THIS HAS BEEN UNSUCCESSFUL WITH THE MOST COMMONLY USED METHOD, TRANSFORMATION OF PROTOPLASTS (CELLS LACKING A CELL WALL).

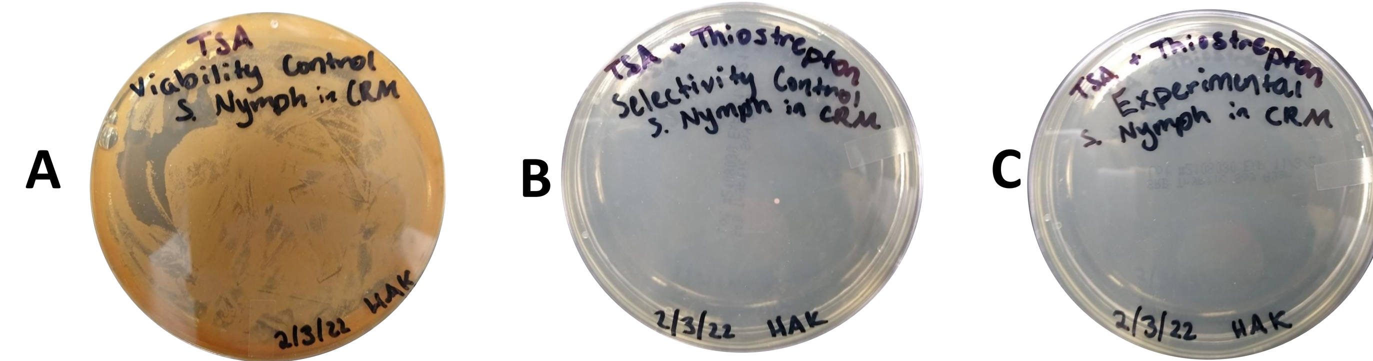
THE GOAL OF OUR RESEARCH WAS TO DEVELOP A METHOD OF INSERTING PLASMID pIJ4083 INTO *S. NYMPHAEIFORMIS* USING AN MORE EFFICIENT ELECTRIC PULSE METHOD CALLED ELECTROPORATION. WE TRIED TO OPTIMIZE THIS PROCEDURE USING DIFFERENT GROWTH STAGES AND ELECTROPORATION VOLTAGES.

FIGURE 1: MAP OF PLASMID pIJ4083



pIJ4083 IS A MULTICOPY PLASMID DESIGNED FOR DETECTING PROMOTER ACTIVITY IN *STREPTOMYCES.* IT CONTAINS A SELECTABLE THIOSTREPTON RESISTANCE (tsr) MARKER AND A PROMOTERLESS *XYLE* REPORTER GENE, WHICH ENCODES CATECHOL DIOXYGENASE. THE *PHAZ* PROMOTER CAN BE INSERTED INTO THE MULTIPLE CLONING SITE (MCS) IMMEDIATELY UPSTREAM FROM *XYLE*, THUS PLACING *XYLE* EXPRESSION UNDER CONTROL OF THE *PHAZ* PROMOTER. 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. FOR SIMPLICITY, WE USED pIJ4083 WITHOUT THE *PHAZ* PROMOTER FOR THE RESEARCH PRESENTED HERE.

FIGURE 2: ELECTROPORATION WITH MID-LOG AND STATIONARY PHASE CELLS



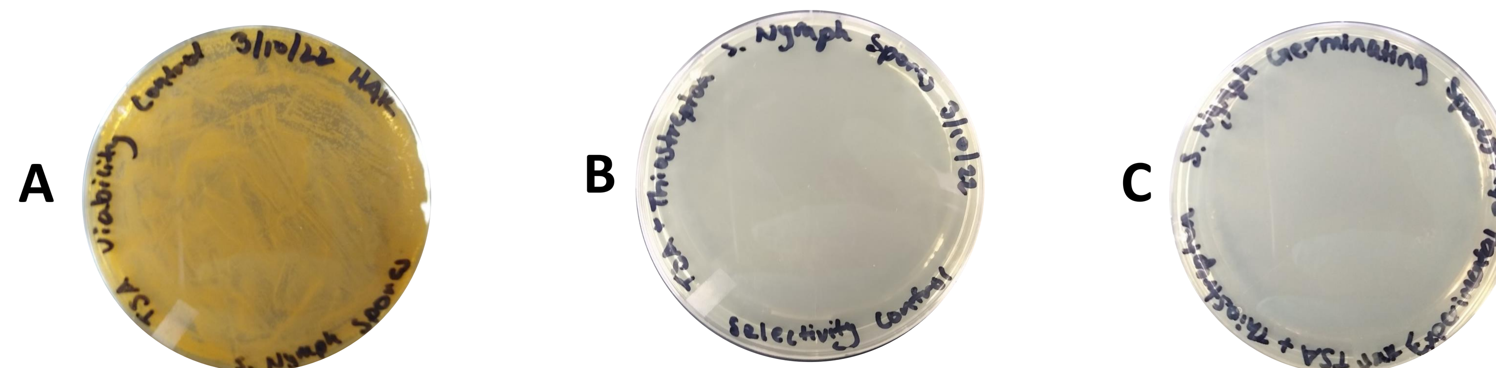
WE GREW CELLS OF *S. NYMPHAEIFORMIS* TO MID LOG OR STATIONARY PHASE (4 AND 16 h, RESPECTIVELY) IN TRYPTIC SOY BROTH CONTAINING 0.5% GLYCINE (TSBG). CELLS WERE HARVESTED BY CENTRIFUGATION AND TREATED WITH LYSOZYME IN ISOTONIC SOLUTIONS TO WEAKEN THE CELL WALL.

TREATED CELLS WERE MIXED WITH 0.5 µl OF pIJ4083 (325 ng) OR WATER, AND PULSED AT 1000-1200 V IN A 1 mm GAP ELECTROPORATION CUVETTE. CELLS WERE INCUBATED FOR 3 h AT 30°C IN ISOTONIC GROWTH MEDIUM TO ALLOW FOR EXPRESSION OF tsr RESISTANCE GENES AND PLATED ONTO TRYPTICASE SOY AGAR (TSA) WITH OR WITHOUT tsr.

RESULTS: STATIONARY PHASE CELLS TREATED WITH: A, WATER, PLATED ON TSA WITHOUT tsr (VIABILITY CONTROL); B, WATER, PLATED ON TSA WITH tsr (SELECTIVITY CONTROL); C, pIJ4083, PLATED ON TSA WITH tsr (EXPERIMENTAL). PARALLEL RESULTS FOR MID LOG PHASE CELLS ARE NOT SHOWN, SINCE THEY WERE SIMILAR.

THE CELLS SURVIVED ELECTROPORATION (A RATHER HARSH PROCEDURE) AS THE VIABILITY CONTROL HAD A FULL LAWN OF GROWTH (A). THIOSTREPTON DID KILL THE WATER-TREATED CELLS (B), BUT CELLS WERE NOT TRANSFORMED BY pIJ4083, AS THE EXPERIMENTAL PLATE (C) HAD NO GROWTH. MID LOG PHASE CELLS GAVE SIMILAR RESULTS, EXCEPT THAT THE VIABILITY CONTROL SHOWED NO GROWTH, POSSIBLY DUE TO CONSIDERABLE CLUMPING DURING TREATMENT, WHICH DID NOT OCCUR WITH STATIONARY PHASE CELLS.

FIGURE 3: ELECTROPORATION OF GERMINATED SPORES



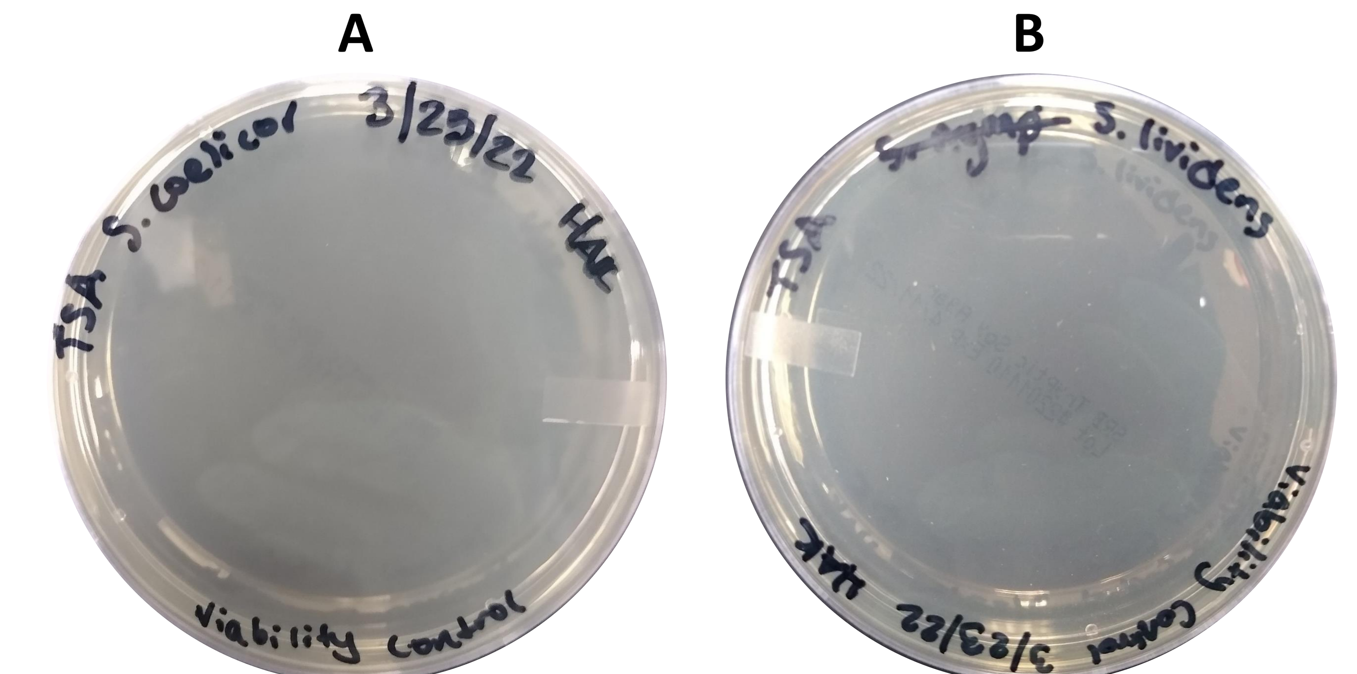
SOME RESEARCHERS HAVE SUCCESSFULLY ELECTROPORATED GERMINATING SPORES OF *STREPTOMYCES* SPP., PRESUMABLY BECAUSE THEIR CELL WALLS ARE THINNER THAN DURING OTHER GROWTH STAGES.

THUS, WE GREW CELLS OF *S. NYMPHAEIFORMIS* TO SPORULATION ON ISP2 PLATES. SPORES WERE COLLECTED, FILTERED THROUGH STERILE COTTON, AND HARVESTED BY CENTRIFUGATION. THE VIABLE COUNT OF THE PREPARATION WAS 3.2 X 10^8 COLONY FORMING UNITS (cfu)/mL. SPORES (5 X 10^8) WERE GERMINATED IN TSBG, HARVESTED BY CENTRIFUGATION, AND RESUSPENDED IN 15% V/V GLYCEROL. THEY WERE THEN MIXED WITH 1µL OF pIJ4083 (650 ng) OR WATER, ELECTROPORATED AT 1500 V, AND DIRECTLY PLATED ONTO TSA PLATES WITH OR WITHOUT tsr.

RESULTS: GERMINATED SPORES TREATED WITH: A, WATER, PLATED ON TSA WITHOUT tsr (VIABILITY CONTROL); B, WATER, PLATED ON TSA WITH tsr (SELECTIVITY CONTROL); C, pIJ4083, PLATED ON TSA WITH tsr (EXPERIMENTAL).

THE RESULTS WERE SIMILAR TO THE OTHER TRIALS. THE CELLS SURVIVED ELECTROPORATION (A), AND tsr WAS SELECTIVE (B), BUT TRANSFORMATION WAS NOT OBTAINED (C).

FIGURE 4: ELECTROPORATION OF DIFFERENT STREPTOMYCETES



DUE TO THE LACK OF RESULTS USING *S. NYMPHAEIFORMIS*, WE ATTEMPTED TO TRANSFORM *STREPTOMYCES COELICOLOR* AND *STREPTOMYCES LIVIDANS,* WHICH EXISTING LITERATURE SUGGESTED WERE TRANSFORMABLE USING ELECTROPORATION METHODS.

WE USED THE SAME METHODS FOR PREPARATION OF CELLS AND ELECTROPORATION OF THESE SPECIES AS IN FIG. 2. HOWEVER, NEITHER *S. COELICOLOR* (A) NOR *S. LIVIDANS* (B) WAS TRANSFORMED WITH A 1000 V ELECTRIC PULSE (VIABILITY AND SELECTIVITY CONTROLS NOT SHOWN).

ONE POSSIBILITY FOR OUR LACK OF SUCCESS IS THAT pIJ4083 MAY HAVE BEEN UNSTABLE IN *S. NYMPHAEIFORMIS*. IN FACT, MANY *STREPTOMYCES* STRAINS HAVE RESTRICTION-MODIFICATION SYSTEMS THAT CUT FOREIGN DNA AT METHYLATED SITES. pIJ4083 COULD CONTAIN SUCH METHYLATED SITES AND WOULD THUS BE CUT WHEN INTRODUCED INTO *S. NYMPHAEIFORMIS.*

ANOTHER POSSIBILITY IS THE TENDENCY OF THE CELLS TO CLUMP TOGETHER, KNOWN AS FLOCCULATION. THE FLOCCULATION COULD HAVE PREVENTED THE ELECTRIC PULSE FROM PENETRATING THE CELLS ON THE INSIDE OF THE CLUMP. IN ADDITION, THE FLOCCULATION MADE PLATING AND PIPETTING THE CELLS MORE DIFFICULT.

SUMMARY

- STATIONARY PHASE CELLS SURVIVED ELECTROPORATION AS EVIDENCED BY VIABILITY CONTROLS.
- THIOSTREPTON WAS SELECTIVE, AS SHOWN BY SELECTIVITY CONTROLS
- TRANSFORMATION WAS NOT OBTAINED WITH *S. NYMPHAEIFORMIS* CELLS FROM ANY OF THE GROWTH STAGES NOR WITH *S. COELICOLOR* OR *S. LIVIDANS.*

FUTURE WORK

- DEVELOP A METHOD TO PREVENT CELL CLUMPING
- ATTEMPT ELECTROPORATION WITH PROTOPLASTS (CELLS LACKING A CELL WALL).
- EVALUATE A STREPTAVIDIN PULL-DOWN ASSAY TO DETECT REGULATORY PROTEINS THAT BIND TO THE *PHAZ* PROMOTER REGION

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