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Genetic Determinants Affecting Macrolide Resistance Conferred by MEGA in *Streptococcus pneumoniae*

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B.S., Clemson University, 2007

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ABSTRACT:

Macrolide antibiotic resistance in *Streptococcus pneumoniae* due to efflux is a major clinical problem and may lead to failure of macrolide treatment of pneumococcal disease. Specifically, macrolide resistance due to efflux is the result of a predicted dual component efflux pump encoded by *mefE/mel* of the mobile genetic element MEGA. A separate operon, *orfs3-6*, frequently associates with *mefE/mel*. o*rfs3-6* were found to influence macrolide resistance levels of clinical isolate GA17457, but did not have any significant effect on the expression of a P*mefE-lacZ* reporter fusion as determined by a β-galactosidase assay. GA17457 was compared to another clinical isolate, GA16242, with nearly three-fold higher macrolide resistance (13 μg/mL compared to 43 μg/mL respectively). A two-fold increase in β-galactosidase expression was observed in the GA16242 reporter construct TS8011 when compared to the GA17457 reporter construct XZ7042. A novel element containing a putative transposase and an uncharacterized open reading frame (*orfsAB)* was identified downstream of MEGA in GA 16242. However, deletion of this region did not influence macrolide resistance or *mefE/mel* in a β-galactosidase expression assay. Results of these studies confirm a role for *orfs3-6* in the macrolide resistance conferred by MEGA but not in the transcriptional regulation of *mefE/mel*. Additionally, the comparison of two strains with different MEGA insertion sites identified significant differences in macrolide resistance and *mefE/mel* expression, but did not elucidate the cause for these observations.

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Table of Contents

INTRODUCTION

History

Brigadier General George Miller Sternberg, the 18th U.S. Army Surgeon General, and French chemist and bacteriologist Louis Pasteur are responsible for independently isolating the major etiologic agent of bacterial pneumonia, *Streptococcus pneumoniae*. The organism was originally named *Diplococcus pneumoniae* due to its typical microscopic appearance and was not given the present name of *S. pneumoniae* until 1974. Pasteur is credited with his discovery of *S. pneumoniae* in a Jan 24, 1881 communication to the Academy of Sciences in Paris. Sternberg reported his discovery the following month [3, 4]. The development of the Gram stain in 1884 allowed for discrimination between the gram-positive *S. pneumoniae* and another kind of bacteria known to cause clinical pneumonia, the Gram-negative *Klebsiella pneumoniae* [5].

The diagnosis and characterization of pneumococci was aided by other discoveries in the early and middle 20th century. The German bacteriologist Friedrich Neufeld observed the lysis of pneumococcal cultures following incubation with ox bile [6, 7]. His observations led to the discovery that pneumococci exist in multiple serotypes determined by differences in their capsular polysaccharide structure. Differentiation between the serotypes was accomplished by agglutination and subsequent prominence of the pneumococcal capsule in the presence of specific antiserum; Neufeld termed this phenomena the Quellung reaction after the German word for swelling [8, 9]. The discoveries made by Neufeld led to Frederick Griffith's experiments in 1928 that uncovered the transformability of pneumococcus in work attempting to design a vaccine. Neufeld's work also led to Thomas Francis and William Tillett's discovery in 1931 that the pneumococcal capsular polysaccharide is immunogenic in humans. Finally, in 1944 Oswald Avery, Colin MacLeod, and Maclyn McCarty discovered that DNA is the source for bacterial

transformation [10-13].

Pathology

Streptococci are spherical Gram-positive bacteria that grow in pairs or chains and include the pathogenic species *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae*. Streptococci are responsible for a wide range of infections including pneumonia, meningitis, and pharyngitis. Streptococci also can be present as colonizing human flora where *S. pneumoniae* is commonly found in the nasopharynx of healthy individuals [14]. Pneumonia is a common disease that affects the lower respiratory tract and is most prevalent in the very young, the very old, and the immuno-compromised. Bacterial pneumonia tends to be the most serious and is commonly due to *S. pneumoniae* [15, 16]. The World Health Organization estimates that nearly 1 million children die each year from pneumococcal disease with most of these deaths occurring in developing countries [17].

Pneumococci are transmitted through the spread of respiratory droplets via sneezing, coughing, or exposure to secretions, and pneumococcal infections are more common in the winter. In addition to causing pneumonia, pneumococci are also responsible for causing sinusitis, otitis media, bronchitis, bacteremia, and meningitis. Pneumococci cause pneumonia when the bacteria gain access to the alveolar spaces and lower respiratory tract via inhalation or aspiration and are not subsequently cleared by the host's local and systemic immune system [18]. Severe complications such as sepsis, respiratory failure, and pleural effusion can occur.

Treatment and Vaccine

Treatment for pneumococcal infections is with antibiotics; however, antibiotic resistance is becoming an increasing problem. Pneumococcal strains resistant to penicillin have appeared in the last two decades and resistance to tetracycline, clindamycin, macrolides, cephalosporins, lincosamides, quinolones, and many other antibiotic classes have been reported with increased frequency. Due to increased resistance in pneumococci, vancomycin and the extended spectrum cephalosporins are frequently used alone or in combination to treat serious pneumococcal infections such as meningitis. Additionally, pneumococcal vaccines have been developed to help protect at-risk groups from infection. The polysaccharide based vaccine, Pneumovax (PPSV23), offers some protection against 23 of more than 90 pneumococcal serotypes. PPSV23 has been given to individuals ages 2 and older that are considered high-risk, the immunocompromised, those living in extended-care facilities, smokers ages 19-64, and those with chronic pulmonary disease. Children under the age of two fail to mount an adequate immune response due to the method of manufacturing for Pneumovax and therefore receive a conjugate vaccine. The newer polysaccharide-protein conjugate vaccines, Prevnar (PCV7 & PCV13), were first introduced in 2000 as a heptavalent vaccine (PCV7) and later (2010) expanded to a thirteen valent vaccine (PCV13). Infants and children ages 2-59 months typically receive PCV13 and on a schedule of 4 doses over the course of a year. It is recommended that children under 70 months of age that have completed the PCV7 dosage schedule receive a booster dose of PCV13

PCV7 consists of the seven most common strains in the United States (4, 6B, 9V, 14, 18C, 19F, and 23F). This vaccine contains the pneumococcal bacterial capsule conjugated to non-toxic proteins of diphtheria toxin, specifically CRM_{197} of *Corynebacterim diptheriae.* Following the introduction of PCV7, remarkable decreases in invasive pneumococcal disease in

both children and adults were documented [19]. However, the decline in invasive serotypes covered by PCV7 was followed to a limited degree by the emergence of replacement serotypes. Due to the serotype replacement that occurred following the introduction of PCV7, six additional capsular polysaccharides (1, 3, 5, 6A, 7F, and 19A) have been added to the conjugate vaccine PCV13 for use in the United States. This should allow better protection against pneumococcal infections, particularly against the multi-antibiotic resistant serotype 19A and other emerging strains. Recently, a decavalent conjugate vaccine, Synflorix, has been approved for use in children in Europe. It covers the same seven serotypes as PCV7 with the addition of 1, 5, and 7F.

Antibiotic Resistance

Antibiotic resistance can arise through a variety of mechanisms that bacteria exploit in order to survive in an antibiotic-containing environment. These mechanisms include, but are not limited to, inactivation/modification of the drug itself, alteration of the drug's target site, alteration of the metabolic pathways targeted by the drug, and reduced accumulation and/or extrusion of the drug. Use and overuse of antibiotics enhances resistance by eliminating sensitive bacteria and selecting for bacteria that carry or acquire resistance genes. Once present, these mechanisms can be selected for and maintained in the presence of the drug. It is not uncommon for bacteria to possess more than once antibiotic resistance mechanism. The genetic mechanisms used by bacteria to acquire antibiotic resistance determinants include horizontal gene transfer mechanisms such as transduction, conjugation, and transformation.

S. pneumoniae are naturally competent bacteria and readily take up DNA from the environment through the process of transformation. The competence of pneumococci can be responsible for the spread of resistance mechanisms such as the chromosomal encoded macrolide

efflux system discussed later. Transformation has been implicated in the increase in resistance of *S. pneumoniae* in patients with middle ear infections that were treated with either a macrolide alone or in addition to another antibiotic prior to infection [20]. As noted, transformation was first documented in *S. pneumoniae* by Griffith in his classic experiments comparing the virulent and avirulent capsules of pneumococci [21].

Efflux Pumps

An important bacterial antibiotic defense mechanism is reduced drug accumulation, which occurs either through reduced permeability of the bacteria to the antibiotic or through efflux of the antibiotic out of the bacterial cell. Bacterial membranes are poorly permeable to hydrophobic and hydrophilic compounds because of the phospholipid bilayer structure; therefore, bacteria utilize nonspecific channels called porins to allow passage of such compounds into and out of the cell. As an example, mutations of OprM decrease permeability and are responsible for increased carbapenam resistance [22]. Reduced accumulation due to rapid efflux of the antibiotic out of the cell has many examples classified into the major facilitator superfamily (MFS), the ATP-binding cassette superfamily (ABC), the small multidrug resistance family (SMR), the resistance-nodulation-cell division family (RND), and the multi-antimicrobial transport and extrusion protein family (MATE).

Pumps belonging to the ABC family of efflux pumps are characterized by an **A**TP **b**inding **c**assette that allows for the hydrolysis of ATP to generate the energy needed to export molecules from the cell [23]. Members of the ABC family possess two transmembrane domains, each of which possesses six transmembrane helices. The two membrane-spanning domains form a pore through the membrane and associate on the cytoplasmic side of the membrane with two nucleotide-binding domains. The nucleotide binding domains bind and hydrolyze the ATP that

drives the pump. An example of a member of the ABC family is LmrA of *Lactococcus lactis*. Additionally, MsrA of the Mel/MsrA efflux pump, discussed in greater detail later, is predicted to be a member of the family of efflux pumps as well.

Pumps belonging to the other four families utilize proton or sodium gradients for energy with the MFS constituting the largest family [23, 24]. Some pumps are able to extrude the antibiotic completely out of the bacterial cell while others are only able to transport the drug into the periplasm [25]. Most members of the MFS possess two transmembrane domains that create a translocation channel through which the antibiotic passes [26]. The SMR family represents oligomeric small hydrophobic efflux proteins [27]. SMR proteins span the membrane four times as alpha-helices, exist as homotrimers, and often form complexes with other proteins [23, 28]. RND efflux pumps are drug antiporters with large periplasmic domains that consist of a tripartite system with adapter or fusion proteins and a channel [29]. The MATE family of efflux pumps topologically mimics the MFS family of efflux pumps but shares no sequence homology [30]. MATE family pumps often uses a Na+ gradient to facilitate transport rather than the proton gradient used by MFS, RND, and SMR families of efflux pumps.

Macrolides

As penicillin resistance emerged in the 1990s among pneumococci and other Gram-positive bacteria, macrolides became increasing popular as clinicians sought alternative and safe ways to treat upper respiratory infections or patients with penicillin allergies. Macrolides belong to the polyketide family of natural antibiotics and consist of a multi-membered macro-cyclic lactone ring possessing one or more deoxy sugars. The macrolides were found to inhibit protein synthesis in gram-positive bacteria by binding to the 50S subunit of the bacterial ribosome and inhibiting the peptidyltransferase reaction. Specifically, by inhibiting the peptidyltransferase,

macrolides prevent translocation of the ribosome and stall chain elongation; this interference with the translocation of the ribosome can lead to premature disassociation of the tRNA from the ribosome and the end of translation of the genetic message [31].

Following the introduction and increase usage of new macrolides, macrolide resistant strains of pneumococcus began to emerge [32]. Three main mechanisms of resistance were identified: modification of the ribosome through methylation of the 23S bacterial ribosomal RNA such that macrolides can no longer bind to the 50S subunit, mutations in the ribosomal RNA such that the binding capacity of the macrolide is reduced or abolished, and employment of an efflux pump to rapidly export macrolides from the inside of the cell into the surrounding environment.

***mef* & *mef* Containing Elements**

Macrolide resistance became a feature of many *S. pneumoniae* in the 1990s, though it may have been acquired first by group A streptococci (*S. pyogenes*) through multiple genetic events [33-36]. The presence, prevalence, and molecular basis of macrolide resistance in streptococci is largely due to either a methylase encoding gene, *ermAM/B,* and/or an efflux pump encoding genetic element, *mefE/A,* which is usually coupled with *msrA/D/mel*. As recently as 2009, around 35% of isolated pneumococcus in the US were resistant to macrolides with *mefE/mel* accounting for a majority of this resistance [37-40].

mef is named as such for **m**acrolide **ef**flux whereas *mel*, the *msrA* homologue, is named for the first three predicted coding amino acids of the gene. Within *mef* there are several subclasses including *mefA,* originally described in *S. pyogenes,* and *mefE,* originally described in *S. pneumoniae* [41, 42]. Additionally, *mef* genes have been found in a variety of other bacteria including gram-positive bacterial species belonging to *Enterococcus* and *Staphylococcus* and in

gram-negative bacterial species belonging to *Acinetobacter* and *Neisseria* [43-46]. Resistance to macrolides that is designated as M in *S. pneumoniae* is associated with the presence of *mef*. Strains designated as the M-phenotype of resistance are sensitive to lincosamide and streptogramin B antibiotics; strains that are resistant to all three are represented as the MLS_B-phenotype and possess *ermAM/B*. Typically strains with the M phenotype exhibit a lower level of resistance (1-32μg/mL) than those strains exhibiting an MLS_B phenotype of resistance (>64μg/ml) [32].

At a nucleotide level *mefA* and *mefE* show 90% homology; this difference in nucleotide homology is the distinction between *mefE*, *mefA*, and other *mef* homologs such as *mefB/G/I* and various mosaic forms [38, 39, 42, 47-50]. However, it has been suggested that the subclasses of *mef* should be based upon the genetic element on which they are carried [50]. *mef* is carried on multiple conserved genetic elements: Tn1207.3 carries subclass *mefA* and is conjugative, Tn1207.1 carries subclass *mefA* and is a non-conjugative derivative of Tn1207.3, and MEGA (**m**acrolide **e**fflux **g**enetic **a**ssembly) carries subclass *mefE* and alone appears to be a non-conjugative element similar to Tn1207.1 (Figure 1) [51-54]. All of these elements also contain in the same operon a *mel/msrD* homolog and various forms of a convergent operon termed *orfs3-6*, both of which are discussed below.

Tn1207.1 was one of the first *mef* carrying elements described, and it, along with a close relative Tn1207.3, preferentially inserts at the site of *celB*, which encodes a DNA binding protein [53, 55]. MEGA has two forms, one of which exists with a 99 bp deletion in the intergenic region between *mefE* and *mel/msrD*. MEGA can insert at different locations in the bacterial chromosome but does not contain genes encoding a recombinase or transposase [33, 45, 56, 57]. Other transposable elements that contain *mef* include Tn2009, containing MEGA inserted within

Tn916, Tn2010, which carries *ermB*, and 5216IQ, which contains *mefI/msrD* without the presence of *orfs3-6*(discussed later) [56, 58, 59] (Figure 1). The presence of MEGA within Tn916 is important due to Tn916's broad host-range and the known occurrence of Tn916-like conjugative transposons in many different bacteria; because of Tn916's ability to form composite structures, Tn916-like elements are postulated to be a direct vehicle for the distribution of antibiotic resistance genes to, but not within, *S. pneumoniae* [52, 60-62].

Conjugal transfer in *S. pneumoniae*, which has highly developed transformation systems, is lacking or inefficient. But in *S. pyogenes* conjugal transfer is often efficient and transformation is not a common event. However, chimeric structures in *S. pyogenes* where *mefA* exists within a prophage have been documented, making it plausible for non-transferable elements to become transferable through transduction [63-65]. Additionally, conjugal transfer of MEGA from *S. salivarius* to *S. pneumoniae* into an novel insertion site has been documented, with such conjugal transfer of MEGA likely made possible by the co-resident Tn3872-like element [66]. Transfer by conjugation allows the spread of non-transformable genetic elements and the spread of antibiotic resistance.

Distribution of *mef* and other macrolide resistance elements

Geography and species identity generally predict what mechanism and what allele of a macrolide resistance gene an isolate may harbor. Pneumococci possessing MEGA tend to be found in the United States, Canada, and the United Kingdom, whereas strains possessing *ermAM/B* are more predominantly found in continental Europe and Asia where macrolide resistance is extremely prevalent [67-76]. The presence of *mefA*, which encodes an efflux pump, has been observed in *S. pyogenes* in the eastern hemisphere and is largely responsible for macrolide resistance in this species [20, 77-79]. Pneumococcal strains harboring *mefA* have been

found to belong to only serotype 14 and confer resistance to only 14- and 15-membered macrolides; whereas, strains harboring *mefE* belong to different serotypes and contained additional resistance mechanisms to other antibiotics [56]. The incidence of *mefE/A* in macrolide resistant *S. pneumoniae* is increasing while *ermAM* remains relatively stable [19, 33, 67, 80-84]. Additionally, increases in the number of dual resistant strains, strains possessing both *ermB* and *mefE,* have been observed [52, 58, 70, 81, 85].

Often, pneumococci are classified into clonal complexes. A clonal complex is established based on the similarity of the allelic identities of housekeeping gene sequences as determined by multi-locus sequence typing (MLST). Two predominant macrolide resistant pneumococcal clonal complexes (CC), CC271 and CC15, accounted for a large majority of the macrolide-resistance isolates in a study in Australia [52]. These are the same clonal complexes that have been shown to be responsible for the increase in *S. pneumoniae* that contain both *ermB* and *mefE* [40, 86, 87]. Serotype 6B is a significant carrier of dual macrolide resistance conferred by the presence of *ermB* and *mefA* and represents a high proportion of isolates in Japan [76]. In addition, serotype 19A and 19F represent a high or increasing proportion of macrolide resistant isolates in multiple geographic locations [81, 83, 88, 89].

Although the MLS_B phenotype predominates in Australia, 22% of an analyzed sample of isolates demonstrated that a substantial minority of the population contained the M phenotype of resistance; these isolates were found to have belong to CC15, a clonal cluster to which much global dissemination of the M phenotype resistance is attributed [52]. Additionally, clonal dissemination of *mefE* containing strains was responsible for an observed increase in macrolide resistant pneumococci in Spain, and *mefE* was responsible for more M type resistance than *mefA* in the strains of *S. pneumoniae* in the sample population [90]. Of interest, many of the *mefE*

containing carrier isolates in this study were unrelated genotypically to each other, suggesting horizontal spread of the resistance gene [50].

MefE and Mel Function

mefE and *msrD*/*mel* encode a proton motive force pump and a putative ATP binding cassette transporter respectively and are co-transcribed as a single operon [33]. Consistent association is found between *mef* and *mel/msrD* in clinical isolates of both *S. pyogenes* and *S. pneumoniae* [47, 72, 91]. The *mefE* gene is predicted to encode a proton motive force pump; experimental evidence supporting this prediction demonstrated that the efflux of radio-labeled erythromycin decreased in the presence of proton motive pump inhibitors [33, 35, 41, 42]. The creation and analysis of *mefE* null mutants also showed increased susceptibility to erythromycin [38, 39, 41, 42].

Both, MefE and Mel are required for maximal efflux-mediated macrolide resistance in the pneumococcus; deletion of either *mefE* or *msrD/mel* in a MEGA containing strain results in significant reduction of the MICs for erythromycin. In a *mel* deletion mutant, *mefE* expression is increased more than 10-fold but this increase in transcription does not restore resistance [92]. The increase in *mefE* expression in a *mel* mutant may suggest a possible regulatory role of *mel* on *mefE* [92]. Additionally, the expression of *mefE* and *mel* are inducible by erythromycin, and induction confers higher levels of resistance equivalent to roughly a four-fold increase [83, 92-94]. In addition to being inducible by erythromycin, *mefE* is also known to be inducible by LL-37, a human antimicrobial peptide, and this induction confers resistance both to macrolides and LL-37. [95]. Induction of the *mefE* promoter in the presence of macrolides or antimicrobial peptides has been predicted to be a possible avenue by which treatment failures occur in “low-level”-macrolide-resistant pneumococci [1, 95].

The *mel* gene is predicted to encode an ATP binding protein that belongs to the ABC family of proteins but, like *msrD*, lacks the hydrophobic segments typically found in ABC transporters [96]. In a macrolide susceptible strain mel homolog msrD, inserted experimentally, was sufficient to provide a modest level of macrolide resistance with debate surrounding if this level of resistance is clinically significant [92, 94], but *msr*D has not yet been found to exist without *mefE* or *mefA* in the environment [94]. The fact that *mel* and *msrD* are able to confer macrolide resistance in a previously macrolide susceptible strain is surprising, given that both proteins lack a transmembrane domain and thus are predicted to require an interaction with a transmembrane associated complex.

Orfs3-6 and the SOS Response

Efflux pumps are often encoded as part of an operon and are subject to control by one or more regulatory genes [97]. A second operon of predicted genes, *orfs3-6*, is found in a convergent orientation to *mefE/mel* in nearly all elements that contain *mefE/mel* [33]. The fact that these genes associate selectively with *mefE/mel* suggests that their presence is important in some way. This operon has homology to transposon-related operons involved in the SOS response, specifically to one present in Tn5252 known to be involved in the SOS response in *S. pneumoniae* [98] (Figure 1). Additionally, the SOS response has been documented in another pathogenic streptococcal species, *S. uberis* [99]. The SOS response mechanism was discovered and named by Miroslav Radman in 1975 and is a response by the bacterial cell to damage of its DNA [100, 101]. The DNA damage that the bacterial cell responds to can be due to exposure to ultra-violet radiation, chemicals, and other damage inducing factors including antibiotics [100]. When this damage is sensed the cell cycle is arrested and error-prone translesion synthesis of the DNA takes place [102].

The SOS response involves several proteins including RecA and LexA, which are its main regulatory proteins. LexA, or a homolog of LexA, is not yet known to exist within streptococcal species; however, streptococcal species are known to possess error prone DNA polymerases characteristic of the SOS response [103-105]. LexA is a repressor of the SOS response, and RecA is involved in inactivation of LexA through auto-cleavage so that error-prone repair can take place [106-108]. RecA is also involved in the cleavage of another important protein involved in the SOS response, UmuD. RecA cleaves UmuD into its active form which then associates with UmuC as $(UmuD')_2C$, a member of the Y-family of DNA polymerases [108, 109]. Under normal growth conditions LexA binds to a 20bp consensus sequence termed the SOS box or the LexA box [110]. This box is located in the operator region of any gene regulated by LexA, and when LexA binds to these boxes transcription of the associated gene/s is repressed. The amount of repression is determined by the affinity of LexA for a given box, with LexA possessing a higher affinity for UmuD and UmuC than other SOS response genes.

The operon identified in Tn5252, like the operon of *orfs3-6* in MEGA, contains four predicted open reading frames. *orfs5* and *6* of MEGA have homology to *orf13* of Tn5252 (see Fig 1). *orf13* shares homology with UmuC and MucB, proteins involved in the SOS response, and was found to be involved in an SOS response in streptococci [98]. An UmuD/MucB homolog, represented by *orf14*, has no homolog in the *orf3-6* operon of MEGA. In the case of the MEGA *orfs3-6* the gene products represent truncated versions of their homologous counterparts and have been predicted to be deficient in their ability to evoke an SOS-like response. However, it is of particular interest that MEGA contains a consensus LexA binding site and a core promoter site recognized by an alternate sigma factor in *orf5* [33, 111, 112].

Additionally, a truncated version of UmuC has been found to play a role in the direct regulation of another gene, *ddrR*, that is induced during DNA damage [113].

Commonly, regulators of bacterial efflux can be assigned to one of four regulatory protein families based on their DNA-binding domain; however, *orfs3-6* exhibit no characterized DNA-binding domains. While these genes may display no DNA-binding domains it is still possible that they in some way regulate the expression of either *mef* and/or *mel*. It is also highly probable that these genes are somehow involved in an SOS-like response in the strains that harbor them; their role in an SOS-like response could be related to that observed in other strains under the exposure of antibiotics [114-117]. Their continual presence and conservation within the MEGA genetic element indicate that they are important in some way to MEGA and potentially its function in conferring antibiotic resistance.

In summary, the non-conjugative genetic element MEGA element confers an M type of macrolide resistance upon the isolates that harbor it and is the most common cause of macrolide resistance in the US, Canada, and the UK. MEGA contains two genes, *mefE* and *mel*, which are involved in the efflux of macrolides and other agents such as certain antimicrobial peptides and their expression is inducible by erythromycin and LL-37. *mefE* and *msrD*/*mel* encode a proton motive force pump and a putative ATP binding cassette transporter respectively and are cotranscribed as a single operon. MEGA also contains an additional operon convergent to *mefE/mel*, *orf3-6*. *orfs5* and *6* of this operon share homology to the SOS response related proteins UmuC/MucB; however, a function has not yet been identified for any of the additional *orfs* contained my MEGA. o*rfs3-6* consistently associate with *mefE/mel*, suggesting involvement by these genes in either some aspect of MEGA's role in macrolide resistance and/or in an SOS-like response which may influence *mefE/mel.*

Investigation into the factors that influence the macrolide resistance conferred by MEGA is needed to further understand how the predicted dual efflux pump MefE/Mel is regulated. Specifically, *orfs3-6* are found to associate frequently as a member of the MEGA element and could be important to the function of the macrolide efflux pump it encodes. Mutant constructs of *orfs3*-6 were made in order to investigate their influence on both macrolide resistance and expression of the predicted dual efflux pump encoded by *mefE/mel*. Additionally, two separate strains (GA17457 and GA16242) and subsequent constructs were assayed for both their macrolide resistance levels and expression of *mefE/mel.*

METHODS

Strains:

Strains of *Streptococcus pneumoniae* used in this study included the clinical isolates and derivative constructs of the macrolide resistant GA17457 (MIC 12 μg/mL) and high-level macrolide resistant GA16242 (MIC 43 μg/mL). These strains and their derivative constructs are detailed in Table 1.

Plasmid and Deletion Construction:

Deletion mutants of the MEGA *orfs3-6* operon were generated by replacement of the operon or the individual genes with an *aphA-3* cassette encoding kanamycin resistance as detailed in Figure 2, Tables 1 & 2. The primers used to amplify the *aphA-3* cassette contained the coding region for a ribosomal binding site (RBS) and a subsequent start site for transcription. This approach was taken so that all mutations created were non-polar to any genes downstream within the same operon. Next, the region surrounding the target deletion site was amplified in two separate reactions, represented as AB for the upstream region and CD for the downstream region. Primers B and C contained overlapping complementary sequence which also encoded a restriction site complementary to the one encoded on both the upstream and downstream flanking DNA of the *aphA-3* cassette.

Following the creation of the two separate AB and CD fragments, overlapping PCR was used to create one cohesive unit, represented as EF, which was gel purified. Primers E and F were designed to be nested from primers A and D, respectively, and encoded different restriction sites to allow for directional cloning into the recipient vector. These constructs were established in pSF151 through restriction digest (SphI and SacI sites were used to create pTS010 and SacI and BamHI sites were used to create pTS019) and T4 DNA ligation. The resulting plasmid was

transformed into chemically competent laboratory prepared *E. coli* DH5α by placing the digested construct and plasmid together on ice for 30 minutes, at 42°C for 90 seconds, on ice for 2 minutes, and then adding 900 μL of LB for recovery in a 37°C shaking incubator for 1 hour. The samples were plated on chloramphenicol selective Luria Bertani agar plates (34 μg/mL), screened for the presence and directionality of the insert, purified by single colony isolation, and glycerol stocked. Isolation of the plasmid was accomplished using a Qiagen (Valencia, CA) plasmid extraction kit. Next, the plasmid was cut with SmaI restriction enzyme, ligated to the *aphA-3* resistance cassette, transformed into *E. coli* DH5α, selected for kanamycin resistance (50 μg/mL), screened, purified, stocked, and isolated as was done for the parental plasmid. The final resistance bearing plasmid was sequenced to confirm correct genetic identity.

The plasmid constructs were transformed into competent laboratory prepared pneumococcal strains. This was done by adding 20 μL of competent cells to 100 μL of pre-warmed competence transformation media (CTM) that was previously incubated for 10 minutes with 0.2 μL of competence stimulating peptide-1 (CSP-1). Up to 1 μg of chromosomal DNA or 5 μg of plasmid DNA was then added, mixed with a pipette tip, and incubated at 37°C for 2.5 hours. Following incubation, 100 μL of the transformation mixture was transferred into an empty Petri dish and combined with 15 mL of trypticase soy agar + 5% sheep blood and any antibiotic required for selection. Plates were incubated at 37°C in 5% CO_2 overnight. Transformants were recovered by sterile pipettes and grown in Todd-Hewitt plus 0.5% yeast extract broth (THY). Mutant constructs were purified by colony isolation and confirmed by gel electrophoresis and sequencing. Both a control without DNA and a chromosomal transformation control were use to monitor random mutation rates and the level of competence for the strains under manipulation.

Reverse Transcriptase PCR (RTPCR)

RNA isolated for RTPCR was obtained using the RNeasy Mini Kit and protocol supplied by Qiagen (Valencia, CA). An alteration to the protocol was made in the final elution step where RNA was eluted in 40 μL of H_2O twice, for a final elution volume of 80 μL. RNA concentration was determined by spectrophotometry. This elutant was then treated with 1 μL DNase per μL RNA (10 μL total for 10 μg of RNA) in the presence of 1 μL rRNasin Ribonuclease Inhibitor, 5 μL buffer and H_2O to 50 μL at 37°C for 30 minutes. Following the 30 minute incubation 1 μL DNase per μg RNA was again added to the reaction and placed at 37°C for another 30 minutes. Following the second incubation, the sample was adjusted to 100 μL with H_2O and purified with a Qiagen (Valencia, CA) RNeasy Mini Kit. 1 μg of RNA was then added to 4 μL of Supermix, 1 μL reverse transcriptase, and brought to a volume of 20 μL with H_2O. RTPCR was then performed according to the manufacturer's protocol. PCR was then performed against both the RTPCR and non-RTPCR control samples for each strain. The quality of the RNA extraction was monitored on agarose gels.

Antimicrobial susceptibility testing: Antibiotic susceptibility was determined using E-test strips (AB Biodisk, Solna, Sweden), following the manufacturer's protocol, and through the use of a disc diffusion assay. Strains were streaked on trypticase soy agar with 5% sheep blood plates in the inducing and noninducing presence of 0.01 μg/mL erythromycin and grown overnight at 37°C in 5% CO_2. Colonies were removed with an inoculating loop and resuspended in Mueller-Hinton broth. OD_{600} was measured, adjusted to 0.5, and the solution was plated by sterile swab, coating the plate three times in different directions to evenly distribute the inoculum. Plates were allowed to dry in a sterile hood and E-test or antibiotic containing discs were applied with forceps. Plates were incubated overnight at 37°C in 5% CO_2 for 20 hours,

quantified, and photographed. Plates containing E-test strips were quantified according to manufacturer's protocol. In the disc diffusion assay, plates containing antibiotic impregnated discs were quantified on the basis of the size in mm of the zone of inhibition. Discs were obtained from Beckman Dickeron & Company (Franklin Lakes, NJ) and contained 15 μg erythromycin.

Dose Response Curve

A dose response curve was conducted to determine the erythromycin concentration best suited to maximally induce cultures for β-galactosidase expression assays. Strains were grown overnight, inoculated at an OD600 ~0.15 in liquid THY, and grown for approximately 2-3 hours in a 37°C water bath until OD_{600} >0.5. Starter cultures were diluted into 100 mL tubes of OD_{600} 0.2 and then partitioned into 9 tubes of 10 mL each. Each tube received a predetermined concentration of erythromycin: 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 20 μg/mL were used. Another experiment was performed where the concentration of erythromycin used was based on 100, 50, 25, 10, 5, 1, and 0.5 % of the MIC. Strains were monitored for growth and harvested when control was predicted to enter late logarithmic growth. Strains were then assayed for β-galactosidase expression as described below.

β-galactosidase Expression Assays: Pre-warmed THY broth was inoculated from plates incubated overnight on trypticase soy + 5% sheep blood at 37°C in 5% CO_2 to an OD_{600} ~0.15. Strains used in this study contained transcriptional reporter fusions (*PmefE-lacZ*) that were present in the *bga* locus. Cultures were incubated in a water bath at 37°C to mid-log phase and diluted into fresh pre-warmed THY broth to an OD_{600} ~ 0.05 with continued incubation. Sub-inhibitory concentrations of erythromycin (~5% MIC) were added to induced cultures at an OD_{600} ~ 0.35. Cells were harvested one hour after induction, centrifuged for 5 minutes at 12,000

x g, and frozen at -30°C. Samples were then lysed, 0.1 mL of the lysate mixed with ONPG, allowed to react for 25 minutes and assayed for β-galactosidase activity. Cultures were monitored throughout the experiment in order to track growth. The amount of β-galactosidase activity, expressed as Miller units (MU), was calculated with the equation: [1000xOD420-(1.75xOD550)]/t*v* OD_{600} where t is the time in minutes of the reaction, v is the volume of lysate in milliliters, and OD_{600} represents the cell density when the culture was harvested [118]. A promoterless control, XZ7049, was used in each experiment to account for the level of false positive responses [119].

Statistics:

Statistical analysis of the data collected was performed using Microsoft Excel. A type 1 two-tailed t-test was used to determine data significance levels; a type 1 test was used because data were of equal variance pertaining to statistical analysis. Significance values are reported as p-values. P-values greater than 0.05 were not reported as significant, and p-values less than 0.05 were reported as significant.

RESULTS

Effect of the MEGA *orfs3-6* on macrolide susceptibility. The erythromycin minimum inhibitory concentrations (MICs) of macrolide resistant strain GA17457 containing a Class I MEGA insertion and its subsequent *orf3-6* deletion constructs were determined by established standards through the use of an E-test strip containing an erythromycin gradient (Table 3). The wildtype MEGA containing strain (GA17457, serotype 19A) and the wildtype MEGA strain containing a *PmefE-lacZ* reporter (XZ7042) placed at the ectopic *bga* locus were tested. This was done to determine a possible effect of the *PmefE-lacZ* reporter fusion on MIC. No significant difference between the MIC of the reporter strain XZ7042 (MIC 12 μg/mL) compared to the wildtype parental strain GA17457 (13 μg/mL) lacking the reporter was observed.

Statistical analysis using a two-tailed t-test was used to determine the effect that different mutations of *orfs3-6* had on macrolide resistance compared to the parental MEGA containing strain. In the reporter strain XZ7042 (MIC 12 μg/mL) background, a significant difference in erythromycin resistance was found when this wildtype was compared to the *orfs3-6* deletion mutant XZ7106 (MIC 4 μg/mL, p-value < .005) and the *orf3* deletion mutant TS8008 (MIC 7 μg/mL, p-value < .05). Specifically, deleting *orfs3-6* resulted in a ~3 fold decrease in erythromycin resistance as determined by MIC. These differences in resistance indicated a role for *orfs3*-6, and specifically *orf3*, in the MEGA mediated macrolide resistance of isolate GA17457. Additionally, a significant difference was also found between the MICs of XZ7106 and TS8008 (p-value <0.005). Due to the intermediate level of macrolide resistance displayed by TS8008 (MIC 7 μg/mL) in comparison to XZ7042 (MIC 12 μg/mL) and XZ7106 (MIC 4 μg/mL), *orf3* appears to contribute to some, but not all, of the loss in macrolide resistance present in the complete *orfs3-6* operon mutant.

To further assess the influence of *orfs3-6* on macrolide resistance, differences in resistance between XZ7042, XZ7016, and TS8008 were observed by subjecting each construct to a disc diffusion plate assay in the presence of a disc containing 15 μg of erythromycin (Figure 3). The zone of inhibition around the discs was measured in millimeters for XZ7042 (11 mm), XZ7106 (14 mm), and TS8008 (12 mm). Comparison of the data collected revealed statistically significant difference between XZ7042 and mutants XZ7106 ($p < 0.005$) and TS8008 ($p<0.05$). Additionally, a significant difference ($p < 0.005$) was observed between the two mutants XZ7106 and TS8008. These observations reflect the differences in MIC data presented above.

D**etermination of erythromycin concentration for the optimal induction of *PmefE-lacZ* expression.** The MefE/Mel efflux pump of MEGA is inducible by erythromycin and other macrolides [92]. The amount of erythromycin needed to evoke the optimal induction response of *PmefE-lacZ* in the macrolide resistance strain GA17457 reporter XZ7042 and its derivative constructs was determined using a dose equivalent response curve. The optimal amount of antibiotic was equivalent to 5% of the erythromycin MIC.

Expression from the *PmefE-lacZ* reporter in derivatives of the macrolide resistance isolate GA17457. Levels of β-galactosidase expression (measured in Miller Units – MU) were monitored using a *PmefE-lacZ* transcriptional fusion in the macrolide resistance wildtype type reporter XZ7042 and its *orfs3-6* deletion derivatives (XZ7106 and TS8008) through a quantitative β-galactosidase assay. A negative control strain, XZ7049, containing a promoterless fusion to *lacZ*, was used and found to have a nominal amount of β-galactosidase expression (data not shown); thus the β-galactosidase expression observed was assumed to be a product of expression from the *PmefE-lacZ* promoter construct. The role of *orfs3-6* on the expression of the predicted dual efflux pump encoded by *mefE/mel* was investigated by comparing expression of

each strain (Figure 4). Strains were exposed to 5% of their erythromycin MIC (Table 3) as discussed above. β-galactosidase expression levels from *PmefE-lacZ* were monitored for the macrolide resistance reporter derivatives XZ7042 (wildtype), XZ7106 (Δ*orfs3-6*), and TS8008 (Δ*orf3*).

No statistical difference using a two-tailed t-test in *mefE* expression was determined to exist between the mutants (XZ7106 – 235 MU, TS8008 – 232 MU) and the wildtype reporter (XZ7042 – 215 MU). These results indicate that *orfs3-6* may not have a direct role in the regulation of expression of the predicted dual efflux pump MefE/Mel. The uninduced controls for all constructs revealed no significant difference when compared to each other (XZ7042 – 6 MU, XZ7106 – 7 MU, TS8008 – 7MU).

Difference in macrolide resistance levels between two separate MEGA classes of insertion in *S. pneumoniae*. The minimum inhibitory concentrations (MICs) of the macrolide resistant strain GA16242 (serotype 6B, class IVa MEGA insertion) and its subsequent constructs were determined by established standards through the use of an E-test strip containing an erythromycin gradient (Table 4). A significant difference in macrolide resistance was found in a comparison between GA16242 reporter construct TS8011 (45 μg/mL) and the GA17457 (12 μg/mL, class I MEGA insertion) reporter construct XZ7042 (p-value < 0.0005). The MIC of TS8011 was 3-fold higher than that of XZ7042. One hypothesis is that this could be related to differences in the MEGA insertion site of these two strains.

An examination of the effect of a deletion of two unique genes found to associate exclusively with the class IVa insertion, *orfsAB* (discussed later), revealed no statistical significance in the difference of resistance between this construct, TS9004, and its wild type reporter parent, TS8011. This observation indicates that *orfsAB* may not be involved in the high-

level resistance observed in strain GA16242 compared to GA17457. As was observed for the reporter construct XZ7042 and GA17457, no significant difference in macrolide resistance was found between the high-level macrolide resistant strain GA16242 (MIC 43 μg/mL) lacking the reporter and its wildtype reporter construct TS8011 (MIC 45 μg/mL).

Expression from *PmefE-lacZ* in derivatives of high-level macrolide resistance isolate GA16242. β-galactosidase expression in the derivatives of GA16242, containing the same *PmefE-lacZ* reporter fusion reported above, were determined.. β-galactosidase expression levels from *PmefE-lacZ* were monitored for the macrolide resistance class I insertion site reporter construct XZ7042 (wildtype) and high-level macrolide resistance class IVa insertion site constructs TS8011 (wildtype, 432 MU) and TS9004 (*ΔorfAB*, 413 MU) (Figure 5). It was assumed that high-level resistant strain GA16242 and its derivatives would exhibit similar kinetics to the dose equivalent response curve presented earlier for the XZ7042 resistant constructs; for this reason they were similarly induced with 5% MIC erythromycin. The role of insertion site in the expression of the predicted dual efflux pump MefE/Mel was investigated by comparing expression levels of TS8011 to expression levels of XZ7042. The absence of a role of *orfsAB* in the increased level of resistance observed in high-level resistance isolate GA16242 was further confirmed through comparison of TS8011 and TS9004 expression levels.

Expression from *PmefE-lacZ* for strains XZ7042 (213 MU), TS8011 (432 MU), and TS9004 (413 MU) is depicted in Figure 5. No statistically significant difference in *mefE* expression was determined to exist between TS8011 and TS9004. This lack of significant difference indicated that *orfsAB* may not play a role in the expression of the predicted dual efflux pump encoded by MEGA and reflects the previous observation that they do not effect the level of resistance in high-level macrolide resistant strain GA16242. Statistical significance was found

in a comparison of XZ7042 and TS8011 (p-value < 0.05) as was found in the comparison of the MICs above. Statistical significance was also found to exist in a comparison of XZ7042 and TS9004 (p-value < 0.05), but this significance was assumed a reflection of the parental strain TS8011 and was unrelated to the absence of *orfsAB*. Uninduced controls for all constructs revealed no significant difference when compared to each other.

DISCUSSION

Macrolide antibiotic resistance in *Streptococcus pneumoniae* due to efflux is a major clinical problem and is caused by a predicted dual component efflux pump (MefE and Mel found on the 5.4or 5.5 kb insertion element MEGA. An operon downstream and divergently transcribed from *mefE/mel*, *orfs3-6*, is found frequently on MEGA. The influence of *orfs3-6* on macrolide resistance and the expression of the predicted dual efflux pump encoded by *mefE/mel* of MEGA was investigated (Table 3, Figures 3 & 4). Clinical isolate GA17457 (MIC~12ug/ml) contains MEGA (but no other macrolide resistant determinant). Deletion of *orfs3-6* resulted in a statistically significant ($p < 0.005$) lower MIC in the mutant (~4 μg/mL) compared to the wildtype. Additionally, a significant ($p < 0.05$) reduction in MIC (~8 μg/mL) in the single gene mutant of *orf3* (TS8008) was observed in a comparison to the wildtype strain.

The reduction in MICs observed in these mutants suggested the involvement of *orfs3-6* in the macrolide resistance conferred by MEGA. Their presence in a strain containing a deletion of *mefE/mel* was not clinically significant (MIC 0.125 μg/mL) compared to the clinical breakpoint for erythromycin (MIC 1 μg/mL), suggesting their dependence on and interaction with MefE/Mel for macrolide resistance [2]. Disc diffusion assays utilizing an antibiotic disc containing erythromycin were performed to confirm the results (Figure 3). As expected, the results obtained in these experiments correlated with the results obtained through MIC testing.

Because the presence of *orfs3-6* in a *mefE/mel* deletion mutant does not confer macrolide resistance, the function of these genes in the expression of *mefE* was investigated through the use of a *PmefE-lacZ* β-galactosidase expression assay. β-galactosidase expression was monitored from the transcriptional reporter fusion in the presence and absence of erythromycin inducing conditions (5% MIC as determined by dose equivalent response assay). The results of the

expression assays indicated no statistically significant difference between the wildtype XZ7042, *orf3* mutant TS8008, and complete operon mutant XZ7106. These data indicate that *orfs3-6* may not play a direct role in the regulation of expression of *mefE*.

While *orfs3-6* may not be involved in the direct regulation of the macrolide efflux pump MefE/Mel, the data indicate they influence macrolide resistance of strains containing MEGA. The coding regions of *orf5* and *orf6* have truncated homology to *orf13* of Tn5252 which is a UmuC/MucB homologue. Thus, *orf5* and *6* are homologues of a gene known to be involved in the SOS response mechanism of bacteria. A potential LexA binding site was identified in *orf5*, upstream of orf6, that shares weak homology to other known binding sites of this SOS-regulating protein [2]. Their influence on macrolide resistance in pneumococci could be a product of an SOS-response like mechanism and should be further investigated for this potential. Additionally, *orf3* appears responsible for some, but not all, of the loss of resistance present in a complete operon mutant ($p < 0.05$). Blast searches against the *orf3* genetic sequence returned no known genes or proteins.

Of additional significance is the MIC data that indicate *orfs3-6* rely on the presence of MefE/Mel to confer additional resistance to macrolides. While there is homology between the orfs and known relatives of the SOS-response, there is the important distinction that *orfs3-6* require the presence of *mefE/mel* in GA17457 to confer macrolide resistance above the clinical breakpoint, and that *mefE/mel* require *orfs3-6* to achieve GA17457's native macrolide resistance level. *orfs3-6* did not confer resistance in the absence of mefE/mel (MIC 0.125 μg/mL) as mefE/mel deletion mutants have been shown to be susceptible to erythromycin [92, 119]. This association of dependence on each other implies that *orfs3-6* somehow interact with MefE/Mel, but not on the level of transcription. Additional experiments investigating their function on the

translational and protein levels of the pump are needed to further understand how *orfs3-6* influence MefE/Mel.

Through previous comparison and classification of clinical pneumococcal isolates, it became apparent that strains carrying MEGA as their only mechanism of macrolide resistance varied in their level of macrolide resistance. Some strains exhibited levels of macrolide resistance resultant of MEGA with MICs between 2-16 μg/ml and others exhibited high-levels of macrolide resistance (MIC >32 μg/ml). A major difference in these strains was the location of MEGA within the pneumococcal genome. In clinical isolate GA17457, which has a macrolide resistance of 12 ug/ml, MEGA is inserted within the gene for HMP-phosphate kinase (SP_1598, TIGR4 annotation). This insertion of MEGA into SP_1598 deletes two nucleotides (CA) and has been termed a class I insertion [33].

In clinical isolate GA16242 (MIC 32-64 ug/ml), MEGA is inserted, in a somewhat complicated manner, into the pneumococcal pathogenecity island 1 (PPI-1), a hotspot for recombination in *S. pneumoniae*. Upstream of MEGA in GA16242 is the remainder of *trmA (SP_1029)* and downstream of MEGA is a small fragment of SP_1049. Downstream of SP_1049 is an additional two gene element termed *orfsAB*. *orfA*, with homology to transposase II of *Streptococcus mitis*, and *orfB*, a hypothetical protein also found in *S. mitis*, are transcribed convergently from each other and found in all isolates manifesting this particular insertion profile, termed a class IVa MEGA insertion. Additionally, strains belonging to class IVa lack nearly the entire PPI-1 region that is present in class I strains. (See Figure 6 for insertion site comparison.)

As anticipated, the comparison of erythromycin resistance between the wildtype class I macrolide resistant GA17457 and the class IVa high-level macrolide resistant GA16242 was significantly different (p-value < 0.005, Table 4). Controls were performed with mutants constructs of these strains lacking MEGA and the results indicated the loss of clinically significant levels of macrolide resistance in both strains. The Class IVa insertion GA16242 exhibited an MIC nearly 3-fold higher than class I insertion GA17457. The differences in macrolide resistance could be a reflection of the differences between the two strains in their insertion sites or other genetic differences associated with the presence of MEGA.

To further explore differences between wildtype strains GA17457 and GA16242, expression assays were conducted to investigate the influence that MEGA insertion site on the transcription of the predicted dual efflux pump encoded by MefE/Mel (Figure 5). β-galactosidase expression was monitored in inducing conditions (5% MIC) from a transcriptional reporter fusion of *lacZ* to the promoter of *mefE*. A comparison between wildtype reporter construct XZ7042 of the class I MEGA insertion GA17457 and TS8011 of the class IVa insertion high-level macrolide resistant GA16242 revealed a significant difference (p-value < 0.05) between the β-galactosidase expression levels of the two classes of strains. The difference in β-galactosidase expression was roughly doubled in the class IVa TS8011 compared to the class I XZ7042. Thus, differences present as a result of the MEGA insertion site may influence the level of macrolide resistance and be responsible for the increased transcription of *mef*E/*mel*; however, more investigation into the specific causes of the differences in macrolide resistance between these classes of insertion sites is needed.

The fold increases observed in macrolide resistance and β-galactosidase expression for both experiments were not equally correlated with each other. In the case of the GA17457 constructs, resistance was reduced in the mutant constructs but their expression levels were unaffected. For GA16242, in comparison with GA17457, resistance was increased three-fold but β-galactosidase expression was only increased 2-fold. While there is no data to suggest that any specific increase in macrolide resistance should provide equal fold increase in expression from *PmefE*, the β-galactosidase expression levels observed in the expression assays could have been influenced by titration of *cis* regulatory elements. This is a possibility because the reporter strain possessed two copies of the *mefE* promoter region, the native promoter and the promoter contained as part of the reporter fusion. The presence of two promoters for *mefE* in the reporter strains could have effectively caused the true amount of expression to be observed at up to half of its normal level.

In an effort to elucidate the involvement in macrolide resistance of the genetic differences and adjacencies between the insertion classes, an *orfsAB* mutant was constructed in GA16242 to determine possible involvement. The Δ*orfsAB* construct TS9004 was tested both for its level of macrolide resistance as well as its level of β-galactosidase expression from *PmefE-lacZ*. In both experiments, deletion of *orfsAB* had no discernable effect on the macrolide resistance of the construct or on the expression of the predicted dual efflux pump MefE/Mel. While it is possible that deletion of both *orfA* and *orfB* masked potentially opposing roles of these gene products, it is predicted that these genes, even individually, do not influence the expression of the MefE/Mel efflux pump. Instead, these genes may be involved in a transposition event or are a residual effect of that event.

In summary, *orfs3-6* appear to play a role in the macrolide resistance mediated by MEGA. Specifically, *orfs3-6* require the presence of *mefE/mel* in MEGA containing strain GA17457 and are responsible for a significant portion of macrolide resistance in this strain. Additionally, a significant portion of the *orfs3-6* conferred macrolide resistance in GA17457 can be attributed to *orf3* alone. Interestingly, data suggest that while *orfs3-6* positively influences the macrolide resistance conferred by *mefE/mel*, *orfs3-6* are not involved in transcriptional regulatory role for *mefE/mel*, but the possibility for roles on the level of translational regulation and protein interaction remains. The homology of *orfs3-6* to proteins known to function in SOS-response mechanism is also of notable interest and more exploration of their involvement in such a role is needed.

Difference in macrolide resistance levels between strains GA17457 and GA16242 was also investigated and found to be significant, with GA16242 (43 μg/mL) exhibiting MICs nearly three-fold higher than GA17457 (13 μg/mL). This is an unusually high level of efflux-mediated erythromycin resistance and these two strains were found to contain different insertion classes of MEGA, class I and class IVa respectively [2]. Deletion of the unique *orfAB* element of GA16242 revealed that they may not be involved in the increased resistance of this strain. Additionally, GA16242 reporter construct TS8011 exhibited *PmefE-lacZ* β-galactosidase expression levels twice that of GA17457 reporter construct XZ7042. This observation is consistent with GA16242's increased level of macrolide resistance, but more investigation is needed to determine what factors are responsible for this increased resistance as it seems unrelated to the unique *orfAB* element.

Figure 1. Alignment of *mef/mel* and *orfs3-6* Containing Elements in *S. pneumoniae.*

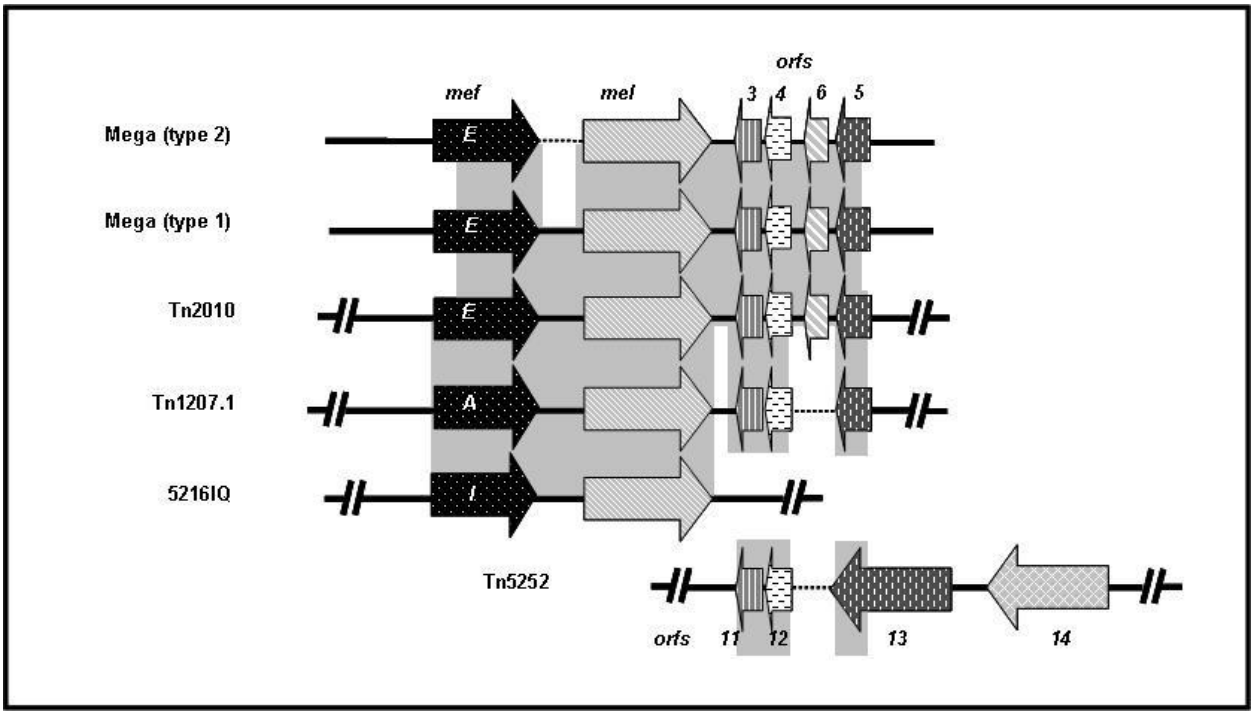


Table 1: Strains

Strain ***S. pneumoniae***	**Genotype**	**Reference**
GA17457	Wildtype; MEGA; EmR	[95]
XZ7042	GA17457-construct, *bgaA::PmefE-lacZ;* MEGA, EmR	[95]
XZ7106	GA17457-construct; *bgaA::PmefE-lacZ; orfs3-6::aphA-3;* MEGA; EmR	[2]
XZ7049	GA17457-construct, bgaA::promoterless-lacZ; MEGA, EmR	[119]
TS8008	GA17457-construct; *bgaA::PmefE-lacZ; orf3::aphA-3;* MEGA; EmR	this study
GA16242	Wildtype, MEGA, EmR	this study
TS8011	GA16242-construct, bgaA::*Pmef*E-lacZ; MEGA, EmR	this study
TS9004	GA16242-construct; bgaA::*Pmef*E-lacZ; *orfsAB::cat;* MEGA; EmR	this study
Plasmid ***E. coli***	**Genotype**	**Reference**
pXZ7016	pEVP3 derivative; *orfs3-6::aphA-3*	[2]
pXZ7032	pPP2 derivative; *bgaA::PmefE-lacZ*	[95]
pTS010	pSF151 derivative; Δ*orf3*	this study
pTS012	pTS10 derivative; *orf3*::*aphA-3*	this study
pTS019	pSF151 derivative; Δ*orfsAB*	this study
pTS020	pTS019 derivative; *orfsAB::cat*	this study

Table 2. Primers

Plasmid	Primer ID	Description	Sequence
pTS010	SC203	Δ*orf3*_A	ggcgttaactggtggtaatgg
pTS010	TS6	Δ*orf3*_B	gtttcacccggggcagaaaaataggagcagttcaattg
pTS010	TS7	Δ*orf3*_C	tttctgccccgggtgaaactcaagctgattcatagagc
pTS010	SC123	Δ*orf3*_D	catggaatcgttgaaacagcaac
pTS010	TS12	Δ*orf3*_E	atagcatgcccaaatgatcttaaaccatgaagaag
pTS010	TS13	Δ*orf3*_F	atatgagctccgtcagagaaaactcaaaacaactg
pTS019	SC2	Δ*orfsAB*_A	aatttgtctcactgcaccagagg
pTS019	TS28	Δ*orfsAB*_B	gcgagtcccgggtcaagactggaatcactgtgtgg
pTS019	TS29	Δ*orfsAB*_C	tcttgacccgggactcgcttcatgtcgaatgtg
pTS019	TS30	Δ*orfsAB*_D	tgccccgtttacggaaatacc
pTS019	TS31	Δ*orfsAB*_E	atatgcatgcactcaccattgttttggggag
pTS019	TS35	Δ*orfsAB*_F	atatgagctcactcaccattgttttggggag
pTS012	SC171	aphA-3_F	aatacccgggtgatgacaaaaagagcaaattttgataaaatagtattagaggattcctgtagaaaagaggaagg
pTS012	SC172	aphA-3_R	taatacccgggcatatgaaaattcctccagtcagacagttgcggatgtacttc
pTS020	TS33	cat_F	atat cccggg tgcaggaattcgatgggttc
pTS020	TS34	cat_R	atat cccggg taccatggcatgcatcgatag

Primers SC203, SC123, SC2, SC171, and SC172 were developed prior to this study [2].

Figure 2. Construction of Mutants

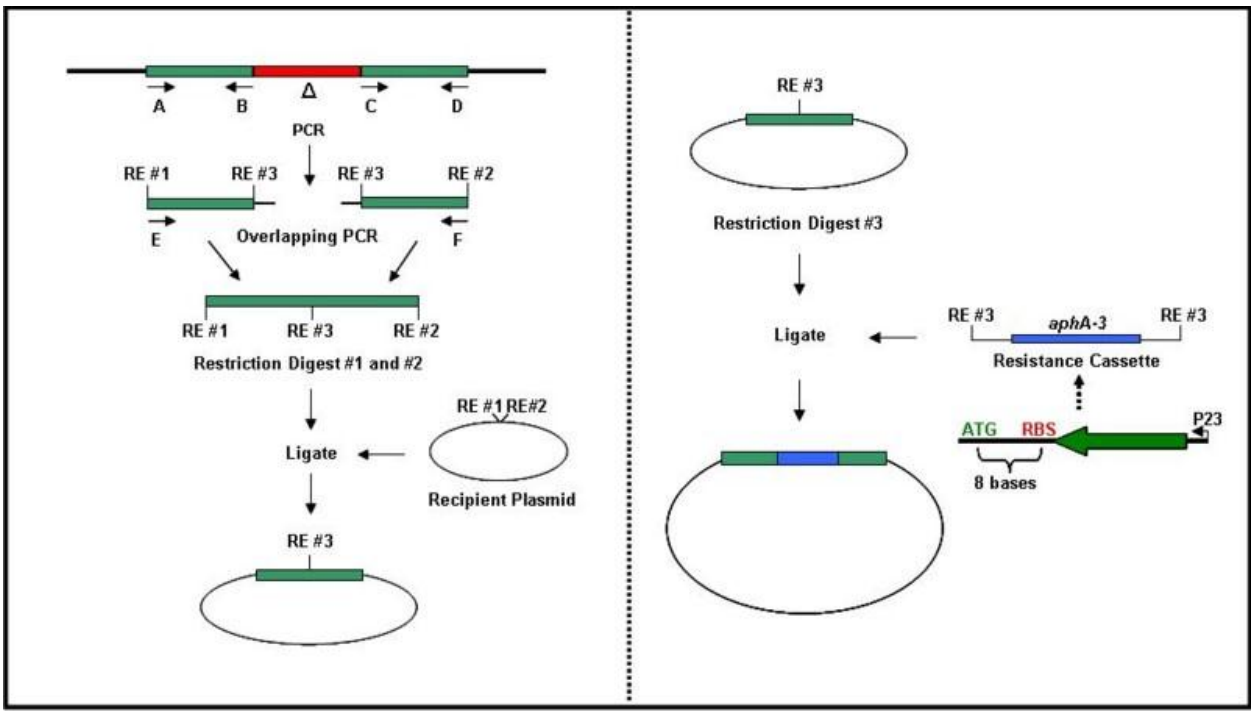


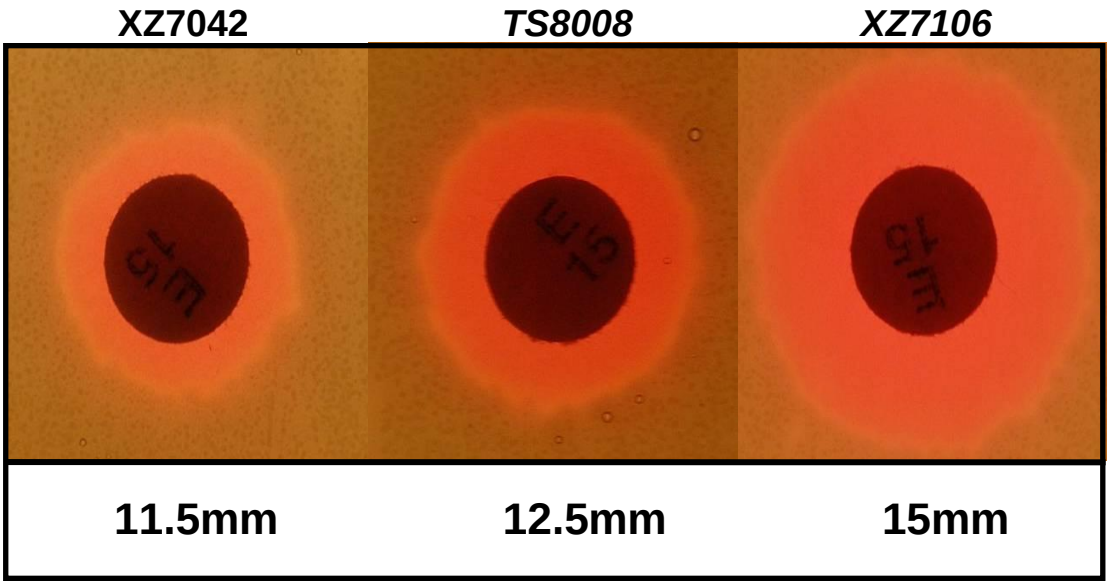
Table 3. MICs observed for erythromycin in MEGA macrolide resistance strain GA17457 and its derivative constructs.

Strain	Genotype	MIC
GA17457	wildtype	13 ± 2 ug/ml
XZ7042	wildtype reporter	12 ± 3* ug/ml
TS8008	Δ*orf3* reporter	7 ± 1** ug/ml
XZ7106	Δ*orfs3-6* reporter	4 ± 0.5*** ug/ml

*****denotes statistical significance ($p<0.005$) in a comparison of XZ7042 and TS8008.
**denotes statistical significance ($p<0.005$) in a comparison of XZ7042 and XZ7106.
***denotes statistical significance ($p<0.05$) in a comparison of XZ7106 and XTS8008.
MICs were determined using erythromycin E-test strips as described in the Methods.

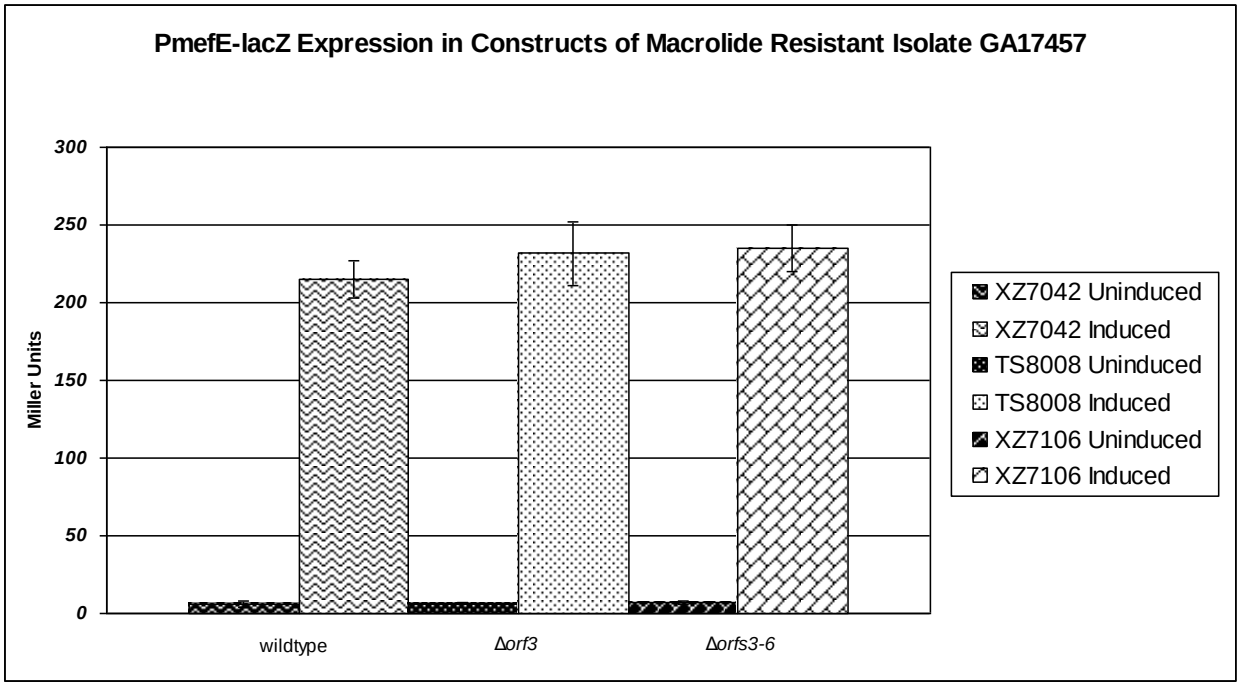
Figure 3. Zone of Inhibition (mm clearing) for erythromycin in MEGA macrolide resistance strain GA17457 and its derivative constructs.

Strain	Genotype	Zone of Inhibition
XZ7042	wildtype reporter	11 ± 0.5*mm
TS8008	Δ*orf3* reporter	12 ± 1**mm
XZ7106	Δ*orfs3-6* reporter	14 ± 1***mm



*****denotes statistical significance ($p<0.005$) in a comparison of XZ7042 and TS8008.
**denotes statistical significance ($p<0.005$) in a comparison of XZ7042 and XZ7106.
***denotes statistical significance ($p<0.05$) in a comparison of XZ7106 and XTS8008.

Figure 4. β-galactosidase Expression from *PmefE-lacZ* reporter (uninduced or induced with erythromycin)



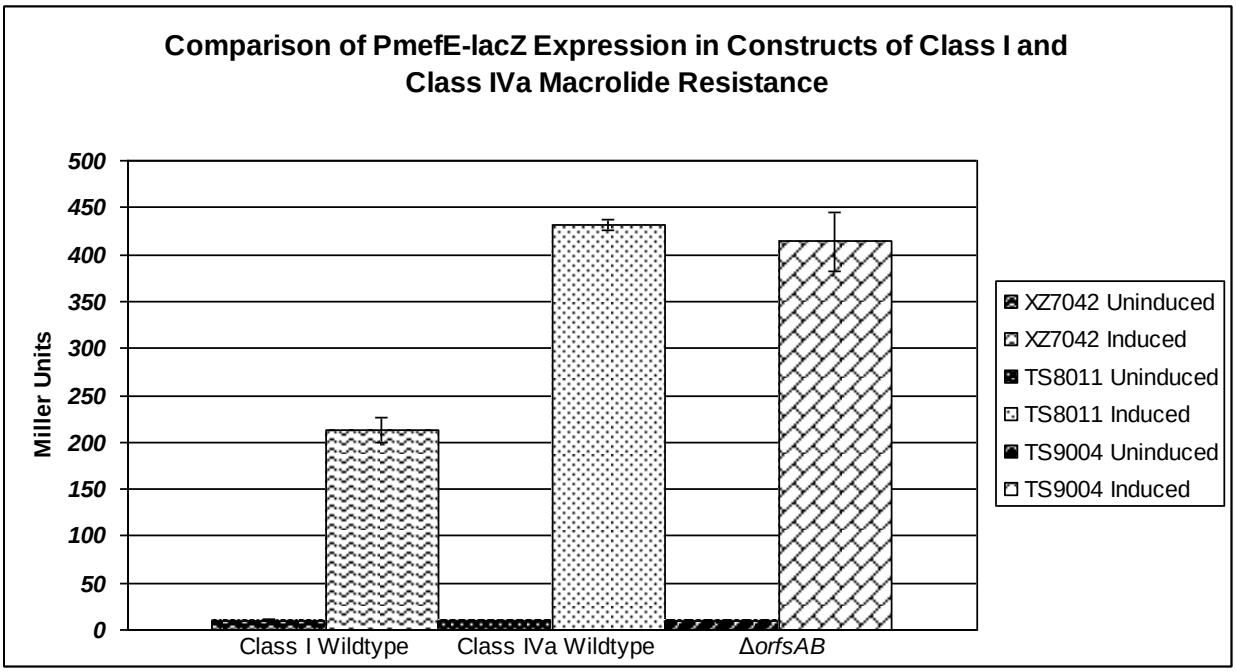
Strains used in this experiment were all reporter construct derivatives of GA17457 and possessed varying forms of *orfs3-6*: wildtype XZ7042, Δ*orf3* TS8008, and Δ*orfs3-6* XZ7106. Shaded bars (black) represent uninduced controls (no antibiotic) for each strain and unshaded bars (white) represent 5% MIC induction (erythromycin) for one hour.

Table 4. MICs for erythromycin observed for MEGA high-level macrolide resistance strain GA16242 and its derivative constructs.

Strain	Genotype	MIC
GA17457	wildtype	13 ± 2 ug/ml
XZ7042	wildtype reporter	12 ± 3 ug/ml
GA16242	wildtype	43 ± 7* ug/ml
TS8011	wildtype reporter	45 ± 4 ug/ml
TS9004	Δ*orfsAB* reporter	40 ± 8 ug/ml

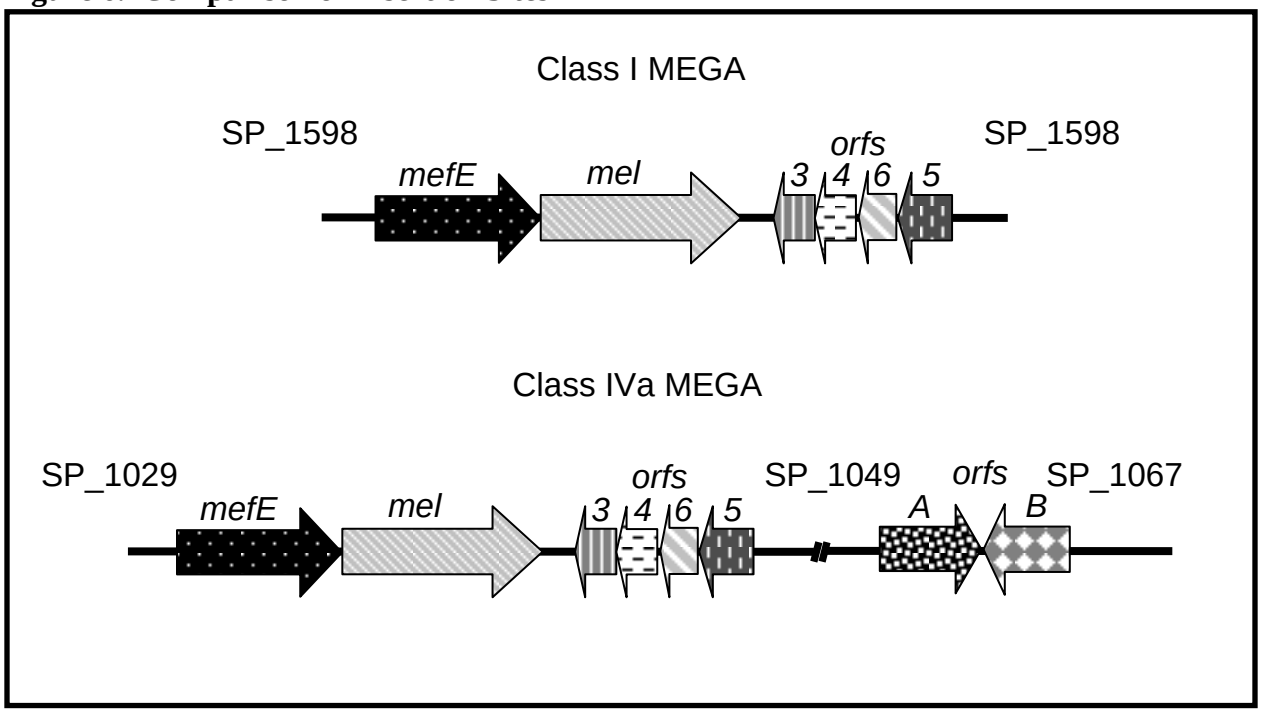
*****denotes statistical significance ($p<0.005$) in a comparison of GA17457 and GA16242. MICs were determined using erythromycin E-test strips containing a concentration range from 0.016 μg/ml to 256 μg/ml. The addition of the *PmefE-lacZ* reporter construct at the *bga* locus of the wildtype strain had no significant impact on the MIC of the construct. The deletion of *orfsAB* had no significant impact on the MIC of the construct.

Figure 5. β-galactosidase Expression from *PmefE-lacZ reporter*.



Strains used in this experiment GA17457(macrolide resistance) reporter construct wildtype XZ7042 and GA16242(high-level macrolide resistance reporter constructs of wildtype TS8011 and Δ*orfsAB* TS9004. Shaded bars (black) represent uninduced controls (no antibiotic) for each strain and unshaded bars (white) represent 5% MIC induction (erythromycin) for one hour.

Figure 6. Comparison of Insertion Sites



Class I MEGA is representative of GA17457. Class IVA MEGA is representative of GA16242.

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