Isolation of bacteria from the ileal mucosa of TNF$\Delta$ARE mice and description of Enterorhabdus mucosicola gen. nov., sp. nov.

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The diversity of bacteria associated with inflamed mucosa was investigated by culturing ileal samples from TNF$\Delta$ARE mice on a selective medium containing mucin. Among eight isolates, two strains (Mt1B3 and Mt1B8$^\top$) belonged to bacterial groups not yet cultured from the mouse intestine. Whereas strain Mt1B3 was identified as a member of the family Planococcaceae and is closely related to Sporosarcina species and Filibacter limicola DSM 13886$^\top$, strain Mt1B8$^\top$ was a novel bacterium. Based on phylogenetic analysis, strain Mt1B8$^\top$ is a member of the family Coriobacteriaceae. The closest relatives with validly published names were Asaccharobacter celatus, Adlercreutzia equolifaciens (<96% similarity) and Eggerthella species (<92%). With respect to Asaccharobacter celatus and Eggerthella, the phylogenetic position of strain Mt1B8$^\top$ was confirmed at the chemotaxonomic level by Fourier-transform infrared spectroscopic analysis. The major fatty acid of strain Mt1B8$^\top$ is C$_{16}:0$ (23.9%). Menaquinones were monomethylated. DNA–DNA relatedness between strain Mt1B8$^\top$ and Asaccharobacter celatus DSM 18785$^\top$ was 28%. Strain Mt1B8$^\top$ is a Gram-positive-staining rod that does not form spores and has a high DNA G+C content (64.2 mol%). Cells are aerotolerant but grow only under strictly anoxic conditions. They are sensitive to cefotaxime, clarithromycin, erythromycin, metronidazole, tetracycline, tobramycin and vancomycin. API and VITEK analysis showed the ability of strain Mt1B8$^\top$ to convert a variety of amino acid derivatives. According to these findings, it is proposed to create a novel genus and species, Enterorhabdus mucosicola gen. nov., sp. nov., to accommodate strain Mt1B8$^\top$. The type strain of Enterorhabdus mucosicola is Mt1B8$^\top$ (=DSM 19490$^\top$ =CCUG 54980$^\top$).

The pioneering work by Dubos & Schaedler (1960) demonstrated the importance of intestinal bacteria to host physiology. Since then, it has been acknowledged that intestinal bacteria play a crucial role in inflammatory bowel diseases (IBD) (Manichanh et al., 2006). Taking into account that the intestinal epithelium is the first line of defence against luminal stimuli and is important for the regulation of innate and adaptive immune responses (Clavel & Haller, 2007), bacteria living in contact with the intestinal mucosa may be of particular interest (Conte et al., 2006; Derrien et al., 2004). Although Mus musculus is one of the most widely used laboratory animals, knowledge of its intestinal microbiota is scant. Recent advances in molecular microbiology have revealed the presence of uncultured novel bacterial groups in the mouse intestine (Apajalahti et al., 2002; Salzman et al., 2002). To date, little
is known about the intestinal microbiota in mouse models of IBD and work has been focused mainly on interleukin-2- and interleukin-10-deficient mice (Bibiloni et al., 2005; Duck et al., 2007; Pena et al., 2004; Schupper et al., 2004; Swidsinski et al., 2005; Ye et al., 2008). Only two of these studies used culture-based techniques (Duck et al., 2007; Pena et al., 2004) and three included analyses of mucosa-associated bacteria (Schupper et al., 2004; Swidsinski et al., 2005; Ye et al., 2008). In that context, the aim of the present study was to isolate mucosa-associated bacteria from inflamed ileal samples obtained from TNFδARE mice (Kontoyiannis et al., 1999). Furthermore, we focused on the genotypic and phenotypic description of the novel bacterium Mt1B8T.

Eight bacterial strains (Mt1B1–Mt1B7 and Mt1B8T) were isolated on a selective mucus-containing medium (Mt1) from two distal ileal samples obtained from TNFδARE C57BL/6 mice. Animal use was approved by the local institution in charge (approval no. 55.2-1-54-2531-74-06; Regierung von Oberbayern). Mice were fed a standard animal diet (V1534-000 R/M-H; Ssniff, Soest). Two female TNFδARE mice were sacrificed by neck dislocation at the age of 12 weeks. Previous experiments performed in our laboratory showed that TNFδARE mice develop mild inflammation in the distal ileum at the age of 12 weeks (mean histological score 5.2 ± 1.3, n = 3). Histological scores, ranging from 0 (not inflamed) to 12 (highly inflamed), were determined by assigning points to specific pathological criteria, as described by Katakura et al. (2005). One distal ileal segment (approx. 3 mm long) from each mouse was stored for 30 min on ice in filter-sterilized PBS (1−1: 8.60 g NaCl, 0.87 g Na2HPO4, 0.40 g KH2PO4, pH 7.2) supplemented with 0.02 % (w/v) peptone from meat (2366; Roth) and 0.05 % (w/v) l-cysteine (PBS-PC). All steps were carried out under sterile conditions. Briefly, PBS-DTT was removed using a pipette and samples were vortexed in 500 μl PBS supplemented with DTT (0.016 %, w/v) for 5 min at room temperature using a Vortex-Genie 2 (Scientific Industries) set to ‘Shake 1’. Subsequently, the PBS-DTT was removed by pipette and replaced by a volume of 500 μl PBS-PC. Eppendorf tubes were shaken by hand for about 15 s. After removal of the PBS-PC, two additional washing steps in PBS-PC were performed. Finally, samples were vortexed in distilled water for 30 min using a Vortex-Genie 2 set to ‘Vortex 4’. Undiluted and 10-fold dilution series (10−1−10−3) of the cell suspensions (100 μl each) were plated in triplicate on the selective medium Mt1 [l−1: 5 g mucin (M1778; Sigma), 500 mg l-cysteine, 1 mg yeast extract, 20 μg folic acid, 20 μg vitamin B12, 50 mmol NaHCO3, 10 mmol sodium acetate, 5 mmol Na2HPO4, 5 mmol NaCl, 3 mmol KH2PO4, 1 mmol CaCl2, 1 mmol MgCl2, 10 μmol FeCl3, 1 % (w/v) agar]. The pH of the medium was 7.7 prior to autoclaving (121 °C, 15 min). Mucin was first dissolved in 100 ml of 5 % (v/v) ethanol in distilled water. The final concentration of ethanol in Mt1 was 0.5 % (v/v). For each animal and each dilution, each of the triplicate plates was incubated at 37 °C under aerobic conditions in a humidified atmosphere containing 5 % CO2, at 30 °C, under micro-aerophilic conditions or at 37 °C under anaerobic conditions in jars using CampyGen or AnaeroGen catalysts (Oxoid). All colony morphology types observed after 9 days of growth were streaked on brain heart infusion (BHI) agar (211059; BD) supplemented with (1−1) 2 g yeast extract and 2 g glucose and 0.05 % (w/v) cysteine (GY-BHI-c) to support better growth and ensure purity. Culture purity was assessed as described previously (Clavel et al., 2007). Cryo-stocks (100 μl) were prepared by mixing bacterial suspensions with equal volumes of Tris-buffered aqueous solution (60 mM) containing 40 % glycerol. Cryo-stocks were stored at −80 °C after snap-freezing in liquid nitrogen. For characterization of strain Mt1B8T, unless otherwise stated, bacteria were grown in GY-BHI-c at 37 °C under strictly anoxic conditions (Attebery & Finegold, 1969). The gas phase was 100 % N2.

For phylogenetic analyses, DNA was extracted from bacterial cell pellets using the QIAamp DNA Stool Mini kit (Qiagen) according to the protocol for isolation of DNA from stools for pathogen detection. When required, the lysis temperature was increased to 95 °C. The 16S rRNA genes were amplified as described previously (Clavel et al., 2005). Amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced with primer 27f (Clavel et al., 2005) using the Qiagen sequencing service. The 16S rRNA gene of strain Mt1B8T was further sequenced using primers 338f, 338r and 1492r (Clavel et al., 2005). Gyrase B (gyrB) genes were amplified as described by Santos & Ochman (2004). Amplicons were purified as described above and sequenced using the primer gyrBBNDN1 (5′-CCGTTTACGTCGGCRTCNC-GYCAT-3′). 16S rRNA gene sequences of organisms closely related to the isolated strains (>90 % similarity) were obtained using the BLAST function of the NCBI server (Altschul et al., 1990) and the Ribosomal Database Project (Cole et al., 2003). Sequences were aligned using BLOSUM software version 7.0.5.3 (Hall, 1999). Percentages of similarity were calculated after unambiguous alignment of each isolated sequence with those of the most closely related species, using the DNADIST DNA distance matrix function.

The partial 16S rRNA gene sequence of strain Mt1B8T (1336 bp) showed 97.6 % similarity with the sequence of bacterium B7 (Duck et al., 2007), 95.9 % similarity with Adlercreutzia equolifaciens FJC-B9T (Maruo et al., 2008) and Asaccharobacter celatus do03T (Minamida et al., 2006, 2008), 95.7 % similarity with strain Julong732 (Wang et al., 2005) and <92 % similarity with Eggerthella species (Kageyama et al., 1999; Lau et al., 2004; Wade et al., 1999).

A rooted phylogenetic tree (Fig. 1) was constructed with CLUSTAL X 1.8 using the neighbour-joining method with bootstrap values calculated from 1000 resamplings. Groupings were confirmed using the maximum-parsimony method.
method. Of note, Adlercreutzia equolifaciens, Asaccharobacter celatus and strain Julong732 cluster together, whereas the sequence of strain Mt1B8T is similar to cloned sequences originating from the mouse intestine (Ley et al., 2005, 2006; Turnbaugh et al., 2006).

Supplementary Fig. S1 (available in IJSEM Online) shows the phylogenetic position of all of the eight newly isolated strains with closely related species and known strains of the mouse intestinal microbiota. Strains Mt1B1 and Mt1B2 and strains Mt1B4–Mt1B7 showed >98 % similarity with Escherichia coli ATCC 25922 and Lactobacillus murinus ASF361, respectively, while Mt1B3 and Mt1B8 belonged to so-far unidentified or uncultured bacterial groups within the mouse intestine. Based on partial 16S rRNA gene sequence analysis (714 bp), strain Mt1B3 was >99 % similar to cloned sequences originating from volcanic soil (GenBank accession no. DQ455577), compost (DQ345490) and sewage sludge (AB241601) (results not shown) and >98 % similar to Sporosarcina soli I80T and Sporosarcina koreensis F73T (Kwon et al., 2007), Sporosarcina aquimarina SAFN-008 (Yoon et al., 2001), Sporosarcina saromensis HG711 (An et al., 2007) and Filibacter limicola DSM 13886T (Maiden & Jones, 1984). Based on a partial sequence analysis of gyrB genes (547 bp), strain Mt1B3 was 79.4 % similar to Eggerthella lenta DSM 2243T. The next most closely related gyrB gene sequences available in the NCBI database were those of Streptomyces species (<60 % similarity) (Hatano et al., 2003). Apart from Eggerthella species, Asaccharobacter celatus DSM 18785T was the only described and related bacterium available as a pure culture. Thus, further analyses for description of strain Mt1B8T were performed using Asaccharobacter celatus DSM 18785T, Eggerthella lenta DSM 2243T and Eggerthella hongkongensis DSM 16106T.

Although there is, so far, no example to show that strains with less than 97 % 16S rRNA gene sequence similarity may exceed the proposed threshold of 70 % DNA–DNA relatedness for distinguishing species (Gevers et al., 2005), DNA–DNA hybridization experiments were carried out at the DSMZ according to standard methods (Cashion et al., 1977; De Ley et al., 1970; Huß et al., 1983; Mesbah et al., 1989; Tamaoka & Komagata, 1984; Visuvanathan et al., 1989). Hybridization of DNA from strain Mt1B8T with DNA from Asaccharobacter celatus DSM 18785T revealed relatedness values of 28 ± 2.1 % from duplicate experiments. These data support the hypothesis that strain Mt1B8T represents a novel bacterial genus. The DNA G + C content (64.2 mol%) is comparable to values reported in the literature for Eggerthella lenta and Asaccharobacter celatus.

Fourier-transform infrared (FT-IR) spectroscopy was used to evaluate phenotypic differences between strain Mt1B8T, Asaccharobacter celatus DSM 18785T, Eggerthella lenta DSM 2243T and Eggerthella hongkongensis DSM 16106T. As a whole-cell fingerprinting technique, FT-IR spectroscopy gives information on the overall biochemical composition of cells, and is a useful tool for species identification and strain typing (Oberreuter et al., 2002; Wenning et al., 2006, 2008). After revival from cryo-stocks, bacteria were cultured twice in 10 ml GY-BHI-c for 24 h. Prior to spectrometric analysis, cells were washed twice (8000 g, 3 min, room temperature) with filter-sterilized distilled water. Two independent experiments with different batches of medium were performed, each experiment with two independent cultures of each bacterium. Spectra were recorded using an IFS 28B spectrometer (Bruker Optics), as described by Kummerle et al. (1998). Spectral similarities were assessed by hierarchical cluster analysis using
OPUS version 5.5 (Bruker). FT-IR data showed clearly that strain Mt1B8\textsuperscript{T} is related less distantly to \textit{Asaccharobacter celatus} than to \textit{Eggerthella lenta} and \textit{Eggerthella hongkongensis} (Fig. 2) and supports the phylogenetic analysis. Additional chemotaxonomic analyses included the determination of whole-cell fatty acid composition by gas chromatography. For that purpose, bacteria were grown anaerobically at 37 °C on chocolate agar (C376; CCUG). Conditions for preparation of cell extracts and gas chromatography analysis are detailed in Sassé (1990) and detailed experimental information is available online (http://www.ccug.se). Fatty acid determination was done in duplicate. Comparison analyses with reference strains were done as described by Eerola & Lehtonen (1988). The complete cellular fatty acid profiles of strain Mt1B8\textsuperscript{T} and related organisms are available online (http://www.ccug.se). Cellular fatty acid analysis revealed that strain Mt1B8\textsuperscript{T} has a unique fatty acid profile within the family \textit{Coriobacteriaceae} (92% similarity with \textit{Eggerthella} species). The major fatty acid was C\textsubscript{16:0} (23.9 ± 1.7% of total fatty acids) (Table 1). In contrast, the major fatty acids in \textit{Adlercreutzia equilifaciens} and \textit{Asaccharobacter celatus} were C\textsubscript{17:1} iso I (24.9%) and C\textsubscript{18:1}\textsuperscript{w9c} (54.0%), respectively. Finally, whole-cell sugar, peptidoglycan, polar lipid and menaquinone analysis were performed at the DSMZ according to standard procedures (Rhuland \textit{et al.}, 1955; Staneck & Roberts, 1974; Whiton \textit{et al.}, 1985). The diaminoo acid in the peptidoglycan was identified as LL-diaminopimelic acid. Galactose and ribose were detected as whole-cell sugars. The major polar lipids were diphasatidylglycerol, phosphatidylglycerol, three unidentified phospholipids and four unidentified glycolipids (Supplementary Fig. S2). The major menaquinone was monomethylmenaquinone-6 (MMK-6) (100%).

For the phenotypic characterization of strain Mt1B8\textsuperscript{T} in liquid media, growth was monitored using McFarland turbidity standards and by measuring optical density at 600 nm. Cell morphology was determined by light microscopy after Gram-staining. The Gram-staining result was confirmed using the KOH lysis test (Gregersen, 1978).

To assess the temperature range for growth, cultures were incubated, in duplicate, at 25–45 °C, at intervals of 5 °C. The pH range for growth was tested, in duplicate, at initial pH values ranging from 5.0 to 9.5 at intervals of 0.5 pH units. The pH of GY-BHI-c was adjusted using aqueous solutions of HCl and NaOH and the medium was filter-sterilized. Spore formation and motility were examined as described previously (Clavel \textit{et al.}, 2007). For spore formation, strain Mt1B3 was used as a positive control. To determine enzymic features, bacterial suspensions were analysed with the Rapid ID 32A test and with ANI cards using the VITEK system (bioMérieux), following the manufacturer’s instructions. For both tests, cell suspensions were prepared from two independent batch cultures. Catalase, coagulase, indole and oxidase activities were tested using commercial reagents (Bactident and James; bioMérieux). Most phenotypic traits of strain Mt1B8\textsuperscript{T} are given below in the species description. In addition, strain Mt1B8\textsuperscript{T} grew on Mt1 agar, in contrast to \textit{Asaccharobacter celatus} DSM 18785\textsuperscript{T}. However, strain Mt1B8\textsuperscript{T} did not grow in GY-BHI-c in the presence of 0.5% (v/v) bile salts (48305; Fluka). \textit{Eggerthella lenta} DSM 2243\textsuperscript{T} was used as a positive control. The procedure that was used for isolation implied that strain Mt1B8\textsuperscript{T} could survive for at least 30 min at room temperature in the presence of atmospheric oxygen. However, strain Mt1B8\textsuperscript{T} did not survive after ethanol or heat treatment. In contrast, strain Mt1B3 survived after ethanol treatment and after 20 min at 60 °C and 10 min at 80 °C. Spores were not observed by microscopic analysis of cells of strain Mt1B8\textsuperscript{T} stained according to Schaeffer & Fulton (1933). The API and VITEK analyses showed that the only substrates that gave positive signals with strain Mt1B8\textsuperscript{T} were \textit{p}-nitroaniline and \textit{β}-naphthylamide derivatives of the following amino acids: L-alanine, L-glycine, L-histidine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine and L-tyrosine. The test for arginine dihydrolase was also positive.

The sensitivity of strain Mt1B8\textsuperscript{T} was tested towards 10 antimicrobial agents. All chemicals were obtained from Sigma and Oxoid. The medium was GY-BHI-c supplemented with 0.05% (v/v) arginine and 2% (v/v) agar. MICs were determined after 48 h at 37 °C in a miniMACS anaerobic workstation using Etest strips (Bio-Stat Diagnostics) according to the approved standard M11-A7 of the CLSI and standard BSAC methods (Andrews, 2007). Each antibiotic was tested in duplicate in three independent experiments and the MIC breakpoint was expressed as the mean of the six replicates. The MIC breakpoints were (\textmu g mL\textsuperscript{-1}): cefotaxime, 1.250 ± 0.112; ciprofloxacin, >32; clarithromycin, <0.016; colistin, >256; erythromycin, 0.048 ± 0.007; metronidazole, 0.034 ± 0.004; oxacillin, 4.667 ± 0.667; tetracycline, 0.115 ± 0.007; tobramycin, 2.667 ± 0.211; and vancomycin, 1.333 ± 0.105. Thus, strain Mt1B8\textsuperscript{T} is resistant to colistin, which interacts with the cytoplasmic membrane of Gram-negative bacteria. It is also resistant to ciprofloxacin, a broad-spectrum antibiotic.
often used in the treatment of IBD (Sartor, 2004). Strain Mt1B8T was susceptible to most of the other antimicrobial agents tested, with the exception of oxacillin, a narrow-spectrum β-lactam antibiotic. In contrast, *Eggerthella hongkongensis* DSM16106T is resistant to cefotaxime (Lau et al., 2004).

Because a number of bacteria belonging to the family *Coriobacteriaceae* convert phyto-oestrogens (Clavel et al., 2006; Minamida et al., 2006; Wang et al., 2005), we investigated the conversion of the isoflavones daidzein and genistein by strain Mt1B8T. Daidzein and genistein were purchased from Acros Organics and Roth, respectively. Stock solutions (20 mmol l\(^{-1}\)) were prepared in DMSO and filter-sterilized (Millex-GV filter; Millipore). Strain Mt1B8T was cultured as described above. BHI was not supplemented with glucose and yeast extract and the gas phase was H\(_2\):CO\(_2\) (80:20, v/v). For conversion experiments, 50 ml of the appropriate isoflavone stock solution and 200 ml of an overnight culture of strain Mt1B8T were added to 10 ml medium. Isoflavones and bacteria were incubated in medium separately as controls. Samples were taken over time with a syringe and centrifuged (14 000 g, 5 min). Supernatants (20 ml) were directly used for reversed-phase HPLC analysis as described by Schoefer et al. (2002). The mobile phases were 98 : 2 water/acetic acid (A) and methanol (B) in a gradient mode (5 to 55 % B over 15 min, 55 % B for 10 min, 55–100 % B over 3 min, 100 % B for 4 min). The flow rate was 0.8 ml min\(^{-1}\) and compounds were detected at 280 nm. Growing cells of strain Mt1B8T transformed daidzein completely within 24 h to form two metabolites, DM 1 and DM 2.

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**Table 1. Traits of strain Mt1B8\(^{\dagger}\) and related species**

Species: 1, *Enterorhabdus mucosicola* gen. nov., sp. nov. (strain Mt1B8\(^{\dagger}\)); 2, *Adlercreutzia equolifaciens* (data from Maruo et al., 2008); 3, *Asaccharobacter celatus* (Minamida et al., 2006); 4, *Eggerthella lenta* (unless indicated, data from Kageyama et al., 1999; Wade et al., 1999). DMA, Dimethylacetal; DMMK, dimethylmenaquinone; MMK, monomethylmenaquinone; +, positive; −, negative; R, resistant; ND, not reported.

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</table>

*MIC breakpoint (µg ml\(^{-1}\)).

†Numbers of 12 strains that are resistant to the given antibiotic at the concentration indicated are given in parentheses (data from Moore & Moore, 1986).

§Data for reference taxa are from *Adlercreutzia equolifaciens* CCUG 54925\(^{\dagger}\), *Asaccharobacter celatus* DSM 18785\(^{\dagger}\) and *Eggerthella lenta* strains CCUG 33760, CCUG 34779 and CCUG 45577 (fatty acid profile varied between strains; ranges are given) (data for CCUG strains from http://www.ccug.se).

§Comparisons were made in this study with *Adlercreutzia equolifaciens* DSM 19450\(^{\dagger}\) and *Asaccharobacter celatus* DSM 18785\(^{\dagger}\).
(Supplementary Fig. S3a). Likewise, genistein was converted completely to GM 1 and GM 2 by growing cells of strain Mt1B8T within 16 h (Supplementary Fig. S3b). The conversion of both daidzein and genistein has not been reported for *Asaccharobacter celatus*, *Adlercreutzia equolifaciens* or strain Julong732 (Maruo et al., 2008; Minamida et al., 2006; Wang et al., 2005).

**Description of Enterorhabdus gen. nov.**

*Enterorhabdus* (En.te.ro.rhab’dus. Gr. n. *enteron* intestine; Gr. fem. n. *rhabdos* a rod; N.L. fem. n. *Enterorhabdus* a rod isolated from the intestine).

Gram-positive-staining rods with a high DNA G + C content (64.2 mol% for the type strain of the type species) that belong to the family *Coriobacteriaceae*. They are distantly related to the genera *Eggerthella* (<92 % similarity based on partial 16S rRNA gene sequence analysis), *Adlercreutzia* and *Asaccharobacter* (<96%). They are mesophilic and grow as single cells under strictly anoxic conditions. Cells are catalase-, coagulase-, indole- and oxidase-negative. Spore formation and motility have not been observed. The major cellular fatty acids are C16:0, C17:0 iso I and C18:1ω9c. Galactose and ribose are detected as whole-cell sugars. The principal respiratory quinone is MMK-6. The diamino acid in the peptidoglycan is LL-diaminopimelic acid. The type species is *Enterorhabdus mucosicola*.

**Description of Enterorhabdus mucosicola sp. nov.**

*Enterorhabdus mucosicola* [mu.co.si’co.la. N.L. n. *mucosa* mucosa from L. adj. *mucus* -a-um mucous; L. suff. -cola (from L. n. *incola*) inhabitant, dweller; N.L. n. *mucosicola* inhabitant of the intestinal mucosa].

The bacterium has the aforementioned features of the genus. Cells are approximately 0.5 × 2.0 μm. Grows at pH 5.5–9.5. Cultures in the stationary phase of growth are characterized by stable pH 6.9–7.1 and a typically low turbidity (≤0.5 McFarland standard). After 48 h of growth at 37°C on GY-BHI-c agar under anaerobic conditions, colonies are circular, entire, pinpoint, raised and translucent to slightly opaque. Grows well in the temperature range 30–40°C. The species is aerotolerant and possesses aminopeptidases but no glycosidases. It is capable of converting the isoflavones daidzein and genistein. The type strain is resistant to colistin and ciprofloxacin. The DNA G + C content of the type strain is 64.2 mol%.

The type strain is Mt1B8T (=DSM 19490T =CCUG 54980T), isolated from the ileal mucosa of a 12-week-old female TNFΔARE mouse.

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**References**


