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Antibiotic Resistances of Starter and Probiotic Strains of Lactic Acid Bacteria[▽]

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The antibiotic resistances of 45 lactic acid bacteria strains belonging to the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, and *Leuconostoc* were investigated. The objective was to determine antibiotic resistances and to verify these at the genetic level, as is currently suggested by the European "qualified presumption of safety" safety evaluation system for industrial starter strains. In addition, we sought to pinpoint possible problems in resistance determinations. Primers were used to PCR amplify genes involved in β-lactam antibiotic, chloramphenicol, tetracycline, and erythromycin resistance. The presence of ribosomal protection protein genes and the *ermB* gene was also determined by using a gene probe. Generally, the incidences of erythromycin, chloramphenicol, tetracycline, or β-lactam resistances in this study were low (<7%). In contrast, aminoglycoside (gentamicin and streptomycin) and ciprofloxacin resistances were higher than 70%, indicating that these may constitute intrinsic resistances. The genetic basis for ciprofloxacin resistance could not be verified, since no mutations typical of quinolone resistances were detected in the quinolone determining regions of the *parC* and *gyrA* genes. Some starter strains showed low-level ampicillin, penicillin, chloramphenicol, and tetracycline resistances, but no known resistance genes could be detected. Although some strains possessed the *cat* gene, none of these were phenotypically resistant to chloramphenicol. Using reverse transcription-PCR, these *cat* genes were shown to be silent under both inducing and noninducing conditions. Only *Lactobacillus salivarius* BFE 7441 possessed an *ermB* gene, which was encoded on the chromosome and which could not be transferred in filter-mating experiments. This study clearly demonstrates problems encountered with resistance testing, in that the breakpoint values are often inadequately identified, resistance genes may be present but silent, and the genetic basis and associated resistance mechanisms toward some antibiotics are still unknown.

Lactic acid bacteria (LAB) have a long and safe history of use in the production and consumption of fermented foods and beverages (5, 29, 50). LAB are consumed in enormous quantities, primarily in fermented foods. According to the International Dairy Federation, the average annual consumption of fermented milk products is 22 kg per capita in Europe (30). In total, this amounts to about 8.5 billion kg of fermented milk per year. However, this figure does not take into account the LAB used in other food fermentations (e.g., vegetable and meat) or probiotic strains, and so the actual amount can thus be expected to be far greater.

Bacteria used as starter cultures for the production of foods could possibly contain antibiotic resistance genes (8, 46). In recent years, studies on the selection for and dissemination of antibiotic resistances have focused mainly on clinically relevant bacterial species. More recently, it was speculated that food bacteria may act as reservoirs of antibiotic resistance genes (13, 26). Fermented foods, therefore, may be important vehicles of enormous amounts of living bacteria, with biotechnical use as starter cultures, into the human body. These may carry transferable antibiotic resistances, which might be transferred to commensal or pathogenic bacteria. Recently, the European Food Safety Authority (EFSA) has taken responsibility to launch the European initiative toward a "qualified presumption of safety" (QPS) concept which, similar to the GRAS system in the United States, is aimed to allow strains with an established history and safety status to enter the market without extensive testing requirements (11). The presence of transmissible antibiotic resistance markers in the evaluation of strains is thus an important safety criterion.

LAB often harbor plasmids of different sizes, and some antibiotic resistance determinants located on plasmids have been reported to occur in *Lactococcus lactis* and various *Lactobacillus* and *Enterococcus* species (14). Among the LAB, antibiotic resistance of the enterococci has been subject to intense study (19, 27, 28), particularly because strains of these bacteria cause numerous and serious infections in humans (32, 34). In contrast, fewer physiological and molecular data are available on the antibiotic resistances of lactobacilli present in fermented foods. Determination of antibiotic resistances among LAB is confounded by problems regarding the use of media and MIC breakpoints for the genera or species. Generally, the choice of medium has been shown to have a profound impact on the MICs of LAB (13, 20). Furthermore, MIC breakpoint values have been shown to be species specific and thus vary between species of the same genera (8). The objective of the present study was not only to determine the spectrum and incidences of antibiotic resistance of LAB starter

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strains but also to verify these resistances with the underlying genetic mechanism. Furthermore, we sought to elucidate mechanisms of LAB resistance to antibiotics such as ciprofloxacin, which thus far have not been intensively investigated. Therefore, the antibiotic resistances of 40 commercial LAB starter strains and 5 probiotic strains were determined by using the E-test, and we attempted to identify the mechanisms for the antibiotic resistance by using PCR amplification of and/or gene probe hybridization with antibiotic resistance genes. The observed physiological resistances were correlated with the genetic data and the reported MIC breakpoint values to pinpoint potential problems in safety evaluations as suggested by the European QPS system.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 45 LAB strains belonging to the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, and *Streptococcus*, including 40 industrial starter cultures used for the manufacture of various fermented foods, and 5 strains that were either being developed as probiotic strains (3 strains) or were from a commercial probiotic product (2 strains), were investigated (Table 1). A total of 43 strains were obtained from Danisco, while two strains were isolated from a probiotic product from Symbio-Pharm. The investigations were done in full cooperation and agreement with the producers of the strains. Strains were routinely grown aerobically at 30°C or, in the case of *S. thermophilus* strains, at 40°C in MRS broth pH 6.0 (Merck, Darmstadt, Germany).

Antibiotic susceptibility testing and MIC determination. E-test strips (Viva Diagnostika, Cologne, Germany) for the determination of either ampicillin, penicillin G, erythromycin, chloramphenicol, gentamicin, streptomycin, tetracycline, or ciprofloxacin resistance were used according to the manufacturer's instructions. Briefly, a bacterial suspension was made by picking colonies from MRS or M17 (Difco, Heidelberg, Germany) agar plates using a sterile loop and then adding these to quarter-strength Ringer's solution (Merck, Darmstadt, Germany) to reach a density corresponding to a McFarland value of 0.5. In a pilot study, we found that in order to ensure the good growth of streptococci, it was necessary to swab the strains onto MRS and M17-agar plates with an inoculum density of McFarland 1 to 2. Using a sterile swab, the suspension was swabbed in three directions onto 4-mm-thick agar plates. Care was taken to use only agar plates with a layer thickness of 4 ± 0.5 mm in order to standardize the diffusion of the antibiotic. The plates were incubated in an anaerobic chamber (95% CO_2, 5% N_2; Don Whitley Scientific, Shipley, United Kingdom) at either 37°C (streptococci) or 30°C (all other LAB strains) for 48 h before reading.

DNA preparation and manipulations. Total genomic DNA from each isolate was extracted and purified by using the method of Pitcher et al. (37) as modified for gram-positive bacteria by Björkroth and Korkeala (3). Small-scale isolation of plasmid-DNA was done as described by van Belkum and Stiles (48). Large-scale plasmid isolation was done by equilibrium centrifugation using the cesium chloride-ethidium bromide gradient centrifugation method as described by Sambrook et al. (38). Agarose gel electrophoresis and Southern blotting were carried out by standard procedures (38). Labeling of DNA probes using a DIG dUTP DNA labeling and detection kit (catalog no. 1093657; Roche, Mannheim, Germany) was performed according to the manufacturer's instructions.

PCR detection of resistance genes. PCR amplification of genes associated with resistance to chloramphenicol (*cat*, the chloramphenicol acetyltransferase gene), β-lactam antibiotics (*bla*, the β-lactamase gene), macrolides (the *ermA*, *ermB*, *ermC*, *msrA*/*B*, *ereA*, *ereB*, *mphA*, and *mefA*/*E* genes), and tetracycline [the ribosomal protection proteins *tet*(M), *tet*(O), *tet*(S), and *tet*(W) or the efflux proteins *tet*(K) and *tet*(L)] was done in 50-μl volumes that contained 30 pmol of each specific primer, 1× *Taq* DNA polymerase buffer (Amersham Biosciences, Freiburg, Germany), each deoxynucleoside triphosphate at a concentration of 200 μM, 1 U of *Taq* DNA polymerase (Amersham Biosciences, Freiburg), and 100 ng of genomic DNA used as a template. The oligonucleotide primers used included those reported previously for *ermA*, *ermC*, *msrA*/*B*, *ereA*, *ereB*, *mphA*, and *mefA*/*E* (43), *ermB* (15), the *tet*(M), *tet*(O), *tet*(S), and *tet*(W) ribosomal protection proteins, and the *tet*(K) and *tet*(L) tetracycline efflux proteins (1, 19), and PCR was performed as described before (1, 15, 19). In addition, custom-designed primers for the *cat* gene (Catfw1 [forward], 5′-TTA GGT TAT TGG GAT AAG TTA-3′, and Catrev [reverse], 5′-GCA TGR TAA CCA TCA CAW AC-3′), and the β-lactamase gene (*bla*) (Bla-forward, 5′-CAT ART TCC GAT AAT ASM GCC-3′; Bla-reverse, 5′-CGT STT TAA CTA AGT ATS GY-3′) were used, which amplified PCR products of 300 and 297 bp, respectively.

PCR amplification was done as described previously (1, 15, 31, 43), or for 35 cycles at annealing temperatures of 48°C (*cat*) or 51°C (*bla*) for 1 min, and extension was done at 72°C for 45 s. A final polymerization step of 5 min at 72°C ended the PCR protocol. The PCR products were subjected to electrophoresis on 1.8% agarose gels, and the products were visualized by staining with ethidium bromide.

PCR amplification and DNA sequencing of parts of antibiotic resistance-associated genes. To investigate whether observed fluoroquinolone resistances were due to mutations in the quinolone resistance-determining regions (QRDR) of the *gyrA* and *parC* genes, the QRDR encoding regions were PCR amplified. The custom-designed primers for the *gyrA* gene were GyrAfw (5′-CAM CGK CGK ATT CTT TAC GGA ATG-3′) and GyrArev (5′-TTR TTG ATA TCR CGB AGC ATT TC-3′), and the primers for the *par*C gene were ParCfw (5′-TAT TCY AAA TAY ATC ATT CAR GA-3′) and ParCrev (5′-GCY TCN

TABLE 1. Strains and plasmids used in this study

Strain category and species (no. of strains) or plasmid	Strain no., antibiotic resistance phenotype, or antibiotic resistance gene (source or reference)
Starter strains[a] and their use for production of:	
Cheese	
Lactococcus lactis subsp. *lactis* (11)	BFE 7400 to BFE 7410
Lactococcus lactis subsp. *diacetylis* (5)	BFE 7411 to BFE 7415
Leuconostoc mesenteroides	BFE 7416
Leuconostoc pseudomesenteroides	BFE 7417
Yogurt	
Streptococcus thermophilus (11)	BFE 7418 to BFE 7428
Lactobacillus acidophilus (1)	BFE 7429
Lactobacillus delbrueckii subsp. *bulgaricus* (2)	BFE 7430, BFE 7431
Lactobacillus helveticus (1)	BFE 7432
Sausage	
Lactobacillus plantarum (2)	BFE 7433, BFE 7440
Pediococcus acidilactici (1)	BFE 7434
Lactobacillus curvatus (1)	BFE 7435
Pediococcus pentosaceus (1)	BFE 7436
Lactobacillus pentosus (1)	BFE 7437
Lactococcus lactis subsp. *lactis* (1)	BFE 7439
Probiotic strains	
Lactobacillus farciminis (1)	BFE 7438
Lactobacillus salivarius (1)	BFE 7441
Lactobacillus rhamnosus (1)	BFE 7442
Lactobacillus acidophilus (1)	BFE 7444
Lactobacillus casei (1)	BFE 7445
Control strains for antibiotic resistance study	
E. faecium FAIR-E 25[b]	Pen^r Cm^r Tet^r Ery^r Ci^r (19)[c]
E. faecalis FAIR-E 85	Pen^r Cm^r Tet^r Ery^r Ci^r Sm^r Gm^r
E. faecalis FAIR-E 260	Pen^r Cm^r Tet^r Ery^r Ci^r Gm^r
E. faecalis FAIR-E 265	Cm^r Tet^r Ery^r Ci^r Sm^r Gm^r
L. lactis MG1363	*lmrA*$^+$
Plasmids	
pMG36e	*ermB* (48)
pUC19	*bla* (New England Biolabs)

[a] Starters were obtained from a major commercial starter strain producer and deposited in our BFE collection.

[b] The enterococci were derived from the EU study Enterococci in Food Fermentations (FAIR-CT97-3078). The FAIR-E culture collection is administered by the BCCM/LMG (Bacteria Collection of the Laboratory of Microbiology, University of Ghent).

[c] Pen^r, penicillin resistant; Cm^r, chloramphenicol resistant; Tet^r, tetracycline resistant; Ery^r, erythromycin resistant; Ci^r, ciprofloxacin resistant; Sm^r, streptomycin resistant; Gm^r, gentamicin resistant.

TABLE 2. Antibiotic resistances of lactic acid bacteria starter strains used for the manufacture of yogurt and cheese[a]

Substrate and starter strain	EUC antibiotic resistance (MIC [μg/ml])				(Multiple) resistances		
	Am, PG, Em, Cl, Te	Ci (DW = >32)	Gm (DW = >128)	Sm (DW = >256)	EUC	DW	Genotype
Yogurt							
S. thermophilus BFE 7418	≤1	0.75	3	3			
S. thermophilus BFE 7419	≤1	>32	16	8	Ci, Gm	Ci	
S. thermophilus BFE 7420	≤1	>32	32	>256	Ci, Gm, Sm	Ci, Sm	*cat*
S. thermophilus BFE 7421	≤1	0.5	3	128	Sm		*cat*
S. thermophilus BFE 7422	≤1	>32	6	16	Ci, Sm	Ci	*cat*
S. thermophilus BFE 7423	≤1	>32	32	48	Ci, Gm, Sm	Ci, Sm	*cat*
S. thermophilus BFE 7424	≤1	>32	12	4	Ci, Gm	Ci	*cat*
S. thermophilus BFE 7425	≤1	0.5	4	6			*cat*
S. thermophilus BFE 7426	≤1	>32	48	64	Ci, Gm, Sm	Ci	*cat*
S. thermophilus BFE 7427	≤1	>32	12	6	Ci, Gm	Ci	*cat*
S. thermophilus BFE 7428	≤1	1	8	48	Gm, Sm		*cat*
L. acidophilus BFE 7429	≤1.5	>32	48	12	Ci, Gm	Ci	*cat*
L. delbrueckii subsp. *bulgaricus* BFE 7430	≤1.5	>32	24	>256	Ci, Gm, Sm	Ci, Sm	*cat*
L. delbrueckii subsp. *bulgaricus* BFE 7431	≤1.5	>32	48	128	Ci, Gm, Sm	Ci	*cat*
L. helveticus BFE 7432	≤1.5	>32	16	3	Ci, Gm	Ci	
Cheese							
L. lactis subsp. *lactis* BFE 7400	≤1.5	3	4	2			
L. lactis subsp. *lactis* BFE 7401	≤1.5	3	6	32	Sm		
L. lactis subsp. *lactis* BFE 7402	≤1.5	4	16	48	Ci, Gm, Sm		
L. lactis subsp. *lactis* BFE 7403	≤1.5	>32	6	24	Ci, Sm	Ci	
L. lactis subsp. *lactis* BFE 7404	≤1.5	8	12	>256	Ci, Gm, Sm	Sm	
L. lactis subsp. *lactis* BFE 7405	≤1.5	6	12	>256	Ci, Gm, Sm	Sm	
L. lactis subsp. *lactis* BFE 7406	≤1.5	>32	8	48	Ci, Gm, Sm	Ci	
L. lactis subsp. *lactis* BFE 7407	≤1.5	>32	24	>256	Ci, Gm, Sm	Ci, Sm	
L. lactis subsp. *lactis* BFE 7408	≤1.5	8	24	6	Ci, Gm		
L. lactis subsp. *lactis* BFE 7409	≤1.5	>32	6	32	Ci, Sm	Ci	
L. lactis subsp. *lactis* BFE 7410	≤1.5	>32	1	12	Ci	Ci	
L. lactis subsp. *diacetylactis* BFE 7411	≤1.5	1.5	12	16	Gm, Sm		
L. lactis subsp. *diacetylactis* BFE 7412	≤1.5	0.5	6	12			
L. lactis subsp. *diacetylactis* BFE 7413	≤1.5	3	6	12			
L. lactis subsp. *diacetylactis* BFE 7414	≤1.5	3	12	>256	Gm, Sm	Sm	
L. lactis subsp. *diacetylactis* BFE 7415	≤1 (Cl = 4)	6	6	16	Ci, Sm		
L. mesenteroides BFE 7416	≤1.5	>32	1	8	Ci, Sm	Ci	*cat*
L. pseudomesenteroides BFE 7417	≤1.5	16	8	192	Ci, Gm, Sm		*cat*

[a] The EU Commission (EUC) breakpoint values as suggested by SCAN (10) and FEEDAP (41) or the values Danielsen and Wind (8) (DW) are given. All strains in Table 2 were not resistant to ampicillin (Am), penicillin G (PG), tetracycline (Te), erythromycin (Em), and chloramphenicol (Cl) based on either the SCAN/FEEDAP or the DW MIC breakpoints; the breakpoints for these antibiotics, together with the breakpoints for ciprofloxacin (Ci), streptomycin (Sm), and gentamicin (Gm), are given in Table 3.

GTA TAA CGC ATM GCC G-3′). The amplification conditions for both the *gyrA* and the *parC* genes consisted of 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 30 s.

Part of the *cat* gene was amplified by using the primers Catfw2 (5′-AGA MAA TTG GRA GAG AAA AGA G-3′) and Catrev (see above). This 568-bp gene fragment was amplified in 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 40 s. A partial 405-bp *ermB* fragment was amplified as described by Gevers et al. (15) and was also sequenced. The resulting PCR products were sequenced bidirectionally at GATC Biotech (Konstanz, Germany), and the deduced amino acid sequences were aligned with those retrieved from the GenBank database by using the DNAStar CLUSTAL W multiple alignment tool. The DNA sequences obtained for the partial *cat* genes from *L. acidophilus* BFE 7429, *L. delbrueckii* subsp. *bulgaricus* BFE 7430, and *S. thermophilus* BFE 7420 were submitted to GenBank and received the accession numbers EF070730, EF070729, and EF070728, respectively. The DNA sequence of the partial *ermB* gene sequence from *L. salivarius* BFE 7441 was also submitted to GenBank and received the accession number EF070727.

Southern hybridization. Large-scale plasmid DNA isolation of the erythromycin-resistant *L. salivarius* BFE 7441 strain was done as described above. Restriction enzyme analysis of plasmid DNA was performed using the restriction enzymes EcoRI, XbaI, PstI, KpnI, BamHI, AvaII, MluI, NotI, SphI, and XmaI (New England Biolabs, Frankfurt am Main, Germany) in separate reactions according to the manufacturer's recommendations. Samples were run on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. DNA was transferred onto a nylon membrane (Hybond N+; Amersham Pharmacia) according to standard methods (38) and then hybridized with an *ermB*-specific probe labeled with digoxigenin (Boehringer, Mannheim, Germany). The probe was obtained by PCR of the *ermB* gene using the oligonucleotide primers and amplification conditions described by Gevers et al. (15) and the DIG dUTP labeling kit for PCR (Roche Diagnostics).

Isolation of total RNA and RNA expression studies. Total RNA was isolated from LAB strains that possess *cat* genes but which were not resistant to chloramphenicol, as well as from positive control strains that were chloramphenicol resistant (Table 1). Total RNA was isolated by using an RNeasy minikit (QIAGEN, Hilden, Germany), with the use of RNA protect solution (QIAGEN) and a DNase (catalog no. 79254; QIAGEN) digest according to the manufacturer's instructions. For RNA isolation, cells were grown until the mid-logarithmic growth phase, and the cell numbers were adjusted to 10^9 CFU/ml, as suggested by the manufacturer. The RNA quality was visually assessed by using denaturing gel electrophoresis, and the RNA quantity was measured spectrophotometrically at 260 nm as described in Sambrook et al. (38). The total RNA was adjusted to 400 ng/μl using diethyl pyrocarbonate-treated water (Ambicon, Huntingdon, United Kingdom), and 600 ng of total RNA was used for reverse transcriptase-PCR (RT-PCR). For RT-PCR, the ready-to-go RT-PCR beads of Amersham Biosciences (Freiburg, Germany) were used. The RT-PCR contained reagents as described above for amplification of antibiotic resistance genes and DNA was amplified after RT of the mRNA at 42°C for 30 min. The *cat* gene was amplified by using primers Catfw1 and Catrev as described above. Housekeeping genes

that were amplified included part of the L-lactate dehydrogenase (*ldhL*) gene, as well as the *gyrA* or the *parC* genes, as described above. The *ldhL* gene fragment was amplified by using the primer LLDHfw (5′-GTT GCY AAC CCA GTT GAT ATC-3′) and the primer LLDHrev (5′-GTA CCA ATG TAA ATG TCG TTC).

Filter-mating experiments. The transferability of the erythromycin resistance of the strain *L. salivarius* BFE 7441 was examined by filter mating. *E. faecalis* JH2-2 (resistant to rifampin at 16 μg/ml), *L. lactis* LMG 19460 (resistant to rifampin at 16 μg/ml), and *E. faecalis* OG1X (resistant to streptomycin at 128 μg/ml) were used as recipients in mating experiments as described previously (19). Transconjugants were spread plated on MRS agar plates containing 128 μg of erythromycin/ml and 32 μg of rifampin/ml. The plates were incubated for 24 to 48 h at 37°C.

RESULTS

Antibiotic resistance phenotypes. The incidence of resistance of the starter and probiotic strains to some antibiotics varied considerably depending on the breakpoints used for determining the MICs (Tables 2 and 3). Generally, more strains were resistant to the antibiotics gentamicin, streptomycin, and ciprofloxacin when the MIC breakpoints suggested by the European Scientific Committee and Panel are used, i.e., SCAN (10) and FEEDAP (41), compared to the number of resistant strains based on the breakpoints proposed by Danielsen and Wind (8), reflecting the fact that the MIC breakpoints indicated by SCAN and FEEDAP are lower for these antibiotics. Interestingly, the incidences of resistance to these three antibiotics was generally very high (>71.1%) as indicated by the SCAN and FEEDAP (10, 41) breakpoint criteria and still noticeably high even by the Danielsen and Wind (8) criteria (Table 4). In contrast, the incidence of resistance to the antibiotics ampicillin, penicillin G, erythromycin, tetracycline, and chloramphenicol was generally low (between 0 and 6.7% of strains; Table 4) using the MIC breakpoint values as proposed by SCAN/FEEDAP (10, 41) or Danielsen and Wind (8). Resistance to Penicillin G, erythromycin, ampicillin, and tetracycline generally did not occur among *S. thermophilus* or *Lactobacillus* strains used as starters in yogurt fermentation or among *L. lactis* or the two *Leuconostoc* strains used as starters for cheese production (Table 2). In contrast, the same strains generally possessed ciprofloxacin, gentamicin, and streptomycin resistances according to the SCAN/FEEDAP (10, 41) breakpoints. When the Danielsen and Wind (8) breakpoints were applied to these latter antibiotics, however, they were found to be mostly ciprofloxacin resistant, whereas some strains were also streptomycin resistant but generally sensitive to gentamicin (Table 2). A different situation was encountered for the starter strains (mostly lactobacilli and two *Pediococcus* strains) used for meat fermentations, as well as for the probiotic strains. All of the ampicillin-, chloramphenicol-, penicillin G-, tetracycline-, and erythromycin-resistant strains, although few in number, occurred among the meat starter and probiotic strains (Table 3). In addition, like the yogurt and cheese starters, numerous strains used as meat starters possessed ciprofloxacin, gentamicin, and streptomycin resistances. In this case, however, resistances to ciprofloxacin, gentamicin, and streptomycin occurred according to both the breakpoint values proposed by SCAN/FEEDAP (10, 41) and the criteria of Danielsen and Wind (8). This indicated that these resistances among the lactobacilli were generally higher than those observed for the most *S. thermophilus* and *L. lactis*

TABLE 3. Antibiotic resistances of lactic acid bacteria starter strains used for the manufacture of fermented sausages and of probiotic strains

Product and starter strain	EUC antibiotic resistance (MIC [μg/ml])[a]							(Multiple) resistances		
	Am (DW = 4)[b]	PG (DW = 4)	Em (DW = 1, 2, or 4)	Cl (DW = 16)	Te (DW 4, 8, or 64)	Ci (DW = >32)	Gm (DW = >128)	Sm (DW = >256)	EUC	DW	Genotype
Sausage											
L. plantarum BFE 7433	0.38	32	1	2	4	>32	>256	>256	Ci, Gm, Sm, PG	PG, Ci, Gm, Sm	
P. acidilactici BFE 7434	2	0.25	1	2	16	>32	128	>256	Te, Ci, Gm, Sm	Te, Ci, Gm, Sm	
L. curvatus BFE 7435	0.38	0.19	1	1	1	>32	>256	>256	Ci, Gm, Sm	Ci, Gm, Sm, Em	*cat*
P. pentosaceus BFE 7436	4	0.75	1	2	16	>32	>256	>256	Am, Te, Ci, Gm, Sm	Am, Te, Ci, Gm, Sm	
L. pentosus BFE 7437	2	1	1.5	4	12	>32	>256	>256	Ci, Gm, Sm, Te	Ci, Gm, Sm	
Lactococcus lactis subsp. *lactis* BFE 7439	0.38	0.25	0.38	1	0.38	>32	24	>256	Ci, Gm, Sm	Ci, Sm	
L. plantarum BFE 7440	0.38	8	1.5	12	16	>32	>256	>256	PG, Ci, Ci, Gm, Sm	PG, Te, Ci, Gm, Sm	
Probiotic strains											
L. farciminis BFE 7438	0.38	0.25	0.38	1.5	1.5	>32	128	>256	Ci, Gm, Sm	Ci, Gm, Sm	
L. salivarius BFE 7441	0.38	0.19	>256	0.5	1.5	>32	64	>256	Em, Ci, Gm, Sm	Em, Ci, Sm	*emB*
L. rhamnosus BFE 7442	0.25	0.19	0.38	2	0.38	6	96	64	Ci, Gm, Sm	-	
L. acidophilus BFE 7444	1	0.19	1.5	2	0.38	16	128	>256	Ci, Gm, Sm	Gm, Sm	
L. casei BFE 7445	1	0.25	0.5	1.5	0.38	>32	>256	>256	Ci, Gm, Sm	Ci, Gm, Sm	

[a] The EU Commission (EUC) breakpoint values as suggested by SCAN (10) and FEEDAP (41) or according to Danielsen and Wind (8) (DW) are given. For ciprofloxacin (Ci) the SCAN breakpoint value of 4 μg/ml was used for all strains except pediococci, for which 16 μg/ml was used. Breakpoints for ciprofloxacin or penicillin G (PG) were not suggested by FEEDAP. The FEEDAP breakpoints (superseding previous SCAN breakpoints) were as follows: ampicillin (Am) and erythromycin (Em), 4 μg/ml for all strains in this study; gentamicin (Gm), 8 μg/ml for obligately homofermentative lactobacilli (HFLB), *L. lactis*, and *S. thermophilus* strains, 4 μg/ml for *Pediococcus* and *Leuconostoc* spp., and 64 μg/ml for *L. plantarum* strains; streptomycin (Sm), 4 μg/ml for *Pediococcus* spp., 8 μg/ml for *Leuconostoc* spp., 16 μg/ml for *L. lactis* and *S. thermophilus* strains and for HFLB, and 64 μg/ml for *L. plantarum* strains; tetracycline (Te), 4 μg/ml for *Pediococcus* and *Leuconostoc* spp., as well as for *L. lactis* and *S. thermophilus* strains, 8 μg/ml for HFLB and 32 μg/ml for *L. plantarum* strains; chloramphenicol (Cl), 4 μg/ml for *Pediococcus* and *Leuconostoc* spp. and for HFLB and 8 μg/ml for *L. lactis*, *S. thermophilus*, and *L. plantarum* strains. Species-specific MIC breakpoints were defined by Danielsen and Wind (8) as follows: for erythromycin, 1 μg/ml for *L. acidophilus*, *L. sakei*, and *L. curvatus*, 2 μg/ml for *L. paracasei* and *L. rhamnosus*, and 4 μg/ml for *L. plantarum* and *L. pentosus*; for tetracycline, 4 μg/ml for *L. paracasei*, *L. acidophilus*, and *L. rhamnosus*, 8 μg/ml for *L. sakei* and *L. curvatus*, and 64 μg/ml for *L. plantarum* and *L. pentosus*.

[b] For pediococci the SCAN (10) MIC breakpoint was stipulated to be 32 μg/ml for streptomycin, 4 μg/ml for gentamicin, and 16 μg/ml for ciprofloxacin.

TABLE 4. Incidence of antibiotic resistance according to MIC breakpoint values of SCAN (10) and Danielsen and Wind (8)

Antibiotic	% Incidence of resistance phenotype according to the MIC breakpoint value from:	
	EU Scientific Commission[a]	Danielsen and Wind (8)
Gentamicin	71.1	20.0
Streptomycin	73.3	40.0
Ciprofloxacin	77.8	60.0
Tetracycline	6.7	6.7
Ampicillin	2.2	4.4
Penicillin G	ND[b]	4.4
Erythromycin	2.2	2.2
Chloramphenicol	4.4	0

[a] SCAN/FEEDAP (10, 41).
[b] ND, not determined since no breakpoint value was suggested for this antibiotic.

strains. However, a relatively low number of species were investigated here, and it would be interesting to determine whether other workers in the field obtain similar data in the future. Even in light of the relatively high MIC breakpoints proposed by Danielsen and Wind (8), some strains, especially the two *Pediococcus* strains, *L. pentosus* BFE 7437, and *L. plantarum* BFE 7440, still showed multiple (four or five different) antibiotic resistances (Table 3).

Detection and characterization of *cat* resistance genes. In an attempt to relate these observed resistances to the presence of a resistance gene, we used PCR amplification or gene probing to detect known resistance genes. From the genomic DNA of both of the two strains showing chloramphenicol resistance (*L. pentosus* BFE 7437 and *L. plantarum* BFE 7440), the *cat* gene could not be amplified. Interestingly, 15 strains (33.3%) possessed the *cat* gene, even though these strains were not chloramphenicol resistant (Tables 2 and 3). Gene fragments were PCR amplified from the genomic DNA of three representative *cat*$^+$ species—*L. delbrueckii* subsp. *bulgaricus* BFE 7430, *L. acidophilus* BFE 7429, and *S. thermophilus* BFE 7420—and sequenced to confirm their identity as part of the *cat* gene. These fragments encoded 171 amino acids, which were identical for *L. delbrueckii* subsp. *bulgaricus* BFE 7430 and *S. thermophilus* BFE 7420. The 171-amino-acid sequence of these latter two strains differed by three amino acids from that of *L. acidophilus* BFE 7429 (results not shown).

The amino acid sequence of the *L. acidophilus* BFE 7429 *cat* gene fragment showed 100% identity to the corresponding region from amino acids 23 to 193 of the 215-amino-acid *cat* gene from plasmid pIP501 of *S. agalactiae* (47) or plasmid pRE25 of *E. faecalis* RE25 (40). The corresponding sequences of *L. delbrueckii* subsp. *bulgaricus* BFE 7430 and *S. thermophilus* BFE 7420 showed 100% identity to the region from amino acids 23 to 193 of the 215-amino-acid *cat* gene from plasmid pC221 of *S. aureus* (42) or plasmid pTZ12 of *B. subtilis* (2).

In order to determine why the strains in the present study did not show a resistance phenotype, the expression of the *cat* gene was studied at the mRNA level for three representative starter strains (*P. acidilactici* BFE 7434, *L. acidophilus* BFE 7429, and *S. thermophilus* BFE 7420), in addition to an *E. faecium* FAIR-E 151 positive control that showed a resistance phenotype in previous studies (19). *P. pentosaceus* BFE 7436, which did not contain a *cat* gene, was used as a negative control. Our RT-PCR results with specific primers for the *cat* gene showed that none of these starter strains investigated expressed the *cat* gene (Fig. 1) under both inducing (culture grown in MRS broth containing 0.015 μg of chloramphenicol/ml at 37°C) and noninducing (culture grown in MRS broth without chloramphenicol at 37°C) conditions. In contrast, the *cat* gene was expressed by the *E. faecium* FAIR-E 151 positive control under such inducing and noninducing conditions. Using our methodology, the expression of two housekeeping genes (either *gyrA* or *ldhL*) could be determined (Fig. 1), indicating the successful RNA isolation and RT of mRNA. Two weak bands of the wrong size were obtained after RT-PCR with the *L. acidophilus* BFE 7429 mRNA and *cat* primers. These bands were sequenced, and a BLAST search indicated homology to a fragment of the thioredoxin reductase gene of *L. acidophilus* NCFM (GenBank accession no. CP000033) but no homology to *cat* genes (results not shown).

Detection of tetracycline and ampicillin genes. The two *Pediococcus* strains, *P. acidilactici* BFE 7434 and *P. pentosaceus* BFE 7436, as well as the *L. pentosus* strain BFE 7437, showed low resistance to tetracycline at 12 to 16 μg/ml. However, when investigated for the presence of resistance genes, neither of the genes encoding ribosomal protection proteins [*tet*(M), *tet*(O), *tet*(S), or *tet*(W)] nor genes encoding the tetracycline efflux pumps [*tet*(K) or *tet*(L)] could be amplified. A probe was developed to search for *tet*(K) and *tet*(L) efflux pump homologue sequences in the total DNA of these strains. Using Southern transfer, followed by hybridization with the digoxigenin-labeled *tet*(K/L) probe, no homologous sequences could be determined in the genomic DNAs of *P. acidilactici* BFE 7434, *P. pentosaceus* BFE 7436, and *L. pentosus* BFE 7437, whereas a positive signal was obtained with a *tet*(K)-positive *E. faecalis* FAIR-E 63 strain (19) (results not shown). In addition, a probe was developed based on an alignment of the *tet*(M), *tet*(O), and *tet*(S) ribosomal protection protein genes of different LAB species. In this case, however, use of the probe led to false-positive hybridization signals in all cases (also with the DNAs of the tetracycline-sensitive, negative control strains *E. faecium* FAIR-E 210 and *E. faecalis* FAIR-E 69), indicating that the probe probably cross-reacted with the sequence of the elongation factor EF-Tu and EF-G genes, which were reported to have considerable sequence homology to ribosomal protection protein genes (7).

As shown in Table 3, only one strain, *P. pentosaceus* BFE 7436 was resistant to ampicillin, and two strains, *L. plantarum* BFE 7433 and BFE 7440, were resistant to penicillin G. In both the ampicillin and the penicillin G resistance cases, the resistance phenotype was low, i.e., equal to or slightly higher than the breakpoint value of the respective antibiotic (Table 3). A β-lactamase gene could not be detected in any of these resistant strains using PCR with specific *bla* gene primers. As a positive control, the *bla* gene could be amplified using the same primers and amplification conditions using plasmid pUC19 (New England Biolabs) DNA as a template, which contains a β-lactamase gene.

Genetic characterization of ciprofloxacin resistance. Ciprofloxacin resistance is known to be associated with mutations in

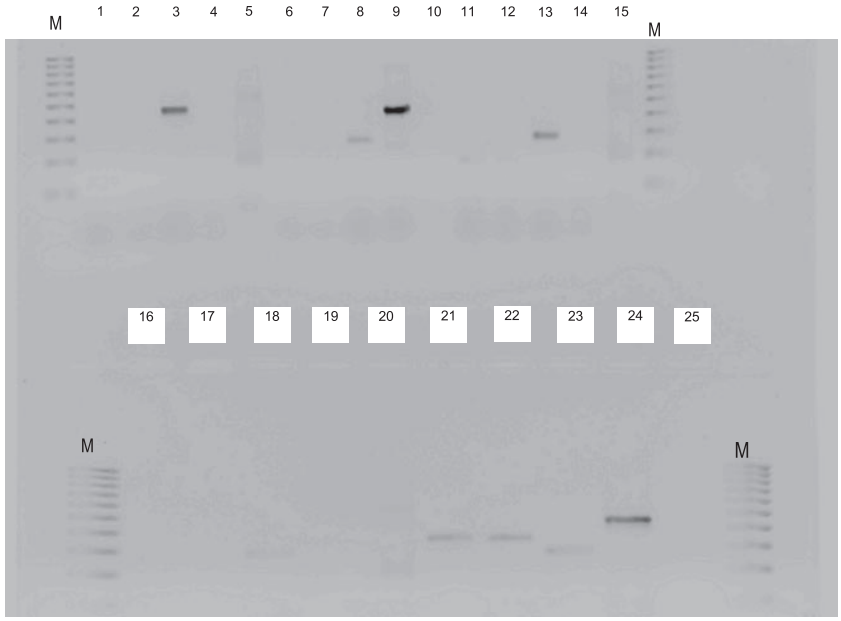

FIG. 1. Results of RT-PCR amplification of chloramphenicol acetyltransferase (*cat*) genes under inducing or noninducing conditions, and selected housekeeping genes. Lanes 1 to 4, PCR of the *P. acidilactici* BFE 7434 (*cat*$^+$, chloramphenicol-sensitive [CL^s]) *cat* gene under noninduced (lane 1) and induced (lane 2) conditions and the L-lactate dehydrogenase (*ldhL*) gene (lane 3) and the *ldhL* gene (negative control, lane 4) after RNA digestion. Lanes 5 and 15, QIAGEN kit RT-PCR positive control. Lanes 6 to 10, PCR of the *P. pentosaceus* BFE 7436 (*cat*$^-$, CL^s) *cat* gene under noninduced (lane 6) and induced (lane 7) conditions and the *gyrA* gene (lane 8), *ldhL* gene (lane 9), and *ldhL* gene (lane 10) after RNA digestion. Lanes 11 to 20, PCR of the *L. acidophilus* BFE 7429 (*cat*$^+$, CL^s) *cat* gene under noninduced (lane 11) and induced (lane 12) conditions, the *gyrA* gene (lane 13) and the *ldhL* gene after RNA digestion (lane 14), the *S. thermophilus* BFE 7420 (*cat*$^+$, CL^s) *cat* gene under noninduced (lane 16) and induced (lane 17) conditions, and the *gyrA* gene (lane 18) and *gyrA* gene (lane 19) after RNA digestion. Lane 20, QIAGEN kit RT-PCR positive control. Lanes 21 to 25, PCR of *E. faecium* FAIR-E 151 (*cat*$^+$, CL^r) *cat* gene under noninduced (lane 21) and induced (lane 22) conditions and the *gyrA* gene (lane 23), the *ldhL* gene (lane 24), and the *ldhL* gene (lane 25) after RNA digestion.

the QRDR of the *gyrA* or the *parC* genes in various gram-positive or gram-negative bacteria, which lead to amino acid substitutions and result in the quinolone resistance phenotype (23, 36). In gram-positive bacteria, different fluoroquinolones have different levels of inhibitory activity against these two enzymes (18), and the findings of several studies suggest that the topoisomerase IV is the primary target of ciprofloxacin in staphylococci, streptococci, and enterococci (4, 17, 39). After PCR amplification and DNA sequencing, the amino acid sequences of the QRDR of selected starter strains with either resistant or sensitive phenotypes were deduced, and these are shown in Table 5. In the case of the ParC subunit of topoisomerase IV, in which the Ser 80 is typically substituted with Leu or Ile, such a substitution could not be observed for any one of the resistant *L. lactis*, *S. thermophilus*, *L. acidophilus*, *P. pentosaceus. L. plantarum*, or *L. curvatus* strains investigated (Table 5). Similarly, a Ser83-to-Arg substitution within the QRDR of the GyrA subunit of DNA gyrase could also not be observed for the resistant strains described above. Moreover, a further possible amino acid substitution in the QRDR of the GyrA subunit associated with quinolone resistance is the Glu87 substitution with either Gly or Lys (36). However, such a substitution also did not occur among the investigated ciprofloxacin-resistant starters in the present study (Table 5). A Glu87-to-Leu substitution was, however, noted for the *L. acidophilus* BFE 7429 strain. Interestingly, for this strain, the *parC* QRDR also showed some amino acid substitutions at positions 74, 84, and 88 (Table 5). However, such substitutions have not yet been reported to be associated with increases in quinolone resistance.

Detection and transfer of *ermB* gene. Only one strain, *L. salivarius* BFE 7441 was resistant (32 μg/ml) to erythromycin (Table 3). This strain was shown to possess the *ermB* gene after PCR amplification with *ermB*-specific primers, but no *ermB* or *ermC* gene could be detected (result not shown). The *ermB* PCR product was sequenced, and the deduced 136-amino-acid sequence showed 100% similarity to amino acids 25 to 160 of the 219-amino-acid *ermB* gene of a group G *Streptococcus* sp. (49) and also 100% similarity to amino acids 45 to 180 of a 237-amino-acid *ermB* gene of *E. faecium* (16). The possibility that the *ermB* gene was located in the plasmid was investigated, and plasmids isolations were attempted on both the small and large scales. Plasmid DNA could not be detected by either of these methods. The possibility of the transfer of the *ermB* gene, for example, by a conjugative transposon, was investigated in filter-mating studies with *L. lactis* LMG 19460, *E. faecalis* JH2-2, and *E. faecalis* OG1X. No transconjugants could be obtained with either of these recipient LAB strains in at least triplicate filter-mating experiments for each strain. In a previous study, this methodology was used successfully to transfer tetracycline resistance genes from a tetracycline-resistant *E. faecalis* strain to the *E. faecalis* OG1X recipient (19).

TABLE 5. CLUSTAL W amino acid alignment of the QRDR of *parC* and *gyrA* of resistant and nonresistant strains published in the literature, as well as lactic acid bacteria strains investigated in this study

Sequence type and strain	Resistance phenotype[a]	Partial ParC or GyrA (QRDR) amino acid sequence[b]	Source or reference
ParC			
E. faecalis ATCC 19433	R	YHPHGDSSIYEAMVRLSQD	36
E. faecalis E52	R	YHPHGD**I**SIYEAMVRLSQD	36
E. faecium ATCC 19434	S	YHPHGDSSIYEAMVRMSQD	36
E. faecium E138	R	YHPHGD**I**SIYEAMVRMSQD	36
S. agalactiae GTC1234	R	FHPHGD**F**SIYDAMVRMSQD	23
L. lactis subsp. *lactis* BFE 7403	R	FHPHGDSSIYEAMIRMSQD	This study
L. lactis subsp. *lactis* BFE 7406	R	FHPHGDSSIYEAMIRMSQD	This study
L. lactis subsp. *lactis* BFE 7409	R	FHPHGDSSIYEAMIRMSQD	This study
L. lactis subsp. *diacetylactis* BFE 7413	S	FHPHGDSSIYEAMIRMSQD	This study
L. lactis subsp. *diacetylactis* BFE 7414	S	FHPHGDSSIYEAMIRMSQD	This study
S. thermophilus BFE 7418	S	FHPHGDSSIYDAMVRMSQD	This study
S. thermophilus BFE 7419	R	FHPHGDSSIYDAMVRMSQD	This study
L. acidophilus BFE 7429	R	**Y**HPHGDSSIYGALVHLSQD	This study
P. pentosaceus BFE 7436	R	FHPHGDSSIYEALVRMSQD	This study
L. plantarum BFE 7433	R	FHPHGDSSIYEAMVRLSQD	This study
L. plantarum BFE 7440	R	FHPHGDSSIYEAMVRLSQD	This study
L. curvatus BFE 7435	R	FHPHGDSSIYEAMVRLSQD	This study
GyrA			
E. faecalis ATCC 19433	S	VMGKYHP HGDSAIYE	36
E. faecalis E52	R	VMGKYHP HGDSAIY**G**	36
E. faecium ATCC 19434	S	VMGKYHP HGDSAIYE	36
E. faecium E138	R	VMGKYHP HGD**R**AIYE	36
E. faecium FE6/NE43	R	VMGKYHP HGDSAIY**G**/**K**	36
L. lactis subsp. *lactis* BFE 7400	R	VMGKYHP HGDSSIYE	This study
L. lactis subsp. *lactis* BFE 7406	R	VMGKYHP HGDSSIYE	This study
L. lactis subsp. *diacetylactis* BFE 7413	S	VMGKYHP HGDSSIYE	This study
L. lactis subsp. *diacetylactis* BFE 7414	S	VMGKYHP HGDSSIYE	This study
S. thermophilus BFE 7418	S	VMGKYHP HGDSSIYE	This study
S. thermophilus BFE 7419	R	VMGKYHP HGDSFRKE	This study
L. curvatus BFE 7435	R	MGKYHP HGDSAIYE	This study
L. plantarum BFE 7433	R	KYHP HGDSAIYE	This study
L. plantarum BFE 7440	R	VMGKYHP HGDSAIYE	This study
L. acidophilus BFE 7429	R	VMGKFHP HGDSSIYL	This study

[a] R, resistant; S, sensitive.
[b] Substituted amino acids that result in a resistant phenotype are indicated in boldface.

DISCUSSION

Knowledge on the antibiotic resistance of LAB is still limited, possibly because of the large numbers of genera and species encountered in this group, as well as variances in their resistance spectra. What is becoming apparent, also from the results of the present study, is that the LAB starter or probiotic strains belonging to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* are generally quite sensitive to clinically relevant antibiotics such as penicillin G, ampicillin, tetracycline, erythromycin, and chloramphenicol. Such resistances were lower than 10% of the isolates studied (6, 8, 21, 22), although Zarazaga et al. (52) reported an unusually high incidence of 26.2% of penicillin-resistant *Lactobacillus* isolates. In contrast, some resistances appear to be intrinsic for lactobacilli. These include resistance to aminoglycosides, quinolones, and glycopeptides (8, 25, 45, 51). In the present study, more than 70% of the isolates were resistant to gentamicin, streptomycin, and ciprofloxacin (Table 4) based on the MIC breakpoint values of SCAN and FEEDAP (10, 41). Similarly, more than 80% of the LAB starter and probiotic strains were previously reported to be aminoglycoside resistant (6, 8), while ciprofloxacin resistance was reported for more than 60% of the LAB strains examined by Zarazaga et al. (52).

The EFSA considers antibiotic resistances, especially transferable resistances, a safety concern and a decision criterion for determining a strain's QPS status (11). Although this step toward a safety evaluation is commendable, we foresee some problems with LAB antibiotic resistance determinations, which can lead to difficulties in safety evaluation. First, there are no approved standards for the phenotypic or genotypic evaluation of antibiotic resistances in food isolates (8, 21). Thus, already the choice of media is problematic, and in a previous study (13) we showed that MIC breakpoints vary considerably depending on the medium and the antibiotic used. Since MRS has been used in most studies and seems to be generally suited for the growth of many LAB and their antibiotic susceptibility determinations, it was used also here. However, MRS agar could not support the growth of streptococci, and thus M17 agar was used. Recently, Klare et al. (24) reported on a "general" broth medium for determining LAB antibiotic susceptibilities. These authors showed that this medium, consisting of Iso-Sensitest (90%) and MRS (10%) broth, optimally supported the growth

of six *Lactobacillus*, two *Pediococcus*, and two *Lactococcus* strains, as well as various *Bifidobacterium* species (24). However, that study did not indicate whether the medium is suitable for the growth of the majority of *Lactobacillus* species (the genus currently consists of >80 species), in addition to *Streptococcus*, *Leuconostoc*, and *Weissella* spp., which were not tested by Klare et al. (24).

A further problem with LAB antibiotic susceptibility determination is the specification of MIC breakpoint values. This is important, since it may affect decisions on whether resistances can be considered to be intrinsic. Again, there are no standards, and the National Committee for Clinical Laboratory Standards (35) does not stipulate MIC breakpoints for LAB, with the exception of *Enterococcus* spp. One problem is the large species variation and the possible resulting variation in MIC values between species and genera (8). Thus, SCAN (10) differentiated between lactobacilli and pediococci with regard to MIC breakpoints for the different antibiotics. To add to the confusion and demonstrating the complexity of the problem, FEEDAP (41) superseded these MIC breakpoints in 2005, now assigning breakpoint values for different LAB groups, species, and strains, i.e., for homofermentative and heterofermentative lactobacilli, enterococci, *Pediococcus* spp., *Leuconostoc* spp., *L. plantarum* strains, and *L. lactis* strains. Danielsen and Wind (8) suggested up to three different breakpoint values based on differences in the resistance of only 14 *Lactobacillus* species. Thus, if the antibiotic resistances of the other (>60) *Lactobacillus* species and of other LAB genera, for which breakpoints were not investigated or specified, are found to differ considerably in future, we may end up with a range of different species- or genus-specific breakpoint values that may increase further the current complexity.

Furthermore, the actual concentration of antibiotic that can be reached in human blood serum was not taken into consideration in LAB resistance determinations. For example, the serum antibiotic concentrations for penicillin, erythromycin, tetracycline, and chloramphenicol that can be reached in humans are 2.5, 2 to 3, 8, and 10 to 15 μg/ml, respectively (51). In this case, determinations of resistances greater than these values would be of academic interest but not of practical relevance.

Resistance to aminoglycoside antibiotics is considered to be intrinsic in LAB (6, 8, 22) and is attributed to the absence of cytochrome-mediated electron transport, which mediates drug uptake (6). In addition, it was shown that when lactobacilli were grown in medium containing bile, they became more sensitive to aminoglycosides, suggesting that membrane impermeability plays an important role in this intrinsic aminoglycoside resistance (9). Our results also showed that the LAB are intrinsically resistant when the SCAN or FEEDAP MIC breakpoints are used (Table 4). However, ≤40% of the strains (Table 4) showed either streptomycin or gentamicin resistance, respectively, when the MIC breakpoints of Danielsen and Wind are used (8). This indicates that these may be set too high. A total of 60 or 77.8% of strains in the present study were resistant to ciprofloxacin according to the MIC breakpoint values of Danielsen and Wind (8) or SCAN (10), respectively (Table 4). This also indicated an intrinsic resistance. However, the basis for this resistance is not clear. For some organisms, e.g., gram-positive cocci, resistance to quinolones has been described as a result of mutation in either *gyrA* of *parC* genes (10, 18, 33, 39). However, no such point mutations in the QRDR of the *gyrA* or *parC* genes could be determined. Although one *L. acidophilus* BFE 7429 strain could be shown to have amino acid substitutions, possibly as a result of point mutations in the QRDR of the *parC* and *gyrA* genes, these substitutions were not the typical ones previously associated with this kind of resistance. Furthermore, only one *L. acidophilus* strain was sequenced in the present study, and therefore it is not clear whether such different amino acid sequences in the QRDR are typical for this species. The present study was the first to investigate whether point mutations in the *gyrA* or *parC* genes may be responsible for fluoroquinolone resistance in LAB other than enterococci and streptococci. However, since this did not appear to be the case, the basis for this resistance could not be established.

In our study, we screened all strains by PCR for known resistance genes and thus were able to determine the presence of *cat* genes in 15 of 46 strains that phenotypically were not resistant to chloramphenicol (Tables 2 and 3). Furthermore, we could show that the *cat* gene was not expressed at the RNA level under both inducing and noninducing conditions (Fig. 1) and that the reason for the chloramphenicol sensitivity was therefore probably not the result of a mutation in the *cat* gene. Thus, speculatively, a mutation in the regulatory region may have resulted in the open reading frame not being expressed. Our study thus warns against the use of only genetic methods, such as PCR amplification or microarray screening, to determine LAB resistances, since this could lead to false assumptions of resistance. However, in many cases such investigations are done on both the phenotypic and the genetic level, which in this case is obviously preferable. Furthermore, the present study is the first to point out that such inactive *cat* genes occur among different LAB genera and species (i.e., the strains of *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *S. thermophilus*, *P. acidilactici*, *L. mesenteroides*, and *L. pseudomesenteroides* used in the present study).

Zarazaga et al. (52) reported a quite high incidence (26.2% of investigated strains) of penicillin-resistant lactobacilli, but the genetic basis for this antibiotic resistance was not elucidated. Gevers et al. (15) isolated tetracycline-resistant lactobacilli (*L. plantarum*, *L. sakei*, *L. curvatus*, and *L. alimentarius* strains) from fermented sausages and found *tet*(M) to be the only resistance genotype. In our study, we found a few strains with low-level resistance to tetracycline and chloramphenicol, as well as to ampicillin and penicillin G. In all cases, the MIC values were equal or close to the MIC breakpoint values, and comparably high resistances as noted by Zarazaga et al. (52) and Gevers et al. (15) were not observed. A close investigation of underlying resistance genes, using either PCR or hybridization with a gene probe, could not determine the presence of any ribosomal protection proteins or efflux genes in the case of tetracycline resistance or β-lactamase genes in the case of β-lactam antibiotic resistance. This could mean that there are underlying resistance mechanisms or genes that have not been described thus far, as may also be the case for the quinolone resistance described above. Kastner et al. (21), using an antibiotic resistance gene-specific microarray, also noticed that some antibiotic resistances could not be traced back to specific genes and hypothesized that this may be the result of possible

unknown resistance genes. The existence of such unknown resistance genes clearly would make verification of the observed phenotypic resistance at the genetic level difficult.

Alternatively, and in our eyes more likely, these MIC breakpoints may be set just too low at 4 μg/ml (8) for the β-lactam antibiotics and 4 to 8 μg/μl (41) for tetracycline. Thus, such breakpoints may allow the determination of some "borderline" resistant strains, which may be resistant as a result of some complex intrinsic features such as cell wall structure or metabolic properties (21). Thus, none of the typically associated resistance genes would be discernible. Such cases can only be critically evaluated when both the phenotypic and the genotypic resistance profiles of LAB starters and probiotic strains are investigated, as was done in the present study. Many of the earlier studies only concentrated on the resistance phenotypes and thus may have had problems in interpreting borderline resistance cases. Again, this may complicate safety determinations and present regulatory drawbacks if such a "borderline" antibiotic resistance has been determined for a particular strain, but none of the typical resistance genes could be identified, leading to confusion as to whether this resistance is acquired and/or transferable.

Only in one *L. salivarius* strain (BFE 7441) in the present study could a typical antibiotic resistance gene, *ermB* involved in erythromycin resistance, be determined. Although plasmid DNA was detected in this strain, it did not hybridize with an *ermB* gene probe. Instead, the gene probe hybridized with genomic DNA (result not shown). This strain showed a very high resistance profile (MIC $>$ 256 μg/ml). Similar to our study, Kastner et al. (21) studied 161 LAB isolates for antibiotic resistance, and only one *L. reuteri* strain SD 2112 showed a high tetracycline resistance phenotype that could be correlated with a *tet*(W) resistance gene (21). Erythromycin resistance genes have been reported to occur on conjugative plasmids in lactobacilli such as plasmid pGT633 from *L. reuteri* strain 100-63 (44) or pLEM3 from *L. fermentum* LEM89 (12). However, the *ermB* gene in *L. salivarius* BFE 7441 appeared to be chromosomally encoded. To investigate the possibility of whether the *ermB* gene is located on a conjugative, integrated plasmid or possibly a transposon, as has been reported for the *E. faecium* strain 160-1 to which the *ermB* gene from our strain showed high homology, we used filter-mating experiments with various sensitive recipient strains. However, conjugative transposition to *E. faecalis* JH2-2, *E. faecalis* OG1X, and *L. lactis* LMG 19460 could not be observed, so that the possibility of transferability by transposon could not be confirmed. However, filter-mating studies that show the involvement of a transpositional event are hampered by many experimental factors and thus are variable in outcome (19). Furthermore, appropriate positive control strains for conjugation and/or transposition experiments and standard protocols for gene transfer are sorely lacking. Thus, another problem associated with safety determinations of starter strains is that once a resistance phenotype and an associated resistance determinant have been identified, it becomes difficult to show that this determinant is not transferable, especially if the resistance gene is not located on a plasmid and no standard protocols for showing genetic transfer are available.

In conclusion, in Europe the adoption of the QPS system for safety evaluation must accommodate such problems in LAB antibiotic resistance determinations and allow flexible interpretation of results and not strict adherence to nonstandardized protocols or breakpoint values. The QPS system should allow leeway for interpretations of results, especially when these relate to the methodology for resistance phenotype determinations; determinations of MIC breakpoints for certain genera, species, or strains; the nondeterminability of a genetic basis of a resistance phenotype; and the transferability of resistance genes.

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