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β-Glucuronidase Activities of Intestinal Bacteria Determined Both In Vitro and In Vivo in Gnotobiotic Rats

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The β-glucuronidase activities of bacterial strains isolated from the rat intestinal tract were studied both in vitro in culture media and in vivo in the intestinal contents of gnotobiotic rats. Only 50 of 407 strains tested were found to be positive in vitro. They belonged to the three genera *Clostridium*, *Peptostreptococcus*, and *Staphylococcus*. The in vitro-negative strains were also negative in vivo. The β-glucuronidase activities of the positive strains were generally greater in vivo than in vitro. The highest in vivo activities were found in the intact bacterial cells and in the soluble fractions prepared from disrupted pellets. There was a discrepancy between the activities obtained from both conventional and gnotobiotic rats harboring selected positive strains, suggesting that the main β-glucuronidase-positive strains have not yet been isolated from the intestines of conventional rats.

Glucuronidation together with sulfation are the most usual forms of conjugation observed during the metabolism processes in mammals and also represent the major pathway for detoxification mechanisms. Thus, hydrolysis of glucuronides by β-glucuronidase enzymes have been considered by pathologists to be an important enzyme reaction in the digestive tract. The β-glucuronidase enzymes were detected both in intestinal tissues and in intestinal bacteria. The activity in the intestinal tissues was found to be optimal at pH 4.5, whereas that of bacteria was best at around pH 6.5 to 7.0 (13). Williams et al. (16) and Rod and Midtvedt (13) clearly established that β-glucuronidase activity was primarily of bacterial origin. However, the authors did not agree on the nature of the bacteria that synthesized the enzyme and on the conditions for obtaining such activity. According to Kent et al. (8), the presence of glucuronides in the culture media was required for the synthesis of the enzyme. These authors detected the enzyme only in the strictly anaerobic strains belonging to the genera *Peptostreptococcus*, *Corynebacterium*, *Propionibacterium*, *Bacteroides*, *Clostridium*, and *Catenabacterium*. By contrast, Hawksworth et al. (7) did not use inductors in their culture media. They observed a β-glucuronidase activity in both facultative and strictly anaerobic strains belonging to the family *Enterobacteriaceae* and the genera *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, and *Bacteroides*. They considered the lactobacilli and the nonsporulating anaerobes to be the most important enzyme producers in the rat intestinal tract. Because of these discrepancies and the significance of β-glucuronidase in numerous physiological and pathological processes, namely in colon cancer (3, 6, 11, 12), it was thought relevant to perform once again a screening of bacterial strains isolated from conventional rats possessing a β-glucuronidase activity and to compare the enzyme activity of selected bacterial strains growing in both culture media (with or without an inductor) and the digestive tract of gnotobiotic rats.

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MATERIALS AND METHODS

Animals. Animals used were male adult gnotobiotic and conventional Fisher 344 rats fed a commercial diet (U.A.R. no. 113; Usine d'Alimentation Rationnelle, Villemoisson-s/orge, France) that was sterilized by autoclaving. Various groups of gnotobiotic rats were caged in pairs in separate Trexler-type isolators (14). They were derived from axenic rats orally and rectally inoculated with 2 ml of an 18-h bacterial culture. The establishment of inoculated strains was checked 3 days later by microscopic examination of the 10^{-2} fecal dilution and by culture techniques.

Bacteriological methods. Strains were cultured in soft agar A medium containing (per liter) Bacto-Peptone, 15g; Bacto-Tryptone, 10 g; yeast autolysate, 10 g (Difco Laboratories, Detroit, Mich.); liver extract, 5 g (Paynes and Byrne, United Kingdom); glucose, 5 g; agar, 1.8 g at pH 7.5, or in the liquid F medium derived from Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) as modified by Aranki et al. (2), to which we added 1% (vol/vol) of a 5% cysteine hydrochloride solution, 1% of a 0.05% menadione alcoholic solution and 1% of decomplemented horse serum. In some trials, this medium was supplemented with an ethanol extract prepared from 8 ml of human urine and containing 2 mg of 3-α[β-D-glucuronyl]hyodeoxycholic acid (14). Bacterial strains were isolated from deoxycholate agar (Difco) from media H and J, described by Dickinson et al. (5), and from nine other media. Six media (B′-N, B′-B, B′-S, B1, ASZ, and C) were prepared from a basic medium (peptone, 15 g/liter; tryptone, 10 g/liter; yeast autolysate, 10 g/liter; agar, 10 g/liter) at pH 6.2 and the following ingredients (per liter): Tween 80 (E. Merck AG, Darmstadt, Federal Republic of Germany), 0.1 g, and neomycin sulfate (International Chemical and Nuclear [ICN] Corp., Cleveland, Ohio), 0.13 g, for medium B′-N; Tween 80, 0.1 g, and bacitracin (ICN), 0.07 g, for medium B′-B; Tween 80, 0.1 g, and streptomycin (ICN), 1.0 g, for medium B′-S; Tween 80, 0.1 g, glucose, 10 g, and sodium azide (Merck), 0.03 g, for medium B1; saccharose, 1.0 g, and sodium azide, 0.03 g (pH 7.5), for medium ASZ; glucose, 20 g, neomycin, 0.13 g, bacitracin, 0.07 g, and streptomycin, 0.09 g, at pH 7 for medium C. The three other

media (D1, E, and K'T) were prepared from the basic medium (peptone, 15 g/liter; tryptone, 10 g/liter; agar, 10 g/liter) and the following ingredients (per liter): liver extract, 5 g; safranin O (Eastman Kodak, Rochester, N.Y.), 0.02 g; sodium azide, 0.02 g at pH 6.8 for medium D1; meat extract (Merck), 15 g; sodium taurocholate (ICN), 1 g; safranin O, 0.02 g at pH 7.0 for medium E; meat extract, 10 g; glucose, 5 g; bromocresol purple (Touzart et Matignon, France), 0.012 g; sodium taurocholate, 10 g at pH 7.7 for medium K'T.

For inoculation of these media, ceca from conventional rats were immediately removed after death, weighed, and introduced into an anaerobic glove box (2). They were homogenized with an Ultra-Turrax (Janke und Kunkel, Staufen i. Brisgau, Federal Republic of Germany), using liquid F medium. Serial 10-fold dilutions were prepared in the glove box. Medium F was inoculated by plating 0.1 ml of 10^{-8} and 10^{-9} dilutions and incubated in the glove box for 7 days at 37°C. The dilutions were then taken out of the glove box, and 1 ml of adequate dilutions was inoculated in 14 ml of media A, B'-N, B'-B, B'-S, B1, D1, E, and K'T poured into tubes (8 by 400 mm) which were immediately cooled in running tap water to ensure prompt solidification (10). Media AZS, H, J, C, and deoxycholate agar were inoculated with 1 ml of adequate dilutions and poured into petri dishes. Tubes and dishes were incubated for 7 days at 37°C.

Strains isolated from the various media were classified as to genera by their shape and motility, Gram stain properties, shape and position of spores (if any), respiratory type, catalase production, and susceptibility to polymyxin B. Susceptibility to atmospheric oxygen of some strains was determined by the method described by Andremont et al. (1). Strains killed by contact for 1 h with the atmospheric oxygen are referred to as oxygen sensitive, those not killed as oxygen tolerant. *Staphylococcus epidermidis* was identified by the method of Dickinson et al. (5); *Clostridium perfringens* and *C. mangenotii* were identified as described in *Bergey's Manual* (4).

Bacteroides spp. were isolated from either F or B'-N, *Clostridium* spp. from F, B'-N, D1, or K'T, *Fusobacterium* spp. from either F or B'-B, *Eubacterium* spp. from F, A, B'-N or B'-S, *Peptostreptococcus* spp. from A, F, or D1, *Veillonella* spp. from E, *Bifidobacterium* spp. and *Lactobacillus* spp. from B1, *Streptococcus* spp. from ASZ, *Staphylococcus* spp. from J1 and H1, *Actinobacillus* spp. and *Escherichia* spp. from deoxycholate agar, and yeast from C.

Determination of β-glucuronidase activity. The assay was performed on bacterial suspensions prepared from either broth culture or feces. The activities found in the feces and in the cultures were called in vivo and in vitro activities, respectively. β-glucuronidase activity was determined spectrophotometrically by looking for the release of phenolphthalein from phenolphthalein mono-β-glucuronide (Sigma Chemical Co., St. Louis, Mo.), as described by Goldin and Gorbach (6). Protein amount was determined by the method of Lowry et al. (9). A quantitative test was used to detect the in vitro activity of the various strains cultured in liquid media F or A for 48 h at 37°C. In some trials, this medium was supplemented with an ethanol extract prepared from 8 ml of human urine and containing 2 mg of 3-α[β-D-glucuronyl]hyodeoxycholic acid to obtain a concentration of 0.1 mg/ml of broth (14a). A 10-ml amount of these cultures was centrifuged at 30,000 × *g* for 20 min at 4°C, and the pellets were suspended in 1 ml of 0.2 M phosphate buffer at pH 6.8. The reaction was performed as described by Goldin and Gorbach (6) and stopped after a 4-h incubation at 37°C. The strains were recorded as positive when pink staining was observed at the end of the incubation and as negative in the absence of any staining. For quantifying both in vivo and in vitro β-glucuronidase activities, the same procedure was used, except that the reaction was incubated for 30 min instead of 4 h. The bacterial pellets were prepared by centrifugation of 20 ml of culture or the 10^{-1} fecal dilution and washed three times with phosphate buffer. The clean-packed cells were suspended in 2 ml of phosphate buffer and transferred to the microchamber of a Sorvall Omnimixer (Sorvall, Newton, Conn.). Glass beads (2 g, 0.10-mm diameter) were added, and the cells were ground for 10 min in an ice-water bath. The mixture was passed through a glass filter to recover the glass beads and centrifuged at 30,000 × *g* for 10 min at 4°C to obtain a soluble and an insoluble fraction. The in vitro activity was measured in soluble fractions of broken pellets prepared from culture media, and the in vivo activity was measured in the whole 10^{-1} dilution of feces, in the supernatant and in the intact pellet of this fecal dilution, in the whole disrupted pellets, and in both soluble and insoluble fractions. Student's *t* test was used for comparison of the mean values.

RESULTS

Screening of β-glucuronidase-positive strains among various strains isolated from ceca and feces of conventional rats. Only 50 strains of the 407 tested were found to be β-glucuronidase-positive by the qualitative test used (Table 1). These positive strains belonged only to the three different genera *Clostridium*, *Peptostreptococcus*, and *Staphylococcus*. In addition, 18 strains belonging to the genera *Bacteroides* (13 strains), *Escherichia* (2 strains), and *Streptococcus* (3 strains) were found to be slightly positive when they were cultured in broth media, whereas the other strains tested, including the predominant ones, were found to be negative.

TABLE 1. Screening of β-glucuronidase-positive strains among various strains isolated from the ceca and feces of conventional rats

Strain, genus, or group	No. of positive strains	No. of strains tested
Bacteroides spp.[a]	0	7
Clostridium spp., OS[a]	0	3
Clostridium spp., OT[a,c]	19	48
Eubacterium spp., OS[a]	0	11
Eubacterium spp., OT[a,b]	0	15
Peptostreptococcus spp.[b]	14	41
Fusobacterium spp.[a]	0	11
Veillonella spp.[c]	0	13
Bifidobacterium spp.[b]	0	7
Nonidentified		
OS spiral-shaped rods[a]	0	1
Lactobacillus spp.[b]	0	64
Streptococcus spp.[b,c]	0	47
Staphylococcus spp.[c]	17	115
Actinobacillus spp.[c]	0	6
Escherichia spp.[c]	0	16
Yeast[c]	0	2

[a] Strains isolated from a 10^{-9} dilution. OS, Oxygen sensitive; OT, oxygen tolerant.
[b] Strains isolated from a 10^{-8} dilution.
[c] Strains isolated from 10^{-7} to 10^{-2} dilutions.

TABLE 2. Comparative β-glucuronidase activities of selected positive strains cultured in vitro, with or without inductor, or in vivo in the intestinal tract of gnotobiotic rats monoassociated with each strain

Strains	In vitro β-glucuronidase activity[a]		In vivo β-glucuronidase activity[b]
	Without inductor	With inductor	
Peptostreptococcus sp. strain 68	5.0–5.3 (2)[c]	2.5 (1)	4.2–18.3 (6)
Peptostreptococcus sp. strain 105	5.3–6.5 (2)	5.2 (1)	3.9–6.0 (6)
S. epidermidis 156×63	0.2–0.4 (2)	2.2–5.8 (2)	3.8–6.3 (6)
Clostridium sp. strain 230×26	1.3 (1)	1.4–3.4 (2)	1.2–2.9 (6)
C. perfringens 215×5	0.2 (1)	0.07–0.13 (2)	1.6–2.6 (6)
C. mangenotii 5×17	0.00 (1)	0.2 (1)	3.4–7.5 (6)

[a] See text for experimental details.
[b] See text for experimental details.
[c] β-Glucuronidase activity was expressed in micrograms of phenolphthalein released per minute by milligrams of proteins of soluble fractions obtained from cultures, *a*, or from feces, *b*. Values are the range of the β-glucuronidase activity. The number of determinations is shown in parentheses.
[d] Sodium taurocholate (1%) was added in medium A to ensure the growth of this strain.

Comparative β-glucuronidase activities of selected positive strains cultured in vitro, with or without inductor, or in vivo in the intestinal tracts of gnotobiotic rats. Large differences in the in vitro activities of the positive strains were found (Table 2). Both strains 68 and 105 of *Peptostreptococcus* sp. exhibited the highest in vitro activity, whatever the medium used, with or without inductor. The in vitro activities of the other strains were much weaker, and when no inductor was added to the medium, no activity could be detected in the extracts obtained from *C. mangenotii*. The inductor present in the urinary medium greatly enhanced the specific activity of strain *S. epidermidis* 156×3 and to a less extent those of strains *Clostridium* sp. strain 230×26 and *C. mangenotii* 5×17. It was ineffective on the other strains.

The in vivo activity of *Peptostreptococcus* sp. strain 68, tested once a week during 16 weeks, was very high but variable, increasing gradually from 0 to 14 units until week 3, decreasing to 6 units until week 7, increasing to 18.3 units until week 10, and then decreasing to 4.2 units. The in vivo activity of *Peptostreptococcus* sp. strain 105 was much less variable. Strains *C. perfringens* 215×5 and *C. mangenotii* 5×17 exhibited in vivo activities which were more than 10-fold higher than the in vitro activities.

Comparative β-glucuronidase activities of various fractions prepared from feces of conventional, axenic, and gnotobiotic rats. These experiments were undertaken to determine whether the positive strains we isolated exhibited the same in vivo β-glucuronidase activity as the complex flora of conventional rats. The β-glucuronidase activities of various fractions prepared from feces of conventional, axenic, and gnotobiotic rats harboring negative or positive strains are compared in Table 3. The results varied according to the microbial status of the rats and the fecal fractions. The feces of axenic group 2 rats exhibited a slight activity only in the 10^{-1} dilution, as did the feces of gnotobiotic group 3 rats associated with the pool of in vitro-negative strains. In group 1 of conventional rats and in the three other groups of gnotobiotic rats, the β-glucuronidase activities were always lower in the supernatant of the 10^{-1} dilution than they were in the intact pellet, and they were lower in the insoluble fraction of disrupted pellet than they were in the soluble fraction. The β-glucuronidase activities of intact pellet and of both whole disrupted pellet and soluble fraction were not significantly different in both groups 1 and 6, but these activities were much higher in group 1 than in group 6. By contrast, the activity of intact pellet was significantly lower than that of both whole disrupted pellet and soluble fraction in groups 4 and 5.

DISCUSSION

Controversial results have been obtained by various authors concerning the β-glucuronidase activities of complex microbial floras or individual bacterial strains. These discrepancies could be due to either the techniques used for determining this activity or the interpretation of the experimental data. For instance, Hawksworth et al. (7) determined the in vitro β-glucuronidase activity of individual strains by using media containing no inductor and the whole culture as a source of enzymes. They estimated the in vivo activity of these strains by multiplying the average activity of individual cells growing in vitro by the number of cells counted in the feces. Kent et al. (8) also performed semiquantitative tests with the intact pellet of centrifuged bacterial cultures in media containing high doses of an inductor. On the contrary, Rod and Midtvedt (13) and Goldin and Gorbach (6) determined the β-glucuronidase activity of soluble extracts pre-

TABLE 3. Comparative β-glucuronidase activities of various fractions prepared from feces of conventional, axenic, and gnotobiotic rats

Group[a]	β-Glucuronidase activities of[b]:					
	Whole 10^{-1} dilution	Supernatant of 10^{-1} dilution	Intact pellet	Whole disrupted pellet	Soluble fraction	Insoluble fraction
1	119 ± 7.0	32 ± 3.0	122 ± 15.0	116 ± 9.0	135 ± 19	28 ± 7.0
2	4.7 ± 0.4	0	0	0	0	0
3	6.0 ± 0.5	0	ND[c]	ND	0	ND
4	33 ± 2.0	4.0 ± 0.2	27 ± 2.0	321 ± 106	294 ± 53	22 ± 6.0
5	10 ± 0.6	1.0 ± 0.2	10 ± 0.8	41 ± 3.0	44 ± 5.0	3.0 ± 0.6
6	28 ± 1.0	0.9 ± 0.5	25 ± 2.0	17 ± 1.0	13 ± 3.0	7.0 ± 0.8

[a] Groups: 1, conventional rats; 2, axenic rats; 3 to 6, gnotobiotic rats harboring different bacteria as follows: 3, 26 negative strains belonging to the genera *Actinobacillus*, *Veillonella*, *Peptostreptococcus*, *Eubacterium*, *Bifidobacterium*, *Clostridium*, *Staphylococcus*, *Lactobacillus*, *Streptococcus*, *Escherichia*, and *Bacteroides*; 4, the six positive strains described in Table 2; 5, *Peptostreptococcus* sp. strain 105; 6, *C. perfringens* 215×5.
[b] β-Glucuronidase activities were expressed in micrograms of phenolphthalein released per minute per gram of fresh feces (mean ± standard deviation of six determinations).
[c] ND, Not determined.

pared by disrupting cecal or fecal suspensions. The substrate used for the test also varied according to the authors. Rod and Midtvedt used *p*-nitrophenyl-β-glucuronide, whereas we and others (6, 8) used phenolphthalein-β-glucuronide.

Our results clearly showed that the estimation of the in vivo β-glucuronidase activity from the values obtained in vitro was erroneous. We isolated a *Bacteroides* strain which exhibited a slight in vitro activity, but no in vivo activity, even though its number exceeded 10^{10} viable cells per g of cecum in gnotobiotic rats. Our results also show that our positive strains belonged to the genera *Peptostreptococcus*, *Clostridium*, and *Staphylococcus*. Such a result is partly in agreement with those of Kent et al. (8), who stated that the main β-glucuronidase-positive strains were strictly anaerobic. However, none of our *Bacteroides* strains were positive, contrary to some of the strains they isolated. We also showed that only one of six strains tested needed an inductor in the culture medium to exhibit β-glucuronidase activity. The comparisons between in vivo and in vitro specific activities is controversial, since fecal pellets might contain some nonbacterial components liable to decrease the measured in vivo specific activities. However, the six strains tested exhibited in vivo specific activities as high as those in vitro, and two of them exhibited much higher ones, suggesting that the intestinal content was the best culture medium for obtaining the highest β-glucuronidase activity.

A surprising fact was observed in the gnotobiotic rats harboring *Peptostreptococcus* sp. strain 68, i.e., an abnormal range of β-glucuronidase activity in the soluble fraction of disrupted fecal bacteria. By time, this range was of the same magnitude as that observed by Goldin and Gorbach (6) in conventional rats fed diets containing more or less meat. However, our rats were fed the same commercial diet and harbored the same bacterial strains throughout the experiment. This result cannot be due to an in vivo emergence of a mutant with a high activity, since the fecal in vivo activity first increased and then decreased. Hypothetically, one can imagine that large in vivo variations in the β-glucuronidase activity of this strain could be related to large variations in the intestinal concentration of bile inductor(s) synthesized by the rat liver.

A striking difference was observed between rats of groups 1 and 6 and those of group 4 harboring the positive strains. In the former, the β-glucuronidase activity of the intact bacterial cells was similar to that of both whole disrupted pellets and their soluble fraction, whereas only the whole disrupted pellets and the soluble fraction of the positive strains present in the feces of group 4 rats exhibited a high β-glucuronidase activity. This discrepancy might be related either to a different permeability of bacterial cells for the substrate used, which could easily enter the fecal bacteria colonizing group 1 or 6 rats but not those colonizing group 4 rats, or to different properties of the β-glucuronidase involved. Nevertheless, it is noticeable that the β-glucuronidases involved are not largely diffused in the supernatant solution or linked to the insoluble extract of disrupted pellets.

Whatever the reasons of such a discrepancy between conventional and group 4 rats, it might be suggested that the β-glucuronidase-positive strains in group 4 rats are not the same as the predominant ones in conventional rats. This hypothesis is supported by the fact that the former strains were not isolated from the fecal 10^{-9} dilution of conventional rats (Table 1). In addition, when the 10^{-2} dilution was observed under a phase-contrast microscope, we noticed that several spiral-shaped bodies were present among the predominant microorganisms but were not cultured in any of the media used. Thus, the hypothesis that the main β-glucuronidase-positive strains of the fecal flora of conventional rats have not yet been isolated is very likely. It may be concluded that study of the mechanisms involved in the dietary modulation of intestinal β-glucuronidase activities is very difficult, since such a study can hardly be performed if the active intestinal strains are unknown.

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