Genetic Basis of Tetracycline Resistance in *Bifidobacterium animalis* subsp. *lactis*[▽]

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All strains of *Bifidobacterium animalis* subsp. *lactis* described to date show medium level resistance to tetracycline. Screening of 26 strains from a variety of sources revealed the presence of *tet*(W) in all isolates. A transposase gene upstream of *tet*(W) was found in all strains, and both genes were cotranscribed in strain IPLAIC4. Mutants with increased tetracycline resistance as well as tetracycline-sensitive mutants of IPLAIC4 were isolated and genetically characterized. The native *tet*(W) gene was able to restore the resistance phenotype to a mutant with an alteration in *tet*(W) by functional complementation, indicating that *tet*(W) is necessary and sufficient for the tetracycline resistance seen in *B. animalis* subsp. *lactis*.

Bifidobacteria are common microorganisms in the gastrointestinal tracts of humans. The intestine of healthy newborns is quickly colonized by bifidobacteria, especially those fed with breast milk, in which they could represent the majority of the total culturable population, typically remaining dominant until weaning (7, 12). In adults, however, the intestinal bifidobacterial population is more variable, although it still constitutes one of the predominant members (29).

Different strains of the genus *Bifidobacterium* are considered health-promoting microorganisms for humans. Among them, *Bifidobacterium animalis* subsp. *lactis* is the most common species included in functional food products (9, 17, 27), and several beneficial effects, such as reduction of the risk of diarrhea in children and allergy relief, have been attributed to this bacterium (27).

Due to the ubiquity of *B. animalis* subsp. *lactis* in foods and dietary supplements, the presence of antibiotic resistance determinants in this bacterium is of key interest, since gut commensals possessing an antibiotic resistance gene could theoretically serve as a reservoir of resistance for intestinal pathogens via horizontal gene transfer. In this regard, resistance to tetracycline has recently been reported as the most common antibiotic resistance in bifidobacteria (1, 3, 4, 8, 18), although one incidence of erythromycin resistance related to the presence of a transposon-associated *erm*(X) gene has also been described previously (32). The most abundant genetic determinants responsible for the tetracycline resistance phenotype among the species of this genus are *tet* genes coding for ribosomal protection proteins (24). The genes *tet*(O), *tet*(M), and *tet*(W) and different hybrid determinants [i.e., *tet*(W/32/O) and *tet*(O/W)] were detected in bifidobacteria, *tet*(W) being the most commonly found (1, 4, 15, 19, 31). Remarkably, *tet*(W) was also found in *Lactobacillus*, *Clostridium*, and other gut bacteria, thus suggesting a wide distribution among bacteria from the mammalian gut (5, 13, 22, 23, 28). In this context, the aim of our research was to investigate the ubiquity of *tet*(W) among *B. animalis* subsp. *lactis* strains isolated from different origins and the influence of gastrointestinal conditions on the antibiotic resistance phenotype and to supply the basis for a correlation between genotype and phenotype.

The presence of *tet*(W) in 26 strains of *B. animalis* subsp. *lactis* from a variety of sources, including functional foods, probiotic supplements, dairy cultures, human feces, and culture collections, was investigated using microarray analysis and PCR. Furthermore, their tetracycline resistance levels were determined using a microdilution method. Briefly, individual colonies on agar plates were suspended in sterile saline until a density corresponding to McFarland standard of 1 was reached. The inoculated suspension was then diluted 1,000-fold in lactic acid bacterial susceptibility test medium (LSM) plus cysteine (90% Iso-Sensitest [IST] broth [Oxoid] plus 10% MRS broth [Oxoid] plus 0.3 g/liter cysteine hydrochloride [Sigma] [16]) containing the appropriate tetracycline concentration, and 100 µl of the diluted bacterial suspension was added to each well. Incubations were carried out at 37°C for 48 h in a Mac 500 anaerobic chamber (Don Whitley Scientific) in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. All the strains displayed a medium level resistance to the antibiotic, close to, or above, reference breakpoints described for this genus (Table 1), suggesting the presence of specific tetracycline resistance determinants (18, 33). The microarray analysis of 24 out of the 26 strains (Table 1) was carried out as previously described (19, 30) with in-house printed DNA chips containing 223 oligonucleotides (50 to 60 nucleotides long), which represent over 430 antibiotic resistance genes, including 2 *otr* and 31 *tet* genes. The results showed, in all cases, positive

TABLE 1. Strains analyzed for tetracycline resistance, tetracycline MICs, and detection of the genes *tet*(W) and
trp by PCR and microarray analysis

Strain[a]	Source or reference	Result[b] by:			MIC (µg/ml)
		Microarray[c]	*trp* PCR	*tet*(W) PCR	
IPLA4549	25	+	+	+	16
4549dOx	25	+	+	+	16
4549dCo	11	ND	+	+	16
IPLAIC1	Dairy culture	+	+	+	16
IPLAIC2	IPLA[d] collection	+	+	+	32
IPLAIC3	IPLA collection	+	+	+	32
A8dOx	IPLA collection	+	+	+	8
M6	Fermented milk	+	+	+	16
M6dCo	11	+	+	+	16
PBT	Probiotic supplement	+	+	+	16
PBTdOx	11	+	+	+	16
658	Fermented milk	+	+	+	16
658dOx	11	+	+	+	8
LMG11580	Chicken feces	+	+	+	8
LMG18314[T]	Yogurt	+	+	+	16
LMG18906	Rabbit feces	+	+	+	8
KCTC3356	Sewage	+	+	+	32
C64	Human feces	+	+	+	16
CECT4550	Nursling stools	ND	+	+	32
Y1	Yogurt	+	+	+	16
Class11	Yogurt	+	+	+	8
Essen2	Fermented milk	+	+	+	8
Dia8	Fermented milk	+	+	+	8
BBI	Fermented milk	+	+	+	16
A12	Dairy starter	+	+	+	32
IPLAIC4	Fermented milk	+	+	+	16
T25	IPLAIC4 Tet[r] mutant	ND	+	+	64
IC4-8658	IPLAIC4 Tet[r] mutant	ND	+	+	128
IC4-9991	IPLAIC4 Tet[s] mutant	ND	+	+	4
IC4-8902	IPLAIC4 Tet[s] mutant	ND	+	+	1
IC4-8902 + pAM1					1
IC4-8902 + pAM-TB					32

[a] IPLAIC4 and mutants derived thereof are in boldface. T, type strain.
[b] + indicates a positive signal in the microarray or the presence of the right amplicon. ND, not determined.
[c] The microarray data displayed are the positive hybridization results with the two oligonucleotides representing *tet*(W) (30).
[d] IPLA, Instituto de Productos Lácteos de Asturias.

hybridization signals for the oligonucleotides representing *tet*(W). Except for the signals with the species-specific control probes (two 60-mer oligonucleotides designed on conserved parts of the 16S rRNA [5′-gtaacggtggaatgtgtagatatcgggaagaac accDatggcgaaggcagDtctctgggc-3′] and *tuf* [5′-gccgtcactcgccSttc ttctcSaactaccgtccgcagttctacttccgYaccaccgacg-3′] gene sequences, respectively, of the genus *Bifidobacterium* (where D is A, G, or T, S is C or G, and Y is C or T), no other hybridization signals were found with any of the remaining spots, indicating the absence of other antibiotic resistance determinants tested for with this microarray. Subsequently, PCR primers were designed based on a 3,696-bp fragment of the chromosomal DNA of *B. animalis* subsp. *lactis* IPLAIC4 (GenBank accession number GU361625) (Table 2 and Fig. 1).

In IPLAIC4, *tet*(W) is flanked upstream by a putative transposase gene (*trp*). Primers were designed in a way that allowed us to amplify the *trp* gene together with the 5′ end of *tet*(W). Thus, a positive 1.47-kb amplicon implies that both genes are organized as they are in IPLAIC4 (Fig. 1). PCRs were carried out according to standard procedures (4). Positive Trp and Tet amplicons were obtained for the 26 strains. Afterwards, 10 Trp and 10 Tet amplicons (from strains IPLA4549, IPLAIC1, A8dOx, M6, PBT, Y1, Class11, Essen2, A12, and C64) (Table

1) were selected for sequencing (Secugen S.L., Madrid, Spain) in both directions with the primer pairs TrpF/TrpR and TetF/ TetR, and they all gave homology with the corresponding sequences of IPLAIC4. This suggests that the structural organizations of *trp* and *tet*(W) are very similar, if not identical, in all our strains. Transposases catalyze the movement of DNA between different locations by recognizing insertion sequences in the DNA and could be involved in the mobilization of *tet* genes in bifidobacteria. In fact, one chromosomal *tet*(W) gene has been shown to transfer, at low frequency, from *Bifidobacterium longum* to *Bifidobacterium adolescentis in vitro*, and the site of chromosomal insertion in *B. adolescentis* is identical to that of the donor strain, consistent with a transposase-mediated site-specific insertion event (13). This suggests that *trp* could have been involved in the acquisition of *tet*(W) by *B. animalis* and opens the question of whether *trp* could participate in the transfer of *tet*(W) to other bacteria.

The ability of IPLAIC4 to transfer *tet*(W) to other bacteria was tested by filter mating on glucose M17 or MRS (both supplied by Difco) plates containing cysteine (Sigma) under anaerobic conditions essentially as previously described (26). Recipient strains included *Lactococcus lactis* MG1614 (resistant to rifampin and streptomycin; 2 experiments), *Bifidobac-*

TABLE 2. Primers and plasmids used in this study

Plasmid or primer	Characteristics[b]	Reference or source
Plasmids		
pAM1	*E. coli-Bifidobacterium* shuttle cloning vector; Ap[r] Em[r]	2
pAM-TB	pAM1 containing the 3.65-kb fragment cloned in the XbaI site; in this construct, the 5′ end of *tet*(W) is located beside the PstI site	This work
Cloning primers		
TTF	GCCGAC*TCTAGA*CTTCAATCCTTTTATCAGAGCATCGC; forward cloning primer for pAM1, XbaI site	This work
TTR	GCCGAC*TCTAGA*TTATCAAGTGATCTTACTGAACATATGG; reverse cloning primer for pAM1, XbaI site	This work
PCR primers		
TrpF	ATTCAGCGACGAACTGGCACAG; forward primer for *trp-tet*(W) amplification	This work
TrpR	CGCTTGAATGGTAATCCCACG; reverse primer for *trp-tet*(W) amplification	This work
TetF	TGGAATTCTTGCCCATGTAGACG; forward primer for *tet*(W) amplification	This work
TetR	GAACATATGGCGCACCTTGTCC; reverse primer for *tet*(W) amplification	This work
RT-PCR[a] primers		
qRT-TrpF	CGACAGCCGCGCATTT	This work
qRT-TrpR	CGTTCGGAACCTGGTTGCT	This work
qRT-TrpIntF	CAGACCCTGTCTGGCAGCTT	This work
qRT-TrpIntR	TGTGGATAACCTCCGAATTTTTTG	This work
qRT-IntF	CGGTGTTGCCTTAAATAAAACTATAATCA	This work
qRT-IntR	GGCAAAAGTATTCTTAAATTGGGTACAA	This work
qRT-IntTetF	TCATCCCCAGTGGTAAAAGTATTTTACT	This work
qRT-IntTetR	CATGGGCAAGAATTCCAATATTG	This work
qRT-Tet1F	GCCCGGCCACATGGAT	This work
qRT-Tet1R	GCCCCATCTAAAACAGCCAAA	This work

[a] RT-PCR, quantitative reverse transcription-PCR.
[b] XbaI sites are in italics and underlined.

terium bifidum CHCC2185 (resistant to trimethoprim; 4 experiments), *Enterococcus faecalis* JH2-2 (resistant to rifampin, fusidic acid, and erythromycin; 1 experiment), and mutants of *B. animalis* subsp. *animalis* ATCC 25527 and ATCC 27672 resistant to both rifampin and fusidic acid (2 experiments). Donors, recipients, and transconjugants were quantitated on plates supplemented with appropriate antibiotics. No transfer of *tet*(W) to any recipient was detected, indicating that the frequency of transfer is less than 10^{-9}/recipient (data not shown). The lack of transfer of *tet*(W) from another *B. animalis* subsp. *lactis* strain has been previously described (18). However, the experimental conditions used do not represent the complex *in vivo* situation in the gut, and the gastrointestinal environment most likely will offer different conditions for antibiotic resistance transfer than those provided *in vitro*.

It is important to remark that a similar genetic context in *B. animalis* E43 was described previously (4), and an identical 3,696-bp fragment is present in the chromosomes of two re-
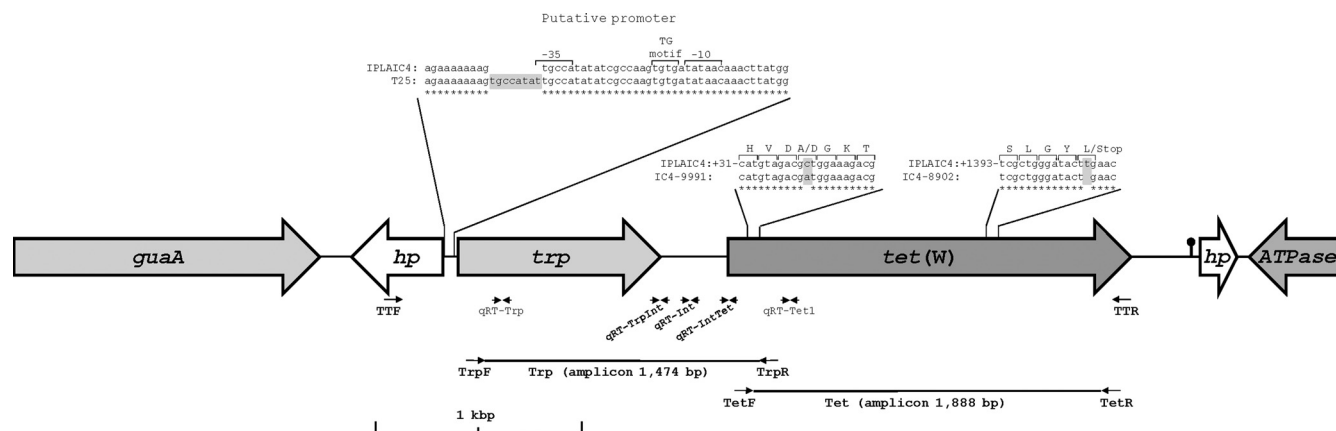

FIG. 1. Organization of the *B. animalis* subsp. *lactis* IPLAIC4 genomic region containing *tet*(W), *trp*, and the upstream and downstream regions. The open arrows indicate the relative positions and directions of transcription of the open reading frames (ORFs). The positions of the primers used for standard PCR and quantitative PCR analysis are indicated. The locations of the mutations found in the IPLAIC4 mutants T25, IC4-9991, and IC4-8902 are detailed. The pin-like symbol indicates a Rho-independent transcription terminator. *hp*, hypothetical protein gene; *guaA*, GMP synthase gene. Asterisks indicate identical bases in the compared sequences.

cently sequenced *B. animalis* subsp. *lactis* strains (GenBank accession numbers CP001606 and CP001515). The strain AD011 also contains an almost identical DNA fragment, differing only in 1 base (GenBank accession number CP001213). The high degree of sequence conservation among our strains and the 3 currently available genome sequences indeed supports the results of Barrangou and coworkers (6) and indicates that this subspecies comprises clonal bacteria which are genomically monomorphic.

Previous studies indicated that the acquisition of a stable bile resistance phenotype or the transient exposure to bile induces an increase in tetracycline resistance in *Bifidobacterium* (14, 20). We tested if this phenotypic change could be correlated with the activity of *tet*(W). For this purpose, IPLAIC4 was incubated in MRSc broth (MRS [Difco] supplemented with 0.05% cysteine [Sigma]) containing 0.8 to 1% bile (Ox-gall; Oxoid) for 5 consecutive days (5 overnight cultures; approximately 50 generations). After that, the culture was plated on MRSc agar containing tetracycline (25, 75, or 125 μg/ml; Sigma). Hardly any colony was able to grow on 75 or 125 μg/ml, but numerous colonies were recovered on 25 μg/ml tetracycline. Interestingly, the frequency of colonies able to grow at this concentration was higher for those cultures previously grown in the presence of bile (one out of 10^2 to 10^4 CFU) than for the control culture grown in the absence of bile (one out of around 10^5 to 10^6 CFU). We analyzed 47 colonies recovered from 25-μg/ml tetracycline plates, and most of them had a transient resistance phenotype (not stable), since they revert to the original resistance level when grown in the absence of bile. This could suggest a temporal activation of *tet*(W) when bile is present, which is lost when bile is removed. In fact, quantitative PCR experiments indicated an induction of *tet*(W) (from 2- to 4-fold at bile concentrations of 0.25 and 0.75%, respectively) when IPLAIC4 was grown in the presence of bile. Also, transcriptional analysis of IPLAIC4 using dedicated genomic microarrays indicated a slight induction of *tet*(W) (between 1.5- and 2-fold upregulation, tested with 4 different probes) in the presence of 0.1% bile (M. Gueimonde and C. Garrigues, unpublished data). Only one of the colonies recovered from the 25-μg/ml tetracycline plates was able to grow consistently in high tetracycline concentrations. This strain was named T25 and was included in further analyses.

Mutants of IPLAIC4, sensitive to tetracycline, were isolated following mutagenesis with UV light (2 exposures of 5-min duration at 70 mJ/cm^2) in the presence of ethidium bromide (20 μg/ml) and a subsequent ampicillin enrichment step. IC4-8902 and IC4-9991 were more sensitive than the original strain to tetracycline (Table 1). A mutant of IPLAIC4 with elevated resistance was isolated following subculturing in medium containing increasing concentrations of tetracycline. Mutant IC4-8658 is resistant to 100 μg/ml tetracycline.

A 3.65-kbp fragment (between primers TTF and TTR) (Fig. 1) containing *tet*(W) was sequenced in these 3 mutants and in T25 (Fig. 1), rendering valuable information on the potential genetic differences that lead to distinct tetracycline resistance levels. The mutant IC4-9991 contains a point mutation in *tet*(W) (cytosine 41 is replaced by an adenine). This introduced a different amino acid at position 14 (A14/D14) in the Walker A motif of the nucleotide binding domain (21), which could influence the activity of the protein, although in our case
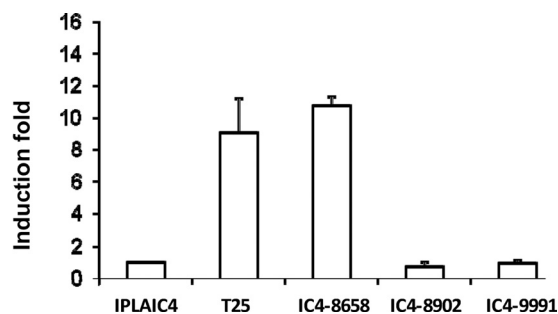

FIG. 2. Relative expression of *tet*(W) from IPLAIC4 and mutants derived thereof, determined by quantitative reverse transcription-PCR. The expression levels in the presence of subinhibitory tetracycline concentrations for each strain (see text) were normalized to those obtained for the control culture (IPLAIC4 grown without tetracycline). Expression levels were calculated by using the ΔΔ*CT* method.

tet(W) seems to retain some residual activity. In the mutant IC4-8902 the thymine at position 1406 of *tet*(W) is missing, which yields a frameshift and a potentially shorter version of the protein (468 residues instead of 639), likely nonfunctional. On the other hand, the 3,696-bp region in the mutant IC4-8658 was 100% identical to the corresponding region in IPLAIC4, suggesting that the mutation(s) responsible for the high-resistance phenotype is located somewhere outside this region. Finally, in the mutant T25 we found a duplication of an 8-bp fragment (TGCCATAT) in the putative promoter (−35 region) of *trp* which could affect promoter activity.

We determined the expression levels of *tet*(W) in the different mutants grown in MRSc in the presence of tetracycline (antibiotic concentrations were selected on the basis of similar levels of growth inhibition among the different strains: 0.4 μg/ml for IC4-8902, 1 μg/ml for IC4-9991, 38 μg/ml for T25 and IC4-8658, and 12 μg/ml for IPLAIC4. Quantitative PCR was carried out as previously described (10) using the primers qRT-Tet1F and qRT-Tet1R (Table 2 and Fig. 2). Expression levels were determined by relative quantification using the ΔΔ*CT* method, in which the expression level in the control culture (IPLAIC4) is arbitrarily set to 1 and the expression levels in the samples are calculated relative to that control. Experiments were carried out in duplicate, each measured in duplicate in two independent PCR runs. This experiment indeed suggested that the increased tetracycline resistance in mutants T25 and IC4-8658 is due to an increased expression of *tet*(W).

A quantitative reverse transcription-PCR (RT-PCR) analysis was carried out in order to ascertain if *trp* and *tet*(W) were expressed independently or in the same transcript. Primers were designed in order to amplify internal fragments of the 2 genes (qRT-TrpF/qRT-TrpR and qRT-Tet1F/qRT-Tet1R), the intergenic region (qRT-IntF/qRT-IntR), the *trp* 3′ end together with the 5′ end of the intergenic region (qRT-TrpIntF/qRT-TrpIntR), and the 5′ end of *tet*(W) together with the 3′ end of the intergenic region (qRT-IntTetF/qRT-IntTetR) (Fig. 1 and Table 2). The analysis was carried out with strains IPLAIC4, T25, and IC4-8902. All primers produced amplification products for the 5 amplicons in the three strains. In addition, the five amplified fragments were found to

be present in equal amounts, indicating that *trp* and *tet*(W) are transcribed together as part of a bicistronic operon.

Finally, in order to unequivocally demonstrate that the tetracycline resistance phenotype in *B. animalis* subsp. *lactis* is conferred by *tet*(W) and not by other genes present in its genome, whose function could have passed unnoticed by us, we cloned the 3.65-kb fragment from IPLAIC4 containing *trp* and its upstream region and the native *tet*(W) gene in the mutant IC4-8902, in which a truncated, nonfunctional version of *tet*(W) is present. The fragment was amplified using *Pfx* DNA polymerase (Invitrogen) and the primers TTF and TTR according to the manufacturer's instructions. The PCR product was digested with XbaI and cloned in the *Escherichia coli*-*Bifidobacterium* shuttle vector pAM1 (2), digested with the same enzyme, to generate the plasmid pAM-TB. Cloning and transformation procedures were carried out as previously described (2). The IC4-8902 strain containing the empty vector (pAM1) displayed a tetracycline MIC of 1 μg/ml, similar to that for IC4-8902. However, IC4-8902 harboring pAM-TB showed a MIC of 32 μg/ml, even higher than that for IPLAIC4; this difference is probably due to the presence of multiple copies of the *tet*(W) gene. This result indicates that tetracycline resistance in *B. animalis* subsp. *lactis* is conferred by *tet*(W) and suggests that no other genetic determinants participate in this process.

In summary, this work shows the wide distribution of *tet*(W) among *B. animalis* subsp. *lactis* strains and its involvement in the tetracycline resistance phenotype of this bacterium. Bile exposure slightly induces *tet*(W) expression, which could influence resistance levels in certain environments. Upstream of *tet*(W) is a transposase gene, which is cotranscribed in tandem. Transposases have been found to be involved in the horizontal gene transfer of genetic elements among bacteria, but to date there is no evidence that *tet*(W) in *B. animalis* subsp. *lactis* is transmissible.

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