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International Journal of Antimicrobial Agents 31 (2008) 544–548

INTERNATIONAL JOURNAL OF Antimicrobial Agents

www.ischemo.org

Short communication

Resistance determinant *erm*(X) is borne by transposon Tn*5432* in *Bifidobacterium thermophilum* and *Bifidobacterium animalis* subsp. *lactis*

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Received 23 October 2007; accepted 24 January 2008

Abstract

The *erm*(X) gene from erythromycin- and clindamycin-resistant *Bifidobacterium* strains was characterised by polymerase chain reaction and sequence analysis, including flanking regions. Results suggest that the resistance determinant was part of transposon Tn*5432* that has been described in several opportunistic pathogens such as *Corynebacterium striatum* and *Propionibacterium acnes.*

Keywords: *erm*(X); Probiotics; *tnp*1249; *tnp*CX; Transferable antibiotic resistance genes

1. Introduction

Probiotics are defined as living microorganisms that upon ingestion in certain numbers exert health benefits [1]. Lactic acid bacteria and bifidobacteria commonly used as probiotics are 'Generally Recognised As Safe' (GRAS). However, they may still cause systemic infections, excessive immune stimulation in susceptible individuals and/or gene transfer [2]. As a consequence, probiotic strains must be characterised before they are legally permitted. Among others things, their antibiotic resistance pattern must be assessed, as microorganisms used for probiotics should not contain any transferable antibiotic resistance genes [2,3].

Presence of the resistance determinant *erm*(X) was demonstrated in six erythromycin- and clindamycin-resistant *Bifidobacterium thermophilum* strains during investigation of a large collection of bifidobacteria that could be potential probiotics [4]. Analysis of additional bifidobacteria revealed that this antibiotic resistance gene was also present in a *Bifidobacterium animalis* subsp. *lactis* strain [5].

In this study, the detected *erm*(X) was analysed in more detail in relation to its potential transmissibility.

2. Materials and methods

2.1. Bacterial strains and antimicrobial susceptibility testing

The strains investigated in this study are shown in Table 1. Bifidobacteria were grown in brain–heart infusion broth containing 0.05% cysteine–HCl in an anaerobic chamber at 37 °C for 48 h.

Minimal inhibitory concentrations (MICs) of the six *B. thermophilum* strains were determined by broth microdilution using VetMIC^{TM} 96-well microtitre plates (National Veterinary Institute, Uppsala, Sweden) containing serial two-fold dilutions of the dehydrated antimicrobial agents clindamycin (0.12–8 μg/mL) and erythromycin (0.12–16 μg/mL) [4]. Cultures were streaked on lactic acid bacteria susceptibility test medium supplemented with cys-

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doi:10.1016/j.ijantimicag.2008.01.025

Table 1
Bifidobacterium strains analysed in the study

Strain #	Species	Origin	Year of isolation	MIC (μg/mL)[a]	
				Erythromycin	Clindamycin
B0196	*B. thermophilum*	Pig faeces	2001	16	>8
B0213	*B. thermophilum*	Pig faeces	2001	>16	>8
B0221	*B. thermophilum*	Pig faeces	2002	>16	>8
B0222	*B. thermophilum*	Pig faeces	2002	>16	>8
B0225	*B. thermophilum*	Surface swab of pig carcass	2002	16	>8
B0252	*B. thermophilum*	Pig faeces	2002	>16	>8
B0456	*B. animalis* subsp. *lactis*	Pig faeces	2001	>256	>256

[a] Minimal inhibitory concentrations (MICs) were determined by broth microdilution [4] for *B. thermophilum* and by the Etest method [5] for *B. animalis* subsp. *lactis*.

teine (LSM-C) [6] and incubated for 48 h at 37 °C in an anaerobic cabinet (80% N_2, 10% CO_2, 10% H_2; Scholzen Technik, Kriens, Switzerland). Inocula were prepared by suspending colonies in 5 mL of 0.85% NaCl solution to a turbidity of McFarland standard 1. The inoculated saline suspension was diluted in LSM-C broth at 1:1000. Subsequently, 100 μL of the diluted inoculum was added to each well of the VetMIC plates. After incubating plates under anaerobic conditions at 37 °C for 48 h, MIC values were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited.

Antimicrobial susceptibility of the *B. animalis* subsp. *lactis* strain was analysed by Etest (AB BIODISK, Solna, Sweden) as described by Mättö et al. [5]. Briefly, the concentration gradient of the tested antimicrobial agents clindamycin and erythromycin on the Etest strips was 0.016–256 μg/mL. Bacterial suspensions with a turbidity equivalent to McFarland standard 1 were prepared as indicated above. Within 15 min after adjusting the turbidity of the inoculum, the cultures were swabbed evenly onto LSM-C agar plates using a sterile cotton swab. After drying the surface of the plates for 15 min, Etest strips were applied. The plates were incubated under the same condition as for the broth microdilution method. MICs were read directly from the test strip according to the instructions of the manufacturer.

2.2. DNA isolation and polymerase chain reaction (PCR)

Total DNA was isolated using the Wizard Genomic DNA isolation kit according to the manufacturer's protocol for Gram-positive bacteria (Promega Benelux BV, Leiden, The Netherlands). The *B. thermophilum* strains were differentiated by the BOX PCR fingerprinting technique as described by Masco et al. [7].

PCR reactions were performed in a total volume of 50 μL containing approximately 20 ng of bacterial DNA, 10 pmol of each primer (Table 2), 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP and 2.5 U of *Taq* DNA polymerase recombinant (Invitrogen BV, Breda, The Netherlands). The following PCR program was used: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 58 °C or 60 °C for 30 s and 72 °C for 30 s or 60 s; and 72 °C for 10 min. The annealing temperature depended on the melting temperature (T_m) of the primer pair. The extension time was determined by the expected product length, i.e. fragments shorter than 1400 bp had an extension period of 30 s, whereas longer products had an extension time of 60 s. The obtained PCR fragments were analysed by electrophoresis on a 1–2% agarose gel, depending on the presumed product size, stained with ethidium bromide and visualised with ultraviolet light.

2.3. Inverse PCR

Inverse PCR was carried out on isolated DNA of the *Bifidobacterium* strains according to the principle described by Ochman et al. [8]. In brief, 20 ng of total DNA was used together with 10 U of endonuclease in the buffer specified by the supplier (New England Biolabs) in a total volume

Table 2
Primers used in the study

Name[a]	Sequence (5′–3′)
BOX[b]	CTACGGCAAGGCGACGCTGACG
ermX_-237F	ATGTTGATTTCAGGTACCGC
ermX_4F	TCTGCATACGGACACGGC
ermX_298R[c]	CCACAATGACGCAGGGAG
ermX_436F[c]	GCTCAGTGGTCCCCATGG
ermX_766R	AGTCACCTGGAAGAGATCG
rplI_87F	CCGCAACTACCTGTTCCCGC
rplI_165F	CGCTCGTCTGGCCAAGGC
tetW_62R	CCGTCAAGGTCGTCTTTCC
tetW_1785F	TTACACCAACGGGCAGAGC
tnp1249_31F	ATGTCGAAGAACCAACCACG
tnp1249_52R	AGCGTGGTTGGTTCTTCGAC
tnp1249_486F	CATCGACGGCGGCCAAGG
tnp1249_503R[c]	CCTTGGCCGCCGTCGATG
tnp1249_1194R	TTGAATGCCGATTGAGTGGG
tnpCX_1F	GTGCAGCCTAACGGAAATGT
tnpCX_573R	TTAACCCAGATTGCACGCGT
tRNA-Lys_21F	CCCTGGACACGCTGATTAAG
tRNA-Lys_41R	TCTTAATCAGCGTGTCCAGG

[a] The number in the primer name indicates the start position of the primer within the specific gene.
[b] See [7].
[c] Primers used for inverse polymerase chain reaction (PCR).