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Original article

Utilization of algal polysaccharides by human colonic bacteria, in axenic culture or in association with hydrogenotrophic microorganisms

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Summary — The ability of different hydrolytic bacteria from the human colon to grow on various algal polymers (carrageenans, *Palmaria palmata* xylan, ulvan, desulphated ulvan and laminaran) was investigated and the interactions between *Bacteroides thetaiotaomicron* and H₂-utilizing microorganisms (one methanogenic archaea and an acetogenic bacterium) were studied during laminaran degradation. None of the algal polysaccharides supported the growth of any of the hydrolytic species tested, except for laminaran, which allowed substantial growth of *B thetaiotaomicron*. This suggested that bacterial consortia were involved in algal polymer breakdown rather than one specific bacterial species. The presence of H₂-utilizing microorganisms did not increase the extent of laminaran degradation by *B thetaiotaomicron*. Whereas the decrease in formate and H₂ concentrations attested to their utilization by both hydrogenotrophic microorganisms, the large increase in acetate production observed in the coculture with acetogenic bacteria was mainly due to acetogenic fermentation of sugars released during laminaran hydrolysis.

human colon / seaweed degradation / *Bacteroides* / H₂-utilizing microorganism / microbial interaction

Résumé — Utilisation de polymères algaux par les bactéries hydrolytiques du côlon humain, en culture pure ou associés à un micro-organisme hydrogénéotrophe. La capacité de différentes espèces bactériennes hydrolytiques du côlon humain à croître avec des polysides algaux (carraghénanes, xylane de *Palmaria palmata*, ulvanes, ulvanes désulfatés et laminaranes) comme source de carbone a été étudiée ainsi que l'effet de deux micro-organismes hydrogénéotrophes (l'un méthano-gène et l'autre acétogène) sur la dégradation in vitro du laminarane par *Bacteroides thetaiotaomicron*. Aucune des espèces hydrolytiques testées n'a été capable de croître en présence d'un des polysides algaux étudiés à l'exception de *B thetaiotaomicron* qui maintient sa croissance avec le laminarane.

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Des consortia bactériens sembleraient donc nécessaires à la dégradation de ces composés algaux plutôt qu'une espèce bactérienne seule. La présence d'un micro-organisme hydrogénéotrophe ne s'est pas traduite par une augmentation de la quantité de laminarane dégradée par *B. thetaiotaomicron*. La diminution des concentrations de formate et de H_2 dans les deux cocultures témoigne de leur utilisation par les deux types de micro-organismes hydrogénéotrophes. En revanche, la forte augmentation d'acétate observée dans la coculture avec la bactérie acétogène s'explique principalement par la fermentation homoacétique des oses issus de l'hydrolyse du laminarane.

côlon humain / dégradation des algues / *Bacteroides* / micro-organisme hydrogénéotrophe / interaction microbienne

INTRODUCTION

Macroalgae are composed of three different classes depending on the nature of their pigments: brown (Phaeophyceae), red (Rhodophyceae) and green (Chlorophyceae) algae. Brown and red algae are the principal sources of commercial interest. They are used both as dietary constituents as well as thickening, gelling and stabilizing agents (alginates, agars, carrageenans) by the colloid industry (Jensen, 1993).

The major components of seaweeds are carbohydrates which account for approximately 50% of their dry matter, and are essentially reserve and cell-wall polysaccharides (Darcy-Vrillon, 1993; Mabeau and Fleurence, 1993). Seaweeds are particularly rich in dietary fibres of which more than 50% are water soluble (Lahaye, 1991). The higher content of soluble dietary fibres in algae compared to that observed in land plants is of particular interest. The soluble polysaccharides recognized as dietary fibres are agars, carrageenans and xylans from red seaweeds, sulfated rhamnoxyloglucuronans from green seaweeds, together with alginates and fucans from brown seaweeds. Most are gel-forming and viscous polymers. The insoluble polymers are essentially cellulose, xylans and mannans (Lahaye, 1991). Laminarans constitute the sole reserve polysaccharides which are classified as fibres.

Therefore, most algal polysaccharides cannot be broken down by human digestive

enzymes, and reach the large intestine where they constitute a source of carbon and energy for the different species of anaerobic bacteria growing here. The anaerobic degradation of organic matter (from both endogenous and dietary origin) in the colon depends on the participation of different groups of microorganisms which have developed complex and diversified interactions constituting a trophic chain. Polysaccharides are broken down by microorganisms into soluble sugars that are fermented by both degrading and fermentative populations. Fermentation of these polysaccharides leads to the production of short chain fatty acids (SCFA), mainly acetate, propionate and butyrate and the gases CO_2 and H_2 (Macfarlane and Cummings, 1991). Hydrogen, which is derived entirely from fermentation reactions, is in part removed through its excretion in breath or flatus. The main pathway of H_2 disposal however involves its reutilisation by H_2 -consuming microorganisms, where it is an electron donor and energy source for several reactions including dissimilatory nitrate reduction, methanogenesis, dissimilatory sulfate reduction and reductive acetogenesis (Durand et al, 1996; Bernalier et al, 1996a). The role of H_2 -utilizing microorganisms is fundamental for the maintenance of the overall process of organic matter degradation in anaerobic ecosystems.

The aim of the present work was to investigate the ability of different hydrolytic bacteria belonging to the dominant human flora

to grow on various algal polymers. Furthermore, the interactions between *Bacteroides thetaiotaomicron*, one of the main polysaccharides-degrading bacteria in the human colon, and two different hydrogenotrophic species, a methanogenic archaea (*Methanobrevibacter smithii*) and an acetogenic bacterium (*Clostridium* sp, strain M5a3) isolated from human feces in our laboratory (Bernalier et al, 1996b) were studied in vitro during laminaran fermentation.

MATERIALS AND METHODS

Sources of organisms

The hydrolytic bacterial species studied were either obtained from culture collections or were isolated from human feces at the Dunn Clinical Nutrition Centre (Cambridge, UK). *Ruminococcus gnavus* 29149 and *Ruminococcus obeum* 29174 came from the American Type Culture Collection. *Bacteroides thetaiotaomicron* 5482, *Bacteroides vulgatus* 4245 and *Bacteroides distasonis* 4243 were kindly provided by Prof AA Salyers (University of Illinois, Urbana-Champaign, USA). Strains 22, 38 identified as bifidobacteria and strains 4ii, 1i, 2i, 5i, 10i, 15i identified as clostridia were a gift from Dr C Michel (INRA, Nantes, France) who isolated them from batch cultures of human feces adapted to ulvan (strain 4ii) or *Ulva* sp.

The H_2/CO_2 -utilizing acetogenic bacterium (strain M5a3) was isolated in our laboratory from a fecal sample of a non-methanogenic subject (Bernalier et al, 1996a). Strain M5a3 (DSM 10510) corresponds to a new species of the genus *Clostridium* (unpublished data). *Methanobrevibacter smithii* 861 came from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Media and growth conditions

The methods used for preparing the prereduced medium and the anaerobic culture techniques were those described by Hungate (1969). The basal medium used was the semi-synthetic modified AC-21 medium (Bernalier et al, 1996c) supplemented with 1 mg/L hemin (AC-21 h). Sup-

plementation of basal AC-21 h with 10% centrifuged rumen fluid was used in some experiments. The culture media were prepared and kept under a 100% CO_2 gas phase and dispensed in 10 mL into serum-capped tubes (Bellco Glass Inc, Vineland, NJ, USA).

Utilization of algal substrates by hydrolytic bacterial species

The utilization of the different algal substrates by colonic microorganisms was determined using basal AC-21 h medium containing 2 g/L of seaweed product as the sole carbon source. The seaweed products tested were lambda, iota and kappa carrageenans, *Palmaria palmata* xylan, ulvan and desulfated ulvan, which were kindly provided by Dr M Lahaye (INRA, Nantes, France) and laminaran (Sigma). Potato soluble starch from (Sigma) was used as a bacterial growth control.

The bacterial inocula consisted of 0.3 mL of a 20 h culture grown on glucose (2 g/L) AC-21 h medium. Cultures were incubated at 37 °C and bacterial growth was followed over 8 days by measuring the absorbance at 600 nm (OD_{600}) with a Spectronic 20D spectrophotometer (Bioblock Scientific, Illkirsh, France). The ability of algal products to support growth of the strains was considered as positive when the culture could be maintained on the same substrate after three transfers. In addition, the OD_{600} of the culture had to be twice that observed on the basal substrate free medium after 48 h incubation at 37 °C.

Coculture studies with *Bacteroides thetaiotaomicron* and H_2/CO_2 -utilizing microorganisms

Coculture experiments were done using the AC-21 h medium containing laminaran (5 g/L) as sole carbon source. Monocultures of *B thetaiotaomicron* and cocultures were inoculated with 0.2 mL of a 4 h culture of the hydrolytic bacterium, exhibiting an OD_{600} equivalent to 0.3 on the laminaran growth medium. The acetogenic strain inoculation was 0.3 mL of a 24 h culture on glucose growth medium. Cocultures with *B thetaiotaomicron* and *Clostridium* sp strain M5a3 were performed by inoculating the two strains

simultaneously. In contrast, the methanogenic strain was inoculated 5 days before the hydrolytic bacterium in the studies with *B thetaiotaomicron* and *M smithii*. The preculture of *M smithii* (0.5 mL inoculum per tube) was carried out under a gas phase composed of H₂/CO₂ (60/40, v/v) at an initial pressure of 202 kPa. When the methanogenic preculture had reached an OD₆₀₀ of 0.2 (after 5 days incubation), the tubes were flushed with 100% CO₂ to eliminate the remaining H₂ and CH₄ produced by the methanogenic organism. The hydrolytic bacterium was subsequently inoculated into these coculture tubes. Monocultures and cocultures were incubated at 37 °C for 2, 3, 4, 6 and 8 days. After incubation, bacterial growth was stopped by rapidly freezing the cultures at -20 °C.

Hydrogen and methane concentrations in the head space gas of each culture tube were measured by gas chromatography as described by Czerkawski and Clapperton (1968). The bacterial cultures were then centrifuged at 12 000 g for 20 min at 4 °C and the supernatants were stored at -20 °C after addition of 10 µL HgCl₂. The soluble fermentation products were measured by capillary gas chromatography, after conversion to tertiary butyldimethylsilyl derivatives according to the method of Richardson et al (1989).

The residual laminaran contained in the supernatants was hydrolysed with H₂SO₄, 15 M at 4 °C for 1 min, then at 20 °C for 2 min and finally at 100 °C for 3 min. The released hexoses and the residual sugars were further measured colori-

metrically using the cysteine-sulfuric acid method described by Dische (1963). The amount of glucose residues remaining in the cultures was determined enzymatically using glucose 6P dehydrogenase (Boehringer-Mannheim).

RESULTS

Utilization of seaweed polysaccharides by hydrolytic bacteria

Algal polysaccharides failed to support the growth of any of the *Bacteroides* and *Ruminococcus* species studied, with the exception of laminaran, which allowed substantial growth of *B thetaiotaomicron* (OD₆₀₀ = 0.695 ± 0.06 on laminaran-AC-21 h medium versus 0.277 ± 0.01 on substrate-free control medium) (table I). However, the growth of this bacterial species on laminaran was significantly slower than that observed on soluble starch. This last polysaccharide also supported the growth of the largest number of bacterial species tested (table I). Among the eight strains isolated from human feces adapted to grow on seaweed-products *Ulva* sp and ulvan, only one *Clostridium* species (10i) could be maintained on xylan from *Palmaria palmata*,

Table I. Growth of human colonic bacterial species on different algal substrates.

Growth substrates	<i>Bacteroides thetaiotaomicron</i>	<i>Bacteroides vulgatus</i>	<i>Bacteroides distasonis</i>	<i>Ruminococcus gnavus</i>	<i>Ruminococcus obeum</i>
Lambda carrageenans	- ^a	-	-	-	nd
Iota carrageenans	-	-	-	-	nd
Kappa carrageenans	-	-	-	-	nd
Laminaran	+ ^b	-	-	-	-
Ulvan	-	-	-	-	-
Desulfated ulvan	-	-	-	-	-
<i>Palmaria palmata</i> xylan	-	-	-	-	-
Potato soluble starch	++ ^c	+	+	+	-

^a-: same OD₆₀₀ in control and test media. ^b+: OD₆₀₀ in test medium is twice the OD₆₀₀ in control medium.

^c++: OD₆₀₀ in test medium is three times higher than OD₆₀₀ in control medium. nd: not determined

Each measurement (OD₆₀₀) was performed three times, with triplicate cultures observed each time. Test medium contained 2 g/L of substrate whereas control medium was a substrate free medium.

ulvan and desulfated ulvan, although growth was poor (data not shown). Ulvan and xylan from *Palmaria palmata* also supported moderate growth of *Clostridium* strain 15i and *Clostridium* strain 2i. In contrast, none of the *Bifidobacterium* strains tested utilized algal polymers as carbon sources. Supplementation of the semi-synthetic modified AC-21 h medium with 10% rumen fluid did not improve the ability of any of the bacteria to grow on algal substrates (data not shown).

From these results, *B thetaiotaomicron* was selected for further studies on the degradation of laminaran, in association with H_2 -utilizing microorganisms.

Laminaran degradation by *B thetaiotaomicron* in axenic cultures and cocultures with H_2/CO_2 -utilizing microorganisms

The extent of laminaran utilization by *B thetaiotaomicron* was not increased by the presence of any of the hydrogenotrophic microorganisms (fig 1). Although the initial rate of laminaran degradation was sig-

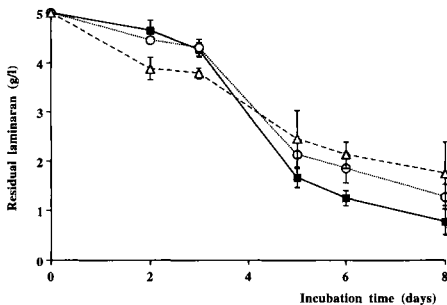


Fig 1. Kinetics of laminaran degradation by *Bacteroides thetaiotaomicron* in monoculture (■) or in cocultures with the acetogenic bacterium, *Clostridium* sp strain M5a3 (○) or with *Methanobrevibacter smithii* (Δ). Each point represents the mean value of three determinations \pm standard error.

nificantly higher in the presence of *M smithii* (1.13 ± 0.23 g of laminaran degraded in the coculture versus 0.38 ± 0.21 g in the monoculture after 2 days of incubation), the final extent of substrate breakdown (8 days of incubation) was significantly lower in the coculture than in the monoculture. In coculture with the H_2/CO_2 -utilizing acetogenic strain, the initial rate of laminaran degradation was similar to that of the monoculture (fig 1). A small decrease in the total amount of substrate used was observed after 8 days of incubation in this coculture compared to the monoculture (fig 1). Glucose did not accumulate in the acetogenic coculture as it did in the monoculture, while this substrate was found in higher concentrations in the presence of the methanogen (0.28 ± 0.08 g/L versus 0.73 ± 0.23 g/L respectively).

In the axenic culture of *B thetaiotaomicron*, the major end-products of laminaran fermentation were acetate, propionate, succinate, formate and H_2 (table II). When *Methanobrevibacter smithii* was cocultivated with *B thetaiotaomicron*, methane was detected in the gas phase of the coculture whereas H_2 and formate did not accumulate (table II). Acetate was found in greater amounts in coculture than in axenic culture. Similarly, acetate production increased while formate and H_2 concentrations decreased in coculture with the acetogenic bacteria (table II). Trace amounts of H_2 however could be detected in the gas phase of the coculture with the acetogenic strain, even after 8 days of incubation, whereas this gas totally disappeared in the presence of *M smithii* (table II). In contrast, higher acetate production was found in coculture with the acetogen than in the methanogenic association.

Analysis of laminaran fermentation products in the *Bacteroides* – acetogen coculture showed that the acetate production mainly increased after 2 days of incubation, while H_2 and formate no longer accumulated. The small amounts of ethanol and lac-

Table II. End-products of laminaran fermentation (obtained after 8 days of incubation) formed by *Bacteroides thetaiotaomicron* in axenic culture or in coculture with *Methanobrevibacter smithii* or with the acetogenic bacterium, *Clostridium* sp strain M5a3.

End-products (mM/100mM of hexoses fermented)	<i>B thetaiotaomicron</i>	<i>B thetaiotaomicron</i> + <i>Clostridium</i> strain M5a3	<i>B thetaiotaomicron</i> + <i>M smithii</i>
Formate	6.0 ± 0.1	0.0	0.0
Acetate	80.5 ± 6.6	124.4 ± 15.0	97.0 ± 9.3
Propionate	48.5 ± 0.6	43.0 ± 5.0	54.5 ± 19.0
Succinate	5.2 ± 2.9	4.0 ± 0.3	6.3 ± 8.6
H ₂	1.6 ± 0.3	0.6 ± 0.1	0.0
CH ₄	0.0	0.0	7.9 ± 0.5
% C recovery ^a	60	69	70

Each value represents the means of three cultures ± standard error. ^a Percentage of carbon recovery did not take into account cell carbon.

tate produced by *B thetaiotaomicron* in monoculture (1.0 and 0.5 mM respectively) were not detected in the two cocultures (data not shown). In contrast, propionate and succinate productions did not appear to be affected by coculture with hydrogenotrophic microorganisms (table II).

DISCUSSION

This study showed that most of algal polysaccharides tested, from three different classes of seaweeds, failed to support the growth of the majority of hydrolytic species from the dominant human microflora. The capacity of bacterial strains to grow, in vitro, with complex algal polysaccharides as carbon sources has not been studied extensively, although more work has been done on the ability of the whole fecal flora to ferment these substrates (Michel and Macfarlane, 1996).

We demonstrated that the three different types of carrageenans from red seaweeds did not support significant growth for any of the bacterial species tested. Similarly, Saly-

ers et al (1977b) have shown the inability of a pure culture of intestinal bacteria to ferment carrageenans in vitro. Our results were consistent with the previous studies reported by Michel and Macfarlane (1996) who demonstrated that the human colonic microflora could chemically modify carrageenans rather than metabolize them.

In contrast, xylan from *Palmaria palmata* appeared to be highly fermented within the first 6 h of incubation with a human fecal microflora (Lahaye et al, 1993). However, only a few *Clostridium* strains (2i, 10i, 15i) showed any ability to grow on this substrate. Salyers et al (1981) observed that oligomers of different sizes were released from non-algal xylan by pure cultures of *Bacteroides ovatus* and *Bacteroides eggerthii*. However, the *Bacteroides* species tested in our study failed to show the same ability to degrade xylan from seaweed.

Adaptation of the fecal microflora to grow on ulvan or desulfated ulvan from *Ulva* sp did not enhance the ability of the predominant bacterial species to use them as growth substrates. Only two clostridia (10i and 15i), isolated from these adapted

microbiotas, were able to grow on these polysaccharides. Similarly, Bobin-Dubigeon et al (1994) and Durand et al (unpublished data) observed that ulvan and desulfated ulvan were poorly fermented by human fecal flora.

Since most of these algal polymers are chemically modified or fermented by mixed populations of intestinal bacteria, while pure cultures of the predominant bacteria in these communities failed to grow with these substrates, it is suggested that breakdown of these algal polysaccharides requires the involvement of more than one bacterial species. Due to the unusual chemical composition of these algal polymers, it is likely that the contribution of several polymer-degrading species may be necessary to significantly degrade these substrates. This would explain our inability to find pure bacterial cultures capable of growing efficiently on these algal polymers.

Laminaran was the sole algal polysaccharide able to support a substantial growth of *Bacteroides thetaiotaomicron*. This substrate has previously been shown to be highly fermented by the human fecal flora (Michel et al, 1996). In earlier studies, laminaran degrading bacteria were mainly found to belong to the genus *Bacteroides* (Salyers et al, 1977a, b). These bacteria possess all of the cell-bound inducible laminarases and β -glucosidases (Salyers et al, 1977c) necessary to fully degrade this polymer which is composed of β -1,3 linked glucans. These have a low degree of polymerization and contain β -1,6 linkages and mannitol residues on the reducing ends (Michel and Macfarlane, 1996). The main laminaran degradation product by strains of *B thetaiotaomicron* was glucose (Salyers et al, 1977c).

The presence of H_2/CO_2 -utilizing microorganisms did not improve laminaran degradation by *B thetaiotaomicron*. Similarly, no enhancement of plant cell wall degradation by *Bacteroides* species was

shown to occur in the presence of *M smithii* (Stewart et al, 1990). While Morvan et al (1996) also reported a similar lack of effect on fermentation with some rumen acetogenic strains, on the rate of cellulose breakdown by *R flavefaciens*, methanogens and acetogens were shown to increase cellulose degradation by rumen fungi and *Ruminococcus albus* respectively (Bernalier et al, 1991; Morvan et al, 1996). This enhancement of polysaccharide utilization by H_2 -consuming microorganisms is known to be due to a reduction in the H_2 partial pressure by H_2 -consuming syntrophy (Wolin and Miller, 1983). In our experiments, the absence of effect of hydrogenotrophic microorganisms on laminaran degradation by *B thetaiotaomicron* could be explained by the fact that this hydrolytic species is a weak H_2 producer. Therefore, the elimination of H_2 in the coculture was probably too low to have a major influence on polysaccharide degradation. Similarly, utilization of H_2 by *M smithii* did not induce metabolic shifts in *B thetaiotaomicron*. Because the methane production remained very low, it is likely that the growth of the methanogen in the coculture was limited, probably due to a low availability of H_2 and formate. It is therefore difficult to assume that the small increase in acetate production by *B thetaiotaomicron* was a consequence of H_2 re-utilization by the methanogens as was demonstrated with *R albus* and rumen fungi (Wolin and Miller, 1983; Bernalier et al, 1991; Morvan et al, 1996). In contrast, large amounts of acetate were observed in coculture with the acetogenic strain. This increase in acetate production however appeared to be mainly due to sugar fermentation by the acetogen. Glucose released from laminaran degradation by *B thetaiotaomicron* could undoubtedly be consumed by the acetogenic organism which was previously shown to form large amounts of acetate from this sugar (Bernalier et al, 1996b). Acetate was also derived in part from the acetogenic metabolism of H_2 and formate, mainly dur-

ing the earlier incubation times of the cocultures. This demonstrated the ability of acetogenic bacteria from the human colon to metabolize H_2 formed by hydrolytic species. However, these acetogenic bacteria appeared less efficient than methanogens in their ability to re-utilize H_2 , since trace amounts of this gas remained in the coculture. The important nutritional versatility of acetogens compared to methanogens, which can only use a restricted range of substrates, may influence their capacity to utilize H_2 . Regulation of H_2 metabolism by organic compounds in colonic acetogenic bacteria may occur as shown with other acetogens such as *Eubacterium limosum* and *Sporomusa termitida* (Sharak-Genthner and Bryant, 1987; Breznak and Blum, 1991). Therefore, the role of acetogenic bacteria in H_2 scavenging in the colon ecosystem may largely be dependent on carbohydrate availability. The influence of organic substrates on the metabolism of H_2 by colonic acetogens is now under study in our laboratory.

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