Preliminary Safety Evaluation of a New *Bacteroides xylanisolvens* Isolate

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Besides conferring some health benefit to the host, a bacterial strain must present an unambiguous safety status to be considered a probiotic. We here present the preliminary safety evaluation of a new *Bacteroides xylanisolvens* strain (DSM 23964) isolated from human feces. First results suggest that it may be able to provide probiotic health benefits. Its identity was confirmed by biochemical analysis, by sequencing of its 16S rRNA genes, and by DNA-DNA hybridization. Virulence determinants known to occur in the genus *Bacteroides*, such as the *bft* enterotoxin and other enzymatic activities involved in the degradation of the extracellular matrix and the capsular polysaccharide PS A, were absent in this strain. The investigation of the antibiotic susceptibility indicated that strain DSM 23964 was sensitive to metronidazole, meropenem agents, and clindamycin. Resistance to penicillin and ampicillin was identified to be conferred by the β -lactamase *cepA* gene and could therefore be restored by adding β -lactamase inhibitors. The localization of the *cepA* gene in the genome of strain DSM 23964 and the absence of detectable plasmids further suggest that a transfer of β -lactamase activity or the acquisition of other antibiotic resistances are highly improbable. Taken together, the presented data indicate that the strain *B. xylanisolvens* DSM 23964 has no virulence potential. Since it also resists the action of gastric enzymes and low-pH conditions, this strain is an interesting candidate for further investigation of its safety and potential health-promoting properties.

Most probiotic strains belong to the genus *Lactobacillus* or *Bifidobacterium*, with only few belonging to other genera like *Enterococcus*, *Escherichia*, or *Streptococcus* (40). The main reason is that *Lactobacillus* and *Bifidobacterium* are commonly found in traditional fermented food and therefore are generally recognized as safe for human consumption. Proposals for the use of nontraditional species in humans generally evoke greater concern about potentially adverse effects. Therefore, current probiotic strains are mainly selected for their mainstream acceptance, a selection process ruling out potential strains with potentially far better health properties. The genus *Bacteroides* accounts for 20% to up to 40% of the human colonic microbiota (19), surpassing *Lactobacillus* and *Bifidobacterium* by a factor of 10,000. However, to our knowledge and with the exception of a *Bacteroides ovatus* strain, which was genetically modified to produce biologically active human KGF in response to the administration of xylan and thereby constraining the development of colitis (17), no other strain of the genus *Bacteroides* has so far been evaluated as a potential probiotic strain. This is surprising since certain *Bacteroides* species are known to possess functions that are beneficial for the human health, including (i) unique metabolic activities involved in the fermentation of carbohydrates, the utilization of nitrogenous substances, and the biotransformation of bile acids and other steroids (31), (ii) prevention of pathogenic organisms from colonizing the intestine (20), (iii) production of short-chain fatty acids (21, 28) that may have cholesterol-reducing, satiety-stimulatory, and anti-carcinogenic properties (16, 21, 56), (iv) immunomodulatory effects involved in the development of the host's immune system and the maintenance of its ability to fight pathogens and diseases (7, 9, 46).

On the other side, however, some *Bacteroides* strains are reported to participate in the development of severe extraintestinal infections under special conditions (29). Therefore, some strains are classified as opportunist pathogens and may raise some safety concern. However, virulence is known to be a strain-specific trait, and the taxonomy of the genus *Bacteroides* has undergone signif-

icant changes during the past few years, leading to a redefinition of the genus *Bacteroides* and to the identification of several new species (2, 3, 5). Some of them, like *B. xylanisolvens*, that are not classified as opportunistic pathogens can be expected to contain strains with a favorable safety profile.

We have isolated a new *Bacteroides xylanisolvens* strain from human feces. Our own preliminary unpublished data suggest that this strain may possess immune-modulatory properties and is able to produce short fatty acid chains (propionate, succinate) with potential health-beneficial effects. Here we present the preliminary safety evaluation of this new *B. xylanisolvens* strain (DSM 23964).

MATERIALS AND METHODS

Abbreviations. BLAST, basic local alignment search tool; dNTP, deoxynucleoside triphosphate; DSMZ, German Collection of Microorganisms and Cell Cultures; IBD, inflammatory bowel disease; KGF, keratinocyte growth factor; MEGA, molecular evolutionary genetics analysis; MIC, minimal inhibitory concentration; NCCLS, National Committee on Clinical Laboratory Standards; OD, optical density; PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA; RT-PCR, reverse transcriptase PCR; SI, sucrase isomaltase; WHO, World Health Organization.

Bacterial strains. *B. ovatus* DSM 1896, *Bacteroides thetaiotaomicron* DSM 2079, *Bacteroides fragilis* DSM 1396, *B. xylanisolvens* DSM 18836, and *Bacteroides finegoldii* DSM 17565 were obtained from the DSMZ (Braunschweig, Germany). The clinical isolates *B. ovatus* MN23 and *B. ovatus* MN7 were provided by Alberto Quesada Molina (Universidad de Extremadura, Cáceres, Spain). All *Bacteroides* strains were cultured in

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TABLE 1 Primer pairs and PCR conditions used to detect virulence genes of strain DSM 23964

PCR	Primers	Sequence (5' to 3')	PCR program ^a	Reference
1	<i>27f</i> <i>519r</i>	AGA GTT TGA TCM TGG CTC AG GWA TTA CCG CGG CKG CTG	45 cycles: 95°C, 5 s; 52°C, 40 s; 72°C, 30 s; 40°C, 30 s	53
2	<i>OPX14</i>	ACA GGT GCT G	35 cycles: 94°C, 60 s; 55°C, 60 s; 72°C, 60 s; 72°C, 10 min	Our work
3	<i>cfiA-F</i> <i>cfiA-R</i>	ATG GTA CCT TCC AAC GGG CAC GAT ATT GTC GGT CGC	25 cycles: 94°C, 60 s; 65°C, 60 s; 72°C, 60 s; 72°C, 3 min	15
4	<i>cepA-F</i> <i>cepA-R</i>	AGC GAY AAY AAY GCN TGY GA GAA GAT CCN GTY TTR TGN CC	35 cycles: 94°C, 60 s; 60°C, 30 s; 72°C, 120 s; 72°C, 10 min	12
5	<i>cfxA-F</i> <i>cfxA-R</i>	ATC GTA GTT TTG AGT ATA GCT TAA AAG CAC TCC GAT AAC GAT	25 cycles: 94°C, 60 s; 65°C, 30 s; 72°C, 120 s; 72°C, 10 min	12
6	<i>bfi-F</i> <i>bfi-R</i>	GAC GGT GTA TGT GAT TTG TCT GAG AGA ATC CCT AAG ATT TTA TTA TTA TCC CAA GTA	35 cycles: 94°C, 60 s; 52°C, 60 s; 72°C, 60 s; 72°C, 5 min	15
7	<i>ompW-F</i> <i>ompW-R</i>	ACG TAT GCT TCC CGC CGA CTC CAA ACA AGT AGT CGT GAA ACC TGC AGT AGC AGT CAG GT	30 cycles: 94°C, 60 s; 65°C, 60 s; 72°C, 60 s; 72°C, 5 min	49
8	<i>upaY</i> <i>orf1</i>	AGA AAA CTC CTG GTC CTT CTT TG ACA CAT ATC ACT TCC GAT GCC	30 cycles: 94°C, 60 s; 65°C, 60 s; 72°C, 120 s; 72°C, 5 min	8
9	<i>upaZ</i> <i>orf1</i>	GTT GAC GGA AAT GAT CGG TAT AG ACA CAT ATC ACT TCC GAT GCC	30 cycles: 94°C, 60 s; 65°C, 60 s; 72°C, 120 s; 72°C, 5 min	8
10	<i>wcfR</i> <i>pdiA</i>	CAC TCT TTG GGA GCA CGT TAC CAG AGT AAT GGT AAA ATC GGG AG	30 cycles: 94°C, 60 s; 65°C, 60 s; 72°C, 120 s; 72°C, 5 min	8
11	<i>wcfS</i> <i>pdiA</i>	AGA TCC GAG AAT AAC TCG TAC C CAG AGT AAT GGT AAA ATC GGG AG	30 cycles: 94°C, 60 s; 65°C, 60 s; 72°C, 120 s; 72°C, 5 min	8
12	<i>nanH-1</i> <i>nanH-2</i>	TGG GTG GTT GCT GCC TGG ACA CA TGT TCG GGT GAC CAA GTC ACA CC	30 cycles: 94°C, 60 s; 68°C, 60 s; 72°C, 60 s; 72°C, 5 min	25
13	<i>SI-F</i> <i>SI-R</i>	CAT CCT ACC ATG TCA AGA GCC A GCT TGT TAA GGT GGT CTG GTT T	35 cycles: 94°C, 60 s; 55°C, 60 s; 72°C, 60 s; 72°C, 10 min	14
14	<i>GAPDH-F</i> <i>GAPDH-R</i>	GAA GGT GAA GGT CGG AGT C GAA GAT GGT GAT GGG ATT TC	35 cycles: 94°C, 60 s; 55°C, 60 s; 72°C, 60 s; 72°C, 10 min	14
15	<i>IRS-1</i> <i>IRS-2</i>	GCT GGA TCA CCT CCT TTC CCT TTC CCT CAC GGT ACT G	30 cycles: 94°C, 60 s; 66°C, 60 s; 72°C, 60 s; 72°C, 5 min	18
16	<i>Bfra-R</i> <i>Bova-R</i> <i>1392A</i>	GCT AAT CCC CCA ATC ATA C AAT AAT GCG TAC TCG AAC AC GTA CAC ACC GCC CGT	35 cycles: 95°C, 20 s; 57°C, 60 s; 72°C, 30s; 72°C, 5 min	27

^a Cycles: denaturation temperature, time; annealing temperature, time; extension temperature, time; final extension temperature, time.

Wilkins-Chalgren broth medium (Oxoid, Wesel, Germany) for 24 to 48 h at 37°C under anaerobic conditions in the Oxoid Atmosphere generation system (Oxoid, Wesel, Germany). The bacterial pellets were washed with 0.85% NaCl, collected, and stored at 37°C under anaerobic conditions shortly before the start of the experiments.

Biochemical identification. The biochemical identification of the isolated strain DSM 23964 was performed with rapid ID 32A and API 20A biochemical test kits (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's protocol.

16S rRNA gene sequencing and phylogenetic analysis. Overnight cultures were harvested by centrifugation for 15 min at 10,000 × g at 4°C and washed three times in 2 ml sodium phosphate buffer (50 mM, pH 7.0). DNA was extracted from the pellet with the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was estimated by absorbance at 260 nm. The 16S rRNA sequencing was performed by Biotecon GmbH (Potsdam, Germany). The 16S rRNA gene sequences (480 bases) of strain DSM 23964 were amplified by PCR (Table 1, PCR no. 1) using universal primers *27f* (5'-AGA GTT TGA TCM TGG CTC AG-3') and *519r* (5'-GWA TTA CCG CGG CKG CTG-3') (53). PCR products were purified with the High Pure PCR product purification kit (Roche, Indianapolis, IN), and the DNA concentration and product sizes were estimated using a low DNA mass ladder (Invitrogen, Carlsbad, CA). PCR products were sequenced by GATC (Biotech AG, Konstanz, Germany): briefly, DYEnamic ET dye terminator cycle sequencing kit (Amersham Bioscience) and ABI PRISM 3100 capillary sequencer (Applied Biosystems) were used for the sequencing process. The identification of phylogenetic neighbors was initially carried out by the BLAST (1) and megaBLAST (55) programs against the

database of type strains with validly published prokaryotic names (6). The 50 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using the global alignment algorithm of the EzTaxon server. The resulting multiple-sequence alignment was corrected manually by using the program MEGA version 4 (45), and a phylogenetic tree was constructed according to the neighbor-joining method (38).

DNA-DNA hybridization. Genomic DNA of strain DSM 23964 and of other closely related strains (*B. xylanisolvens* DSM 18836, *B. ovatus* ATCC 8483, *B. thetaiotaomicron* ATCC 29148 and *B. fingoldii* DSM 17565) was isolated as described above. DNA-DNA hybridizations were performed by the DSMZ Identification Service.

RAPD pattern and genotype. The arbitrary oligonucleotide primer *OPX-14* (5'-ACA GGT GCT G-3') from Eurofins MWG Operon (Ebersberg, Germany) was used for the amplification of template DNA (Table 1, PCR no. 2). The PCR mixture (50 µl) contained 16 mM 10× *Taq* buffer, 2.5 mM MgCl₂, 0.25 mM each dNTP, 1 µM primer, 5 U of *Taq* DNA polymerase, and 2 µl of template DNA. The PCR conditions were as follows: predenaturation at 95°C for 5 min, 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension for 10 min at 72°C. A 10-µl sample of amplification products was analyzed by electrophoresis in 1% agarose gel (Bioline).

Effect of gastric and intestinal juice on viability of strain DSM 23964. The tolerance to gastric and intestinal juice of strain DSM 23964 was analyzed by using modified simulated gastric juice (5 M HCl, 2 g/liter NaCl, 3.2 g/liter pepsin, pH 2) and small intestine juice (6.8 g/liter KH₂PO₄, 10 g/liter pancreatin, 0.1 M NaOH, pH 8.0) as previously described (24). Cells of an overnight culture were harvested by centrifuga-

tion for 15 min at $5,000 \times g$ at 4°C , washed, resuspended in PBS, mixed with PBS (positive control), and gastric and/or intestinal juice to an OD of 1.0 and incubated at 37°C . Bacterial concentration (bacteria/ml) was determined microscopically. Aliquots were removed immediately after dilution (t_0) and after 5, 15, 30, 60, 120, and 180 min for intestinal juice. For intestinal juice tolerance, the aliquots were removed immediately after dilution (t_0) and after 5, 60, 120, and 240 min. For each point, appropriate dilutions were also done and 200 cells plated on Wilkins-Chalgren agar and incubated for 48 h at 37°C under anaerobic conditions. The survival rate was determined by comparing the number of cells at each point to the number of cells at t_0 . *Lactobacillus acidophilus* DSM 9126 was used as a positive control.

MIC. MICs of different antibiotic agents for the isolated strain DSM 23964 were determined by the agar dilution method according to CLSI (formerly NCCLS) standards (34) using Wilkins-Chalgren agar (Oxoid, Wesel, Germany). The following antibiotics or combinations were used: ampicillin (Merck, Darmstadt, Germany), $4 \mu\text{g/ml}$; penicillin G (Merck), $0.5 \mu\text{g/ml}$; mezlocillin (Oxoid), $8 \mu\text{g/ml}$; ampicillin + sulbactam (Oxoid), $16 \mu\text{g/ml}$; piperacillin + tazobactam (Oxoid), $64 \mu\text{g/ml}$; meropenem (Oxoid), $8 \mu\text{g/ml}$; clindamycin (Oxoid), $8 \mu\text{g/ml}$; metronidazole (Oxoid), $16 \mu\text{g/ml}$. Sulbactam and tazobactam were used as inhibitors of chromosomal class A β -lactamases. The breakpoints of the antibiotics used are shown (see Table 3). Wilkins-Chalgren agar plates containing serial dilutions of the antimicrobial agents ranging from $0.125 \mu\text{g/ml}$ to $256 \mu\text{g/ml}$ were prepared and used on the same day. Colonies were resuspended to a turbidity of 0.5 McFarland standard (approximately 10^7 bacteria). A $50\text{-}\mu\text{l}$ portion of the bacterial suspension was plated and incubated overnight in an anaerobic chamber at 37°C . The MIC was defined as the lowest concentration of antimicrobial agent that caused complete inhibition of growth, a barely visible haze, or one colony only (39).

Determination of plasmids. Overnight cultures (100 ml) of *B. xylanisolvens* DSM 23964, *Escherichia coli* DSM 3876 (plasmid RP4, 60 kb), and *E. coli* DSM 6202 (plasmid pSC 101, 9.4 kb) were harvested by centrifugation for 10 min at $13,000 \times g$ at 4°C and washed three times in 2 ml sodium phosphate buffer (50 mM, pH 7.0). Plasmid DNA was extracted with the Qiagen plasmid midikit (Qiagen) according to the manufacturer's protocol. According to the Qiagen manufacturer, the plasmid purification kit used in this study was able to purify plasmids up to approximately 150 kb. The analysis of plasmid DNA was performed by a modification of the procedure as previously described (4). The quantitative analysis of plasmid DNA was done by horizontal agarose gel electrophoresis in 1% agarose gel.

Detection of potential virulence genes by specific PCR. PCRs were performed with $2 \mu\text{l}$ of the DNA extract, $1 \mu\text{M}$ each primer, 2 mM MgCl_2 (Bioline, Luckenwalde, Germany), 0.2 mM dNTPs (Bioline), and 2.5 U of *Taq* polymerase (Bioline) in a total reaction volume of $50 \mu\text{l}$ with buffer provided by the manufacturer. The primers used, provided by BioTeZ (Berlin-Buch, Germany), and the PCR conditions are given in Table 1. At the end of the PCR processes, $10 \mu\text{l}$ of each amplified product was analyzed in a 1% (wt/vol) agarose gel (Bioline). The following genes were analyzed by specific PCR.

(i) ***cfiA* gene.** A modified PCR method based on the procedure as described by Gutacker et al. (15) was used to detect the *cfiA* gene with a fragment length of 353 bp (Table 1, PCR no. 3). *B. fragilis* TAL 3636 was used as a control strain.

(ii) ***cepA* and *cfxA* genes.** Based on the procedure described by Garcia et al. (12), PCR no. 4 and 5 were used to detect *cepA* (338 bp) and *cfxA* (1,011 bp) genes as described in Table 1. *B. fragilis* DSM 1396, *B. ovatus* MN23, and *B. ovatus* MN7 were used as control strains.

(iii) ***bft* gene.** Based on the procedure described by Gutacker et al. (15), PCR no. 6 as described in Table 1 was used to detect the *bft* gene (294 bp). *B. fragilis* ATCC 43858 was used as a positive control, and *B. fragilis* DSM 2151 was used as a negative control.

(iv) ***ompW* gene.** PCR no. 7 as described in Table 1 shows the primer and the PCR conditions for the detection of the TonB-linked outer mem-

brane protein encoding gene *ompW* with a fragment length of 468 bp (49). *Bacteroides caccae* DSM 19024 was used as a control strain.

(v) ***upaY*, *upaZ*, *wcfR*, and *wcfS* genes.** The primer pairs and their sequences for biosynthesis locus genes *upaY* and *upaZ* for the capsular polysaccharide A, *wcfR* for the aminotransferase, and *wcfS* for the glycosyltransferase are shown in Table 1. Modified PCR procedures (PCR no. 8 to 11) were used for the detection of *upaY* (1,636 bp), *upaZ* (2,208 bp), *wcfR* (1,895 bp), and *wcfS* (932 bp) genes as described by Coyne et al. (8). *B. fragilis* DSM 1396, *B. fragilis* ATCC 43858, and *B. fragilis* DSM 2151 were used as positive-control strains, and *B. fragilis* TAL 3636 was used as a negative-control strain.

Determination of extracellular enzymes and pathogenic factors. The extracellular enzyme activities of *B. xylanisolvens* DSM 23964 were determined as follows.

(i) **DNase activity.** DNase agar plates (Oxoid, Wesel, Germany) were used to determine DNase activity of bacterial strains as described by Porschen and Sonntag (33). Samples ($50 \mu\text{l}$) of the overnight cultures were dropped onto different places of the agar surface, incubated for 24 to 48 h at 37°C under anaerobic conditions, and tested by flooding the plates with 1 N HCl. *B. thetaiotaomicron* ATCC 29148 was used as a positive control.

(ii) **Chondroitinase and hyaluronidase activities.** The agar plates for the chondroitinase and hyaluronidase test were prepared as described by Smith and Willett (42). *B. thetaiotaomicron* ATCC 29148 was used as a positive control.

(iii) **Hemolysin activity.** The analysis of hemolytic activity of *B. xylanisolvens* DSM 23964 was performed as described previously by Robertson et al. (35). Samples ($100 \mu\text{l}$) of the overnight cultures were streaked onto sheep blood agar and incubated for 24 h at 37°C under anaerobic conditions. *B. ovatus* ATCC 8483 was used as a positive control.

(iv) **Collagenase activity.** The activity of collagenase was determined by a modification of the method described previously by Steffen and Hentges (43). Wilkins-Chalgren broth (5 ml) containing 5 mg autoclaved collagen was inoculated with the test strains and incubated for 2 weeks under anaerobic conditions. The specificity of this procedure was confirmed by adding trypsin and protease to separate Wilkins-Chalgren broth including collagen under identical conditions. Sterile collagenase solution added to a Wilkins-Chalgren broth including collagen served as a positive control. *B. ovatus* ATCC 8483 was also used as a positive control.

(v) **Neuraminidase activity.** Based on the procedure described by Kuwahara et al. (25), PCR no. 12 as described in Table 1 was used to detect the neuraminidase-encoding gene *nanH* (648 bp). The following bacterial strains were used as positive controls: *B. fragilis* ATCC 43858, *B. fragilis* DSM 1396, and *B. fragilis* TAL 3636. *B. ovatus* DSM 1896 was used as a negative control.

Determination of cell adhesion of strain DSM 23964 to Caco-2 cells. The human colonic carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). The cultivation and differentiation of Caco-2 cells were performed as previously described by Wang and Evers (48). Monolayers of Caco-2 cells were collected on different days and analyzed for the expression of GAPDH (226 bp) and sucrase isomaltase (196 bp) by RT-PCR as previously described by Gu et al. (14). The primer and PCR conditions used are described in Table 1 (PCR no. 13 and 14). On the day of analysis, a final concentration of 2×10^8 bacterial cells per ml was added to the monolayer of Caco-2 cells and the cells were incubated at 37°C for 2 h under anaerobic conditions. *L. acidophilus* DSM 9126 and *B. fragilis* DSM 1396 were used as positive-control strains. After 2 h, the wells were washed six times with PBS. Samples from each wash step were centrifuged, and the cell pellets were harvested for a species-specific PCR analysis (18, 27) as described in Table 1 (PCR no. 15 to 16). For *B. xylanisolvens*, the primers and conditions described for the species *B. ovatus* revealed a single and *B. xylanisolvens*-specific band of about 820 bp.

TABLE 2 Biochemical characteristics of the strain DSM 23964 and related members of the genus *Bacteroides*

Characteristic	Result for strain ^a					
	1	2	3	4	5	6
Indole formation	–	–	–	+	+	–
Enzymatic activities						
<i>N</i> -Acetyl- β -glucosaminidase	+	+	+	–	+	+
Glutamic acid decarboxylase	+	+	+	–	+	+
α -Fucosidase	+	+	–	+	+	+
Indol production	–	–	–	+	+	+
Arginine arylamidase	–	–	–	–	+	–
Phenylalanine arylamidase	–	–	–	–	+	+
Leucine arylamidase	–	–	–	+	+	+
Tyrosine arylamidase	–	–	–	–	+	+
Glycine arylamidase	–	–	–	–	+	–
Histidine arylamidase	–	–	–	–	+	+
Glutamyl glutamic acid arylamidase	+	+	+	–	+	+
Serine arylamidase	–	–	–	–	+	–
Acid production from:						
D-Mannitol	+	+	–	–	–	–
Salicin	+	+	+	+	–	–
Glycerol	+	+	–	–	–	–
D-Melezitose	+	+	–	–	–	–
D-Sorbitol	+	+	–	–	+	–
D-Trehalose	+	+	–	+	+	–
Catalase activity	–	–	+	+	+	+

^a Strains: 1, strain DSM 23964; 2, *B. xylanisolvens* DSM 18836; 3, *B. fingoldii* DSM 17565; 4, *B. ovatus* DSM 1896; 5, *B. thetaiotaomicron* DSM 2079; 6, *B. fragilis* DSM 1396. +, positive reactivity; –, negative reactivity.

RESULTS

Microbiological and biochemical properties. The strain DSM 23964 was identified by its strictly anaerobic, non-spore-forming, nonmotile, and Gram-negative cells. The short rods or rod-shaped cells were 0.4 to 0.5 μ m in width and variable in length (generally in the range of 1 to 2 μ m). After 18 h, colonies grown on Wilkins-Chalgren agar were 2 to 3 mm in diameter with a circular, milky, raised, and convex surface. Initial biochemical analyses showed a 91% similarity to *B. ovatus* species (Databank bio-Mérieux). In contrast to *B. ovatus*, the strain DSM 23964 was unable to utilize starch or to produce indole, and it did not show catalase activity. We therefore directly compared the biochemical profile of strain DSM 23964 with other phylogenetically related *Bacteroides* species. The results depicted in Table 2 suggest a greater biochemical relationship to *B. xylanisolvens* than to other *Bacteroides* species.

16S rRNA gene sequencing. Based on a 480-bp sequence from the 16S rRNA genes, we constructed a phylogenetic tree for strain DSM 23964 (Fig. 1A). According to this, strain DSM 23964 had a 16S rRNA sequence similarity amounting to 100% and formed a single cluster with *B. xylanisolvens* DSM 18836. The relationships to other related species were of significantly lower levels, with 97.5%, 94.2%, and 92.2% similarity to *B. ovatus* ATCC 8483, *B. thetaiotaomicron* ATCC 29148, and *B. fingoldii* DSM 17565, respectively.

DNA-DNA hybridization. In order to definitively identify strain DSM 23964, DNA-DNA hybridization experiments were performed. Confirming the results of the 16S rRNA gene sequencing, a similarity of 98.65% was identified between strain DSM 23964 and *B. xylanisolvens* DSM 18836. Similarity to *B. ovatus* ATCC 8483, *B. thetaiotaomicron* ATCC 29148, and *B. fingoldii* DSM 17565 reached 26.9%, 28.65%, and 25.2%, respectively.

FIG 1 Phylogenetic representation and RAPD profile of *B. xylanisolvens* DSM 23964. (A) Phylogenetic representation of different *Bacteroides* strains based on 16S rRNA gene sequences. The scale bar represents 0.01 substitutions per nucleotide position. Bootstrap values (>50%) based on 1,000 replications are listed as percentages at branching points. (B) RAPD amplification profile with primer OPX-14. Lanes: 1 and 5, 1-kb DNA ladder (Bioline); 2, *B. xylanisolvens* DSM 23964; 3, *B. xylanisolvens* DSM 18836; 4, negative control (water).

TABLE 3 Resistance profile of *B. xylanisolvens* DSM 23964 toward antibiotics

Agent(s)	MIC ($\mu\text{g/ml}$)	Breakpoint ($\mu\text{g/ml}$)	Sensitivity ^a
Penicillin G	4	0.5	R
Mezlocillin	8	8	R
Ampicillin	8	4	R
Ampicillin + sulbactam	0.25	16	S
Piperacillin + tazobactam	2	64	S
Meropenem	0.25	8	S
Clindamycin	0.5	8	S
Metronidazole	0.25	16	S

^a R, resistant; S, sensitive.

RAPD fingerprinting of *B. xylanisolvens* DSM 23964. RAPD profiling using arbitrary 10-mer oligonucleotide primers was used to characterize strain DSM 23964 and to distinguish it from *B. xylanisolvens* DSM 18836 down to the strain level. As shown in Fig. 1B, strain DSM 23964 presented a distinct RAPD profile compared to the reference strain *B. xylanisolvens* DSM 18836, indicating that DSM 23964 is a new strain.

Effect of gastric and intestine juice on viability of strain DSM 23964. To examine the effect of a passage through the gastrointestinal tract, the bacteria were subjected to the action of simulated gastric and intestinal juices. The survival rate for the *B. xylanisolvens* DSM 23964 strain in gastric juice was above 90% after 180 min and above 96% after 240 min of exposure to intestinal juice (data not shown).

Antibiotic resistance of *B. xylanisolvens* DSM 23964. The analysis of the MIC of several antibiotics revealed that the *B. xylanisolvens* DSM 23964 strain was resistant to β -lactam drugs like penicillin G, ampicillin, and mezlocillin. These resistances are based on the presence of β -lactamase activity as demonstrated by restoration of full sensitivity following addition of β -lactamase inhibitors. Furthermore, *B. xylanisolvens* DSM 23964 was sensitive to the antibiotics metronidazole, meropenem, and clindamycin (Table 3).

Detection of plasmid(s) in *B. xylanisolvens* DSM 23964. To investigate the potential presence of plasmid(s) in strain DSM 23964, plasmid DNA material was isolated from strain *B. xylanisolvens* DSM 23964 and from two control strains, *E. coli* DSM 3876 and *E. coli* DSM 6202, which contain the low-copy-number plasmids RP4 (60 kb) and pSC101 (9.4 kb), respectively. These strains were specifically used as control because most plasmids previously identified within strains of the genus *Bacteroides* ranged between 3 and 80 kb. Measurements of the plasmid DNA concentration at 260 nm revealed the absence of DNA in the plasmid preparation of strain DSM 23964. Running the plasmid preparations on an agarose gel confirmed the isolation of both low-copy-number plasmids from the control strains and the absence of detectable plasmid material from *B. xylanisolvens* DSM 23964. We conclude that strain DSM 23964 contains no plasmid with a size within the isolation limit of the kit (Table 4).

Identification of the β -lactamase activities. In order to characterize the β -lactamase activity of strain DSM 23964, specific PCR assays were run for each of the β -lactamase genes, *cfiA*, *cfxA*, and *cepA*, known for the genus *Bacteroides*. The results presented in Table 4 indicate that the strain *B. xylanisolvens* DSM 23964 contains only the *cepA* gene.

TABLE 4 Detection of virulence factors of *B. xylanisolvens* DSM 23964

Virulence factor	Result for <i>B. xylanisolvens</i> DSM 23964	Positive control(s)	Negative control
<i>bft</i>	—	<i>B. fragilis</i> ATCC 43858	—
<i>wcfR</i>	—	<i>B. fragilis</i> DSM 1396, <i>B. fragilis</i> ATCC 43858, <i>B. fragilis</i> DSM 2151	—
<i>wcfS</i>	—	<i>B. fragilis</i> DSM 1396, <i>B. fragilis</i> ATCC 43858, <i>B. fragilis</i> DSM 2151	—
<i>ompW</i>	—	<i>B. caccae</i> DSM 19024	—
<i>cfiA</i>	—	<i>B. fragilis</i> TAL 3636	—
<i>cfxA</i>	—	<i>B. ovatus</i> MN7 and <i>B. ovatus</i> MN23	—
<i>cepA</i>	+	<i>B. fragilis</i> DSM 1396, <i>B. ovatus</i> MN23	—
Plasmid(s)	—	<i>E. coli</i> DSM 3876 (plasmid RP4, 56.4 kb), <i>E. coli</i> DSM 6202 (plasmid pSC101, 9 kb)	—

Genes encoding virulence factors in the genus *Bacteroides*. In order to investigate the presence of the *B. fragilis* enterotoxin (Bft), we ran a PCR specific for the *bft* gene. In the case of the capsular polysaccharide PS A, specific PCRs for the highly conserved open reading frames *upaY* and *upaZ* (located upstream of the biosynthesis genes of PS A) and for the most important genes, *wcfR* (encoding an aminotransferase), and *wcfS* (encoding a glycosyltransferase), were designed. As shown in Table 4, strain *B. xylanisolvens* DSM 23964, in contrast to *B. fragilis* ATCC 43858, does not possess the *bft* gene. Also, the genes *wcfR* and *wcfS* (Table 4) and the open reading frames *upaY* and *upaZ* could not be detected (data not shown).

We further analyzed the presence of the gene *ompW* encoding the virulence factor “TonB-linked outer membrane protein,” which may be involved in the development of IBD (49). As shown in Table 4, no *ompW* gene could be detected in strain *B. xylanisolvens* DSM 23964.

Determination of extracellular enzymes and pathogenic factors. Besides neuraminidase, several strains of the genus *Bacteroides* were described to produce unwanted exoenzymes, including collagenase, DNase, and some proteases that could participate in infection processes. The most relevant exoenzyme activities were analyzed by means of PCR (neuraminidase) or with enzymatic assays. *B. xylanisolvens* DSM 23964 showed no DNase, chondroitinase, hyaluronidase, or neuraminidase activities and only very weak β -hemolytic and collagenase activities.

Adhesion of *B. xylanisolvens* DSM 23964 to Caco-2 cells. Cultivation and differentiation induction of Caco-2 cells were performed as previously described (22, 32). The results, as presented in Fig. 2A, demonstrate that the expression level of GAPDH was constant during differentiation, whereas the level of sucrase isomaltase increased during differentiation. Microscopic observation confirmed that the Caco-2 cells were well differentiated as a monolayer after 14 days. The binding of bacteria to differentiated Caco-2 cells after 3 h of incubation under anaerobic conditions was analyzed by means of a species-specific PCR performed on supernatants from successive washing steps and finally on the scraped Caco-2 cells. As depicted in Fig. 2B and C, *B. xylanisolvens*

FIG 2 Molecular analysis of the binding of strain DSM 23964 to Caco-2 cells. (A) GAPDH and sucrose isomaltase. Lanes: 1, 100-bp DNA ladder (Bioline); 2, first day; 3, third day; 4, sixth day; 5, eighth day; 6, 10th day; 7, 13th day; 8, 14th day; 9, negative control. (B) Detection of strains *B. xylanisolvans* DSM 23964 and *B. fragilis* DSM 1396. Lanes: 1, 100-bp DNA ladder; 2, *B. xylanisolvans* DSM 23964 after first wash step; 3, *B. xylanisolvans* DSM 23964 after sixth wash step; 4, *B. xylanisolvans* DSM 23964 + Caco-2 cells; 5, *B. fragilis* DSM 1396 after first wash step; 6, *B. fragilis* DSM 1396 after sixth wash step; 7, *B. fragilis* DSM 1396 + Caco-2 cells; 8, Caco-2 cells; 9, strain DSM 23964; 10, *B. fragilis* DSM 1396; 11, negative control. (C) Detection of *L. acidophilus*. Lanes: 1, *L. acidophilus* DSM 9126 after first wash step; 2, *L. acidophilus* DSM 9126 after sixth wash step; 3, *L. acidophilus* DSM 9126 + Caco-2 cells; 4, Caco-2 cells; 5, *L. acidophilus* DSM 9126; 6, negative control; 7, 100-bp DNA ladder.

DSM 23964 cells could of course be detected in the supernatant of the first washing step but not in later supernatants or on scraped Caco-2 cells, indicating that the *B. xylanisolvans* DSM 23964 cells did not attach to epithelial cells of the human colon.

DISCUSSION

A prerequisite for a bacterial strain to be considered a probiotic strain is an unambiguous safety status. As a consequence, *Lactobacillus* and *Bifidobacterium*, which are generally recognized as safe for human consumption, have so far attracted the most attention from probiotic research and industry. Other genera, like the genus *Bacteroides*, which is the second most frequently represented genus in the human intestine (exceeding *Lactobacillus* and *Bifidobacterium* by up to four orders of magnitude), may embody great potential for the isolation and development of new probiotic strains. Thus, two recent studies presented specific *Bacteroides* strains isolated from human feces for increasing the production of mucosal IgA (54) and removing cholesterol from enterohepatic cycling (13). However, the unknown or even ambiguous safety status of some *Bacteroides* species may have prevented the probiotic community from investigating their potential.

We have isolated from human feces a new *Bacteroides xylanisolvans* strain with potential probiotic properties (data not shown). Since the species *Bacteroides xylanisolvans* is not classified

as an opportunistic pathogen, the new strain could be reasonably expected to present a favorable safety profile. Furthermore, as this species displays a high xylanolytic activity, it may offer the advantage of specifically potentiating the assumed health-promoting effect of the strain through the administration of xylan (5, 17).

Following recommendations for the initial safety assessment of a new strain without a history of use in the food industry (10, 52), we have run an extensive taxonomical examination of this strain and investigated its virulence potential in detail.

The initial biochemical characterization of this new strain advocated an affiliation to the genus *Bacteroides*, especially to the species *B. xylanisolvans*. This was confirmed by the analysis of its 16S rRNA sequence, which revealed 100% similarity with *B. xylanisolvans* DSM 18836. Subsequent DNA-DNA hybridization, the “gold standard” for the definitive assignment of a strain to the correct taxonomic unit, definitively established its identity. Through RAPD genotyping, strain DSM 23964 was further characterized down to the strain level and identified as a new strain of the species *B. xylanisolvans*.

B. fragilis is the most notable opportunistic pathogen species of the genus *Bacteroides* (51). Its most prominent virulence factors are thought to be the *bft* enterotoxin and the capsular polysaccharide PS A (8, 15). This species contains three distinct capsular polysaccharides, PS A, B, and C (50). PS A, to which have been attributed positive immunomodulatory properties (9, 36), seems to be an essential factor responsible for the development of abscesses in infections. Indeed, strains lacking PS B or PS C are not attenuated in their ability to induce abscesses (8). Furthermore, although purified PS B may induce some abscess formation, the dose required was much higher than for PS A (47).

We have demonstrated that the strain *B. xylanisolvans* DSM 23964 is free from genes involved in the biosynthesis of both *bft* and PS A. We have also shown that this strain does not possess measurable exoenzyme activities like collagenase, neuraminidase, DNase, hyaluronidase, chondroitin sulfatase, or hemolytic activity, which are known to be involved in the development of the infection process through their role in the degradation of the extracellular matrix (33, 37, 43). A further important prerequisite for a microorganism to exert pathogenic effects is its binding to the cells of the host (41). Using the Caco-2 model, we have demonstrated that strain *B. xylanisolvans* DSM 23964 does not stably bind to human intestinal epithelial cells. Notably, the adhesion of probiotic to the gut epithelium is considered an important property for some probiotic effects (26). However, colonization of probiotic may not be requested for attaining probiotic effects, as long as regular bacteria intake occurs (36). Therefore, the absence of adhesion to the gut would not alter the probiotic potential of our strain *B. xylanisolvans* DSM 23964.

The danger of developing or increasing resistance to antibiotics is another important safety aspect for a probiotic strain. Interestingly, although clindamycin resistance occurs in about 30% of isolates of the *B. fragilis* group (30), the *B. xylanisolvans* DSM 23964 strain did not present resistance toward this agent. Furthermore, this strain was also sensitive to metronidazole and meropenem, confirming that it may be easily and effectively eliminated if necessary. Since *Bacteroides* species are almost invariably resistant to penicillin and β -lactam antibiotics (44), it was not surprising to find that *B. xylanisolvans* DSM 23964, too, was resistant to penicillin and ampicillin. However, as already described for other *Bacteroides* species (23), full susceptibility to β -lactam agents

could be restored by the addition of β -lactamase inhibitors like sulbactam and tazobactam. In addition, we have demonstrated that the β -lactamase activity of the strain *B. xylanisolvens* DSM 23964 was encoded only by the gene *cepA*. This is important because of the three β -lactamase genes described in *Bacteroides* species, only *cfxA* was reported to be horizontally transmitted as a consequence of its association with the conjugative transposon Tn4555, a genetic element that drives its own transfer (11). Since *cfxA* is absent from strain DSM 23964, and since the *cepA* gene was identified in the genomic DNA of this strain, the possibility of a transfer of β -lactamase activity to other bacteria can be dismissed. Furthermore, the most common mechanism involved in gene transfer between bacteria is the horizontal gene transfer of plasmid materials. The fact that we did not detect any plasmid material in the strain *B. xylanisolvens* DSM 23964 is a further criterion to support its general safety.

Taken together, our data indicate that the *B. xylanisolvens* strain DSM 23964 is free of any virulence factors that may preclude it from being considered for further safety and health-promoting evaluation, and this work may eventually lead to the development of the first natural probiotic strain of the genus *Bacteroides*.

Perspective view. With over 100 trillion bacteria, the human intestinal microbiota contains an extraordinary and almost unexplored richness of potential probiotic entities and functions. Indeed, until today only a tiny fraction has been considered and evaluated for possible probiotic properties. Developing the first probiotic *Bacteroides* strain may lead to the identification of novel probiotic properties and to an expansion of application fields of probiotics and thereby push the frontiers of probiotic research beyond traditional lines.

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