Investigation of hsdSA1-A3's Impacts On *Streptococcus Pneumoniae*'s Phase Variation Between Opaque and Transparent Phenotypes

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ABSTRACT

Streptococcus pneumoniae causes contagious and potentially severe pneumococcal infections, including pneumonia, septicemia, and meningitis. Despite the introduction of vaccines, pneumococcal infections are still prevalent due to its remarkable adaptive and invasive abilities, which are enabled partially by *S. pneumoniae*'s phase variation between opaque colonies (colonies with higher resistance to host clearance) and transparent colonies (colonies with higher airway adherence). It is known that pneumococcal type I restriction-modification system, cod locus, consists of six genes: an endonuclease R subunit hsdR, a methyltransferase M subunit hsdM, three homologous specificity S subunits (hsdSA, hsdSB, and hsdSC), and a tyrosine site-specific recombinase psrA. Two copies of R subunit, two copies of M subunit, and one S subunit form pentameric complex REase/MTase, which performs DNA endonuclease and methyltransferase activities that confer bacterial defending machinery. Active expression of hsdSA but not hsdSB and hsdSC indicates that hsdSB and hsdSC do not produce functional proteins for DNA inversion and instead act as templates for recombination by psrA. Besides, hsdS inversions are mediated by three pairs of inverted repeats (IR1, IR2, IR3). DNA inversions between the IRs are catalyzed by psrA to generate six different hsdS alleles, resulting in diverse genome methylation patterns where pneumococci carrying hsdSA1 allele form opaque colonies while those lacking hsdSA1 produce transparent colonies. This study is conducted to testify hsdSA1-3's impacts on *S. pneumoniae*'s phase variation.

Keywords: *Streptococcus pneumoniae*; phase variation; type I restriction-modification system; DNA inversion

INTRODUCTION

Streptococcus pneumoniae, or pneumococcus, is a Gram-positive, opportunistic pathogen that colonizes the mucosal surfaces of the human upper respiratory track and invades sites like the middle ear spaces, lungs, bloodstream and meninges [1]. Taking advantage of hosts' weak immune systems, *S. pneumoniae* exhibits greater incidence rates among children under the age of two, the immunocompromised, and the elderly [2].

S. pneumoniae evades vaccine-induced immunity and overcomes pressures from a wide range of antibiotics, such as beta-lactams, macrolides, lincosamides, flouroquinolones, tetracyclines, and trimethoprim-sulfamethoxazole (TMP-SMX) [3]. Studies conducted between 2001 and 2016 estimated that *S. pneumoniae* colonization rate in UK children under 5 years old was between 48%-52% despite the introduction of vaccines [4]. With a 20% prevalence of resistance to most antibiotics, pneumococcus was enlisted as one of the nine bacteria of international concern in a report on global antibiotic resistance published by World Health Organization (WHO) in 2014 [3, 5]. *Streptococcus pneumoniae* is the leading cause of various pneumococcal diseases, including community-acquired pneumonia, sepsis, and meningitis, and is a major cause of death of children under 5 years old worldwide [1, 5, 6]. Up to 27%-65% of children and <10% of adults are carriers of *S. pneumoniae* [1]. Specifically, the nasopharyngeal carriage rate of pneumococcus ranges from 20%-50% in children and 5%-30% in adults. In bacterial meningitis, pneumococcus accounts for 16% to 37% of mortality rates [5].

Streptococcus pneumoniae has a variety of properties that contribute to its exceptional adaptive and invasive capacities. For instance, pneumococcus possesses a polysaccharide capsule that plays a critical role in virulence by interfering with host clearance mechanisms [7]. Furthermore, *S. pneumoniae* is able to remodel its genome through incorporation of exogenous DNA from other pneumococci, allowing it to confer a wide scope of antibiotic resistance [1]. Importantly, *S. pneumoniae* demonstrates phase variation between opaque and transparent phenotypes [8].

Phase variation in *S. pneumoniae* is dictated by epigenetic switch driven by DNA inversions among three methyltransferase hsdS genes in the Spn556II type I-restriction modification (R-M) locus: hsdSA, hsdSB, and hsdSC [9, 10], as shown in **Fig. 1**. Pneumococcal type I R-M system, cod locus, consists of a specificity S subunit (hsdS) for recognizing a specific DNA sequence, two DNA methyltransferase M subunits (hsdM) for catalyzing the methylation reaction, and two restriction endonuclease R subunits (hsdR) for DNA cleavage [11]. Together, the RMS subunits form a pentameric complex REase /MTase that performs

DNA endonuclease and methyltransferase activities. According to the specific sequence binding by S subunit, the MTase adds a methyl group to the adenosine base at the nitrogen-6 position [12, 13, 14]. Meanwhile, hsdS inversions are mediated by three pairs of inverted repeats (IR1, IR2, IR3) and are catalyzed by psrA tyrosine recombinase. Among the three hsdS genes, only hsdSA is actively expressed——hsdSB and hsdSC remain silent and act as templates for sequence switching of hsdSA [15]. Since hsdSA encodes the specificity subunit S of the type I R-M system, site-specific recombinations produce variable hsdSA alleles and diverse genome methylation patterns. For instance, pneumococci carrying hsdSA1 allele form opaque colonies while those lacking hsdSA1 allele form transparent colonies [9]. Phase variation between the two different phenotypes enables *S. pneumoniae* to adjust its adaptive and invasive abilities accordingly: The opaque phenotype consists of a thicker capsule and is specialized in invasive infection and evasion of host clearance, while the transparent phenotype possesses a thinner capsule and thereby allows higher airway adherence and colonization capacity [16].

Owing to *S. pneumoniae*'s complex, interchangeable biological properties, the treatment of pneumococcal diseases is now in the face of difficulties. To effectively resolve such challenges, the biological mechanisms of pneumococci's regulation of phase variation must be well understood. This study aims to assess hsdSA1, hsdSA2, and hsdSA3's impacts on *S. pneumoniae*'s phase variation between opaque and transparent phenotypes and subsequently looks into psrA's role in pneumococci's DNA inversions.

RESULTS

The knock-out and revertant strains were successfully constructed through the insertion and excision of replacement gene Janus cassette (JC). **Fig 1** shows the genetic arrangement in the Spn556II locus. The methylated bases are highlighted with red characters. The gene order and other components in the Spn556 locus of three pneumococcal strains (ST556, TIGR4 and D39) are displayed, with arrowheads representing the orientations of the coding sequences and dashed lines indicating the identical hsdS segment sequences. With kanamycin resistance marker and a counter-selection marker conferring streptomycin-sensitivity (rpsL), JC replaced the allele of interest through homologous recombination in streptomycin background with selection for kanamycin resistance, as shown in **Fig. 2**. The cassette was then replaced with an allele, leading to mutants that were kanamycin sensitive and streptomycin resistant. After selective cultivation of bacteria on antibiotic plates, the transformed colonies were successfully collected and preserved. **Fig 3** shows the JC replacement strain and the revertant strain on streptomycin plates. During the reversion process, the transformed strains mutated to exhibit false-positive resistance to streptomycin, resulting in a particularly large amount of bacterial colonies shown in **Fig. 4**.

The wild-type colonies exhibited both opaque and transparent phenotypes (**Fig. 5A**). After constructing hsdSA allele-locked revertant mutants, the strains with hsdSA allele fixed at hsdSA1 displayed completely opaque phenotype (**Fig. 5B**), while the strains with hsdSA allele fixed at hsdSA2 and hsdSA3 were entirely transparent (**Fig. 5C, Fig. 5D**). Such monotonicity in the respective phenotypes reflects the deactivation of the site-specific recombinase psrA, which was disabled from catalyzing hsdS inversions. PCR results showed that the sizes of all three revertants matched those of the original strain. Two colonies of each revertant strain was sent for DNA sequencing and the results confirmed that the construction of revertants was successful.

DISCUSSION

The biological advantages of *S. pneumoniae*'s diverse phase variation have been well documented: by reversibly switching the "opaque ON-or-OFF" methylation patterns of pneumococcal genome, wild-type *S. pneumoniae* enhances its adaptive or invasive ability according to the specific condition or environment [16]. The original wild-type genotype is shown to produce both opaque and transparent colonies (**Fig. 5A**), implying that in natural conditions, *S. pneumoniae* possesses the capacity to reversibly translate between the two different phenotypes to increase its chance of survival and invasive ability. The A1 genotype, where psrA is inactive and hsdSA allele is fixed at hsdSA1, is shown to confer opaque phase, indicating that the pneumococci carrying hsdSA1 can produce opaque colonies with more specialized ability in invasive infection and higher resistance to host clearance (**Fig. 5B**). The A2 and A3 genotypes, where psrA is inactive and hsdSA allele is fixed at hsdSA2 and hsdSA3 respectively, are shown to confer transparent phase, which enables pneumococci to display greater airway adherence and colonization capacity (**Fig. 5C, Fig. 5D**) [16]. As wild-type S. *pneumoniae* bacteria exhibits diverse phase variation due to constant psrA-catalyzed inversion of DNA methyltransferase hsdS genes, the colonies display monotonous phenotypes (either all opaque or all transparent) after deactivation of psrA and fixation of hsdSA at hsdSA1, hsdSA2, or hsdSA3. This suggests that by deactivating psrA, a key component responsible for pneumococcal DNA inversions, it is possible to singularize the phenotype and counter *S. pneumoniae*'s advantage with a specific phase.

Moreover, as *S. pneumoniae* displays remarkable ability in taking up foreign DNA under stimulation by stress response, the challenge of overcoming *S.pneumoniae*'s diverse antibiotic resistance is now a problem of concern [17]. Further, it has been known that *S. pneumoniae's* polysaccharide capsule is a significant virulence factor that performs various functions, such as helping the escape of phagocytosis, allowing adherence and colonization of the nasopharynx, and inhibiting recognition by immunoglobulins. This reveals that the polysaccharide capsule can be used as the primary research object for vaccine development. However, *S. pneumoniae* has evolved various characteristics, such as interchangeable capsules and diverse capsule types, to escape attack from the host's immune response, posing difficulties for the treatment of pneumococcal infections [2]. In addition, it has been experimentally shown in this work that the use of replacement gene Janus cassette has led to a high number of false-positive transformed colonies, therefore a modified cassette with additional counter-selection markers may be used instead to increase efficiency.

While the workings of *S. pneumoniae*'s phase variation between opaque and transparent phenotypes have been well understood, wild-type *S. pneumonie*'s directionality and self-regulation of the process remains unknown. Specifically, how wild-type *S. pneumoniae* detects whether it is situated at a location for colonization or is ready for invasion is still a question that demands future study.

MATERIALS AND METHODS

Antibiotics

-Streptomycin resistant (Sm): 150 µg/mL

-Kanamycin resistant (Kan): 400 µg/mL

Competence Stimulating Peptide (CSP)

-CSP-1&2 mixture stock: 10 µg/mL (respectively)

THY Preparation

30 grams of Todd Hewitt Broth (BD), 5 grams of yeast extract, and 1000 mL of H2O were mixed together. The mixture solution was autoclaved at 115°C for 20 minutes.

Kana/Sm Plates

40 grams of Tryptic Soy Agar (TSA) were mixed with 1000 mL of H2O. The solution was autoclaved at 121°C for 15 minutes and was allowed to cool down in 52°C water bath for over 1 hour. 4% sheep blood and the respective antibiotic were then added to the solution by mixing well. The Kan plates were used to select kanamycin-resistant JC-replacement strains. The Sm plates were used to select streptomycin-resistant revertant strains.

Polymerase Chain Reaction (PCR) System Preparation

The primers used in this work are listed in **Table 1** and **Table 2**. 1000 µl PCR system was prepared by mixing 3 µl of each primer, 50 µl of master mix (P123 PCR mix Vazyme), 2 µl of template DNA, and 42 µl of H2O.

Purification of DNA

-Vazyme gel DNA extraction mini kit

C+Y Procedure

S. Pneumoniae was grown in THY to 0.5 OD. Bacteria was transferred into 6 mL C+Y while the initial OD was about <0.03. Bacteria was then cultured at 37°C and grown to 0.04~0.06 OD. 200 µl of bacteria solution was collected in 1.5 mL tube. 5 µL CSP and <40 µL DNA were added. Solution was mixed well with tips (negative control: only CSP without DNA) and

incubated at 37°C for 40 minutes to 1 hour. Cells were transferred on selective plates containing antibiotics and spread with a glass spreader.

Construction of Mutants

The up- and down-stream fragments of respective strains were amplified. The JC fragment was amplified with JC-F/R. The Up-JC-Down fragments were ligated with overlapping PCR and transformed into ST606 strain. Bacteria was cultured on streptomycin and kanamycin plates overnight. xx::JC bacteria that survived on kanamycin plates was cultured. The up- and down-stream fragments were amplified from ST556 genome and fused to transform into xx::JC. Wild-type (WT) gene was amplified from ST556 genome and fused to transform into the xx::JC to construct the revertant strain. Bacteria was cultured on streptomycin plates overnight. Two xx^{rev} colonies that survived on streptomycin plates were sent for DNA sequencing.

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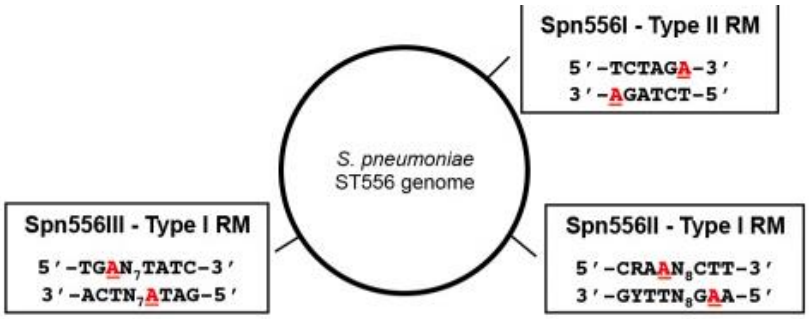
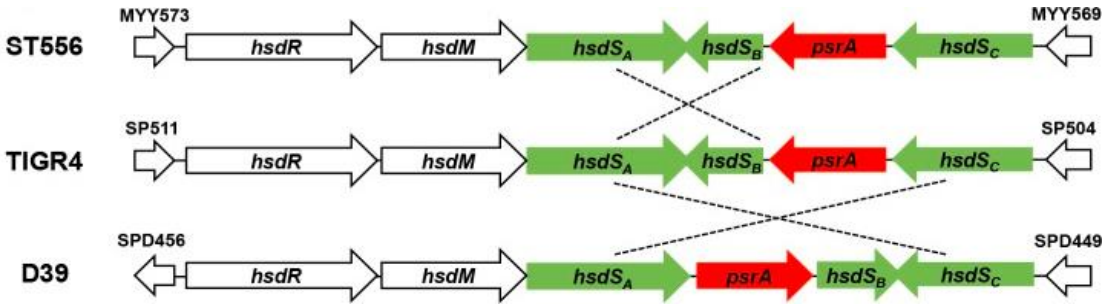
FIGURES AND TABLES



A



B

https://pubmed.ncbi.nlm.nih.gov/27427949/

Fig 1. Genetic arrangement in the Spn556II locus. The methylated bases are highlighted with red characters (A). The gene order of the three pneumococcal strains (ST556, TIGR4 and D39) are displayed in (B).

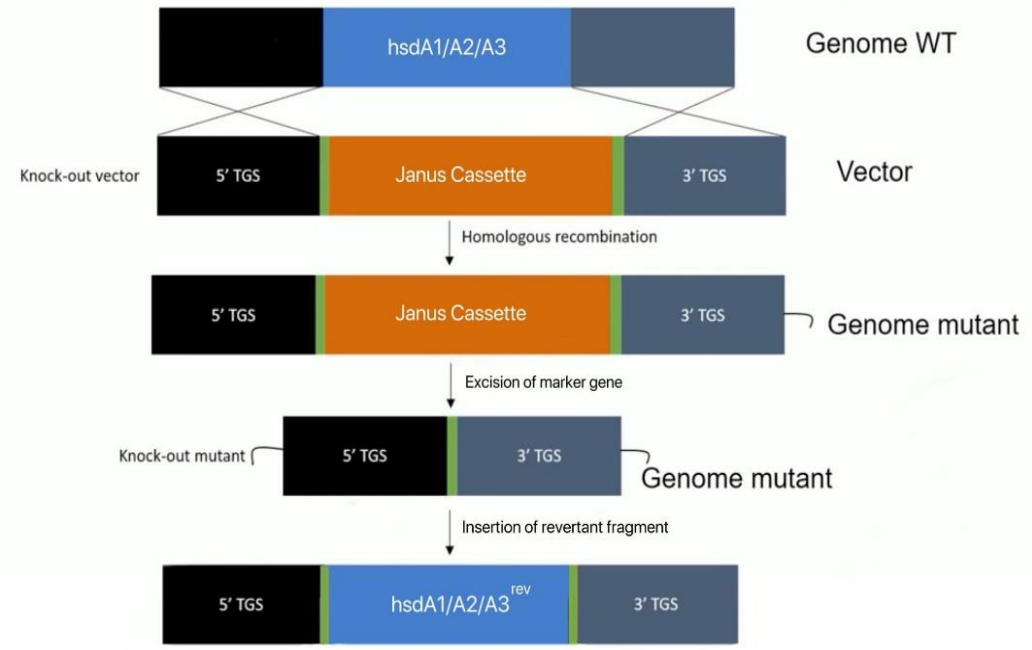
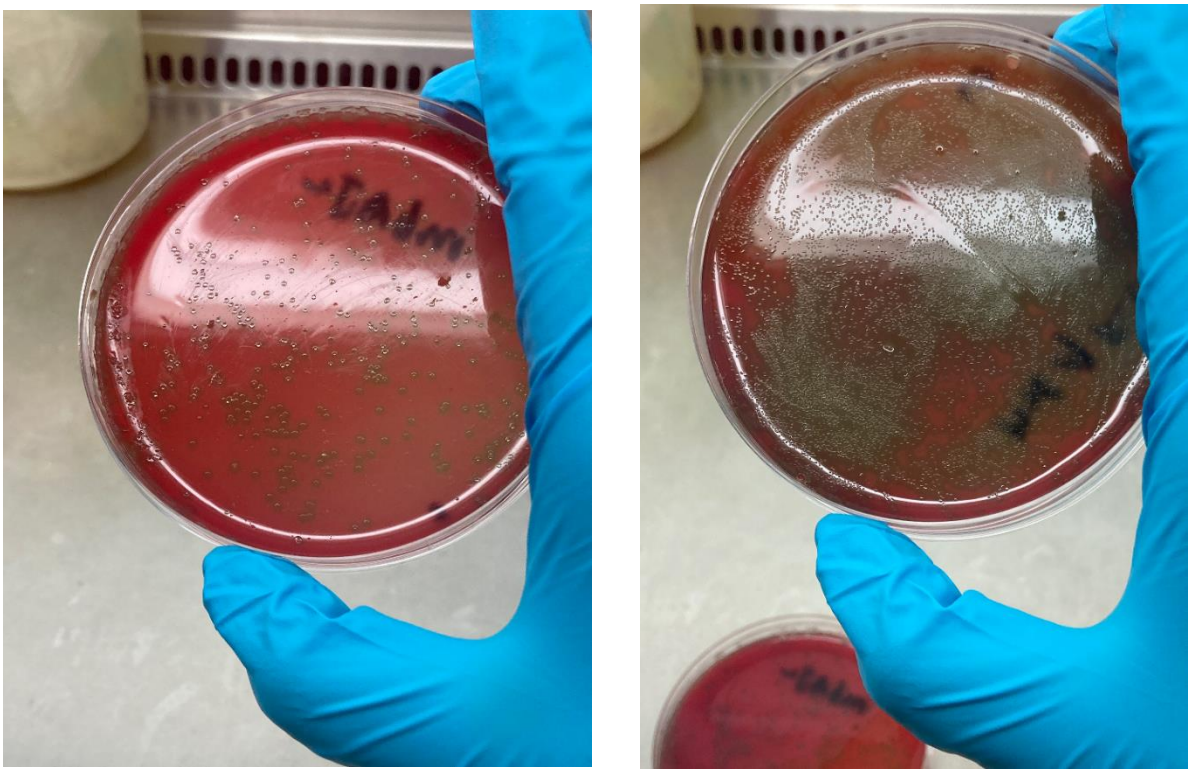


Fig. 2 Diagram of the Mutant Construction. The knock-out and revertant mutants were constructed through the insertion and excision of Janus cassette using homologous recombination.



A B

Fig 3. JC replacement strain on streptomycin plate (A) and revertant strain on streptomycin plate (B). The reverent strain grew large amounts of false-positive colonies due to spontaneous mutation.

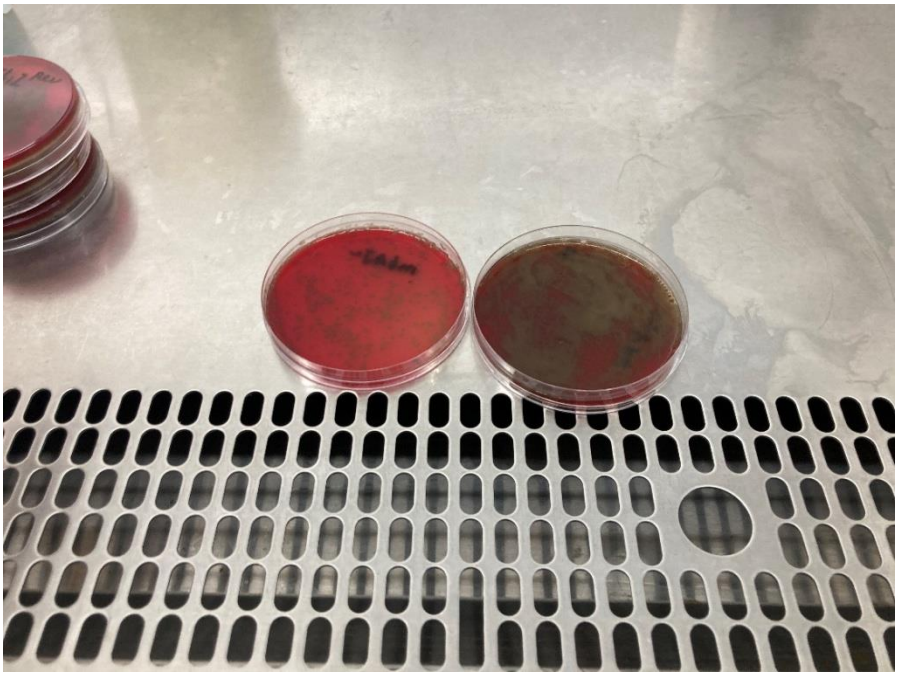
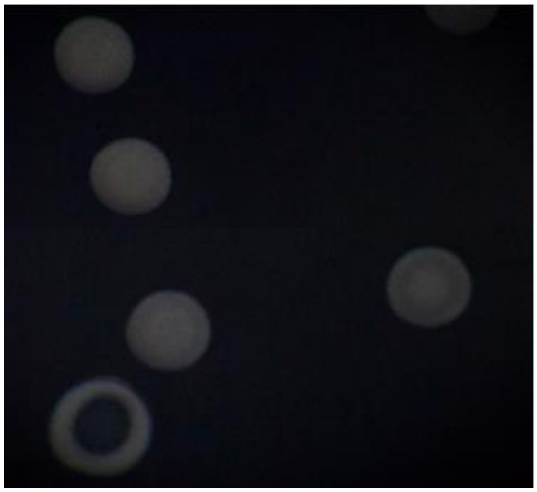
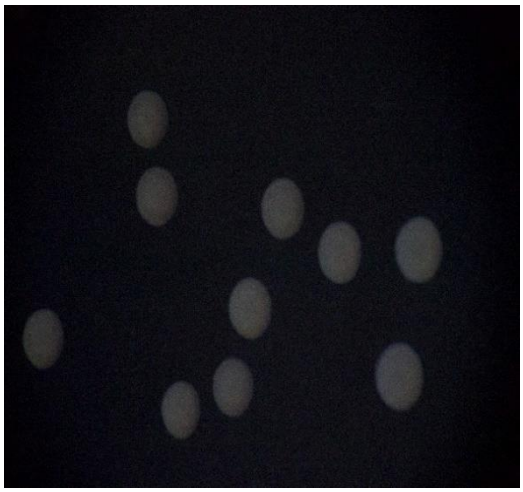


Fig 4. JC replacement strain VS. revertant strain on streptomycin plates. The number of colonies of the revertant strain was significantly greater than that of the replacement strain.



A



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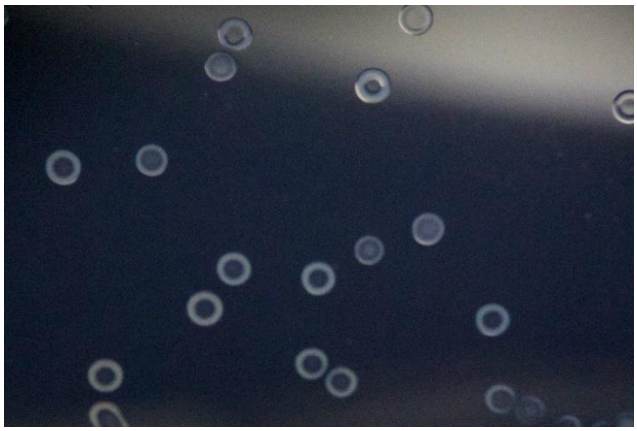

C

D

Fig 5. Comparison of wild-type phenotype (A), A1 phenotype (B), A2 phenotype (C) and A3 phenotype (D). The wild-type strain exhibited both opaque and transparent phenotypes. The A1 phenotype was entirely opaque. The A2 and A3 phenotypes were entirely transparent.

Table 1. Fragment preparation: mutant

Strain	Fragment	Primer	Template	Fusion	Receipt
Δ0620-JC	Up	Δ0620-UF/0620-JC-UR	556	Δ0620-UF/Δ0620-DR	556
	JC	0620-JCF/0620-JCR			
	Down	0620-JC-DF/Δ0620-DR	556		
Δ0620	Up	Δ0620-UF/Δ0620-UR	556	Δ0620-UF/Δ0620-DR	Δ0620-JC
	Down	Δ0620-DF/Δ0620-DR	556		
Δm6A-JCMYY0450	Up	Δm6A-UF/m6A-JC-UR	556	Δm6A-UF/Δm6A-DR	556
	JC	m6A-JCF/m6A-JCR			
	Down	m6A-JC-DF/Δm6A-DR	556		
Δm6A	Up	Δm6A-UF/Δm6A-UR	556	Δm6A-UF/Δm6A-DR	Δm6A-JC
	Down	Δm6A-DF/Δm6A-DR	556		
ΔThil-JCMYY1318	Up	ΔThil-UF/ΔThil-JC-UR	556	ΔThil-UF/ΔThil-DR	556
	JC	Thil-JCF/Thil-JCR			
	Down	ΔThil-JC-DF/ΔThil-DR	556		
ΔThil	Up	ΔThil-UF/ΔThil-UR	556	ΔThil-UF/ΔThil-DR	ΔThil-JC
	Down	ΔThil-DF/ΔThil-DR	556		
ΔCshA-JCMYY1516	Up	ΔCshA-UF/ΔCshA-JC-UR	556	ΔCshA-UF/ΔCshA-DR	556
	JC	CshA-JCF/CshA-JCR			
	Down	ΔCshA-JC-DF/ΔCshA-DR	556		
ΔCshA	Up	ΔCshA-UF/ΔCshA-UR	556	ΔCshA-UF/ΔCshA-DR	ΔCshA-JC
	Down	ΔCshA-DF/ΔCshA-DR	556		
Δ1259-JC	Up	Δ1259-UF/Δ1259-JC-UR	556	Δ1259-UF/Δ1259-DR	556
	JC	1259-JCF/1259-JCR			
	Down	Δ1259-JC-DF/Δ1259-DR	556		
Δ1259	Up	Δ1259-UF/Δ1259-UR	556	Δ1259-UF/Δ1259-DR	Δ1259-JC
	Down	Δ1259-DF/Δ1259-DR	556		

Table 2. Fragment preparation: revertants

Strain	Fragment	Primer	Template	Receipt
Δ0620 rev	Up	Δ0620-UF/Δ0620-DR	556	Δ0620-JC
	Down		556	
Δm6A	Up	Δm6A-UF/Δm6A-DR	556	Δm6A-JC
	Down		556	
ΔThil	Up	ΔThil-UF/ΔThil-DR	556	ΔThil-JC
	Down		556	
ΔCshA	Up	ΔCshA-UF/ΔCshA-DR	556	ΔCshA-JC
	Down		556	
Δ1259	Up	Δ1259-UF/Δ1259-DR	556	Δ1259-JC
	Down		556	