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Research Article

Genetic diversity of β-glucuronidase activity among 14 strains of the dominant human gut anaerobe *Ruminococcus gnavus*

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Abstract

Bacterial β-glucuronidase activity in the gut increases the enterohepatic circulation of toxic compounds and plays a major role in the etiology of colon cancer. Previously, we had found that the *gus* gene, which codes for β-glucuronidase in a dominant anaerobic species of the gut microbiota, *Ruminococcus gnavus* strain E1, is transcribed as part of an operon that includes three ORFs that code for β-glucoside permeases of the phosphotransferase systems. This genetic organization had never been described. We have now compared β-glucuronidase activity and the genetic environment of the *gus* gene in 14 strains of *Ruminococcus gnavus*.We found that five out of the seven glucuronidase-positive *R. gnavus* strains possessed another glucuronidase gene different from the *gusA* operon of *R. gnavus* E1. This dominant commensal intestinal species appears to have a high degree of genetic diversity in the genes that control β-glucuronidase activity.

Key words : Ruminococcus gnavus, glucuronidases, digestive microbiota.

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Introduction

Glucuronidation is a major detoxification process that converts a large number of xenobiotic and endogenous substances into more hydrophilic metabolites (Tephly and Burchell,1990; Gueraud and Paris,1998). Parts of the glucuronides are secreted through the biliary route into the intestine. They are poorly reabsorbed into the bloodstream and are efficiently eliminated from the body, unless they are hydrolyzed by intestinal β-glucuronidase enzymes. However, most of the β-glucuronidase activity in the caecum and in the large intestine of rats has been attributed to bacterial enzymes (Rod and Midtvedt, 1977). β-glucuronidase activity increases the enterohepatic circulation of toxic compounds. Consequently, it plays a major role in the generation and maintenance of toxic and carcinogenic metabolites in the body, which can promote tumor formation at various sites, including the large bowel (McBain and MacFarlane,1998; Arimochi et al.,1999). We previously identified and cloned a new β-glucuronidase gene, *gus*, in an anaerobic strain of the dominant gut microorganism, *Ruminococcus gnavus* strain E1 (Beaud et al.,2005). This *gus* gene was transcribed as part of an operon, including three ORFs, ORF2, ORF3 and ORF5. ORF2 and ORF3 products presented strong similarities with many β-glucoside permeases of the phosphoenolpyruvate β-glucoside phosphotransferase systems (PTS), such as BglC in *Escherichia coli*, BglP in *Bacillus subtilis* and PTS enzyme II in *Bacillus halodurans*. ORF5 product presents strong similarities with the amino-terminal domain of *Clostridium acetobutylicum* β-glucosidase (bglA). Because of the significance of β-glucuronidase in numerous physiological and pathological processes, including colon cancer, we compared β-glucuronidase activity and the genetic environment of the corresponding gene in 13 *R. gnavus* strains and the original strain E1, isolated from human gut.

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Material and Methods

Ruminococcus gnavus strains and culture conditions

The 14 *R. gnavus* strains were isolated from 14 donors - (Table 1). These strains were identified at the species level on the basis of the nucleotide sequence of the DNA encoding the 16S RNA (Marcille *et al*., 2002). The strains

Table 1 - Strains of *Ruminococcus gnavus*.

R. gnavus strains	Origin/ Isolated from human fecal samples of :	Reference or source
B53	adults with chronic pouchitis.	Our collection [a]
B57	healthy adults(type strain)	ATCC 29149
B70	patient with Crohn disease.	Our collection [a]
B74	patient with Crohn disease.	Our collection [a]
G25	healthy children.	Our collection [a]
E1	healthy adults.	(Ramare F., 1993)
V42	adults with chronic pouchitis.	Our collection [a]
V50	adults with chronic pouchitis.	Our collection [a]
V51	adults with chronic pouchitis.	Our collection [a]
V62	healthy adults.	Our collection [a]
V66	adults with chronic pouchitis.	Our collection [a]
V58	adults with chronic pouchitis.	Our collection [a]
V95	healthy children	Our collection
V99	healthy children	Our collection

[a]Unité d' Ecologie et de Physiologie du système digestif, Institut National de la Recherche Agronomique, Jouy en Josas, France. All-*R. gnavus* strains from our collection were identified by 16S rDNA sequencing.

were grown in an anaerobic chamber at 37 °C in reduced BHI broth (Difco) supplemented with 5g/L of yeast extract (Difco) and 5 mg/L of haemin (Sigma-Aldrich).

DNA Techniques

Procedures for DNA manipulations were performed essentially as described in Sambrook et al. (1989). Total cellular DNA were extracted from a *R. gnavus* strains as previously described by Beaud *et al.* (2005). *R. gnavus* DNAs were digested with *Eco*RV according to the manufacturer's specifications. Digested genomic DNA (10 µg) was separated by electrophoresis through a 1% agarose gel (Tebu, Le-Perray-en-Yvelines, France) and transferred by capillary blotting to Hybond-N*** nylon membranes (Amersham Biosciences) The blots were hybridized with three different [α-^{32}P]dCTP-labelled PCR products obtained with oligonucleotides complementary to the coding strand of the *gus* gene (5'-AAATCTGCAAAATTCCA-3' and 5'-TCATTATCCTTATGCAGAAGA-3'), ORF2 (5'-CGGTTCTGCTCTGGTAGATG-3' and 5'-CAATGCCG CAAATGCAAATC-3') and ORF3 (5'-CAGCCGACGT CGGAAATGG-3' and 5'-ACTGATATCGTGTATTC TC CTC-3').of strain E1. PCR amplifications were performed on genomic DNA of the 14 strains as template, using a pair of primers specific for *gus* gene (5'-AAATCTGCAAAA TTCCA-3' and 5'-TCATTATCCTTATGCAGAAGA-3'). PCR reactions were performed in a 50 µL volume with TaKaRa Ex Taq polymerase (Takara Shuzo) under the following conditions: 94 °C for 2 min; 30 cycles of 94 °C for 15 s, 44 °C for 30 s, and 72 °C for 1 min; and a final elongation step at 72 °C for 7 min.

Enzyme assays

β-glucuronidase activity was assessed spectrophotometrically in cell extracts prepared by centrifugation of the cell pellet and disrupting it with 106 µm glass beads (Sigma-Aldrich). The enzyme activity was measured as the rate of release of para-nitro-phenol (at λ = 400nm) from para-nitro-phenyl-β-D-glucuronide (PNPG) (Sigma-Aldrich). One unit of activity is defined as 1 nmol of para-nitro-phenol formed per min per milligram of total protein. Each value was calculated as the average of at least three independent determinations (Beaud *et al.*, 2005).

Results

Seven out of 14 *R. gnavus* strains possessed β-glucuronidase activity (Figure 1). PCR amplifications and hybridization experiments showed that two out of the seven glucuronidase-positive strains (B70, E1) possessed the *gus* operon (Figure 2, Table 2).

Surprisingly, two (B53, V66) out of the seven glucuronidase inactive *R. gnavus* strains possessed a *gus* operon homolog (Figure 2, Table 2). The five other strains (B57, B74, G25, V62, V95), including *R. gnavus* type strain (B57), lacked both β-glucuronidase activity and the *gus* operon homolog. Out of the 14 *R. gnavus* strains isolated from humans, seven presented β-glucuronidase activity and four possessed the E1 strain *gus* operon or a homolog (Table 2).

Discussion

Out of the seven glucuronidase-positive *R. gnavus* strains, five (V42, V50, V51, V58 and V99) were found to possess a glucuronidase gene quite different from the *gusA* operon of *R. gnavus* E1. In order to be sure that these five β-glucuronidase strains did not possess a *gusA* homolog, lower stringency hybridizations were performed under the same conditions. The same results were obtained, so we

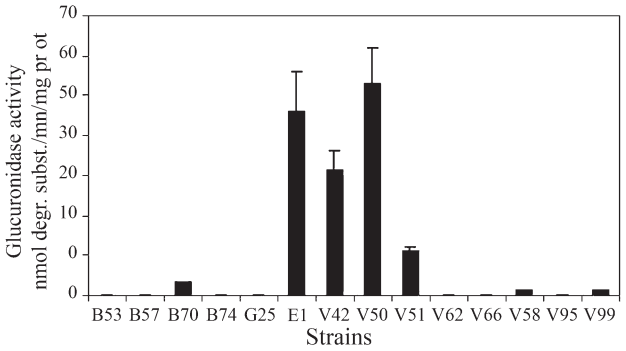

Figure 1 - β-glucuronidase activity of *Ruminococcus gnavus* strains, after 24 h of growth, based on the ability of cell extracts to hydrolyse PNPG. β-glucuronidase activity was expressed in nmol of degraded substrate/ min/mg of total proteins. Each value presented is the average of results from at least three independent experiments.

Table 2 - Summary of β-glucuronidase activity, PCR amplifications and hybridizations for each strain. All strains are *Ruminococcus gnavus* strains, listed in Table1.

			Hybridizations (probes used)		
R. gnavus strains	β-glucuronidase activity	*gus* PCR	Gus	ORF2	ORF3
B53	-	+	+	+	-
B57	-	-	-	-	-
B70	+	+	+	+	-
B74	-	-	-	-	-
G25	-	-	-	-	-
E1	+	+	+	+	+
V42	+	-	-	-	-
V50	+	-	-	-	-
V51	+	-	-	-	-
V62	-	-	-	-	-
V66	-	+	+	+	-
V58	+	-	-	-	-
V95	-	-	-	-	-
V99	+	-	-	-	-

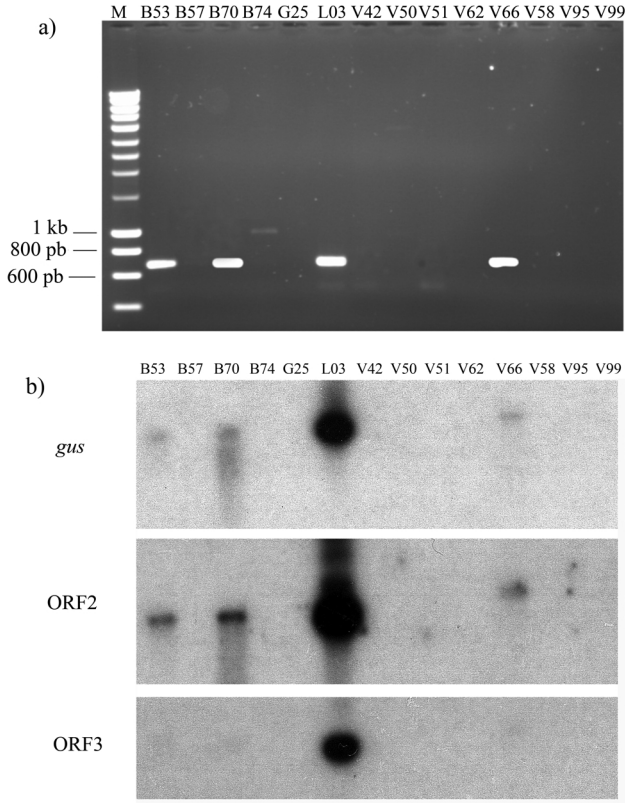

Figure 2 - Genetic analysis of the gus gene environment for strains of *Ruminococcus gnavus.* A. Agarose gel electrophoresis to analyze the PCR products. Lane M corresponds to the molecular size marker 1kb ladder (Gibco).The other lanes correspond to PCR performed on genomic DNA from 14 *R. gnavus strains.* The primers used were specific for the *gus* gene (685 bp). B. Southern hybridization to identify the genomic fragments carrying the gene *gus*, ORF2 and ORF3. DNA from *R. gnavus* strains (Table1) was restricted by *Eco*RV and hybridized with ^{32}P-labelled *gus*, ORF2 and ORF3 probes after electrophoresis. One unique *Eco*RV fragment of 5 kb was shown to hybridize to these probes. Previous studies have shown that this fragment comprises these three complete ORFs (Beaud *et al.*, 2005).

concluded that these strains have no *gus* gene homolog. The unexpected observation that a number of β-glucuronidase-negative *R. gnavus* strains possess *gusA* and ORF2 homologs raises further questions.Isolates of *E. coli* O157:H7 with nucleotide sequences for the *uidA* gene had no β-glucuronidase activity (Feng and Lampel,1994). This gene has a small number of mutations that result in the production of an inactive β-glucuronidase enzyme, which was still recognized by the anti-β-glucuronidase antibody. Further experiments, such as DNA sequencing and analysis, would be necessary to elucidate the true distribution of *gusA* and ORFs homologs in these *R. gnavus* strains. Her possibility could explain the preshence of *gusA* homologs without β-glucuronidase activity in these two strains could be the techniques used to measure the activity. We determined the *in vitro* β-glucuronidase activity of individual strains by disrupting a pellet of centrifuged bacterial cultures in media containing sugar but no inducer. The *gus* gene in *R. gnavus* E1 was transcribed as part of an operon that included three ORFs, ORF2, ORF3 and ORF5. The ORF2 and ORF3 products presented strong similarities with many β–glucoside permeases of the phosphoenolpyruvate β-glucoside phosphotransferase systems (PTS). The structural organization of the *gus* gene suggests a regulation of β-glucuronidase activity by catabolite repression, dependent on sugar availability. As no chemically-defined medium was available for *R. gnavus* growth, it was not possible to study catabolite repression in this species. Further experiments with media containing inducers, such as methyl-glucuronide or bile extract, should be made with the β-glucuronidase-negative *R. gnavus* strains.

The true distribution of the functional *gusA* operon in human gut *R. gnavus* strains and what kind of advantage GusA expression provides to *R. gnavus* have yet to be determined. A number of important natural compounds, including bilirubin and endogenous hormones, are routinely excreted as glucuronide conjugates via bile into the gastrointestinal tract; consequently, *R. gnavus* could use glucuronides for energy production. But the natural substrates of *R. gnavus* are yet unknown. This information may provide clues for dietary control of β-glucuronidase activity of the intestinal microbiota.

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