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Whole-Genome Sequencing Reveals a Link Between β-Lactam Resistance and Synthetases of the Alarmone (p)ppGpp in *Staphylococcus aureus*

Michael M. Mwangi,[1,2] Choonkeun Kim,[1] Marilyn Chung,[1] Jennifer Tsai,[3] Govindan Vijayadamodar,[3,4] Michelle Benitez,[3] Thomas P. Jarvie,[3] Lei Du,[3] and Alexander Tomasz[1]

The overwhelming majority of methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates exhibit a peculiar heterogeneous resistance to β-lactam antibiotics: in cultures of such strains, the majority of cells display only a low level of methicillin resistance—often close to the MIC breakpoint of susceptible strains. Yet, in the same cultures, subpopulations of bacteria exhibiting very high levels of resistance are also present with variable frequencies, which are characteristic of the particular MRSA lineage. The mechanism of heterogeneous resistance is not understood. We describe here an experimental system for exploring the mechanism of heterogeneous resistance. Copies of the resistance gene *mecA* cloned into a temperature-sensitive plasmid were introduced into the fully sequenced methicillin-susceptible clinical isolate *S. aureus* strain 476. Transductants of strain 476 expressed methicillin resistance in a heterogeneous fashion: the great majority of cells showed only low MIC (0.75 μg/ml) for the antibiotic, but a minority population of highly resistant bacteria (MIC >300 μg/ml) was also present with a frequency of $\sim 10^{-4}$. The genetic backgrounds of the majority and minority cells were compared by whole-genome sequencing: the only differences detectable were two point mutations in *relA* of the highly resistant minority population of bacteria. The *relA* gene codes for the synthesis of (p)ppGpp, an effector of the stringent stress response. Titration of (p)ppGpp showed increased amounts of this effector in the highly resistant cells. Involvement of (p)ppGpp synthesis genes may explain some of the perplexing aspects of β-lactam resistance in MRSA, since many environmental and genetic changes can modulate cellular levels of (p)ppGpp.

Introduction

Introduction of the semisynthetic β-lactam antibiotic methicillin into clinical practice in 1961 was followed rapidly by the appearance of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates,[4,12,26,30] which were shown to carry an extra penicillin-binding protein—PBP2A[17]—that was added to the four native staphylococcal PBPs—a group of membrane anchored extracytoplasmic proteins that synthesize the cell wall peptidoglycan. In contrast to the native PBPs, PBP2A was shown to have extremely low affinity to all β-lactam antibiotics and was able to continue catalyzing peptidoglycan biosynthesis even in the presence of high concentrations of antibiotics that inhibited native PBPs. The genetic determinant of PBP2A—*mecA*—was also identified[5] as an acquired gene associated with heterologous staphylococcal chromosomal cassettes (SCC*mec*), which incorporate into the chromosome of MRSA strains at a unique chromosomal site.[22] While the carrier cassettes—SCCs—were shown to exhibit considerable structural variation,[23] the sequence of the *mecA* determinant is invariant in most MRSA isolates with the exception of some recently identified strains that carry a new *mec* gene homologue.[15,28]

In contrast to the rapid clarification of biochemical and genetic basis of methicillin resistance, the mechanism of the phenotype of β-lactam resistance has remained unclear. It has been known for some time that to express methicillin resistance it was necessary, but not sufficient, that the MRSA strain carried a functional copy of *mecA* since variations in the temperature, pH value, or salt concentration during exposure of the same MRSA strain to methicillin were shown to cause profound effects on the level of antibiotic resistance.[9,18,38] Also, clinical isolates of MRSA exhibit large variations in the resistance level, which cannot be simply explained by the activity of regulatory genes (*mecI/mecR* or *blaI/blaR*) that control the transcription of the *mecA* determinant.

[1]Laboratory of Microbiology, The Rockefeller University, New York, New York.
[2]Department of Veterinary and Biomedical Sciences, Penn State University, University Park, Pennsylvania.
[3]454 Life Sciences, a Roche Company, Branford, Connecticut.
[4]BioXcel Corporation, Branford, Connecticut.

An even more complex feature of the resistant phenotype of MRSA is its heterogeneity detectable in most clinical isolates. The great majority of MRSA strains grown from single cell inocula produce cultures in which, most (over 99%) of the cells exhibit only modest or low-level resistance, sometimes approaching the breakpoint of susceptible strains. However, the same MRSA cultures also contain highly resistant cells with a frequency and degree of resistance, which are characteristic of the particular MRSA clone.[18,35] Plotting the number of bacterial cells capable of growing at various concentrations of the antibiotic against the concentration of the antibiotic in the agar medium produces population analysis profiles (PAPs) the shape of which seems to represent a unique signature of the particular MRSA clone.

The purpose of studies described here was to develop a model system that would allow a better understanding of the mechanism that controls the heterogenous β-lactam-resistant phenotype in *S. aureus*.

Materials and Methods

Population analysis profiles

The susceptibility of *S. aureus* strains to oxacillin (a β-lactam antibiotic, similar to methicillin, resistant to penicillinase) was determined by PAPs. Population analysis was carried out on tryptic soy agar (TSA) plates containing increasing concentrations of oxacillin and supplemented with 20 μg/ml of chloramphenicol for 476(*pmecA*) and 476^{mut} (*pmecA*) as described previously.[10,28] Colony forming units were counted after 48 hr of incubation of the plates at 30°C.

Introduction of plasmid-borne copies of the mecA *gene into the oxacillin-susceptible* S. aureus *strain 476*

The recombinant plasmid pSPSW2C, which carries the 3,737-bp region of *S. aureus* COL *mecA*, was introduced into *S. aureus* strain RN4220 by electroporation, and then transduced by phage 80α into *S. aureus* strain 476 to yield strain 476(*pmecA*) as described previously.[2,41]

Whole-genome sequencing

Chromosomal DNA preparations isolated from the plasmid-free strains named "476^{mut}-cured-small" and "476^{mut}-cured-large" were sequenced and compared to the DNA sequence of strain 476. The complete genomic sequence (no contig gaps) for 476 is available at the NCBI's Website*. The 454 platform was used for the re-sequencing of the genome of strain 476—a stock of which was available in our laboratory's strain collection. The same 454 platform was also used for the sequencing of the genome of 476^{mut}-cured-small and *de novo* assemblies of the reads were done. The same stock of strain 476 also served as the recipient of the *pmecA* in all the experiments described in this communication.

The Illumina platform was used for the sequencing of the genome of 476^{mut}-cured-large, and a template-based assembly of the reads was done using the NCBI's complete genomic sequence for 476 as the reference. The methods for the sequencing, assemblies, and polymorphism detection were described previously.[7,19,36,39]

*http://www.ncbi.nlm.nih.gov/genome/154?project_id=57841

Measurement of (p)ppGpp accumulation

The level of (p)ppGpp (guanosine 5′-diphosphate, 3′-diphosphate) was determined by P^{13} labeling as previously described.[34,37] A 3 ml culture of cells was harvested at OD 0.4 and extracted with 40 μl of 5 M formic acid by repeating freeze/thaw cycles four times, followed by incubation for 30 min on ice. Cell debris were removed by centrifugation at the maximum speed for 5 min at 4°C. The extracted samples were spotted on polyethyleneimine-cellulose F thin layer chromatography (TLC) plates pretreated with 10% sodium chloride and 100% methanol.[8] The plates were developed with 1.5 M monopotassium phosphate (pH 3.5) for separation of the phosphorylated nucleotides. $^{32}P_i$-labeled nucleotides were visualized with a Typhoon9400 image scanner, and nucleotide spots were quantified by an ImageQuant software. Amounts of (p)ppGpp were described as fractions of total guanine nucleotides, including GTP and (p)ppGpp. The sample extracted from 476(*pmecA*) treated with 60 μg/ml of mupirocin (an agent that can induce the stringent stress response), 15 min before harvest, was used as a positive control for identification of (p)ppGpp spots on TLC plates.

Results and Discussion

Search for a simple experimental model to study the mechanism of heterogeneous resistance

While all MRSA strains carry the methicillin resistance gene *mecA* on a chromosomally located SCC*mec* cassette, experimental manipulation of these structures is difficult. Previous studies from our lab[2] and other labs[27] have shown that methicillin resistance can be introduced and expressed in susceptible strains of *S. aureus* by plasmid-borne copies of the *mecA* gene. It was shown that introduction of a plasmid-borne *mecA* into the *S. aureus* strain COL-S (a derivative of the highly resistant MRSA strain COL from which the SCC*mec* cassette was removed by precise excision), produced transductants with high and homogeneous oxacillin resistance indistinguishable from that of the original COL strain.[2] Similar findings were obtained when the same plasmid was introduced into strain COL in which the resident *mecA* was inactivated by transposon insertion.[32] Interestingly—and in contrast—introduction of the same *mecA* carrying plasmid into several methicillin-susceptible *S. aureus* (MSSA) strains produced transductants with heterogeneous methicillin resistance, similar to the phenotypes of most clinical MRSA strains, suggesting that at least some of the determinants of β-lactam resistance reside in the genetic background of the bacteria that received the plasmid-borne *mecA* determinant (results not shown).

Oxacillin-resistant transductants of MSSA strain 476

After testing a number of methicillin-susceptible clinical isolates of *S. aureus*, we chose the fully sequenced MSSA strain 476 (sequence type—ST1),[20] to construct a model for studying heterogeneous resistance. A heteroresistant MRSA strain—MW2—with the same sequence type 1 carrying SCC*mec* type IV was also sequenced recently.[3]

Upon transduction of the thermosensitive plasmid *pmecA* into strain 476, a heterogeneously resistant strain was obtained in which the majority of transductants—named 476(*pmecA*)—showed only low-level resistance to oxacillin

(MIC 0.75 μg/ml). However, a more detailed population analysis indicated that the same culture also contained an apparently single subpopulation of a highly resistant mutant—bacteria named 476^{mut}(*pmecA*)—with an approximate frequency of 10^{-4} and an extremely high oxacillin MIC value of >3 mg/ml (Fig. 1A). The high level resistance was specific for β-lactam antibiotics such as methicillin, oxacillin, cefuroxime, imipenem, while the bacteria remained susceptible to other antibiotics such as vancomycin, fosfomycin, rifampicin, or linezolid.

A single colony of 476^{mut}(*pmecA*) picked from a TSA plate containing 200 μg/ml oxacillin was used as an inoculum to generate an overnight culture in tryptic soy broth (TSB). Upon plating for population analysis, this culture produced bacteria that were homogeneously and highly resistant to oxacillin (Fig. 1A).

The poorly resistant majority cells—476(*pmecA*)—and the highly resistant mutant— 476^{mut}(*pmecA*)— selected as homogeneous cultures were next compared for a number of properties.

The two subpopulations of bacteria differed in colony size and growth rate: in contrast to 476(*pmecA*), the highly resistant mutant 476^{mut}(*pmecA*) produced small colonies when plated on TSA (Fig. 1B) and determination of the corresponding growth rates of liquid cultures in TSB at 30°C showed mass doubling times of about 32 min for 476(*pmecA*) and 55 min for 476^{mut}(*pmecA*) (Fig. 1B).

Growth of the highly resistant 476^{mut}(*pmecA*) cells at 42°C, a temperature nonpermissive for plasmid replication produced bacteria that were free of the plasmid and plating of such cured (plasmid-free) bacteria on TSA produced primarily (over 95%) small colonies (named 476^{mut}-cured-small) similar to the size of the original 476^{mut}(*pmecA*). A small proportion of the cured bacteria produced somewhat larger colonies (named 476^{mut}-cured-large) (Fig. 2). Both populations of the cured bacteria regained susceptibility to oxacillin with MIC values identical to that of the parental strain 476 (oxacillin MIC = 0.4 μg/ml).

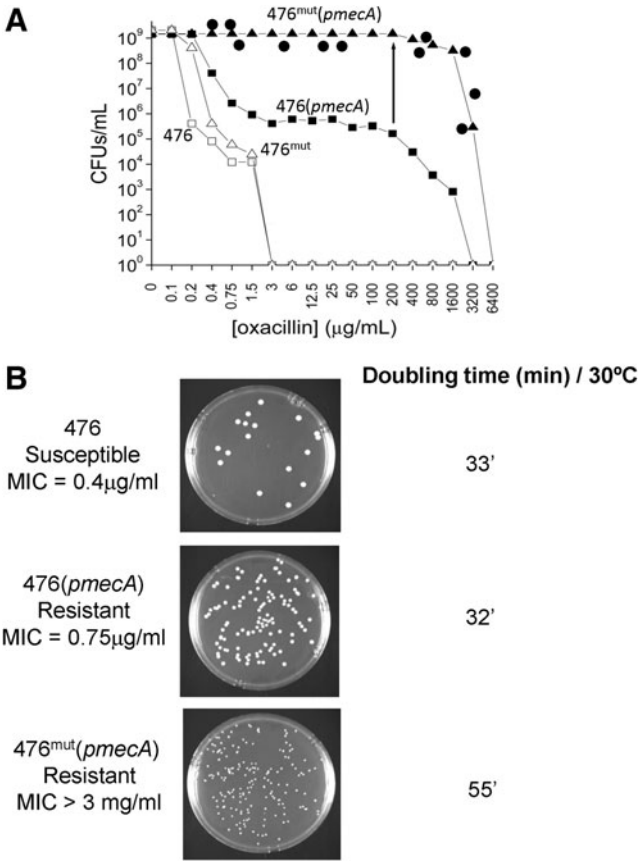

FIG. 1. Properties of *Staphylococcus aureus* strain 476 and its laboratory constructs carrying plasmid-borne copies of the *mecA* gene. The *mecA* gene was introduced into strain 476 by transduction using the temperature-sensitive plasmid pSTSW-2C—as described in the "Materials and Methods" section. **(A)** shows the oxacillin-susceptibility profile of the transductant 476(*pmecA*) (■) as determined by its population analysis profile (PAP). Also shown are the PAPs of the cultures of the susceptible recipient strain 476 (□); the highly resistant mutant 476^{mut}(*pmecA*) (▲), which was obtained by picking a rare colony present in the culture of 476(*pmecA*), and the PAP of strain 476^{mut} (Δ), which was generated from 476^{mut}(*pmecA*) by deletion of the thermosensitive *mecA* plasmid. The PAP of 476(*pmecA*) was also determined by supplementing each oxacillin-containing plate with 0.03 μg/ml mupirocin (•). **(B)** shows colony sizes of strains 476, 476(*pmecA*), and 476^{mut}(*pmecA*) on TSA plates incubated at 30°C for 48 hr. Also shown are growth rates (mass doubling times) of the same cultures grown in TSB at 30°C.

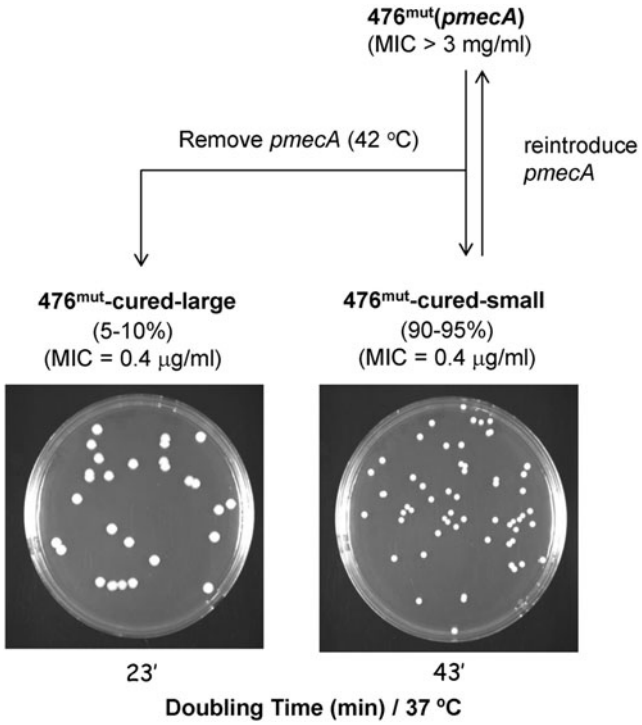

FIG. 2. Schematic representation of methods used to generate various derivatives of the transductants of strain 476. 476^{mut} cells free of the *pmecA* plasmid were produced by incubation of 476^{mut}(*pmecA*) at 42°C, resulting in 95% small colonies (named 476^{mut}-cured-small) and 5% large colonies (named 476^{mut}-cured-large). Reintroduction of the *pmecA* plasmid into 476^{mut}-cured-small cells produced bacteria with homogenous and high-level resistance to oxacillin indistinguishable from that of the original 476^{mut}(*pmecA*) transductants.

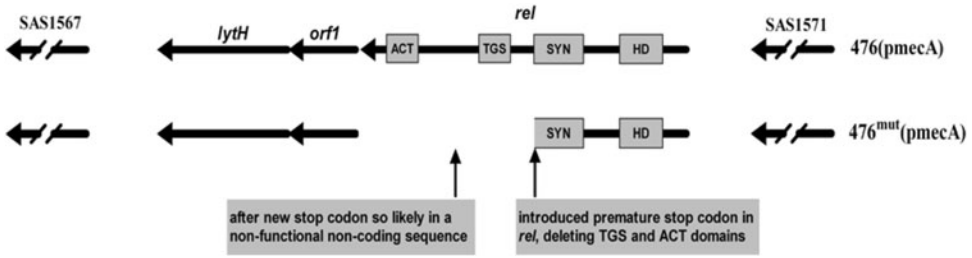

FIG. 3. Structure of the *relA* gene in *Staphylococcus aureus* strains 476(*pmecA*) and location of the two-point mutations in the *relA* gene of 476^{mut}(*pmecA*). In the 736 aa-long wild-type RelA protein, the Pfam search found an aa 57–156 match hydrolase domain (HD; E-score = 10^{-17}); an aa 247–357 match synthetase domain (SYN; E-score = 10^{-51}); and an aa 402–461 and 661–727 match, respectively, TGS and ACT (respective E-scores = 10^{-26} and 10^{-7}). In the 2,211-bp-long *relA* gene, the actual base changes are g→a at the 1,052nd base introducing a new premature stop codon and g→c at the 1,546th base. The former substitution caused the mutant RelA protein lack of TGS and ACT domains.

Reintroduction of the *pmecA* plasmid into the small-size cells in the second round of transduction produced a homogenous culture of highly oxacillin-resistant bacteria indistinguishable from the original 476^{mut}(*pmecA*) transductants—both in the antibiotic resistance level and in their small colony size.

These observations clearly indicate that important determinant(s) that define the level of oxacillin resistance (MIC value) in strain 476^{mut}(*pmecA*) must reside in the genetic background of the bacteria.

Identification of the mutations in the highly resistant bacteria

To identify the mutation(s) responsible for the high oxacillin resistance of 476^{mut}(*pmecA*), whole-genomic sequencing was done using the Genome Sequencer FLX from Roche 454.[31] The only mutations found in the highly resistant mutant were two single-nucleotide substitutions that occur in the *relA* gene on the chromosome[a] (Fig. 3). PCR sequencing confirmed that these mutations were not sequencing or assembly errors.

The *relA* gene has been partially characterized experimentally in a study unrelated to antibiotic resistance[16] and is involved in the production of the alarmone (p)ppGpp. When faced with nutrient starvation, virtually all bacterial species synthesize high levels of (p)ppGpp,[25] with *S. aureus* being no exception.[8] The effector (p)ppGpp then goes on to initiate a complex cellular program called the stringent response that modulates the expression of many genes and slows down growth.[25]

To better understand the effects of the mutations in *relA*, a Pfam search[13] was done that predicted that the wild-type Rel protein consists of at least four domains (Fig. 3). Listed in order from the amino- to carboxy-terminus, the domains are as follows: hydrolysis domain (HD), responsible for hydrolyzing/degrading (p)ppGpp; synthetase (SYN), responsible for synthesizing (p)ppGpp; and TGS and ACT, whose functions are poorly understood.

Of the two single-nucleotide substitutions in the highly resistant mutant, one introduced a premature stop codon in *relA* that deleted the TGS and ACT domains. The other mutation occurs after the new stop codon in what is likely to be a noncoding nonfunctional sequence. That only the TGS and ACT domains were deleted may be significant as a larger disruption of *relA* was proposed to be lethal.[16]

Studies of orthologs of *relA* in other bacterial species offer some insight into the functions of the TGS and ACT domains. Blasts[33] of sequences in GenBank[6] indicate that *relA* has a very close ortholog rel_{Msm} in *Mycobacterium smegmatis* MC2 155.[b] The Rel_{Msm} protein is known to have the same domain organization as depicted in Fig. 3, and in an *in vitro* system, deletion of the TGS and ACT domains has been found to increase in the net rate of (p)ppGpp synthesis by this protein.[24] An increased cellular level of (p)ppGpp in the highly resistant mutant would be consistent with the mutant's observed reduced growth rate.

A partial revertant with reduced resistance carries an extra mutation in a gene relQ *that codes for another (p)ppGpp synthetase*

Removal of the *pmecA* plasmid from strain 476^{mut}(*pmecA*) by growing bacteria at 42°C produced antibiotic-susceptible cells named 476^{mut}-cured-small most of which (95%) have retained the small colony size characteristic of 476^{mut} (*pmecA*). Nevertheless, a fraction of the cured cells (about 5%) produced larger colonies called 476^{mut}-cured-large.

Reintroduction of the *pmecA* plasmid into the majority of the cured cells, which had the small colony size (476^{mut}-cured-small) produced transductants indistinguishable from 476^{mut}(*pmecA*) both in the resistance level and colony size. Reintroduction of the plasmid-borne *mecA* into the rare larger colonies (476^{mut}-cured-large) also produced homogeneous oxacillin-resistant cultures, but the actual oxacillin MIC value was somewhat less (about 800 μg/ml) compared with the majority 476^{mut}(*pmecA*) cells (MIC > 3 mg/ml).

PCR sequencing of the *relA* gene of 476^{mut}-large and 476^{mut}-small showed that both strains carried the same two-nucleotide substitutions in *relA* (Fig. 3). To identify the presumed additional mutation in 476^{mut}-cured-large, whole-genome sequencing was done using a shortread technology. The only mutation found between the highly resistant mutant and the partial revertant was a single-nucleotide

[a]The gene *relA* has the identifier SAS1570 in the annotation for the 476 genomic sequence produced by the Sanger Institute.

[b]The two proteins were found to be excellent best blastp reciprocal matches (E-scores < 10^{-160}, with 41% aa identity extending over their entire lengths).

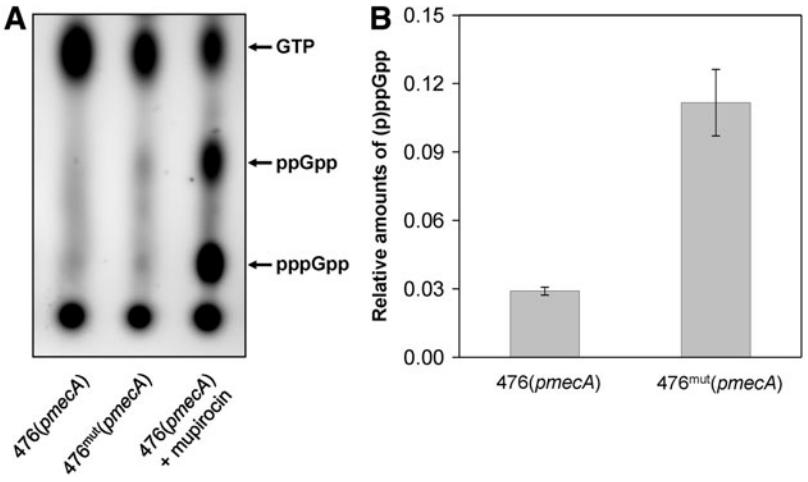

FIG. 4. Oxacillin resistance and cellular amounts of (p)ppGpp. **(A)** Cell extracts were obtained from identical amounts of strains 476(*pmecA*) and 476^{mut}(*pmecA*). The relative amounts of (p)ppGpp were determined by thin layer chromatography followed by autoradiography of extracts from ^{32}P-labeled bacteria. The sample from 476(*pmecA*) + mupirocin was used as a control for the (p)ppGpp spots on TLC plates. **(B)** Relative amounts of (p)ppGpp are expressed as a fraction of the total ^{32}P-labeled guanine phosphate precursor pool.

substitution in the latter strain in the gene *relQ*, which is separated from *relA* by 728,007 bases on the chromosome. PCR sequencing confirmed that the base change in *relQ* was not a sequencing or assembly error.

The *relQ* gene has not been characterized experimentally, but its function can be inferred using standard bioinformatics tools. A Pfam search[13] found that the wild-type RelQ protein consists of a (p)ppGpp synthetase domain SYN.[c] The base change in the partial revertant converted a valine to a glycine in the middle of SYN, possibly affecting (p)ppGpp synthesis.[d] Relative to the highly resistant mutant, a decrease in the cellular (p)ppGpp level in the partial revertant would be consistent with the strain's observed increased growth rate and reduced antibiotic resistance.

Interestingly, blasts[33] of sequences in GenBank[6] reveal that the two genes *relA* and *relQ* (and another gene *relP* also in *S. aureus* 476) have very close orthologs ($relA_{Smu}$, $relQ_{Smu}$, and $relP_{Smu}$ respectively), in another gram-positive bacterium *Streptococcus mutans* UA159.[1,e] Very recently, these three genes in *S. mutans* were all shown experimentally to code for (p)ppGpp synthetases.[29]

That the extra gene found to be mutated in the partial revertant, is *relQ*, is highly statistically significant. There are roughly 2,800,000 bp and 2,500 predicted genes in the *S. aureus* genome, so it is exceedingly unlikely that it is a mere coincidence that the one extra gene found to be mutated in the partial revertant also codes for a (p)ppGpp synthetase.

[c]In the 211 aa long wildtype *relQ* protein, aa 43-162 match SYN (E-score = 10^{-43}).

[d]In the 636 bp long *relQ* gene, the actual base change is t→g at the 299th translated base.

[e]The gene *relP* has the identifier SAS2394 in the annotation for the 476 genomic sequence. The *rel* and rel_{strep} proteins were found to be excellent best blastp reciprocal matches (E-scores = 0, with 50% identity over their entire lengths). Ditto for the *relQ* and $relQ_{strep}$ proteins (E-scores < 10^{-68}, 58% aa identity over entire lengths). Ditto for the *relP* and $relP_{strep}$ proteins (E-scores < 10^{-27}, 39% aa identity over entire lengths).

The role of stress response in the expression of β-lactam resistance

The findings described in this communication suggest that expression of high-level resistance in our model system was connected to mutations in the *relA* and/or *relQ* genes, which should cause production of increased amounts of (p)ppGpp in the resistant bacteria. To test this, we determined the cellular amounts of this effector. Figure 4 shows that the model strain 476^{mut}(*pmecA*) indeed had larger relative amounts of (p)ppGpp—approximately fourfold—as compared to strain 476(*pmecA*). The fractions of (p)ppGpp in strains 476(*pmecA*) and 476^{mut}(*pmecA*) were 0.029 and 0.113, respectively. Increased relative level of (p)ppGpp in 476^{mut}(*pmecA*) is similar to that in the small colony variant of MRSA JKD6210, which carries a point mutation in the *relA* gene causing reduction of the RelA hydrolase activity and constitutive activation of the stringent response.[14]

Involvement of a stress response in the β-lactam-resistant phenotype of MRSA has been proposed earlier.[11] A direct effect of the stringent stress response on the β-lactam resistance level is shown in Fig. 1A. Plating strain 476(*pmecA*) on oxacillin-containing agar plates (see curve with solid square symbols) produced a heterogeneous phenotype in which, the overwhelming majority of bacteria expressed only very low levels of antibiotic resistance (MIC around 0.75 μg/ml). When the same population analysis was repeated using agar plates that also contained a sub-MIC concentration of mupirocin (see curve with solid circle symbols in Fig. 1A), culture strain 476(*pmecA*) was able to express homogenous and high-level resistance indistinguishable from the resistance level obtained in strain 476^{mut} *pmecA*, that is, the strain that carried the *relA* mutation.

Of the roughly 2,500 predicted genes in the *S. aureus* genome, the only two genes found to be mutated in the highly resistant mutant and its partial revertant both code for (p)ppGpp synthetases. Involvement of (p)ppGpp may explain many of the peculiar properties of β-lactam resistance in MRSA, since variables such as shifts in temperature, salt

concentration, and pH, which were shown to impact on β-lactam resistance in MRSA, are also known to affect cellular (p)ppGpp levels in several bacterial species.[21,40] Any one of the many mutations in any one of the many genes can also be expected to modulate (p)ppGpp levels, including genes important for the uptake and utilization of nutrients.[25]

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Alexander Tomasz, PhD
Laboratory of Microbiology
The Rockefeller University
New York, NY 10065

E-mail: tomasz@mail.rockefeller.edu