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Mosaic Tetracycline Resistance Genes and Their Flanking Regions in *Bifidobacterium thermophilum* and *Lactobacillus johnsonii*▽

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For the first time, mosaic tetracycline resistance genes were identified in *Lactobacillus johnsonii* and in *Bifidobacterium thermophilum* strains. The *L. johnsonii* strain investigated contains a complex hybrid gene, *tet*(O/W/32/O/W/O), whereas the five bifidobacterial strains possess two different mosaic *tet* genes: i.e., *tet*(W/32/O) and *tet*(O/W). As reported by others, the crossover points of the mosaic *tet* gene segments were found at similar positions within the genes, suggesting a hot spot for recombination. Analysis of the sequences flanking these genes revealed that the upstream part corresponds to the 5′ end of the mosaic open reading frame. In contrast, the downstream region was shown to be more variable. Surprisingly, in one of the *B. thermophilum* strains a third *tet* determinant was identified, coding for the efflux pump Tet(L).

Tetracycline is an antimicrobial agent that is active against a wide range of gram-positive as well as gram-negative bacteria. Consequently, it is used therapeutically in the treatment of various infections in both humans and animals. Furthermore, it was also often applied as a growth promoter in several countries inside and outside Europe (7). However, since the European Union ban on antibiotics as growth promoters, tetracycline has been used primarily as a therapeutic antimicrobial. In comparison to other antibiotics like trimethoprim/sulfonamides and macrolides, sales of tetracycline have increased the most since the prohibition in 1999 (5). Therefore, it is not surprising that tetracycline-resistant bacteria are very prevalent in all kinds of habitats: e.g., oral cavities, soils, and intestinal tracts of humans and animals (1, 7). Resistance to tetracycline can be mediated by different mechanisms: the most common are efflux pumps, ribosome protection proteins, and enzymatic inactivation (23).

New *tet* genes keep appearing in literature: for example, very recently *tet*(41) was reported (27). Currently 40 different tetracycline resistance genes (including oxytetracycline-resistant determinants) have been identified (7, 22, 23). Furthermore, during the last decade also mosaic *tet* genes, primarily encoding for ribosomal protection proteins (RPP), have been characterized in different bacterial species. For instance, intraclass mosaic structures have been described in *tet*(M) (10, 21). Moreover, multiple interclass hybrid genes originating from *tet*(O) and *tet*(W) have been discovered in *Megasphaera elsdenii* (24, 25) and a *tet*(O/32/O) gene was identified in *Clostridium* strain K10, although initially described as *tet*(32) (18, 25). It remains unclear what the actual role of these interclass RPP mosaic genes is. It has been suggested that these hybrid genes might be restricted to only a very small group of (anaerobic) bacteria (23); however, recently they have been shown to be widespread and abundant (22).

In a recent study, tetracycline-resistant *Bifidobacterium thermophilum* strains from animal sources have been described (17). PCR analysis revealed the possible presence of both *tet*(O) and *tet*(W) in some of the strains analyzed. In this study, these strains, together with *Lactobacillus johnsonii* L0077, a strain from human intestine which was also suspected to harbor a mosaic gene in a previous survey (2), were analyzed in more detail to investigate the potential presence of hybrid genes composed of *tet*(O) and *tet*(W) segments.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. The strains used in this study are indicated in Table 1. The bifidobacteria were grown in brain heart infusion broth containing 0.05% cysteine-HCl. The *Lactobacillus johnsonii* L0077 strain was grown in MRS broth supplemented with 0.03% cysteine-HCl. Both were incubated in an anaerobic chamber at 37°C for 48 h. DNA was isolated using the Wizard Genomic DNA isolation kit according to the manufacturer's protocol for gram-positive bacteria (Promega Benelux, Leiden, The Netherlands).

The *B. thermophilum* strains were differentiated by the BOX primer PCR fingerprinting technique as described by Masco et al. (15).

PCR. PCRs with specific primers for *tet*(32), *tet*(O), and *tet*(W) were performed in a total volume of 50 μl containing approximately 40 ng of bacterial DNA, 10 pmol of each primer (Table 2), 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), and 2.5 U *Taq* DNA polymerase recombinant (Invitrogen BV, Breda, The Netherlands). The following PCR program was used: 95°C for 3 min and then 35 cycles of 95°C for 30 s, 58 or 60°C for 30 s, and 72°C for 30 or 60 s, ending with 72°C for 10 min. The annealing temperature depends on the melting temperature (T_m) of the primer pair, whereas the extension time was determined by the expected product length: i.e., fragments shorter than 1,400 bp had an extension period of 30 s and longer products had an extension period of 60 s. The obtained PCR fragments were analyzed by electrophoresis on a 1 to 2% agarose gel, depending on the product sizes, stained with ethidium bromide, and visualized with UV light.

PCR with the DI-DII primers was performed according to Clermont et al. (8) in a total volume of 50 μl containing approximately 40 ng of bacterial DNA.

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TABLE 1. Bacterial strains analyzed in this study

ACE-ART strain no.	Original no.	Species	Origin	Yr of isolation	Phenotypic tetracycline MIC (μg/ml)
B0219	B173	*B. thermophilum*	Environmental sample from pig slaughterhouse	2001	128[a]
B0241	B187	*B. thermophilum*	Pig feces	2002	128[a]
B0242	B290	*B. thermophilum*	Pig feces	2002	256[a]
B0253	B226	*B. thermophilum*	Pig feces	2002	128[a]
B0256	B315	*B. thermophilum*	Pig feces	2002	128[a]
L0077	G41	*L. johnsonii*	Human feces	2001	>256[b]

[a] Determined by broth microdilution (17).
[b] Determined by Etest (2).

PCR-RFLP analysis. PCR-restriction fragment length polymorphism (RFLP) was performed on 2 μl of DI-DII PCR product using 10 U of the chosen restriction enzyme (New England Biolabs) and the recommended buffer at the appropriate temperature for 2 h. The restriction fragments were separated on a 1.5% agarose gel. The size of DNA fragments was estimated in comparison with two markers: a 100-bp DNA ladder (New England Biolabs) and a 500-bp DNA ladder (Invitrogen BV, Breda, The Netherlands).

Inverse PCR. The inverse PCR was carried out on the *B. thermophilum* strains according to the principle described by Ochman et al. (20). In total, 12 different restriction enzymes were used: i.e., BclI, ClaI, HindIII, KpnI, NcoI, NheI, NsiI, PvuI, SalI, TaqI, XbaI, and XmnI. In the digestion, 20 ng of genomic DNA was used together with 10 U of the endonuclease in the buffer specified by the supplier (New England Biolabs) in a total volume of 20 μl. Intramolecular ligation was performed using 5 μl digested DNA, 1× ligation buffer, and 200 U T4 DNA ligase (New England Biolabs) in a total volume of 200 μl at 4°C for at least 16 h. The ligated DNA was precipitated, collected by centrifugation, and dissolved in 100 μl sterile water. The inverse PCR was carried out in a total volume of 50 μl using 2 μl of ligated DNA, 10 pmol of each primer (various divergent primer pairs were used, as indicated in Table 2), 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, and 2.5 U *Taq* DNA polymerase recombinant (Invitrogen BV, Breda, The Netherlands). The following PCR program was used: 95°C for 3 min and 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 3 min, and 72°C for 10 min.

Sequence analysis. The various (inverse) PCR fragments were cloned in the pGEM-T Easy vector (Promega Benelux BV, Leiden, The Netherlands) and transformed into *Escherichia coli* XL2-Blue ultracompetent cells (Stratagene Europe, Amsterdam, The Netherlands). Plasmid DNA was isolated with a QIAprep Spin Miniprep kit (QIAGEN Benelux B.V., Venlo, The Netherlands). DNA sequencing was carried out with the GenomeLab methods development kit dye terminator cycle sequencing chemistry protocol and analyzed on a CEQ 2000 DNA analysis system (Beckman Coulter [Nederland] B.V., Mijdrecht, The Netherlands). Multiple clones were analyzed for each strain and PCR fragment. The Seaview software program (9) freely available by anonymous FTP at http://pbil.univ-lyon1.fr/software/seaview.html was used to align the various sequenced fragments.

Nucleotide sequence accession number. The nucleotide sequences of the *tet*(O/W) and *tet*(W/32/O) genes and their flanking regions from the five *B. thermophilum* strains have been deposited in the EMBL nucleotide sequence database under accession no. AM889118 to AM889122 and AM710601 to AM710605, respectively. The sequence of the *tet*(O/W/32/O/W/O) gene of *L. johnsonii* L0077 has also been submitted (DQ525023).

TABLE 2. Primers used in this study

Primer[a]	Sequence (5′→3′)
BOX[b]	CTACGGCAAGGCGACGCTGACG
DI[c]	GAYACICCIGGICAYRTIGAYTT
DII[c]	GCCCATWAIGGRTTIGGIGGIACYTC
tet32 1107F	TGATACAGACCCTCTTTTGC
tet32 1254R*	AACCGAAGGCTCTTTCATAG
tetO −372F	ACAACCGATTAGTGGCAGG
tetO −204F	AAGTAGCAGTCCCGTTTCAC
tetO 14F	ACTTAGGCATTCTGGCTCAC
tetO 144F	GAGCGTCAAAGGGGAATC
tetO 161R	ATTCCCCTTTGACGCTCC
tetO 1368F*	CGGAGTGCAGTATGAAAGC
tetO 1798F*	CAGGGAGTCTGCTTGACAG
tetO 1917R	GCTAACTTGTGGAACATATGC
tetW −609F	CGCCAGCACTACACTATTC
tetW −207F	ATAGCTCCTTTTGTAGGGGC
tetW-Fw$_d$	GAGAGCCTGCTATATGCCAGC
tetW 61R*	CCGTCAAGGTCGTCTTTCC
tetW 384F*	CAAGATCGACCAGGCTGGCG
tetW 589R	GGCTGATTGGTTCTCCTGCG
tetW 1278F	AGCAGCCAGCCACACCATC
tetW 1757R	ATACAGCGGGCGGGAATCTC
tetW 1890R	TTGTCCAGGCGGTTGTTTGG

[a] The number in the primer name indicates the start position of the primer within the specific gene. Primers with an asterisk were used for inverse PCR.
[b] See reference 15.
[c] See reference 8.

RESULTS

Identification of mosaic genes. The mosaic tetracycline resistance genes identified in the first instance by PCR in five *B. thermophilum* strains and one *L. johnsonii* strain were sequenced. A complete open reading frame (ORF) was found for these mosaic genes, and they contained parts of *tet*(O), *tet*(W), and/or *tet*(32). In the *B. thermophilum* strains, the mosaic structure was *tet*(W/32/O). In contrast, the gene identified in the *L. johnsonii* L0077 strain was far more complex, *tet*(O/W/32/O/W/O). Homology analysis revealed that the *tet*(W/32/O) genes are very similar (>99.8%) or even identical (B0242 and B0253). BOX-PCR, a DNA fingerprinting technique targeting repetitive genomic elements, was used to exclude clonality of the strains B0242 and B0253. The obtained patterns clearly demonstrated that these two strains are different, as are the other three *B. thermophilum* strains (results not shown). The few dissimilarities between the *tet*(W/32/O) of the five *B. thermophilum* bacteria seem to be randomly distributed within the mosaic gene, with one exception: the stop codon of strain B0241 is TGA, whereas the others end with TAA, as do all other known *tet*(O)/*tet*(W) hybrids.

A schematic representation of the identified mosaic *tet* genes clearly demonstrates that the *tet*(W/32/O) and *tet*(O/W/32/O/W/O) have similar crossover points (Fig. 1).

Upstream region of the *tet*(O/W/32/O/W/O) gene. The 5′-end flanking sequence of the *L. johnsonii* strain was investigated by various PCR tests. Two PCR tests using primers directed against sequences at approximately 400 and 200 nucleotides (nt), respectively, upstream of known *tet*(O) genes in combination with a reverse primer binding to sequences located within the mosaic gene resulted in the amplification of the

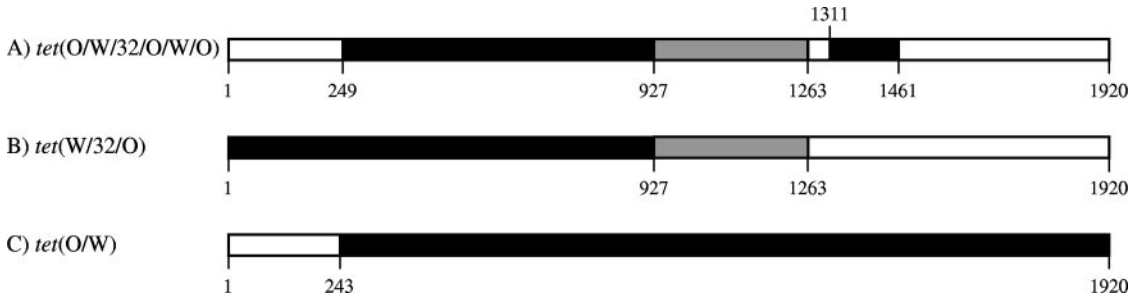

FIG. 1. Schematic depiction of the different mosaic *tet* genes identified in *L. johnsonii* (A) and *B. thermophilum* (B and C). Black bars indicate regions with very high sequence identity to known *tet*(W) genes. Gray sections represent parts with high homology to *tet*(32), whereas open bars symbolize regions with high sequence identity to known *tet*(O) genes. The different crossover positions are shown.

correct PCR fragments (Table 3, PCR tests 1 to 4 and 17). In contrast, PCRs identifying approximately 600 bp and 200 bp of the upstream region of *tet*(W) did not result in any amplicons (results not shown).

Flanking regions of the *tet*(W/32/O) genes. Inverse PCR was used to determine up- and downstream sequences of *tet*(W/32/O) in the five *B. thermophilum* strains. From the 12 different restriction enzymes tested only with HindIII and TaqI, fragments of the flanking regions were retrieved. The DNA sequences obtained are schematically represented in Fig. 2. The upstream regions of four *tet*(W/32/O) genes are identical and very similar to the approximately 600-bp region commonly found in front of various *tet*(W) genes (12). Unfortunately, inverse PCR did not give sequence data of this upstream part in the B0241 strain. Moreover, in contrast to the other four strains, PCR tests amplifying 600 bp and 200 bp, respectively, of the 5′ flanking region of *tet*(W) genes did not result in the amplification of the expected PCR fragments (data not shown), indicating that this part is different in B0241.

The *B. thermophilum* strain isolated in 2001 (B0219) has clearly different downstream sequences from the isolates from the year 2002. Inverse PCR demonstrated that besides the *tet*(W/32/O) gene, this strain also seems to possess another tetracycline resistance gene, coding for the efflux pump Tet(L) (Fig. 2). This result was confirmed by microarray analysis (16, 29), PCR (data not shown), and sequence analysis of a complete *tet*(L) ORF. In between these two resistance genes, an ORF is present which has a high sequence identity (99%) with the *cpp2* gene found on the tetracycline resistance plasmids of two different *Campylobacter* species, where it is localized downstream of *tet*(O) (3).

In contrast, the *tet*(W/32/O) gene of the *B. thermophilum* strains B0241, B0242, and B0256 is followed by two ORFs coding for a so-far-unknown protein and a transposase gene, respectively. Unfortunately, this sequence information was not retrieved for the B0253 strain.

PCR-RFLP. Amplified DNA fragments of 1.1 kb obtained with the primer pair DI-DII (localized at approximately 219

TABLE 3. PCR results with various *tet*(32), *tet*(O), and *tet*(W) primers

PCR test	Forward primer	Reverse primer	Annealing T_m (°C)	Product length (bp)	Presence/absence of strain(s)			
					B. thermophilum	*L. johnsonii*	*tet*(W)	*tet*(O)
1	tetO −372F	tetO 161R	58	533	+	+	−	+
2	tetO −204F		58	365	+	+	−	+
3	tetO −372F	tetW 589R	58	961	+	+	−	−
4	tetO −204F		58	793	+	+	−	−
5	tetO 144F		58	445	+	+	−	−
6	tetO −372F	tetW 1757R	58	2,129	+	−	−	−
7	tetO −204F		58	1,961	+	−	−	−
8	tetW-Fw		60	1,695	−	−	+	−
9	tetO 144F		58	1,613	+	−	−	−
10	tetW 384F		60	1,373	+	−	+	−
11	tetW 1278F		60	479	+	−	+	−
12	tetO −204F	tetW 1890R	58	2,094	+	−	−	−
13	tetO 14F		60	1,876	+	−	−	−
14	tetO 144F		58	1,746	+	−	−	−
15	tetW 384F		60	1,506	+	−	+	−
16	tetW 1278F		60	612	+	−	+	−
17	tetO −204F	tetO 1917R	58	2,121	−	+	−	+
18	tetO 14F		60	1,903	−	+	−	+
19	tetW-Fw		60	1,855	+	−	−	−
20	tetO 144F		58	1,773	−	+	−	+
21	tetW 384F		60	1,533	+	+	−	−
22	tet32 1107F		58	810	+	+	−	−
23	tetW 1278F		60	612	−	−	−	−

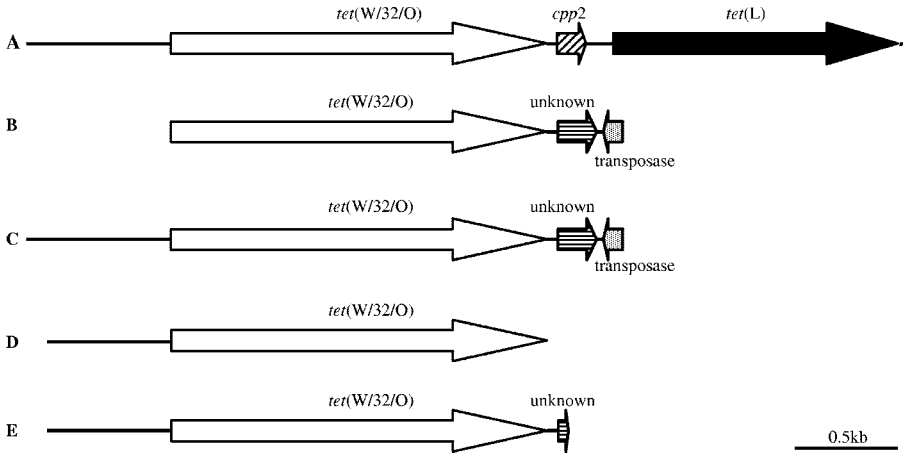

FIG. 2. Schematic representation of *tet*(W/32/O) and flanking regions in different *B. thermophilum* strains: A, B0219; B, B0241; C, B0242; D, B0253; and E, B0256. The overall sequence lengths determined were as follows: B0219, 4,339 nt; B0241, 2,290 nt; B0242, 2,991 nt; B0253, 2,520 nt; and B0256, 2,623 nt.

and 1,328 bp from the start codon of the RPP genes) generated a slightly different pattern after gel electrophoresis for the *B. thermophilum* strains: i.e., several vague larger fragments (results not shown). This phenomenon was further investigated by RFLP of the generated fragments. Besides the *B. thermophilum* and *L. johnsonii* strains, two other bacteria were included: i.e., *B. thermophilum* LMG 21813^T containing *tet*(W) and *Bifidobacterium bifidum* B0045 with *tet*(O) (2, 17). The RFLP of the DI-DII products of the *L. johnsonii tet*(O/W/32/O/W/O), *B. thermophilum tet*(W), and *B. bifidum tet*(O) only generated fragments that were expected based on the analysis of the gene sequence using the REBsites software program (http://tools.neb.com/REBsites; results not shown). In contrast, RFLP of the DI-DII products of the five *B. thermophilum* strains generated fragments corresponding to the expectations for the *tet*(W/32/O) gene; however, a *tet*(W) restriction pattern was also found in these bacteria. To investigate this in more detail, a number of different PCR tests were performed using various different *tet*(32), *tet*(O), and *tet*(W) primers (Table 2). A summary of the PCRs performed is shown in Table 3. The presence of the *tet*(W/32/O) was indicated by PCR tests 19, 21, 22, and 23, whereas all other PCR tests clearly demonstrated the existence of an additional tetracycline resistance gene in the *B. thermophilum* strains with a probable mosaicism of *tet*(O/W) (Fig. 1) preceded by a region similar to 5′ flanking sequences of *tet*(O) (PCR tests 1 to 4, 6, 7, and 12). The absence of *tet*(32) sequences in this mosaic gene was shown by RFLP analysis of the fragments obtained with PCR tests 3, 4, and 12 (Table 3) using more than 10 different restriction enzymes. All digestion results corresponded with a pattern expected for a *tet*(O/W) gene (results not shown). This was confirmed by sequence analysis revealing a crossover point at position 243 (Fig. 1), similar to the previously described *tet*(O/W)-*2* gene in *Megasphaera elsdenii* (26).

DISCUSSION

Tetracycline resistance genes have been identified in both gram-positive and gram-negative bacteria. Besides the 40 thoroughly characterized *tet* genes, mosaic genes also have been reported. For example, more than 10 different *tet*(O)/*tet*(W) hybrid genes have been currently recognized (22, 24–26). Most of the bacteria harboring these mosaic genes were isolated from the intestinal tract of pigs. The *B. thermophilum* strains investigated in this study also originate from pig intestines, while the *L. johnsonii* isolate came from human feces. The DNA sequence of the mosaic gene determined in *L. johnsonii* was identified as *tet*(O/W/32/O/W/O), whereas in five *B. thermophilum* strains a *tet*(W/32/O) ORF was characterized. Subsequently and very surprisingly, a second mosaic gene, *tet*(O/W), was demonstrated in these bifidobacteria. The more complex *tet*(O/W/32/O/W/O) gene was nearly identical to a mosaic *tet* gene recently recovered from a *tet*(O)-based clone library of pig feces by Patterson et al. (22): i.e., 99.9% identity on the DNA sequence level and 99.7% identity on the deduced amino acid level. Phylogenetic analysis of the mosaic *tet* sequences identified in this study with related RPP genes including other hybrid genes indicated that they are closely related. The nearest nonmosaic RPP relative is *tet*(W), whereas *tet*(O) and *tet*(32) are more distantly related (data not shown).

The investigation of the upstream sequences showed that this region corresponds to the first part of the mosaic ORF. For example, the *tet*(O/W/32/O/W/O) and *tet*(O/W) genes are preceded (i.e., nearly 400 bp) by sequences found in front of several *tet*(O) genes (Table 3). Since Wang and Taylor (30) described that this DNA sequence upstream of the *tet*(O) gene is required for full expression of tetracycline resistance, it seems likely that the mosaic genes *tet*(O/W/32/O/W/O) and *tet*(O/W) are expressed. A similar situation was identified for the *tet*(W/32/O) genes. In four out of five strains, the gene is flanked at the 5′ end by sequences (up to 600 bp) nearly 100% identical and commonly found upstream of *tet*(W) in several different bacterial species (4, 12) and required for full expression (18). The upstream region of the *tet*(W/32/O) gene of the B0241 strain appeared to be different, and furthermore, the stop codon of this gene also differed from the rest (TGA versus TAA). These results could indicate that the other identified hybrid gene, *tet*(O/W), might be the most active component in B0241; however, this was not supported by the phenotypes of

the bifidobacteria investigated (Table 1). Based on the obtained results of the flanking sequences, it is most likely that these hybrid genes arose from the interclass recombination within the coding regions of the RPP genes *tet*(O) and *tet*(W). Detailed characterization of the coding sequences confirms this fact and reveals preferential crossover positions (Fig. 1), which was also demonstrated by other studies (22, 25, 26).

Various methods (several PCR tests, PCR-RFLP, and sequencing) demonstrated the unexpected presence of an additional mosaic tetracycline resistance gene in the *B. thermophilum* strains investigated, showing a mosaicism of *tet*(O/W) (Fig. 1). To our knowledge, this is the first description of multiple mosaic *tet* genes within one bacterium, although this could also be the case in the study by Patterson et al. (22), since individual bacteria were not isolated from the pig and human feces, with the exception of the *tet*(32) gene-containing human oral strain, *Streptococcus salivarius*. Very surprisingly, one of the *B. thermophilum* strains (i.e., B0219), besides the two mosaic RPP genes also seems to possess an additional *tet* determinant, *tet*(L), coding for an efflux pump (Fig. 2). However, the presence of three *tet* genes did not result in an extremely high phenotypic tetracycline resistance profile in this isolate (Table 1).

The large diversity of mosaic genes identified in bacteria isolated from the intestinal tract of pigs (references 22 and 24 and this study) and the fact that these strains demonstrate a high level of tetracycline resistance (for resistance levels of the parent genes, see reference 26) clearly suggest the need to have a closer look at the use of tetracycline in pig husbandry as also shown by other studies of swine production facilities and pigs' waste treatment systems (6, 11, 14). Furthermore, since, cooking procedures for meat, even to "well done," cannot be relied on to completely inactivate even the more heat-sensitive tetracyclines (13, 19, 28), consumption of tetracycline-containing meat and meat products might further drive selection of tetracycline-resistant bacteria in the human intestines.

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