

Isolation, Identification and Characterization of Rabbit Caecal Mucinolytic Bacteria

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Abstract

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The aim of our study was to isolate, identify and characterize mucinolytic bacteria from the rabbit caecum. Two hundred and thirty mucin-grown colonies from the caecal contents of 7 rabbits were examined microscopically after the Gram staining. Gram-negative irregular rods were the most numerous mucinolytic isolates (34.1%), followed by gram-negative cocci and short rods (22.5%), gram-positive rods (17.3%), gram-positive cocci (16.6%) and gram-positive sporeforming rods (9.5%). An attempt was done to identify 31 typical isolates on basis of their morphology, biochemical characteristics and production of metabolites. In addition, bacterial cells were hybridized with fluorescently labelled probes for *Bacteroides/Prevotella* and *Clostridium* genus. Four isolates were identified at the species level as *Mitsuokella multiacidus*, three isolates as *Bacteroides capillosus* and one isolate as *Actinomyces izraeli*. All isolates except the last one belong to the *Bacteroidaceae* family. One strain could be assigned to the *Bacteroides/Prevotella* genus on basis of the hybridization test. Other mucinolytic isolates were not identified as their characteristics did not correspond to any previously described bacterial species. No *Clostridium* sp. strain was detected. In two *M. multiacidus* strains high activities of extracellular mucin lyases were found. The pH-optimum of lyases was 6.2. Calcium cations were necessary for their optimal function. This work extends general knowledge about fermentation of carbohydrate and nitrogen substrates in rabbit caecum.

Rabbit, caecum, mucin, bacteria

Mucin is a glycoprotein present in the mucosal layer lining mammalian gastrointestinal, respiratory and reproductive tract. The carbohydrate moiety can be either an acidic mucopolysaccharide containing uronic acids and hexosamines or an oligosaccharide containing L-fucose and sialic acid (Salyers and Leedle 1983). Mucin composition is tissue specific and differs in different animal species. Mucin is a part of the defense mechanism as it prevents bacteria, viruses and dietary lectins from attaching to the intestinal or other epithelium. Another mucin function is the protection of the mucosal epithelium against acidic and proteolytic damage in the stomach and intestine. In the digestive tract, mucin insures a smooth passage of the foodpulp. Holmes et al. (1974) observed in pigs that large amounts of mucin were not digested in the small intestine, but fermented in the hindgut. Microorganisms responsible for mucin fermentation in the human colon are partially known. Salyers et al. (1977) isolated from the human colon several *Bacteroides* strains able to degrade mucins and plant polysaccharides. Bayliss and Houston (1984) identified some mucin fermenters as members of the *Bacteroides* genus, other resembled *Bifidobacterium* sp., *Clostridium septicum*, and *Eubacterium* sp. Crociani et al. (1994) found that only

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Bifidobacterium bifidum strains fermented gastric mucin among 29 species of bifidobacteria tested. Mucin fermenters from the digestive tract of herbivores are almost unknown. Hill (1986) has shown that bacteria isolated from the rabbit caecum belonging to the *Bacteroides* genus were most active in digestion of mucin *in vitro*. In *in vitro* incubations of the rabbit caecal contents, mucin was converted into mixture of metabolites, yielding more volatile fatty acids and methane than plant proteins and casein (Marounek et al. 2000). The aim of our study was to isolate, identify and characterize mucinolytic bacteria from the rabbit caecum.

Materials and Methods

Animals and diets

Three rabbits were purchased from a commercial farm with 200 does (Hyplus genotype). Four crossbred rabbits were obtained from a small farm (backyard system). Commercial rabbits were fed *ad libitum* a granulated feed containing alfalfa meal, barley, oat, wheat bran and extracted oilseed meals as the principal components. Two crossbred rabbits were fed with wheat (30 - 40 g/day), apples (200 - 300g/d) and meadow hay *ad libitum*. Another two crossbred rabbits were fed with wheat (30 - 40g/d) and grass *ad libitum*.

Enumeration, isolation and characterization of bacteria

Rabbits were killed by cervical dislocation, their caecal contents were aseptically collected, serially diluted and plated on Wilkins-Chalgren agar (Oxoid), containing 10% caecal extract prepared according to Emaldi et al. (1979) and 0.4% porcine gastric mucin (Fluka). The plates were incubated anaerobically for 2 days at 39 °C in anaerostats (Anaerobic Plus System, Oxoid) under CO₂/H₂ (9:1) atmosphere. The colonies were counted and the mean and standard deviation calculated. A total of 230 mucin-grown colonies were picked up at random and examined microscopically after the Gram staining. Typical isolates (31 strains) were tested for growth in 20% bile, 5% NaCl and for catalase production (Faddin 1976). Their biochemical characteristics were determined using commercial tests Anaerostest 23 and Enterotest 24 from Lachema (Brno, Czech Rep.). End-products of glucose fermentation were estimated in cultures of bacteria grown in the Wilkins-Chalgren broth.

Fluorescence *in situ* hybridization (FISH)

An attempt was done to identify some isolates using the *in situ* hybridization. Two fluorescently labelled probes, 10-MC-H008 for *Bacteroides/Prevotella* and 10-MC-H009 for *Clostridium* genus were purchased from Microscreen BV (Groningen, The Netherlands). *Bacteroides thetaiotamicron* ATCC 29741 and *Clostridium perfringens* CNCTC 5459 were the control organisms. Overnight cultures were centrifuged and cells resuspended in 10 mM phosphate buffer (pH = 6.5) containing cysteine.HCl at 0.5 g/l. The "Fixative" (0.1 ml) was added to resuspended cells (0.9 ml) and incubated for 4h at 4 °C. After fixation, the cells were centrifuged and the pellet resuspended in 5.0 ml of "MCPXI" buffer. Ten µl of the suspension were pipetted onto a microscope slide and spreaded over the glass surface. The spot was dried for 15 min at 45 °C and dehydrated by submerging the slide in a graded ethanol series (50, 75 and 100% ethanol), for 2 min each. Ten µl of "Hybridization mixture" were pipetted to the cell spot and covered with a cover glass. The microscope slide was placed in the prewarmed hybridization chamber and hybridized for 16 h at 45 °C in the dark. After hybridization, the cells were washed for 20 min at 45 °C by submerging the slide in 40 ml of "MCW1" buffer. The surface was rinsed with sterile water and the slide dried in air. Six µl of "Mounting Fluid" were applied to the cells and covered with a cover glass. The samples were viewed by the E-800 Nikon epifluorescence microscope using 1000 × magnification and appropriate excitation and emission filters for FITC (fluorescein thiosocyanate; 495/520 nm). Cells stain green due to the fluorescence of FITC.

Analyses

Volatile fatty acids were determined by gas-liquid chromatography on a column of Chromosorb WAW with 15% SP 1220 and 1% H₃PO₄ (Supelco). Lactate, fumarate and succinate were methylated and estimated on a column of Supelcoport with 3% SP 2340 from the same firm. Concentration of protein was determined by the Lowry method (Herbert et al. 1971). The optical density of cultures was measured at 640 nm.

Enzyme assays

Bacteria were grown in a vitamin-mineral medium prepared according to Marounek et al. (1993), supplemented with 0.1% yeast extract and 10% caecal extract. The medium contained 0.4% mucin as the substrate. Two-day bacterial cultures were centrifuged at 5000 rpm for 40 min, and cell-free supernatant collected for extracellular enzymes activity assays. Equal amounts of the supernatant and 2% mucin solution in 0.1 M phosphate buffer were mixed and incubated at 39 °C with or without CaCl₂ addition (0.5% final concentration). Activity of mucin lyase was assayed according to Linker (1966), and expressed as nmol of unsaturated products released per min. per mg of protein. In accordance with Colmer et al. (1988), we supposed that the molar extinction coefficient of unsaturated products (double bonds) was 4600 dm³·mol⁻¹·cm⁻¹. Activity of mucin hydrolase was assayed by spectrophotometric determination of reducing sugars released from mucin using the method of Lever (1977).

The *t*-test was used to evaluate differences in proportions of morphological types of mucinolytic bacteria isolated from Hyplus and crossbred rabbits.

Results and Discussion

The number of mucinolytic bacteria in caecal samples was $5.96 \pm 3.79 \times 10^6$ cfu per g. Relatively low counts of mucinolytic bacteria contrast with high fermentability of mucin in mixed cultures of rabbit caecal microorganisms (Marounek et al. 2000). Most of mucinolytic bacteria in the rabbit caecum, thus, may be non-cultivable, i. e. not able to grow in pure cultures, without interactions with other microbes. Gram-negative irregular rods were the most numerous mucinolytic isolates in this study, followed by gram-negative cocci and short rods, gram-positive rods, gram-positive cocci and gram-positive spore-forming rods (Table 1). Differences between commercial (Hyplus) and crossbred rabbits were mostly small in spite of the fact that rabbits were fed different diets. Significantly higher proportion of gram-positive sporeforming rods, however, was found among mucinolytic isolates from caeca of Hyplus rabbits ($P < 0.05$).

Nine strains out of 31 isolates examined were identified at the species level. Their biochemical characteristics are shown in Table 2. According to current taxonomic criteria, gram-negative isolates MB2, MB4, MB5 and MB9 were identified as *Mitsuokella multiacidus* (formerly *Bacteroides multiacidus*), and strains MA2, MA5, MK6 and MK7 as

Table 1
Proportions of various morphological types of mucinolytic bacteria in caeca of rabbits

Type	Hyplus rabbits	Crossbred rabbits	All rabbits
Gram-negative irregular rods	0.349 ± 0.052	0.335 ± 0.018	0.341 ± 0.032
Gram-negative cocci and short rods	0.206 ± 0.056	0.240 ± 0.031	0.225 ± 0.041
Gram-positive rods	0.143 ± 0.091	0.196 ± 0.047	0.173 ± 0.066
Gram-positive cocci	0.163 ± 0.020	0.169 ± 0.024	0.166 ± 0.021
Gram-positive sporeforming rods	0.137 ± 0.024^a	0.064 ± 0.041^b	0.095 ± 0.040

Mean values for 3 Hyplus and 4 crossbred rabbits \pm SD; total number of isolates 230

^a^bSignificant difference between Hyplus and crossbred rabbits ($P < 0.05$)

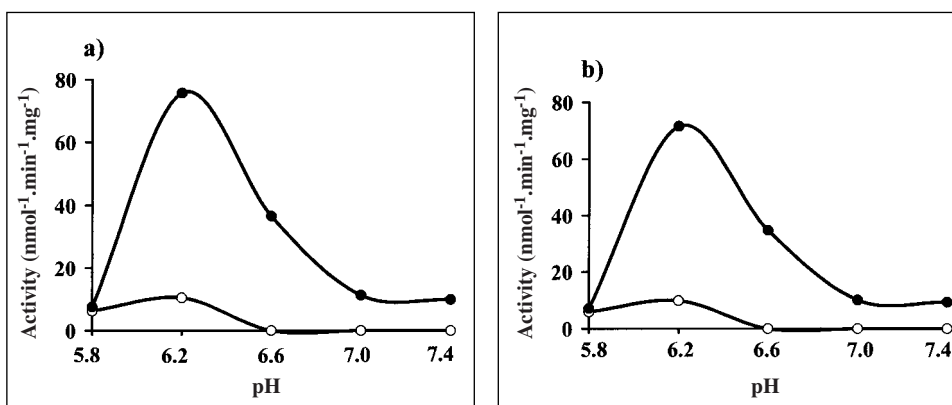


Fig. 1. Effect of pH and Ca^{++} ions on activity of mucin lyases in *Mitsuokella multiacidus*, strains MB2 (a) and MB9 (b). Enzymatic reactions were carried out with (●) or without (○) CaCl_2 addition. Activity was expressed as nmol of unsaturated products released per min. per mg of protein.

Bacteroides capillosus. Nucleic acids of the MK7 isolate, however, did not hybridize with the *Bacteroides/Prevotella* fluorescent probe. The taxonomy of this isolate, thus, remains uncertain. One gram-negative isolate, unidentified at the species level (MA1 strain), hybridized with fluorescently labelled *Bacteroides/Prevotella* probe. Identification of gram-positive bacteria was successful in one isolate only. Strain MA3 was identified as

Table 2
Biochemical characteristics^a of rabbit mucinolytic isolates

Parameter	MA2	MA3	MA5	MB2	MB4	MB5	MB9	MK6	MK7
Gram staining	-	+	-	-	-	-	-	-	-
H ₂ S production	-	-	-	-	-	-	-	-	-
Indol production	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	+	+	±	+	-	-
Aesculin	+	+	+	+	+	+	+	+	+
Mannose	+	+	-	+	+	+	+	-	-
Maltose	-	+	-	+	+	+	+	-	-
Salicin	-	+	-	+	+	+	+	-	-
Raffinose	-	-	-	+	+	+	+	-	-
Fructose	-	+	-	+	+	+	+	-	-
Trehalose	-	+	-	+	+	+	+	-	+
Cellobiose	-	+	-	+	+	+	+	-	-
Galactose	-	+	-	+	+	+	+	-	-
Mannitol	-	+	-	+	+	+	+	-	-
Xylose	-	+	-	+	+	+	+	-	-
Lactose	-	-	-	+	+	+	+	-	-
Rhamnose	-	-	-	+	+	-	+	-	-
Arabinose	-	+	-	+	+	+	+	-	-
Melezitose	-	-	-	+	+	+	+	-	-
Sorbitol	-	+	-	-	+	-	+	-	-
Melibiose	-	-	-	+	+	+	+	-	-
Urease	+	-	+	-	+	-	-	+	+
Catalase	+	-	+	+	+	+	+	+	+
Growth in bile	+	-	+	+	+	+++	+	+	+
End - products ^b	a, l, p, s	A, l, S	a, l, p, s	L, A, S	L, A	L, A, S	L, A, S	a, l, s	a, l, p, s

^a See Materials and Methods

^b Metabolites of glucose: a - acetate, l - lactate, p – propionate;
capital letters indicate concentrations > 10 mM, small letters < 10 mM.

Actinomyces izraeli. Only this strain does not belong to *Bacteroidaceae*. No strain belonging to the *Clostridium* genus was found.

Mucinolytic bacteria produce a range of proteolytic and glycosidic enzymes that play a role in the degradation of host-derived glycoproteins (e.g. Rafay et al. 1996). Fig. 1 shows activity of lyases involved in the degradation of mucin in two bacteria growing on mucin to the highest optical density. Activities of lyases in *M. multiacidus* strains MB2 and MB9 were similar, with the pH-optimum at 6.2. Calcium ions were necessary for their function. Activity of mucin hydrolase was not found in these strains.

It follows from our results that bacteria *M. multiacidus*, *B. capillosus* and *A. izraeli* participate in the fermentation of mucin, which is one of substrates coming to the hindgut from the small intestine and secreted by goblet cells. As shown in Table 2, *B. capillosus* isolates were less versatile (i.e. fermenting a limited number of substrates) than those of *M. multiacidus* and *A. izraeli*. Attempts to identify other mucinolytic isolates failed as their phenotypic characteristics did not correspond to any previously described species. In our opinion, information on the complex microflora in the rabbit coecum is far from complete and novel species may be present in the rabbit digestive tract. This has similarly been shown

to be the case for the equine large intestine, as the analysis of 16S ribosomal RNA gene sequences from samples of intestinal wall and lumen contents showed that only 5% of the sequence corresponded to known organisms (Daly et al. 2001).

Isolace, identifikace a charakterizace mucinolytických bakterií ze slepého střeva králíků

Z obsahů slepých střev sedmi králíků jsme získali 230 bakteriálních isolátů, schopných růst v médiu s mucinem ze žaludku prasete. Největší zastoupení měly gram-negativní nepravidelné tyčinky (34,1%), následované gram-negativními koky a krátkými tyčinkami (22,5%), gram-positivními tyčinkami (17,3%), gram-positivními koky (16,6%) a gram-positivními sporotvornými tyčinkami (9,5%). Třicet jeden isolát jsme se pokusili identifikovat na základě morfologie, výsledků biochemických testů a tvorby metabolitů. Navíc jsme bakteriální buňky hybridizovali s fluorescenčně značenými sondami pro rody *Bacteroides/Prevotella* a *Clostridium*. Čtyři isoláty se podařilo identifikovat na úrovni druhu jako *Mitsuokella multiacidus*, tři jako *Bacteroides capillosus* a jeden jako *Actinomyces izraeli*. Vyjma posledního, identifikované kmeny přísluší k čeledi *Bacteroidaceae*. Jeden isolát jsme na základě výsledku hybridizační zkoušky mohli přiřadit k rodu *Bacteroides/Prevotella*, bez bližšího druhového určení. Ostatní isoláty se nepodařilo identifikovat, neboť jejich fenotypické charakteristiky neodpovídaly známým bakteriálním druhům. Mezi neidentifikovanými isoláty nebyl žádný zástupce rodu *Clostridium*. U dvou kmenů *M. multiacidus*, které na mucinu rostly nejlépe, jsme zjistili vysokou aktivitu mucin lyas. Optimální pH jejich účinku bylo 6,2. Aktivita byla podmíněna přítomností Ca^{++} iontů.

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