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Alternative Environmental Roles for Cellulose Produced by *Acetobacter xylinum*

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The cellulose-producing bacterium *Acetobacter xylinum* has been considered a strict aerobe, and it has been suggested that the function of cellulose is to hold cells in an aerobic environment. In this study, we showed that *A. xylinum* is capable of growing microaerophilically. Cellulose pellicles provided significant protection to *A. xylinum* cells from the killing effects of UV light. In experiments measuring colonization by *A. xylinum*, molds, and other bacteria on pieces of apple, cellulose pellicles enhanced colonization of *A. xylinum* on the substrate and provided protection from competitors which use the same substrate as a source of nutrients. Cellulose pellicles produced by *A. xylinum* may have multiple functions in the growth and survival of the organism in nature.

Acetobacter xylinum is a gram-negative, aerobic bacterium that secretes cellulose fibrils as part of its normal metabolic activity (for a review, see reference 6). The substrate for cellulose synthesis is glucose, which is the major constituent of the medium in which *A. xylinum* is cultivated. Microorganisms within the genus *Acetobacter* are obligate aerobes usually found on fruits and vegetables and in vinegar, fruit juices, and alcoholic beverages. *A. xylinum* has been reclassified as *Acetobacter hansenii* (11). However, the new species name has not been used widely in recent literature; *A. xylinum* will be used here. *A. xylinum* is typically found in nature on decaying fruit. When *A. xylinum* is cultivated statically in broth culture in the laboratory, a pellicle of cellulose is formed and cells of the bacterium are trapped within it. These pellicles can vary in thickness and tensile strength, depending on which strain of *A. xylinum* is cultivated. When a broth culture is shaken or stirred, *A. xylinum* grows rapidly; however, less cellulose is produced, and the cellulose fibrils do not form a well-organized pellicle. In a rotatory shaker, round balls of cellulose form in the broth. The cellulose pellicle is composed of bundles of cellulose microfibrils that are extruded through pores located in the lipopolysaccharide layer of *A. xylinum*. These cellulosic ribbons intertwine to form the pellicle (8). The enzyme apparently responsible for polymerization of glucose, cellulose synthetase, has previously been shown to be associated with the cell membrane of *A. xylinum* (1).

It has been proposed that cellulose serves to hold the bacterium in an aerobic environment (3, 15), probably on the surface of the decaying fruit that it uses for nutrients. In this report, we describe experiments that suggest possible alternative roles for cellulose. We propose that cellulose may act as an extracellular matrix to enhance colonization by the bacterium of its substrate in nature and to prevent potential competitors from gaining access to the decaying substrates that *A. xylinum* uses for nutrients. Another possible role for cellulose may be to provide protection for the cells from damage by UV light. In addition, the pellicle may also aid in moisture retention to prevent drying of the natural substrates for *A. xylinum* while the bacterium is growing on them. Our results raise the possibility that the environmental role of cellulose production by *A. xylinum* is more complex than was previously recognized.

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MATERIALS AND METHODS

Bacteria. The wild-type strain *A. xylinum* ATCC 23769 was generously provided by R. Malcolm Brown, Jr., of the Botany Department at the University of Texas at Austin. The cellulose-negative strain of *A. xylinum* (S. Anderson, G. Keesler, J. Warren, and R. Cannon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H-70, p. 156) was selected after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of the wild-type strain (17). The strain is stable and nonreverting after 3 years of cultivation. A cellulose-overproducing strain designated Thick (an *A. xylinum* isolate from nature) was also generously provided by R. M. Brown, Jr.

Culture conditions. Bacteria were cultured in Schramm and Hestrin medium (9), either in broth or in solid medium at 25°C. The solid medium contained 0.01% Tinopal (CIBA-GEIGY Corp.), which is a fluorescent brightener that causes cellulose-producing colonies of *A. xylinum* to fluoresce under UV light (12). Molds were assayed by plating on Sabouraud dextrose agar (Difco Laboratories). Other bacteria were grown on tryptic soy agar (Difco). Cultures of *A. xylinum* were grown on shakers and statically. Standing broth cultures in petri dishes were used to produce cellulose pellicles for colonization experiments. Petri dishes (60 by 15 mm) were filled with 5 to 7 ml of broth; larger petri dishes (100 by 15 mm) were filled with 10 to 12 ml of broth. The broth was inoculated with 10^6 to 10^7 cells per ml. The pellicles used in the experiments formed after 7 days of incubation.

To examine the effect of various oxygen regimes on growth, *A. xylinum* cultures were incubated in candle jars, in jars gassed with 5% carbon dioxide and air, and in Bio-Bags (Marion Scientific) that are designed to provide microaerophilic conditions. Under these conditions, oxygen concentration was approximately 6%, compared with the 21% oxygen concentration in ambient air. The bacterial growth and cellulose production of aerobic and microaerophilic cultures were compared by colony counts and dry weight

determinations of cellulose pellicles produced after 7 days of incubation.

Colonization experiments. Slices of apple (Golden Delicious variety) weighing between 15 and 20 g were washed with equal volumes of 70% alcohol and sterile distilled water. The apples slices were composed of both skin and pulp but had been cored. The slices were placed in sterile, covered 250-ml beakers. Samples (1 g each) of apple including both skin and pulp were shaken in 9 ml of phosphate-citric acid buffer (10) with glass beads to determine via standard plate count whether *A. xylinum*, molds, or other bacteria were present on the fruit naturally. Molds were assayed by plating on Sabouraud dextrose medium. Other bacteria were assayed by plating on Schramm and Hestrin medium containing Tinopal and on tryptic soy agar plates.

For the colonization experiments, either Thick or wild-type pellicles were aseptically placed over individual slices of apple. The cellulose pellicles had been formed in petri dishes (100 by 15 mm). Also, 3-ml portions of shaken broth cultures from wild-type, Thick, or cellulose-negative strains were pipetted over individual slices of apple (10^6 cells per ml). Uninoculated apple slices served as controls. All apple samples were incubated at 25°C and observed frequently to note any differences among the various samples. After 2 weeks of incubation, the experiment was terminated. Pellicles were removed from apple slices, and 1-g apple samples were taken from both control and experimental apples to be assayed for *A. xylinum*, molds, and other bacteria. The samples were taken from an area previously covered by pellicles.

In a second series of colonization experiments, the same protocol was followed except that the apple slices were not surface sterilized.

In a third series of colonization experiments, 1-g samples of apple were covered with broths from the various cultures or pellicles formed in petri dishes (60 by 15 mm) and were processed as described above.

Whole Golden Delicious apples were also used in colonization experiments. Controls were apples that had been rinsed with sterile distilled water before being placed in sterile beakers. Experimental apples were washed as the controls had been. Apples were placed in sterile beakers and inoculated individually with 7 ml of broth cultures (10^7 cells per ml) from Thick, wild-type, or cellulose-negative strains, a volume sufficient to moisten the whole apple. After 7 days of incubation, 1-g samples were taken from all the apples and assayed for *A. xylinum* and other microorganisms.

UV-light experiment. Cellulose pellicles produced by the wild-type strain were formed in 10 ml of broth in small petri dishes after 7 days of incubation. Wet pellicles averaged 1 mm in thickness. Broth culture (5 ml) of wild-type *A. xylinum* at a density of 10^7 cells per ml was pipetted into the bottom of a small petri dish. A preformed pellicle was placed aseptically over the broth. Samples were exposed to a germicidal UV light (MR-4; George Gates and Co.) with a G8T5 bulb (General Electric Co.) placed 15 cm from the surface of the pellicle for 1, 15, 30, and 60 min. After irradiation, the pellicle was removed and the broth below the pellicle was assayed for viable cells. Broth cultures irradiated without pellicles and control cultures not treated by UV light were sampled and assayed at the same times.

Determinations of wet and dry weights of pellicles. Pellicles were formed in small petri dishes (60 by 15 mm) containing 10 ml of broth medium and inoculated with 1 ml of *A. xylinum* at 10^7 cells per ml. The plates were incubated at room temperature for 7 days. To determine the wet weight of a pellicle, the pellicle was lifted from the plate with a bent glass rod and allowed to drip for 30 min; the pellicle was then placed in a preweighed plastic weighing boat. The weighing boat and pellicle were then placed in a drying oven for 6 h at 80°C. After being dried and removed from the oven, the weighing boat and pellicle were weighed immediately. Pellicles were also washed with running tap water prior to the wet and dry weight determinations. There were no percentage differences between the wet and dry weight comparisons of pellicles that had been washed before being allowed to drip and the wet and dry weight comparisons of pellicles that had not been washed before being allowed to drip for 30 min.

RESULTS

A. xylinum grew well microaerophilically in a candle jar, a jar gassed with a mixture of 5% carbon dioxide and air and tightly sealed, and a Bio-Bag. The Bio-Bag provides an immediate CO_2-enriched atmosphere based on a reaction between hydrochloric acid and sodium bicarbonate and has been shown to permit growth of fastidious organisms like *Neisseria gonorrhoeae* (2). Growth rates under these conditions of limited oxygenation were not significantly different from those of aerobic controls in either standing or shaking cultures (measured by daily plate counts). There was no significant difference in the amounts of cellulose produced (assayed by dry weight determination) under either microaerophilic or aerobic conditions. Though it may be a strict aerobe, *A. xylinum* can be grown under environmental conditions of reduced oxygen. Attempts to culture *A. xylinum* under anaerobic conditions were unsuccessful.

Colonization experiments in which surface-sterilized apple samples were covered with preformed pellicles from wild-type and Thick strains of *A. xylinum* and portions from broth cultures of the same strains and of a cellulose-negative mutant were devised. After incubation for 7 to 14 days, the pellicles were removed and the apples were assayed for *A. xylinum*, molds, and other bacteria. Before a quantitative assessment of the effects of pellicles on the surface of apple slices is presented, qualitative observations of the differences among the various preparations will be described. Controls that received surface sterilization before being placed in the sterile beakers dried out over the course of each experiment and showed little or no macroscopic mold or bacterial growth. Samples inoculated with broth culture from the cellulose-negative strain also dried over the course of the experiment and supported luxuriant mold growth. Samples that received portions of either wild-type or Thick pellicle-producing broth cultures remained moist throughout the incubation period. After approximately a week of incubation, apple slices displayed macroscopic cellulose pellicle formation. Mold contamination was observed occasionally, although it was not as extensive as that seen on the apple slices inoculated with the cellulose-negative strain. Samples covered with either wild-type or Thick pellicles remained moist. Apple slices covered completely by pellicles never appeared macroscopically colonized by molds or other microorganisms. When the apples were not surface sterilized, there was mold growth on all samples; slices of apple covered by pellicles had less growth than other slices did.

When either Thick or wild-type pellicles covered the 1-g apple slices, the density of *A. xylinum* on the apple after 1 week of incubation was greater than the density of non-*A. xylinum* organisms, which included both molds and other bacteria. When apples were covered by Thick or wild-type pellicles, *A. xylinum* predominated (Fig. 1). When broth

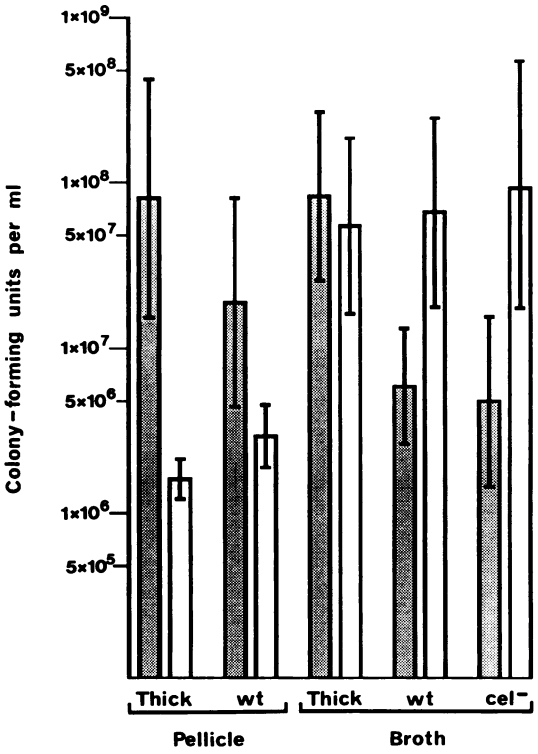

FIG. 1. Cell densities of *A. xylinum* versus non-*A. xylinum* organisms for 1-g samples of apple covered by Thick pellicle, wild-type (wt) pellicle, Thick broth culture, wild-type broth culture, and cellulose-negative (cel$^-$) broth culture and incubated at room temperature for 2 weeks. Uninoculated, alcohol-sterilized controls showed little or no growth of either *A. xylinum* or non-*A. xylinum* organisms. Non-*A. xylinum* organisms include molds and other bacteria. Symbols: ▨, *A. xylinum*; □, non-*A. xylinum* organisms; bars, standard errors of the mean. Values shown are means of six replicates.

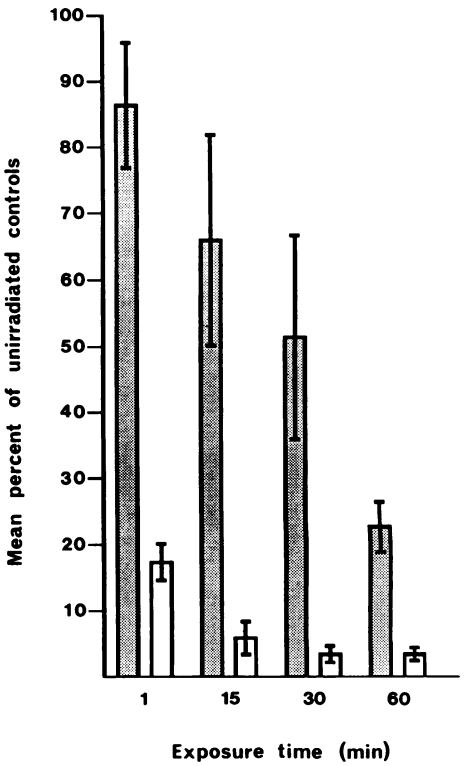

FIG. 2. Effects of UV light on viability of *A. xylinum* with and without cellulose pellicle protection. Pellicles formed during 1 week of incubation before being used. Unirradiated control samples taken at 1, 15, 30, and 60 min showed no changes in cell densities over the course of replicate experiments and were normalized to 100% in each experiment. Values shown are from five replicates. Symbols: ▨, UV exposure with *A. xylinum* pellicle; □, UV exposure without *A. xylinum* pellicle; bars, standard errors of the mean.

cultures were inoculated over the apple slices, the Thick strain and non-*A. xylinum* organisms grew equally well; however, when wild-type and cellulose-negative broths were inoculated over the apple slices, non-*A. xylinum* organisms proliferated. Uninoculated, alcohol-sterilized controls showed little or no growth (less than 10^2 CFU/g of apple sampled) of either *A. xylinum* or non-*A. xylinum* organisms.

When whole apples were tested for colonization by *A. xylinum*, uninoculated controls showed no growth of *A. xylinum* and very low cell counts of non-*A. xylinum* microorganisms. Control samples dried out, unlike experimental samples. Experimental samples that were inoculated with either wild-type or Thick broth had pellicles forming after 1 week of incubation. Apples inoculated with cellulose-negative broth had a film of growth but were drier than were apples covered by newly forming pellicles. Quantitatively, these results were comparable to those obtained with assays of 1-g apple samples (data not shown).

The cellulose pellicle had a substantial effect on the susceptibility of *A. xylinum* to UV light. Figure 2 shows the results of exposing *A. xylinum* to UV light for various time intervals with and without a pellicle. Data are presented as the mean percentage of unirradiated controls, with five replicates for each UV-light exposure time. During 60 minutes of UV-light exposure, the mean percent viable cells of *A. xylinum* protected by pellicles declined from 100 to 22.8% of unirradiated controls, while cell densities of unprotected samples dropped from 100 to 3.15%.

The dry weights of cellulose pellicles averaged 4% of the wet weights of pellicles. These results are based on measurements of 25 pellicles.

DISCUSSION

Cellulose microfibrils that have formed pellicles are quite strong and resilient. When wet, the pellicles resist tearing; when dry, they have the consistency of paper. The cellulose produced by *A. xylinum* is structurally identical to that found in eucaryotic plants. One role of cellulose may be to hold bacteria in the aerobic environment (3, 15). However, our results show that *A. xylinum* can grow and produce cellulose under microaerophilic conditions, which suggests that a strictly aerobic environment is not required by this organism.

In our experiments, apple slices covered by pellicles remained moist, unlike uninoculated controls or samples that were treated with small portions of liquid culture. The cellulose pellicles can hold considerable amounts of water, since the dry weights of the pellicles were only 4% of the wet weights. Perhaps cellulose serves to retain moisture near the bacterial cells as they decompose rotting fruit. Because of their opacity, pellicles also provided significant protection from the damaging effects of UV light.

Another role of cellulose might be to enhance colonization of rotting substrates by *A. xylinum*. When broth cultures of wild-type and Thick strains were inoculated on apple slices, macroscopically visible pellicles began to form within a week. Apple slices inoculated with a cellulose-negative strain dried during the period of incubation and showed growth of other organisms. A possible mechanism for enhancement of colonization by cellulose synthesis might involve cellulose-mediated attachment of the bacteria to the substrate. For *Agrobacterium tumefaciens*, another cellulose-synthesizing bacterium, cellulose synthesis is an important part of successful attachment to cultured carrot tissue (13, 14). Cellulose fibrils have also been shown to enhance flocculation of gram-negative bacteria isolated from activated sludge (5). The cellulose pellicle may also reduce the opportunity for organisms other than *A. xylinum* to compete successfully for a limited resource, i.e., rotting fruit. Our experiments showed that under certain conditions, cellulose pellicles can prevent or reduce colonization of a natural substrate (apple slices) by molds and other bacteria. When cellulose pellicles or Thick broth culture were placed on pieces of apple, *A. xylinum* predominated; however, when broth cultures of wild-type and cellulose-negative strains were placed on the apples, molds and other bacteria flourished and *A. xylinum* grew poorly. Our apple sterilization procedures were quite effective, since there were usually no bacteria or molds detected on uninoculated controls. When whole apples were used as experimental substrates, the absolute numbers of bacteria and molds on the surfaces of the apples were reduced. Apparently, unbroken skin is an effective barrier to colonization of apples by microorganisms. In the whole-apple experiments, pellicles began to form with wild-type and Thick broths, whereas less growth was detected with the cellulose-negative strain. Preliminary studies (data not shown) have shown that *A. xylinum* was rarely isolated from apples still on the tree but was found frequently on apples on the ground that were beginning to decompose.

There are innumerable decomposers found in the soil, waiting for substrates to consume. In order to be successful, these saprophytes must find and hold on to limited resources in nature. We recognize that the results which we have obtained and the conclusions which we have drawn may not reflect events that occur in microhabitats in which interactions between organisms are complex and in which diverse abiotic factors can play much more complicated roles than can be controlled or manipulated in the laboratory. In nature, *A. xylinum* may not reach a density equivalent to that used in our experiments and may not form pellicles with the same thicknesses and densities as those formed by laboratory-grown cultures. Thompson et al. (16) observed with scanning electron microscopy that cellulose microfibrils produced by *A. xylinum* formed tunnel-shaped structures with diameters large enough for bacteria to move within them. These structures could have many environmental roles, assuming that they are formed by organisms growing in the natural environment.

An additional complication in hypothesizing roles for cellulose in the growth of *A. xylinum* in the environment is that variations in environmental conditions may actually affect the levels of cellulose synthesis by this organism. Previous investigators (3, 15) have reported isolation of cellulose-negative or cellulose-deficient mutants by cultivating *A. xylinum* with vigorous aeration (shaking). These mutants revert to wild-type at a high rate, although cellulose-negative mutants that have been selected through chemical mutagenesis are quite stable. We have observed similar transient cellulose negativity when wild-type *A. xylinum* was cultivated on a defined, minimal medium (E. R. Forng, S. M. Anderson, and R. E. Cannon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I-18, p. 175). These are not mutants in the strict genetic sense of the term but are more likely cells that have turned off cellulose synthesis to direct metabolic activity elsewhere. Dudman (7) demonstrated that ethanol could stimulate cellulose production by *Acetobacter acetigenum* if other carbon sources were also available. Ethanol and other volatile substrates would normally be present in decaying fruit and could have a natural, stimulatory effect on cellulose biogenesis by *A. xylinum*.

The ability to colonize surfaces is vital to the environmental success of many microorganisms, both pathogenic and saprophytic. The formation of biological films (biofilms) may enhance resistance to environmental hazards including drying, pH changes, and toxic chemicals (4). Many of the chemicals that play roles in the formation of extracellular matrixes are polysaccharides. Cellulose is an extracellular polymer that is well organized structurally by glucan linkages. Cellulose might not be considered a biological film or adherence exopolysaccharide in the strictest sense, but it may play a similar environmental role in protecting the organisms from dessication and UV irradiation, in promoting colonization, and in aiding competition for substrates.

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