Final Report

Microbial Cellulose Assembly in Microgravity

NAG10-143

PI: R. Malcolm Brown, Jr.

Summary

Based on evidence indicating a possible correlation between *hypo-gravity* conditions and alteration of cellulose production by the gram negative bacterium, *Acetobacter xylinum,* a ground-based study for a possible long term Space Shuttle flight has been conducted. The proposed experiment for *A. xylinum* aboard the Shuttle is the BRIC (Biological Research in a Canister), a metal container containing spaces for nine Petri plates. Using a common experimental design, the cellulose production capability as well as the survivability of the *A. xylinum* strains NQ5 and AY201 have been described. It should now be possible to use the BRIC for the first long term microgravity experiments involving the biosynthesis of cellulose.

Introduction

This study began, in part, from evidence from earlier NASA-supported work citing a possible correlation between *hypo*-gravity conditions and cellulose synthesis by the obligate aerobe, *Acetobacter xylinum* (Brown, 1992). These preliminary studies were accomplished on board NASA's KC 135 parabolic flight program for short term microgravity applications.

A. xylinum possesses a row of cellulose synthase complexes along its cell membrane that produce individual glucan chain aggregates which associate to form microfibrils which then aggregate into a cellulose. When A. *xylinum* was flown on the National Aeronautic and Space Administration's (NASA) Reduced Gravity Laboratory, a KC-13S aircraft that produces 20 seconds of microgravity at the height of a parabolic dive, evidence indicated some part of the flight path (the 20 second microgravity phase, the 20 second 2 x g, or a combination of both) was responsible for an alteration in the structure of biosynthesized cellulose (Brown, 1992). From the possible correlations between a change in gravity (reduced or enhanced), a flight on the Space Shuttle was proposed. NASA's Space Biology Program was created to study the role of gravity in biological activities and involves both plant and animal research . NASA approved a preliminary proposal by Dr. R. Malcolm Brown, Jr. for a 12-month based ground-based study to test the feasibility of an experiment on cellulose biosynthesis for shuttle flight.

This ground-based study had three main objectives: (a) preparation of the experimental system for flight; (b) manipulation of the system during flight along with simultaneous ground controls; and, (c) post-flight analysis (Brown, 1994). The first of these objectives, preparation of the experimental system for flight, was based on a BRIC (Biological Research in a Canister) experiment (NASA, 1993). The BRIC is a Petri dish canister which holds standard sized Petri dishes. There are two types of canisters, 82 mm or 120 mm in diameter, both 312 mm long. We were provided with two of 120 mm diameter BRICs for ground-based study. Each BRIC can hold nine Petri dishes. BRIC experiments are placed in the Shuttle's mid-deck locker and are passive. The metal canisters can be sealed or vented to the temperature and humidity conditions of the mid-

deck. This ground-based study has focused on the preparation of the BRIC for flight. *A.. xylinum* growth, and cellulose biosynthesis in the BRIC was studied with special attention to the optimum inoculating concentrations of *A. xylinum* as well as the effect of a sealed BRIC environment and the hypothesized limited oxygen availability. In addition, a novel system, the silicon bilayer growth system (SBGS), for *A. xylinum* growth in Petri plates was developed.

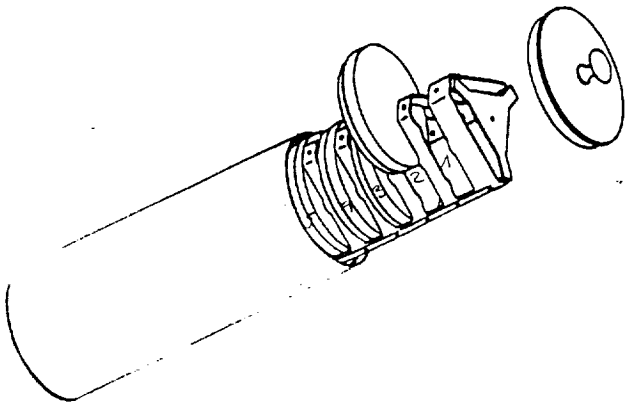


Figure 1. The BRIC canister (NASA, 1993).

The second and third objectives of this ground-based study, the manipulation of the system during flight, and post-flight analysis, are addressed in the discussion section of this report. The A. *xylinum* cultures used in this ground based study were the NQ5 and AY201strains. Each of these strains has unique characteristics (Brown, 1992). NQ5 produce compact cellulose, synthesize cellulose in tunnels in agar medium, and periodically reverse the direction of cellulose synthesis. AY201 forms a spectrum of different colony morphologies and does not undergo reversals in the synthesis of cellulose.

Materials and Methods

Experimental Design

One basic type of experiment was used to investigate the growth of *A. xylinum* inside the BRIC. Petri plates containing Shramm-Hestrin agar or liquid media were inoculated with either NQ5 or AY201 *A. xylinum* cultures. The Petri plates were then placed in the BRIC (variable) or aside the BRIC (control). Temperatures of 4°C or 28°C were used as starting environments for this growth. At 4°C, *A. xylinum* stops cellulose production and becomes lethargic; at 28°C, it grows and proliferates.

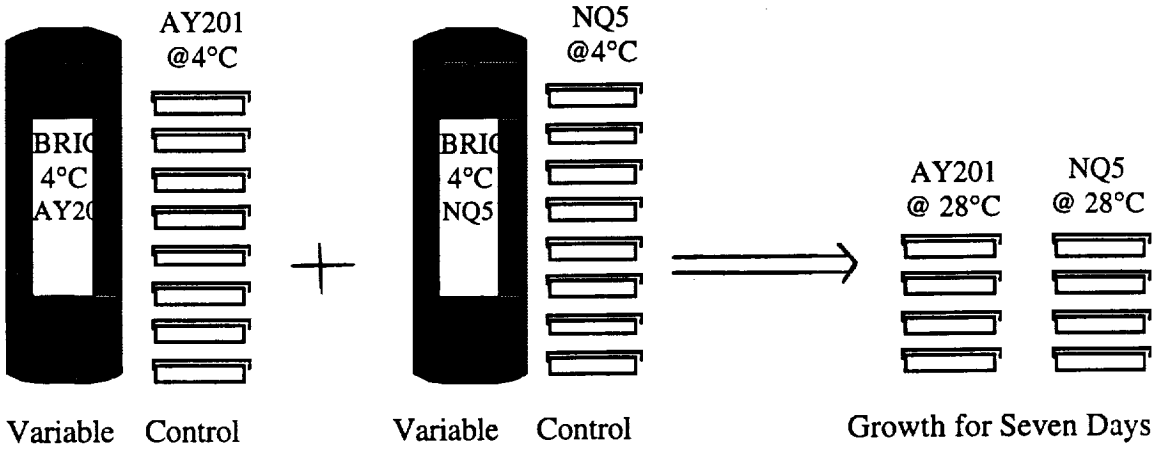


Figure 3. Common Experimental Setup

Recall from the Introduction that these experiments focused on evaluating the optimum inoculating concentrations of *A. xylinum* as well as the effect of a sealed BRIC environment and hypothesized limited O_2 availability. Using the common experimental setup mentioned above, the number of colonies produced per concentration of inoculum and the rate of growth or death of the cultures could be observed.

Results

Cellulose Production by Agar and Liquid Cultures after Cold (4°C) Initial Conditions

2-days @4°C+five days @28°C (liquid culture plates): A two-day-old single colony culture tube was used to inoculate NQ5 and AY201 cultures. The liquid culture plates were grown at 4°C for two days and then at 28°C for five days.

The AY201 liquid cultures produced thick pellicles as did the NQ5 liquid culture.

NQ5--liquid culture plate	**AY201--liquid culture plate**
2 days @4°C, 5 days @28°C: thick pellicle	2 days @4°C, 5 days @28°C: thick pellicle

One control for AY201 and another for NQ5 were grown at 28°C during the duration of the experiment. They produced thick pellicles.

5-days @4°C+12-days @28°C ([10^{-2}] and liquid); 7-days @4°C+10-days @28°C ([10^{-2}] agar plates and liquid culture plates): A 10^{-2} dilution of two-day-old single colony tube culture was prepared. Two agar NQ5 and two agar plates of AY201 (for a total of eight plates) were inoculated with 100mL of this 10^{-2} dilution. Two liquid plates of NQ5 and two liquid plates of AY201 were inoculated with 1 mL of this 10^{-2} dilution.

One of the agar plates from the pair of each strain was kept at 4°C for five days then transferred and grown at 28°C for 12 days. The other agar plate

The NQ5 and AY201 inoculating cultures were prepared by inoculating 10 mL of Schramm-Hestrin media in test tubes with single colonies of NQ5 or AY201. The test tube cultures were grown for approximately 3 days at 28°C. Then, the test tubes were vortexed to free the cells from the pellicle and the liquid from the tube was diluted to concentrations of 10^{-1}, 10^{-2}, 10^{-3}. The dilution were used to inoculate Petri plates of agar or liquid Schramm-Hestrin (SH) media.

Cellulose Production by Agar and Liquid Cultures after Cold (4°C) Initial Conditions

These experiments aimed to determine the effects of cold temperature (4°C) starting conditions on AY201 and NQ5 *Acetobacter xylinum* cultures. The cultures were grown in two types of Shramm-Hestrin media: agar and liquid. The agar-plate cultures were grown for a period (5, 6, 7, 8, 10 days) in 4°C and then grown at 28°C until the number of surviving colonies could be counted. The liquid cultures were treated similarly to the agar cultures except for different periods (2, 7, 10 days) at 4°C.

The Petri plates were kept at 4°C for 5, 6, 7, 8 or 10 day periods then transferred to 28°C for growth. The seven and ten day period experiments were repeated. The other experiments were not.

The dilution concentrations yielding a uniform spread and adequate size of colonies on agar plates as well as concentrations yielding thick pellicles in liquid medium were noted.

Survivability of NQ5 and AY201 inside the BRIC

The same experimental setup was used. The number of colonies surviving on agar parallel plates inside and outside the BRIC were counted. These numbers were used to determine whether a significantly greater amount of cells died inside the BRIC than outside, a phenomenon attributable to the sealed environment of the BRIC.

from the pair was kept at 4°C for seven days and then transferred and grown at 28°C for 10 days.

In the NQ5 10^{-2} agar plate kept for five days at 4°C, 20 large normal colonies were present. In the NQ5 10^{-2} agar plate kept for seven days at 4°C, only two colonies were present. In the AY201 10^{-2} agar plate kept for five days at 4°C, growth was normal, and rough colonies were produced. In the AY201 10^{-2} agar plate kept for seven days at 4°C, growth was normal and rough colonies were also produced.

NQ5--agar plate	**AY201--agar plate**
5 days @4°C, 12 days @28°C: ++ growth	5 days @4°C, 12 days @28°C: ++ growth
7 days @4°C, 10 days @28°C: -- growth	7 days @4°C, 10 days @28°C: ++ growth

In both the NQ5 liquid culture plates kept for five days and seven days at 4°C and twelve and 10 days at 28°C, respectively, a nice pellicle was produced. The same was true for AY201.

NQ5--liquid culture plate	**AY201--liquid culture plate**
5 days @4°C, 12 days @28°C: nice pellicle	5 days @4°C, 12 days @28°C: nice pellicle
7 days @4°C, 10 days @28°C: nice pellicle	7 days @4°C, 10 days @28°C: nice pellicle

10-days @4°C+5-days @28°C (only [10^{-1}] agar plates--contaminated the rest): A no dilution plate and three dilution plates, 10^{-1},10^{-2}, 10^{-3}, of four-day-old single colony tube cultures of NQ5 and AY201 were prepared. Thus, types of plates for each strain were grown. A total of four

sets were used: 4°C inside BRIC, 4°C outside BRIC, 28°C inside BRIC, 28°C outside BRIC. 100mL from the appropriate dilution tube was used to inoculate each individual plate. The 10^{-3} plates at 4°C were moved to 28°C after six days and grown at 28°C for nine days. The 10^{-2} plates at 4°C were moved to 28°C after eight days and grown at 28°C for seven days, and the 10^{-1} plates were moved to 28°C after 10 days and grown at 28°C for five days.

For the AY201 plate left in the BRIC at 4°C for 10 days and grown at 28°C for ten days, 1mm colonies were produced. The colonies were morphologically smooth at the bottom and bumpy and rough in the center. The control for this plate also showed the same morphology. For the NQ5 plate left in the BRIC at 4°C for 10 days, small but typical NQ5 colonies were produced. The control showed the same behavior.

NQ5--agar plate	AY201--agar plate
10 days @4°C, 5 days @28°C: ++ growth	10 days @4°C, 5 days @28°C: +++^{D}growth

^{D}= smooth at bottom, rough in center

In the following two experiments, BRICs were kept sealed for the duration of the growth period.

7 -days @ 4°C + 14-days @28°C ([10-2] agar and liquid cultures): Two [10^{-2}] dilution plates and two liquid culture plates were prepared for both NQ5 and AY201 from a 5-day-old single colony tube. 250mL of inoculum was used to prepare the NQ5 agar plate. 100mL was used to prepare the AY201 agar plate. The liquid culture plates were inoculated with 1mL from the single colony tube. The plates were kept for seven days at 4°C and

fourteen days at 28°C before BRICs were opened and observations were recorded.

The AY201 [10^{-2}] agar plates grew nice, rough colonies. The NQ5 agar plates made too densely packed colonies.

NQ5--agar plate	**AY201--agar plate**
7 days @4°C, 14 days @28°C: ++ growth	7 days @4°C, 14 days @28°C: ++ growth

The AY201 liquid culture plate formed a nice pellicle as did the NQ5 culture plate.

NQ5--liquid culture plate	**AY201--liquid culture plate**
7 days @4°C, 14 days @28°C: nice pellicle	7 days @4°C, 14 days @28°C: nice pellicle

10-days @4°C+10-days @28°C ([10^{-2}] agar plates); 10-days @4°C+10-days @28°C ([10^{-3}] AY201 liquid, [10^{-2}] NQ5 liquid): Two [10^{-2}] agar plates were prepared for both NQ5 and AY201. Both NQ5 and AY201 plates were inoculated with 100mL of culture from a single colony tube. For the liquid culture plates, different concentrations than previously used were made. For AY201, a [10^{-3}] liquid culture plate was made from 10mL of inoculum to 1mL of SH liquid medium. For NQ5, [10^{-2}] liquid culture plates were made in the same fashion. All of the plates were grown for 10 days at 4°C and 10 days at 28°C.

For the [10^{-3}] AY201 agar plate kept at 4°C for 10 days, "doughnut" shaped colonies were observed. That is, the colonies were smooth at the bottom

but had a large hump in the center. For the [10^{-2}] NQ5 colonies, good growth was observed with normal colony growth.

NQ5--agar plate	AY201--agar plate
10 days @4°C, 10 days @28°C: +++ growth	10 days @4°C, 10 days @28°C: ++° growth

°="doughnut" shape

For the [10^{-3}] AY201 liquid culture plate, a nice pellicle was formed. For the [10^{-2}] NQ5 liquid culture plates, a few thin and separate floating pellicles were formed with liquid medium remaining.

[10-2] NQ5--liquid culture plate	AY201--liquid culture plate
10 days @4°C, 10 days @28°C: scattered pellicles	10 days @4°C, 10 days @28°C: thick pellicle

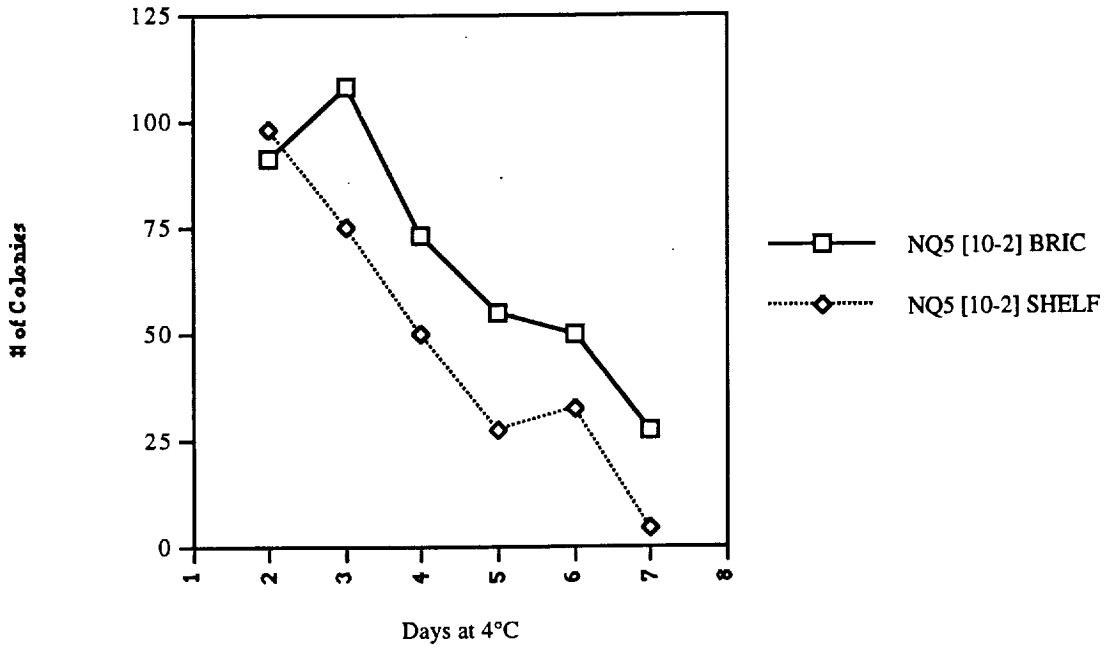
Survivability of NQ5 and AY201 inside the BRIC

These experiments were conducted using the common experimental setup and measured the number of colonies surviving after varying days inside and outside the BRIC at 4°C. Dilution concentrations of 10^{-2} were used. The results are shown in the tables and graphs below:

NQ5 Survivability

# of Days in 4°C BRIC	BRIC 4°C Plate (# of colonies)	Shelf 4°C Plate (# of colonies)
2	91	98
3	108	75
4	73	50
5	55	27
6	50	32
7	27	4

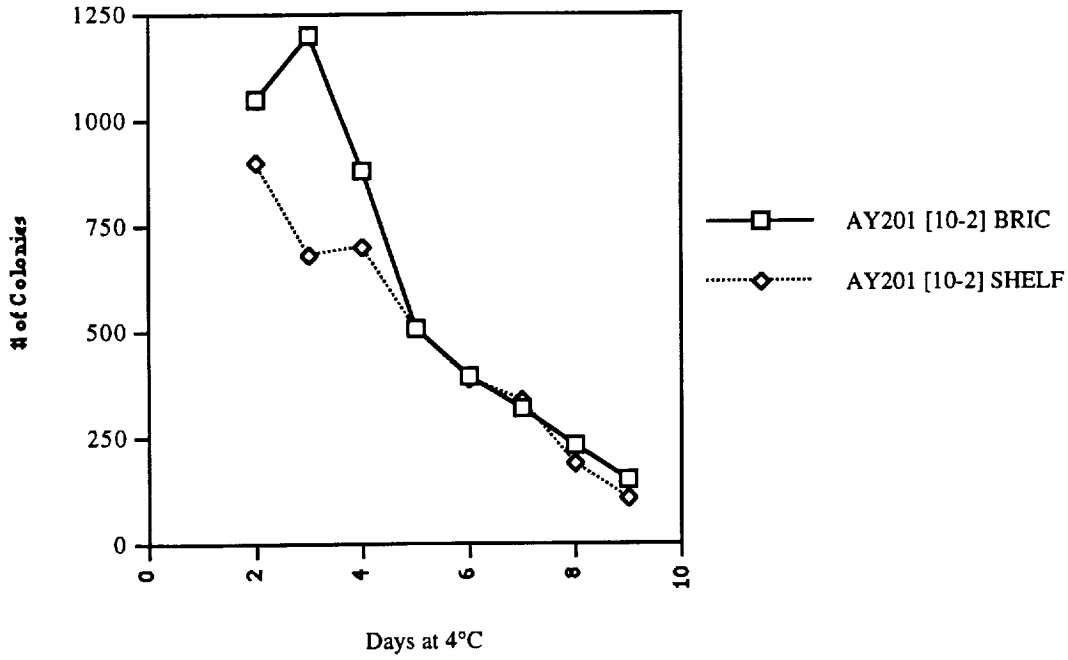
Number of NQ5 Colonies vs. Days at 4°C + Seven Days at 28°C



AY201 Survivability

# of Days in 4°C BRIC	BRIC 4°C Plate (# of colonies)	Shelf 4°C Plate (# of colonies)
2	1050	900
3	1200	680
4	880	698
5	504	506
6	390	384
7	364	334
8	231	183
9	147	106

Number of Colonies vs Days at 4°C + Seven Days at 28°C



Discussion

The experiments investigating the effects of temperature on the growth revealed that both solid agar culture and liquid suspensions *of A. xylinum* survive in the BRIC for up to seven days in a 4°C BRIC environment. The liquid suspensions survive for up to ten days in a 4°C BRIC environment. These data support the possibility of A. *xylinum* surviving a seven-to-ten day mission aboard the space shuttle (depending on the mid-deck locker temperatures).

A second set of experiments indicated the BRIC's sealed environment did not have a significant effect on *A. xylinum* growth. This was determined by comparing the survivability of *A. xylinum* both inside and outside of the BRIC. The similar death rate or survivability of colonies both inside and outside of the BRIC indicate that the BRIC's sealed environment is not a significant limiting factor in the growth of *A. xylinum* strains.

In summary, from these studies, it has been shown that *A. xylinum* strains NQ5 and AY201 may persist in a BRIC environment for a period of at least ten days, unrestricted by the sealed environment. The results of these findings may be applied to a possible Shuttle flight. Knowing the optimum concentrations of the AY201 and NQ5 cultures as well as the positive findings that A. *xylinum* grows as well inside the BRIC as outside, an experiment could be designed which would produce as much cellulose as possible, given the environmental conditions of the mid-deck locker.

The manipulation of the system during flight and post-flight analysis are areas for additional investigation. The BRIC system could be manipulated during flight by a small amount of attention from the mission specialist. The experiment could be terminated by either placing the BRIC in a 4°C refrigerator (preferable) or by freezing the cultures.

Chemical fixation also has been suggested as a termination mechanism. Since this is a passive system, it would require the least amount of attention from a mission specialist; however, the goals are to obtain sufficient quantity of cellulose synthesized under prolonged microgravity conditions, and to accomplish this may require further manipulations and/or additional monitoring. In this regard, it was suggested during preliminary discussions on the BRIC experiment that an electronic, battery-operated device be used to monitor BRIC conditions over time during the Shuttle flight. The idea was abandoned upon consideration of outgassing by the batteries. Currently, the idea for the BRIC experiment is a passive one designed to send mostly liquid-based systems as well as a few agar-containing Petri plates.

Post-flight analysis is rather straightforward. The microgravity-produced, never dried cellulose will be subjected to intense investigation covering such applications as light and electron microscopy, diffraction analysis, NMR analysis. We will look for changes which may be caused by the microgravity environment. To ascertain if these changes are due solely to such a conditions, careful controls of ground-based experiments will need to be maintained, and preferably, a 1 x g centrifuge on board the Shuttle. The latter could come once significant and interesting results are secured by the first experiments.

The significance of hypergravity conditions on cellulose production has yet to be described. One idea is a centrifuge experiment designed to centrifuging agar-containing Petri plates parallel to the arms of the rotor at a force that does not disrupt the agar solid surface. Theoretically, this would cause the A. *xylinum* colonies, that were inoculated at the center of the plate, to radiate toward the rim of the Petri plate, producing a ribbon of

cellulose from the center of the plate to the outside edge. The ribbon could be analyzed using light and electron microscopy.

The BRIC experiments have demonstrated the feasibility for a series of exciting and interesting experiments on board future Shuttle or Space Station flights. Because plant growth is a gravity-driven process and because cellulose is the major component of plant cell walls, it seems inevitable that a quest for knowledge on microgravity effects on cellulose biosynthesis will be sought. Using the model system with *Acetobacter xylinum* could pave the way for future experiments of *in vitro* cellulose synthesis experiments on board the Shuttle or Space Station. As we learn more about which genes control this process, it may be possible in the future to dissect and produce efficient plant growth in microgravity for long term missions where food, fiber, and materials all will be limited. We appreciate the opportunity to design and conduct the BRIC experiments, and now we would be anxious and very much looking forward to the application of these results for long term microgravity flights.

References

Brown, Jr. R. M. , K. Kudlicka, S. K. Cousins, and R. Nagy. 1993. Gravity effects on cellulose assembly. *Amer. J. Bot.* **79**: 1247-1258.