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ABSTRACT

Background: MRSA CM05 is the first clinical isolate documented to carry the *cfi* gene, which confers resistance to many antibiotics targeting the ribosome. Resistance results from methylation of nucleotide A2503 in the peptidyl transferase center (PTC). The *cfi* gene is typically plasmid-borne and associated with transposons in many veterinary and clinical isolates; however, in CM05 it is located on the chromosome and is co-expressed with *ermB* as a part of the *mfr* operon. The chromosomal location of *cfi* in the CM05 was unknown, association with mobile genetic elements was unclear, and stability of the region had not been evaluated.

Methods: Sequencing of the CM05 genome and targeted PCR analysis were used to elucidate the chromosomal integration site of *cfi* and plasmid-associated sequences. Single colony analysis was employed to evaluate the stability of *cfi* in the CM05 genome.

Results: The *cfi*-containing *mfr* operon in CM05 genome is associated with a 15.5 kb plasmid insertion into an *rna* locus. A second *ermB* gene was identified downstream of the *mfr* locus and *istAS*. MIC analysis of several individual CM05 colonies revealed two distinct populations having linezolid (LZD) MICs of either 8 µg/ml or 2 µg/ml. In the LZD-susceptible colonies, sequencing revealed evidence of recombination between the two *ermB* genes, resulting in deletion of the *cfi* and the 3' flanking region.

Conclusions: The genetic environment of *cfi* in CM05 is conducive to its rapid spread. Redundancy of *rna* alleles opens the possibility that copy number of *mfr* can be increased by gene conversion. Association of *mfr* with transposon and plasmid elements may further increase the mobility of *mfr* and its potential for horizontal transfer. However, the spontaneous deletion of *cfi* and 3' flanking sequences suggests that either the gene or, more likely, the associated genetic elements are deleterious in the absence of antibiotic selection.

INTRODUCTION

cfi is an acquired ribosomal methyltransferase gene which confers resistance to multiple classes of antibacterial agents targeting the 50S peptidyl transferase center (PTC) including the oxazolidinone linezolid (LZD), phenicols, lincosamides, pleuromutilins, and streptogramin A

CM05 was the first Cfr-positive LZD^R MRSA clinical isolate (Colombia - 2005) (Toh *et al.*, 2007)

cfi is constitutively co-expressed with *ermB* and chromosomally located in CM05 (Smith and Mankin, 2008)

Here we define the sequence and site of the insertion as well as the stability of the locus

METHODS

MIC values were determined by broth microdilution (CLSI)

Pyrosequencing and targeted PCR were used to define the sequence of the insertion

Growth curves were performed in MHB with triplicate cultures

In vitro passaging was done through plating on MHA media or MHA antibiotic gradient plates containing LZD or TR-700

Animal passage was performed through IP challenge in BALB/c mice

RESULTS

I. *cfi* is contained within a 15.5 kb insert in *S. aureus* CM05



- Sequencing revealed that *cfi* lies within a 15.5 kb plasmid-like insertion into the CM05 chromosome
- The insertion occurs in a 23S rRNA gene between bases 1,266 and 1,257
- Much of the insertion is identical to the streptococcal pSM19035 plasmid (Dixon and Lipinski, 1972)
- The plasmid insertion occurred precisely within the pSM19035 β-recombinase *six* site I recognition site (GTATAC) previously shown to be critical for strand exchange (Canosa *et al.*, 1996)
- Previously characterized *mfr* and IS21-558-like sequences are shown for reference (Toh *et al.*, 2007)

II. The 15.5 kb *cfi*-containing insert disrupts the 23S rRNA gene of *rna4*



III. Differences in plating efficiency reveal a heterogeneous population of LZD^R and LZD^S CM05

Drug name [µg/ml]	CFU	CFU 10 ⁻⁷ 00
0	89	89
0.25	64	63
0.5	41	0
1	68	0
2	16	0
4	21	0
8	0	0
16	0	0
32	0	0

• A 80°C glycerol stock of CM05 was grown to mid-log phase in MHB, diluted, and plated on MHA plates containing increasing concentrations of LZD and TR-700 (a novel second generation oxazolidinone)

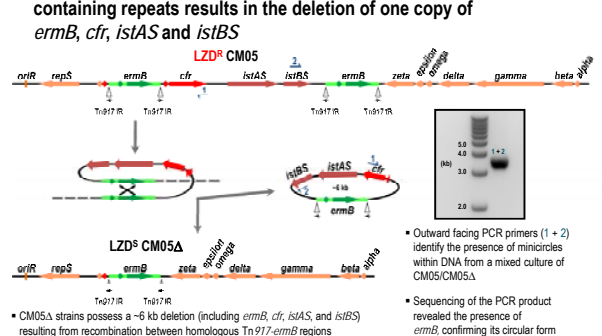
• Although the MIC vs LZD is 8 µg/ml, only ~20% of CFU plated grew in the presence of 2 µg/ml LZD

• TR-700, unaffected by Cfr methylation due to replacement of the acetamide group with the smaller hydroxyl group, produced growth inhibition trends consistent with the reported MIC of 0.5 µg/ml

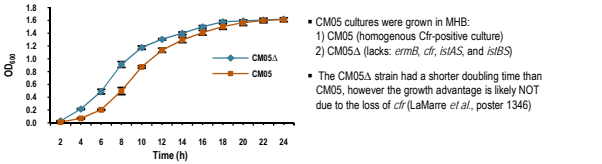
Colony #	MIC LZD (µg/ml)	MIC TR-700 (µg/ml)	PCR for <i>cfi</i> genes
LZD ^R	8	0.5	+
LZD ^S	2	0.5	-

• PCR and MIC analysis of individual colonies revealed two distinct populations with and without the *cfi* gene, corresponding to LZD MICs of 8 or 2 µg/ml, respectively

IV. A recombination/deletion event between the two 1.5 kb *ermB*-containing repeats results in the deletion of one copy of *ermB*, *cfi*, *istAS* and *istBS*



V. CM05Δ has a growth advantage over CM05



VI. CM05Δ has a competitive advantage over CM05 through non-selective passage *in vitro* and *in vivo*

- Three CM05 cultures were passaged 10 times on MHA media and individual colonies were analyzed for LZD MIC:
 - Cfr-positive CM05: all colonies tested remained at 8 µg/ml indicating maintenance of *cfi*
 - CM05Δ: all colonies tested remained at 2 µg/ml
 - Mixed Cfr-positive CM05/CM05Δ: colonies in the first passage were 1:1 mix of CFU with LZD MICs of 8 and 2 µg/ml, however, by passage 10 a homogenous culture of 2 µg/ml CM05Δ CFU was obtained
- In vitro* serial passage of a mixed Cfr-positive CM05/CM05Δ culture in the presence of LZD maintained the Cfr-positive CM05 following 30 passages whereas the CM05Δ strain predominated following selection with TR-700 (torezolid) (Locke *et al.*, 2009)
- A CM05Δ population resulted after 3 serial passages of the mixed Cfr-positive CM05/CM05Δ culture in BALB/c mice
 - Without selective pressure to maintain *cfi*, CM05Δ strains outcompete Cfr-positive CM05, however, the initial loss of the *ermB-cfr-istAS-istBS* region from CM05 does not appear to be a frequent event and is under further investigation

SUMMARY

- CM05 contains a 15,511 bp plasmid-like insertion into the *rna4* 23S rRNA gene
- Nearly 60% of the insert is identical to pSM19035, a broad host range plasmid, including plasmid maintenance genes
 - Insertion likely mediated by the β-recombinase
- Selective plating discrepancies revealed a heterogeneous CM05 population of *cfi*⁺ and *cfi*⁻ strains with LZD MICs of 8 and 2 µg/ml, respectively
- CM05 *ermB-cfr-istAS-istBS* deletion (Δ) was identified in LZD^S CM05 colonies present in a CM05 glycerol stock
 - Recombination occurred between large 1,545 bp Tn917-derived repeated elements that include the *ermB* gene
- CM05 grows more slowly than CM05Δ likely due to factors OTHER THAN *cfi* (see LaMarre *et al.*, poster 1346) and out competes CM05 in non-selective *in vitro* or *in vivo* passage
- The *ermB-cfr-istAS-istBS* deletion event appears to be rare

CONCLUSIONS

- Association with pSM19035 sequences suggests potential for rapid horizontal spread of *cfi* among gram-positive pathogens
- Minicircles may enable additional spread of resistance genes by conjugation, transposition or insertion
- The 2nd generation oxazolidinone TR-700 (torezolid) maintained 16-fold potency advantage over LZD against Cfr-positive CM05 and a 4-fold advantage over Cfr-negative CM05Δ strains

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