Utilization of Cellulose Oligosaccharides by *Cellvibrio gilvus*

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ABSTRACT

SCHAFER, MARION L. (Virginia Polytechnic Institute, Blacksburg), AND KENDALL W. KING. Utilization of cellulose oligosaccharides by *Cellvibrio gilvus*. J. Bacteriol. **89**: 113-116. 1965.—The hypothesis that oligosaccharides of the cellulose polymer series can be absorbed by cellulolytic bacteria, prior to hydrolysis to the level of glucose or cellobiose, has been tested. Resting-cell suspensions of *Cellvibrio gilvus* removed oligosaccharides of one to six monomer units from solution at a rate providing the cells with 37×10^6 to 42×10^6 molecules of glucose per cell per minute. There was no concurrent extracellular hydrolysis of the oligosaccharides. The fact that the rate of uptake was constant indicates that an active absorption system is involved. Filtrates from washed-cell suspensions before or after exposure to the oligosaccharides were incapable of hydrolyzing the sugars. In media where the carbohydrate concentration was growth-limiting, the larger members of the oligosaccharide series supported greater final cell densities than the smaller sugars, but there were no recognizable differences in the growth rates during the logarithmic-growth phase.

Recent reviews of microbial utilization of cellulose as an energy and carbon source have considered primarily the extracellular events involved in solubilization of the substrate (Cascoigne and Cascoigne, 1960; King, 1961; Cowling, 1963). Although there are a great many uncertainties regarding the extracellular hydrolysis of cellulose, the notion that the end products for absorption by the cell are glucose or cellobiose is firmly established. Careful review of the literature, however, reveals that degradation to the glucose or cellobiose level prior to absorption is an assumption with no experimental basis.

Because larger chain fragments are demonstrated water-soluble intermediates during cellulose degradation by many microorganisms, the possibility of their absorption, prior to hydrolysis, to glucose or cellobiose seems reasonable. Indeed, the fact that the glucosyl-bond energy is lost to the cell during hydrolysis, but could be conserved by intracellular phosphorylation, makes oligosaccharide absorption seem advantageous. The phosphorylolytic conservation of energy would appear to be particularly likely among bacteria such as *Clostridium thermocellum*, *Ruminococcus flavefaciens* (Sijpesteijn, 1951), and *Cellvibrio gilvus* (Hulcher and King, 1958a) which have been shown to possess an intracellular cellobiose

phosphorylase (Ayers, 1958; Sih and McBee, 1955; Hulcher and King, 1958a, b).

In the present report, the ability of *C. gilvus* to absorb and utilize cellulose oligosaccharides as large as the hexasaccharide has been measured directly, and evidence is presented indicating that the absorption and utilization of the oligosaccharides, without prior extracellular hydrolysis, occur at rates which meet the nutrient demands of both resting and growing cells as well as either glucose or cellobiose does.

MATERIALS AND METHODS

The culture and media used were those described by Hulcher and King (1958a), except that the vitamins were omitted and cellobiose was included at 0.2% (w/v). Routinely and prior to each experiment, the culture was tested for purity by examining Gram-stained smears.

For preparation of resting-cell suspensions, cells from 24-hr broth cultures were washed once by centrifugation ($13,300 \times g$) for 5 min at 25 C in 0.067 M potassium phosphate buffer (pH 7.0). The washed cells were suspended in the same buffer and stirred for 10 min with a magnetic stirrer to disperse clumps. The suspension was diluted with the buffer to give a final Klett-Summerson turbidity value of 250 corresponding to 2.6×10^9 cells per ml; samples of the diluted suspension were placed in 250-ml Erlenmeyer flasks fitted with cotton stoppers. After a 30-min starvation period at 25 C to reduce endogenous reserves, substrates

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FIG. 1. Absorption of oligosaccharides by resting-cell suspensions.

TABLE 1. Failure of resting-cell suspensions and filtrates from resting-cell suspensions to alter the degree of polymerization of oligosaccharides

Oligosaccharide	Observed degree of polymerization			
	Incubated with cells		Incubated with filtrate only	
	0 hr	1 hr	0 hr	1 hr
Cellobiose	2.1*	2.0	1.9	2.0
Cellotriose	3.2	3.1	3.1	2.9
Cellotetraose	3.9	4.2	3.8	4.2
Cellopentaose	5.7	4.9	5.4	5.6
Cellohexaose	5.5	5.4	—	—

* Degree of polymerization is the number of glucose moieties per molecule of oligosaccharide.

were added at a final concentration of $0.5 \mu\text{moles/ml}$ in a total volume of 10 ml. The flasks were then incubated at 25°C on a mechanical shaker. Immediately after addition of oligosaccharide, and at intervals thereafter, samples were removed from the flasks and filtered through a $0.65\text{-}\mu$ Millipore filter. The filtrates were analyzed for sugar by the phenol-sulfuric acid procedure (Dubois et al., 1956) as modified by Timell (1960), and for degree of polymerization (DP) of the sugar by the borohydride reduction procedure (Timell, 1960).

Growth experiments were carried out in the basal medium (Hulcher and King, 1958a) with the following modifications: casein hydrolysate was added as 0.05% of the medium (w/v), and no yeast extract was included. Glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, or cellohexaose was added at a final concentration of 0.03 mmole/ml on a hexose basis. The basal medium was

sterilized by autoclaving for 15 min at 121°C . The vitamin supplement and cellulodextrins were sterilized by filtration through a $0.45\text{-}\mu$ Millipore filter, and added aseptically to the basal medium. Tubes containing 5 ml of the broth were inoculated with 0.1 ml of a dilution (1:50) of a 24-hr broth culture, and incubated at 25°C on a mechanical shaker. Turbidity was measured with a Klett-Summerson colorimeter by use of the green filter. Cell concentrations were then determined by reference to a standard curve established from appropriate dilutions of a 24-hr broth culture, the cell concentration of which had been determined by use of cells stained with crystal violet in a Levy-Hausser hemacytometer.

The cellulodextrins were prepared and purified by the method of Miller, Dean, and Blum (1960) as modified by Storvick, Cole, and King (1963). The glucose was obtained from the National Bureau of Standards.

RESULTS

Absorption of oligosaccharides. A description of the disappearance of cellulodextrins from the medium in the presence of a resting-cell suspension is given in Fig. 1. The rate of disappearance of each oligosaccharide was constant, and decreased as the number of glucose units per molecule increased. To determine the nature and magnitude of extracellular metabolism of the oligosaccharides, filtrates taken from resting-cell suspensions after the 30-min starvation period were incubated with the oligosaccharides in 250-ml Erlenmeyer flasks for 1 hr on a mechanical shaker, and then boiled for 5 min to terminate enzymatic activity. Analyses of these filtrates indicated that no appreciable change in either the concentration of cellulodextrins or in their DP occurred during the incubation. Similarly, there was no detectable change in the DP of the unabsorbed oligosaccharides recovered after incubation of cellulodextrins with cell suspensions for 1 hr. Both sets of control data on DP appear in Table 1.

The experiments measuring oligosaccharide uptake permit calculation of the number of glucosyl moieties metabolized per cell per unit time (Table 2). On a hexose basis, approximately equivalent amounts of each oligosaccharide were absorbed per cell per minute.

Growth response to oligosaccharides. Growth in media containing each of the oligosaccharides is described in Table 3. Although the growth rate in each of the carbohydrate media was essentially the same during the log phase, the final cell densities increased as the DP of the oligosaccharide increased. Subtracting the maximal growth on the carbohydrate-free control medium from the others, and expressing the resulting

carbohydrate-dependent growth on each sugar as the percentage of the growth on cellobiose, gave values of 68, 85, 85, 86, and 93% for glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose, respectively.

DISCUSSION

These data demonstrate that oligosaccharides larger than cellobiose are actively removed from a culture medium at a rate which appears to be controlled by the respiration of the cell. The linearity of oligosaccharide uptake (Fig. 1) indicates that the removal is by an active absorption mechanism. If simple diffusion accounted for the disappearance, an exponential curve would be expected. From the control experiment (Table 2), it is evident that the cells are necessary for removal of oligosaccharide, and that there is no β -(1,4)-glucan hydrolase in the medium during the brief incubation times that have been used. That the oligosaccharides were removed intact is confirmed by the constancy of the DP of the oligosaccharides in the presence of cells (Table 2).

The conclusion that the rate of uptake is con-

trolled by the rate of respiration of the cell is indicated by the results in Table 1. Independent of the DP of the carbohydrate, the same number of glucose molecules was removed from the medium per cell per unit time. This behavior is strongly reminiscent of the behavior of *Streptococcus faecalis* with which Abrams (1960) obtained data indicating that the absorption of starch oligosaccharides was dependent upon the rate of glycolysis.

It is evident (Table 3) that *C. gilvus* is capable of utilizing the oligosaccharides as sources of energy for growth. Although provided with an equivalent amount of carbohydrate on a hexose basis, the cells were able to metabolize larger dextrins with greater efficiency, suggesting that the degradation of oligosaccharide once absorbed may begin with a phosphorylytic attack. Other factors, however, must be involved in the increasingly efficient utilization of the larger oligosaccharides, because the added energy yield per mole of hexose resulting from phosphorylysis can account for only a small portion of the energy demand to support the marked increases in cell yield.

TABLE 2. Oligosaccharide absorption rates by resting cells of *Cellvibrio gilvus*

Oligosaccharide	Molecules absorbed*	Molecules of glucose provided*
Glucose	39	39
Cellobiose	20	41
Cellotriose	14	43
Cellotetraose	11	42
Cellopentaose	9	45
Cellohexaose	6	37

* Results expressed as millions per cell per minute.

TABLE 3. Growth of *Cellvibrio gilvus* on cellulose oligosaccharides*

Carbohydrate source	Incubation time (hr)									
	0	3	6	9	12	15	18	21	24	30
None	1	4	9	12	30	70	80	83	83	79
Glucose	1	4	16	59	163	263	318	355	377	384
Cellobiose	1	4	14	47	151	251	328	388	427	458
Cellotriose	1	4	15	45	152	251	324	385	426	460
Cellotetraose	1	6	13	45	153	241	318	383	431	463
Cellopentaose	1	5	5	41	155	243	333	390	433	493
Cellohexaose	1	7	13	41	155	252	366	465	518	523

* Turbidities expressed in Klett-Summerson (KS) units. The standard curve of KS units vs. number of cells per milliliter is approximately linear through 500 KS units equivalent to 5.2×10^9 cells per milliliter.

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