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Mechanism of Resistance to Macrolide-Lincosamide-Streptogramin Antibiotics in *Streptococcus thermophilus*

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Abstract

Resistance to macrolide-lincosamide-streptogramin (MLS) group antibiotics in the dairy bacterium *Streptococcus thermophilus* (ST) is documented but the mechanism of resistance has not been elucidated. MIC values for erythromycin (Erm), azithromycin (Azm), tylosin (Tyl), spiramycin (Spm), pristinamycin (Prm) and virginiamycin S (Vir) were determined by the disk diffusion method. PCR products were obtained with primer pairs for the L4, L22 and 23S rDNA (domain V) genes. The sequencing results ruled out mutations in the L4 and L22 ribosomal proteins and the presence of rRNA methylase, efflux, and inactivating genes. However, sequencing of domain V in each of the six ribosomal alleles detected by *Eco*RI/I-*Ceu*I digestion in ST mutants identified three types of mutations that led to MLS resistance. Type A mutants, induced by Erm, had high resistance to 14- and 15-membered ring macrolides (Erm, Azm) and streptogramin B antibiotics (Prm, Vir), moderate resistance to 16-membered ring macrolides (Tyl, Spm), but remained susceptible to lincomycin. In Type B mutants, also induced by Erm, resistance was high to Erm, Tyl and Spm, and moderate to lincomycin but sensitivity was retained to Prm. Type C mutants, induced by Prm, showed high resistance to 16-membered ring macrolides but remained sensitive to Erm, Azm and lincomycin. The three identifiable resistance patterns were apparently due to point mutations in domain V of the 23S rRNA gene, resulting in three phenotypes among resistant *S. thermophilus* isolates. Type A phenotype mutants had a C2611G mutation in five of the six ribosomal alleles, Type B phenotypes had a A2058G mutation in five alleles, and Type C variants had a A2062C mutation in all six alleles. Resistance to MLS antibiotics in *S. thermophilus* was inducible by 14- and 15-membered ring macrolides and streptogramin B type antibiotics but not by 16-membered ring macrolides or lincosamides.

Keywords: macrolide antibiotic resistance, *Streptococcus thermophilus*

Abbreviations: ST-*Streptococcus thermophilus*; Erm-erythromycin; Azm-azithromycin; Tyl-tylosin; Spm-spiramycin; Prm-pristinamycin; Vir-virginiamycin; Cli-clindamycin; MLS-macrolide-lincosamide-streptogramin

1. Introduction

Streptococcus thermophilus is a widely used food-grade lactic acid bacterium responsible for carrying out essential biocatalytic functions in the industrial production of yogurt and other fermented dairy foods, including Italian and Swiss style cheeses. Previously, several reports identified *S. thermophilus* as a potential carrier of antibiotic resistance determinants, including MLS antibiotic resistance genes (Sozzi & Smiley, 1980; Wang et al., 2005; Tosi et al., 2007). Since dairy fermentations also provide a suitable environment for the growth of pathogenic bacteria, there has been a persistent concern about the possible interchange of antibiotic resistance genes between *S. thermophilus* and pathogens that may proliferate in the human gastrointestinal tract. Although the erythromycin resistance phenotype in *S. thermophilus* is documented in the literature, the mechanism of

resistance to antibiotics of the macrolide-lincosamide-streptogramin (MLS) group in this important industrial species has not been studied at the molecular level.

In clinical isolates of streptococci, resistance to macrolide antibiotics is orchestrated predominantly by two well-known resistance mechanisms, target site modification and macrolide-specific efflux. In target site modification, an acquired *erm*B gene specifically methylates A2058 in 23S rRNA and blocks the binding of macrolides by causing conformational changes in the 50S ribosomal subunit, resulting in cross-resistance to 14-(erythromycin), 15-(azithromycin), and 16-membered (tylosin) macrolides. In macrolide-specific efflux that is mediated by a membrane protein encoded by the *mef* (A) gene, resistance develops to 14- and 15-membered macrolides but not to 16-membered macrolides, lincosamides and streptogramins (Depardieu & Courvalin, 2001). Other known macrolide resistance mechanisms involve mutations in the ribosomal proteins L4 and L22, and also the 23S rRNA (Tait-Kamradt et al., 2000; Pihlajamaki et al., 2002).

In this study, the mechanism of resistance to MLS antibiotics was elucidated in mutants of *S. thermophilus* ST113 displaying different phenotypes that were isolated following exposure to macrolide antibiotics.

2. Methods

2.1 Bacterial Strains and Induction of MLS Resistance

The identity of *S. thermophilus* strain ST113 (deposited as NRRL B-59386 in the Agricultural Research Service Culture Collection, NCAUR-USDA, Peoria, IL, http://nrrl.ncaur.usda.gov) was confirmed by 16S rRNA gene sequence analysis (MIDI Labs, Inc., Newark, DE). The culture was maintained in tryptone-yeast extract-lactose (TYL) medium at 37 °C (Somkuti & Steinberg, 1986). MLS resistance was induced out by growing ST113 on TYL agar plates supplemented with 14-membered lactone ring (erythromycin, Sigma Chemical Co., St. Louis, MO), 15-membered lactone ring (azithromycin, Pfizer Inc., New York, NY) or 16-membered lactone ring (spiramycin and tylosin, Rhone-Poulenc Rorer, Paris, France) macrolide antibiotics, streptogramin B-type antibiotics (pristinamycin and virginiamycin S, gifts from B. Weisblum, University of Wisconsin, Madison), or lincosamide antibiotics (clindamycin and lincomycin, Sigma Chemical Co.), using each at 15 μg mL^{-1}. After incubation for 72 h at 37 °C, single colonies were picked into TYL/antibiotic broth and passaged daily for 3 days before plating serially diluted cultures on TYL/antibiotic agar plates. Antibiotic sensitivity of isolates was evaluated by the disk diffusion assay method.

2.2 Detection of MLS Resistance Phenotypes

The resistance patterns in ST113 mutants was detected by a paper disk-agar diffusion technique. This was done by applying 150 μL of each antibiotic stock solution (1,00 μg mL^{-1}) to filter paper disks and placing them in a grid pattern on agar films. Each plate was inoculated with 2×10^6 cfu mL^{-1} cells of ST113 cultures that were grown from single cfu-s isolated following exposure to MLS antibiotics. Plates were incubated at 37 °C for 24 h.

2.3 Determination of MIC Values

The minimum inhibitory concentration (MIC) of each antibiotic was tested by a paper disk agar diffusion method. Samples of serial twofold dilutions of antibiotics (final concentrations from 0.2 to 1,000 μg mL^{-1} were soaked into sterile paper disks and placed on the surface of plates inoculated with putatively identified phenotypic mutants of ST113.

2.4 Detection of MLS Resistance Determinants

PCR amplification was used to detect the presence of *erm*B and *mef*A genes in mutant *S. thermophilus* clones. The primers for *erm*B were 5'-GAAAAGGTACTCAACCAAATA-3'(F) and 5'-AGTAACGGTACTTAAATTTAC-3'(R), whereas *mef*A was detected with the primers 5'-AGTATCATTAATCACTAGTGC-3'(F) and 5'-TTCTTCTGGTACTAAAAGT-GG-3'(R) (Sutcliffe et al., 1996). Mutations in the L4 and L22 ribosomal proteins that also cause macrolide resistance in clinical streptococci were detected with primer pairs described previously (Canu et al., 2002).

PCR was performed with a Perkin-Elmer Thermal Cycler (Model 9700) using the following cycling conditions: 95 °C for 5 min, followed by 30 cycles of (95 °C for 1 min, 50, 55 or 60 °C for 1 min, 72 °C for 1 min), followed by 1 cycle of 72 °C for 7 min and a 4 °C soak. PCR products were cleaned up for sequencing using Ampure reagent (Agencourt, www.agencourt.com). Sequencing was performed in an ABI 3730 DNA Analyzer (Applied Biosystems), and sequences were trimmed and aligned using Sequencher software (Genecodes Corp., Ann Arbor, MI). Nucleotide sequences were compared to sequences present in the NCBI database using BLASTn at http://www.ncbi.nlm.nih.gov/.

2.5 DNA Sequencing of Ribosomal Alleles

Genomic DNA was isolated from single clones taken from TYL-agar plates and the number of ribosomal alleles was determined by co-restriction with I-*Ceu*I (New England Biolabs), an intron-encoded enzyme from *Chlamydomonas eugametos* with a unique 26-bp recognition sequence present only within 23S rRNA genes (Liu et al., 1993), and *Eco*RI, which cuts outside of 23S rRNA. After I-*Ceu*I/*Eco*RI co-digestion, DNA fragments were resolved by gel electrophoresis and transferred to nylon membranes using an Oncor Probetech I automated Southern blot system. The probe was a purified 1,003 bp PCR product derived from the 3'end of the 23S rRNA, biotinylated with the BioNick Labeling System (BRL Life Technologies). Hybridization conditions, subsequent washes, streptavidin and phosphatase treatment, and staining with nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate were done according to the supplier (Oncor, Inc.).

For sequencing 23S rDNA (domain V) of *S. thermophilus* (Ludwig et al., 1992), primers were designed using the Genbank sequence (accession number X68429.1) to generate the following primer pairs:

GGTTAAGTTAATAAGGGCGCACGGT(F)	
GGAGGCGACCGCCCCAGTC(R)	PCR product: 2271 bp
CCTAGCAGTATCCTTTGAGTACGGCG(F)	
GGAGGCGACCGCCCCAGTC(R)	PCR product: 1884 bp
CCTAGCAGTATCCTGAGTACGGCG(F)	
CGCTCCCCAFCACAGCTCAATG(R)	PCR product: 1173 bp
CCTAGCAGTATCCTTTGAGTACGGCG(F)	
GGATAAGTCCTCGAGCTTATTAGTATTAG(R)	PCR product: 2519 bp

3. Results and Discussion

3.1 Induction of Resistance to MLS Antibiotics

S. thermophilus ST113 colonies appeared within 72 h on TYL/Erm plates during incubation at 37 °C. A total of 100 cfu-s were randomly picked for establishing resistance patterns to MLS-type antibiotics, using the grid shown in Figure 1. Based on the results of these bioassays, the Erm resistant mutants were grouped in 2 categories: Type A mutants were highly resistant to Erm, Prm and VirS, with increased resistance to the 16-membered lactone macrolides Spm and Tyl but remained sensitive to Cli and Lin. Type B mutants were similar in resistance to Erm, Spm and Tyl and VirS but remained sensitive to Cli and Lin, as well as Prm.

Incubation of *S. thermophilus* ST113 in the presence of the streptogramin B-type pristinamycin resulted in dozens of cfu-s which uniformly displayed the Type C resistance pattern: they were highly resistant to the 16-membered lactone ring macrolides Spm and Tyl but remained sensitive to Erm as well as Cli and Lin. Similar results were obtained when ST113 was grown in the presence of VirS.

After exposure to the 15-membered lactone ring Azm, only two resistant clones were present after 72 h. These clones displayed resistance to Spm and Tyl but remained sensitive to Erm, Cli and Lin, as well as to the streptogramin-type Prm (data not shown).

Several attempts to induce resistance in *S. thermophilus* ST113 with 16-membered lactone macrolides Spm and Tyl were unsuccessful.

3.2 Molecular Basis for Macrolide Resistance

Several preliminary experiments were performed to define the molecular basis of the apparently MS phenotype in *S. thermophilus* ST113. First, the wild-type ST113 was cured of its 4.13 kb resident plasmid (Somkuti & Steinberg, 1986) by treatment with ethidium bromide (25 μg mL^{-1}, 24 h at 37 °C) ruling out the presence of plasmid borne MLS resistant genes. Screening total DNA extracted from randomly selected clones by Southern blotting with a biotinylated plasmid-based probe yielded several plasmid-free mutants which displayed the same MLS resistance pattern as the wild-type parent culture (Figure 1).

Subsequent screening for the presence of rRNA methylase *(erm*B) indicated the absence of this resistance mechanism. Since the resistance pattern induced with Erm showed the retention of a Cli and Lin sensitive phenotype, the possible involvement of an active efflux (*mef*A) gene known to be operational in *S. pneumoniae*

and *S. pyogenes* (Sutcliffe et al., 1996) was tested in *S. thermophilus* ST113 by PCR techniques. The results of these trials were negative. The absence of *mef*A involvement in macrolide resistance in ST113 was further confirmed by the addition of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (25 μM) to test media, which failed to influence the outcome of bioassays (Giovanetti et al., 2002).

Next, various domains in the ribosomal architecture were checked for features that are known to be involved in MLS resistance in other species of streptococci. Specific areas encoding L4 and L22 ribosomal proteins were checked for mutations by PCR but DNA sequencing data did not detect any alterations in amplification products. This indicated that the type of macrolide resistance detectable in *S. pneumoniae* (Canu et al., 2002) was absent in *S. thermophilus* ST113.

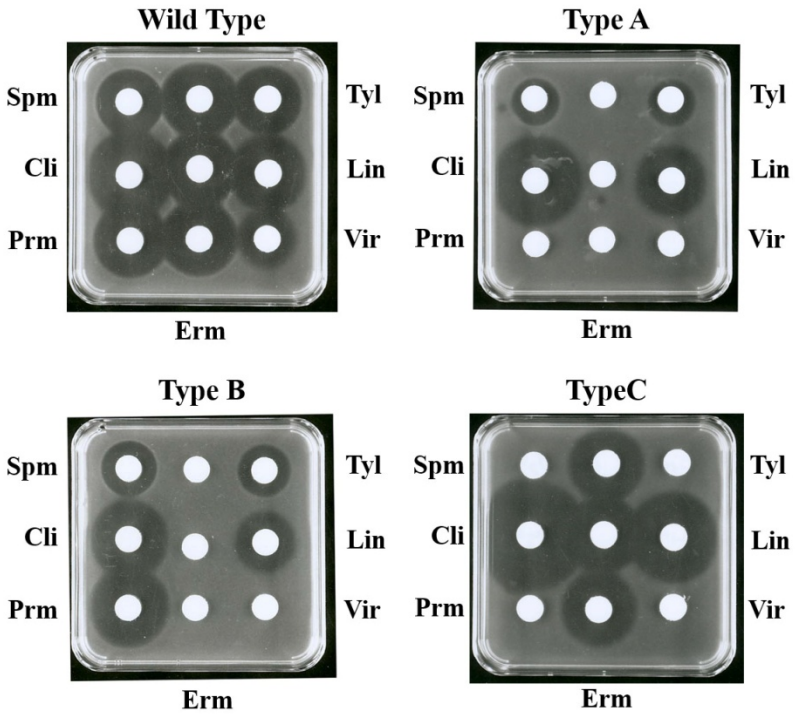

Figure. 1. Resistance patterns detectable in *S. thermophilus* ST113 mutants after exposure to MLS antibiotics. Each paper disk contained 15 μg of antibiotic. Spm: spiramycin, Tyl: tylosin, Cli: clindamycin, Lin: lincomycin, Prm: pristinamycin, Vir: virginiamycin S; Erm: erythromycin

3.3 Detection of Mutations in Domain V of 23S rRNA

After ruling out the presence of *erm*B and *mef*A genes as well as mutations in the L4 and L22 ribosomal proteins, we targeted domain V of 23S rRNA which is the center of peptidyl transferase activity in the ribosomes and is responsible for the elongation of the nascent peptide chain. Several studies demonstrated that resistance to MLS antibiotics may also develop in streptococci and other bacteria by specific base substitutions in domain V of 23S rRNA (Vester & Douthwaite, 2001; Pihlajamaki et al., 2002; Jalava et al., 2004; Haanpera et al., 2005).

PCR analysis of genomic DNA from *S. thermophilus* ST113 identified three specific point mutations in 23S rRNA, involving nt2058, nt2062 and nt2611 (Figure 2). However, in some cases the exact base calls were ambiguous which indicated that not all ribosomal alleles may have the same base substitutions. To resolve the ambiguity and clearly establish the extent of domain V involvement in the macrolide resistance phenotype displayed by *S. thermophilus* ST113, the number of ribosomal alleles in the genome was determined. Genomic DNA double-digested with I-*Ceu*I and *Eco*RI was resolved by AGE and Southern

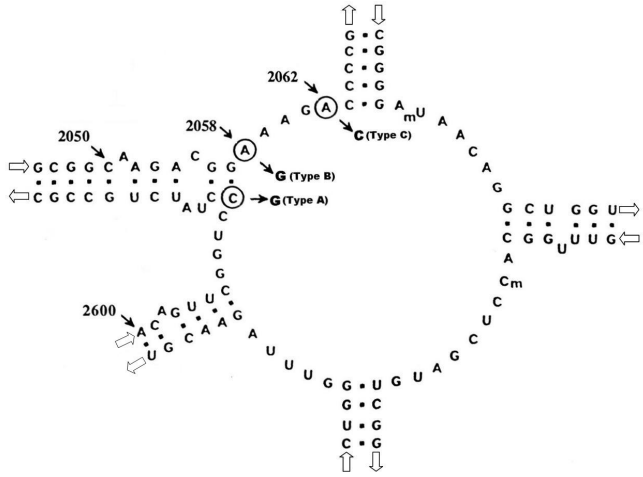

Figure 2. Secondary-structure model of the peptidyl transferase center in domain V of 23S rRNA. The circled nucleotides indicate the positions where base substitutions occurred conferring different types of MLS antibiotic resistance patterns in *S. thermophilus* ST113

hybridyzation tests revealed 6 distinct bands with estimated molecular masses of 23 kb, 13 kb 9.4 kb, 7 kb, 6.2 kb and 5.2 kb, respectively (Figure 3).

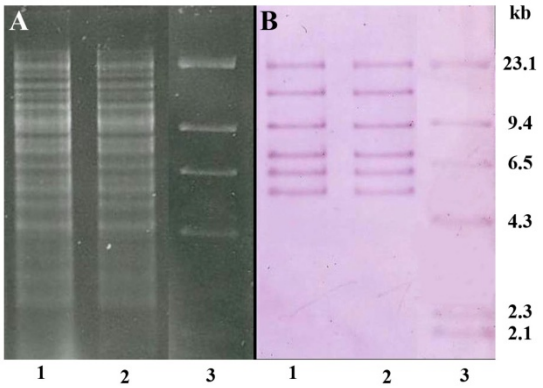

Figure 3. Detection of ribosomal alleles in *S. thermophilus* ST113. Genomic DNA digested with I-*Ceu*I and *Eco*RI was resolved by AGE (A). Southern blots with a biotinylated fragment of 23S rRNA as the probe (B); lane 1, MLS-sensitive ST113; lane 2, MLS-resistant ST113; lane 3, MW markers: biotinylated *Hin*dIII fragments of λ DNA

Bands were extracted from gels and sequenced to determine the exact nature of base substitutions in domain V of each of the 6 identifiable ribosomal alleles. The results of the DNA sequencing data permitted the identification of three types of domain V mutants (Table 1). Type A phenotype mutants had a specific C2611G mutation in 5 of the 6 ribosomal alleles. Type B phenotype mutants had a A2058G mutation in 5 alleles, while Type C mutants were homozygous with all 6 ribosomal alleles having a A2062C mutation. A2058G and C2611G mutations are frequently detected in clinical isolates of *S. pneumoniae* (Vester & Douthwaite, 2001; Canu et al., 2002; Pihlajamaki et al., 2002). On the other hand, there is one report on a A2062C mutation (Type C phenotype in ST113) resulting in resistance to 16-membered macrolides (Tyl, Spm) and streptogramins while retaining sensitivity to the 14-membered lactone Erm (Depardieu & Courvalin, 2001).

Table 1. Distribution of base substitutions in domain V of six ribosomal alleles in *S. thermophilus* ST113

Alleles		nt #2058	nt #2062	nt #2611
Wild Type		A	A	C
Type	A-1	A	A	G
	A-2	A	A	C
	A-3	A	A	G
	A-4	A	A	G
	A-5	A	A	G
	A-6	A	A	G
Type	B-1	G	A	C
	B-2	A	A	C
	B-3	G	A	C
	B-4	G	A	C
	B-5	G	A	C
	B-6	G	A	C
Type	C-1	A	C	C
	C-2	A	C	C
	C-3	A	C	C
	C-4	A	C	C
	C-5	A	C	C
	C-6	A	C	C

Site specific mutations resulting in three identifiable phenotypes of *S. thermophilus* ST113 led to substantial changes in the MIC values when the allelic mutants were challenged

with antibiotics. Although Type A and Type B mutants had different base substitutions at different sites in domain V of the ribosome, both strains showed over a 1,000-fold increase in Erm resistance (Table 2). On the other hand, only the Type A allelic mutant had a substantially increased resistance to pristinamycin and virginiamycin B. Type C mutants that were induced by exposure to pristinamycin, remained sensitive to Erm but displayed a moderately high level of resistance to the 16-membered macrolides spiramycin and tylosin, while remaining sensitive to the licosamide antibiotics lincomycin and clindamycin.

Table 2. Antibiotic susceptibilities of *S. thermophilus* ST113 parent and mutant strains

Strain	Site of ribosomal		MIC ($\mu g\ mL^{-1}$)*						
	mutation	alleles	Erm	Spm	Cli	Pri	Tyl	Lin	Vir
Parent	none	none	0.78	6.25	0.4	12.5	3.12	1.56	12.5
Type A	C2611G	5	>1000	40	1.56	>1000	18	5	>1000
Type B	A2058G	5	>100	400	12.5	12.5	400	400	400
Type C	A2062C	6	3.12	200	0.4	125	750	2.5	400

* Erm: erythromycin; Spm: spiramycin; Cli: clindamycin; Pri: pristinamycin; Tyl: tylosin; Lin: lincomycin; Vir: virginiamycin S.

The results demonstrated that in the dairy fermentation bacterium *S. thermophilus* ST113, resistance to macrolide antibiotics and streptogramins but not to lincosamides was inducible with erythromycin (14-member lactone) and azithromycin (15-member lactone) but not with the 16-member lactone macrolides tylosin and

spiramycin. Streptogramins (pristinamycin and virginiamycin S) induced resistance in *S. thermophilus* ST113 to 16-member lactone macrolides but not to erythromycin (14-member lactone ring) or lincosamides.

The DNA sequencing data of domain V in the six identified ribosomal alleles in this bacterium revealed specific point mutations at nt2058, nt2062 and nt2611 that were apparently involved in the resistance mechanism and the display of the three detectable phenotypes. The ribosomal alleles had a heterozygous profile in the case of Type A and Type B mutants, but were homozygous in mutants displaying a Type C phenotype.

Since *S. thermophilus* is a widely used bacterium in industrial-scale dairy food production and may come into contact with antibiotic resistant pathogenic streptococci in a production plant environment, further work is needed to elucidate its potential for the possible transmission of antibiotic resistance to other bacteria.

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