APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2008, p. 1469–1477 Vol. 74, No. 5
0099-2240/08/$08.00+0 doi:10.1128/AEM.02064-07

Isolation and Characterization of Human Intestinal Bacteria Capable of Transforming the Dietary Carcinogen 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine▽

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Received 10 September 2007/Accepted 27 December 2007

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a carcinogenic heterocyclic aromatic amine formed in meat products during cooking. Although the formation of hazardous PhIP metabolites by mammalian enzymes has been extensively reported, research on the putative involvement of the human intestinal microbiota in PhIP metabolism remains scarce. In this study, the in vitro conversion of PhIP into its microbial derivate, 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3′,2′:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1), by fecal samples from 18 human volunteers was investigated. High-performance liquid chromatography analysis showed that all human fecal samples transformed PhIP but with efficiencies ranging from 1.8 to 96% after 72 h of incubation. Two PhIP-transforming strains, PhIP-M1-a and PhIP-M1-b, were isolated from human feces and identified by fluorescent amplified fragment length polymorphism and *pheS* sequence analyses as *Enterococcus faecium* strains. Some strains from culture collections belonging to the species *E. durans*, *E. avium*, *E. faecium*, and *Lactobacillus reuteri* were also able to perform this transformation. Yeast extract, special peptone, and meat extract supported PhIP transformation by the enriched *E. faecium* strains, while tryptone, monomeric sugars, starch, and cellulose did not. Glycerol was identified as a fecal matrix constituent required for PhIP transformation. Abiotic synthesis of PhIP-M1 and quantification of the glycerol metabolite 3-hydroxypropionaldehyde (3-HPA) confirmed that the anaerobic fermentation of glycerol via 3-HPA is the critical bacterial transformation process responsible for the formation of PhIP-M1. Whether it is a detoxification is still a matter of debate, since PhIP-M1 has been shown to be cytotoxic toward Caco-2 cells but is not mutagenic in the Ames assay.

Diet is a major risk factor in human cancer (14). Epidemiological studies indicate that the consumption of cooked meat and meat products predisposes individuals to neoplastic disease, particularly of the colon (13). Cooked muscle meats contain potent genotoxic carcinogens belonging to the heterocyclic aromatic amine (HAA) class of chemical compounds (31). Of the 19 heterocyclic amines identified so far, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass-abundant heterocyclic amine produced during the cooking of beef, pork, and chicken (15, 40). Experimentally, PhIP is a potent mutagen and genotoxin and has been shown to produce mammary gland, prostate, and colon tumors in rats (23, 39). For humans, less is known about the potential role of PhIP and related heterocyclic amines in tumor development. Several studies have shown that individuals who eat "well-done" meat have an increased risk of breast (52) and colorectal (18) cancers.

To determine the potential health risks associated with heterocyclic amines, several dietary studies have been conducted on the metabolism and disposition of these compounds in humans. So far, most investigations have focused on the activation and detoxification of heterocyclic amines by mammalian enzymes. The genotoxic/carcinogenic effect of heterocyclic amines is closely related to a highly complex metabolism involving xenobiotically induced enzymes generating very reactive metabolites as well as detoxified derivatives (1). On the other hand, the involvement of the intestinal microbiota in the digestive fate of heterocyclic amines remains poorly investigated (27). Recent research showed that PhIP metabolites excreted in 0- to 24-h urine represented 17% ± 10% of the ingested PhIP in a meat matrix (28). In an earlier study with patients administered PhIP in capsules, 90% of the ingested dose was recovered in the urine (29), indicating that PhIP provided in capsule form is more bioavailable than that via meat ingestion. The nonbioavailable PhIP fraction reaches the colon in an intact form and is there in contact with the resident microbiota. Direct binding of heterocyclic amines to the cell walls of intestinal bacteria has been reported and is currently considered as a detoxification mechanism, since it prevents absorption of heterocyclic amines through the intestinal mucosa (5, 44). However, results of IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline)-induced genotoxicity assays in germfree and conventional rodents showed that the presence of intestinal microbiota is essential to the induction of DNA damage in colon and liver cells (19, 24). These findings suggest that the intestinal microbiota plays a significant role in the biocon-

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▽ Published ahead of print on 11 January 2008.

version of HAAs into harmful metabolites. Indications exist that hydrolysis of HAA glucuronides by bacterial β-glucuronidase may release mutagenic intermediates (36).

Information on the bacterial metabolism of native HAAs is still scarce. Nevertheless, researchers have shown that incubation of the heterocyclic amine IQ with mixed human feces under anaerobic conditions results in the formation of the hydroxy metabolite 7-OH IQ (3, 7), and recent research identified 10 bacterial strains able to perform the transformation of IQ to 7-OH-IQ: *Bacteroides thetaiotaomicron* ($n = 2$), *Clostridium clostridioforme* ($n = 3$), *C. perfringens* ($n = 1$), and *Escherichia coli* ($n = 4$) (22). However, little has been done to characterize PhIP metabolism by human intestinal microbiota, although our early work examined the in vitro transformation of PhIP by human fecal microbiota (48). In this study, one major microbial metabolite of PhIP (PhIP-M1) was identified using electrospray ionization-tandem mass spectrometry and one- and two-dimensional nuclear magnetic resonance as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3′,2′:4,5]-imidazo[1,2-*a*]pyrimidin-5-ium chloride. This compound was subsequently detected in human urine and feces following consumption of well-done chicken meat and showed no mutagenic potency in the Ames test (47).

This study presents the isolation and identification of individual intestinal bacteria from human feces capable of transforming PhIP into its microbial derivate, PhIP-M1. Representative culture collection strains isolated from the intestine were screened for their PhIP transformation potentials, and the nutritional requirements for microbial PhIP-M1 formation were clarified. In addition, the microbial and chemical mechanisms for this carcinogenic transformation were elucidated.

MATERIALS AND METHODS

Chemicals. PhIP was purchased from Toronto Research Chemicals (Ontario, Canada). For incubation purposes, it was dissolved in dimethyl sulfoxide. The constituents of the culture media, namely, tryptone, yeast extract, and meat extract, were obtained from AppliChem (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich (Bornem, Belgium). Acrolein was purified by distillation at 53°C. The hydroxypropionaldehyde (HPA) system (3-HPA and its aqueous derivates) was produced as described by Vollenweider et al. (49) by use of *Lactobacillus reuteri* ATCC 533608. The solvents for high-performance liquid chromatography (HPLC) and LC-mass spectrometry analysis were of HPLC grade and purchased from Acros Organics (Geel, Belgium).

Collection and preparation of human fecal samples and fecal matrix. Fecal samples were obtained from 18 healthy volunteers between the ages of 20 and 65. Donors were on a Western-type diet; none had a history of digestive pathology, and they had not received antibiotics during the 3 months prior to sample delivery. Fecal slurries of 20% (wt/vol) fresh fecal inocula were prepared by homogenizing the feces with phosphate-buffered saline (0.1 M, pH 7), containing 1 g/liter sodium thioglycolate as a reducing agent (33). The particulate material was removed by centrifugation for 2 min at 400 × *g*.

Fecal matrix was prepared by autoclaving fecal slurries for 20 min at 121°C and centrifuging for 10 min at 8,000 × *g*.

PhIP-M1 production by human fecal microbiota. Bacterial incubations were performed by transferring 1 ml fecal inoculum to a penicillin flask containing 9 ml autoclaved TY broth (tryptone at 30 g/liter, yeast extract at 20 g/liter; pH 7.0) supplemented with 0.5 g L-cysteine/liter and 5 μM PhIP. The flasks were made anaerobic by flushing the headspace with nitrogen gas during 15 cycles of 2 min each at 80-kPa overpressure and 90-kPa underpressure and incubated at 37°C while shaking at 140 rpm for 72 h. Daily samples were taken for HPLC analysis to verify PhIP-M1 production. Incubations were performed in triplicate.

PhIP-M1 production by inactivated human fecal microbiota. Bacterial incubations of 72-h-grown fecal communities obtained from the human volunteer with the highest PhIP transformation efficiency were subjected to several treatments to verify the involvement of the colonic bacteria and fecal matrix constituents in the transformation of PhIP. During a first treatment, the overall fecal microbiota was filtered over a 0.22-μm filter to remove the bacterial cells from the suspension but withhold the extracellular protein fraction, a treatment hereafter referred to as FS. A second treatment consisted of a consecutive filter sterilization and pasteurization for 30 min at 60°C in a warm water bath to achieve removal of microbial biomass and degradation of heat-sensitive enzymatic activity, a treatment hereafter referred to as FS-PS. During the third treatment, the feces-grown microbial communities were autoclaved for 20 min at 121°C. All treatments were performed in triplicate, and data were compared using Student's *t* test.

Effect of pH, surfactants, and protease inhibitors on PhIP metabolism. The sensitivities of the active substances involved in PhIP-M1 formation to surfactants, protease inhibitors, and pH were tested on cell-free supernatants of a 72-h-grown mixed fecal community from a high-PhIP-transforming individual that were incubated at 37°C in TY broth under anaerobic conditions. The cells were harvested by centrifugation (8,000 × *g*, 10 min, 4°C), and the cell-free supernatant adjusted to pH 6.0 with 6 M NaOH.

The surfactants tested were sodium dodecyl sulfate, Tween 80, and Triton X-100 at final concentrations of 0.1% (wt/vol). The protease inhibitor EDTA was added to the cell-free supernatant to yield a final concentration of 5 mM.

The sensitivity of the active substance to different pH values (from 1 to 12) was tested by adjusting the cell-free supernatants from pH 1.0 to 12.0 (at increments of 1 pH unit) with sterile 1 M NaOH or 1 M HCl. The pH values were measured before and after the 72-h incubation and remained constant during the entire incubation period.

Untreated cell-free supernatants were used as controls. All treatments and controls were incubated anaerobically at 37°C for 72 h. Samples were taken every 24 h for HPLC analysis. The different treatments were executed in triplicate, and data were compared using Student's *t* test.

Effect of nutrition on microbial PhIP metabolism. One milliliter of human fecal inoculum from the individual with the highest PhIP transformation capacity was transferred in 10 ml minimal medium (composition per liter was 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.12 g $MgSO_4$, 0.01 g $CaCl_2$, and 0.5 g L-cysteine) supplemented with 10 g/liter of different feed sources covering the main nutritional components relevant for the colon: yeast extract, tryptone, special peptone, protease peptone, meat extract, fibers, glucose, and olive oil (extra virgin; Delhaize). The suspensions were prepared in 50-ml penicillin flasks and incubated anaerobically at 37°C and 140 rpm for 72 h in the presence of 5 μM PhIP. At the end of this incubation period, samples were taken for HPLC analysis and pH measurements were made. Samples were kept at −20°C prior to analysis. The different treatments were executed in triplicate, and data were compared using Student's *t* test.

Isolation and identification of PhIP-transforming bacterial strains. The fecal slurry from the two human volunteers with the highest PhIP transformation capacity was diluted in a 10-fold dilution series (10^{-1} to 10^{-8}) in TY broth supplemented with fecal matrix (10% [vol/vol]) and PhIP (5 μM). Dilutions were incubated at 37°C under anaerobic conditions for 3 days and assayed at 24-h intervals for residual PhIP and PhIP-M1 formation. At the same time intervals, samples from all dilutions were spread onto TY agar plates supplemented with PhIP (5 μM) to maintain a continuous exposure of the bacteria to the substrate. Following incubation at 37°C under an atmosphere of nitrogen-hydrogen-carbon dioxide (84/8/8), five colonies per plate that differed, whenever possible, in size, shape, and color were picked up, subcultured in TY broth supplemented with fecal matrix (10% [vol/vol]) and PhIP (5 μM), and then stored as stock cultures at −80°C after the addition of glycerol (20% [vol/vol]). Identification of the biotransforming strains was performed phenotypically by microscopic examination and genetically by sequence comparison of the amplification products of the cloned 16S rRNA genes. Total DNA was extracted from 24-h cultures in TY broth by using the QIAamp DNA mini stool kit (Qiagen Benelux B.V., Venlo, The Netherlands). Denaturing gradient gel electrophoresis, using universal bacterial primers, was performed according to the method of Possemiers et al. (33). The entire 16S rRNA genes of the isolated strains, amplified using primers 63r and 1378f (6), were cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Sequencing of the 16S rRNA gene fragments was performed by ITT Biotech (Bielefeld, Germany). Analysis of DNA sequences and sequence identity searches were completed with standard DNA-sequencing programs and the BLAST server of the National Center for Biotechnology Information using the BLAST algorithm and the BLASTN program for the comparison of a nucleotide query sequence against a nucleotide sequence database (2).

Using the former approach, identification of the bacterial strains was achieved at the genus level. Identification at the species level was obtained by fluorescent

TABLE 1. Abilities of individual bacterial strains originating from the human digestive tract to convert PhIP to PhIP-M1[a]

Bacterial species and strain	Origin	Source or reference	% of initial PhIP converted[b]
E. durans LMG 20231	Farm, human feces	LMG[c]	93
E. durans LMG 16891	Human blood	LMG	65
E. faecium LMG 8147	Human feces	LMG	2.4
E. avium LMG 10744	Human feces	LMG	90
E. fecalis LMG 7937	Human feces	LMG	0.0
E. faecium PhIP-M1-a	Human feces	This study[d]	91
E. faecium PhIP-M1-b	Human feces	This study[d]	86
L. reuteri LMG 13557	Human feces	LMG	97
L. reuteri ATCC 53608	Human feces	ATCC	96

[a] For incubation, cell suspensions of the strains in TY broth were supplemented with 5 μM PhIP (anaerobic conditions, 37°C, 140 rpm).
[b] At the end of the incubation (72 h), the PhIP and PhIP-M1 concentrations were determined by HPLC analysis.
[c] LMG, BCCM/LMG Bacteria Collection.
[d] Among the 65 strains isolated from the human fecal samples and assayed for PhIP transformation, only the 2 biodegradative strains are indicated in this table.

amplified fragment length polymorphism (FAFLP) and partial *pheS* sequence analysis. DNA was prepared according to the method of Gevers et al. (17). FAFLP is a PCR-based technique for whole-genome DNA fingerprinting via the selective amplification of restriction fragments (50) and was performed as described by Vancanneyt et al. (46), except that the BioNumerics software package version 4.61 (Applied Maths, Belgium) was used. A fragment of the *pheS* gene was amplified and sequenced following the protocol of Naser et al. (32) with an ABI Prism 3130XL genetic analyzer (Applied Biosystems). Sequence assembly was obtained via the AutoAssembler program (Applied Biosystems, Foster City, CA). Phylogenetic analysis was performed using the BioNumerics software package, version 4.61, after alignment of the consensus *pheS* sequences with in-house-determined *pheS* sequences of reference strains of lactic acid bacterium taxa currently covered by the database of the Laboratory of Microbiology, BCCM/LMG Bacteria Collection.

Strains from culture collections. Six strains from the collection of "Unité d'Ecologie et de Physiologie du Système Digestif" (INRA; Jouy-en-Josas, France) were kindly provided by Sylvie Rabot. All of them originated from human feces or intestinal contents and were isolated locally. The strains were strictly anaerobic gram negatives belonging to *Bacteroides* or gram positives belonging to the *Clostridium*, *Eubacterium*, and *Bifidobacterium* genera. A further 14 strains from human origin were selected from the BCCM/LMG Bacteria Collection. They were microaerophilic gram positives belonging to the lactic acid bacteria *Enterococcus*, *Pediococcus*, and *Lactobacillus*. One *Lactobacillus reuteri* strain of human origin was purchased from the ATCC culture collection (Table 1). The cells were stored at −80°C in physiological solution (8.5 g/liter NaCl) supplemented with sterile glycerol (20% [vol/vol]).

Incubation conditions for isolates and culture collection strains. All strains were inoculated in penicillin flasks containing 50 ml autoclaved TY broth supplemented with 0.5 g L-cysteine/liter and incubated for 24 h at 37°C. Subsequently, 9-ml portions of the 24-h-grown cultures were transferred to a penicillin flask containing 1 ml fecal matrix and 5 μM PhIP. The flasks were incubated anaerobically at 37°C under shaking at 140 rpm for 72 h; daily samples were taken for HPLC analysis. All strains were incubated in triplicate.

A negative control, 1 ml fecal matrix incubated in 9 ml TY broth supplemented with 5 μM PhIP was included to exclude the possibility that physicochemical interactions of the fecal matrix components are at the origin of the disappearance of PhIP.

Effect of nutrition on PhIP metabolism by *E. faecium* PhIP-M1-a. Fifty microliters of thawed *E. faecium* PhIP-M1-a stock was transferred in 10 ml of minimal medium supplemented with 10 g/liter yeast extract, tryptone, special peptone, protease peptone, meat extract, fibers, sugars (glucose, dextrose, lactose, sucrose, maltose, mannose, ribose, and fructose), carbohydrates (starch and cellulose), or olive oil or combinations thereof. The suspensions were prepared in 50-ml penicillin flasks, the headspace was replaced by nitrogen gas, and the flasks were incubated at 37°C under shaking at 140 rpm for 72 h in the presence of 5 μM PhIP. Then, 1 ml was sampled from each flask for HPLC analysis, and incubation continued for 72 h upon supplementation with 10% (vol/vol) fecal matrix. At the end of this incubation period, samples were taken for HPLC analysis and pH measurements were made. Samples were kept at −20°C prior to analysis. The different treatments were executed in triplicate, and data were compared using Student's *t* test.

Elucidation of fecal matrix constituents required for PhIP metabolism. The fecal slurry from the human volunteer with the highest PhIP transformation capacity was diluted using serial 10-fold dilutions (10^{-2} to 10^{-4}) in 10 ml of TY broth supplemented with 10 g/liter of glucose, dextrose, lactose, sucrose, maltose, mannose, ribose, fructose, starch, cellulose, glycerol, fumarate, succinate, or pyruvate and 5 μM PhIP. In addition, an amount of 50 μl of thawed *E. faecium* PhIP-M1-a or *L. reuteri* ATCC 53608 was transferred into 10 ml of TY broth added with the same supplements.

Dilutions and pure cultures were incubated in triplicate in penicillin flasks at 37°C under shaking at 140 rpm under anaerobic conditions for 72 h. Every 24 h, samples were taken for PhIP and PhIP-M1 analysis and pH measurements were made. Samples were kept at −20°C prior to analysis.

The highest-PhIP-transforming fecal dilution (10^{-4}) and *L. reuteri* ATCC 53608 were subsequently incubated in 50 ml of 10 g/liter meat extract supplemented with 10 g/liter glycerol at 37°C and 140 rpm under anaerobic conditions for 72 h. Every 24 h, samples were taken for 3-HPA analysis and derivatized as described below.

Abiotic synthesis of PhIP-M1. The potential glycerol metabolites or derivates of interest, i.e., the HPA system and acrolein, were supplemented in concentrations of 0.01, 0.1, 1, 10, and 100 mM into penicillin flasks containing 10 ml of 10 g/liter meat extract and 5 μM of PhIP. Flasks were incubated at 37°C under shaking at 140 rpm for 36 h, and samples were taken every 12 h for 3-HPA and PhIP-M1 analysis. Incubations were performed in triplicate.

Acrolein and 3-HPA analysis. The concentration of the HPA system (3-HPA and derivates) during synthesis was determined by using a colorimetric method containing tryptophan adapted from the work of Circle et al. (8) by Vollenweider et al. (49).

The concentration of acrolein and 3-HPA during batch incubation experiments was determined by preparing the more stable 2,4-dinitrophenyl hydrazine (DNPH) derivates. DNPH derivatization was carried out according to methods described in the literature (53) by adding 500 μl DNPH reagent solution to 5 ml of bacterial medium or bacterial medium dilution. The reagent solution was prepared by dissolving 20 mg DNPH in 15 ml HCl-water-acetonitrile at 2:5:1 (vol/vol) according to methods described in the literature (26). The reaction time was at least 12 h at room temperature. The acidified samples were extracted and preconcentrated by solid-phase extraction on Oasis HLB cartridges (60 mg sorbent; Waters, Milford, MA). The cartridges were preconditioned with methanol (3 ml), acetonitrile (3 ml), and MilliQ water (4 ml). For extraction, the acidified samples (5 ml) were sucked through the preconditioned sorbent at a flow rate of approximately 5 ml/min. After sample extraction, the adsorbent was washed with MilliQ water (1 ml) and the adsorbed compounds were subsequently eluted with acetonitrile (3× 2 ml). Before measurement, the samples were evaporated to dryness with a gentle stream of nitrogen, and the residue was dissolved in acetonitrile-MilliQ (50:50 [vol/vol]). Preconcentration factors of 1 to 25 were achieved. HPLC analysis was performed on a Dionex system (Sunnyvale, CA) comprising an ASI-100 autosampler, a P580 pump series, and an STH585 column oven coupled to a UVD340S UV-visible light detector. A 20-μl volume of the sample was injected and separated over a Genesis C_{18} column (150 mm by 4.6 mm; 5 μm; Jones Chromatography). The temperature was set at 35°C and the flow rate was maintained at 1 ml/min. Solvents were designated A (water-acetonitrile-tetrahydrofuran-isopropanol [59:30:10:1]) and B (acetonitrile-water [65:35]). The elution gradient was 100% A at 0 min to 60% A at 12 min to 40% A at 17 min and back to 100% A at 20 min. Absorbance was monitored at 365 nm. Linear calibration curves for acrolein and 3-HPA spiked in 10 g/liter meat extract, extracted with solid-phase extraction, and redissolved in an equal amount of acetonitrile-MilliQ were obtained in a concentration range of 0.75 to 90 mg/liter.

PhIP and PhIP-M1 analysis. One hundred microliters of each sample was diluted 10-fold with acetonitrile-0.01% formic acid (75:25), vortexed rigorously, and centrifuged (10,000 × *g*, 2 min). The supernatant was transferred to an HPLC vial and stored at 4°C until analysis.

PhIP and PhIP-M1 analyses were performed according to the method of Vanhaecke et al. (48) on a Dionex HPLC system (Sunnyvale, CA).

Nucleotide sequence accession numbers. Partial sequences of the 16S rRNA genes have been deposited at GenBank under accession numbers EF373550 for *Enterococcus* sp. PhIP-M1-a and EF373551 for *Enterococcus* sp. PhIP-M1-b.

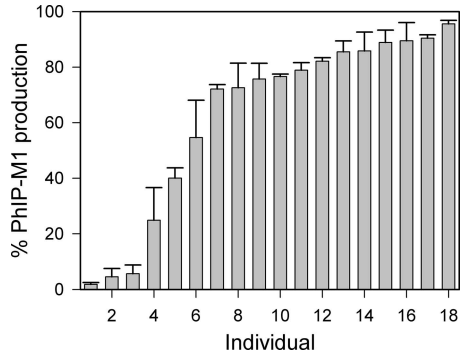

FIG. 1. Conversion of PhIP into PhIP-M1 by intestinal bacteria from 18 different human individuals incubated for 72 h with 5 μM PhIP. The individuals' data were arranged by increasing PhIP-M1 production. Values are means ± standard deviations (SD) ($n = 3$).

RESULTS

PhIP-M1 production by human fecal microbiota. The capacities of mixed microbial cultures obtained from 18 human stool samples to transform the food carcinogen PhIP were tested by incubating the obtained overall human fecal microbiota with 5 μM PhIP for a period of 3 days (Fig. 1). All human fecal samples transformed PhIP, though with different efficiencies ranging for the produced PhIP-M1 between 1.8 and 96% for the lowest- and highest-transformation microbiotas, respectively. Based on these results, two high-PhIP-converting microbiotas were selected for elucidation of the nature of PhIP metabolism and isolation and identification of the PhIP-transforming species.

PhIP metabolism by inactivated human fecal microbiota. The production of PhIP-M1 during 3-day periods following different inactivating conditions is presented in Fig. 2A. The control reached an average PhIP-M1 formation of 96% ± 0.1% after 3 days, which decreased significantly ($P < 0.05$) to 73% ± 5.0% or 35% ± 16%, upon filter sterilization alone or combined with pasteurization, respectively. The difference in PhIP-M1 formation between FS and FS-PS treatments was however not significant. After autoclaving of the bacterial suspension, only a very limited PhIP-M1 production was detected.

Effect of surfactants, protease inhibitors, and pH on PhIP metabolism. The production of PhIP-M1 following 3 days of incubation of supernatants prepared from overall fecal microbiota and supplemented with different surfactants and protease inhibitors is depicted in Fig. 2B. The control incubation revealed an average PhIP-M1 production of 78% ± 0.6% after 72 h, while with sodium dodecyl sulfate a significant ($P < 0.01$) increase in PhIP-M1 formation of up to 94% ± 2.7% was observed. Treatment with EDTA (54% ± 1.5%) and Triton X-100 (42% ± 1.2%) significantly ($P < 0.01$) decreased the PhIP-M1 production. No significant ($P > 0.05$) effects could be observed upon Tween 80 addition.

The transformation of PhIP into PhIP-M1 measured at different pH values ranging from 1 to 12 revealed a maximum efficiency of 93% at pH 6 and no PhIP-M1 production below pH 2 and above pH 9.

Effect of nutrition on PhIP metabolism by human fecal microbiota. The capacity of the highest-PhIP-transforming

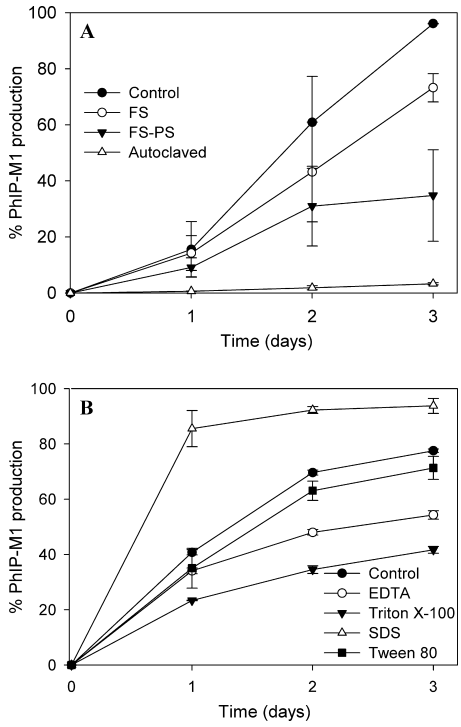

FIG. 2. Conversion of PhIP into PhIP-M1 by 24-h-grown human fecal microbiota exposed to different inactivating treatments (A) and enzyme inhibitors and surfactants (B). Data are presented as means ± SD ($n = 3$). SDS, sodium dodecyl sulfate.

mixed fecal microbiota to transform the food carcinogen PhIP under different nutritional conditions was tested by incubating 5 μM PhIP for a period of 3 days in the presence of minimal medium supplemented with different protein sources, glucose, starch, cellulose, fibers, and olive oil. It was observed that supplementation with protein-rich feed sources such as meat extract, yeast extract, and special peptone also containing traces of sugars and carbohydrates led to a significant production of PhIP-M1 (Fig. 3A), while protein-rich feed sources such as tryptone and protease peptone exclusively containing amino acids and peptides did not support PhIP-M1 formation. Glucose supplementation, however, drastically decreased the PhIP transformation efficiency ($P < 0.01$) (Fig. 3A). The carbohydrates starch and cellulose and the fiber-rich medium did not sustain any PhIP-M1 formation (data not shown). Supplementation with olive oil allowed intermediate transformation efficiency (Fig. 3A). Concomitant supplementation with yeast extract and glucose, yeast extract, and carbohydrates and yeast extract and fibers did not significantly ($P > 0.05$) affect the transformation efficiency observed after yeast extract supplementation (data not shown).

Isolation and characterization of PhIP-transforming bacteria. When PhIP was incubated with serial 10-fold dilutions in TY broth of the highest-PhIP-converting fecal bacterial community, only 10^{-1} and 10^{-2} concentrations demonstrated PhIP transformations of up to 95% and 84%, respectively. Because it did not seem probable that bacteria are present at this low order of magnitude in fecal suspensions, the assumption was made that diluting the fecal inoculum led to the concurrent

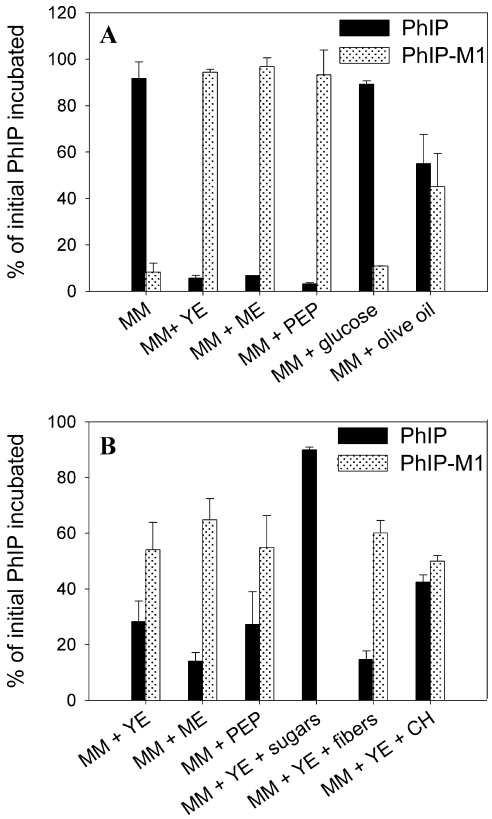

FIG. 3. Conversion of PhIP into PhIP-M1 by mixed fecal microbiota (A) and *E. faecium* PhIP-M1-a (B) grown under different nutritional conditions for 72 h, supplemented with 10% (vol/vol) fecal matrix, and incubated for another 72 h. Values are means ± SD (n = 3). MM, minimal medium; YE, yeast extract; ME, meat extract; PEP, special peptone; CH, carbohydrates.

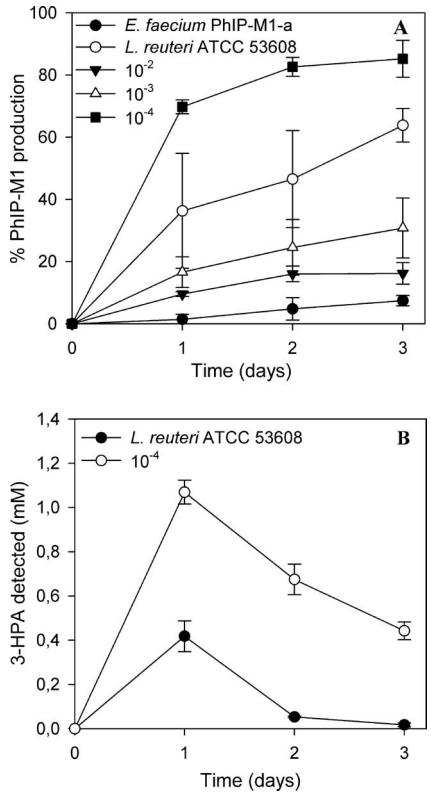

FIG. 4. Formation of PhIP-M1 (A) and 3-HPA (B) by 10-fold dilutions of mixed fecal microbiota, *E. faecium* PhIP-M1-a, and *L. reuteri* ATCC 53608 supplemented with 10 g/liter glycerol. Values are means ± SD (n = 3).

dilution of an unidentified fecal matrix component essential for sustaining microbial PhIP metabolism. Therefore, new fecal dilution series from the two most efficient PhIP-converting individuals were again tested, but with additional supplementation with 10% cell-free fecal matrix (vol/vol). Cosupplementation with this fecal matrix allowed PhIP-M1 formation to occur at lower dilutions (up to 10^{-5}), confirming our hypothesis (data not shown). Therefore, the enrichment procedure was performed in the presence of fecal matrix. Among the 65 colonies picked from plates on which serial dilutions of the PhIP-M1-producing fecal microbiota were plated, two colonies were retrieved that transformed PhIP upon subculturing, as measured by HPLC analysis of culture supernatants (Table 1).

The identities of the isolated strains were confirmed by comparing the sequence of the 16S rRNA gene of each strain within a database. Both isolates were shown to have a 100% sequence similarity with the genus *Enterococcus*. Definite identification of the isolates at species level was achieved by FAFLP and partial *pheS* sequence analysis. Cluster analysis of the FAFLP profiles of these strains with FAFLP profiles of reference strains of lactic acid bacterium taxa (including bifidobacteria) identified both strains as *Enterococcus faecium*. Cluster analysis of the consensus *pheS* sequences of these strains with *pheS* sequences of reference strains of lactic acid bacterium taxa also identified both strains as *Enterococcus faecium* strains. However, distinct profiles were observed for the PhIP-M1-a and PhIP-M1-b strains, and this was true for both the FAFLP and *pheS* sequence phylogenetic fingerprints (data not shown).

PhIP metabolism by bacterial strains of fecal origin. As the new biodegradative strains were identified as members of the genus *Enterococcus*, selected strains belonging to the *Enterococcus* genus and *Lactobacillaceae* family were tested for their PhIP-transforming capacities (Table 1). Among the 20 collection strains that were assayed in the present experiment, 6 were able to produce PhIP-M1, as shown by HPLC analysis with fluorescence detection (Table 1). Most of them belonged to the genus *Enterococcus*, and two *Lactobacillus* strains were capable as well.

Effect of nutrition on PhIP metabolism by *E. faecium* PhIP-M1-a. The percent transformation of PhIP into PhIP-M1 after incubation of *E. faecium* PhIP-M1-a under different nutritional conditions is presented in Fig. 3B. Incubation of the strain in minimal medium did not result in PhIP-M1 formation. Supplementation of the medium with a protein-rich feed source, such as yeast extract, meat extract, or special peptone, low in sugar content resulted in significant PhIP-M1 production. In the absence of a protein-rich feed source or in the presence of protein sources not containing traces of sugar, no transformation could be observed (data not shown). Cosupplementation

TABLE 2. Abiotic synthesis of PhIP into PhIP-M1 by addition of HPA or acrolein to sterile bacterial growth medium containing 5 μM of PhIP

Concentration (mM)	% (± SD) PhIP-M1 production after 24 h with:	
	Acrolein	HPA
0.1	1.5 ± 0.8	1.2 ± 0.8
1	11.3 ± 0.9	8.6 ± 1.3
10	69 ± 0.6	71 ± 0.5
100	89 ± 5.3	78 ± 0.8

with yeast extract and easily degradable sugars, such as glucose, sucrose, mannose, and maltose, etc., completely ($P < 0.01$) inhibited microbial metabolite formation. The addition of carbohydrates or fibers to the yeast extract-containing medium did not significantly alter the microbial PhIP-M1 production ($P < 0.05$).

Elucidation of fecal matrix constituents required for PhIP metabolism. Supplementation with different diet-relevant components or systemic metabolites to the most efficient PhIP-transforming fecal community, *E. faecium* PhIP-M1-a or *L. reuteri* ATCC 53608, in TY broth showed that glycerol allows significant PhIP transformation. No other supplement sustained microbial PhIP-M1 production. PhIP-M1 formation was detected in mixed microbial cultures, and a clear increase in PhIP transformation could be observed with increasing fecal dilution (Fig. 4). Upon incubation of *E. faecium* PhIP-M1-a in a glycerol-enriched medium, only a limited percentage of PhIP-M1 conversion was measured, while *L. reuteri* ATCC 53608 showed an intermediate transformation efficiency (Fig. 4) compared to its high transformation efficiency after supplementation with fecal matrix (Table 1).

Incubation of the highest-PhIP-transforming fecal dilution (10^{-4}) and *L. reuteri* ATCC 53608 in the presence of glycerol gave rise to the formation of 3-HPA (Fig. 4B). The 3-HPA concentration, however, decreased with longer incubation durations.

Abiotic synthesis of PhIP-M1. Incubation of *L. reuteri* ATCC 53608 in 200 mM of glycerol led to the formation of 3-HPA and its aquatic derivates (HPA system), as measured colorimetrically with the method of Circle et al. (8). Supplementation of the HPA system with PhIP in a protein-rich matrix gave rise to the formation of PhIP-M1 for an HPA concentration ranging from 0.1 to 100 mM (Table 2). The addition of acrolein also significantly induced PhIP-M1 production for the same concentrations (Table 2). During the acrolein synthesis experiments, no detectable amounts of acrolein could be measured. Equivalent concentrations of 3-HPA were detected, however. During incubation with 3-HPA, significant decreases in the 3-HPA concentration could be observed. After 24 h of incubating 100 mM 3-HPA in 10 g/liter of meat extract supplemented with PhIP, only 11% ± 1.7% of the initial 3-HPA dose could be detected. Incubating 10 mM 3-HPA for 24 h resulted in the detection of only 3.3% ± 0.4% of the initial 3-HPA dose.

DISCUSSION

In this study, we have isolated two individual strains capable of transforming the food carcinogen PhIP into its derivate PhIP-M1 from human fecal samples and examined the production of PhIP-M1 upon the inoculation of the isolated strains under different nutritional conditions. Moreover, we investigated the interindividual variation in PhIP metabolism between 18 human gut microbiotas. In addition, we contributed to the mechanistic basis for this transformation (Fig. 5) by incubating mixed fecal microbiota under different inactivating conditions and identifying the nutritional requirements for PhIP conversion.

Like many other environmental carcinogens, PhIP requires metabolic activation to exert toxic effects. Previous studies indicate that PhIP is converted into two primary products: 2-hydroxyamino-PhIP (N^2-OH-PhIP) and 4′-hydroxyamino-PhIP (4′-OH-PhIP), the former being highly mutagenic, and the latter being nonmutagenic (9, 45). These metabolites may subsequently be conjugated with acetyl, glucuronide, glutathione, or sulfate to form secondary phase II metabolites. While PhIP is biotransformed into a large number of derivatives in the liver, the human intestinal microbiota selectively converts PhIP into one major metabolite (48). However, strong individual variations occurred among the 18 human fecal samples screened in this study with regard to their PhIP-transforming capabilities. Such metabolic variations can be attributed to commonly encountered interindividual differences in microbial community activity and structure. A striking example is the microbial conversion of the dietary phytoestrogen daidzein

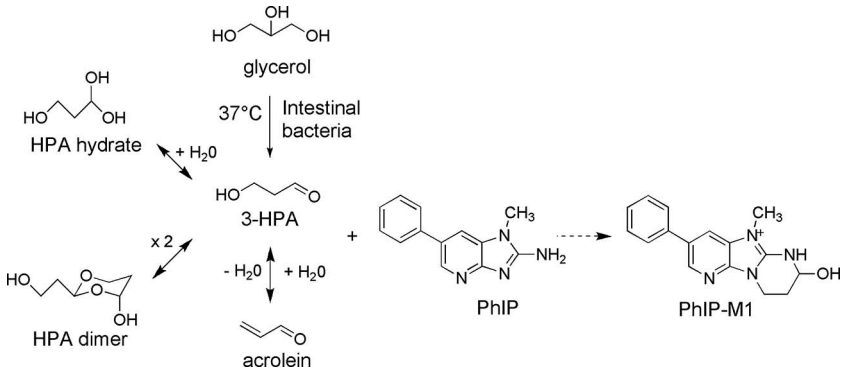

FIG. 5. Reaction mechanism for microbial PhIP-M1 formation through fermentation of glycerol to 3-HPA by the isolated human intestinal bacteria *E. faecium* PhIP-M1-a and PhIP-M1-b. Symbols: →, enzymatic reaction; ↔ , equilibrium reaction; ----➤, chemical reaction.

(11, 35). Intensive research has shown that only approximately one-third of humans harbor an intestinal microbiota capable of transforming daidzein into equol (35). In addition, as our experimental results have shown, the nutritional composition and concentration of required cofactors for transformation by the individual human feces might greatly influence the individual PhIP metabolism. The differences in PhIP transformation capacity may thus well be linked with individual diet and gastrointestinal absorption, metabolism, and excretion.

Until now, the metabolic nature leading from PhIP to PhIP-M1 was unknown. Liver cytochrome P450 in humans and rats is able to perform several hydroxylations and subsequent glucuronidations of the PhIP molecule. The addition of a ring substituent as observed with PhIP-M1 is however unseen. Our results have clearly shown that this metabolite cannot be produced in the absence of intestinal bacteria, i.e., upon autoclaving of the incubation suspension. Filter sterilization and pasteurization of the fecal slurry significantly decreased metabolite formation, confirming the role of actively fermenting bacteria in PhIP-M1 formation by the production of an extracellular substance through an enzymatic process. Reduction of PhIP-M1 formation after supplementation with the enzyme inhibitor EDTA and the surfactant Triton X-100 may be explained by the involvement of an enzymatic reaction in the PhIP transformation process. Moreover, we have observed that PhIP-M1 production takes place only in the presence of a nitrogen-rich food source containing trace amounts of sugars and carbohydrates. These nutritional requirements were shown for mixed fecal microbiota as well as for the *E. faecium* PhIP-M1-a transforming strain. This underlines the importance of a specific bacterial fermentation pattern for PhIP-M1 formation to occur. Besides the nutritional composition of the bacterial medium, additional components or cofactors present in the fecal matrix, not influenced by autoclaving, are required for PhIP transformation to take place. These fecal constituents, identified during our study as glycerol and its fermentation products and the potential cofactors required by enterococci to perform the glycerol fermentation reaction, are not generally included in culture media for intestinal bacteria. From a nutritional point of view, glycerol may be considered as a relevant colonic nutritional constituent, since it is liberated from dietary fat (triglycerides) in the intestinal tract (30). Glycerol is a small hydrophilic solute and, until recently, it was generally believed to be absorbed mainly by paracellular passive transport from the intestine. However, recent research shows that glycerol absorption is saturable in the rat small intestine in situ (51) and in the HCT-15 human colon cancer cell line (16) and involves carrier-mediated transport (16, 25). This creates the opportunity for intraluminal glycerol, depending on the fat intake of the individual, to become available for intestinal microbial metabolism by fermenting strains or fecal excretion.

The eight PhIP-M1-producing individual bacterial strains that we discovered in the mixed fecal contents of humans ($n = 2$) and in culture collections ($n = 6$) are all, except for *L. reuteri*, members of the genus *Enterococcus* and belong to three different species, *E. durans*, *E. faecium*, and *E. avium*. All of the strains converted PhIP solely into PhIP-M1, regardless of the extent of substrate consumption (range, 2.4 to 96%). The enterococci phylogenetically belong to the clostridial subdivision of the gram-positive bacteria and are detected in adult human feces at concentrations of 6.1 ± 0.7 $\log_{10}$ CFU/g (21). *L. reuteri* is also a resident of the gastrointestinal tract of humans and animals and is one of the dominant heterofermentative species in this ecosystem (34). Under anaerobic conditions, several lactobacilli, as well as other bacterial species (*Klebsiella*, *Clostridium*, *Enterobacter*, and *Citrobacter* genera), have been shown to use glycerol as an external electron acceptor (37, 38, 43). Our study is however the first to relate bacterial species of the genus *Enterococcus* to this anaerobic pathway of glycerol dissimilation. During this fermentation, glycerol is converted by a coenzyme B_{12}-dependent dehydratase to 3-HPA. 3-HPA is normally an intracellular intermediate that does not accumulate but is reduced by an NAD^+-dependent oxidoreductase to 1,3-propanediol (PPD) (4, 10). *L. reuteri* is unique among the lactobacilli in that the glycerol metabolite 3-HPA is excreted in amounts higher than is the case for other lactobacilli forming the HPA system (3-HPA and its aqueous derivates), also known as reuterin, a potent bacterial inhibitor (42). The regulation of the PPD pathway is dependent on the availability of fermentable carbohydrates, in particular glucose. In the absence of glucose, PPD formation is the rate-limiting step and 3-HPA may accumulate (12). In the present study, we have observed that easily degradable sugars inhibit PhIP-M1 production. This may be linked to the absence of 3-HPA and its aqueous derivates under these conditions.

The addition of the HPA system to our bacterial medium significantly enhanced PhIP-M1 formation. The 3-HPA dehydration product acrolein was also potent in producing PhIP-M1 but was as a consequence of its instable nature in aqueous environments immediately converted to 3-HPA. Another remarkable finding was the relatively fast disappearance of 3-HPA when spiked into a protein-rich bacterial medium. This can be explained by the tendency of 3-HPA and its derivates, molecules which all have aldehyde groups, to react with amino groups in biological tissues (41). This tendency of 3-HPA to react with free amino groups thus very well may be responsible for the PhIP-to-PhIP-M1 conversion. Incubation of a high-PhIP-transforming mixed fecal dilution and *L. reuteri* strain ATCC 53608 with glycerol clearly gave rise to the formation and detection of 3-HPA, even though a large part of the total amount of 3-HPA produced by the bacteria was undetectable as a result of interactions with cellular material and medium components. Although reuterin (the HPA system) is currently accepted as an antibiotic produced by probiotic strains such as *L. reuteri*, the risk involved with 3-HPA and its binding potency toward biological tissue components and procarcinogens such as PhIP should be taken into account.

PhIP-M1 has been investigated for its potential mutagenic/genotoxic activity. It does not act as a direct mutagen in the Ames test, but a small increase in mutagenicity is observed after the addition of S9 liver fraction (48). On the other hand, it has been shown that the intestinal microbiotas are essential to the induction of DNA damage by PhIP in human fecal flora-associated rats (20), and recent investigations (L. Vanhaecke, L. Derycke, F. Le Curieux, S. Lust, D. Marzin, W. Verstraete, and M. Bracke, unpublished data) indicate that PhIP-M1 exerts cytotoxic and apoptotic effects toward Caco-2 cells in vitro. Such contrasting data highlight the necessity of identifying the metabolites produced by microbial processes

from important known procarcinogens in our diet and of further evaluating their genotoxic/carcinogenic activity.

ACKNOWLEDGMENTS

We thank Cosucra N.V. for supporting this work. We also acknowledge Cindy Snauwaert (BCCM/LMG Bacteria Collection) for performing the *pheS* sequence analyses. Birger Bocxstael and Petra Van Damme are gratefully acknowledged for performing the denaturing gradient gel electrophoresis analyses and cloning reactions.

This research was funded by a doctoral fellowship for Lynn Vanhaecke from the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT). Tom van de Wiele is a postdoctoral fellow of the Fund for Scientific Research-Flanders (FWO-Vlaanderen). The BCCM/LMG Bacteria Collection is supported by the Prime Minister's Services—Federal Office for Scientific, Technical and Cultural Affairs, Belgium.

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