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Effect of Surface Attachment on Synthesis of Bacterial Cellulose†

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Abstract

Gluconacetobacter spp. synthesize a pure form of hydrophilic cellulose that has several industrial specialty applications. Literature reports have concentrated on intensive investigation of static and agitated culture in liquid media containing high nutrient concentrations optimized for maximal cellulose production rates. The behavior of these bacteria on semisolid and solid surfaces has not been specifically addressed. The species *Gluconacetobacter hansenii* was examined for cellulose synthesis and colony morphology on a range of solid supports, including cotton linters, and on media thickened with agar, methyl cellulose, or gellan. The concentration and chemical structure of the thickening agent were found to be directly related to the formation of contiguous cellulose pellicules. Viability of the bacteria following freezer storage was improved when the bacteria were frozen in their cellulose pellicules.

Index Entries: *Gluconacetobacter hansenii;* cellulose; pellicule; gellan; agar.

Introduction

In the majority of laboratory studies and industrial fermentations, bacteria are cultivated in liquid medium. However, in the natural environment, bacteria adhere to surfaces and grow in various semisolid and liquid-solid microenvironments. Under such conditions, communal synchronized behavior coordinated by quorum-signaling mechanisms is observed in the reaction of the bacterial population to environmental stimulus. This behavior is important for bacterial survival as well as interactions, both symbiotic and pathogenic, with host organisms. Two commonly observed, interrelated

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responses to growth on solid and semisolid surfaces are biofilm production and change in mode of motility from swimming to swarming. Changes in size, number, and position of flagella induced by environmental stimuli and propagated by quorum signaling enable the switch from swimming to swarming *(1,2)*. Swarming is a type of surface translocation that depends on close cell contact and multiple flagella and is typified by rapid spread of a bacterial colony across the surface of agar media. These responses are found to be induced by changes in the viscosity of the media and may also be influenced by the presence or absence of certain nutrients, depending on the species. In general, low concentrations of agar (0.4%) and lower viscosity of media induce swarming behavior *(3–5)*.

The bacteria of the species *Gluconacetobacter hansenii* and *Gluconacetobacter xylinus*, formerly classified as species of *Acetobacter hansenii* and *Acetobacter xylinum* (or *xylinus,* depending on one's interpretation of Latin grammar as applied to species names) *(6)*, are renowned for their production of thick hydrated membranes or pellicules composed of pure cellulose *(7–10)*. In nature, the bacteria are found on the surfaces of rotting fruit and spoiled fruit juice or beer. The pellicule is a type of biofilm that is formed at the air-liquid interface of the culture medium when the bacteria are cultivated under static conditions. *G. xylinus* and G. *hansenii* lack flagella but move rapidly through liquid culture media propelled by the nascent cellulose microfibrils that they continuously extrude at a rate of 2 μm/min *(9)*. Adherence of the microfibrils to the surface of nematically ordered cellulose has been observed during cellulose synthesis. The cellulose synthesis activity of *Gluconacetobacter* is not stable during long-term, continuous cultivation under agitated conditions. The spontaneous appearance of these cellulose-synthesis-deficient cel^- mutants complicates attempts to produce bacterial cellulose in large-scale fermentation. The loss of cellulose synthesis is also correlated with the morphologic change from small, rough colonies to large, smooth ones that is observed when cultures are streaked on agar plates, because smooth colonies are found to be cel^- when cultivated in liquid media *(9,11)*.

In the present study, the effects of surface properties on the cellulose-synthesizing bacterium *G. hansenii* were investigated by examining behavior on low-viscosity solid media, adhesion to plant-derived cellulose, and cryoprotection by cellulose formation.

Materials and Methods

Chemicals

Phytagel™, bovine serum albumin (BSA), carboxymethylcellulose (CMC) (D.S. 0.65–85, medium viscosity), *p*-nitrophenyl β-D-glucopyranoside (PNPG), and *p*-nitrophenyl β-D-cellopryanoside (PNPC) were obtained from Sigma-Aldrich (St. Louis, MO). Methyl cellulose (D.S. 1.6) was obtained from BDH (Poole, England). Other reagents were purchased from VWR (Westchester, PA).

Table 1
Media Formulations Used in Experiments.

Schramm-Hestrin (pH 6.2)	Modified synthetic (pH 6.2)	1-Mannitol (pH 6.2)
2% Glucose	2% Glucose	1.5% Mannitol
0.5% Yeast extract	0.1% Ammonium chloride	0.5% Yeast extract
0.5% Peptone	0.115% Citric acid	0.3% Peptone
0.27% Disodium phosphate	0.33% Sodium dihydrogen phosphate	
0.115% Citric acid	0.01% Potassium chloride	
	0.025% Magnesium sulfate	
	100 mg/L Niacinamide	
	100 mg/L Calcium pantothenate	
	100 mg/L Thiamine	

Stain, Media, and Cultivation.

The cellulose-producing bacterial strain *G. hansenii* ATCC 10821 (formerly classified as *A. xylinum*) was obtained from the American Type Culture Collection (ATCC). All cultivations were carried out at 23°C under aerobic conditions. The bacteria were grown in liquid culture in Schramm-Hestrin medium *(12)*, and in modified Schramm-Hestrin medium with mannitol substituted for glucose. Soybean peptone was substituted for beef-derived bactopeptone. Synthetic medium *(9)* was modified by addiing of 0.1 mg/mL of niacinamide, thiamine, and calcium pantothenate, and increasing the sugar concentration to 2% (see Table 1). For cellulose production, precultures of the bacteria were diluted 10-fold with fresh media and cultivated for 7–21 d under static conditions. Culture dishes 6 or 10 cm in diameter were used for routine cellulose production.

ATCC recommends a different medium, 1-mannitol agar, for propagation of *Gluconacetobacter* spp. Mannitol has been reported to stimulate maintenance of cellulose production *(7)*. For experiments examining behavior on solid media, 1-mannitol medium (Table 1) was prepared with the addition of methylcellulose (5%), agar (0.4–1.5%), or gellan (0.4–2%). Aliquots (100 µL) of a culture of *G. hansenii* were pipetted into the center of each plate and the plates were incubated at 23°C as before. The colony size was measured after 2 wk of growth. The biofilms were harvested and cleaned twice with boiling Millipore water, followed by one wash at 80°C for 1 h with 0.1 *M* sodium hydroxide. Neutralization was carried out with one-fifth volume of 0.5 *M* sodium acetate, pH 4.5. The samples were soaked in two changes of Millipore water. Finally, they were dried on a gel drier (Hoeffer) for 30 min at 60°C under vacuum.

Determination of Protein Concentration

Protein concentrations were determined with the Coomassie Blue protein assay reagent (Bio-Rad, Hercules, CA), using BSA as a standard.

Enzyme Assays

Enzymes were extracted by shaking pellicules grown on 5% cellobiose synthetic medium under static conditions for 11 d with 20 m*M* sodium phosphate buffer, pH 5.5, for 1.5 h at 23°C. The extracts were precipitated with 65% ammonium sulfate. The pellets were dialyzed against 20 m*M* sodium phosphate, pH 5.5, for 18 h at 4°C. All enzyme assays were carried out at 23°C with 0.050 mg/mL of the dialyzed protein extract. Control reactions without enzyme were carried out for all assays. β-Glucosidase activity was determined for reaction mixtures containing 10 m*M* PNPG in 20 m*M* sodium phosphate buffer, pH 5.5, by removing a 100-µL aliquot for each time point; mixing it with 1 mL of 1 *M* sodium carbonate, pH 11.0, and measuring the absorbance of the released *p*-nitrophenol at 402 nm. Endoglucanase activity was assayed in a similar fashion with the synthetic substrate PNPC. Assays for hydrolysis of CMC were carried out at 1% CMC in 20 m*M* sodium phosphate, pH 6.0. Reducing sugar was determined by the dinitrosalicylic acid method *(13)*. The net hydrolysis of substrate was calculated by subtracting the absorbance values for the control reactions without protein extract. Absorption measurements were carried out with a Spectronic 21 ultraviolet-visible spectrophotometer.

Results and Discussion

The effects of medium viscosity were first examined on agar plates. The bacteria were grown by spotting a 2-wk-old culture grown on Schramm-Hestrin mannitol liquid medium on duplicate 1-mannitol plates with agar concentrations of 0.4, 0.6, 0.8, 1.0, and 1.5%. The large colonies were harvested by removing from the agar with a spatula; extracted with 1% Triton X-100, 100 m*M* sodium phosphate, pH 6.5, buffer to remove proteins; and then treated with sodium hydroxide to purify the cellulose. The colonies grown on 0.4% agar formed small hydrated pellicules, whereas the colonies from the higher concentrations of agar did not and contained only small, discontinuous fragments of cellulose. The amount of protein extracted was similar for all of the colonies, indicating a similar number of cells in each (Fig. 1).

A striking change in morphology was noted when the agar substitute gellan gum (Phytagel™) was substituted for agar (Fig. 2). Experiments were carried out in triplicate for each concentration. At 2.0 and 1.5% (Fig. 2A ,B), the colonies resembled those that formed on the agar plates. At 1.0% (Fig. 2C), bacterial colonies appeared to branch out through channels in the gellan matrix from the main colony at the inoculation point, forming a

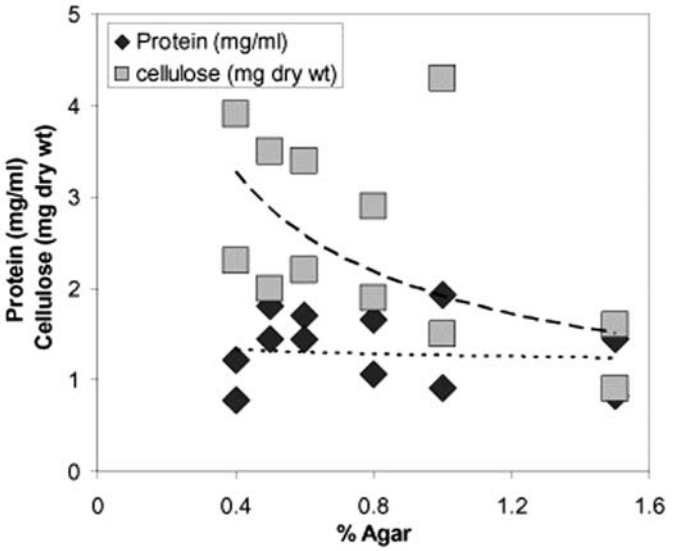

Fig. 1. Dependence of protein and cellulose production by *G. hansenii* on concentration of agar in 1-mannitol plates.

halo-like effect. When the concentration was dropped to 0.4 and 0.5% gellan (Fig. 2D), the bacteria formed a large hydrated pellicule on the top of the media similar to those formed in liquid medium. The amount of cellulose formation and the size of the pellicules were much larger for the low concentration of gellan than for the low concentration of agar (Figs. 3 and 4). The yields of cellulose and the size of pellicules obtained on 0.5% gellan averaged 50–70% of those expected from similar volumes of liquid media. The yield of dried cellulose obtained with four control cultures grown on liquid medium was 1.09 ± 0.0408 g/L, whereas the cultures grown on 0.5% gellan produced 0.743 ± 0.121 g/L, assuming 30 mL of medium in one 10-cm culture dish. This lower yield is not surprising, given that when grown on liquid media, the pellicule will grow to cover the entire surface of the media to the edges of the culture dish and extend into the liquid for 1 to 2 cm.

The striking difference in pellicule morphology on gellan vs agar plates is likely a result of the structural differences between these two polysaccharides. The chemical structures of agar and gellan differ in sugar composition, net charge, and glycosidic linkage. Agar is an uncharged polysaccharide derived from various species of seaweed and is composed of repeating disaccharide units with the sequence β-(1 → 4)-D-galactose-3,6-anhydro-α-L-galactose. Gellan is an extracellular polysaccharide secreted by the bacterium *Pseudomonas elodea* and is composed of a repeating tetrasaccharide unit with the sequence β-(1 → 3)-2-L-glyceryl-6-acetyl-D-glucose-β (1 → 4)-D-glucuronic acid-β (1 → 4)-D-glucose-α

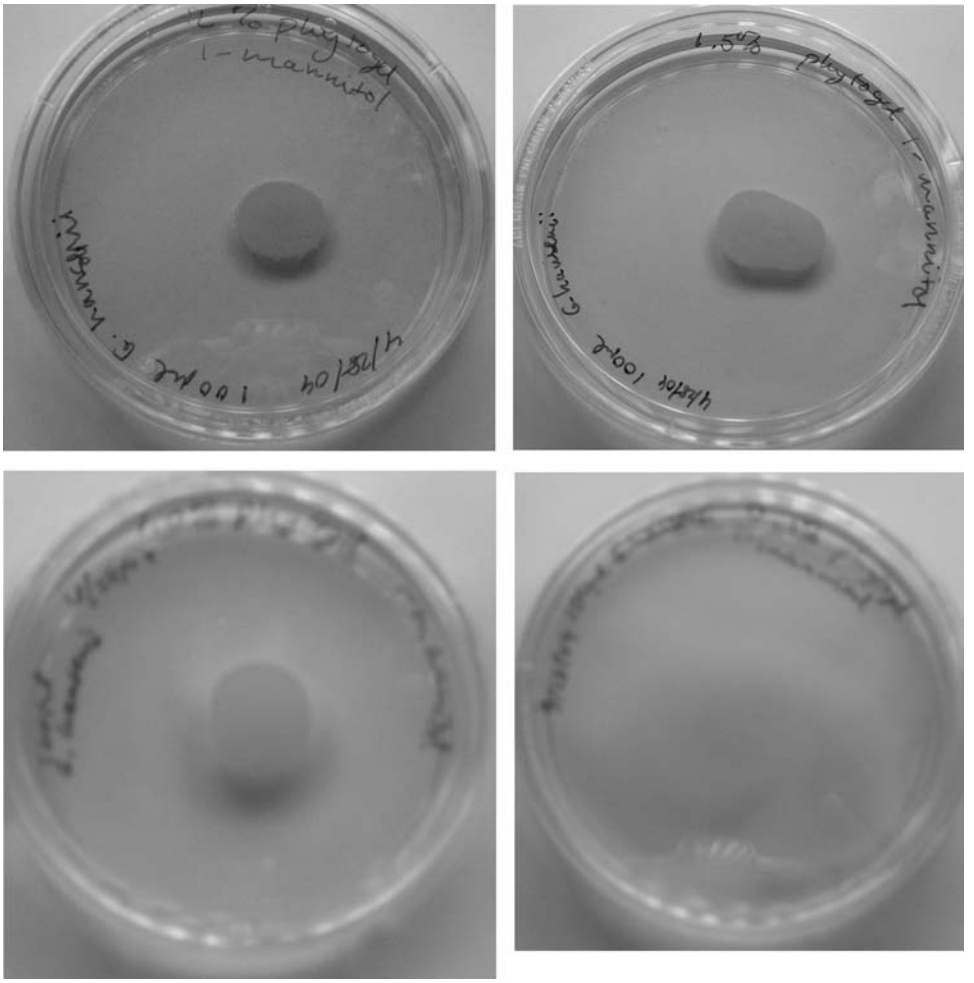

Fig. 2. Appearance after 15 d of growth of *G. hansenii* spotted on 1-mannitol plates containing **(A)** 2.0%, **(B)** 1.5%, **(C)** 1.0%, and **(D)** 0.5% gellan.

(1 → 4)-L-rhamnose *(12)*. The structure of gellan resembles that of cellulose, which is a polymer of β-(1 → 4)-D-glucose, more than that of agar *(14)*. The bacteria have been shown to track along stretches of nematically ordered cellulose prepared from cotton *(15)*.

Cultivation of the bacteria on plates prepared from 1-mannitol medium containing 5% methylcellulose resulted in the formation of large clumps and dispersed fibers of cellulose that were not connected into a contiguous pellicule (data not shown). These results are consistent with the observation of disruption of the network of crystalline cellulose in liquid medium containing soluble cellulose derivatives *(16–18)*.

The bacteria were observed to adhere to cellulosic supports and synthesize pellicules on these surfaces. Attachment of the bacteria and growth of cellulose were observed on cotton fabrics with loose weave, filter paper,

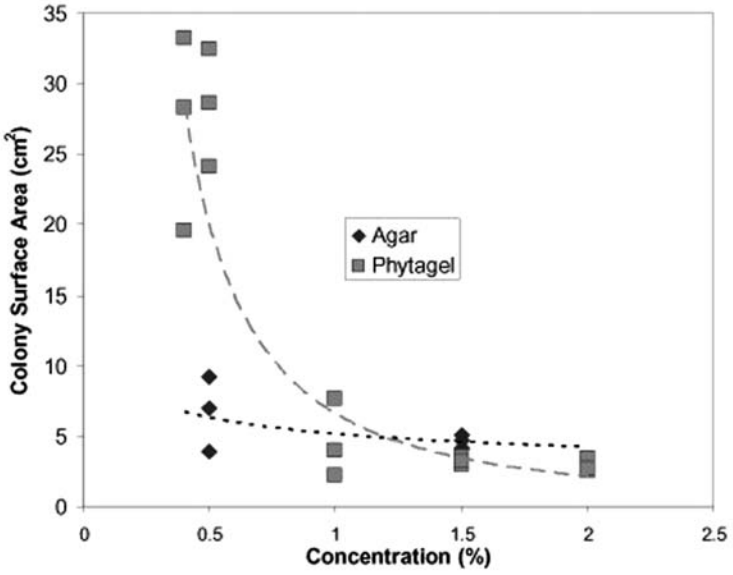

Fig. 3. Comparison of colony surface area for *G. hansenii* grown for 15 d on 1-mannitol medium thickened with gellan or agar at indicated concentrations.

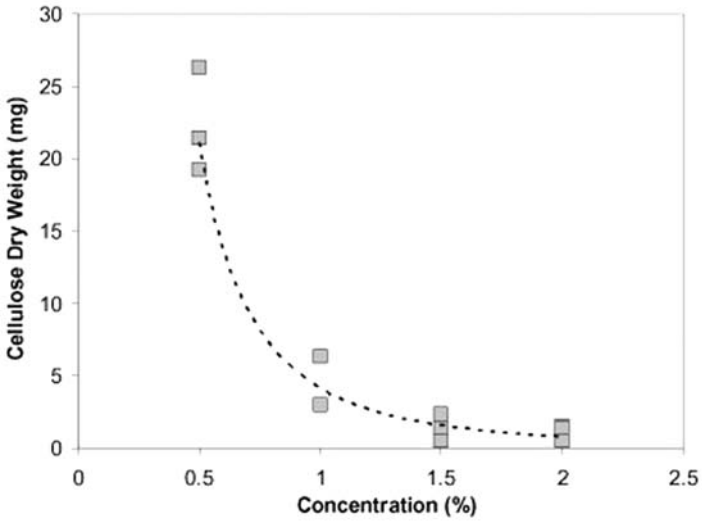

Fig. 4. Dependence of cellulose production on gellan concentration for *G. hansenii* grown for 15 don 1-mannitol medium.

wheat bran, and cotton linters. Little attachment was observed for cotton knit fabric and newsprint. No attachment was observed for polypropylene screens or carbon paper. It was observed that attachment appeared to be increased under conditions of nutrient stress, such as synthetic media or media without sugars. No net gain in cellulose weight was observed for the

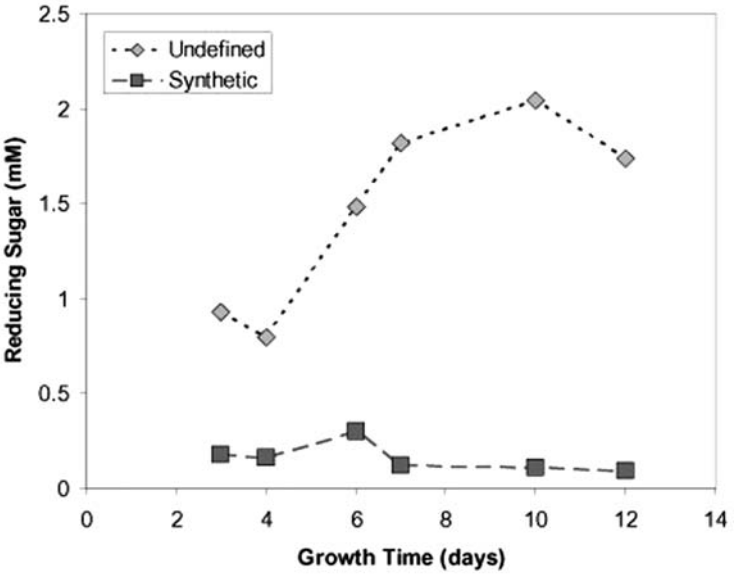

Fig. 5. Reducing sugar produced by *G. hansenii* incubated with 5% (w/v) cotton linters in undefined (Schramm-Hestrin) and synthetic culture media prepared without glucose.

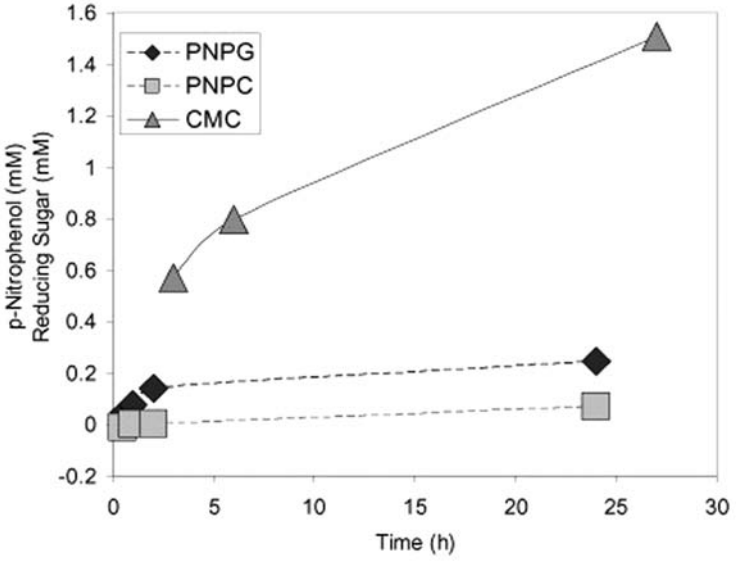

Fig. 6. Hydrolytic activity of extracts of *G. hansenii* grown on cellobiose on the β–glucosidase substrate PNPG endoglucanase substrates PNPC and CMC.

samples following purification, and slight losses in weight of 1 to 2% were observed. Small amounts of reducing sugar were detected during growth on media without sugars in the presence of 5% (w/v) cotton linters (Fig. 5). To investigate possible expression of endoglucanase under nutrient stress

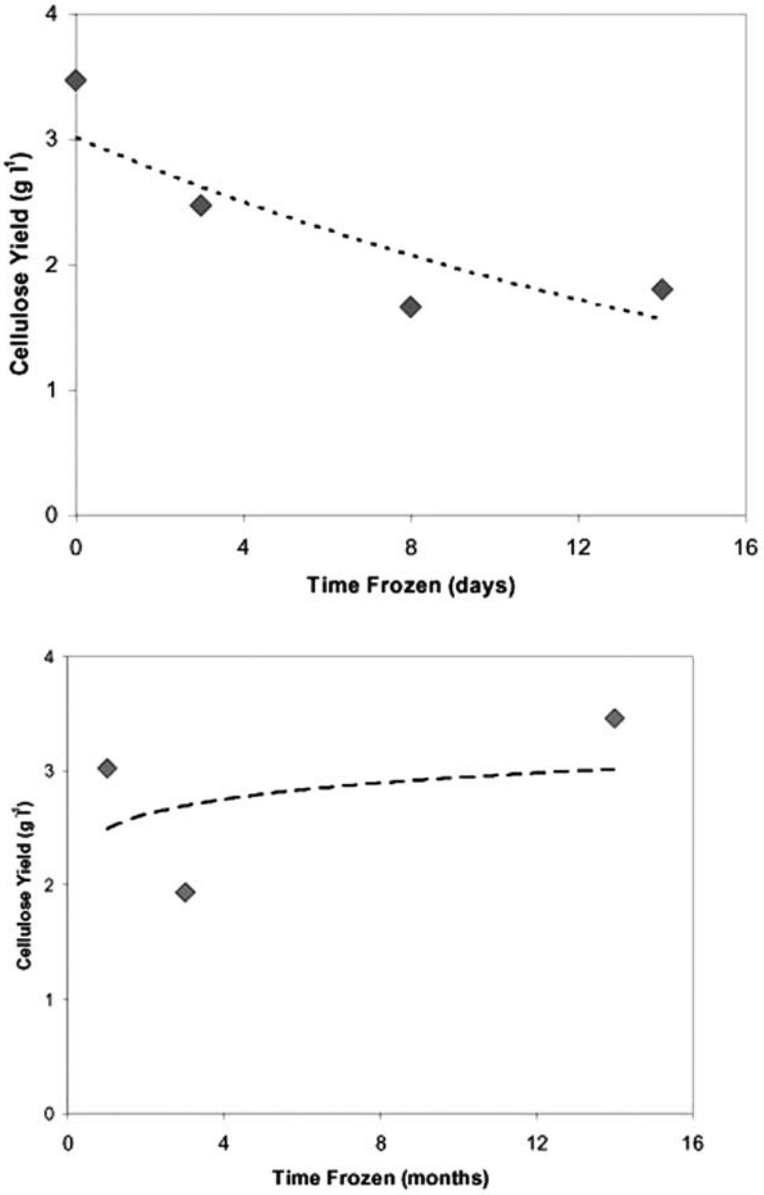

Fig. 7. Effect of storage at −20°C on viability of *G. hansenii:* **(A)** production of cellulose by bacteria frozen as free cells; **(B)** production of cellulose by bacteria frozen in cellulose pellicules.

conditions, cultures were grown in synthetic media with and without 2% glucose in the presence of 0.5-g cotton linters. The synthetic substrate PNPC was added to the cultures to a final concentration of 2 m*M*. The cultures were incubated for 24 h at 23°C. The control containing 2% glucose and cotton linters hydrolyzed 6.62% of the PNPC, whereas the culture with cotton

Table 2
Effects of Carbon Source on Viability of *G. hansenii* During 4 mo of Storage at Low Temperature

Original growth medium	Cellulose yield (g/L)		
	Storage at 4°C	Storage at −20°C (duplicate samples)	
Glucose synthetic	0.365	0	0
Fructose synthetic	2.65	1.64	1.80
Mannitol synthetic	2.03	1.80	1.78

linters but no glucose hydrolyzed 65.6% of the PNPC. Growth on cellobiose was found to induce low levels of β-glucosidase and endoglucanase activity (Fig.6). This low level of hydrolytic activity is consistent with reports of genes encoding β-glucosidase and endoglucanase in the genome of *G. xylinus (19–21)*.

Freezer storage of the bacteria following removal of cellulose and freeze-drying has been reported to result in a 90% reduction in viability *(12)*. The cryoprotective properties of the cellulose pellicule were examined by comparing the viability of cells removed from the cellulose pellicule with that of cells left *in situ* after cellulose formation. The bacteria maintained viability at −20°C for months when frozen after formation of the cellulose pellicule, whereas cells removed from the pellicules before freezing suffered a loss of 50% viability (Fig. 7A,B). Maintenance of viability at low temperatures was found to be strongly influenced by the sugar or other carbon source used in the culture media. Bacteria were grown in 6-cm culture dishes on synthetic media containing 2% glucose, fructose, or mannitol for 2 wk, during which time all cultures formed pellicules. One set of duplicate cultures was then stored at −20°C, and another set was stored at 4°C. After 4 mo, 10 mL of Schramm-Hestrin medium was added to each culture. After 2 wk of growth at 23°C, the cellulose pellicules were harvested, cleaned, and dried. It was found that the bacteria grown on glucose did not maintain viability during low-temperature storage, whereas cultures grown in the same medium with fructose or mannitol substituted for glucose survived quite well (Table 2). The formation of a brown pigment in the cellulose pellicules was observed for cultures grown in synthetic media containing fructose or mannitol, whereas little color formation was observed with glucose medium (data not shown).

Conclusion

Bacteria of the genus *Gluconacetobacter* have developed cellulose synthesis machinery that allows them to adapt to their environment

and increase their survival. Although they lack flagella, they are able to demonstrate a response resembling swarming when grown on low-viscosity media. We found that adherence to and growth on solid supports was dependent on the structural similarity of the substrate to cellulose. Formation of the cellulose pellicule appeared to provide protection against freeze damage at low temperatures. A surprising observation was the loss of viability during low-temperature storage of cultures grown on glucose in synthetic medium. This effect may be owing to greater production of gluconic and ketogluconic acids during growth on glucose, and the concomitant reduction in pH compared to growth on fructose or mannitol.

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