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Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rabbit caecal bacterium *Bifidobacterium pseudolongum*

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Aims: In a rabbit caecal bacterium *Bifidobacterium pseudolongum*, metabolites of pectin and glucose, and activities of enzymes involved in the degradation of pectin were assayed. Simultaneously, activities of these enzymes were assayed in a rumen pectinolytic strain of *Streptococcus bovis*.

Methods and Results: A strain *B. pseudolongum* P6 which grew best on pectin was selected among bifidobacteria isolated from the rabbit caecum. Cultures of *B. pseudolongum* P6 grown on pectin produced significantly less formate, lactate and ethanol, and more acetate and succinate than cultures grown on glucose. No CO_2 production on pectin was observed. Pectin macromolecule was degraded by extracellular pectinase (EC 3.2.1.15). Cell extracts possessed the activity of 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14). *Streptococcus bovis* X4, possessed activity of exopectate lyase and pectinase, but not that of KDPG aldolase.

Conclusions: Our results are consistent with the assumption that in *B. pseudolongum* P6 acidic products of pectin degradation are catabolized via a modified Entner–Doudoroff pathway, as shown previously in rumen pectin-utilizing bacteria. The missing KDPG aldolase activity in *Strep. bovis* X4 seems to be the reason for the absence of growth of this bacterium on pectin.

Significance and Impact of the Study: Information on polysaccharide metabolism in bifidobacteria is fragmentary. This study extends the knowledge on pectin metabolism in intestinal bacteria.

INTRODUCTION

Anaerobic bacteria belonging to the *Bifidobacterium* genus are ubiquitous in the mammalian and avian digestive tract, insect intestine and sewage. Bifidobacteria have attracted the interest of microbiologists due to their abundance in the digestive tract of humans, assumed health-promoting activities, and the specifics of their carbohydrate metabolism. In bifidobacteria, glucose is degraded by the fructose-6-phosphate shunt, in which fructose-6-phosphoketolase (EC 4.1.2.2) cleaves fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate (Scardovi and Trovatelli 1965). End-products of the carbohydrate metabolism are acetate, lactate, formate and ethanol (De Vries and Stouthamer 1968). Bifidobacteria utilize a variety of mono- and oligosaccharides (Scardovi 1986). Most strains of 29 species of bifidobacteria fermented amylopectin, amylose and/or some other polysaccharides in the study of Crociani *et al.* (1994). However, studies designed to investigate bifidobacterial metabolism of polysaccharides other than fructans are scarce. Degnan and Macfarlane (1995) described utilization of arabinogalactan, a plant cell wall polysaccharide, by cultures of *Bifidobacterium longum*. Marounek *et al.* (1998) compared fermentation of starch and glucose in rabbit caecal strains of *Bifidobacterium globosum*. A number of bifidobacterial isolates ferment pectin. The results of Crociani *et al.* (1994) showed that pectin was fermented by 10% of strains of bifidobacteria tested for their ability to ferment complex carbohydrates. Metabolism of pectin in bifidobacteria has

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not been studied adequately. Thus this report presents metabolite profiles and activity of enzymes involved in the degradation of pectin in a pectinolytic strain of *Bifidobacterium pseudolongum*, isolated from the rabbit caecum. Simultaneously, the activities of pectin-degrading enzymes were assayed in a pectinolytic strain of *Streptococcus bovis*, and in a *Bifidobacterium* sp. strain that did not degrade pectin. Particular attention was given to the reaction route in which D-galacturonate, the principal pectin monomeric component, was catabolized.

MATERIALS AND METHODS

Bacteria

Two Silver grey breed rabbits, 5 months old, were fed oats and meadow hay *ad libitum*. Rabbits were killed, their caeca emptied and caecal contents serially diluted and plated on Wilkins–Chalgren agar (Oxoid, UK) containing 5% caecal extract prepared according to Emaldi *et al.* (1979) and 0·4% pectin. Plates were incubated at 37 °C for 3 d in anaerostats (Anaerobic Plus System, Oxoid, UK) under CO_2/H_2 atmosphere. Representative colonies were picked up at random and screened for fructose-6-phosphate phosphoketolase activity (Biavati *et al.* 1992). Phosphoketolase-positive strains were assigned according to their biochemical characteristics (Scardovi 1986; Biavati *et al.* 1992), using the API 50 CHL tests (BioMerrieux, France). The isolate which grew best on pectin was identified as *B. pseudolongum* and chosen for further study (strain P6). *Bifidobacterium* sp. N13, a nonpectinolytic strain, was isolated from the caeca of a chick at the Czech Agricultural University, Department of Microbiology and Biotechnology, Prague. *Streptococcus bovis* X4 was isolated from the rumen fluid of a sheep at this Institute. *Pseudomonas fluorescens* DBM 3056, a bacterium with enzymes of the Entner–Doudoroff pathway, was obtained from the culture collection of the Department of Biochemistry and Microbiology of the Institute of Chemical Technology, Prague. Bacteria were maintained in 20% (v/v) glycerol at −40 °C.

Pectin, media and growth conditions

Apple pectin was supplied by Pektin, Ltd (Smiřice, Czech Republic). It was purified by ethanol extraction (75% v/v). Pectin contained (in mg g^{-1} of dry matter): methoxylated polygalacturonate, 506; calcium polygalacturonate, 9; polygalacturonate, 417; neutral sugars, 68.

Bifidobacteria and *Strep. bovis* X4 were grown on a medium containing (in g l^{-1}): $K_2HPO_4 \cdot 3H_2O$, 5·9; KH_2PO_4, 4·5; $NaHCO_3$, 3·0; $(NH_4)_2SO_4$, 2·9; NaCl, 0·9; $MgSO_4 \cdot 7H_2O$, 0·09; $CaCl_2$, 0·09; yeast extract, 1·0; pancreatic casein hydrolysate, 1·0; caecal extract, 100 ml. A trace metal solution (Clark and Holms 1976) and a vitamin solution (Scott and Dehority 1965) were also added, 1 ml l^{-1} of each. The medium was reduced by 0·05% cysteine.HCl. *Bifidobacterium pseudolongum* P6 was grown on pectin and glucose. Substrates were added at 4 g l^{-1} final concentration. *Bifidobacterium* sp. N13 and *Strep. bovis* X4 were grown on the mixed substrate: pectin and glucose were added at 2 g l^{-1} each. *Pseudomonas fluorescens* DBM 3056 was cultivated on glucose (4 g l^{-1}) in the medium of Van Dijken and Quayle (1977).

Medium for *B. pseudolongum* P6 was distributed in 15-ml aliquots into CO_2-gassed 20-ml flasks, closed by rubber stoppers and autoclaved at 110 °C for 1 h. Inoculated cultures were grown at 39 °C overnight in seven replicates. For enzyme assays, bacteria were cultivated in 500-ml batch cultures at 39 °C overnight (bifidobacteria, *Strep. bovis* X4), or at 28 °C for 2 d (*Ps. fluorescens* DBM 3056). The pseudomonad was grown aerobically.

Carbon dioxide production in the strain P6 was measured in 30-ml cultures cultivated in 100-ml flasks, sealed with rubber stoppers. Cultures were grown under N_2 atmosphere on the above medium, except that sodium bicarbonate was omitted and concentration of phosphates doubled. Pectin- and glucose-supplied cultures were cultivated for 2 and 1 d, respectively. Substrate-free control cultures were cultivated simultaneously.

Analyses and calculations

Ethanol and acetate were determined by gas–liquid chromatography on a column of the Chromosorb WAW with 15% SP 1220/1% H_3PO_4 (Supelco, USA). Lactate and succinate were methylated and determined on a programmed (120–185 °C) capillary column with DB-FFAP stationary phase, 30 m × 0·53 mm (J & W Scientific, Folsom, CA, USA). The cell dry weight was determined after centrifugation of cultures, washing with rinsing solutions and drying at 105 °C overnight. Formate was estimated colorimetrically (Sleat and Mah 1984), residual pectin by 3-phenylphenol reagent (Blumenkrantz and Asboe-Hansen 1973), and residual glucose enzymatically by means of a commercial kit (Lachema, Czech Republic). The CO_2 content in headspace gas was determined by gas chromatography using a chromatograph equipped with a thermal conductivity detector and a column of Carboxen 100 (Supelco, USA). Control, pectin- and glucose-grown cultures of the strain P6 were acidified with 4 mol l^{-1} HCl (2 ml per flask), and 1 ml of gas taken by means of a gas-tight syringe A-2 (Dynatech, USA). Separation was carried out at 90 °C.

To determine activities of pectin-degrading enzymes, cultures were centrifuged, and the cells were washed and disrupted by sonication using a 600-W ultrasonic processor

VC 601 (Sonics & Materials Inc., USA). Supernatant fluids were dialysed at 4 °C for 24 h. Intra- and extracellular activity of poly(1,4-α-D-galacturonide) exolyase (EC 4.2.2.9) and pectinase (EC 3.2.1.15) was determined as described previously (Dušková and Marounek 2001). The activity of 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14), an enzyme unique for the Entner–Doudoroff pathway, was determined as described by Marounek and Dušková (1999), with KDPG and 6-phosphogluconate as substrates.

Production of metabolites was related to the amount of substrate utilized. Enzyme activities were expressed in nanomoles of substrate split or product liberated per minute per mg of protein. Protein content in extracts was determined by the Lowry method (Herbert *et al.* 1971). The significance of differences was evaluated by the *t*-test.

RESULTS

Bifidobacterium pseudolongum P6 utilized almost all glucose (3·92 ± 0·1 g l^{-1}) and *ca* 76% of pectin. The composition of fermentation end-products differed in cultures with pectin and glucose. Cultures grown on glucose produced significantly more formate, lactate and ethanol per gram of substrate used, and less acetate and succinate than corresponding cultures grown on pectin (Table 1). Production of CO_2 was 0·02 ± 0·15 and 0·96 ± 0·32 mmol g^{-1} substrate used in pectin- and glucose-grown cultures, respectively. Yields of dry matter and protein were nonsignificantly higher in cultures grown on glucose.

In cultures of *B. pseudolongum* P6 pectin macromolecules were degraded by the action of extracellular pectinase. Exopectate lyase activity was not present in cells or culture supernatant fluids of this bacterium (Table 2). *Streptococcus bovis* X4 possessed both exopectate lyase and pectinase activity. Activity of pectinase, however, was very low.

Table 1 Metabolite profiles and cell yields of the rabbit caecal bacterium *Bifidobacterium pseudolongum* P6 grown on pectin and glucose*

	Pectin	Glucose
Metabolites (mmol g^{-1} substrate used)		
Formate	3·22 ± 0·23†	3·63 ± 0·38
Acetate	6·01 ± 0·64	5·81 ± 0·45
Lactate	1·58 ± 0·25†	2·42 ± 0·17
Succinate	0·66 ± 0·10†	0·48 ± 0·09
Ethanol	0·32 ± 0·10†	0·51 ± 0·12
Yields (mg g^{-1} substrate used)		
Dry matter	154 ± 37	168 ± 40
Protein	71 ± 29	84 ± 41

*Means of six cultures ± S.D.

†Significantly different from the glucose value ($P < 0·05$).

Activity of KDPG aldolase was found both in pectin- and glucose-grown cells of *B. pseudolongum*. Contrary to *Ps. fluorescens* DBM 3056, the cell extracts of *B. pseudolongum* did not metabolize 6-phosphogluconate. The KDPG aldolase activity was not found in cells of *Strep. bovis*. None of the enzymatic activities assayed was found in the strain N13 of *Bifidobacterium* sp.

DISCUSSION

The hexose fermentation in bifidobacteria theoretically should yield acetate and lactate in a 1·5 : 1 molar ratio (Kandler 1983). When *B. pseudolongum* P6 was grown on glucose this ratio was 2·4 : 1. When grown on pectin the fermentation pattern was altered in favour of acetate and succinate at expense of formate, lactate and ethanol. Similar fermentation shifts in response to a change of substrate (glucose *vs* pectin) were observed in rumen bacteria *Butyrivibrio fibrisolvens*, *Prevotella ruminicola* and *Lachnospira multiparus* in our previous experiments (Marounek and Dušková 1999; Dušková and Marounek 2001). These bacteria produced more lactate and ethanol, and less acetate when grown on glucose rather than on pectin. Contrary to the indications in *Bergey's Manual* (Scardovi 1986) *B. pseudolongum* P6 produced low but measurable amount of CO_2 when grown on glucose.

Both pectin- and glucose-grown cells of *B. pseudolongum* P6 possessed activity of KDPG aldolase, the enzyme which catalyses the final reaction step of the Entner–Doudoroff pathway of galacturonate metabolism (Rombouts and Pilnik 1980). This activity was absent in the nonpectinolytic *Bifidobacterium* sp. N13 strain. The KDPG aldolase activity has been reported in rumen pectin-utilizing bacteria *Treponema saccharophilum* (Paster and Canale-Parola 1985), *Butyrivibrio fibrisolvens*, *Prevotella ruminicola* (Marounek and Dušková 1999), *Lachnospira multiparus* (Dušková and Marounek 2001), and in various saprophytic bacteria and phytopathogens. Similarly to rumen pectin-utilizing bacteria, 6-phosphogluconate was not metabolized by cell extracts of *B. pseudolongum*, presumably because of the lack of 6-phosphogluconate dehydrase (EC 4.2.1.12) activity.

Streptococcus bovis X4 degraded pectin to unsaturated oligogalacturonides, not further utilized. The ability to degrade pectin was observed in 126 out of 134 strains of *Strep. bovis* examined by Ziolecki *et al.* (1972). This allows the organism to recover utilizable sugars associated with the pectin fraction in plants. The lack of pectin utilization in *Strep. bovis* X4 may result from the lack of KDPG aldolase activity in this bacterium.

There has been no evidence for a reaction sequence suggested by Leng (1970) and Van Soest (1983), in which D-galacturonate is decarboxylated to a pentose and metabolized via the pentose phosphate pathway. There was no

Table 2 Specific activities of exopectate lyase (EL), pectinase (P), 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPGA), and 6-phosphogluconate dehydrase (PGD) plus KDPGA in cells and culture supernatant fluids of *Bifidobacterium pseudolongum* P6, *Pseudomonas fluorescens* DBM 3056, *Streptococcus bovis* X4 and *Bifidobacterium* sp. N13

		Substrate				
		Pectin	Glucose		Pectin + glucose	
Enzyme activity*	Sample	*B. pseudolongum*	*B. pseudolongum*	*Ps. fluorescens*	*Strep. bovis*	*Bifidobacterium* sp.
EL	Cell extract	0	0		0	0
EL	Supernatant	0	0		102 ± 22	0
P	Cell extract	3·7 ± 0·2	0		3·5 ± 0·3	0
P	Supernatant	87·4 ± 16·5	11·4 ± 0·6		11·6 ± 0·3	0
KDPGA	Cell extract	919 ± 274	254 ± 194	562 ± 72	0	0
PGD + KDPGA	Cell extract	0	0	80 ± 27	0	0

*Expressed in nanomoles of substrate split or product released per minute per miligram of protein. Means of four or five cultures ± S.D.

production of CO_2 in pectin-grown cultures of *B. pseudolongum* P6, although the decarboxylation of pectin used in this study theoretically should yield 5·08 mmol of CO_2 per g. In addition, metabolites of pectin in the strain P6 and other bacteria so far examined were more oxidized than those of glucose, indicating that the oxidized part of the D-galacturonate molecule was involved in the end-product formation.

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Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rabbit caecal bacterium *Bacteroides caccae*

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ABSTRACT

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Aims: To compare fermentation pattern in cultures of *Bacteroides caccae* supplied with pectin and glucose, and identify enzymes involved in metabolism of pectin.

Methods and Results: A strain KWN isolated from the rabbit caecum was used. Fermentation pattern, changes of viscosity and enzyme reactions products were determined. Cultures grown on pectin produced significantly more acetate and less formate, lactate, fumarate and succinate than cultures grown on glucose. Production of cell dry matter and protein per gram of substrate used was the same in pectin- and glucose-grown cultures. The principal enzymes that participated in the metabolism of pectin were extracellular exopectate hydrolase (EC 3.2.1.67), extracellular endopectate lyase (EC 4.2.2.2) and cell-associated 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14). The latter enzyme is unique to the Entner–Doudoroff pathway. Activities of pectinolytic enzymes in cultures grown on glucose were low. Activity of KDPG aldolase was similar in pectin- and glucose-grown cells.

Conclusions: Metabolites and activities of pectin-degrading enzymes differed in cultures of *B. caccae* KWN grown on pectin and glucose. Yields of dry matter and protein were the same on both substrates.

Significance and Impact of the Study: Information on metabolism of pectin in animal strains of *Bacteroides* is incomplete. This study extends the knowledge on metabolism in bacteria from the rabbit caecum.

Keywords: *Bacteroides caccae*, caecum, metabolism, pectin, rabbit.

INTRODUCTION

Pectin is a fibre component, occurring in the middle lamella and primary cell wall of higher plants. Like other fibre constituents, pectin is not degraded by endogenous enzymes in the stomach and small intestine of man and other animals, but fermented in the hindgut (Bacon 1978). Among pectinolytic bacteria isolated from the lower intestinal tract, members of the *Bacteroides* genus are probably the most important, taking into account their high numbers and nutritional versatility (Bayliss and Houston 1984; Macfarlane *et al.* 1997). *Bacteroides* counts in fresh faeces increased greatly when rats were fed diets containing pectin (Dongowski *et al.* 2002). Bacteria belonging to the *Bacteroides* predominated over other identified pectinolytic organisms in the rabbit caecum (Sirotek *et al.* 2001).

Pectin degradation pattern has been determined in *Bacteroides* from the human intestine. In strains examined, pectin was converted to a mixture of oligogalacturonides, mainly unsaturated products of pectate lyase activity (McCarthy *et al.* 1985; Jensen and Canale-Parola 1986; Dongowski *et al.* 2000). Oligogalacturonides were metabolized intracellulary with acetate as the main fermentation product. Little work has been published on the metabolism of pectin in *Bacteroides* from other habitats. There are

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studies on metabolism of pectin in *Bacteroides ruminicola* (Wojciechowicz 1971; Szymański 1981), however, according to current taxonomic criteria this rumen bacterium is assigned to the genus *Prevotella* (Avguštin *et al.* 1994). Thus, the aim of our study was to elucidate metabolism of pectin in a strain of *Bacteroides caccae* from the rabbit caecum. The isolate KWN grew well on pectin and its pectinolytic activity was higher than that of other *Bacteroides* strains tested in a previous study (Sirotek *et al.* 2001).

MATERIAL AND METHODS

Bacteria

Bacteroides caccae KWN was isolated by Dr V. Rada (CUA Prague) from the caecal contents of a rabbit fed with oats and meadow hay *ad libitum*. Preliminary identification of the isolate was based on its phenotypic characteristics (API 50 CHL tests; Biomérieux, Marcy l'Etoile, France). To determine the taxonomy of the strain KWN more precisely, DNA was isolated according to Gregg *et al.* (1994). The 16S rDNA fragments were obtained after amplifying bacterial DNA using FP27 (5′-AGA GTT TGA TCC TGG CTC AGG A-3′, *E. coli* position 8–29) and 515R (5′-TTA CCG TGA CTG GCA C-3′, *E. coli* position 520–538) primers on a thermocycler (Kopečný *et al.* 2001). These 500 bp fragments were sequenced with the ABI 310 capillary sequencer (Perkin-Elmer, Boston, MA, USA). After editing, 16S rDNA sequences were compared with published sequences of related bacteria from the EMBL (EBI) and GenBank (NCBI) nucleotide databases using BLAST and with data from the Ribosomal Database Project (Maidak *et al.* 1994).

Two control organisms were used: *Streptococcus bovis* X4, a bacterium with an endo-type of pectin-depolymerizing activity (Wojciechowicz and Ziolecki 1984) and *Pseudomonas fluorescens* DBM 3056, a bacterium with enzymes of the Entner–Doudoroff metabolic pathway (Preiss and Ashwell 1963). *Streptococcus bovis* X4 was isolated from the rumen fluid of a sheep at this Institute. *Pseudomonas fluorescens* DBM 3056, was obtained from the culture collection of the Department of Biochemistry and Microbiology of the Institute of Chemical Technology, Prague. Bacteria were maintained in 20% (v/v) glycerol at −40°C.

Pectin

Apple pectin was supplied by Pektin Ltd (now Danisco Czech Republic, Smiřice, Czech Republic). It was purified by ethanol extraction (75% v/v) to remove low-molecular weight contaminants. Uronic acid content of pectin was determined by the 3-phenylphenol method (Blumenkrantz and Asboe-Hansen 1973), and neutral monosaccharide composition by gas chromatography (Marounek and Dušková 1999). To determine methanol, methoxyl groups were hydrolysed in an alkali milieu (0·5 mol l^{-1} NaOH), and methanol estimated by gas-liquid chromatography on a 2·4 m column of Chromosorb W AW with 15% SP1220/1% H_3PO_4 (Supelco, Bellefonte, PA, USA), operated at 100°C. Carbon content of pectin was determined using a Perkin Elmer 2400 elemental analyser (Perkin-Elmer). Pectin contained neutral sugars and carbon at 68 and 419 mg g^{-1} of dry matter, respectively, and 52·4% of its carboxyl groups were methylated.

Media

Throughout the study, *B. caccae* KWN and *Strep. bovis* X4 were grown anaerobically on a medium supplemented with clarified caecal extract, yeast extract and pancreatic casein hydrolysate (Slováková *et al.* 2002). The medium was reduced by 0·05% cysteine·HCl. *Bacteroides caccae* KWN was grown on pectin or glucose. Substrates were added at 4 g l^{-1}, final concentration. *Streptococcus bovis* X4 was cultivated on the mixed substrate: pectin and glucose were added to the medium at 2 g l^{-1} each. *Pseudomonas fluorescens* DBM 3056 was grown on the medium of Van Dijken and Quayle (1977) with glucose (4 g l^{-1}). The pseudomonad was grown aerobically.

Metabolites and cell yields

Medium for *B. caccae* KWN was distributed in 15-ml amounts into CO_2-gassed 20-ml flasks, closed by rubber stoppers, and autoclaved at 110°C for 1 h. One-day culture (0·3 ml) was used to inoculate the growth medium. Inoculated cultures were grown at 39°C overnight (16 h) in six replicates. The culture pH fell from 6·7–6·8 to about 6·0 (pectin) or 5·8 (glucose) in the course of the incubation. Methods for determination of cell dry matter, protein, residual pectin and glucose and analysis of fermentation products were as described previously (Marounek and Dušková 1999; Slováková *et al.* 2002). Carbon content was determined in freeze-dried cells harvested from an overnight culture. Methanol and carbon were determined as described above.

Hydrogen production in *B. caccae* KWN was measured in 100-ml flasks hermetically closed with butyl rubber stoppers. Inoculated cultures (30 ml) were grown in six replicates at 39°C for 16 h. Samples of the headspace gas were taken with a gas-tight syringe and analysed by gas chromatography at 100°C, using a chromatograph equipped with a thermal conductivity detector and a column of Carboxen 100 (Supelco).

Enzyme assays and calculations

For enzyme assays, *B. caccae* KWN was grown on pectin or glucose in 500-ml batch cultures at 39°C for 16 h. One-day

cultures (2 ml) were used to inoculate the growth medium. Cells were collected by centrifugation from an early stationary phase, washed, and disrupted by sonication (see Marounek and Dušková 1999 for details). Culture supernatant fluids were dialysed at 4°C for 24 h. Cell extracts and dialysed supernatant fluids were used for determination of cell-associated and extracellular activity, respectively, of pectate lyase and pectinase (Dušková and Marounek 2001). Pectate lyase releases products with an unsaturated residue at the nonreducing end. Pectinase cleaves the macromolecule by hydrolysis. The activity of the former enzyme can be assayed by determination of the absorbance at 232 nm, and activity of the latter enzyme by determination of the concentration of reducing sugars (Collmer *et al.* 1988).

Endo- and exo-acting polysaccharidases differ in the rate of the decrease in viscosity of the substrate solution (Rombouts and Pilnik 1980). Lyases have an alkaline pH optimum and require divalent cations. Hydrolases have pH optimum 6·0 or lower and do not require divalent cations (Collmer *et al.* 1988). Lyase and hydrolase activity assays, thus were performed at pH 7·5 or 5·6, with or without calcium chloride addition, respectively. This arrangement enabled to distinguish between hydrolase and lyase type of pectin-degrading activity (McCarthy *et al.* 1985). To distinguish between endo-and exo-type of pectin-depolymerizing activity of lyase and hydrolase, cultures of *B. caccae* KWN and *Strep. bovis* X4 were grown on pectin for 16 h (strain KWN), and on pectin + glucose for 12 h (strain X4). Cells were harvested by centrifugation and culture supernatants dialysed. A reaction mixture was prepared consisting of 100 ml of 1·2% (w/v) pectin in 0·1 M Na-acetate buffer (pH 7·5 or 5·6) and 20 ml of dialysed supernatant fluid. One half of the mixture was used for viscosity measurements at 39°C in a Hoeppler viscosimeter B3 (VEB MLW; Prüfgeräte-Werk, Medingen, Germany). The time of fall of a ball in the reaction mixture, hermetically closed, was measured at intervals according to the manufacturer's instruction. Simultaneously, the second half of the reaction mixture was incubated anaerobically at 39°C and samples were taken for the estimation of reducing sugars (Lever 1977), or unsaturated products measured as absorbance at 232 nm. The reaction mixture in lyase activity assay was supplemented with $CaCl_2$ at 7·5 mmol l^{-1}, final concentration.

The activity of 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14), an enzyme unique to the Entner–Doudoroff pathway, was determined as described by Marounek and Dušková (1999), with KDPG as the substrate. To test the method, the activity of KDPG aldolase was determined in glucose-cultivated cultures of *Ps. fluorescens* DBM 3056. The growth medium (500 ml in 1·5-l flasks) was inoculated with 2 ml of 2 day-culture of the pseudomonad, and cultivated aerobically at 28°C on a shaking water bath for 48 h. In both bacteria, the activity of 6-phosphogluconate dehydrase (EC 4.2.1.12) and KDPG aldolase was determined in a coupled reaction with 6-phosphogluconate as the substrate.

Production of metabolites, cell dry matter and protein was related to the amount of substrate utilized. Carbon recovery was calculated from the metabolic products and C content of the cells (46·4%). Enzyme activities were expressed in nanomoles of substrate split or product liberated per minute per milligram of protein. The significance of differences was evaluated by the *t*-test.

RESULTS

Molecular-genetic analysis identified the KWN isolate as a strain of *B. caccae* with 98% identity of 16S rDNA sequence. *Bacteroides caccae* KWN utilized almost all glucose and 81% of pectin. Cultures grown on pectin produced significantly more acetate and less formate, lactate, fumarate and succinate than cultures grown on glucose. As expected, methanol was found only in former cultures (Table 1). Production of hydrogen was very small. Yields of dry matter and protein were the same in cultures grown on pectin and glucose. Carbon recovery did not differ greatly in these cultures.

Pectin macromolecule was degraded by the action of pectate lyase and pectinase. Specific activities of both enzymes were higher in culture supernatants than in cell extracts, and *ca* 10-times lower in cultures grown on glucose than on pectin (Table 2). Action pattern of pectic enzymes

Table 1 Metabolite profiles and cell yields of the rabbit caecal bacterium *Bacteroides caccae* KWN grown on pectin and glucose*

	Pectin	Glucose
Substrate used (g l^{-1})	3·25 ± 0·19†	3·99 ± 0·01
Metabolites (mmol g^{-1})		
Formate	0·9 ± 0·3†	2·5 ± 0·6
Acetate	24·9 ± 0·7†	17·7 ± 0·5
Propionate	7·6 ± 0·7	7·8 ± 0·8
Lactate	0†	4·5 ± 0·9
Fumarate	0·3 ± 0·1†	1·3 ± 0·5
Succinate	5·4 ± 0·9†	9·6 ± 1·7
Methanol	11·4 ± 1·7†	0
Hydrogen	0·12 ± 0·01	0·13 ± 0·03
Cell dry weight (g l^{-1})	1·06 ± 0·17†	1·31 ± 0·06
Cell yields (g g^{-1} substrate used)		
Dry matter	0·33 ± 0·05	0·33 ± 0·02
Protein	0·10 ± 0·01	0·10 ± 0·01
C-recovery (%)	121·0	127·2

*Means of six cultures ±S.D. The results are differences in amounts between the beginning and end of the incubation.
†Significantly different from the glucose value ($P < 0·01$).

Table 2 Specific activities of PL, P, KDPGA, and PGD plus KDPGA in cells and culture supernatant fluids of *Bacteroides caccae* KWN and *Pseudomonas fluorescens* DBM 3056. Bacteria were grown on pectin or glucose

Enzyme activity*	Sample	Pectin	Glucose	
		B. caccae	*B. caccae*	*Ps. fluorescens*
PL	Cell extract	2·9 ± 0·5	0·3 ± 0·1	–
PL	Supernatant	30·2 ± 5·1	2·8 ± 0·7	–
P	Cell extract	5·5 ± 1·1	0·5 ± 0·1	–
P	Supernatant	45·9 ± 7·9	4·5 ± 0·3	–
KDPGA	Cell extract	662 ± 55	719 ± 31	468
PGD + KDPGA	Cell extract	0	0	94

*Expressed in nanomoles of substrate split or product released per minute per milligram of protein. See Material and methods for substrates of enzymatic reactions.
Means of two (enzymes of *Ps. fluorescens*) or four (other enzymes) cultures ±S.D.
–, not determined; PL, pectate lyase; P, pectinase; KDPGA, 2-keto-3-deoxy-6-phosphogluconate aldolase and PGD, 6-phosphogluconate dehydrase.

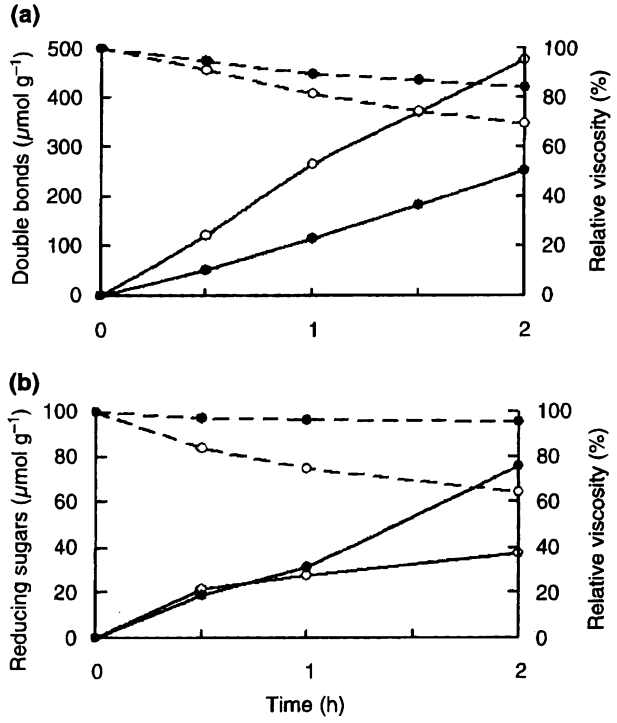

Fig. 1 Formation of unsaturated products (a) and production of reducing sugars (b) in reaction mixture of culture supernatant and solution of pectin. Dashed lines show relative viscosity of reaction mixture. Closed symbols: *Bacteroides caccae* KWN; open symbols: *Streptococcus bovis* X4

was determined by viscosimetric and reaction product analyses. Figure 1a shows that the formation of unsaturated products and drop in viscosity were more rapid in the culture supernatant of *Strep. bovis* X4 than in that of *B. caccae* KWN. The ratio of both parameters (micromoles of double bonds per gram per 1% of the relative viscosity decrease), however, was almost the same in both bacteria: 15·6 and 16·0 in *Strep. bovis* and *B. caccae*, respectively. A great increase of the reducing sugar concentration in the *B. caccae* KWN culture supernatant supplied with pectin was accompanied by a negligible reduction of viscosity (Fig. 1b). Contrary to this, a relatively small increase of the reducing power and a substantial loss of viscosity were observed in the culture supernatant of *Strep. bovis* X4.

Both pectin- and glucose-grown cells of *B. caccae* KWN possessed 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14) activity. Phosphogluconate was metabolized by the cell extract of *Ps. fluorescens*, but not by the cell extract of *B. caccae*.

DISCUSSION

Pectin is a more oxidized substrate than glucose, thus its metabolites should be less reduced than those of glucose. Indeed, production of acetate, the formation of, which does not require reducing equivalents, was higher by 40·7% in cultures grown on pectin compared with those supplied with glucose (by 72·7% when expressed per gram of substrate used). However, the production of lactate, fumarate and succinate, the synthesis of which from pyruvate requires metabolic hydrogen, was lower on pectin. Lactate was produced only in cultures supplied with glucose. In accordance with Johnson *et al.* (1986), only a trace of hydrogen was detected in the headspace gas. Yields of cell dry matter and protein suggest that the gain of energy on both substrates was similar. C-recoveries, however, exceeded 100%, indicating that also compounds other than pectin and glucose were used for the synthesis of cell matter.

Depolymerases of pectin split the glycosidic bonds either by β-elimination (lyases) or by hydrolysis (hydrolases). Enzymes of both types can degrade the macromolecule in a random fashion, or liberate galacturonate residues by a terminal attack on the polymer (Rombouts and Pilnik 1980). Comparison of the time course of the concentration of reducing sugars and relative viscosity (Fig. 1b) indicates that the pectin hydrolase has an exo-type mode of action (terminal cleavage of the polymer) in *B. caccae* KWN, but an endo-type mode in *Strep. bovis* X4 (random cleavage). The lyase activity of *Strep. bovis* X4 was twofold higher than that of *B. caccae* KWN. Action patterns of lyases, however, were similar in both bacteria (Fig. 1a). Wojciechowicz and Ziolecki (1984) identified the

pectin-degrading enzyme of *Strep. bovis* 13E as endopolygalacturonate (i.e. endopectate) lyase (EC 4.2.2.2). We propose that an enzyme of the same type is produced in pectin-grown cultures of the strain KWN. Specific activity of extracellular pectinolytic enzymes of the strain KWN was by one order higher than that of cell-associated enzymes. Although a part of the extracellular pectinolytic activity may be released into the environment by cell lysis, we believe on basis of light microscopy examinations that most of this activity was produced by nonlysed cells. This has similarly been shown to be the case for human colonic bacteria *B. pectinophilus* and *B. galacturonicus* (Jensen and Canale-Parola 1986). On the contrary, polygalacturonic acid (PGA) lyase and PGA hydrolase of *B. thetaiotamicron* were cell-associated (McCarthy *et al.* 1985). The distinction between extracellular and cell-associated enzymes, however, is somewhat artificial as in natural ecosystems an extracellular polysaccharidase is trapped between the bacterium and the plant particle, making the enzyme effectively cell-associated (Salyers and Leedle 1983).

High concentration of methanol in pectin-grown cultures of *B. caccae* KWN suggests that methoxyl groups of pectin were hydrolyzed. Products of pectin degradation are catabolized intracellulary. Cells of the strain KWN possess the activity of KDPG aldolase, the key enzyme of the Entner–Doudoroff pathway of uronate metabolism, as has been shown also in other pectin-utilizing rumen and rabbit caecal bacteria (Paster and Canale-Parola 1985; Marounek and Dušková 1999; Dušková and Marounek 2001; Slováková *et al.* 2002), and in various saprophytic micro-organisms and plant pathogens (Rombouts and Pilnik 1980). Phosphogluconate, a product of the glucose metabolism, was not metabolized by the cell extracts of *B. caccae* KWN. This indicates that phosphogluconate dehydrase (EC 4.2.1.12), the enzyme producing KDPG from 6-phosphogluconate (Touster 1969) was absent. Consequently, the conventional Entner–Doudoroff pathway of glucose metabolism cannot operate in *B. caccae*.

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