First Report of Macrolide Resistance Gene *erm*(T) Harbored by a Novel Small Plasmid from *Erysipelothrix rhusiopathiae*

Chang-Wen Xu, An-Yun Zhang, Chun-Mei Yang, Yun Pan, Zhong-Bin Guan, Chang-Wei Lei, Lin-Yao Peng, Qing-Zhou Li, Hong-Ning Wang

College of Life Sciences, Sichuan University; Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province; Key Laboratory of Bio-resources and Eco-environment, Ministry of Education; 985 Project Science Innovative Platform for Resource and Environment Protection of Southwestern China, Chengdu, Sichuan, People's Republic of China

The macrolide resistance gene *erm*(T) was identified for the first time in a porcine *Erysipelothrix rhusiopathiae* isolate from swine in China. The novel 3,749-bp small plasmid pER29, which carries *erm*(T), had a G+C content of 31% and four distinct open reading frames. The presence of pER29 increased by at least 128-fold the MICs of clindamycin and erythromycin for *E. rhusiopathiae*. The fitness cost of pER29 could be responsible for the low frequency of *erm*(T) in *E. rhusiopathiae*.

Erysipelothrix rhusiopathiae is a Gram-positive bacillus that causes a variety of diseases in many species, including birds, mammals, and humans (1–3). In pigs, it can cause swine erysipelas, which may occur as acute septicemia or chronic endocarditis and polyarthritides. Penicillins are the antibiotics of choice for treatment of *E. rhusiopathiae* infection, with macrolides recommended as alternatives. Macrolide resistance is commonly mediated by *erm* genes and complemented by *mef* genes and *msr* genes (4–6). The *erm*(T) gene, since its first identification in *Lactobacillus reuteri* (7), has been detected in isolates of the genera *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Haemophilus* (5, 8–12). The *erm*(T) gene in some cases confers cross-resistance to both lincosamides and streptogramins and therefore limits the effectiveness of these two classes of agents in the treatment of disease caused by *erm*(T)-positive strains (5). The resistance of *E. rhusiopathiae* to macrolides was first reported in 1984 (13). However, the genetic basis of macrolide resistance in *E. rhusiopathiae* is still unclear. The aim of this study was to determine the genetic basis responsible for the phenotypic resistance in *E. rhusiopathiae*.

A total of 51 *E. rhusiopathiae* strains were isolated from sick swine from 35 unrelated pig farms in China between 2011 and 2014. Species-specific identification was performed by 16S rRNA gene sequencing and then confirmed by using the BD Phoenix-100 system (Becton Dickinson, USA) as previously described (14). Antimicrobial susceptibility testing of the isolates was performed by agar dilution using Mueller-Hinton plates supplemented with 5% sheep blood, according to the protocols of the CLSI guidelines (15, 16). *Staphylococcus aureus* ATCC 29213 served as the quality control strain. A single *E. rhusiopathiae* isolate, ER29, obtained from the spleen of a pig in Sichuan Province, exhibited high MIC values for erythromycin (>64 µg/ml) and azithromycin (>64 µg/ml) (Table 1).

All strains were investigated for the presence of the genes *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(G), *erm*(X), and *erm*(T), which have been reported to be responsible for high-level resistance to macrolides in Gram-positive bacteria, by PCR using specific primers (see Table S1 in the supplemental material) (5, 17). Amplicons were sequenced by Shanghai Sangon Bioengineering Co., Ltd., using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification. Only an *erm*(T)-specific PCR product was obtained from

strain ER29 and then was confirmed by sequence analysis. The obtained sequence showed 100% identity to the *erm*(T) gene of *Streptococcus agalactiae* plasmid pCCH208 (KJ778678). This is the first report of the *erm*(T) gene in *E. rhusiopathiae*.

To investigate whether the *erm*(T) gene of ER29 was plasmid borne, plasmid DNA was extracted as previously described (18). Purified plasmid DNA was transformed into *S. aureus* RN4220 and *E. rhusiopathiae* vaccine strain G4T10 by electrotransformation (19). Transformants were selected on brain heart infusion (BHI) agar supplemented with erythromycin (10 µg/ml) and 5% sheep blood. A transformant (designated G4T10+pER29) using *E. rhusiopathiae* G4T10 as the recipient was successfully obtained, and a small 3.7-kb plasmid (designated pER29) was also obtained. The antimicrobial susceptibilities of strains ER29, G4T10, and G4T10+pER29 were determined. Compared to G4T10, the G4T10+pER29 strain exhibited at least 128-fold-higher MICs of clindamycin (64 µg/ml), lincomycin (>64 µg/ml), erythromycin (>64 µg/ml), and azithromycin (>64 µg/ml) (Table 1).

To determine the sequence of plasmid pER29, a PCR assay was performed using the specific primers P1 and P2 (see Table S1 in the supplemental material) that were located within the *erm*(T) gene and using plasmid DNA purified from ER29 as the template. The 3,312-bp PCR product was obtained and sequenced using primer walking. Then, another PCR assay was performed using the specific primers P3 and P4 (see Table S1) located within the known 3,312-bp sequence. An expected 1,006-bp PCR product

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Address correspondence to Hong-Ning Wang, whongning@163.com.

C.-W.X. and A.-Y.Z. contributed equally to this article.

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TABLE 1 Comparative analysis of MICs of the original *erm(T)*-carrying strain ER29 and the *E. rhusiopathiae* G4T10 and G4T10+pER29 transformants

Bacterial isolate	MIC ($\mu\text{g/ml}$) of antimicrobial agent ^a											
	TIA	VAL	CLI	LIN	ERY	AZM	CIP	GEN	STR	FFC	VAN	PEN
ER29	32	2	64	64	>64	>64	8	>64	512	8	128	0.125
G4T10	2	0.25	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.063	32	8	>8	256	0.125
G4T10+pER29	2	0.25	64	>64	>64	>64	≤ 0.063	32	8	>8	256	0.125

^a TIA, tiamulin; VAL, valnemulin; CLI, clindamycin; LIN, lincomycin; ERY, erythromycin; AZM, azithromycin; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; FFC, florfenicol; VAN, vancomycin; PEN, penicillin.

was also sequenced. The two sequences were assembled using SeqMan computer programs, and the complete sequence of pER29 was obtained. Sequence analysis of pER29, performed with the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>) and the ORF Finder program (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), revealed that plasmid pER29 had a G+C content of 31% and four distinct open reading frames (ORFs) (Fig. 1a). The *erm(T)* gene region and the 44 downstream nucleotides showed a high level of homology (identity, 100%) to *Streptococcus agalactiae* plasmid pCCH208 (KJ778678) (20), *S. aureus* ST398 plasmid pUR2940 (HF583292) (21), *Haemophilus parasuis* plasmid pFS39 (KC405064) (8), and *Lactobacillus* sp. plasmid p121BS (AF310974) (22) (Fig. 1b). However, the remainder of the sequence of pER29 was not similar to any known sequences. Downstream of the *erm(T)* gene, ORF1, which is designated *mobE*-like, predicts a protein similar (identity, 58%) to mobilization (Mob) proteins from *Bacillus thuringiensis* (WP_029437116). However, ORF2, next to ORF1, predicts a 173-amino-acid protein that was not similar to any known proteins. ORF3, which contains a *rep-3* domain, and thus designated *repE*-like, predicts a protein which has low similarity (identity, 33%) to replication proteins such as *Lactococcus lactis* replication initiator protein (WP_011669056).

The growth kinetics of *E. rhusiopathiae* G4T10 and *E. rhusio-*

pathiae G4T10+pER29 in the absence of erythromycin and the fitness cost of pER29 were determined as previously described in three independent experiments (23). The strains were grown in BHI broth supplemented with 5% sheep blood, and the proportion of resistant colonies was deduced by replica plating of 100 colonies on BHI agar plates supplemented with erythromycin (50 $\mu\text{g/ml}$) and 5% sheep blood. A slight fitness burden of *E. rhusiopathiae* G4T10+pER29 in logarithmic growth phase was observed and led to a lower concentration in stationary phase (Fig. 2a). From day 2 on, a constant decrease in the proportion of pER29-carrying strains was observed. At day 13, all the colonies tested were pER29 free (Fig. 2b). *E. rhusiopathiae* G4T10+pER29, i.e., bearing pER29, presented a competitive disadvantage of ca. 2.8% per 10 generations relative to *E. rhusiopathiae* G4T10. This slight fitness cost of pER29 may help the dissemination of *erm(T)* in *E. rhusiopathiae*.

In conclusion, the macrolide resistance gene *erm(T)* was identified for the first time in a porcine *E. rhusiopathiae* isolate from swine in China. The presence of the *erm(T)*-harboring plasmid pER29 greatly increased the MICs of macrolides and lincosamides. Moreover, *E. rhusiopathiae* can infect birds, mammals, and humans; the high-level resistance gene *erm(T)* in this Gram-positive organism will likely decrease the cure effect. Although the

FIG 1 (a) Diagram of pER29 (GenBank accession number KM576795). The primer pair used and the relevant amplicon are shown. The ORFs, represented by arrows pointing in the direction of transcription, are white, except for that for the *erm(T)* gene, which is black. (b) Comparative analysis of the genetic environment of the *erm(T)* gene in plasmid DNA from *Staphylococcus*, *Lactobacillus*, *Haemophilus*, *Streptococcus*, and *E. rhusiopathiae*. Regions with 100% nucleotide sequence identity are shaded gray.

FIG 2 Fitness cost of pER29 in *E. rhusiopathiae*. (a) Comparison of the growth kinetics of strains *E. rhusiopathiae* G4T10 and *E. rhusiopathiae* G4T10+pER29 without erythromycin. Error bars represent the standard deviations of the means. Growth curves represent the average results of three independent experiments. (b) Growth competition between *E. rhusiopathiae* G4T10 and *E. rhusiopathiae* G4T10+pER29. The initial ratio was 1:1. The relative percentages of each strain at different time points are shown. Competition curves represent the average results of three independent experiments. (c) The selection coefficient (s) was calculated from the competition experiment. s is the slope of the linear regression model, $\ln(CI)/\ln(d)$, where CI is the ratio between the CFU counts of the resistant and susceptible populations at t_1 divided by the same ratio at t_0 , and d is the dilution factor.

fitness cost showed us a way to get rid of this drug resistance plasmid, the risk of dissemination of *erm*(T) should not be underestimated.

Nucleotide sequence accession number. The sequence of the 3,749-bp sequence of the *erm*(T)-carrying plasmid pER29 has been deposited in the GenBank database under accession no. [KM576795](#).

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