Mutations in *apt* (adenine phosphoribosyltransferase) affect vancomycin susceptibility in vancomycin-intermediate *Staphylococcus aureus*

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Abstract

The gene *apt* encoding a purine salvage enzyme adenine phosphoribosyltransferase catalyzes the conversion of adenine and phosphoribosyl pyrophosphate into AMP and it also plays a role in the uptake of adenine. We had previously reported the laboratory selection of stable vancomycin-intermediate *Staphylococcus aureus* (VISA) mutants (MM66-3 and MM66-4) from a hetero-VISA strain (MM66) and now we are reporting that one mutation observed in the VISA mutants characterized was in *apt*. Compared to MM66, growth of MM66-3 and MM66-4 in the presence of adenine and 2-fluoroadenine was less impaired than the MM66 VISA mutants, which indicated a greater reduction in the accumulation of these toxic compounds in the VISA mutants compared to MM66. In addition to increased adenine and 2-fluoroadenine resistance, we also noted that several genes required for purine (*purLFMNHD*) and pyrimidine (*pyrABFE*) biosynthesis were upregulated in both MM66 VISA mutants compared to MM66. It has been reported that the selection with 2-flouroadenine resulted in 2-flouroadenine reduced susceptibility (FARS) mutants harboring mutations in *apt*. To ascertain a role for *apt* mutations on vancomycin susceptibility, FARS mutants of MM66 were selected and their *apt* regions were sequenced. Suspected MM66 FARS mutants arose on media containing 5 mM 2-flouroadenine at a mutation frequency of 4.7 X 10^{-7} and all randomly selected MM66 colonies demonstrated higher 2-flouroadenine MICs than MM66. In addition, all FARS MM66 mutants harbored one of the variety of mutations (e.g., deletion, insertion and nonsense mutations) within the *apt* gene. FARS mutants MM66-FARS-1 and MM66-FARS-6 demonstrated identical growth curves and slightly decreased tolerance to 5 mM adenine growth inhibition compared to the parent strain MM66. Furthermore, compared to MM66, all MM66-FARS mutants demonstrated increased resistance to Congo red, similar to the *apt* mutants previously reported. In contradiction to these findings, MM66-3 and MM66-4 demonstrated reduced resistance to Congo red compared to parent strain MM66. The FARS MM66 mutants also demonstrated increased distances grown on the vancomycin gradients when investigated. These findings suggests that the *apt* mutations can alter vancomycin susceptibility suggesting a role for altered purine metabolism in a VISA mechanism.

Introduction

Staphylococcus aureus is a notorious human pathogen that is associated with both hospital- and community-acquired infections. Since the emergence of multidrug-resistant methicillin-resistant *S. aureus* (MRSA), the treatment of infections caused by these organisms have become challenging [1]. The glycopeptide antibiotic vancomycin remains a clinically proven drug for the treatment of serious MRSA infections [2]. The increased use of vancomycin, in large part due to increased incidence of MRSA infections, eventually led to the selection of *S. aureus* strains that demonstrated reduced susceptibility and resistance to vancomycin. Based on vancomycin MICs, *S. aureus* isolates are classified as vancomycin-susceptible *S. aureus*, (vancomycin MIC ≤ 2 µg/ml) vancomycin-intermediate *S. aureus* (VISA) (MIC ≥ 4 µg/ml), and vancomycin-resistant *S. aureus* (VRSA) (MIC ≥ 16 µg/ml) [2]. The VISA phenotype however is unrelated to the *van*-mediated VRSA mechanism and the VISA mechanism is supported by chromosomal mutation(s) that are strain dependent and variable [2,3]. VISA mutations in turn lead to alterations in peptidoglycan metabolism and structure, and increased peptidoglycan thickness is common among VISA [3]. The overproduction and accumulation of cell wall material in VISA strains, and thus free-D-ala-D-ala binding sites, is hypothesized to sequester vancomycin away from its target at the plasma membrane [2,3]. Based on a comparison of VISA strain physiology and mutational analysis, it has been surmised that the acquisition of the VISA phenotype can occur via multiple evolutionary trajectories [3].

During the early days of VISA characterization, it was hypothesized that the altered regulation of genes involved with purine biosynthesis played a role in a VISA mechanism [4]. Following this suggestion, another study could not confirm a link between altered purine biosynthetic gene expression and reduced vancomycin susceptibility [5]. The gene *apt* encoding purine salvage enzyme adenine phosphoribosyltransferase catalyzes the conversion of adenine and phosphoribosyl pyrophosphate into AMP [6,7] and plays a role in the uptake of adenine [8,9]. It was reported previously that an *apt* mutation was present in a laboratory-derived VISA strain derived from an MRSA strain [10], although the impact of *apt* mutation was not fully explored.

Our laboratory reported on the isolation of stable VISA strains from a clinical hetero-VISA strain (MM66) via vancomycin selection [11]. During the investigation of the VISA mechanism of MM66 VISA mutants, we discovered that these mutants demonstrated the exact same *apt* point mutation (submitted for publication). In this presentation, we are providing evidence that supports that the *apt* mutations in MM66 VISA mutants lead to a loss of Apt function. In order to further understand an association of the *apt* mutation with the MM66 VISA mechanism, we characterized MM66 2-fluoroadenine reduced susceptibility (FARS) mutants harboring *apt* mutations. The research completed adds to the literature on VISA mechanisms and we are also providing evidence that the *apt* mutations can support reduced susceptibility to vancomycin.

Methods

Growth curves and conditions: All bacteria were cultured in Luria Bertani broth (LB) (Difco, Detroit, MI) with shaking (200 rpm, 37ºC) or on LB agar (LBA) as required. All overnight cultures were initiated with single colonies and then allowed to grow at 37ºC (200 rpm) overnight. Working stock LBA cultures were kept at 4ºC and all the strains were stored in LB containing 20% glycerol at -80ºC. Overnights were diluted to initial OD_{580} of 0.04 and the OD_{580} was recorded over time with triplicate cultures. Growth curves were performed in LB and in LB containing 5 mM adenine or 5 mM 2-fluoroadenine.

Isolation and characterization of FARS MM66 mutants:

Aliquots (100 µl) of overnight grown cultures were spread on the LB agar containing 5 mM 2-fluoroadenine (2-FA) and incubated at 37°C for 24 h. Colonies appeared on 2-FA containing plates were then selected for MICs determination. In order to sequence and detect mutations in the *apt* genes of the MM66 FARS mutants a primer set (apt100U-F and apt100D-R) was designed that would amplify 100 bp upstream and downstream of the *apt* gene. Sequencing of the *apt* amplicons was done using ABI 3100 Genetic Analyzer (Applied Biosystem, USA) and *apt* mutation were detected with DNASTAR SeqMan Pro, (Version 9.1.0 (109), 2011).

Antimicrobial susceptibility testing: Vancomycin gradient plates were inoculated with overnight grown culture and incubated at 37ºC and read after 24h incubation. Susceptibility was determined by measuring the lengths of confluent growth in mm on the gradient plates following 48h of growth at 37ºC. Congo red susceptibility assays were performed by spotting 10-fold dilutions of overnight cultures onto LBA and LBA supplemented with 0.1% (wt/vol) Congo red. Plates were incubated at 37ºC overnight and imaged.

Results and Discussions

It has been reported that *apt* plays a role in the uptake of exogenous adenine [12,13] and adenine inhibits the growth of *S. aureus* [14]. Also, *apt* mutants of *Bacillus subtilis* exhibited increased tolerance to the toxic adenine analogue 2-FA [13]. We therefore hypothesize that the *apt* mutation in the MM66 VISA mutants would reduce adenine and 2-FA accumulation and toxicity compared to MM66. As expected, MM66 grew slightly slower than MM66-3 and MM66-4 in the presence of 5 mM adenine (Fig. 1) and the growth of MM66 was limited by the addition of 2-FA, while MM66-3 and MM66-4 grew in the presence of this toxic adenine analog (Fig. 2). Note that the MM66 VISA mutants and MM66 exhibited almost identical growth curves in drug-free media (Figs. 1 and 2). In addition to the increased 2-FA and adenine growth resistance, we also noted that several genes required for purine (*purLFMNHD*) and pyrimidine (*pyrABFE*) biosynthesis were upregulated in both MM66 VISA mutants compared to MM66 (unpublished).

To ascertain a role for *apt* mutations on vancomycin susceptibility, FARS mutants of MM66 were selected and the *apt* regions were sequenced. Suspected MM66 FARS mutants (MM66-FARS-1 through MM66-FARS-6) arose on media containing 5 mM 2-FA at a mutation frequency of 4.7 X 10^{-7} and all randomly selected MM66 colonies demonstrated higher 2-FA MICs than MM66 (Table 1). All FARS MM66 mutants harbored one of a variety of mutations (e.g., deletion, insertion and nonsense mutations) within the *apt* gene (Table 2). FARS mutants MM66-FARS-1 and MM66-FARS-6 demonstrated identical growth curves and slightly decreased tolerance to 5 mM adenine growth inhibition compared to the parent strain MM66 (Fig. 3). Furthermore, all MM66-FARS mutants investigated demonstrated increased resistance to Congo red, similar to the *apt* null mutants previously reported on (Fig. 4) [15]. All FARS MM66 mutants demonstrated increased distance when grown on the vancomycin gradients compared to MM66 (Table 1).

Conclusions

1. VISA mutants of MM66 harbored an *apt* loss of function mutation which supported the growth of these mutants in the presence of toxic purines and was associated with the altered expression of purine biosynthesis genes.
2. MM66 FARS mutants were easily isolated, did not seem to demonstrate growth deficiencies in laboratory media, and harbored one of the variety of *apt* mutations.
3. MM66 FARS mutants demonstrated increased 2-FA MICs, slightly decreased susceptibility to adenine growth inhibition, increased resistance to Congo red, and increased distance when grown on the vancomycin gradients.
4. We conclude that *apt* loss of function mutations can contribute to reduced vancomycin susceptibility in MM66.

Results and Discussions contd.

Table 1. 2-FA MICs and distances (mm ± SD, n = 3) grown on vancomycin gradient plates

Strains	2-FA MIC (mM)	Vancomycin gradients (mm)	
		0 - 2 µg/ml	0 - 2.5 µg/ml
MM66	5	26 ± 2.00	17 ± 1.00
MM66-FA-1	>7	47 ± 0.58*	33 ± 2.52*
MM66-FA-2	>7	48 ± 3.05*	33 ± 2.65*
MM66-FA-3	>7	49 ± 3.06*	36 ± 1.00*
MM66-FA-4	>7	52 ± 0.58*	37 ± 0.00*
MM66-FA-5	>7	55 ± 2.52*	38 ± 1.00*
MM66-FA-6	>7	53 ± 1.53*	36 ± 0.58*

*p-value ≤ 0.05 in comparison to MM66

Table 2. *apt* mutations in MM66 2-FA reduced susceptibility mutants

2-FA^{RS} mutant(s)	*apt* mutation*	Effect of mutation on Apt
MM66-FA-1	Contiguous 45 bp deletion between $A^{1720937}$ — $A^{1720891}$	M^{103}→I^{103} and H^{104} — D^{118} (HKDAIKPGQRVLITD) internal deletion
MM66-FA-2	Contiguous 45 bp deletion between $A^{1720937}$ — $A^{1720891}$	M^{103}→I^{103} and H^{104} — D^{118} (HKDAIKPGQRVLITD) internal deletion
MM66-FA-3	C insertion after GCACC^{1721018}	Frameshift after P^{76}
MM66-FA-4	Non-contiguous 15 bp deletion between $C^{1721039}$ — $A^{1721020}$	M^{70} — F^{74} (MGIGF) internal deletion
MM66-FA-5	ATGGG insertion after TGGGG^{1701031}	Frameshift after G^{71}
MM66-FA-6	$T^{1720975}$→$A^{1720975}$	Y^{90}→stop codon (TAA)

*Based on nucleotide positions in NC_002951

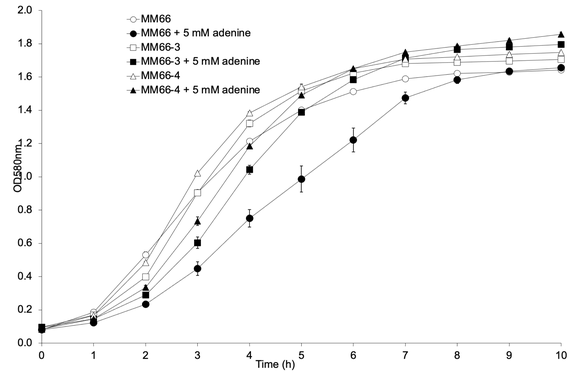

Figure 1. Growth curves. Open symbols represent control and closed symbols represent growth with 5 mM adenine. ○ and ●, MM66; □ and ■ MM66-3; △ and ▲ MM66-4. (Error bars represent standard deviation (n =3))

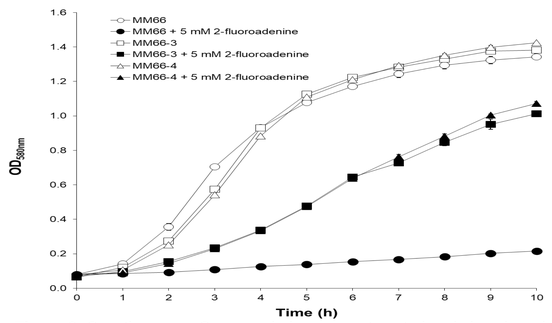

Figure 2. Growth curves. Open symbols represent control and closed symbols represent growth with 5 mM 2-fluoroadenine. ○ and ●, MM66; □ and ■ MM66-3; △ and ▲ MM66-4.

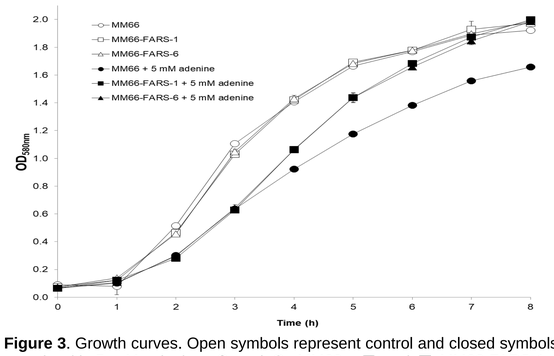

Figure 3. Growth curves. Open symbols represent control and closed symbols represent growth with 5 mM adenine. ○ and ●, MM66; □ and ■ MM66-FARS-1; △ and ▲ MM66-FARS-6.

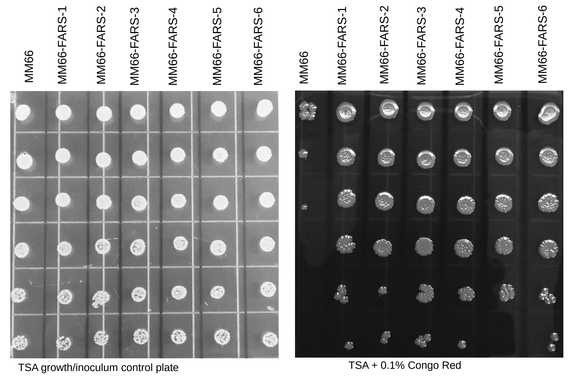

Figure 4. Growth on TSA and TSA + 0.1% Congo red.

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