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Molecular Characterization of Intrinsic and Acquired Antibiotic Resistance in Lactic Acid Bacteria and Bifidobacteria

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Key Words
Lactic acid bacteria · Bifidobacteria · Antibiotic resistance · Antibiotic resistance genes · Multidrug resistance

Abstract
The minimum inhibitory concentrations (MICs) of 6 different antibiotics (chloramphenicol, clindamycin, erythromycin, streptomycin, tetracycline and vancomycin) were determined for 143 strains of lactic acid bacteria and bifidobacteria using the Etest. Different MICs were found for different species and strains. Based on the distribution of these MIC values, most of the strains were either susceptible or intrinsically resistant to these antibiotics. However, the MIC range of some of these antibiotics showed a bimodal distribution, which suggested that some of the tested strains possess acquired antibiotic resistance. Screening for resistance genes was performed by PCR using specific primers, or using a DNA microarray with around 300 nucleotide probes representing 7 classes of antibiotic resistance genes. The genes identified encoded resistance to tetracycline [*tet*(M), *tet*(W), *tet*(O) and *tet*(O/W)], erythromycin and clindamycin [*erm*(B)] and streptomycin [*aph*(E) and *sat*(3)]. Internal portions of some of these determinants were sequenced and found to be identical to genes described in other bacteria. All resistance determinants were located on the bacterial chromosome, except for *tet*(M), which was identified on plasmids in *Lactococcus lactis*. The contribution of intrinsic multidrug transporters to the antibiotic resistance was investigated by cloning and measuring the expression of *Bifidobacterium breve* genes in *L. lactis*.

Introduction

The resistance of bacteria to antibiotics is an increasingly important public health problem worldwide. There is a pressing need to limit the spread of resistance genes, since these could be transferred to opportunistic and pathogenic bacteria [Blázquez et al., 2002]. Antibiotic resistance can be 'intrinsic' or 'acquired' [Anadón et al., 2005]. Intrinsic or 'natural' resistance is inherent to a bacterial species and involves the absence of the target or the

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presence of low-affinity targets, low cell permeability, antibiotic inactivation of the antibiotics and the presence of efflux mechanisms. The acquisition of antibiotic resistance occurs via the mutation of pre-existing genes or by horizontal transmission. With some exceptions, intrinsic resistance and resistance by mutation are unlikely to be disseminated; horizontally transferred genes, particularly those carried on mobile genetic elements, are those most likely to be transmitted [Normark and Normark, 2002].

The food chain has been recognized as one of the main routes of transmission of antibiotic resistance from animal to human bacterial populations [Teuber et al., 1999; Witte, 2000]. More specifically, fermented products that are not heat treated before consumption provide a vehicle for transmission from the indigenous microbiota of animals to the bacteria of the human gastrointestinal tract (GIT) [Bates et al., 1994; Nikolich et al., 1994].

Either added as a starter or present in raw materials, many lactic acid bacteria (LAB) species participate in the manufacture and preservation of fermented foods and feed products. LAB are also commonly found, together with bifidobacteria (LAB&B), among the resident microbiota of the GIT of humans and animals. LAB&B have considerable potential as probiotics based on their long history of safe use and a growing body of evidence supporting their positive health-promoting effects [Ouwehand et al., 2002]. However, attention is currently being paid to commensal LAB&B with respect to their potential role in the spread and transmission of antibiotic resistance determinants in food matrices and into the GIT [Teuber et al., 1999]. This interest is strengthened by the fact that a large number of these bacteria are extensively used for large-scale feed, food and probiotic industrial manufacture. Further, the number of papers reporting the isolation of antibiotic-resistant LAB&B strains is increasing [Ahn et al., 1992; Danielsen, 2002; Fons et al., 1997; Perreten et al., 1997; Scott et al., 2000; Tannock et al., 1994]. Therefore, there is a need to establish clear cutoff values for separating susceptible and resistant bacteria, to distinguish between intrinsic and acquired forms of resistance, and to study the molecular mechanisms responsible for the spread of resistance among the LAB&B community.

The aim of this study was to assess the antibiotic resistance patterns of a large collection of LAB&B strains from dairy and intestinal sources and to characterize the genetic determinants responsible for the resistances. The role of intrinsic multidrug resistance (MDR) transporters in antibiotic resistance was also addressed.

Results and Discussion

Antibiotic Susceptibility Patterns of LAB&B Strains

Tables 1 and 2 show the minimum inhibitory concentration (MIC) ranges of 6 antibiotics for 143 LAB&B strains isolated from different environments when clustered into 7 groups: *Lactococcus lactis* (50), *Lactobacillus plantarum* (29), *Lactobacillus acidophilus-Lactobacillus delbrueckii* (1 *L. acidophilus*, 1 *Lactobacillus amylovorus*, 7 *L. delbrueckii*, 3 *Lactobacillus gasseri*, and 2 *Lactobacillus johnsonii*), other lactobacilli (3 *Lactobacillus brevis*, 1 *Lactobacillus casei*, 1 *Lactobacillus fermentum*, 1 *Lactobacillus helveticus*, 2 *Lactobacillus paracasei*, 1 *Lactobacillus pentosus*, 1 *Lactobacillus reuteri*, 3 *Lactobacillus rhamnosus*, 1 *Lactobacillus sakei* and 1 *Lactobacillus vaginalis*), *Bifidobacterium longum* (17), *Bifidobacterium bifidum* (6) and other bifidobacteria (1 *Bifidobacterium adolescentis*, 2 *Bifidobacterium animalis*, 1 *Bifidobacterium breve*, 4 *Bifidobacterium pseudocatenulatum*, 2 *Bifidobacterium pseudolongum* and 1 *Bifidobacterium thermophilus*).

The distinction between natural and acquired resistance is of great importance, since only the latter has a serious chance of being transferred [Anadón et al., 2005]. Analysis of MICs and their distributions helps differentiate between these 2 resistance mechanisms. The MIC distribution of a given antibiotic for a single bacterial species in the absence of resistance mechanisms should approach statistical normality [Murray et al., 2003]. As an example, figure 1 shows the MIC distributions of erythromycin (normal distribution) and tetracycline (bimodal distribution, thus suggesting the possession of acquired resistance) for the *L. lactis* strains.

Both the LAB and bifidobacteria were susceptible to chloramphenicol (MIC range 0.032–12 μg/ml; table 1). These results agree with data published elsewhere [Danielsen and Wind, 2003; Delgado et al., 2005; Flórez et al., 2005; Zarazaga et al., 1999; Zhou et al., 2005]. According to the clinical breakpoint of this drug (≥32 μg/ml) [Clinical and Laboratory Standards Institute, 2004], none of the strains could be considered resistant. However, at least 4 lactobacilli could be considered resistant (1 strain each of *L. plantarum*, *L. johnsonii*, *L. rhamnosus* and *L. vaginalis*) if MICs were compared to the breakpoints recently proposed by the FEEDAP Panel [Anadón et al., 2005]. The unimodal distribution of the MICs, however, does not support the presence of acquired resistance (data not shown).

The MICs of erythromycin and clindamycin for most of the LAB&B strains assayed (table 1) were within the

Table 1. Distribution of MICs of chloramphenicol, clindamycin and erythromycin (antibiotics that inhibit protein synthesis; target 50S ribosomal subunit) for LAB&B species from different environments

Anti-biotic[a]	Species	Strains n	Isolates with the following MICs (in μg ml^{-1}), n													
			<0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥256
Cm	*L. lactis*	50						3	30	12	5					
	L. plantarum	29							6	16	6	1				
	L. acidophilus/L. delbrueckii	15	1					1	5	7	1					
	Other lactobacilli	15						4	4	5	2					
	B. longum	17					2	10	5							
	B. bifidum	6						2	4							
	Other bifidobacteria	11					3	6	2							
Ery	*L. lactis*	50	3	10	30	4	3									
	L. plantarum	29			2	11	13	2	1							
	L. acidophilus/ L. delbrueckii	15	3	2	2	6	1									1
	Other lactobacilli	15		3	4	2	2	2								2
	B. longum	17		1	2	6	1				2	5				
	B. bifidum	6	1		3	2										
	Other bifidobacteria	11	1	1	6	2						1				
Clin	*L. lactis*	50	4	10	14	18	4									
	L. plantarum	29	4	2	5	3	6	7	2							
	L. acidophilus/ L. delbrueckii	15	4	1	2			1	1		2	2	1			1
	Other lactobacilli	15	2	1	4		2	1	2	1				1	1	
	B. longum	17	3	2	1	4										7
	B. bifidum	6	2	2	2											
	Other bifidobacteria	11	4	4				1	1							1

[a] Cm = Chloramphenicol; Ery = erythromycin; Clin = clindamycin.

normal range of susceptibility [Clinical and Laboratory Standards Institute, 2004]. Variations in the MICs of erythromycin and clindamycin similar to those obtained in the present work have been reported by other authors [Danielsen and Wind, 2003; Delgado et al., 2005]. Moderate levels of clindamycin resistance (8–32 μg/ml) have previously been observed in *L. gasseri* [Danielsen and Wind, 2003]. Based on these results, the raising of the cut-off value for clindamycin in this species should be considered. Nevertheless, the bimodal distribution of MICs strongly suggests that certain intestinal isolates possess acquired resistance mechanisms. Resistance to both antibiotics was usually observed in the same strain, suggesting a common resistance mechanism [the so-called macrolide-lincosamide-streptogramin (MLS) phenotype].

The LAB&B strains showed a wide range of streptomycin MICs (2 to >256 μg/ml; table 2), as reported elsewhere [Delgado et al., 2005; Katla et al., 2001]. However, a clear streptomycin cut-off MIC for the LAB&B strains is still to be defined. Indeed, the highest MIC for this aminoglycoside was moderate compared to the high clinical breakpoint for enterococci (≥1,024 μg/ml) [Clinical and Laboratory Standards Institute, 2004]. All *B. bifidum* isolates showed a streptomycin MIC of >256 μg/ml. Such resistance might be intrinsic in this species. In addition, several other strains showing an MIC higher than 256 μg/ml (table 2) were suspected of possessing acquired resistance to this drug (2 strains of *B. pseudocatenulatum*, 2 of *L. rhamnosus*, and 1 strain each of *B. longum*, *Bifidobacterium pseudolongum* subsp. *globosum* and *B. thermophilus*).

Although most strains were susceptible to tetracycline (table 2), their MIC distributions suggested that 11 *B. longum*, 5 *B. bifidum*, 3 *L. lactis*, and 1 strain each of *B. ani-*

Table 2. Distribution of MICs of streptomycin and tetracycline (antibiotics that inhibit protein synthesis; target 30S ribosomal subunit), and of the peptidoglycane synthesis inhibitor vancomycin, for LAB&B species from different environments

Antibiotic[a]	Species	Strains n	Isolates with the following MICs (in μg ml^{-1}), n													
			<0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥256
Tc	*L. lactis*	50			30	14	3							2	1	
	L. plantarum	29								3	9	10	6			1
	L. acidophilus/L. delbrueckii	15			1		4	4	4			1				1
	Other lactobacilli	15			2		3	3		2	1	4				
	B. longum	17					3	3				6	5			
	B. bifidum	6					1						5			
	Other bifidobacteria	11					7	1		1		1		1		
Str	*L. lactis*	50							5	6	8	20	11			
	L. plantarum	29									2	10	13	3	1	
	L. acidophilus/L. delbrueckii	15							4		7	2	2			
	Other lactobacilli	15							2		1	2	5	1	2	2
	B. longum	17									4	3	3	5	1	1
	B. bifidum	6														6
	Other bifidobacteria	11											3	1	3	4
Van	*L. lactis*	50			2	9	35	3	1							
	L. plantarum	29														29
	L. acidophilus/ L. delbrueckii	15					3	6	6							
	Other lactobacilli	15					1								1	13
	B. longum	17				1	13	3								
	B. bifidum	6							6							
	Other bifidobacteria	11					7	4								

[a] Tc = Tetracycline; Str = streptomycin; Van = vancomycin.

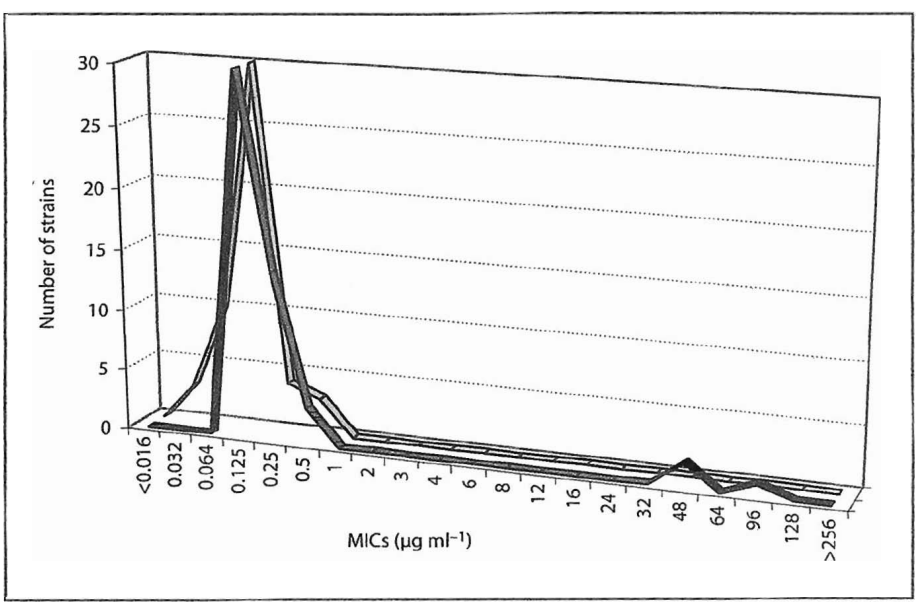

Fig. 1. Distribution of tetracycline MICs for the *L. lactis* strains (in black), showing a clear bimodal distribution, and of erythromycin MICs (in grey), showing a unimodal distribution.

Table 3. Results of screening selected LAB&B strains for antibiotic resistance genes by PCR and DNA microarray analysis

Species	Strains examined, n	Relevant phenotype	Genes detected by	
			PCR	DNA microarrays
B. animalis	1	Str^r, Tet^r	*tet*(W)	ND
B. bifidum	1	Str^r	–	*aph*(E), *tet*(O)
B. bifidum	5	Str^r, Tet^r	*tet*(W)	*tet*(W)
B. longum	2	susceptible	–	*tet*(O)
B. longum	4	$Clin^r$, Ery^r, Str^r	*tet*(W)[1]	*tet*(W)
B. longum	3	$Clin^r$, Ery^r, Str^r, Tet^r	*tet*(W)	*tet*(W)
B. longum	6	Str^r, Tet^r *tet*(W)	*tet*(W)	
B. longum	1	Str^r, Tet^r *tet*(W)	*tet*(W), *aph*(E)	
B. longum	1	Str^r, Tet^r *tet*(W)	*tet*(W), *aph*(E), *sat*(3)	
B. pseudocatenulatum	1	susceptible	–	–
B. pseudocatenulatum	2	Str^r	–	*aph*(E)
B. pseudocatenulatum	1	$Clin^r$, Ery^r, Str^r	–	–
L. lactis subsp. *lactis*	3	Tet^r	*tet*(M)	ND
L. brevis	1	susceptible	–	–
L. johnsonii	1	$Clin^r$, Ery^r, Tet^r	*erm*(B), *tet*(W/O)	*erm*(B), *tet*(W)
L. plantarum	1	susceptible	–	–
L. plantarum	2	Tet^r		
L. rhamnosus	1	susceptible		
L. rhamnosus	2	$Clin^r$, Ery^r, Str^r		
L. vaginalis	1	Tet^r		

$Clin^r$, Ery^r, Str^r and Tet^r stand for clindamycin, erythromycin, streptomycin and tetracycline resistance, respectively. ND = Not done.

[1] The *tet*(W) gene detected in these strains was found to be non-functional.

malis, B. pseudolongum subsp. *globosum, L. johnsonii, L. plantarum,* and *L. vaginalis* possessed potentially acquired resistance. Indeed, resistance to tetracycline has been widely reported in LAB&B species [Danielsen and Wind, 2003; Delgado et al., 2005; Flórez et al., 2005; Moubareck et al., 2005; Temmerman et al., 2003].

Heterofermentative lactobacilli were intrinsically resistant to vancomycin (table 2), as reported by other authors [Hamilton-Miller and Shah, 1998]. The resistance of these species to vancomycin is intrinsic, due to the presence of D-Ala-D-lactate in their peptidoglycan instead of the normal dipeptide D-Ala-D-Ala [Klein et al., 2000]. The obligate homofermentative lactobacilli, lactococci and bifidobacteria, however, proved to be very susceptible.

Multiresistance is not a common trait in LAB&B, but the 2 *L. rhamnosus* isolates shown to be resistant to 2 antibiotics (erythromycin and clindamycin) and the *L. johnsonii* isolate proved to be resistant to 3 antibiotics (erythromycin, clindamycin and tetracycline) are worthy of note.

Screening for Resistance Genes by PCR and Sequencing

Isolates on the right side of the tetracycline MIC distribution curves were all screened for tetracycline resistance determinants using universal primers for genes encoding ribosome protection proteins [Barbosa et al., 1999; Clermont et al., 1997]. A single PCR product of the same size with both of the primer pairs used (DI-DII and tet1-tet2) was obtained for 11 *B. longum,* 5 *B. bifidum,* 3 *L. lactis,* 1 *B. animalis* and 1 *L. johnsonii* strains (table 3). More particularly, the sequences from the amplicons of *L. lactis* proved to be 100% identical to the *tet*(M) gene present in the Tn*916* transposon of *Enterococcus faecalis* [Herzog-Velikonja et al., 1994]. A section of the nucleotide sequence of the amplicon obtained from *L. johnsonii* showed 99% homology with *tet*(W) genes, while another region matched the sequence of *tet*(O) genes, suggesting the determinant for this strain may be mosaic (data not shown). A single PCR product of around 1,250 bp was obtained for all the 17 tetracycline-resistant bifidobacteria, which was identical to the recently reported *tet*(W)

gene [Barbosa et al., 1999; Scott et al., 2000]. On the contrary, no amplification was obtained with *L. plantarum* and *L. vaginalis* when using specific primers for *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S) and *tet*(W). Preliminary results suggested the possibility of this apparent resistance being an effect of the size of the inoculum (data not shown).

Total DNAs of the MLS-resistant isolates were amplified with primers specific for the *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A) genes [Luna et al., 2000; Roberts et al., 1999]. Among the 11 previously detected resistant isolates, a positive amplification product of 639 bp was obtained when using the DNA of the *L. johnsonii* strain as a template plus specific primers for *erm*(B). The nucleotide sequence of the amplicon showed 99% homology with the plasmid-encoded *erm*(B) gene of a *L. fermentum* strain (GenBank acession No. U48430). No amplification was obtained with any other erythromycin- or clindamycin-resistant strains, indicating they might harbour other MLS resistance mechanisms.

Screening for Resistance Genes Using DNA Microarrays

Selected susceptible and resistant isolates were analysed by DNA microarray analysis to corroborate the results obtained by PCR screening, trying to identify previously undetected antibiotic resistance genes. The DNA microarray used contained nearly 300 oligonucleotide probes specific for the majority of antibiotic resistance gene classes. Table 3 shows a summary of the results obtained by this technique, and a comparison with those previously obtained by PCR. In general, the results of the 2 methods correlated well. Indeed, the presence of *tet*(W) and *erm*(B) was confirmed by the microarray analysis. Further, the DNA of 3 susceptible strains used as controls (2 *B. longum* and 1 *B. bifidum*) was found to hybridize with *tet*(O) (2 strains) and *tet*(W) (1 strain). In this last strain, fragments of *tet*(W) were also amplified by PCR; however, no amplification was obtained with primers encompassing the whole *tet*(W) gene, suggesting the existence of a non-functional (interrupted) *tet*(W) gene.

Finally, the microarray analysis also detected *aph*(E) and/or *sat*(3) genes in 1 *B. bifidum*, 2 *B. pseudocatenulatum*, and 3 streptomycin-susceptible isolates of *B. longum* and *B. pseudocatenulatum*.

Genetic Location of Resistance Genes

Among the erythromycin- and tetracycline-resistant isolates, only 4 strains were found to harbour plasmids (the 3 *L. lactis* strains and a single *B. bifidum* strain). Consequently, most resistance traits were deemed to be encoded on the bacterial chromosome. Internal segments of *tet*(M) (1.5 kb), *tet*(W) (1.2 kb) and *erm*(B) (639 bp) genes obtained by PCR were digoxigenin-labelled and hybridized against total and plasmid DNA digested with the restriction enzymes *Eco*RI and *Hin*dIII. Southern blot assays confirmed the chromosomal location of *tet*(W) in bifidobacteria, as well as the *erm*(B) and *tet*(O/W) genes in *L. johnsonii*. These assays showed a plasmid location for the *tet*(M) gene in all 3 *L. lactis* strains (results not shown).

MDRs of Bifidobacterium and Antibiotic Resistance

Besides dedicated antibiotic resistance genes, several intrinsic mechanisms have been suggested to contribute to antibiotic resistance in bacteria, including the thickness and compactness of the cell wall [Cui et al., 2003], defective cell wall autolytic systems [Kim et al., 1982] and MDR transporters [Price et al., 2006; Putman et al., 2001]. Recent studies have reported that, among the different *Bifidobacterium* species, *B. breve* possesses resistant levels to several antibiotics that are higher than those of other *Bifidobacterium* species [Moubareck et al., 2005], indicating that it could have a stronger intrinsic resistance. Therefore, we selected *B. breve* UCC2003 as a model organism to study the involvement of different MDR-like proteins in antimicrobial resistance.

Three genes encoding proteins with homology to MDR proteins were cloned and expressed in *L. lactis* (*bbmR*, *bbmA* and *bbmB*). BbmR exhibited characteristics reminiscent of proton motive force-driven MDR proteins, and its heterologous expression in *L. lactis* has been previously shown to confer resistance to macrolides [Margolles et al., 2005]. By contrast, recent experimental evidence and homology comparison suggested that BbmA and BbmB form a functional heterodimeric MDR transporter belonging to the ABC superfamily (data not shown). Consequently, the effect of separated or coordinated expression of *bbmA* and *bbmB* was also studied. The resistance levels of control and recombinant *L. lactis* cells to different antibiotic classes are summarized in table 4. As previously reported [Margolles et al., 2005], the expression of BbmR in *L. lactis* increased its resistance levels to macrolides by a factor of 3–6. MICs of aminoglycosides and antibiotics in the other groups were also increased. Separated expression of BbmA or BbmB did not influenced MIC of the antibiotics in *L. lactis* as much as coordinated expression of these 2 polypeptides in the same cell, further supporting their complementarity.

Table 4. Resistance levels to different antibiotic groups of control *L. lactis* cells and cells expressing the MDR proteins BbmR, or jointly BbmA and BbmB

L. lactis	Antibiotic class				
	β-lactams	tetra-cyclines	amino-glycosides	macro-lides	others
Control	–	–	–	–	–
BbmR	–	–	+/–[a]	+	+
BbmA/BbmB	–	–	–	–	+/–[c]; +[d]

– = Cells without increase in MIC compared with the control; + = cells with more than 3-fold increase in MIC compared with the control; +/– = cells in which the MIC increased between 2- and 3-fold compared with the control.

[a] Only for streptomycin. [b] Only for vancomycin.

[c] Only for ciprofloxacin. [d] Only for polymyxin B.

Cells containing BbmA and BbmB increased the MIC more than 2-fold to ciprofloxacin, and more than 10-fold to polymyxin B. However, in all these cases, MIC levels were still low compared to those obtained by acquired mechanisms [Clinical and Laboratory Standards Institute, 2004; Teuber et al., 1999].

Conclusions

Antibiotic resistance is not a common trait of dairy and intestinal LAB&B species. However, several strains were considered to be resistant to some antibiotics, and were thought to harbour acquired resistance genes. Moreover, genes identical to those described in other bacteria from the same environments [*tet*(M), *tet*(O/W), *tet*(W), *erm*(B)] were identified in dairy and intestinal LAB&B strains. Microarray hybridization was found to be a powerful screening technique able to screen in a single step for hundreds of genes of the most common antibiotic resistance families, although positive results require confirmation by other methods (hybridization, sequencing). When present, all resistance determinants were located on the bacterial chromosome, except for *tet*(M) which was identified on plasmids in *L. lactis*. Non-functional resistance genes were observed in some strains, while some others may harbour resistance determinants not yet characterized. Intrinsic mechanisms, such as those involving the membrane MDR transporters studied in this work, might contribute to broaden the antibiotic MIC ranges. Determination of the molecular mechanisms underlying the transfer of resistance in LAB&B species would be essential for the control of their spread via the food chain.

Experimental Procedures

Bacterial Strains, Growth Media and Culture Conditions

A total of 143 strains, isolated from different origins and geographical locations and thought to be representative of a majority of the LAB&B species, were surveyed for antimicrobial resistance. Most were isolated in the period of 1998–2003, but several were collected during the so-called pre-antibiotic era [Teuber et al., 1999]. The 31 *L. lactis* and 22 *L. plantarum* strains were isolated as part of the dominant populations of artisanal starter-free cheeses [Flórez et al., 2005]. The 12 intestinal lactobacilli and 27 bifidobacteria belonged to the dominant populations of the faeces and intestinal mucosa of healthy individuals [Delgado et al., 2005]. Nineteen *L. lactis*, 18 lactobacilli and 7 bifidobacteria were provided by the Belgian Co-ordinated Collections of Microorganisms (BCCM), University of Ghent, Ghent, Belgium. Finally, 7 *L. plantarum* strains were obtained from the Colección Española de Cultivos Tipo (CECT), University of Valencia, Valencia, Spain.

Culture media were all purchased from Oxoid (Basingstoke, UK). Cryopreserved cultures in glycerol were first recovered on Mueller-Hinton agar (for *L. lactis*), MRS agar (for dairy lactobacilli and lactobacilli of vegetable origin) or MRS agar supplemented with 0.3 g/l cysteine-HCl (for intestinal lactobacilli and bifidobacteria; Merck, Darmstadt, Germany). Isolated colonies were then streaked onto Mueller-Hinton plates (for *L. lactis*) or onto lactic acid bacteria susceptibility test medium (LSM; 90% Iso-Sensitest and 10% MRS [Klare et al., 2005] for lactobacilli), and incubated for 24 h at 30°C. Colonies of intestinal lactobacilli and bifidobacteria were streaked onto LSM supplemented with 0.3 g/l cysteine, and incubated at 37°C in an anaerobic chamber (Mac500; Down Whitley Scientific, Shipley, UK; atmosphere 10% H_2, 10% CO_2, 80% N_2) for 48 h.

L. lactis NZ9000 was used for the heterologous cloning and expression of MDR genes from *B. breve* UCC2003. For this purpose, appropriate nisin-inducible expression vectors of the pNZ8000 series and previously reported conditions were utilized [de Ruyter et al., 1998]. Control and recombinant *L. lactis* cells were grown at 30°C in M17 broth (Oxoid) with 0.7% (w/v) glucose (GM17). Antibiotics (chloramphenicol, erythromycin or both) at 5 μg/ml were added to the media when necessary.

MICs Determined by the Etest

Individual colonies from the agar plates were suspended in 2–5 ml of sterile saline (Oxoid) until a density corresponding to McFarland standard 1 or its spectrophotometric equivalent (≈3 × 10^8 CFU/ml) was obtained.

Bacterial suspensions were plated onto the surface of Mueller-Hinton (or LSM) agar with a sterile cotton swab, and the plates were allowed to dry for approximately 15 min before applying the Etest strips (AB Biodisk, Solna, Sweden). MICs were recorded following the manufacturer's recommendations after 48 h of incubation at 30 or 37°C. The susceptibilities of the LAB&B strains were established using the clinical MIC breakpoints defined by the

Table 5. List of the antibiotic resistance genes represented by oligonucleotides on the microarray used in this study

Class of antibiotic	Genes
Aminoglycosides	*aac(3')-Ib*; *aac(3')-IIIb*; *aac(3')-IIIc*; *aac(3')-IV*; *aac(6')-aph(2')*; *aac(6)-II*; *aac(6')-Ib*; *aac(6')-Ic*; *aac(6')-Iy*; *aac(6')-Il*; *aac(6'')-Iq*; *aacA1*; *aacA4*; *aacA7*; *aacC1*; *aacC2*; *aacC3*; *aacC7*; *aacC8*; *aacC9*; *aadA1*; *aadA2*; *aadA3*; *aadA6*; *aadA8*; *aadB*; *aadD*; *aadE*; *aph(2'')-Ib*; *aph(2'')-Ic*; *aph(2'')-Id*; *aph(3')-IIa*; *aphA1*; *aphA-Iab*; *aphA2*; *aphA3*; *aphA6*; *aphA7*; *aph*(E); Kn^R; *nptII*; *sat1*; *sat2*; *sat3*; *sat4*; *strA*; *strB*
Extended spectrum β-lactamases[1]	bla_{ACC}-01–03; bla_{ACT}-01; bla_{CARB}-01–08; bla_{CMY}-01–12; $bla_{CTX\text{-}M}$-01–38; bla_{DHA}-01–02; bla_{FOX}-01–06; bla_{IMP}-01–11; bla_{KPC}-01–03; bla_{LAT}-01–04; bla_{MIR}-01–02; bla_{MOR}-01; bla_{MOX}-01–02; bla_{OXA}-01–35; 40; bla_{PER}-01–02; bla_{PSE}-01–02; 04–05; bla_{ROB}-01; bla_{SHV}-01–30; bla_{TEM}-01–90; bla_{UOE}-01; bla_{VIM}-01–07
Chloramphenicol	*cat*; *catI*; *catII*; *catIIH*; *catIII*; *catA1*; *catA3*; *catB*; *catB1*; *catB2*; *catB3*; *catB4*; *catB5*; *catB6*; *catB7*; *catB8*; *catB9*; *catD*; *catP*; *catQ*; *cmlA*; *cmlA1*; *cmlA2*; *cmlA4*; *cmlA5*; *cmlA6*; *cmlA7*; *cmlA*-like; *cmlB*; *flo*R
Macrolides, lincosamides and streptogramins	*ere*(A); *ere*(A2); *ere*(B); *erm*; *erm*(2); *erm*(33); *erm*(A); *erm*(AM); *erm*(AMR); *erm*(B); *erm*(BC); *erm*(BP); *erm*(BZ); *erm*(C); *erm*(D); *erm*(F); *erm*(G;) *erm*(GM); *erm*(GT); *erm*(IP); *erm*(IM); *erm*(J); *erm*(K); *erm*(M); *erm*(Q); *erm*(T); *erm*(TR); *mef*(A); *mef*(E); *mph*(A); *mph*(B); *msr*(A); *msr*(B); *sat*(G); *vat*; *vat*(B); *vat*(C); *vat*(D); *vat*(E); vga(A); *vgb vgb*(B)
Sulfonamides	*sul1*; *sul2*; *sul3*; *sulA*
Tetracyclines	***otr*(A)**; ***otr*(B)** ***tet*(30)**; *tet*(31); *tet*(32); *tet*(33); *tet*(34); *tet*(35); *tet*(36); *tet*(37); *tet*(A); *tet*(B); *tet*(C); *tet*(D); *tet*(E); *tet*(G); *tet*(H); *tet*(J); *tet*(K); *tet*(L); *tet*(M); *tet*(O); *tet*(O/W); *tet*(A(P)); *tet*(B(P)); *tet*(Q); *tet*(S); *tet*(T); *tet*(U); *tet*(V); *tet*(W); *tet*(X); *tet*(Y); *tet*(Z)
Trimethoprim	*dfrA*; *dfrA1*; *dfrA2*; *dfrA3*; *dfrA5*; *dfrA6*; *dfrA7*; *dfrA8*; *dfrA9*; *dfrA10*; *dfrA12*; *dfrA13*; *dfrA14*; *dfrA15*; *dfrA16*; *dfrA17*; *dfrA18*; *dfrA20*; *dfrA21*; *dfrB*; *dfrB2*; *dfrB3*; *dfrC*; *dfrD*; *fol*A
Vancomycin	*vanA*; *vanB*; *vanC1*; *vanC2/C3*; *vanD*; *vanE*

[1] For example: bla_{ACC}-01–03 means that oligonucleotides representing bla_{ACC}-01, bla_{ACC}-02 and bla_{ACC}-03 are present on the microarray. The nomenclature for genes encoding for extended spectrum β-lactamases has been standardized by adding a number to the gene name [for review, see Paterson and Bonomo, 2005].

Clinical and Laboratory Standards Institute [2004], and compared to the microbiological breakpoints proposed by the FEEDAP Panel [Anadón et al., 2005].

DNA Techniques

Genomic DNA was isolated using a commercial kit (GenElute bacterial genomic DNA kit; Sigma-Aldrich Co., St. Louis, Mo., USA). Plasmid DNA extraction was performed as previously described [O'Sullivan and Klaenhammer, 1993]. *Eco*RI- and *Hin*dIII-digested DNA was transferred to nylon membranes and hybridized with probes obtained by PCR using oligonucleotide primers internal to specific genes. Hybridization and detection were all performed using the digoxigenin DNA-labelling and detection kit (Roche Molecular Biochemicals, Lewes, UK) as recommended by the manufacturer.

The presence of tetracycline resistance genes encoding ribosomal protection proteins was checked by PCR with 2 pairs of degenerate primers: DI-DII [Clermont et al., 1997] and tet1-tet2 [Barbosa et al., 1999]. Additional PCR assays were performed with primers tetWF and tet2, specific for *tet*(W) [Scott et al., 2000], and primers DI and TetMR, specific for *tet*(M) [Clermont et al., 1997]. The set of primers included specific oligomers for *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S) and *tet*(W) [Gevers et al., 2003; Scott et al., 2000]. The presence of macrolide, lincosamine and streptogramin resistance genes was checked with primers specific for *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A) genes [Luna et al., 2000; Roberts et al., 1999]. Purified amplicons were sequenced by cycle extension in an ABI 370 DNA sequencer (Applied Biosystems, Foster City, Calif., USA).

The microarray assay was performed with an array containing 300 oligonucleotides (50–60 bp long) specific for 250 antibiotic resistance genes of the following classes: aminoglycosides, extended-spectrum β-lactamases, chloramphenicol, MLS, sulfonamides, tetracyclines, trimethoprim and vancomycin (table 5). The spotting of the oligonucleotides, hybridization conditions and the analysis of the microarrays were as previously described [van Hoek et al., 2005].

Cloning of MDR Genes from B. breve *and Expression in* L. lactis

To study the role of MDRs in antibiotic resistance, a first draft of the *B. breve* UCC2003 genome sequence was searched for homologous genes to reported MDRs. Based on this homology, 3 genes *(bbmB, bbmR and bbmA)* were selected, amplified by PCR, and cloned in pNZ8000 vectors. In this way, constructs pN38, pNAbcA and pNAbcB, expressing BbmR, BbmA and BbmB, respectively, were obtained.

MIC of a large series of antibiotics in control and recombinant cells was assayed by Etest. The antibiotic classes analysed included β-lactams (ampicillin, benzylpenicillin, ceftazidime, cephalothin and meropenem), tetracyclines (tetracycline, doxycycline and minocycline), aminoglycosides (kanamycin, gentamicin and streptomycin), macrolides (erythromycin, azithromycin, dirithromycin and clarithromycin) and others (clindamycin, polymyxin B, quinupristin-dalfopristin, rifampicin, trimethoprim-sulfamethoxazole, vancomycin and ciprofloxacin).

Computer Analyses

DNA and protein sequences were analysed using the Clone Manager 5 computer program (Scientific and Educational Software, Durham, N.C., USA). Homology searches were carried out using the BLAST server of the NCBI (http://www.ncbi.nlm.nih.gov/) and the software available on the Pôle Bioinformatique Lyonnais web page (http://pbil.univ-lyon1.fr/).

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