Investigating Prophage (φSa2, φSa3) and PICI (SaPI5) Induction Dynamics in a Community-Associated Methicillin-Resistant (CA-MRSA) *Staphylococcus aureus* Strain USA300

Via Mitomycin C (MC) induced SOS activation in reporter systems

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Table of Contents

Abstract

Mobile genetic elements (MGEs) such as prophages and phage-inducible chromosomal islands (PICIs) play key roles carrying virulence & pathogenicity factors, driving horizontal gene transfer, and potentially mobilizing antimicrobial resistance (AMR) genes in *Staphylococcus aureus*. These elements' mobilisation is often triggered by the bacterial SOS response to DNA damage. This study investigates the induction dynamics of two resident prophages (Sa2, Sa3) and the PICI SaPI5 in the Community-Associated Methicillin Resistant *Staphylococcus aureus* (CA-MRSA) strain USA300 under SOS-inducing conditions. β-lactamase (*blaZ*) promoter–reporter systems (pAH0871, pAH0872, pAH0873) were constructed for sensitive quantification of promoter activity. Reporter plasmids were cloned in *E. coli* IM01B, electroporated into *S. aureus* RN4220 to bypass restriction barriers, and introduced into USA300 via phage-mediated transduction. Cultures were treated with mitomycin C (MC) to trigger the SOS response, and reporter activity was monitored over time. Study hypothesised that SaPI5 would activate earlier and/or more strongly than its helper prophages under MC treatment, reflecting differences in regulatory control. This work focuses on how MGEs are regulated differently in lab (RN4220) versus clinical strains (USA300) of *S. aureus* drawing insights on how prophage–PICI interactions could influence the dissemination of virulence and AMR determinants in clinically relevant *S. aureus* lineages.

1. Introduction

1.1. Background Information

1.1.1. Mobile Genetic Elements (MGE) in *Staphylococcus aureus*

Mobile genetic elements (MGEs) are segments of DNA that can move within or between genomes, and they play a central role in the adaptability and evolution of *Staphylococcus aureus* (1). The *S. aureus* genome consists of a conserved core and a diverse accessory gene pool, the latter largely contributed by MGEs acquired via horizontal gene transfer (1). Common MGEs in *S. aureus* include plasmids, transposons, insertion sequences, temperate bacteriophages (prophages), pathogenicity islands, and integrative conjugative elements (1). Many of the accessory genes carried on these MGEs encode factors that enhance bacterial fitness – for example, virulence toxins and enzymes or antibiotic resistance determinants.

Among the MGEs of *S. aureus*, two particularly important classes are prophages and pathogenicity islands. Prophages are bacteriophages that have integrated into the bacterial chromosome (lysogeny) and can later enter the lytic cycle, producing phage particles often killing host cells. *S. aureus* prophages often carry genes for virulence or immune evasion, a phenomenon known as lysogenic conversion (2) (1). Prophages are maintained in a dormant state (lysogeny) under the control of a phage-encoded repressor (such as the CI repressor), which silences phage lytic functions until induction signals arise. Pathogenicity islands, especially the *Staphylococcus aureus* Pathogenicity Islands or SaPIs, are smaller (~15–17 kb) chromosomal islands that also reside integrated at specific at sites (1). SaPIs typically harbour genes for superantigen toxins (e.g. TSST-1 or enterotoxins) which can greatly enhance virulence (1). Unlike prophages, SaPIs do not encode their own phage structural proteins or autonomous transfer machinery; instead, they hijack “helper” phages for mobilization. During a helper phage’s lytic cycle, SaPIs excise, replicate, and get packaged into phage-like particles, enabling their high-frequency transfer between bacteria (3). As with prophages, SaPI genomes are kept quiescent in the host by dedicated repressors (e.g. Stl or ImmR proteins) that prevent expression of the island’s mobilization functions (4). Together, temperate phages and phage-related islands disseminate antibiotic resistance and virulence

genes across *S. aureus* populations, directly contributing to the emergence of hyper-virulent and drug-resistant strains (2).

1.1.2. MGE Interactions and Antimicrobial Resistance

MGEs in *S. aureus* do not operate in isolation; they often interact in ways that facilitate horizontal gene transfer and can contribute to antimicrobial resistance. An example of MGE interaction is the phage–SaPI relationship. SaPIs are phage-dependent “molecular parasites”: when a SaPI-bearing bacterium is infected by (or induces) a specific helper phage, a phage-encoded protein with a dual role (“moonlighting protein”) binds and inactivates the SaPI repressor (5). This derepression triggers SaPI excision and replication (4). Once mobilised, SaPIs hijack the phage’s replication and packaging machinery, simultaneously interfering with phage reproduction - for example, by redirecting capsid assembly and reducing phage yield - ensuring that SaPI genomes are efficiently encapsidated and transferred.

The interplay of MGEs is a major driver in the spread of antibiotic resistance genes. *S. aureus* MGEs frequently carry resistance determinants – for example, plasmids encoding β-lactamase (penicillinase) or transposons carrying *mecA* (methicillin resistance) (1). Phage’s can facilitate the dissemination of these genes by generalized transduction: they occasionally package host DNA (plasmids or chromosomal fragments) and transfer them to new bacterial hosts. Classic studies have shown that phage φ11 can transduce a penicillinase plasmid (pI258 carrying *bla* genes) and other resistance plasmids between staphylococci (1). Through such mechanisms, virulence and resistance genes located on SaPIs, plasmids, or chromosomal fragments can rapidly spread in the staphylococcal population. Antibiotic-induced activation of prophages has been documented to promote the dissemination of SaPI-encoded superantigen genes (6). MGEs work in concert- a phage infecting a new host may mobilize a SaPI or plasmid, or occasionally package other DNA during transduction, thereby moving resistance determinants between strains and even across species.

1.1.3. The SOS Response, Mitomycin C and Induction Triggers

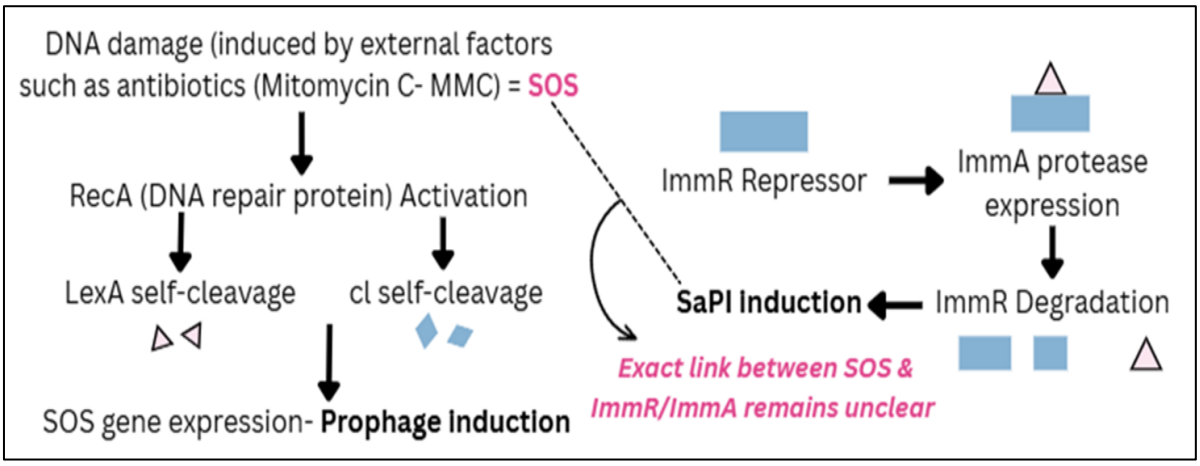
Bacteria have a stress response to DNA damage known as the SOS response. In *S. aureus*, the SOS network is controlled by two regulators: LexA, a transcriptional repressor, and RecA, a recombinase that senses DNA damage. Many temperate phage's encode a repressor protein like LexA that maintains the phage in the lysogenic state by repressing lytic functions (7). Under normal conditions (no DNA damage), LexA dimers bind to specific operator sequences (SOS boxes) in the promoters of SOS-response genes, thereby repressing their transcription (7). DNA-damaging agents (such as UV irradiation, fluoroquinolone antibiotics, or crosslinking chemicals) cause replication forks to stall and generate single-stranded DNA, which activates RecA. RecA interacts with the phage CI repressor, triggering it to autocleave (analogous to LexA cleavage) (7). This inactivation of the phage repressor flips the switch from lysogeny to lysis: the phage's lytic genes (e.g., for DNA replication, head and tail proteins, and cell lysis) are expressed, and the phage begins to replicate, ultimately lysing the host cell. Thus, the bacterial SOS response provides the cue for prophages to escape a doomed host and enter the lytic cycle (2).

Phage–SaPI interactions illustrate a more complex regulatory link to SOS. In many SaPIs, the master repressor – Stl is not itself a substrate of RecA; instead, induction relies on a *helper phage* protein to bind and inactivate the repressor (4). However, SaPI3 and SaPI5 (the native SaPI in USA300) differ: their repressor belongs to the ImmR family, which is *not* directly cleavable by RecA/LexA proteolysis (8). Instead, these elements encode a metalloprotease called ImmA that specifically cleaves the ImmR repressor to trigger the excision of the element (8). The precise trigger for ImmA activity remains unresolved, but SOS-induced phage activation is thought to provide the conditions that permit ImmA function. Thus, whereas prophage repressors are directly cleaved by RecA, SaPIs rely on either phage-encoded anti-repressors (Stl-regulated SaPIs) or the ImmR/ImmA system (SaPI3/5).

Mitomycin C (MC) is a DNA-crosslinking agent that potently triggers the SOS response in *S. aureus* by stalling replication forks, activating RecA, and driving LexA autocleavage. This leads to prophage excision, lytic replication, and, in the presence of helper phage's, SaPI excision and packaging. Because of its strong and reproducible effect, MC is widely used as

a classic laboratory inducer of SOS and MGE mobilization, comparing responses to DNA damage in RN4220 and USA300 (9).

Figure 1. Prophage & SaPI induction mechanism (image made on PowerPoint)



DNA damage, caused by antibiotics such as mitomycin C (MC), activates the bacterial SOS response. Single-stranded DNA generated at stalled replication forks promotes RecA filament formation, which in turn stimulates autocleavage of the LexA repressor. Loss of LexA repression leads to derepression of SOS regulon genes. In parallel, RecA can also trigger autocleavage of phage CI-type repressors, releasing lytic promoters and initiating prophage induction, genome replication, and particle assembly. SaPIs respond more indirectly to SOS activation. SaPI5 uses an ImmR/ImmA regulatory switch. Here, ImmR maintains the island in a repressed state, while the metalloprotease ImmA specifically cleaves ImmR to permit SaPI excision and replication. Although SOS-triggered phage induction is thought to promote conditions for ImmA activity, the precise mechanism by which SOS signals connect to ImmR/ImmA regulation remains unclear.

1.1.4. *Staphylococcus aureus* strain- USA300

USA300 refers to a hyper-virulent clone of *Staphylococcus aureus* that emerged in the early 2000s as the predominant cause of community-associated MRSA (CA-MRSA) infections in North America (1). This clone is notable for its enhanced transmissibility and virulence, which have been attributed in part to a unique complement of mobile genetic elements acquired by the strain. USA300 belongs to sequence type 8 and carries a type IVa SCCmec, conferring methicillin resistance, integrated into the orfX locus of the chromosome (1).

Adjacent to SCCmec IV, USA300 also harbours the ACME (Arginine Catabolic Mobile Element) type I – a genomic island encoding an arginine deiminase pathway (arcA-D) and an oligopeptide permease (opp3) cluster (1). ACME is thought to enhance fitness by enabling better colonization of skin (through ammonia production and pH modulation via arginine catabolism)(1), and its presence in USA300 is a distinguishing feature (most hospital MRSA strains lack ACME).

Crucially, USA300 contains multiple prophages and SaPIs that contribute to its virulence arsenal. It typically carries a SaPI5 island, which encodes the staphylococcal enterotoxin genes *sek* and *seq* (1). These superantigen toxins can cause severe systemic effects (e.g. septic shock) and heighten USA300's pathogenic potential. In addition, USA300 is lysogenized by at least two significant prophages: ΦSa2 and ΦSa3. ΦSa2USA300 encodes the Panton–Valentine leukocidin, a bicomponent pore-forming toxin that targets leukocytes and is epidemiologically linked to necrotizing skin and soft-tissue infections (1). The PVL genes on this phage are thought to contribute to the aggressive skin infections characteristic of early USA300 outbreaks. Meanwhile, ΦSa3USA300 carries immune evasion genes such as *sak* (staphylokinase) and *chp* (chemotaxis inhibitory protein of staphylococci) (1). These proteins help the bacterium evade the host immune system: staphylokinase activates plasminogen to degrade fibrin clots and certain immune components, and CHIP blocks chemokine receptors on neutrophils, impairing chemotaxis (1).

The combination of these MGEs in USA300 underlies this strain's success as a pathogen. Importantly, each of these elements is subject to regulatory controls and induction cues. For instance, the PVL phage can be induced under SOS conditions (leading to toxin release during host stress), and SaPI5 can potentially be mobilized by helper phage's if present. USA300 therefore serves as a model of a highly mobilized, clinically relevant strain, in contrast to laboratory strains that often lack many of these MGEs. This study specifically compares USA300's responses to induction stimuli with those of a lab strain RN4220 to discern how the rich MGE complement of USA300 influences outcomes like gene expression from MGE-associated promoters.

1.1.5. Rationale

Laboratory strain RN4220, derived from phage-cured NCTC 8325, lacks many MGEs and prophage-encoded virulence factors found in clinical isolates like USA300 (10). USA300 instead carries multiple inducible elements (e.g. prophages, SaPI5) that may respond strongly to SOS signals (11). These genomic differences suggest the two strains regulate MGEs differently when exposed to the same DNA-damaging stimulus.

To investigate this, we constructed plasmid-based reporter systems in which MGE-linked promoters drive expression of the *blaZ* gene. Expression of β-lactamase converts the chromogenic substrate nitrocefin (from yellow to red), providing a quantitative measure of promoter activity. By transferring the plasmids into RN4220 and then USA300, we can measure induction kinetics in two contrasting genomic contexts: one largely devoid of MGEs and one densely populated with prophages and SaPIs.

This comparative approach allows us to test whether promoter activity is regulated differently in the two strain backgrounds and to identify potential interactions between MGEs that are only apparent in the clinical isolate. In doing so, the study provides insight into how DNA damage and antibiotic stress may mobilize virulence and resistance determinants in pathogenic *S. aureus*.

1.3. Hypothesis

We hypothesize that mobile genetic elements are regulated differently in laboratory (RN4220) versus clinical (USA300) strains of *Staphylococcus aureus*.
Specifically, activation of the SOS response will drive a qualitatively different induction of MGE-associated promoters in USA300, whereas RN4220, lacking many inducible MGEs, may show a more limited response.

1.2. Aims

- **Aim 1:** Construct individual plasmid-based reporters in which MGE-associated promoters (SaPI5, φSa2USA, φSa3) drive *blaZ* expression.

- **Aim 2:** Introduce these reporters into RN4220 and assess *blaZ* promoter induction following mitomycin C treatment.

- **Aim 3:** Introduce the same reporter plasmids into USA300 and compare induction kinetics to RN4220 to determine whether clinical isolates exhibit altered regulatory responses.

For this experiment, promoter regions - SaPI5, φSa2USA, and φSa3USA300 were PCR-amplified and cloned upstream of the reporter gene in the respective plasmid constructs for SOS induction assays. Their characteristics are as follows:

Table 1: Key MGEs and their characteristics studied in this paper.

MGE	Full Name	Key Function	Representative Genes	Known SOS Induction Association
SaPI5 (12)	*Staphylococcal Pathogenicity Island 5*	Encodes super-antigenic enterotoxins (Sek, Seq); mobilized by helper phage's	*sek*, *seq*	Strongly mobilized upon SOS induction via helper phage activation
φSa2USA (13)	PVL-encoding bacteriophage	Encodes Panton-Valentine leukocidin, associated with severe skin and lung infections	*lukS-PV*, *lukF-PV*	Induction triggered by SOS response and DNA-damaging stressors
φSa3 (14)	Immune evasion cluster (IEC)-converting bacteriophage	Carries immune evasion genes (e.g., *scn*, *chp*, *sak*); can integrate into *hlb* gene	*scn*, *chp*, *sak*	SOS induction can promote excision and horizontal transfer

2. Materials and Methodology

2.1. Approach

Prophage replication in *S. aureus* is maintained in lysogeny by CI/ImmR repressors, which block transcription from lytic promoters (*cro*/*cro*'). Upon DNA damage, the SOS response activates RecA, driving LexA and repressor autocleavage. This relieves *cro*/*cro'* repression, initiating transcription of phage genes, genome replication, and particle packaging (15).

Induction can be monitored by traditional methods such as plaque enumeration; however, this approach is complicated in strains carrying multiple prophages, since it is often not possible to attribute plaques to a specific prophage. To overcome this limitation, we employed plasmid-based promoter–reporter fusions for SaPI5, φSa2USA, and φSa3, each driving expression of *blaZ*. Reporter activity, normalized to optical density, was therefore used as a more specific proxy for MGE induction.

(All experiments were conducted under sterile conditions and in accordance with risk assessments.)

2.2. Strains

The bacterial strains and associated plasmids used in this study are listed in **Table A1** (Section 8. Appendix). *S. aureus* strains were cultured in tryptic soy broth (TSB) or on tryptic soy agar (TSA) plates, and *E. coli* strains were grown in Luria–Bertani (LB) broth or on LB agar. Antibiotic selection was applied where appropriate: ampicillin (100 µg/mL), chloramphenicol (10 µg/mL), and erythromycin (10 µg/mL).

Ampicillin-resistant *E. coli* IM01B derivatives were used to propagate plasmid backbones (e.g. pAH0198) prior to transfer into *S. aureus*. For promoter–reporter assays, two *S. aureus* strains were employed: (i) RN4220, a restriction-deficient laboratory strain lacking most

endogenous MGEs, used to validate reporter construct function in the absence of native repressors or antirepressors (10); and (ii) USA300 (AH3352), a clinically relevant CA-MRSA strain carrying SaPI5, φSa2USA, and φSa3, used to assess promoter activity in the native genetic context, including regulation by the ImmR/ImmA system (SaPI5) and helper phage antirepressors (11).

All the strains were used/made in Haag Labs by my supervisor Dr. Andreas Haag.

2.3. Cloning and Reporter Construct Preparation

1. **Inoculation for Cloning**

   Ampicillin-resistant *E. coli* donor strains (AH2900,) carrying the pAH0198 shuttle vector were streaked on LB (Luria-Bertani medium) agar with Ampicillin (10ml LB added to sterile 50ml flasks and supplemented with antibiotic ampicillin to a final concentration of 100 μg ml^{-1}) and incubated overnight at 37 °C to obtain single colonies. Single colonies were inoculated into LB broth supplemented with ampicillin and cultured with shaking. Cells were pelleted and plasmid DNA was isolated using a GenElute Miniprep Kit (Sigma-Aldrich), yielding 100μl of purified pAH0198 for downstream restriction digestion and cloning.

2. **Promoter Amplification via PCR**

   To amplify promoter regions from *S. aureus* USA300 genomic DNA for cloning into shuttle vectors driving the *blaZ* reporter, plasmids and primers used are listed in Section 8 (Appendix), Table A2. Promoter fragments from *S. aureus* USA300 genomic DNA were amplified with PrimeSTAR GXL polymerase (Takara) using primers carrying SalI and BamHI overhangs (Table A2). PCR reactions were prepared and cycled according to the manufacturer's instructions. Products were verified on a 1% agarose gel, purified with the GenElute PCR Cleanup Kit (Sigma-

Aldrich), and quantified by NanoDrop.

3. Restriction Enzyme Digest of pAH0198 and Ligation of Inserts

To linearise the pAH0198 shuttle vector and generate compatible cohesive ends, PCR-amplified promoter inserts (SaPI5, φSa2USA, φSa3) were digested with SalI-HF and BamHI-HF and ligated into SalI/BamHI-digested, rSAP-treated pAH0198 (*blaZ* reporter backbone).

Purified pAH0198 vector and promoter PCR products were digested with SalI-HF and BamHI-HF in rCutSmart® buffer (NEB) as determined by the NEB RE-Cutting Calculator. Reaction volumes were adjusted with Milli-Q water to 50 µl for the double digest and 10 µl for controls. Post digestion, the double-digested pAH0198 backbone was dephosphorylated with rSAP to prevent self-ligation. Digested products were purified using a PCR cleanup kit. DNA concentration measured using a NanoDrop spectrophotometer. Inserts were ligated into digested vectors at a 3:1 insert:vector molar ratio using T4 DNA ligase. Reaction products were subsequently used for transformation.

4. *E. coli* IM01B Transformation

To generate chemically competent *E. coli* (IM01B derivatives) for uptake of ligation products and recover transformants on selective media. *E. coli* is not naturally transformable; divalent cations (e.g., $CaCl_2$) neutralize electrostatic repulsion between the negatively charged DNA and the cell envelope, and a brief 42 °C heat-shock creates a transient permeability window that allows plasmid entry.

Chemically competent *E. coli* IM01B derivatives were prepared by the $CaCl_2$ method. Overnight starter cultures were grown in LB broth (5 ml, 37 °C, 200 rpm) and diluted 1:50 into fresh LB (50 ml). Cells were harvested at mid-log phase (OD_{600} ≈ 0.3–0.5 after ~1.5–2 h), chilled on ice, and pelleted (3,200 × g, 10 min, 4°C). Pellets were

resuspended in ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30–60 min. Following a second centrifugation step, cells were gently resuspended in 0.1 M $CaCl_2$ containing 15% glycerol, aliquoted (50–100 µl), and stored at −80°C until use.

For transformation, competent cells were thawed on ice and mixed with ligation products (~10 µl reaction mixture or ~100 ng circular plasmid). After 15 min on ice, cells were heat-shocked at 42°C for 60 s, then immediately returned to ice for 5 min. LB broth (1 ml) was added, and cells were recovered at 37°C for 1 h with shaking. Transformants were plated on LB agar supplemented with ampicillin (100 µg ml^{-1}) and incubated overnight at 37°C.

Control plates confirmed transformation efficiency and antibiotic selection: LB-only (bacterial lawn), LB+ampicillin without DNA (no colonies), and vector-only ligation (low colony background, ~10–20 colonies). Experimental ligation plates produced multiple colonies for pAH0871, pAH0872, and pAH0873, consistent with successful insert–vector ligation.

5. **Colony PCR screening and Sequencing of Transformants**

E. coli transformants carrying recombinant pAH0198 constructs were screened by colony PCR to rapidly identify correct promoter insertions prior to miniprep. Individual colonies were picked with sterile pipette tips, briefly inoculated into 25 µl LB, and dabbed into 25 µl PCR master mix containing DreamTaq DNA polymerase (Thermo Fisher), 10× DreamTaq buffer, dNTPs (10 mM), vector-flanking forward primer pKX-US-F and reverse primer pCN41-R, and 5 µl of cell colony as template. Amplification products were resolved on 1% agarose gels; purified positive colonies yielded high-quality DNA, with representative concentrations of colony 1 of each plasmid of 396.4 ng/µl (pAH0871), 352.4 ng/µl (pAH0872), and 387.1 ng/µl (pAH0873).

Sanger sequencing confirmed insert identity and orientation, data aligned in Benchling to verify both insert identity and orientation. Confirmed plasmids were subsequently transferred into *Staphylococcus aureus* RN4220 via electroporation and into USA300 by φ11-mediated transduction.

6. **Electroporation of Recombinant Plasmids into RN4220**

Electrocompetent *S. aureus* cells were prepared to enable uptake of verified promoter–reporter plasmids. Single colonies were inoculated into 5 ml tryptic soy broth (TSB) and incubated overnight at 37 °C with shaking (120 rpm). Overnight cultures were diluted 1:100 into 100 ml fresh TSB and grown to mid-log phase (OD_{600} ≈ 0.5; ~2.5–3 h). Cells were harvested by centrifugation (3,200 × g, 10 min, 4 °C), washed twice with ice-cold 0.5 M sucrose, and resuspended in 10 ml sucrose. After a 30-min incubation on ice, cells were pelleted once more and finally resuspended in 2–5 ml sterile 0.5 M sucrose. Aliquots (300 µl) were stored at −80 °C until electroporation.

For transformation, competent aliquots were thawed on ice and mixed with plasmid DNA (500 ng–2 µg in ≤10 µl volume). Suspensions were transferred to pre-chilled electroporation cuvettes (2 mm, 200 µl) and pulsed at 2.1 kV, 100 Ω, 25 µF using Bio-Rad Gene Pulser. Immediately afterwards, 1 ml TSB was added and cultures incubated at 37 °C with shaking (120 rpm) to allow recovery. 200µl of recovered cells were plated on TSB agar with chloramphenicol (10 µg ml^{-1}) and incubated overnight at 37 °C.

Appropriate controls behaved as expected: viability controls (TSB only) showed confluent growth, while selection controls (TSB + antibiotic, no plasmid) showed no colonies. Recombinant plasmids displayed variable efficiencies: pAH0871 yielded multiple transformants, pAH0198 produced a single colony, while pAH0872 did not produce colonies under initial conditions. Backup cultures maintained on selective media by supervisor- Dr. Haag ensured successful continuation of assays.

7. **Phage-Mediated Transduction**

Reporter plasmids introduced into USA300 by phage-mediated transduction according to protocol by Krausz & Bose (16). Donor strains of RN4220 carrying pAH0871, pAH0872, or pAH0873 were cultured overnight in TSB supplemented with chloramphenicol (10 µg ml^{-1}). Staphylococcal phage (φ11) was propagated on these

donors in TSB containing 10 mM $CaCl_2$ to improve adsorption, and lysates were clarified by sterile filtration.

Recipient USA300 cultures were prepared by diluting overnight cultures 1:100 in TSB and growing at 37 °C, 250 rpm to mid-log phase (OD_{600} ≈ 0.5). Recipient cells were mixed with phage lysates at a multiplicity of infection sufficient for plasmid transfer, incubated briefly at room temperature to permit adsorption, and then shifted to 30 °C to allow transfer. Following adsorption, cells were pelleted, resuspended in fresh medium, and recovered for 1h at 37°C with shaking.

Aliquots were plated on TSB agar with chloramphenicol to select plasmid-containing transductants. Colonies obtained were screened by colony PCR to confirm carriage of the correct reporter plasmid and to exclude contamination with phage particles.

2.4. β-Lactamase Activity Assays

Induction dynamics of MGEs were quantified in *S. aureus* via colour change nitrocefin β-Lactamase (*blaZ*) Activity Assay and normalized to bacterial growth (OD_{600}). *BlaZ* hydrolyses nitrocefin, turning it yellow to red. Verified RN4220 and USA300 transformants carrying pAH0198 and promoter–*blaZ* plasmids (pAH0871–pAH0873) were confirmed free of contaminating phages by PCR.

Cultures were grown in TSB with the appropriate antibiotic (chloramphenicol for RN4220, erythromycin for USA300), diluted 1:50 into fresh medium, and incubated to OD_{600} ≈ 0.25. At this point, cultures were split into untreated controls and SOS-induced samples supplemented with mitomycin C (MC, 2 μg ml^{-1}). For RN4220, assays were performed in 5 biological replicates; for USA300, 4 replicates were carried out following φ11 mediated plasmid transduction.

Aliquots (200 μl) were removed at 30-min intervals and snap-freezed over a 2-h induction window (0–120 min; timepoints T0 to T4), mixed with 800 μl potassium phosphate buffer, and stored at –80 °C until assay conducted. For the assay, samples were thawed and

incubated with nitrocefin substrate, and hydrolysis was monitored at 490nm over 30 minutes using a CLARIOstar Plus microplate reader by BMG Labtech. Promoter activity was calculated from the slope of the linear reaction phase and expressed as OD_{486}/OD_{600} to account for cell density. Plasmids were first validated in RN4220, then tested in USA300.

2.5. Data Processing

β-lactamase activity was expressed as the rate of nitrocefin hydrolysis normalized to cell density, dilution, and assay volume. The slope of absorbance over time ($dA490/dt$) was calculated and divided by the optical density of the culture at the sampling timepoint (OD_{600}), the dilution factor (DF=0.2), and the assay volume (50µl, V=0.05ml). The calculation is:

$$X = \frac{dA_{490}}{dt\,(h)} \frac{1}{OD_{540} * DF * V}$$

Where X is the normalised β-lactamase activity.

To assess statistical significance, Welch's unpaired two-tailed t-test was performed to compare promoter activity with and without mitomycin C (MC). A significance threshold of α= 0.05 was applied, with p-values < 0.05 considered significant.

Data were manually filtered to exclude plateau regions at the end of reactions, which reduced slope accuracy, and to remove early anomalies caused by air bubbles, identified as sharp OD fluctuations. Goodness of fit (R^2 values) for each slope was calculated to ensure reliable linearity. For all biological replicates, mean and standard deviation were calculated, and error bars representing standard deviation were plotted on clustered bar graphs comparing promoter activity ±MC across all timepoints for each plasmid.

3. Results

3.1. Validation of Reporter Constructs

Plasmid-based *blaZ* reporters were generated by cloning promoter regions from SaPI5, φSa2USA, and φSa3 upstream of the *blaZ* gene (Figure 2). Sequence verification confirmed correct insert orientation and integrity. These constructs provided the basis for subsequent induction assays in RN4220 and USA300.

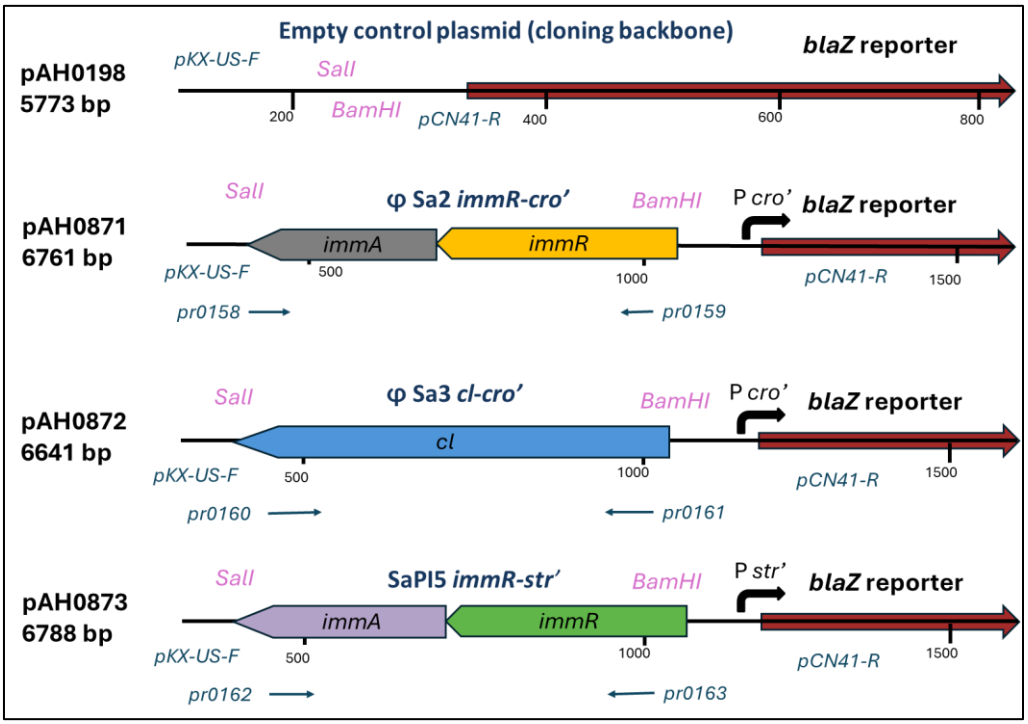


Figure 2. Plasmid reporter constructs (Designed on MS PowerPoint)

Maps of reporter plasmids generated for this study. pAH0198 served as an empty vector backbone. Promoter regions from (i) φSa2 (immR–cro'), (ii) φSa3 (cI–cro'), and (iii) SaPI5 (immR–str') were cloned upstream of the promoterless blaZ reporter. These plasmids provided quantitative readouts of promoter activity in subsequent induction assays.

3.2. Promoter Activity Assay Results of RN4220 and USA300

To assess how individual prophage- and SaPI-associated promoters respond to SOS induction, we introduced *blaZ* reporter plasmids (pAH0198, pAH0871–pAH0873) into the lab strain RN4220 and the clinical isolate USA300. RN4220 provides a background devoid of native MGEs, allowing promoter induction to be measured without interference, while USA300 enables assessment in the natural genetic context containing multiple prophages and SaPI5. Exponential cultures were treated with or without mitomycin C (MC, 2 µg ml^{-1}) to trigger the SOS response, and promoter activity was quantified by nitrocefin hydrolysis normalized to OD_{600}.

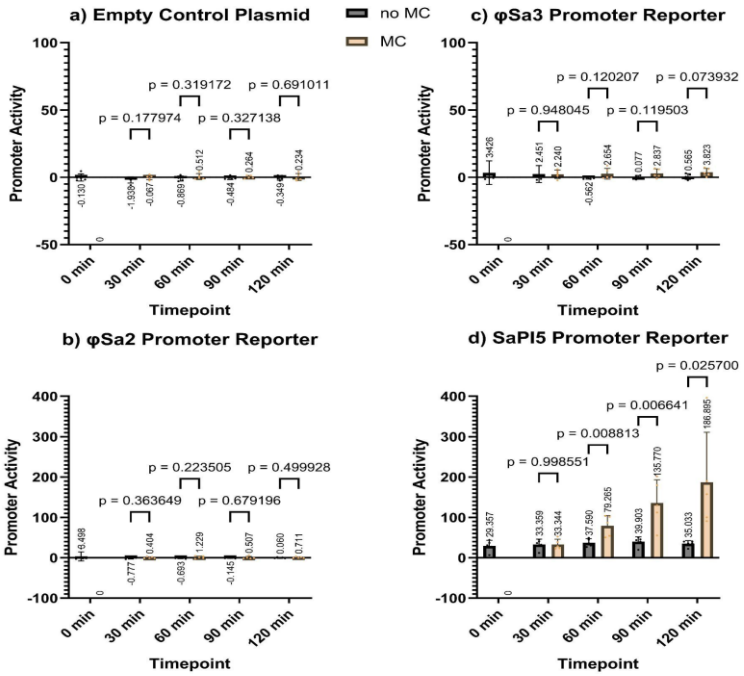


Figure 3. Promoter activity of prophages and SaPI5 in RN4220 blaZ reporter strains.

Cultures were grown to OD_{600} ≈ 0.25 and split ± MC (2 µg ml^{-1}). Promoter activity was quantified by blaZ nitrocefin assay normalized to OD_{600}. Bars represent mean ± SD of n = 5 biological replicates. Dots show individual values. Error bars represent standard deviation. p-values from unpaired two-tailed t-tests compare MC vs. untreated at each timepoint.

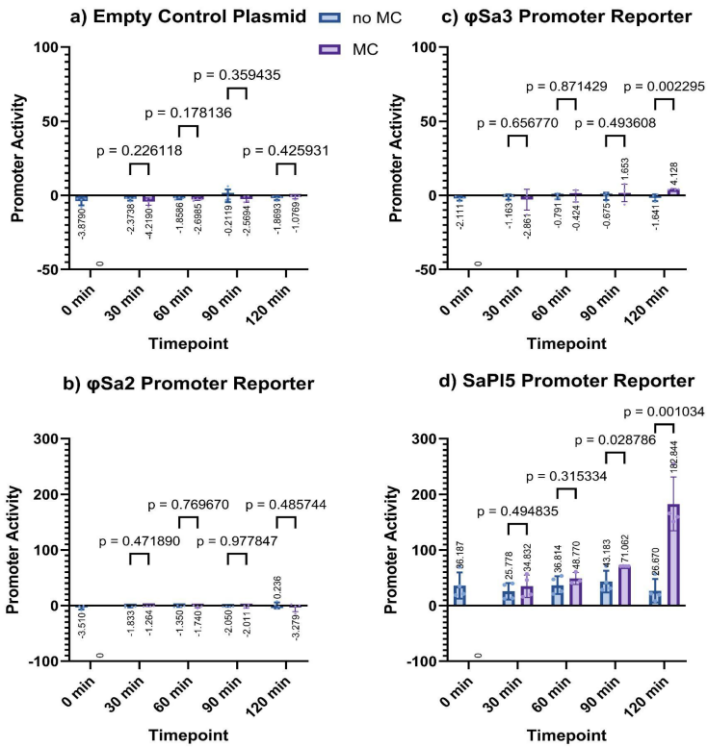


Figure 4. Promoter activity of prophages and SaPI5 in USA300 blaZ reporter strains.

Cultures were grown to OD_{600} ≈ 0.25 and split ± MC (2 µg ml⁻¹). Promoter activity was quantified by blaZ nitrocefin assay normalized to OD_{600}. Bars represent mean ± SD of n = 4 biological replicates. Dots show individual values. Error bars represent standard deviation. p-values from unpaired two-tailed t-tests compare MC vs. untreated at each timepoint.

3.3. Analysis of Promoter Activity Assay Results

In both RN4220 and USA300, similar trends were observed. Welch's two-tailed unpaired t-test was performed to identify plasmids showing significant promoter activity with and without Mitomycin C (MC), as demonstrated in Table A3 in Section 8.

In **RN4220**, the empty vector control (pAH0198) showed no increase in promoter activity following MC treatment (Figure 3), confirming that induction required a cloned promoter. The φSa2 reporter (pAH0871) also remained unresponsive, with no significant differences across the 0–120 min induction window ($p \gg 0.05$). The φSa3 reporter (pAH0872) displayed only low-level activity and did not reach statistical significance at any timepoint. By contrast, the SaPI5 reporter (pAH0873) demonstrated strong MC-dependent induction, becoming

significant at 60, 90, and 120 min ($p < 0.05$). These findings establish that in a prophage-cured, restriction-deficient background, SaPI5 is the most responsive element, while φSa2 and φSa3 remain inactive.

In **USA300**, the empty vector control (pAH0198) again showed no induction (Figure 4), confirming promoter dependence. The φSa2 reporter (pAH0871) exhibited no measurable induction ($p \gg 0.05$), similar to RN4220. The φSa3 reporter (pAH0872), however, showed weak but significant induction at the 120 min timepoint ($p = 0.002$). The SaPI5 reporter (pAH0873) was again the strongest responder, with significant induction detected at 90 and 120 min ($p < 0.05$). Notably, the amplitude of induction in USA300, albeit strong, was lower and delayed compared to RN4220, suggesting that in the clinical strain, competition or crosstalk with endogenous MGEs reduces the apparent responsiveness of SaPI5.

Overall, these results indicate that:

- SaPI5 is strongly inducible in both RN4220 and USA300, though induction is earlier and stronger in RN4220.
- φSa3 shows weak, late induction only in USA300.
- φSa2 is unresponsive under the tested conditions.
- Differences between the two strains likely reflect the presence of additional MGEs in USA300 that compete for resources or influence regulatory pathways during SOS induction.

4. Discussion

4.1. SOS-Induced Promoter Activation Patterns

As per results, SaPI5 promoter (pAH0873) induction in RN4220 was statistically significant as early as 60 min with further increases. In USA300, induction, although strong, was delayed, reaching significance only at 90 and 120 min ($p<0.05$). These differences underscore SaPI5's role as a highly SOS-responsive island with strain-specific changes in activity.

The φSa3 promoter (pAH0872) showed weak induction. No significant activity was seen in RN4220, whereas in USA300 a slight increase was observed. This late, weak response suggests φSa3 lytic genes are tightly repressed under normal conditions and only partially derepressed by SOS. The φSa2 promoter (pAH0871) did not respond to MC in either strain, consistent with either a non-SOS-regulated promoter or failure of repressor inactivation under these conditions. The vector control (pAH0198) remained at baseline near zero throughout, confirming the validity of the experiment.

These observations indicate that among USA300's MGEs, SaPI5 is the most transcriptionally responsive to SOS induction, φSa3 is weakly inducible, and φSa2 appears inert in this context.

The differences can be explained by phage and SaPI regulation. Staphylococcal prophages employ CI-like repressors (ImmR family) to maintain lysogeny and Cro-like proteins to drive lytic switch upon SOS induction (17). RecA activation stimulates cleavage of LexA and CI-like repressors, permitting transcription from lytic promoters (see section 1.1.3.) (18).

The regulatory link between SaPIs and the SOS response is indirect. Unlike prophages, most SaPIs are not directly SOS-inducible. In SaPI5, repression is mediated by ImmR, which is degraded by the ImmA metalloprotease upon SOS induction. Though, how ImmA is activated remains unclear. RecA indirectly induces ImmA (4). DNA damage promotes RecA activation, which in turn stimulates ImmR cleavage (or ImmA protease activity), derepressing SaPI5 promoters. This explains why SaPI5 displayed strong MC-dependent induction in our assays without requiring a specific phage helper, distinguishing it from other SaPIs that typically require helper phage proteins for derepression. The weak φSa3 induction in USA300 suggests partial CI derepression, while φSa2's unresponsiveness may reflect repressor resistance to cleavage, or regulatory inputs not mimicked by MC.

A consistent observation was the greater amplitude and earlier onset of SaPI5 induction in RN4220 compared to USA300. RN4220 is a laboratory strain cured of endogenous prophages, providing a "clean" background where SOS activation primarily targets the plasmid reporter constructs (10). In contrast, USA300 carries multiple prophages which are themselves induced by SOS. USA300 prophages also encode derepressor-like proteins that

can interact with SaPI repressors, but specificity varies by phage–SaPI pair. In RN4220, by contrast, no competing prophages interfere, allowing direct derepression of the reporter. This competition likely explains why SaPI5 induction in USA300 was weaker and delayed.

4.2. Clinical and Biological Implications

The SOS responsiveness of SaPI5 has important clinical implications. SaPI5 encodes super-antigenic toxins (e.g., SEK2, SEQ2), and its activation during antibiotic stress could exacerbate disease severity. DNA-damaging antibiotics induce SOS and thereby risk triggering SaPI excision and toxin release. Once derepressed, SaPI5 can hijack phage capsids for horizontal transfer, disseminating virulence determinants across strains.

Ranc et al. (2025) (19) documented a USA300 lineage in which SaPI5 had been lost prior to a prolonged period of transmission within a low-antibiotic, closed hospital environment. Their phylogenetic reconstruction suggested that the loss occurred several years before the outbreak, and the absence of strong selective or SOS-inducing pressures may have prevented reacquisition. Taken together, these findings highlight two complementary points: (i) SaPI5 is highly inducible under SOS stress and thus may be a major driver of virulence dissemination in settings with frequent exposure to SOS-inducing agents, and (ii) sustained environments with low antibiotic pressure could promote its long-term loss, potentially reducing virulence potential.

These findings highlight how antibiotic therapy can mobilize virulence elements (20). Prior work has shown β-lactams and fluoroquinolones increase SaPI transfer frequency via SOS induction. The results support this, demonstrating that SOS strongly upregulates SaPI5 transcription. The limited inducibility of φSa2 and φSa3 suggests they may contribute less to immediate SOS-driven virulence activation, though φSa3 encodes immune evasion proteins that, when mobilized, could still impact host-pathogen interactions (21).

4.3. Data Filtering, Quality Control, and Limitations

For pAH0873, absorbance curves plateaued at later timepoints (60, 90, 120 min in MC-treated samples). Readings after plateau were excluded, consistent with best practice for enzymatic assays. Including measurements beyond this plateau could misrepresent the

active phase of the reaction – artificially flattening or skewing calculated rates (22). Early anomalies, such as spikes due to air bubbles, were also removed. These adjustments improved linear regression fits, yielding higher R^2 values and more accurate slope estimates.

In the fourth USA300 replicate of pAH0873, readings at later timepoints with MC differed from prior trends, likely reflecting localized lysis or plate reader error. These points were excluded to prevent skewing means, while early data from the same replicate were retained. Controls remained near zero throughout, confirming minimal baseline drift.

A potential limitation in the experiment could be the age of the plate reader. High standard deviations were observed owing to defective plate reader function. Old plate readers are prone to lamp intensity decline and temperature instability. Even a 1°C drift can affect enzyme kinetics by >10%, potentially confounding long assays (23). Additionally, plasmid-based reporters may not fully simulate chromosomal regulation. Nevertheless, relative differences between treated and untreated conditions, and between strains, remain accurate.

5. Conclusion

This study demonstrates that the SaPI5 promoter is inducible by SOS activation, showing strong MC-dependent induction in both RN4220 and USA300. Induction was earlier and stronger in RN4220 than in USA300. In contrast, φSa2 remained non-responsive, and φSa3 showed only weak induction (significant in USA300).

These results align with known ImmR/cro′ and LexA/RecA regulatory logic: SOS-induced derepression activates prophage and SaPI promoters, with SaPI5 behaving like an SOS-controlled island (4). Strain-specific differences highlight how prophage presence in USA300 dampens SaPI5 activation compared to the clean RN4220 background.

Clinically, the findings stress that SOS-inducing antibiotics may mobilize SaPI5, releasing toxins and enabling horizontal transfer, with potential to exacerbate infections and spread virulence. Data filtering ensured accurate kinetic interpretation improving reliability despite limitations from the aging plate reader.

Overall, the results reinforce the view that staphylococcal pathogenicity islands are rapidly mobilised under stress, linking host- and antibiotic-induced SOS responses directly to virulence and gene transfer. Future work should validate these findings in chromosomal SaPI5 contexts, dissect ImmR/ImmA regulatory mechanisms, and explore SOS-targeting therapeutics to mitigate mobilization of virulence determinants.

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8. Appendix

Table A1. The table illustrates strains used in this study, including strain number, species, plasmid carried, antibiotic resistances, and relevant notes.

Strain Number	Species	Derivative of	Plasmid	Antibiotic resistances	Notes
AH3463	*E. coli*	IM01B	pAH0871	100 µg/mL ampicillin	USA300 Sa2 *immR to cro'* with blaZ reporter
AH3464	*E. coli*	IM01B	pAH0872	100 µg/mL ampicillin	USA300 Sa3 *cI to cro* with blaZ reporter
AH3465	*E. coli*	IM01B	pAH0873	100 µg/mL ampicillin	USA300 SaPI5 *reporter immR to cro'* with blaZ reporter
AH3477	*S. aureus*	RN4220	pAH0198	10µg/mL chloramphenicol	blaZ reporter control
AH3478	*S. aureus*	RN4220	pAH0871	10µg/mL chloramphenicol	USA300 *Sa2 immR to cro'* with blaZ reporter
AH3479	*S. aureus*	RN4220	pAH0872	10µg/mL chloramphenicol	USA300 Sa3 *cI to cro* with blaZ reporter
AH3480	*S. aureus*	RN4220	pAH0873	10µg/mL chloramphenicol	USA300 SaPI5 reporter *immR to cro'* with blaZ reporter
AH3484	*S. aureus*	AH3352	pAH0198	10µg/mL chloramphenicol, 10µg/mL erythromycin	USA300 with pAH0198 blaZ reporter control
AH3485	*S. aureus*	AH3352	pAH0871	10µg/mL chloramphenicol, 10µg/mL erythromycin	USA300 Sa2 *immR to cro'* with blaZ reporter
AH3486	*S. aureus*	AH3352	pAH0872	10µg/mL chloramphenicol, 10µg/mL erythromycin	USA300 Sa3 *cI to cro* with blaZ reporter
AH3487	*S. aureus*	AH3352	pAH0873	10µg/mL chloramphenicol, 10µg/mL erythromycin	USA300 SaPI5 reporter *immR to cro'* with blaZ reporter

Table A2. Primer Sequences and Amplicon Sizes for pAH0871, pAH0872, and pAH0873

Plasmid	Primer	Primer sequence (5'-3')	Amplicon	Size (bp)
pAH0871	pr0160	ctgcaggtcgacttataaaataacttttccaattaacctcacac	A	898
	pr0161	acccggggatccagtcctcaactcttttaatgttttc	A	898
	pKX-US-F	CCGGCTCGTATGTTGTGTGG	colony PCR/sequencing	1107
	pCN41-R	ctctttggcatgtgaactgtttg	colony PCR/sequencing	1107
pAH0872	pr0162	ctgcaggtcgacttatatttctttatatttaaaaactctcaacgg	A	1045
	pr0163	acccggggatccctcaatctttttaaatttagtaccacgttac	A	1045
	pKX-US-F	CCGGCTCGTATGTTGTGTGG	colony PCR/sequencing	1254
	pCN41-R	ctctttggcatgtgaactgtttg	colony PCR/sequencing	1254
pAH0873	pr0158	ctgcaggtcgacgtttatatttccttatatttaaaaactctcaacgg	A	1018
	pr0159	acccggggatccattttcttacggataattgttcc	A	1018
	pKX-US-F	CCGGCTCGTATGTTGTGTGG	colony PCR/sequencing	1227
	pCN41-R	ctctttggcatgtgaactgtttg	colony PCR/sequencing	1227

Table A3. Plasmids showing significant promoter activity ($p<0.05$) with and without Mitomycin C (MC) using Welch's unpaired two-tailed t-test.

Plasmid	Timepoint(min)	Condition	P Value
RN4220 pAH0873	60	no MC	0.008813
RN4220 pAH0873	60	MC	
RN4220 pAH0873	90	no MC	0.006641
RN4220 pAH0873	90	MC	
RN4220 pAH0873	120	No MC	0.025700
RN4220 pAH0873	120	MC	
USA300 pAH0872	120	No MC	0.002295
USA300 pAH0872	120	MC	
USA300 pAH0873	90	No MC	0.028786
USA300 pAH0873	90	MC	
USA300 pAH0873	120	No MC	0.001034
USA300 pAH0873	120	MC	