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Bacterial Cellulose as a Substrate for Microbial Cell Culture

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Bacterial cellulose (BC) has a range of structural and physicochemical properties that make it a particularly useful material for the culture of bacteria. We studied the growth of 14 genera of bacteria on BC substrates produced by *Acetobacter xylinum* and compared the results to growth on the commercially available biopolymers agar, gellan, and xanthan. We demonstrate that BC produces rates of bacterial cell growth that typically exceed those on the commercial biopolymers and yields cultures with higher titers of cells at stationary phase. The morphology of the cells did not change during growth on BC. The rates of nutrient diffusion in BC being higher than those in other biopolymers is likely a primary factor that leads to higher growth rates. Collectively, our results suggest that the use of BC may open new avenues in microbiology by facilitating bacterial cell culture and isolation.

The introduction of agar as a substrate for bacterial culture in 1882 was an innovation that continues to have a major impact on the microbiology field over a century later (1). A major component of the cell wall of red algae (e.g., the genera *Gelidium* and *Gracilaria*), agar consists of monomers of 3,6-anhydro-L-galactose, D-galactose, and L-galactose that are connected by β-(1,4) and α-(1,3) bonds. The preparation of agar for microbial cell culture involves decreasing the temperature of a solution of agar below its melting temperature (T_m) (typically ~50°C), which produces a disordered gel network in which a large volume fraction of water is bound to the hydrophilic polymer chains of agar. Agar has a range of physicochemical characteristics that have made it a widely used material for the culture of microbes, namely, (i) thermal stability, (ii) optical transparency, (iii) biocompatibility, (iv) resistance to degradation by many microorganisms, and (v) availability (2).

Agar is by no means an ideal material for all microbiological studies, and its application has been limited by several salient characteristics. First, a number of bacterial species grow poorly or slowly on agar, including *Edwardsiella tarda*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Shigella boydii*, *Shigella flexneri*, *Staphylococcus saprophyticus*, *Yersinia enterocolitica*, *Haemophilus parainfluenzae*, *Sinorhizobium meliloti*, and *Bradyrhizobium japonicum* (3, 4). Second, agar is a heterogeneous polymer due to variability in the chain length and composition of the polysaccharide. This characteristic can be problematic, as the physicochemical properties of agar vary between manufacturers and batches, which complicates experimental outcomes and reproducibility in phenotypic studies (5, 6). Third, agar is not inert to all bacteria—several genera of bacteria, including *Streptomyces*, *Vibrio*, *Pseudoalteromonas*, *Thalassomonas*, *Alteromonas*, *Agarivorans*, and *Microbulbifer*, produce and secrete extracellular galactosidases that degrade the hydrogel, which limits its application in bacterial isolation and culture (7). Finally, the costs of harvesting algae and the subsequent processing to produce bacteriological-grade agar make it expensive. To circumvent the limitations of agar and increase the repertoire of materials available for bacterial culture, several polysaccharides have been used, including xanthan, gellan, Eladium, and guar (4, 8–10). These polymers can be used to culture organisms that do not grow or grow poorly on agar and may increase growth rates (8, 9), which may ultimately have applications for culturing the microbial majority (11). New materials for culture may revolutionize microbiology by improving access to organisms previously recalcitrant to culture and facilitating medical microbiology by decreasing the time to culture and detection.

Bacterial cellulose (BC) is an exopolysaccharide produced and secreted by the aerobic, Gram-negative bacterium *Acetobacter xylinum* (12). As a homopolymer of D-glucose connected by β-(1,4) glycosidic bonds, this polymer is chemically well defined and is identical to plant cellulose; however, its macromolecular structure and properties have several salient characteristics that distinguish it from the plant polymer: (i) it consists of a nanoscopic three-dimensional network of fibers—with diameters of ~40 to 60 nm and lengths of ~1 to 10 μm—with a characteristically high degree of crystallinity; (ii) it is produced in high purity, typically free of biogenic compounds that are present in plant-derived cellulose and are removed by chemical processing steps; (iii) it absorbs water readily; and (iv) it displays large values of mechanical strength (13, 14). The biocompatibility and moldability of BC has fueled an interest in using it in biomedical applications, including in tissue engineering, as wound dressings, and in drug delivery systems (15–18).

Here, we introduce BC as a substrate for the growth and study of bacteria. By studying the growth of 14 different bacterial genera on BC and benchmarking the material against agar, gellan, and xanthan, we demonstrate that BC produces rates of cell growth that typically exceed those on the biopolymers and produces cultures with higher titers of cells at stationary phase.

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MATERIALS AND METHODS

Production and purification of BC. We cultured *A. xylinum* ATCC 53582 in Hestrin and Shramm (H&S) broth (2% [wt/vol] glucose, 0.5% [wt/vol] peptone, 0.5% [wt/vol] yeast extract, 0.27% [wt/vol] Na_2HPO_4, and 0.15% [wt/vol] citric acid, pH 5.0). Agar plates were prepared by adding 1.5% (wt/vol) agar to H&S broth. We picked isolated colonies on agar plates, inoculated 50 ml of H&S broth with cells, and cultured them for 2 days at 30°C under static conditions. We inoculated a 5-ml aliquot of the preinoculum into 50 ml of H&S broth, transferred the diluted cell suspension into a 15-ml Falcon tube or a well in a 24-well plate, and incubated it for 7 days at 30°C. After 7 days of growth, we removed the BC hydrogels from the 15-ml Falcon tubes or wells of a 24-well plate and treated them with 1% (wt/vol) NaOH at 90°C for 30 min to lyse attached cells. We rinsed the BC hydrogels extensively with distilled water until the pH of the eluent was ~7.0 and autoclaved the substrates for 30 min at 120°C before using them for microbial culture.

Preparation of BC substrates for bacterial cell culture. We placed the BC hydrogels into wells of 24-well plates and overlaid the polymer with a volume of lysogeny broth (LB) (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl, pH 7.0) that was approximately half of the volume of the gel and incubated it for 1 h at 25°C. To ensure that the BC was saturated with undiluted LB, we decanted the LB and repeated the process four times. A fifth overlay step was performed overnight at 25°C. Excess liquid was decanted, and the gels were incubated for 2 h in a laminar flow hood with the lid removed. We sterilized the BC substrates by exposure to UV light for 15 min in a laminar flow hood.

Preparation of agar, gellan, and xanthan gum substrates for cell culture. We prepared solutions of agar (1% [wt/vol]), gellan (1% [wt/vol] containing 0.1% [wt/vol] $MgCl_2 \cdot 6H_2O$), and xanthan gum (0.7% [wt/vol] xanthan admixed with 1% [wt/vol] agar) in deionized water. Each solution was pipetted into either a 15-ml Falcon tube or a well of a 24-well plate and solidified for 30 min at 25°C. The hydrogels were dried in air, sterilized by exposure to UV light for 15 min in a laminar flow hood, overlaid with a volume of LB that was approximately half of the volume of the gel, and incubated for 1 h at 25°C; we repeated the process four times. We performed a fifth overlay step overnight at 25°C. Excess liquid was decanted, and the gels were incubated for 2 h with the lid removed. We sterilized the substrates by exposing them to UV light for 15 min in a laminar flow hood. We used the same procedures for preparing all the substrates to make it possible to compare growth rates under the same conditions.

Bacterial cell growth. We selected 14 phylogenetically diverse bacterial strains to cultivate on the substrates described in this study. *Escherichia coli* strain BW25113, *Pseudomonas aeruginosa* strain PAO1, *E. tarda* (no strain information available), *S. boydii* (no strain information available), and *S. saprophyticus* and *K. pneumoniae* (both isolated from Medical Microbiology and Immunology stock cultures at the University of Wisconsin, Madison, WI) were grown in LB at 37°C. As *Agrobacterium tumefaciens* strain GV3101 contains a rifampin resistance cassette in its genome and *Agrobacterium rhizogenes* strain Arqual contains a tetracycline resistance cassette, we cultured these organisms at 30°C in LB supplemented with 50 μg/ml rifampin and 2.5 μg/ml tetracycline, respectively. *S. meliloti* strain Rm2011 (wild type), *S. meliloti* strain Rm2011 (Δ*nodA*), *Agrobacterium* sp. strain IRBG74 (wild type), and *Agrobacterium* sp. IRBG74 (Δ*nodA*) were cultured in TY medium (0.6% [wt/vol] tryptone, 0.3% [wt/vol] yeast extract, and 0.05% [wt/vol] $CaCl_2$) at 30°C. We cultured *B. japonicum* strain USDA110 and *S. meliloti* strain USDA6489 on AG medium (0.1 [vol/vol] HEPES-MES [morpholineethanesulfonic acid] buffer, pH 6.9, 0.067% [wt/vol] $FeCl_3 \cdot 6H_2O$, 1.8 [wt/vol] $MgSO_4 \cdot 7H_2O$, 0.13 [wt/vol] $CaCl_2 \cdot 2H_2O$, 2.5% [wt/vol] $NaSO_4$, 3.2% [wt/vol] NH_4Cl, and 1.25% [wt/vol] Na_2HPO_4). Cells of each strain were grown in culture medium for 20 h at 37°C or 30°C with shaking at 200 rpm. The culture was diluted 1:100 in fresh medium and grown at 37°C or 30°C with shaking at 200 rpm to an absorbance of 0.6 ($\lambda = 595$ nm).

Inoculation of various substrates. We grew cell cultures to an absorbance of 0.60 ($\lambda = 595$ nm) as described above, removed a volume of cell culture (~20% of the total volume of the substrate), pipetted it on the surface of the hydrogel, and incubated it for 1 min. Excess liquid was removed using a pipette, followed by incubating the substrate for 20 min with the lid removed in a laminar flow hood. This step brought the cells into contact with the polymer surface. Hydrogels inoculated with bacteria were incubated at 37°C. When viewed by phase-contrast optical microscopy, cells growing on hydrogel surfaces were short (i.e., undifferentiated) and nonmotile, indicating that they were in direct contact with the substrate surface. For xanthan, agar, and gellan, we did not observe cells growing within the hydrogel, indicating that they were confined to the gel surface. We noticed that a population of the cells growing on BC partially or completed penetrated into the BC polymer. We did not observe cells ~2 μm below the BC surface, suggesting that the pores enable cells to penetrate into the gel yet largely restrict cell growth to the polymer surface.

Plate reader growth curves. We prepared BC and other hydrogels in a disc-shaped form (total volume, 550 μl; diameter, 16 mm; and height, 4 mm) in the wells of a 24-well Costar culture dish (Corning Inc., Corning, NY) as described above. In each of the six rows of wells, one well was used as a negative control and contained a hydrogel substrate that was not inoculated with bacteria. The substrates in the other five wells in each row were inoculated as described above. Immediately following the inoculation of hydrogel substrates with cells, we inserted the plate into a Tecan Infinite M500 plate reader (Tecan Group, Ltd., Männedorf, Switzerland), incubated it at 30 or 37°C, and measured the absorbance of each well at a λ of 595 nm every 5 min for 20 h. Prior to analyzing the data, we measured the average absorbance of the wells in each row that were not inoculated and subtracted it from the absorbance for each inoculated well.

Determination of cell number. We used the drop plate method to determine the number of cells growing on the substrates. Briefly, a saturated culture of each strain was grown in different media at 30°C or 37°C for 20 h with shaking at 200 rpm. The culture was diluted 1:100 in fresh medium and grown at 30°C or 37°C with shaking at 200 rpm to an absorbance of 1.5 ($\lambda = 595$ nm). We used these cultures to prepare a series of cell suspensions with absorbance values that ranged from 0.05 to 1.0 (in 0.05 intervals) by dilution in sterile 1× phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). We divided the surfaces of agar plates into eight quadrants in which each quadrant corresponded to one dilution in the series. Three, 10-μl drops of each dilution of the cell suspension were spaced evenly on the agar surface of each quadrant. This process was repeated for every cell concentration in the dilution series. After the drops had dried on the plates, we inverted the plates and incubated them at 30°C or 37°C for 1 to 2 days. We counted the colonies in each quadrant, determined the number of CFU for each cell dilution, plotted the data for CFU versus absorbance ($\lambda = 595$ nm), and fitted the data. The fit enabled us to convert growth measurements of absorbance to CFU.

Determination of cell doubling time. We determined the doubling time ($t_{1/2}$) for cells in each experiment using the following equation: $t_{1/2} = \ln(2)/\{[\ln(A_2) - \ln(A_1)]/(t_2 - t_1)\}$, where A_1 and A_2 represent successive absorbance measurements and t_1 and t_2 are the times at which measurements were performed. Doubling times reflect the growth rate during the exponential phase of growth.

Young's modulus of hydrogels. Agar, gellan, xanthan, and BC hydrogels were prepared immediately prior to tensile testing and stored in distilled H_2O to keep them hydrated. We used a type IV sample for tensile strength measurements described in ASTM D 638-03 (33) and scaled it up to a sample thickness of ~3.2 mm, which better approximated the hydrogels used for our experiments. We used an Instron Model 5566 tensile tester (Instron, Norwood, MA, USA) equipped with a 10-N load cell to measure the tensile properties of the substrates. For each sample, we measured the mechanical properties of 3 duplicate hydrogels by elongating them at a constant rate of 5 mm/min until fracture. The Young's modulus (*E*; the intrinsic stiffness of materials, defined as the slope of stress-strain

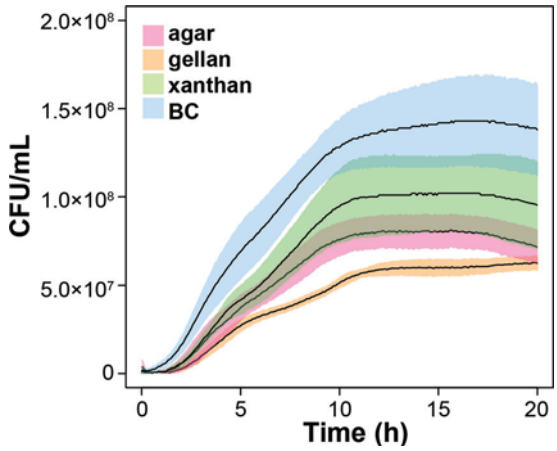

FIG 1 Numbers of cells of *P. aeruginosa* strain PAO1 on different substrates. The same substrates were cast in single rows of wells of a 24-well plate, inoculated with *P. aeruginosa* strain PAO1, and incubated at 37°C for 20 h. We measured the absorbance ($\lambda = 595$ nm) at 5-min intervals using a plate reader. The black lines represent the mean numbers of CFU ($n = 5$) of bacteria on substrates, and the shaded areas indicate the standard deviations of the mean CFU values.

curves) of the gel was determined by calculating the slope of the linear response region of the stress-versus-strain curve.

Diffusion rates of nutrients through hydrogels. To compare the diffusion of nutrients through agar, gellan, xanthan, and BC hydrogels, we prepared 10-ml solutions of agar (1% [wt/vol]), xanthan gum (0.7% [wt/vol] xanthan admixed with 1% [wt/vol] agar), and gellan (1% [wt/vol] containing 0.1% [wt/vol] $MgCl_2 \cdot 6H_2O$) in 15-ml Falcon tubes and solidified them as described above, and we placed BC hydrogels into 15-ml Falcon tubes. We pipetted 200 μl of a solution of fluorescein (100 mM) in water on top of each hydrogel and photographed the gels every 10 min for 2.5 h. We measured the distance the fluorophore had traveled at each time point and used these data to calculate the diffusion rate of small molecules through the hydrogel.

Cell phenotypes on the surfaces of substrates. To test the phenotype of one organism growing on each gel, we selected *S. meliloti* strain Rm2011 (Δ*nodA*) and grew it in TY broth for 20 h at 30°C with shaking at 200 rpm. The culture was diluted 1:100 in fresh TY broth and grown at 30°C with shaking at 200 rpm to an absorbance of 0.6 ($\lambda = 595$ nm). We prepared a suspension of cells by diluting the culture 1:20 into fresh culture medium and then removed a volume of cells (~20% of the total volume of the substrate) and inoculated it on the substrate surface for 1 min. Excess liquid was removed as described above. The substrates were placed in an incubator at 30°C for 4 h and imaged using a Nikon Eclipse TE2000 inverted microscope (Nikon Instrument Inc.) equipped with a Photometrics Cool Snap charge-coupled-device (CCD) camera.

RESULTS

BC as a substrate for the bacterial cell culture. BC substrates can be fabricated in a range of shapes and physical dimensions that are dictated by the shape of the container in which *A. xylinum* is cultured. We used wells of 24-well plates to cast BC hydrogels for the experiments described here. After preparing BC substrates, we placed the gels into wells of a 24-well plate and found that they fit snugly.

Using *P. aeruginosa* PAO1 as a model organism for growth experiments, we inoculated a suspension of cells on the surfaces of 4 different hydrogels, including BC, and compared the growth rates. Our choice of *P. aeruginosa* centered on three characteristics: (i) it is a model bacterium, and much is known about its physiology and behavior; (ii) it grows under a wide variety of conditions; and (iii) in cystic fibrosis, it grows in the lungs in contact with hydrogels consisting largely of mucus (19, 20, 31). The growth of *P. aeruginosa* PAO1 on all of the substrates displayed three prominent phases that are characteristic of the growth of bacteria in liquid culture: (i) lag phase, (ii) exponential phase, and (iii) stationary phase (Fig. 1). The number of cells of *P. aeruginosa* PAO1 on BC after growth for 20 h was larger than on the other substrates and suggests that the polymer has a higher carrying capacity for cell growth. The growth rate of *P. aeruginosa* PAO1 on BC was also higher than on the other substrates (the values are tabulated in Table 1). In particular, we found that the number of *P. aeruginosa* cells that grew on 1% agar substrates with a diameter of 16 mm (7.9×10^7) was lower than on BC hydrogels (1.4×10^8). The growth rate of cells on agar substrates ($t_{1/2} = 76$ min) was also lower than on BC ($t_{1/2} = 47$ min).

Xanthan is a microbial polysaccharide produced by *Xanthomonas campestris* that forms viscous solutions and does not exhibit a gel phase. In previous studies, agar (0.3% [vol/wt]) was admixed with xanthan (0.7% [wt/vol]) to support bacterial cell growth on the surface of the composite material (8). This composite material, however, has physical properties that make it challenging to overlay it with nutrients—an important step in preparing it for cell culture experiments—without physically disrupting the smooth surface of the gel. To prepare xanthan substrates in a manner compatible with our method of infusing BC gels with LB, we used gels consisting of xanthan (0.7% [wt/vol]) admixed with agar (1% [wt/vol]) for cell culture experiments. Substrates consisting of xanthan (0.7% [wt/vol]) admixed with agar (1% [wt/vol]) displayed the second highest number of *P. aeruginosa* cells (1.02×10^8) and the second highest growth rate ($t_{1/2} = 70$ min) (Fig. 1 and Table 1).

Gellan is a heterosaccharide secreted by *Pseudomonas* spp. that forms hydrogels in the presence of exogenous cations added to culture media. In this study, we prepared gellan substrates consisting of 1% (wt/vol) gellan containing 0.1% (wt/vol) $MgCl_2 \cdot 6H_2O$ and found that it produced the lowest number of cells and lowest growth rate of the substrates we tested after 20 h of incubation. To explore whether differences in the physical properties of the gels influenced the rate of nutrient diffusion through the hydrogel compared to other substrates—and thus the nutrients available to *P. aeruginosa* cells to support their growth—we measured the diffusion rates for each polymer. Of the substrates that we studied, fluorescein had the lowest diffusion rate through gellan (Fig. 2). In contrast, the diffusion rate of fluorescein through BC was higher than through the other polymers. In general, there was a small but consistent correlation between an increase in the diffusion rate of fluorescein in hydrogels, the number of *P. aeruginosa* cells, and their growth rate (Fig. 1 and 2).

To explore whether differences in the stiffness of the gels influ-

TABLE 1 The doubling time of *P. aeruginosa* PA01 depends on the substrate

Parameter	Value on substrate:			
	Agar	Gellan	Xanthan	BC
Composition	1.0%	1.0%; 0.1% $MgCl_2 \cdot 6H_2O$	0.7%; 1.0% agar	1.0%
Doubling time (min)	76 ± 14	120 ± 17	70 ± 26	47 ± 6

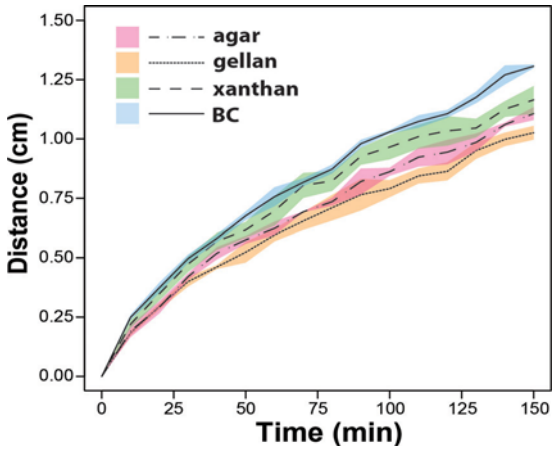

FIG 2 Diffusion rates of nutrients through hydrogels. Hydrogels consisting of agar, gellan, xanthan, and BC were placed in 15-ml Falcon tubes, overlaid with a solution of fluorescein, and imaged every 10 min for 2.5 h using a digital camera. The distance that the fluorescein had migrated was measured at each time point and was plotted as a function of time. The data are shown as the mean values determined from three independent experiments, while the shaded areas indicate the standard deviations of the mean distance values.

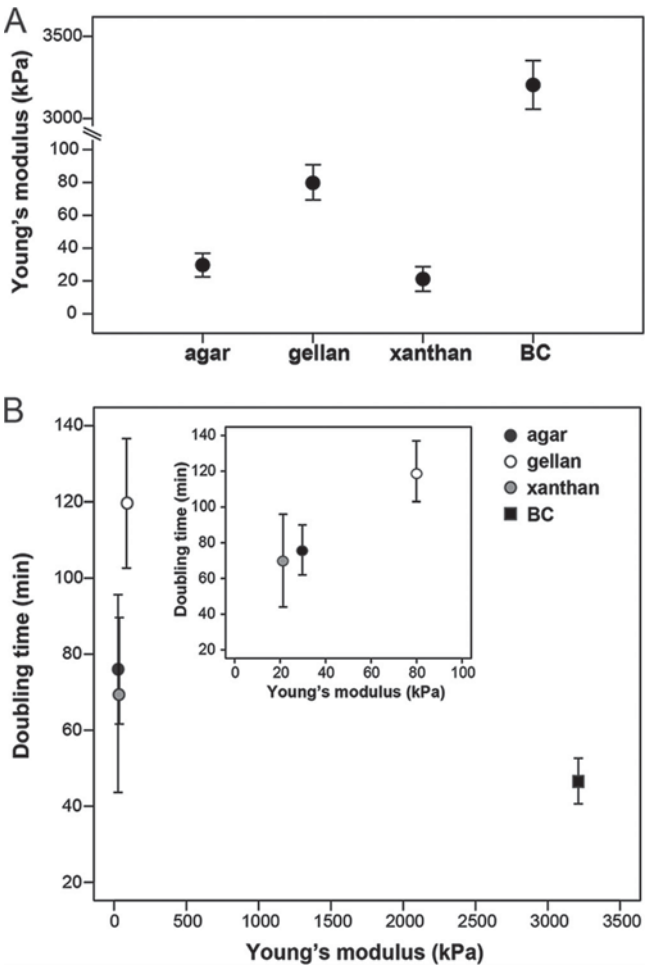

FIG 3 Relationship between the substrate stiffness and the doubling time of *P. aeruginosa* strain PAO1 cells grown on the surfaces of substrates. (A) Stiffness of each substrate. (B) Doubling time of *P. aeruginosa* strain PAO1 cells grown on each substrate compared to the Young's modulus of the hydrogel. The inset displays the relationship between the growth rate of *P. aeruginosa* strain PAO1 cells and the stiffness of agar, gellan, and xanthan. The data indicate the mean doubling times, and the error bars represent the standard deviations of the mean ($n = 4$).

enced the growth rate of *P. aeruginosa* cells—and thus the nutrients available to the cells to support growth—we measured the Young's modulus for each polymer. The correlation between material stiffness and eukaryotic cell growth and behavior has been described previously (21–23). In contrast, very little is known about the response of bacteria to surface stiffness (24, 25). Agar, gellan, xanthan, and BC had *E* values of 29.8, 79.9, 21.3, and 3,205.5 kPa, respectively (Fig. 3A). A plot of the *P. aeruginosa* doubling time versus *E* for each material did not highlight an obvious relationship (Fig. 3B).

Bacterial cell titer and growth rate on BC. To expand our preliminary studies with *P. aeruginosa*, we measured the total numbers of CFU (in quintuplicate) for cells of 14 bacterial strains growing on each of the substrates for 20 to 45 h (Table 2). To determine the numbers of CFU, we chose different time intervals due to the various growth rates of cells on each substrate. The data highlight the fact that the mean numbers of CFU of the 14 different bacterial strains growing on BC substrates were higher than for bacterial growth on agar. With the exception of *S. meliloti* Rm2011 (wild type), these bacterial strains had lower numbers of CFU on gellan after 20 h. However, *S. boydii*, *A. rhizogenes* Arqual, *Agrobacterium* sp. IRBG74 (wild-type and Δ*nodA* strains), *S. meliloti* USDA6489, and *B. japonicum* USDA110 had lower numbers of CFU on BC than on substrates gelled with xanthan gum (0.7% [wt/vol]) and agar (1% [wt/vol]).

Growth rates of bacterial strains on different hydrogel substrates. We determined the growth rates (i.e., the doubling times) of the 14 bacterial strains on the 4 different substrates (Table 3). The data in Table 3 reflect the mean doubling times determined by averaging 5 independent measurements on different gels. Of the 14 strains tested, 5 strains, i.e., *P. aeruginosa* PAO1, *E. tarda*, *K. pneumoniae*, *S. meliloti* Rm2011 (Δ*nodA*), and *Agrobacterium* sp. IRBG74 (Δ*nodA*), had shorter mean doubling times on BC than on the other substrates. BC not only supports more total cell growth than the other substrates, it also increases the growth rates of a number of bacterial strains; it is not obvious that growth rates and total cell numbers should be correlated. The mean doubling times for *S. saprophyticus*, *A. rhizogenes* Arqual, *Agrobacterium* sp. IRBG74 (wild type), and *B. japonicum* USDA110 were higher on xanthan than on BC. The growth rates of *E. coli* BW25113, *S. boydii*, and *A. tumefaciens* GV3101 were higher on agar substrates than on the other substrates. *S. meliloti* Rm2011 (wild type) and *S. meliloti* USDA6489 displayed the shortest doubling time on gellan compared to the other hydrogels.

DISCUSSION

In this study, we introduce BC as a new hydrogel substrate for culturing and studying bacteria. Over the past century, agar has been the most widely used substrate for microbial culture. Despite its convenience and success, several bacterial species are unable to grow or grow very slowly on agar (3, 4). The heterogeneity of agar also poses challenges to building connections between cell physiology and cell biology experiments performed on the material by different laboratories. These limitations suggest new opportuni-

TABLE 2 Total cell numbers of bacterial strains growing on different substrates

Bacterial strain	No. of CFU[a] (10^6) on substrate:			
	Agar	Gellan	Xanthan	BC
P. aeruginosa PA01	79 ± 8.8	62 ± 3.9	102 ± 21	**140 ± 23**
E. coli BW25113	47 ± 8.8	51 ± 3.3	60 ± 3.1	**62 ± 6.5**
E. tarda	90 ± 8.6	170 ± 10.8	225 ± 11.9	**400 ± 10.4**
S. boydii	78 ± 7.8	90 ± 6.9	**150 ± 12.6**	105 ± 21.2
S. saprophyticus	90 ± 12.7	115 ± 6.1	130 ± 5.7	**150 ± 17.5**
K. pneumoniae	125 ± 16.5	175 ± 15.4	180 ± 18.4	**275 ± 15.5**
A. tumefaciens GV3101	458 ± 18.6	155 ± 12.6	375 ± 20	**529 ± 98**
A. rhizogenes Arqual	167 ± 21	138 ± 80.4	**354 ± 46.1**	204 ± 104
S. meliloti Rm2011 (wild type)	11 ± 2.3	**34 ± 33.6**	19 ± 2.5	12 ± 0.5
S. meliloti Rm2011 (Δ*nodA*)	61 ± 9.2	67 ± 34	171 ± 61.3	**474 ± 18.4**
Agrobacterium sp. IRBG74 (wild type)	117 ± 9.1	51 ± 4.0	**336 ± 17.2**	150 ± 33.5
Agrobacterium sp. IRBG74 (Δ*nodA*)	9.8 ± 3.2	5.3 ± 0.8	**19 ± 6.6**	13 ± 1.4
S. meliloti USDA 6489	27 ± 3.9	23 ± 3.0	**36 ± 3.7**	30 ± 1.1
B. japonicum USDA 110	19 ± 3.4	16 ± 4.1	**59 ± 8.8**	34 ± 6.5

[a] The values represent the total numbers of cells (means and standard deviations) after 20 to 45 h at 37°C or 30°C, depending on the strain. The highest number of CFU for each strain is highlighted in boldface to indicate the substrate on which growth was maximized.

TABLE 3 Doubling times of bacterial strains growing on different substrates

Bacterial strain	Doubling time[a] (min) on substrate:			
	Agar	Gellan	Xanthan	BC
P. aeruginosa PA01	76 ± 14	120 ± 17	70 ± 26	**47 ± 6**
E. coli BW25113	**38 ± 4**	46 ± 2	40 ± 2	41 ± 7
E. tarda	58 ± 18	71 ± 8	39 ± 12	**39 ± 11**
S. boydii	**48 ± 2**	73 ± 6	48 ± 3	52 ± 6
S. saprophyticus	81 ± 8	129 ± 11	**72 ± 3**	81 ± 3
K. pneumoniae	31 ± 3	43 ± 3	33 ± 4	**28 ± 4**
A. tumefaciens GV3101	**89 ± 6**	91 ± 9	101 ± 5	99 ± 8
A. rhizogenes Arqual	114 ± 5	115 ± 7	**103 ± 9**	118 ± 14
S. meliloti Rm2011 (wild type)	227 ± 12	**79 ± 10**	133 ± 23	107 ± 9
S. meliloti Rm2011 (Δ*nodA*)	186 ± 21	178 ± 19	125 ± 10	**103 ± 8**
Agrobacterium sp. IRBG74 (wild type)	75 ± 6	86 ± 8	**69 ± 2**	80 ± 10
Agrobacterium sp. IRBG74 (Δ*nodA*)	101 ± 24	179 ± 68	72 ± 3	**69 ± 3**
S. meliloti USDA6489	111 ± 8	**93 ± 15**	119 ± 10	129 ± 10
B. japonicum USDA110	285 ± 15	321 ± 13	**277 ± 38**	293 ± 46

[a] The values represent the doubling times (means and standard deviations) for the growth of each strain on the substrates. The shortest doubling time for each strain is highlighted in boldface to indicate the substrate on which growth was maximized.

ties for the introduction of other biocompatible polymers that stimulate cell growth and decrease cell culture time. Polysaccharides produced by bacteria—namely, gellan, xanthan, and Eladium—have attracted attention as substrates for microbial cell culture (4, 8, 9), as these exopolysaccharides support cell growth and facilitate colony formation. The omission of BC from the list of exopolysaccharides produced by bacteria that have been explored in the context of cell culture substrates is surprising and motivated us to test its properties.

BC is an exopolysaccharide produced by *A. xylinum* with interesting physical properties that made it an attractive substrate for mammalian cell biology studies (16, 17). BC hydrogels with centimeter length scales are produced as a pellicle by *A. xylinum* after fermentation for 5 to 7 days. Conveniently, the gel accumulates at the air-liquid interface and is easy to remove. Unlike other polysaccharides produced by bacteria, BC does not require extraction, purification, and solidification, which simplifies its production and reduces costs for its preparation for microbial cell culture. BC is stable at high temperatures (~300°C), to a broad range of pH values, and in various organic solvents (25, 26). Although it is slightly more opaque than agar and agarose, BC is suitable for imaging studies, as cells growing on the surface are easily observed by microscopy.

We tested 14 different bacterial strains that were selected randomly and demonstrated that most of these strains produce more cells and have higher growth rates on BC hydrogels than on other polysaccharides used as substrates for bacterial growth and studies. As the physical properties of the environment are recognized to play a role in bacterial behavior (24, 25, 27, 28, 32), we investigated two factors that may produce differences in cell numbers and growth rates on each substrate. We measured *E*—the parameter most commonly used to characterize the stiffness of a material—and the diffusion rate of fluorescein—as a surrogate of nutrients—for agar, gellan, xanthan, and BC substrates. The results demonstrate that the growth rate of bacterial strains is independent of substrate stiffness and may depend on the diffusion rate of nutrients through the substrates for most test cells (Fig. 2 and 3).

BC has a three-dimensional nanofiber network structure that is different from those of agar, gellan, and xanthan, which may influence the rate of nutrient and metabolite diffusion and provide a unique mechanical environment. *A. xylinum* extrudes nanofibrils of crystalline BC, which assemble into ribbons that have characteristic dimensions: 40 to 60 nm wide and 1 to 10 μm long. The presence of nanoscale ribbons in BC creates a polymer with a large surface area and provides numerous channels for the transport of ions and small molecules (28, 29), which may increase the delivery of growth factors to cells and enhance growth rates.

A variety of cellular changes are triggered when bacteria are inoculated on substrates to facilitate cell attachment to surfaces and influence their growth (6, 30). To test whether changes in cell phenotypes may be responsible for differences in cell numbers and growth rates for each substrate, we imaged *S. meliloti* Rm2011 (Δ*nodA*) growing on the surface of each substrate after 4 h of incubation using phase-contrast microscopy (Fig. 4). We chose *S. meliloti* Rm2011 as a model organism, as it displayed the largest differences in doubling rate ($t_{1/2}$ = 103 to 186 min) and cell numbers on polymer substrates (6×10^7 to 47×10^7 cells). With the exception of higher densities of *S. meliloti* Rm2011 cells on BC surfaces, we found no obvious differences in cell phenotypes on the substrates. Interestingly, we observed up to ~50% of *S. meliloti* Rm2011 cells on the surfaces of BC substrates partly penetrating into the hydrogel (see Fig. S1 in the supplemental material). The cells penetrated less than ~2 μm into the gel. Apparently, the porous nanofiber structure of BC provides a network of channels that may facilitate attachment to surfaces, improve mass transport of growth factors and metabolic waste, and facilitate cell growth.

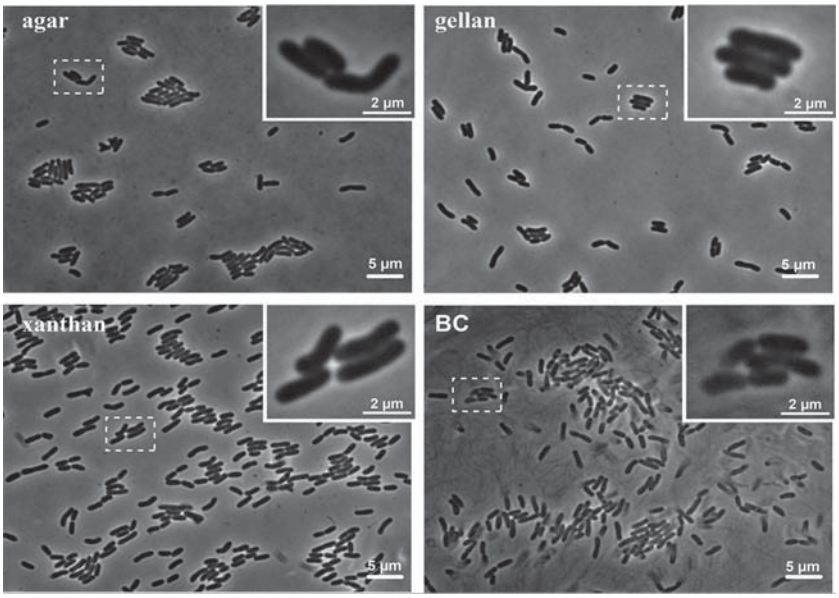

FIG 4 Microscopy images of cells of *S. meliloti* strain Rm2011 (Δ*nodA*) after growth on each substrate for 4 h. We pipetted 110 μl of a suspension of cells of *S. meliloti* strain Rm2011 (Δ*nodA*) on the substrate surfaces, incubated it at 30°C for 4 h, and imaged cells using phase microscopy. The insets are higher-magnification images of the cells in the dashed boxes.

In summary, the introduction of BC polymers infused with nutrient media expands the limited repertoire of substrates available for bacterial cell culture. Although BC is not "tunable" in the same sense as agar, gellan, and xanthan, which can be prepared at a range of concentrations and by admixing other soluble polymers, *A. xylinum* mutants may be used or engineered to produce BC with different physical properties. The simplicity and reproducibility with which substrates can be prepared—and the enhancement of bacterial growth on these materials—suggest that BC may open new doors in microbiology, including culturing organisms that have been recalcitrant to culture and isolation and facilitating medical microbiology by decreasing the time to culture and detection.

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