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The curdlan-type exopolysaccharide produced by *Cellulomonas flavigena* KU forms part of an extracellular glycocalyx involved in cellulose degradation

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Abstract

The genus *Cellulomonas* is comprised of a group of Gram-positive, soil bacteria capable of utilizing cellulose as their sole source of carbon and energy. *Cellulomonas flavigena* KU was originally isolated from leaf litter and subsequently shown to produce large quantities of a curdlan-type (β-1,3-glucan) exopolysaccharide (EPS) when provided with an excess of glucose or other soluble carbon-source. We report here that curdlan EPS is also produced by *Cellulomonas flavigena* KU when growing on microcrystalline cellulose in mineral salts-yeast extract media. Microscopic examination of such cultures shows an adherent biofilm matrix composed of cells, curdlan EPS, and numerous surface structures resembling cellulosome complexes. Those *Cellulomonas* species that produce curdlan EPS are all non-motile and adhere to cellulose as it is broken down into soluble sugars. These observations suggest two very different approaches towards the complex process of cellulose degradation within the genus *Cellulomonas*.

Abreviations: CMCase – carboxymethyl cellulase; EPS – exopolysaccharide; PTYE – peptone-tryptone-yeast extract medium

Of the many roles frequently proposed for bacterial exopolysaccharides (EPSs), the adhesion of cells to surfaces is one of the most common. EPSs are frequently associated with other extracellular materials in the form of a glycocalyx surface layer (Costerton and Irvin 1981). Glycocalyces often have a net negative charge due to the presence of anionic polysaccharides (Sutherland 1990) or proteinaceous materials, and can typically be visualized by electron microscopy when stained with cationic stains such as ruthenium red or cationized ferritin. These surface layers are often responsible for the adherence of cells to each other, causing the formation of flocs or aggregates of bacteria, and for the adherence of cells to solid surfaces, resulting in the formation of adherent microcolonies which eventually develop into larger biofilms (Costerton 1987). In nature, the biofilm mode of growth is usually the predominant one and is potentially important in the digestion of insoluble substrates such as cellulose (Costerton and Irvin 1981; Costerton 1987). Encapsulated biofilm residents are believed to be protected from harmful biological and chemical agents. Within biofilms, the glycocalyx layer may function like an ion-exchange resin to concentrate nutrients from the surrounding aqueous environment and/or to trap nutrients such as cellulose breakdown

products (Costerton 1987). As an example, rumen bacteria such as *Fibrobacter succinogenes* (Groleau and Forsberg 1981; Gong and Forsberg 1989), *Ruminococcus albus* (Patterson et al. 1975), and *Ruminococcus flavefaciens* (Latham et al. 1978) all grow in very close association with cellulose fibers while producing cell-bound cellulases associated with a glycocalyx-type of surface layer.

Lamed et al. (1987) showed that several cellulose degrading bacteria, including *Cellulomonas uda* (ATCC 21399), produce bulbous surface protuberances resembling the cellulosome structures first discovered on the surface of *Clostridium thermocellum* (Lamed et al. 1983). Cellulosomes are large protein complexes containing both adhesive and enzymatic peptide components involved in the multi-step process of cellulose degradation (Mayer et al. 1987; Béguin et al. 1992; Felix and Ljungdahl 1993; Bayer et al. 1998; Doi et al. 2003). These structures are often visualized by electron microscopy using cationized ferritin and are sometimes associated with EPSs. Thus, cellulosomes are, in some cases, part of a complex glycocalyx layer on the surface of the bacterial cell.

Cellulomonas flavigena strain KU (ATCC #53703) was isolated by Angelo et al. (1990) as the result of a search for cellulose degrading soil bacteria. When cultures of this isolate were grown in minimal medium containing an excess of glucose and a growth-limiting amount of ammonium chloride (high C/N source ratio), the total amount of dry biomass obtained from the culture was much greater than that from cultures grown in tryptone broth (Voepel and Buller 1990). It was discovered that most of this difference in weight was due to the carbohydrate content of the biomass. Phase-contrast microscopy of cultures grown in the high C/N media revealed large cellular aggregates and transmission electron microscopy (TEM) showed the presence of a thick capsule (Buller and Voepel 1990). A water-insoluble polysaccharide could be isolated from the cellular aggregates by extraction with 1 N NaOH followed by acid precipitation (Buller and Voepel 1990; Kenyon and Buller 2002). The purified EPS had solubility and physical properties very similar to the β-1,3-D-glucan known as curdlan (Harada et al. 1966, 1968; Saito et al. 1968) and was later proven to be identical in structure to curdlan (Kenyon and Buller 2002).

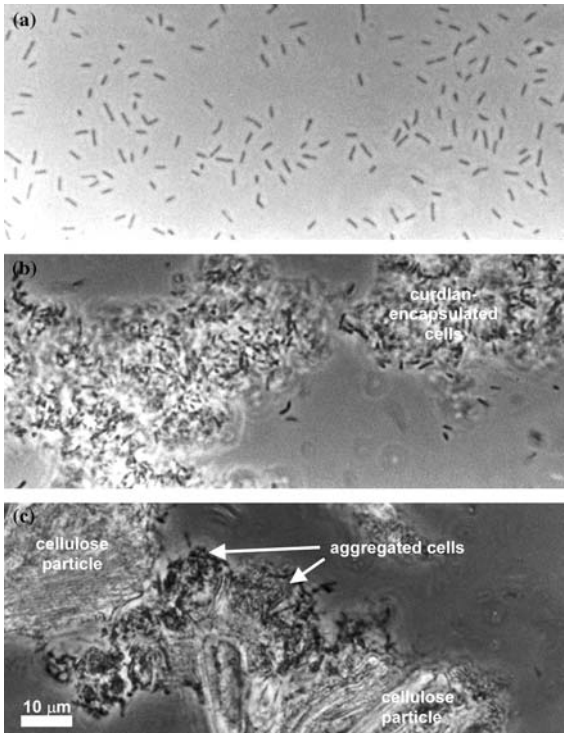

Figure 1. Phase-contrast micrographs of *Cellulomonas flavigena* KU grown on glucose and microcrystalline cellulose. Non-aggregated *C. flavigena* KU cells grown for 1 day in M9-yeast extract medium containing 0.4% glucose (a), aggregated cells grown for 3 days in M9-yeast extract medium containing 1% glucose (b), and aggregated cells grown for 4 days in M9-yeast extract medium containing 1% Avicel microcrystalline cellulose (c). All cultures were grown aerobically at 30 °C. The bar represents 10 μm for all three panels.

Curdlan EPS can also be extracted from cultures grown in M9 minimal medium supplemented with 0.05% yeast extract (Angelo et al. 1990; Buller and Voepel 1990) and containing 1% microcrystalline cellulose (Avicel, FMC Corporation) as the only source of carbon and energy. When such cultures are viewed by phase-contrast microscopy, the cellular aggregates typically associated with curdlan EPS production can be seen (compare Figure 1c to a and b). We unexpectedly discovered that most of these aggregates appeared to be very closely associated with microscopic particles of cellulose. When observed over the course of several days, the cellulose particles in these cultures were first colonized at their rough ends where the cellulose fibers are presumably more amorphous and easier to digest. The cellulose particles were then split into progressively smaller pieces held together by

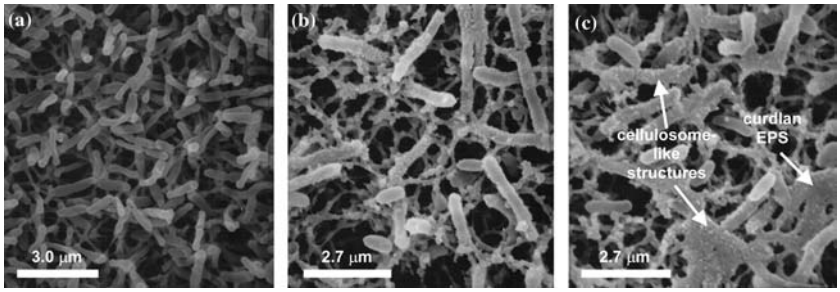

Figure 2. Scanning electron micrographs of *Cellulomonas flavigena* KU grown on soluble substrates. Non-aggregated *C. flavigena* KU cells grown for 3 days in PTYE medium (a), aggregated cells grown for 3 days in M9-yeast extract medium containing 1% glucose (b), and aggregated cells grown for 3 days in M9-yeast extract medium containing 1% cellobiose (c). All cultures were grown aerobically at 30 °C. Scanning electron microscopy was performed as described by Lamed et al. (1987).

extensive aggregation. As mentioned above, transmission electron micrographs of thin sections of encapsulated *C. flavigena* KU had previously revealed the presence of a polyanionic glycocalyx layer (Voepel and Buller 1990). These facts prompted the further examination of aggregates by scanning electron microscopy, using the stain cationized ferritin (Lamed et al. 1987).

Scanning electron micrographs of *C. flavigena* KU grown in the nutrient rich peptone-tryptone-yeast extract (PTYE) medium (Vladut-Talor et al. 1986; Atlas 1993), with or without cellulose, show bacterial cells with very smooth surfaces (Figures 2a and 3a). In contrast, cultures grown in M9-yeast extract medium, containing either 1% glucose (Figure 2b), cellobiose (Figure 2c), or Avicel microcrystalline cellulose (Figure 3b and 3c), show cells encased in a curdlan EPS matrix. In cultures grown on cellulose, most of the cells are attached to the microscopic cellulose particles as encapsulated microcolonies. The surfaces of these microcolonies are covered with many surface protrusions very similar in size and appearance to cellulosomes (Lamed et al. 1987). It is possible that these polyanionic, and presumably proteinaceous, cellulosome-like surface structures are responsible for the electron-dense layer surrounding the curdlan EPS capsule seen in the transmission electron micrographs of Voepel and Buller (1990). These structures are likely denatured during preparation of samples for TEM.

Similar observations were made by Vladut-Talor et al. (1986) while studying the growth of *Cellulomonas* sp. (NRCC 2406) on different types of cellulose. They discovered a sudden fall in colony forming units accompanied by macroscopic aggregation of cells on the second day of incubation. Phase-contrast microscopy of such cultures

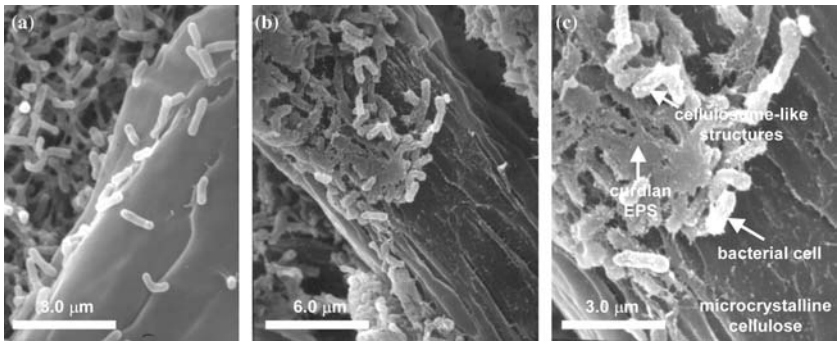

Figure 3. Scanning electron micrographs of *Cellulomonas flavigena* KU grown in the presence of microcrystalline cellulose. Non-aggregated *C. flavigena* KU cells grown for 3 days in PTYE medium containing 1% Avicel cellulose (a) and aggregated cells grown for 4 days in M9-yeast extract medium containing 1% Avicel cellulose (b). A higher magnification of (b) is shown in (c). All cultures were grown aerobically at 30 °C. Scanning electron microscopy was performed as described by Lamed et al. (1987).

revealed cells which were ''closely associated with cellulose fibers; some seemed to be growing within the fibers, or to form long strands in which the fibers were trapped''. When glucose was added to these cellulose cultures it prevented attachment of the cells to cellulose. In a similar fashion, cultures of *C. flavigena* KU grown in M9-yeast extract media containing an excess of both glucose and cellulose resulted in cellular aggregates which were not associated with microcrystalline cellulose particles (William J. Kenyon, unpublished data). Using TEM, Vladut-Talor et al. (1986) observed a thick outer layer surrounding cellulose-grown cells. Like the layer on the outer surface of encapsulated *C. flavigena* KU cells, this layer stained well with ruthenium red, suggesting that it was polyanionic, and was described by them as a glycoprotein glycocalyx involved in attachment of the cells to cellulose. It is not known how closely related *Cellulomonas* sp. (NRCC 2406) is to *C. flavigena* KU, but the similarities observed in the process of cellulose degradation are strikingly similar.

Involvement of such glycocalyces in the adhesion of cells to cellulose and in the degradation of cellulose is not restricted to members of the genus *Cellulomonas*. For example, *Ruminococcus albus* was shown by Patterson et al. (1975) to form a polysaccharide-containing coat layer which stained with ruthenium red and seemed to mediate attachment of the cells to cellulose, and Latham et al. (1978) showed that *R. flavefaciens* produced a periodate-reactive (carbohydrate containing) coat layer which was also involved in adhesion to cellulose fibers. In addition, *R. albus* was shown to produce cellulosome-like protuberances on its cell surface when grown on cellobiose (Lamed et al. 1987).

It should be noted that the curdlan glycocalyx produced by *C. flavigena* KU also forms during growth on other insoluble substrates. For example, scanning electron micrographs of cells growing on hemicellulosic polysaccharides such as xylan are very similar in appearance (Chen 1995), and the cellular aggregates that form in these cultures are also closely associated with the insoluble growth substrate. This is perhaps not surprising considering the fact that cellulosomes of *Clostridium thermocellum* have been shown to contain multiple glycanase activities (Bayer et al. 1998).

Table 1. Distribution of total protein and carboxymethyl-cellulase (CMCase) activity in *Cellulomonas flavigena* KU cultures grown on microcrystalline cellulose

Culture fraction	% of total protein	% of total CMCase activity
Aggregated biomass and cellulose particles (1)–(2)	58.6	96.4
Free-floating cells (2)	2.50	0.375
Cell-free (3)	38.9	3.50

C. flavigena KU was grown in M9 medium containing biotin and thiamine (1 μg/ml each) to support growth and 1% microcrystalline cellulose (Avicel, FMC Corporation). Cultures were incubated aerobically at 30 °C for 5 days, and then divided into three fractions. Culture fraction (1) was simply a sample of the whole culture. Culture fraction (2) was obtained by allowing aggregated biomass and cellulose particles to sediment, and then removing a sample from the supernatant fluid. Culture fraction (3) was the cell-free supernatant obtained after centrifugation of culture fraction 2 ($14{,}500 \times g$ at 4 °C for 10 min). The amount of total protein (Markwell et al. 1978) and CMCase activity (Wood and Bhat 1988) was then determined for each fraction. Results from a representative experiment are shown.

These observations suggest that the curdlan EPS produced by *C. flavigena* KU probably serves more than one physiological function for this organism. In addition to its role as an extracellular carbon and energy reserve (Voepel and Buller 1990), it appears to be part of a complex glycocalyx layer involved in the establishment of bacterial microcolonies that adhere to cellulose and hemicellulosic materials. The biofilm that forms appears to sequester cellulose breakdown products because very little soluble sugar is released into the culture supernatant. In addition, this mode of growth effectively concentrates most of the cellulase activity at the substrate surface. In fact, over 96% of the total cellulase activity in cellulose grown cultures is associated with macroscopic aggregates of cells and cellulose particles (Table 1).

Interestingly, all of the *Cellulomonas* species capable of producing curdlan EPS are non-motile and closely adhere to cellulose as it is broken down into sugars that can be transported into the cell (Table 2). In contrast, cells of *Cellulomonas gelida* (ATCC 488), a motile *Cellulomonas* strain, were shown to be present very near cellulose fibers but not to be directly attached to the fibers (Hsing and Canale-Parola 1992). This strain exhibits chemotaxis towards cellobiose as well as other soluble cellulose and hemicellulose breakdown

Table 2. Curdlan production and motility characteristics of cellulomonads

Cellulomonas species	ATCC #	Curdlan production and aggregation in media with high C/N source ratio[a]	Motility[b]
C. flavigena KU	53703	+	−
C. uda	21399	+	−
C. uda	492	+	−
C. flavigena	482	+	−
C. gelida	488	−	+
C. fimi	15724	−	+
C. fimi	484	−	+
C. biazotea	486	−	+

[a] Buller and Voepel (1990) and personal observations.
[b] Stackebrandt and Keddie (1984) and personal observations.

products that are produced by the action of its secreted (cell-free) hydrolytic enzymes. Thus, there appear to be two different modes of cellulose degradation employed by members of the genus *Cellulomonas*. One strategy appears to involve cellulose degradation by firmly attached, curdlan-encapsulated cells possessing cellulosome-type surface complexes. The other approach involves cells which remain close to cellulose because of a chemotactic response to the soluble sugars that are released by the action of their secreted cellulases (Table 2).

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