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Tetracycline Resistance Mediated by *tet*(W), *tet*(M), and *tet*(O) Genes of *Bifidobacterium* Isolates from Humans▽

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MICs of tetracyclines were determined for 86 human *Bifidobacterium* isolates and three environmental strains. The *tet*(O) gene was found to be absent in these isolates. *tet*(W) and *tet*(M) were found in 26 and 7%, respectively, of the *Bifidobacterium* isolates, and one isolate contained both genes. Chromosomal DNA hybridization showed that there was one chromosomal copy of *tet*(W) and/or *tet*(M).

Bifidobacteria are gram-positive anaerobic bacteria found in the gastrointestinal tracts of humans and animals. Strains belonging to the genus *Bifidobacterium* have been reported to have several health-promoting effects (15, 16, 21), explaining why they are increasingly used as probiotics in a wide range of functional foods (9). For probiotic safety (20), guidelines have recently recommended that probiotic bacteria should not harbor transmissible genes encoding resistance to antibiotics that are used clinically (17). For bifidobacteria two molecular antibiotic resistance determinants have been described, the *bbmr* gene of *Bifidobacterium breve*, which confers moderate resistance to macrolides (10), and *tet* genes coding for ribosomal protection proteins involved in resistance to tetracyclines (3). Although different types of acquired tetracycline resistance genes have been found in anaerobes (3, 18), only the *tet*(M) and *tet*(W) genes encoding ribosomal protection proteins have been selectively found in bifidobacteria (6, 8, 11, 12, 22). In order to better understand resistance mechanisms in human bifidobacterial strains, we investigated the prevalence and distribution of the *tet*(M), *tet*(W), and *tet*(O) genes encoding ribosomal protection proteins involved in acquired tetracycline resistance in this human commensal genus.

Eighty-nine strains of bifidobacteria belonging to nine species were included in our study (Table 1). Eighty-six of these strains were isolated, as described previously (2), from feces of healthy humans (adults and newborns), and three were environmental strains (laboratory collection). Bacteria were assigned to the genus *Bifidobacterium* on the basis of their anaerobic requirement, cellular morphology, Gram staining, and fructose-6-phosphate phosphoketolase activity and by PCR (7). Species were identified using a validated multiplex PCR (13) which included control strains. Isolates whose identities were not clear-cut were not included in the study.

Most of the anaerobe efflux proteins confer resistance to tetracycline but not to minocycline (3). Therefore, the phenotypic patterns of resistance to tetracyclines of *Bifidobacterium* isolates were determined using three tetracyclines: tetracycline, minocycline, and doxycycline (Sigma-Aldrich, Saint Quentin Fallavier, France). MICs were determined by the agar dilution method, as described previously (12), following the CLSI (formerly NCCLS) recommendations (14). In general, the MICs of tetracycline were one- to twofold higher than those of the two other molecules tested (Table 1).

To differentiate resistant strains from susceptible strains, we use the wild-type/nonwild-type definition from EUCAST (http://www.escmid.org/sites/index.aspx), which allows strain differentiation based on the presence or absence of resistance genes. Therefore, purified genomic DNA (23) of all 89 strains was used as a template for PCR amplification of the *tet*(M), *tet*(W), and *tet*(O) genes using sense and antisense primers as described previously (4, 19, 22). Our results showed that the prevalence of tetracycline-resistant *Bifidobacterium* strains was 33% (Table 2). PCR results showed that the *tet*(O) determinant was not present in any of the *Bifidobacterium* isolates tested, while 29 of the 89 isolates carried either *tet*(W) or *tet*(M) or both. *tet*(W) was the most widely distributed gene among *Bifidobacterium* species and was found in 83% of the tetracycline-resistant isolates, while the prevalence of *tet*(M) was lower (21%).

Based on the CLSI anaerobic bacterium tetracycline breakpoints (14), two isolates carrying a *tet*(M) gene were clinically susceptible to tetracylines (MICs, ≤4 mg/liter) and four isolates carrying *tet*(W) were determined to be intermediate strains (MICs, 8 mg/liter). These results suggest that when bifidobacteria are categorized as clinically intermediate for tetracycline resistance, they should be screened genetically for the presence of *tet* genes.

We report here for the first time the presence of the *tet*(M) gene in the human species *Bifidobacterium bifidum*, *Bifidobacterium longum*, and *Bifidobacterium breve*. The fact that *tet*(W) has a G+C content (50 to 55%) closer to the average G+C content of the *Bifidobacterium* chromosome (58% G+C) is a possible explanation for the spread of *tet*(W) in this genus at the expense of *tet*(M) (32 to 40% G+C). Interestingly, one *B. breve* tetracycline-resistant isolate contained both the *tet*(W) and *tet*(M) genes (MIC of tetracycline, 64 mg/liter), an uncommon feature that has not been described previously for the genus *Bifidobacterium*.

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TABLE 1. MICs of tetracycline, minocycline, and doxycycline for *Bifidobacterium* isolates

Antibiotic	*Bifidobacterium* sp.	No. of isolates	No. of isolates inhibited at a concn (mg/liter) of:									
			≤0.125	0.25	0.5	1	2	4	8	16	32	64
Tetracycline	*B. longum* longum type	30		1	2	11	4	2	2	2	2	4
	B. longum infantis type	4			1			2			1	
	B. pseudocatenulatum	12				5	2					5
	B. breve	14		1	1	9	1			1		1
	B. angulatum	2				2						
	B. bifidum	12				4	4			1	1	2
	B. adolescentis	3			1	2						
	B. dentium	5			3	1	1					
	B. animalis subsp. *animalis*	3						1		1		1
	B. animalis subsp. *lactis*	4							1	3		
Minocycline	*B. longum* longum type	30	1	7	10	2	2	2	1	5		
	B. longum infantis type	4	1				2			1		
	B. pseudocatenulatum	12		4	2	1				4	1	
	B. breve	14	1	4	7				1	1		
	B. angulatum	2			2							
	B. bifidum	12		4	3	1			1	3		
	B. adolescentis	3		2	1							
	B. dentium	5	1	2	2							
	B. animalis subsp. *animalis*	3				1				1	1	
	B. animalis subsp. *lactis*	4							3	1		
Doxycycline	*B. longum* longum type	30		1	8	10	1	1	3	5	1	
	B. longum infantis type	4			1			2		1		
	B. pseudocatenulatum	12			4	2	1			2	3	
	B. breve	14	2		7	3				2		
	B. angulatum	2			2							
	B. bifidum	12			5	3		1		2	1	
	B. adolescentis	3			2	1						
	B. dentium	5		2	3					1	1	
	B. animalis subsp. *animalis*	3					1			1	1	
	B. animalis subsp. *lactis*	4							2	2		

The presence of both these *tet* genes was not associated with an MIC that was higher than the MICs for all strains that contained only *tet*(W) or *tet*(M), for which the MIC of tetracycline was 64 mg/liter. This suggests that a need for an increased level of tetracycline resistance is not the selective pressure for the presence of more than one gene and is consistent with genetic events in the dissemination of *tet* resistance genes that are independent of antibiotic pressure.

TABLE 2. Distribution of *tet* genes in *Bifidobacterium* isolates

Bacteria	No. of isolates	No. (%) of isolates with the following resistance gene(s):			
		tet(W)	*tet*(M)	*tet*(O)	*tet*(W) and *tet*(M)
All *Bifidobacterium* isolates	89	23 (26)	5 (6)	0	1 (1)
Human *Bifidobacterium* isolates					
B. longum longum type	30	6 (20)	2 (6)	0	0
B. longum infantis type	4	1 (25)	0	0	0
B. pseudocatenulatum	12	5 (41)	0	0	0
B. breve	14	1 (7)	2 (14)	0	1 (7)
B. angulatum	2	0	0	0	0
B. bifidum	12	4 (33)	1 (8)	0	0
B. adolescentis	3	0	0	0	0
B. dentium	5	0	0	0	0
B. animalis subsp. *lactis*	4	4 (100)	0	0	0
Environmental *Bifidobacterium* isolates					
B. animalis subsp. *animalis*	3	2 (67)	0	0	0

Partial sequencing (495 nucleotides) of the *tet*(W) genes of 12 tetracycline-resistant isolates revealed that the nucleotide sequences exhibited 98 to 100% identity to an internal fragment (nucleotides 330 to 825) of the *tet*(W) genes of *Butyrivibrio fibrisolvens* (1) and *B. longum* (5). The partial sequences (500 nucleotides) of the *tet*(M) genes of two tetracycline-resistant isolates exhibited 97% identity with *Enterococcus faecalis* and *Streptococcus pneumoniae tet*(M) genes (GenBank accession no. AY466395 and AJ585081, respectively). The high level of sequence identity between the *tet*(W) genes of bifidobacteria and the rumen anaerobe *B. fibrisolvens* or between the *tet*(M) genes of bifidobacteria and *E. faecalis* suggests that horizontal gene transfer occurred.

The *tet*(W) or *tet*(M) locus is thought to be located on the bacterial chromosome since when strains were found to harbor plasmids, no *tet* genes could be amplified by PCR (data not shown). For all strains, chromosomal localization of *tet*(W) or *tet*(M) was assessed under standard conditions by hybridization of PvuII-digested total DNA using a 1,200-bp PCR fragment of *tet*(M) or *tet*(W) as a chemiluminescently labeled probe (ECL kit; Amersham, Sacley, France). Southern blots contained single hybridization bands at 3,000 to 5,000 bp for tetracycline-resistant *Bifidobacterium* strains carrying *tet*(W) (Fig. 1) or

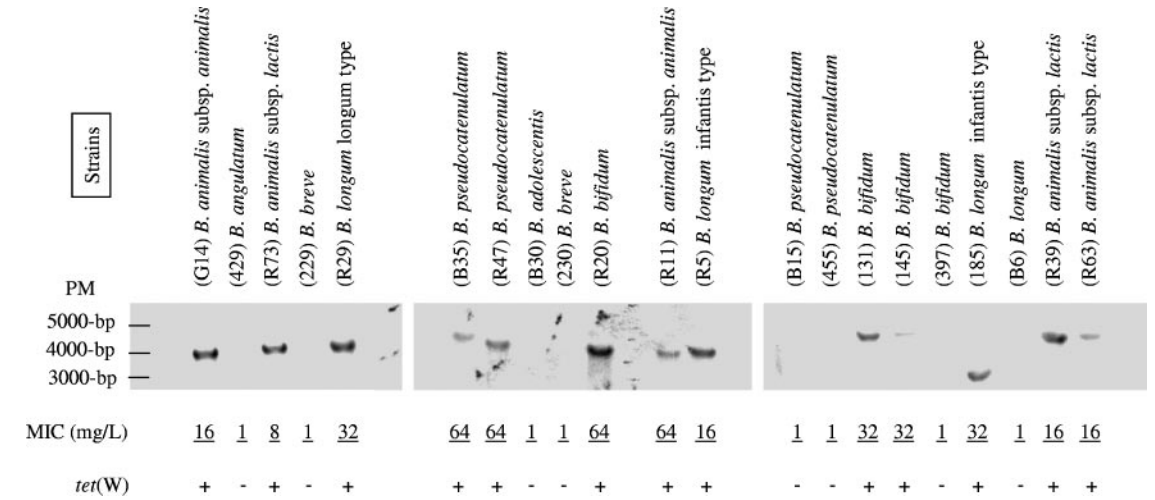

FIG. 1. MICs of tetracycline and *tet*(W)-specific hybridization patterns of PvuII-restricted chromosomal DNA of *Bifidobacterium* isolates. Not all strains analyzed in this study are included. PM, molecular weight.

tet(M) (data not shown), which was consistent with the MICs and the PCR results. The fact that the *tet*(W) hybridization signal appeared at different positions suggests that there is variability in the DNA region containing the *tet* genes and/or in the genetic transfer mechanism(s).

This study is the first study showing a high prevalence and wide distribution of acquired resistance to tetracyclines due to ribosomal protection proteins in human *Bifidobacterium* isolates and three strains from the environment. The findings suggest that bifidobacteria in the human gastrointestinal tract have access to tetracycline resistance genes and may be involved in their dissemination. However, when we investigated the possible transfer of *tet*(W) among *Bifidobacterium* isolates by conducting conjugations experiments, preliminary results showed that there were no transconjugants (data not shown). How *tet* genes are maintained and disseminate through bifidobacteria needs to be addressed. Indeed, *Bifidobacterium* is of special interest because several *Bifidobacterium* strains are used as probiotics and because of general concern concerning the safety of probiotics (i.e., the potential transferability of antibiotic resistance determinants).

Nucleotide sequence accession numbers. The accession numbers for the partial nucleotide sequences of the *tet*(W) genes that have been deposited in the GenBank database are as follows: DQ988358 and DQ988363 for *Bifidobacterium animalis* subsp. *animalis*; DQ988360, DQ988361, and DQ988362 for *B. animalis* subsp. *lactis*; DQ988353 and DQ988359 for *B. longum* longum type; DQ988357 for *B. longum* infantis type; DQ988352 for *B. breve*; and DQ988354, DQ988355, and DQ988356 for *B. bifidum*.

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