ROLE OF PROPHAGES IN *Staphylococcus aureus* VIRULENCE AND PATHOGENICITY

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ABSTRACT

Prophage, a temperate phage embedded in a bacterium's genome, affect bacterial fitness in multiple ways, including infectivity, toxin secretion, virulence regulation, surface modification, immune evasion, and microbiome competition. Studies have revealed that prophages significantly impact the distribution and spread of virulence and antimicrobial resistance. Lysogenic conversion by prophages introduces novel accessory functions to bacteria, enhancing bacterial fitness, host adaptation and persistence in different econiches. Prophages can also be triggered by stress conditions, such as exposure to ultraviolet (UV) radiation, antibiotics, or other chemical agents. Antibiotic-mediated prophage induction is known to cause a high frequency of prophage mobilization, implying that certain clinical strains carrying virulent prophages may have unintended consequences from antibiotics. For example, the hlb-converting prophage (also known as φSa3int) of *Staphylococcus aureus* encodes exotoxins and immune modulatory molecules that can inhibit human innate immunity, increasing the bacterium's pathogenicity. This property contributes to chronic infections and inflammation, such as chronic rhinosinusitis (CRS). Moreover, sub-lethal concentrations of fluoroquinolones, trimethoprim, and β-lactams are known to trigger prophage induction in *S. aureus*, accelerating the dissemination of prophage-encoded virulence factors to avirulent strains.

In this study, our first goal was to identify and describe prophages found in *S. aureus* isolates recovered from CRS patients and to examine their relationship to CRS disease phenotype and severity. We also aimed to determine whether these prophages could produce active reinfecting phage particles under sub-lethal concentrations of commonly used antibiotics and steroids. Furthermore, we explored links between inducible and non-inducible prophage in *S. aureus* and factors such as biofilm formation, metabolic activity, and CRS disease severity.

Finally, we investigated the genomic and phenotypic plasticity of *S. aureus* and changes in its extracellular proteome following the acquisition of the φSa3int prophage, which was one of the most frequently found prophages in CRS with nasal polyp (CRSwNP) patients.

To achieve our goals, we first used various computational tools to identify prophage regions in *S. aureus* genomes (N = 66) primarily isolated from CRS patients' sinonasal cavities. We then detected virulence and antibiotic resistance genes within the prophage regions of these bacteria. To measure the disease severity of CRS patients, we used computed tomography Lund Mackay scores. We determined antibiotic resistance patterns using the broth microdilution method and identified the minimum inhibitory concentration (MIC). Using sub-MIC concentrations of antibiotics and steroids, we induced the prophages and assessed their infectivity, biofilm biomass and metabolic activity in relation to prophage inducibility. Furthermore, we observed the beta-hemolysis activity of the isolates on sheep blood agar to understand its prevalence in human-adapted *S. aureus.* Moving forward, we then, induced a φSa3int prophage from one of the high biofilm-forming isolates (SA333) and transduced it into another Sa3int-prophage-free *S. aureus* (SA222, relatively low biofilm forming) isolate to obtain a laboratory-generated 'lysogen'. We confirmed the successful integration of φSa3int prophage into *hlb*-gene and stable lysogenic conversion by short- and long-read sequencing. We then compared the growth kinetics, biofilm biomass, and metabolic activity between the parent and the laboratory-generated lysogen by establishing growth curves, crystal violet and resazurin assays. Finally, we identified and quantified exoproteins secreted by parent strains and lysogens using mass spectrophotometry to understand the virulence factors encoded and secreted by φSa3int prophage carrying *S. aureus*.

All *S. aureus* clinical isolates obtained from CRS patients (N = 57) and control patients (N = 9) carried at least one prophage (average = 3.6 prophages/isolate), with prophages contributing up to 7.7% of the bacterial genome. Based on the completeness scores, we found that nearly 85% (56/66) of *S. aureus* clinical isolates had at least one intact prophage that were likely inducible. Prophages belonging to type 3 integrase (φSa3int-type) were the most prevalent (40%), followed by φSa2int (14%). The prophages harbored a distinct set of virulence genes: φSa3int-group often encoded human immune evasion cluster genes like *sak, scn, chp,* and *sea*, while φSa2int-group often harbored leukocidins like *luk*E/D. Intact prophages were more frequently found in *S. aureus* isolated from CRS with nasal polyp (CRSwNP) patients than in CRS without nasal polyp (CRSsNP) patients (p = 0.0021). Similarly, intact prophages belonging to the φSa3int-group were more frequent in CRSwNP than in CRSsNP (p = 0.0008). Spontaneous prophage induction (SPI) was observed in around 26% (17/66) of the *S. aureus* clinical isolates, while mitomycin C dependent induction was observed in almost 52% (34/66) of the clinical isolates. Most of the *S. aureus* clinical isolates showing prophage induction harbored at least one intact prophage(s).

Exposure of exponentially growing bacteria to sub-inhibitory concentrations of antibiotics enhanced the prophage induction compared to SPI in almost 50% of active lysogens. Among antibiotics tested, ciprofloxacin was the most potent prophage inducer inducing prophages from 51% of the isolates, followed by amoxicillin, doxycycline, mupirocin, clindamycin, and azithromycin, all of which enhanced the release of prophage in > 40% of the isolates. There was no correlation between *S. aureus* harboring active prophages and inactive prophages with their biofilm biomass and metabolic activity. However, the disease severity score of patients harboring inducible prophage within *S. aureus* was significantly lower, implying the role of

active lysogeny in CRS disease. In addition, beta-hemolysin activity was absent in almost 92% of *S. aureus* isolated from the sinonasal cavities of chronic rhinosinusitis patients.

Integration of a ~43.8 kb φSa3int prophage in one of the hemolysin-producing clinical isolates (SA222) down-regulated the beta-hemolysin expression, implying the role of Sa3int-type prophages in the disruption of beta-hemolysin production. There was no change in bacterial growth kinetics, biofilm formation, adhesion to primary human nasal epithelial cells, and the metabolic activity in a biofilm after φSa3int prophage integration. However, the acquisition of φSa3int prophage significantly altered the expression of various secreted proteins, both bacterial and prophage encoded. Altogether, thirty-eight exoproteins were significantly differentially regulated in the laboratory-generated lysogen, compared to its recipient strain SA222. Among these proteins, there was significant upregulation of 21 exoproteins (55.3%), including staphylokinase (sak), SCIN (scn), and intercellular adhesion protein B (icaB), and downregulation of 17 exoproteins (44.7%), including beta-hemolysin (hlb/sph) and outer membrane porin (phoE). Most of the upregulated proteins are known for human immunomodulation which helps *S. aureus* escape human innate immunity and cause chronic infection.

In summary, our research has expanded the understanding of prophage distribution in *S. aureus* among patients with chronic rhinosinusitis (CRS) and their potential impact on the development of the disease. We identified diverse types of prophages in *S. aureus* within a limited geographic area and among a specific population suffering from CRS. This suggests the presence of a range of prophages that contribute to the adaptability and virulence of the bacteria. Our findings also shed light on the prevalence of active lysogeny in clinical *S. aureus* isolates and the impact of commonly used antibiotics on prophage mobilization. This can

affect both virulence and the spread of antimicrobial resistance. Therefore, our research underscores the importance of minimizing the unnecessary use of antibiotics and the potential hazards associated with exposure of bacteria to sub-lethal antibiotics. Such exposure can promote not only antimicrobial resistance but also accelerates the development of virulent strains. Finally, we also caution against poly-lysogeny, which can worsen the pathogenicity of an isolate through an accumulation of auxiliary phage-encoded traits.

Keywords:

prophage, Sa3int prophage, Sa2int prophage, lysogenic conversion, chronic rhinosinusitis, hlb-converting phages, *Staphylococcus aureus*, virulence factors

DECLARATION

I, Roshan Nepal, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University *of* Adelaide.

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Roshan Nepal

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PUBLICATIONS DURING THE CANDIDATURE

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1. **Nepal R**, Houtak G, Shaghayegh G, Bouras G, Shearwin K, Psaltis AJ, Wormald PJ, Vreugde S. (2021). Prophages encoding human immune evasion cluster genes are enriched in *Staphylococcus aureus* isolated from chronic rhinosinusitis patients with nasal polyps. *Microbial Genomics*, 7(12): 000726. https://doi.org/10.1099/mgen.0.000726

2. **Nepal R**, Houtak G, Wormald PJ, Psaltis AJ, Vreugde S. (2022). Prophage: a crucial catalyst in infectious disease modulation. *The Lancet Microbe,* 3 (3), e162-e163. https://doi.org/10.1016/S2666-5247(21)00354-2

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10. Houtak G, Bouras G, **Nepal R,** Shaghayegh G, Cooksley C, Psaltis A, Wormald P, Vreugde S. (2023). The intra-host evolutionary landscape and patho-adaptation of persistent *Staphylococcus aureus* in chronic rhinosinusitis. *bioRxiv.* http://doi:10.1101/2023.03.28.534496 (preprint)

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PRESENTATIONS ARISING FROM THIS THESIS

National conferences/seminars

1.	30th The Queen Elizabeth Hospital Research Expo (TQEH Expo 2020), October 15-16, 2020 | Adelaide, SA, Australia	Oral
2.	31st The Queen Elizabeth Hospital Research Expo (TQEH Expo 2021), October 14-15, 2020 | Adelaide, SA, Australia	Oral
3.	Adelaide Immunology Retreat (AIR-16), 18-19 October 2021 | Adelaide, SA, Australia	Oral
4.	Adelaide Immunology Retreat (AIR-17), 19-20 September 2022 | Adelaide, SA, Australia	Oral
5.	32nd The Queen Elizabeth Hospital Research Expo (TQEH Expo 2022), October 15-16, 2020 | Adelaide, SA, Australia	Oral
6.	50th Annual Scientific Meeting of the Australian and New Zealand Society for Immunology (ASI2022), 29 Nov-02 Dec 2022 | Melbourne, VIC, Australia	Poster

International conferences/seminars

7.	32nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID2022), 23-26 April 2022 | Lisbon, Portugal	Poster
8.	Global Rhinology Network: Topical Research Symposium, 6 December 2022 | virtual	Oral
9	Microbiology Society Annual Conference, 17-20 April 2023 | Birmingham, UK	Poster

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3. **AMS/Biomedicine Publication Award - October 2021** | AMS/Biomedicine Research Committee, Adelaide Medical School, The University of Adelaide

4. **ESCMID Postgraduate Education Course Award for Pre-ECCMID Postgraduate Course on Antimicrobial Stewardship** (21-22 April 2022). Carcavelos, Portugal

5. **ECCMID 2022 Outreach Grant, Bill and Melinda Gates Foundation** | 32nd ECCMID (23-26 April 2022) | Lisbon, Portugal

6. **THRF/BHI Travel Award** for 32nd ECCMID (23-26 April 2022). Lisbon, Portugal

7. **FEMS Meeting Attendance Grant** for 50th Annual Scientific Meeting of the Australian and New Zealand Society for Immunology – ASI2022 (29 Nov-02 Dec 2022) Melbourne, VIC, Australia

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ABBREVIATIONS

Abi	: Abortive Infection
BREX	: Bacteriophage Exclusion
CRISPR/Cas	: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated
CRS	: Chronic Rhinosinusitis
CRSsNP	: Chronic Rhinosinusitis sine (without) Nasal Polyposis
CRSwNP	: Chronic Rhinosinusitis with Nasal Polyposis
DISARM	: Defense Island System Associated with Restriction-Modification
DNA	: Deoxyribonucleic Acid
GEPARD	: GEnome PAir - Rapid Dotter
HGT	: Horizontal Gene Transfer
IS	: Insertion Sequence
LMK	: Lund MacKay Score (CRS disease severity score)
MAFFT	: Multiple Alignment using Fast Fourier Transform
MGEs	: Mobile Genetic Elements
MLST	: Multilocus Sequence Typing
MMC	: Mitomycin C
MRSA	: Methicillin-resistant *Staphylococcus aureus*
MSSA	: Methicillin-sensitive *Staphylococcus aureus*
NCBI	: National Center for Biotechnology Information
PHASTER	: PHAge Search Tool – Enhanced Release
PHASTEST	: PHAge Search Tool with Enhanced Sequence Translation
SaPIs	: Staphylococcal Pathogenicity Islands
SCC	: Staphylococcal Cassette Chromosome
SPI	: Spontaneous Prophage Induction
SI	: Superinfection Immunity
Tn	: Transposons
VGT	: Vertical Gene Transfer
VRSA	: Vancomycin-resistant *Staphylococcus aureus*

GLOSSARY

Active lysogen	:	bacterial strain harboring identifiable prophage sequence and releasing reinfecting phage particles.
Incomplete prophage	:	a prophage DNA with a low completeness score (<90) as identified by the PHASTER web tool, irrespective of its inducibility
Intact prophage	:	a prophage DNA with a high completeness score (>90) as identified by the PHASTER web tool, irrespective of its inducibility
Lysogen	:	bacterial cell containing one or more prophages within its genome.
Lysogenic conversion	:	change in the properties of a bacterial host cell resulting from the stable integration of a prophage.
Lytic phage	:	bacterial virus, which, upon entering its host, produces offspring and lyses the bacterial cell to release its progeny.
Non-lysogen	:	bacterial strains lacking any identifiable prophage sequence in their genome
Passive lysogen	:	bacterial strains harboring identifiable prophage sequence but not releasing actively reinfecting phage particles
Poly-lysogen	:	a bacterium infected or lysogenized by more than one prophage
Prophage	:	temperate phage DNA that is stably integrated into the bacterial genome.
Sa2int prophage	:	a prophage having type 2 integrase often carrying Panton-Valentine leukocidins (PVL genes)
Sa3int prophage	:	a prophage having type 3 integrase often carrying human immune evasion cluster (IEC) genes
Temperate phage	:	bacterial virus that can integrate into a bacterial genome (or be maintained extra-chromosomally), become stabilized in this way, and, upon receiving a cue, can excise and propagate as a phage particle.
Transduction	:	process of horizontal gene transfer, wherein a region of a bacterial genome is packaged into phage particles that, upon release and entrance into a new host, is inserted into the genome of the latter.
Virulent phage	:	an obligate lytic phage that causes a productive infection and lysis of the host bacterium.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Chronic rhinosinusitis (CRS) is a complex multifactorial heterogeneous disease of the sinuses and is characterised by inflammation of the mucosa of the nasal cavity and paranasal sinuses. Chronic rhinosinusitis occurs in > 10% of adults in Europe and the USA and poses a substantial global health and economic burden (Lee and Lane, 2011). It can significantly impact the quality of life (QoL), affecting sleep, exercise, and other daily-life activities. Intranasal antibiotics and steroids treatments are effective for most people but do not guarantee the cure and need to be used correctly and regularly. Although etiopathogenesis of the CRS condition remains elusive, major external factors implicated in the development of CRS include superantigens (SAgs), abnormal inflammatory cytokine cascade, abnormal cell-mediated immune response and the existence of microbial biofilms (Maina et al., 2018).

Traditionally and for clinical purposes, CRS phenotypes are differentiated into a disease sine (without) nasal polyps (CRSsNP) and a disease with nasal polyps (CRSwNP) based on nasal endoscopic findings (Fokkens et al., 2012). Both CRSsNP and CRSwNP have distinct symptoms, CT findings, treatments, and risks of recurrence and comorbid asthma (Laidlaw et al., 2021). Inflammation in both CRS types displays a cellular infiltrate of neutrophils, macrophages, and lymphocytes and numerous proinflammatory cytokines associated with helper T-cell inflammation (Lee and Lane, 2011). In addition, both of the CRS phenotypes are characterised by structural remodeling (with collagen and fibrin deposition) and inflammation, and have combinations of type 1, 3 and/or type 2 inflammatory signatures that vary between diseases (Akdis et al., 2013).

Lately, greater emphasis is being placed upon inflammation rather than infection because inflammatory signatures determine the CRS endotype, which affects disease severity, comorbidity, prognosis and also a response to treatment (De Greve et al., 2017; Kim and Cho, 2017; Staudacher et al., 2020). Although not universal, CRSsNP is usually characterised by a T-helper type 1 (Th1)-mediated inflammation, while CRSwNP has a greater propensity for a T-helper type 2 (Th2)-mediated immune response (Maina et al., 2018). These two endotypes may require different pharmacological and, eventually, biological treatments in addition to different surgical approaches.

Although the etiopathogenesis of CRS is likely influenced by multiple genetic and environmental factors, *Staphylococcus aureus,* a Gram-positive biofilm-forming opportunistic pathogen, is often isolated from CRS patients, more often in CRSwNP compared to CRSsNP, and is regarded as one of the disease modifiers that impacts the outcome of CRS severity and post-operative recovery (Kim and Cho, 2017). Further, microbiome, as well as culture-based studies in CRS, have supported the notion that microbial dysbiosis, particularly enrichment of *Staphylococcus aureus* and its persistent colonisation, plays a cardinal role in exacerbation, pathophysiology and recovery of CRS (Drilling et al., 2014; Psaltis et al., 2022; Rom et al., 2019). Despite prolonged antibiotic treatment, persistent colonisation of *S. aureus* is a common manifestation of CRS and *S. aureus* superantigens (SAgs), and virulence factors like pore-forming toxins, phenol-soluble modulins and exfoliative toxins are known potent inflammatory agents resulting in an inflammatory cascade (Chen et al., 2022). Clinically, CRSwNP has greater morbidity than CRSsNP owing to greater disease severity and a higher number of surgeries or medication exposure. Further, in CRSwNP, *S. aureus* is usually enriched that has been shown to bind to toll-like receptor-2 (TLR-2), leading to an increase in type 2 cytokine

production. Further, *S. aureus* enterotoxin (SE) amplified the type 2 reaction acting as a superantigen leading to SE-IgE production (Ahern and Cervin, 2019). However, the role of *S. aureus* on CRS and subsequent symptom persistence appears conflicting because of the heterogeneity of the disease itself and the bacterium *S. aureus*. Although, we do not completely understand the aetiology of the CRSwNP, a relatively more severe form of CRS, chronic colonisation by a virulent strain of *S. aureus* is often suspected to play a role in exacerbating CRS disease and its recovery leading to CRSwNP.

Recently, prophage-mediated pathogenicity has been linked with various chronic diseases like cystic fibrosis (CF), inflammatory bowel diseases (IBD) and overall gut health (Davies et al., 2016; Fortier and Sekulovic, 2013). Also, the release of filamentous phage (Pf) from a lysogenic *Pseudomonas aeruginosa* is known to induce an antiviral immunity preventing bacterial phagocytosis and delaying wound healing resulting in a chronic infection (Bach et al., 2022; Burgener et al., 2019). Whilst *S. aureus* is known to play a role in CRS pathophysiology, there is not enough evidence on which factors in bacteria, such as biofilm, metabolic activity, virulence, or toxins, exactly contribute to the inflammation and chronicity of CRS. Thus, it is important to determine whether the presence of *S. aureus* encoding specific virulence factors, toxins and/or mobile genetic elements (MGEs) contributes to the CRS exacerbations or can be a biomarker for CRS exacerbation risk. There is emerging evidence that bacteria infected by a phage (a lysogen) is more pathogenic to humans, particularly in chronic diseases, as it provides bacteria survival fitness such as immune evasion, biofilm formation, and toxin secretion (Busby et al., 2013; Hacker and Carniel, 2001). As such, it is important to study how the aforementioned factors contribute to bacterial pathogenicity and its contribution to CRS pathophysiology.

In line with the latest discoveries, we found that more severe chronic rhinosinusitis patients often harbored *S. aureus* with specific prophage DNA encoding human immune evasion cluster (IEC) genes that help bacteria evade the human immune system and persist (Nepal et al., 2021). Further, domestication of prophage by *S. aureus* is associated with elevated biofilm, a gain of antibiotic resistance, toxins and virulence factors leading to higher pathogenicity (Fernandez et al., 2018). It is becoming more evident that bacterial virulence is combinatorial and involves both core genome and flexible MGEs that increase competitiveness/fitness in an unfavorable niche. However, as prophages are extremely mobile, transient and mosaic, their role in pathogenesis and disease epidemiology is largely underestimated in clinical settings. Understanding prophage biology, the underlying mechanisms that promote lysogenic conversion and identifying the key toxins responsible for heightened pathogenicity would not only be cardinal in understanding the impact of *S. aureus* in CRS but would also advance our knowledge in elucidating other prophage-encoded bacterial pathogenesis and implication of lysogenization in disease progression.

In this research, we study the prevalence of lysogenic S. *aureus* in CRS disease, focusing on φSa3int prophages that encode human immune evasion cluster (IEC) genes. We further investigate if sub-lethal concentrations of antibiotics often used in CRS treatment have any impact on prophage activation. Finally, we conclude the study by investigating the role of φSa3int prophage by transducing it into a Sa3int-prophage-free bacteria isolated from the same patient and quantifying the virulence in the secretome of the bacteria.

Our findings, which align with the latest discoveries on prophage-encoded virulence, open a novel unexplored area for future investigations in prophage-mediated pathophysiology in chronic disease. This will help uncover tripartite associations between prophage-bacteria- and human that ultimately impacts human health. These findings collectively reinforce researchers and clinicians to consider prophage-encoded virulence and its consequences in bacterial infection epidemiology and the development of diagnostic tools to accurately predict mobile genetic elements of a bacteria that alter the pathogenicity of the bacteria.

1.2 AIMS AND HYPOTHESIS

In this research, we aim to achieve the following goals and expand our understanding of the contribution of prophage in *S. aureus* host adaptation, virulence and pathogenicity in relation to CRS disease.

Aim 1 | Characterization of prophage in *S. aureus* isolated from chronic rhinosinusitis patients

Objectives:
a. Screen prophages in the chromosome of *S. aureus* isolated from CRS patients,
b. Genomic profiling of identified prophages,
c. Identification of prophage-encoded virulence and antimicrobial-resistance genes.

Rationale: Understand the impact of prophage-associated virulence on CRS severity.

Aim 2 | Study antibiotic-dependent prophage induction and assess released phage's re-infectivity

Objectives:
a. Identify inducible/active prophages,
b. Assess antibiotic-dependent inducibility of prophages,
c. Correlate biofilm formation with prophage status and types.

Rationale: Understand prophage behaviours in presence of sub-lethal drugs.

Aim 3 | Prophage mobilization and its implications in bacterial virulence

Objectives:
a. Construct a lysogen by transducing prophage from one bacterium into another,
b. Compare genotypic and phenotypic characteristics between a genetically modified lysogen and a parent strain,
c. Identify prophage-encoded virulence and its expression.

Rationale: Understand the impact of prophage (φSa3int) integration in *S. aureus* virulence modulation.

Hypothesis

Prophages have a significant impact on host adaptation, virulence and pathogenicity of *Staphylococcus aureus* thereby modulating chronic rhinosinusitis pathophysiology.

1.3 FORMAT AND OUTLINE OF THIS THESIS

This thesis is formatted as a thesis by publication and is composed of six chapters that elucidate the original research undertaken by **Mr. Roshan Nepal** during his higher degree by research (PhD in Medicine) candidature at The University *of* Adelaide. One of the result chapters (Chapter 3) and a commentary synthesis on the literature review (Chapter 2) included in this thesis are already published in highly esteemed peer-reviewed journals in the field of microbial genomics and clinical microbiology: *Microbial Genomics*, *The Lancet Microbe*. Chapter 4 from the result section is written in a manuscript style and will shortly be submitted to an appropriate journal for publication. Chapter 5 from the result section is also written in a manuscript style and is published as a preprint on bioRxiv.

Briefly, this thesis deals with estimating prophages (phage DNA) in *Staphylococcus aureus* bacteria isolated from chronic rhinosinusitis (CRS) and control patients. The study further investigates the impact of sub-lethal concentrations of antibiotics on prophage activation and its impact on biofilm formation. Finally, the study explores the role of φSa3int prophage on *S. aureus* virulence and pathogenicity by transducing the φSa3int prophage into a φSa3int-prophage-free strain of *S. aureus*.

CHAPTER 1 | This chapter introduces the thesis and outlines the background of the research, its core arguments, rationale, aims, hypotheses, and methodology overview. It briefly gives an overview of the concepts that underpin the subsequent chapters. It is subdivided into four sections. The first section deals with chronic rhinosinusitis and the implications of S*. aureus* in CRS. It further discusses prophage biology in brief and identifies the research gap. The second section deals with the aims and hypothesis of

the research. The third section gives an outline and format of this thesis, and the fourth section briefly outlines the methodology implemented in this study.

CHAPTER 2 | This chapter is a comprehensive review of the literature and evidence available on prophage-encoded virulence and its impact on bacterial disease. It further explores the impact of *S. aureus* on the pathophysiology of CRS disease. The chapter reviews the literature on chronic rhinosinusitis, the role of *S. aureus* in CRS pathophysiology, the diversity of prophage elements in *S. aureus* and its contribution to bacterial pathogenesis. It also includes a comprehensive review of studies on prophages of *S. aureus*, particularly the φSa3int, known to encode a cluster of genes responsible for human immune evasion. The section concludes with a published peer-reviewed commentary article entitled "Prophage: a crucial catalyst in infectious disease modulation" discussing the synthesis of the reviewed literature.

CHAPTER 3 | This constitutes a published peer-reviewed original research article entitled "Prophages encoding human immune evasion cluster genes are enriched in *Staphylococcus aureus* isolated from chronic rhinosinusitis patients with nasal polyps." This study reports a higher prevalence of intact (or complete) prophages and prophage-encoded immune evasion cluster (IEC) genes in *S. aureus* isolated from severe chronic rhinosinusitis patients with polyps. Building upon the findings, we further proposed that prophage-encoded genes may equip bacteria with human immune-evading functionalities that play a crucial role in the modulation of chronic diseases like chronic rhinosinusitis.

CHAPTER 4 | This chapter is a research manuscript entitled "Sub-inhibitory antibiotics enhance prophage induction and release of infective phage in patient-derived

Staphylococcus aureus." The study elaborates on the impact of different antibiotics and steroids in prophage induction and their release as infective transducing phage particles. We also study the role of temperature in prophage induction. The chapter further explores the difference in biofilm development and metabolic activity of bacteria in relation to prophage status and behavior.

CHAPTER 5 | This chapter is a manuscript entitled "Prophage acquisition by *Staphylococcus aureus* contributes to the expansion of Staphylococcal immune evasion" available in a public database (bioRxiv) as a preprint. This study discusses the genotypic and phenotypic implications of φSa3int prophage in the *S. aureus* clinical strain by modifying a φSa3int-free clinical strain by inserting φSa3int prophage. The section details the change in bacterial growth kinetics, biofilm formation, metabolic activity, and extracellular toxin secretion in a clinical strain after lysogenization by a temperate Sa3int group (pro)phage isolated and purified from different clinical isolates collected from a severe CRS patient.

CHAPTER 6 | This section concludes the thesis and reflects upon the findings. It further discusses the future directions in *S. aureus* prophage research and implications of lysogenic conversion in bacterial disease modulation.

1.4 METHODOLOGY OUTLINE

In this study, we employed *in silico* as well as *in vitro* approaches to justify our key research questions. In brief, we first employed descriptive bioinformatical approaches to study the hypothesis. We then implemented various experimental approaches to justify it. To achieve our goal, we first retrospectively analysed the whole genomes of *S. aureus* (N = 66) isolated from chronic rhinosinusitis and control patients to identify prophage sequences using a web-based bioinformatics tool called PHASTER. We then characterized the prophage genome using various bioinformatics tools like VFDB, CARD, MAFTT in Geneious Prime to correlate the prophage-encoded virulence genes with CRS disease phenotype and severity. We found that *S. aureus* collected from CRS patients with nasal polyps have a significantly higher abundance of intact prophages in their genome. Further analysis showed that most of the prophages identified in those isolates belonged to a specific group of prophages called 'Sa3int or beta-converting phage' known to encode human immune evasion cluster genes.

Secondly, we confirmed the inducibility and re-infectivity of the intact prophages using mitomycin C (MitC) as an inducing agent. We then stratified the clinical isolates based on the inducibility of the prophage they harboured (isolate with active/inducible prophage vs isolate with inactive/non-inducible prophage and studied all the clinical isolates' biofilm biomass and metabolic activity. We further used sub-lethal concentrations of various antibiotics (amoxicillin, azithromycin, ciprofloxacin, clindamycin, doxycycline, mupirocin, rifampicin) to activate the prophage. We found that sub-lethal antibiotic concentration significantly induces prophages from the *S. aureus* strains harboring 'intact' prophages. Although the prophage abundance and prophage type were correlated with CRS phenotype and severity, the prophage

inducibility was not correlated with CRS phenotype or CRS disease severity. Further, the prophage inducibility was also not correlated with biofilm formation and metabolic activity, implying prophages have minimal impact on the growth kinetics of the host bacteria.

Finally, we studied the impact of φSa3int prophage by genetically incorporating a φSa3int prophage into a genetically close Sa3int-prophage-free *S. aureus* and analyzing its protein expression. For this, we first purified an induced φSa3int prophage and inserted it into another *S. aureus* strain. We confirmed the successful integration of φSa3int prophage by culture method as well as sequencing the whole genome. We then studied the growth kinetics, metabolic activity and biofilm formation of genetically modified lysogen. Further, we collected the secretome of lysogen grown in optimal conditions and identified the additional secreted proteins by gas chromatography-mass spectrometry (GC-MS).

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CHAPTER TWO

LITERATURE REVIEW

"**prophage** (ˈprō-ˌfāj -ˌfäzh): an intracellular form of a bacteriophage (or phage) in which it is harmless to the host, is usually integrated into the hereditary material of the host, and reproduces when the host does" – Merriam-Webster Dictionary

2.1 RHINOSINUSITIS

Rhinosinusitis is an inflammation of the nasal cavity and the paranasal sinuses. There are four pairs of sinus cavities – frontal, maxillary, sphenoid and ethmoid through which clean air circulates before entering the lungs. These sinuses are covered by a muco-cilliary membrane that helps in cleansing, humidifying and thermoregulating the air that circulates in these sinuses (Gudis et al., 2012). The mucus filters and traps the particulate that passes through these sinuses, and the cilia, with their unidirectional movement, sweep the mucus towards the back of the nasal cavity to be swallowed and sanitized in the stomach acid (Fahy and Dickey, 2010). When this muco-ciliary cleansing is dysfunctional (infection, ciliary dysfunction, abnormal mucus) the cavity is blocked, and the membranes become inflamed. The inflammation may be caused by various intrinsic (genetic predisposition) as well as extrinsic (viral, bacterial, fungal, dust, pollen) factors (Van Crombruggen et al., 2011). It can be loosely classified into following four subsets:

a. **Acute rhinosinusitis (ARS):** Acute rhinosinusitis (also called acute sinusitis) is symptomatic inflammation of the nasal cavity and paranasal sinuses that lasts for less than 4 weeks (Desrosiers et al., 2011). The ARS symptoms include thick, yellow to green discharge from the nose, nasal congestion and facial pain, pressure or fullness. Symptoms can also include fever, fatigue, cough, reduced sense of taste and smell, ear pressure, headache, upper jaw or teeth ache, and bad breath (Sharp et al., 2007). In most cases, these symptoms develop over the course of one day and begin to improve in 7 to 10 days. Acute rhinosinusitis can further be classified into the following three spectrums (EPOS, 2020):

i. **Acute viral rhinosinusitis (AVRS):** Also known as the 'common cold', the most common cause of ARS is a viral infection associated with the upper respiratory tract, such as rhinovirus (common cold virus). The duration of symptoms is often < 10 days (but less than 12 weeks).

ii. **Acute post-viral rhinosinusitis:** Acute post-viral rhinosinusitis is defined by an increase of symptoms after five days or persistence of symptoms after ten days but less than 12 weeks duration.

iii. **Acute bacterial rhinosinusitis (ABRS):** Acute bacterial rhinosinusitis **is** defined by the presence of at least three of the following symptoms: discoloured mucus, severe local pain, fever > 38°C, raised CRP/ESR and 'double' sickening. Bacterial rhinosinusitis is less prevalent and occurs in only 0.5-2% of viral upper respiratory tract infections, usually due to complications of viral rhinosinusitis (Hoffmans et al., 2018). A viral or post-viral ARS generally precedes ABRS.

b. Sub-acute rhinosinusitis: Sub-acute rhinosinusitis is a continuum of acute rhinosinusitis but less than 12 weeks (> 4 weeks but < 12 weeks). There is a complete resolution of sub-acute rhinosinusitis after effective medical therapy.

c. Recurrent acute rhinosinusitis (RARS): Recurrent ARS is four or more episodes of acute rhinosinusitis per year with symptom-free intervals. Each episode must be at least seven days' duration and meet the criteria for acute post-viral (or bacterial) rhinosinusitis.

d. Chronic rhinosinusitis (CRS): Chronic rhinosinusitis is clinically defined as the presence of two or more cardinal symptoms, one of which should be either nasal

blockage/congestion or nasal discharge (anterior/posterior nasal drip) and ± facial pain/pressure or ± reduction or loss of smell for at least 12 consecutive weeks, in addition to objective evidence (e.g., mucopurulent drainage, edema, polyps in the middle meatus) (Fokkens et al., 2020). The objective evidence of CRS is obtained by either physical examination (anterior rhinoscopy, endoscopy) or sinus radiography (computed tomography). Common symptoms include facial pain or pressure, nasal discharge, congestion, and hyposmia or anosmia. Patients with CRS may also have other inflammatory airway conditions, such as asthma and allergic rhinitis.

2.2 CHRONIC RHINOSINUSITIS (CRS)

Chronic rhinosinusitis is a multifactorial disease that involves genetic predisposition, environmental factors and polymicrobial interactions in the nasal microenvironment (Foreman et al., 2010; Psaltis et al., 2022; Psaltis et al., 2008). The classification of CRS has progressed from phenotypic to endotype-based in recent years as this may be more appropriate in disease diagnosis and establishing the therapies (Kato et al., 2022; Staudacher et al., 2020).

2.2.1 Classification of chronic rhinosinusitis

Traditionally, according to The European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS2012), chronic rhinosinusitis phenotypes are broadly classified into two groups based on the presence or absence of nasal polyposis tissue (Fokkens et al., 2012) (Figure 2.1).

a. **CRS sine (without) nasal polyps (CRSsNP):** Chronic rhinosinusitis without nasal polyp (representing about 80% of the total CRS patients) has been attributed to mechanical obstruction of the ostiomeatal complex and is characterised by histologic abnormalities, including basement membrane thickening (fibrosis), and goblet cell hyperplasia (Cho et al., 2016; Leung et al., 2011; Van Bruaene et al., 2009). Traditionally, CRSsNP has been associated with a type 1 inflammatory profile. However, recent findings have suggested it to be a heterogeneous condition and is often referred to as non-type 2 (mainly a mix of type 1 and type 3) inflammation with a significant neutrophilic infiltration regulated by an elevated level of IL-6, IL-8, IL-17 and TNF-a (Ahern and Cervin, 2019; Delemarre et al., 2021).

b. **CRS with nasal polyps (CRSwNP):** Chronic rhinosinusitis with nasal polyp (representing about 20% of the total CRS patients) is regarded as a diffuse eosinophilic-based mucosal disease and is strongly skewed towards a type 2 inflammation in American and European patient cohorts (Kato, 2015). This view was enhanced by the often-observed eosinophil accumulation in nasal polyp tissue of CRSwNP patients and elevated type 2 cytokines (Hamilos et al., 1996; Kato, 2015; Van Zele et al., 2006). Nasal polyps are benign typically, bilateral inflammatory lesions originating from the ethmoid sinus projecting into the nasal airway. CRSwNP is frequently associated with asthma, allergic rhinitis, nonsteroidal anti-inflammatory drug-exacerbated respiratory disease, and chronic colonisation of *S. aureus* in the upper airway leading to biofilm formation, immune dysregulation,

inflammation, and barrier dysfunction, contributing to recalcitrant disease (Bendouah et al., 2006; Psaltis et al., 2008; Van Zele et al., 2004).

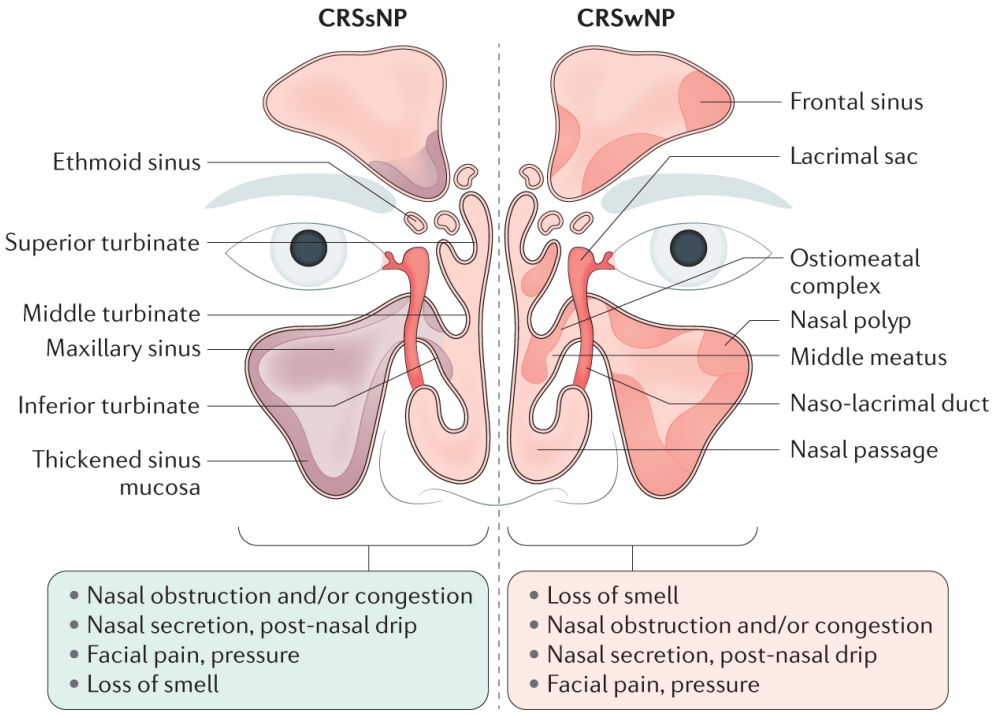

Figure 2.1 | Anatomy of the paranasal sinuses and the nasal passage. Anatomical changes in chronic rhinosinusitis without nasal polyps (CRSsNP) and chronic rhinosinusitis with nasal polyps (CRSwNP). CRSwNP is characterized by the presence of polyposis tissues while CRSsNP is devoid of polyposis. #Reproduced from Bachert et al. (2020)

However, it is still unclear, whether these CRS phenotypes represent different aetiologic and pathophysiologic entities, or rather different stages and courses of one underlying disease (Kato et al., 2022). To address this concern, an updated classification proposed by Grayson et al. was adopted and proposed in The European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS2020) (Fokkens et al., 2020; Grayson et al., 2020) based on the anatomical distribution of the CRS (Figure 2.2).

a. **Primary CRS:** Primary CRS is defined as a 'primary inflammatory disorder of the airway or respiratory system'. Patients are defined as having primary CRS if they have a disorder that is limited to their airway or respiratory system only (Grayson et al., 2020). Thus, patients who have CRS in the setting of immunodeficiencies (eg, selective immuno-globulin deficiency), autoimmune conditions (eg, granulomatosis with polyangiitis or sarcoidosis), genetic abnormalities (eg, cystic fibrosis), odontogenic sinusitis, or local neoplasm do not have primary CRS, as their sinonasal mucosal disease is secondary to another process. They may be localized (unilateral) or diffused (bilateral). The endotypic dominance of primary CRS is type 2 and non-type 2 (primarily a mix of type 1 and 3).

b. **Secondary CRS:** Secondary CRS are CRS not intrinsically because of the inflammatory responses seen in primary CRS. These causes indirectly result in symptoms of CRS, whether from a localized disease or a systemic process. A common cause of secondary CRS is odontogenic in origin, most linked to prior dental procedures, such as dental extractions, implant placement or 'sinus lifts.' Intrinsic periodontal disease may also result in odontogenic sinusitis. Mycetomas, or fungal balls, are other common causes of secondary CRS. The symptoms are essentially identical to those of other forms of CRS. Other causes of secondary CRS include benign and malignant neoplasms that lead to post-obstructive phenomena in the sinus, which may lead to the patient's initial symptoms. Other important causes of secondary CRS include genetic disorders, immunodeficiencies and autoimmune inflammatory disorders. Secondary CRS is simply an expression of

another condition. The secondary CRS are usually unilateral and bilateral in distribution. The mechanisms involved, however not exclusive, may include local pathologies (fungal infection, tumor), mucocilliary disturbance (cystic fibrosis), autoimmune diseases (eosinophilic granulomatosis with polyangiitis) and immunodeficiencies (Grayson et al., 2020).

2.2.2 Severity scoring in chronic rhinosinusitis

The most commonly used and validated scoring system of sinonasal inflammatory change remains the Lund-MacKay score (LMK) which gives a maximum score of 24 (Lund and Mackay, 1993). The LMK staging involves scoring 6 bilateral areas of sinus (anterior ethmoid, posterior ethmoid, maxillary, frontal, and sphenoid sinuses) opacification from 0 to 2 according to the following scale: 0 (no opacification), 1 (partial opacification), or 2 (complete opacification). The ostiomeatal complex is scored as 0 (not occluded) or 2 (occluded). The Left and right sides are staged separately, and the scores are summed so that each patient's total LMK score may range from 0 to 24. An LMK of 2 or less has an excellent negative predictive value, and an LMK of 5 or more has an excellent positive predictive value, strongly indicating a true disease (Bhattacharyya et al., 2004).

2.2.3 Aetiology of CRS

Chronic rhinosinusitis is a heterogeneous condition with multifactorial aetiologies that include genetic, anatomical, and environmental factors that may or may not be infectious. Infectious factors like bacterial, viral, and fungal infections that lead to

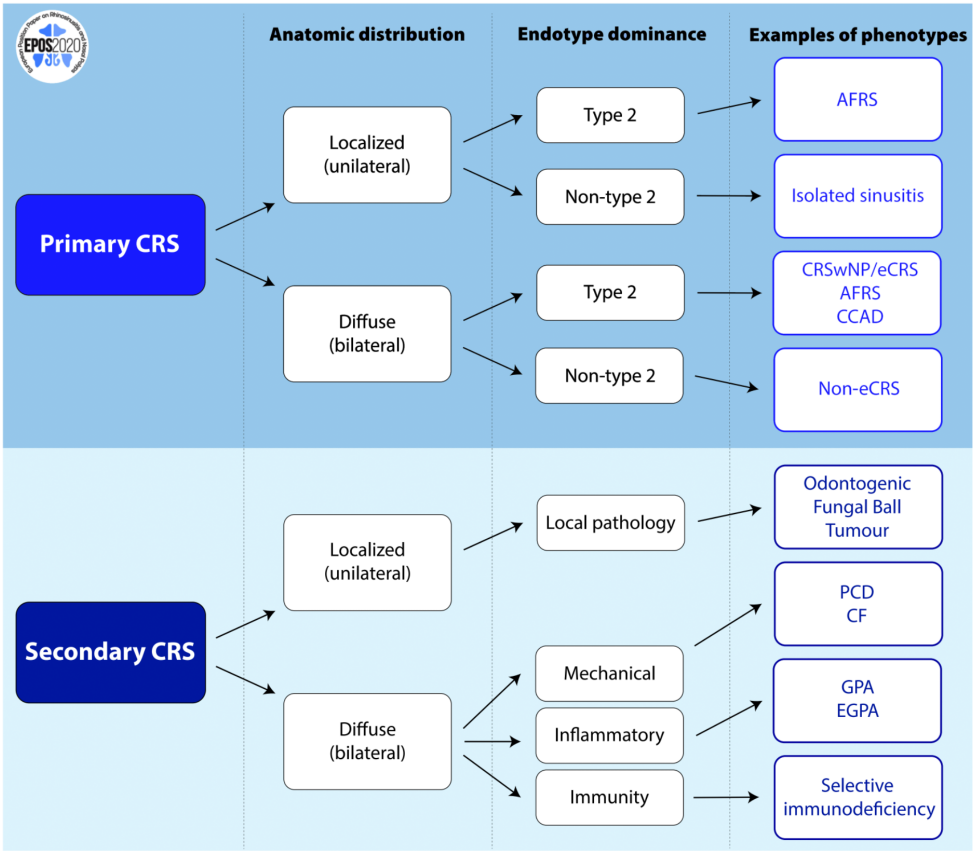

Figure 2.2 | Classification of primary and secondary chronic rhinosinusitis. AFRS = allergic fungal rhinosinusitis; CCAD = central compartment allergic disease; CRSwNP = chronic rhinosinusitis with nasal polyps; eCRS = eosinophilic CRS. CF = cystic fibrosis; EGPA = eosinophilic granulomatosis with polyangiitis (Churg-Strauss disease); GPA = granulomatosis with polyangiitis (Wegener's disease); PCD = primary ciliary dyskinesia. #Reproduced from Fokkens et al (2020).

microbiome dysbiosis, and non-infectious factors like genetic disorders (autoimmune diseases, immunodeficiencies, gastroesophageal reflux disease), rhinitis (allergic, nonallergic), anatomic abnormalities (septal deviation, concha bullosa), ciliary disorders (cystic fibrosis), metabolic derangements (aspirin sensitivity), comorbidities (asthma), mucosal barrier dysfunction, are often considered potential risk factors for CRS (Ramakrishnan et al., 2013; Shaghayegh et al., 2022). Among many aetiologies implicated in CRS, bacterial infection, biofilm formation, and its persistence is considered one of the cardinal triggers that are linked to higher disease severity, chronicity, CRS with nasal polyps and poor post-sinus surgical outcome which negatively

affects patient's health-related quality of life and productivity (Foreman et al., 2010; Psaltis et al., 2008; Shaghayegh et al., 2022). Further, any interruption in the balance of the nasal microbiome can cause a pathological state. An imbalanced sinus microbiome or loss of microbiome diversity appears to be a crucial factor in CRS; however, whether this dysbiosis is a causative or propagative mechanism of inflammation remains controversial. Dysbiosis might stimulate an inflammatory response, whereas inflammation can establish an environment that encourages alterations in the local bacterial residents. Common bacteria often harboured from the nasal cavities associated with CRS include *Streptococcus pneumonia*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus, and Pseudomonas aeruginosa* (Shaghayegh et al., 2022).

Even though the single pathogen hypothesis in the pathology of CRS has not been proven yet, *S. aureus* and its enterotoxins have been long implicated with severe outcomes. *S. aureus* has been reported in about 64% of CRSwNP compared to only 33% and 20% of CRSsNP and healthy control subjects, respectively (Vickery et al., 2019). Patients with chronic rhinosinusitis colonized with specific pathogenic strains of *S. aureus* retain the same strain over time rather than harbouring different isolates over time, suggesting either pathogenic resistance to therapeutic interventions or the existence of a reservoir for recolonization (Drilling et al., 2014). Several unique virulence factors and immune-modulatory actions of *S. aureus* have been described, which remain essential to the currently accepted theories about CRS pathogenesis. Further, *S. aureus* is a strong biofilm former, and biofilms consist of bacteria in a specialised protective polysaccharide gel impermeable to antibiotics. Increasingly, *S. aureus* colonization is

recognized as a disease modifier, promoting immune dysregulation, barrier dysfunction, and bacterial dysbiosis, leading to biofilm formation and recalcitrant CRS disease.

2.2.4 Role of Staphylococcus aureus in chronic rhinosinusitis

Historically, *S. aureus* infection was thought to be a causative agent in the pathogenesis of CRS, with recurrent infections selecting for increasingly virulent and antibiotic-resistant strains (Vickery et al., 2019). Although specific *S. aureus* type or clones are not yet associated with CRS disease pathophysiology, chronic colonization of *S. aureus* and its biofilm is correlated with the development of persistent severe inflammation in CRS, particularly CRSwNP – a more severe case of CRS (Bardy et al., 2018; Vickery et al., 2019). *S. aureus* has been isolated from almost 65% of CRSwNP patients compared to 33% CRSsNP and 20% of healthy controls suggesting a role of *S. aureus* in the exacerbation of CRS (Van Zele et al., 2004). Patients colonized with specific virulent strains of *S. aureus* seem to retain the strain over time suggesting resistance to therapeutic intervention or evolutionary adaptation (Drilling et al., 2014). It is further noted that *S. aureus* containing several auxiliary factors or genetic backgrounds that enhance their virulence or enable them to cause particular clinical symptoms are usually enriched in more severe cases of CRS, including CRSwNP (Shaghayegh et al., 2022). As such, *S. aureus* is often regarded as a CRS disease modifier that promotes immune dysregulation, barrier dysfunction, and bacterial dysbiosis leading to biofilm formation and recalcitrant disease rather than a pathogenic factor in CRS (Vickery et al., 2019). Several unique virulence factors (enterotoxin B, protein A, lipoteichoic acid, lipopolysaccharide) and immune-modulatory actions of *S. aureus* have been described, which remain essential to the

currently accepted theories about CRS pathogenesis (Braga et al., 2019; Patou et al., 2008). As such, understanding the mechanisms *S. aureus* utilizes to prolong tissue inflammation, promote nasal polyp formation, and bacterial dysbiosis are essential in our efforts to identify new therapeutic approaches to resolve the chronic inflammatory pathways that are activated in CRS.

2.3 STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is a Gram-positive, non-motile, facultative anaerobe coccus, often catalase positive, capable of colonizing diverse ecological niches within its hosts – upper respiratory tract, skin, and nasal passages in humans. Although *S. aureus* is considered one of the commensals in the human microbiome and 20-30% human population are long-term carriers, they also cause a range of infections and are one of the five most prevalent pathogens in hospital-acquired infections, particularly causing wound infection post-surgery (Etter et al., 2020; Frank et al., 2010). *S. aureus* is one of the most successful invasive human pathogens entering the body through skin breaches or mucous membranes, where the innate immune system immediately confronts it as the first line of defense. To counteract innate immunity, *S. aureus* expresses several virulence factors like biofilm and immune-modulating proteins. Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies. The versatile host adaptation and successful pathogenicity of *S. aureus* are strongly influenced by the acquisition of virulence factors encoded in the mobile genetic elements (MGEs) such as prophages, plasmids, pathogenicity islands and genomic islands (Moon et al., 2015).

Staphylococcus aureus is considered a pathogen with 'high' priority by World Health Organization (WHO) because of its increasing incidence of infections caused by strains resistant to antibiotics like methicillin and vancomycin. A recent study on the global burden of antimicrobial resistance estimated that in the year 2019, methicillin-resistant *S. aureus* (MRSA) was a leading cause of death (> 100,000 deaths) attributable to a single pathogen-drugs combination and the second leading cause of death attributable to an antimicrobial resistant pathogen (Antimicrobial Resistance, 2022). Recent studies have shown that colonization of nares is a potential risk factor for subsequent *S. aureus* infection as almost in 80% of bacteremia, the infecting strain was identical to the nasal colonizing strain (Frank et al., 2010).

2.3.1 Genomic plasticity and virulence factors of Staphylococcus aureus

Accessory mobile genetic elements (MGEs) significantly introduce genetic diversity and phenotypic traits in microorganisms, particularly bacteria. A wide range of environmental conditions, including interspecies competition within a particular econiche and antibiotic selective pressure, select the organisms that have acquired MGEs as they presumably have better survival fitness (Foxman, 2012; Levin, 1993). The composition of bacterial genomes is altered rapidly and radically through various processes, including horizontal gene transfer (HGT) (Juhas et al., 2009; Lerner et al., 2017). Horizontal gene transfer entails incorporating genetic elements from another organism directly into the genome where they form 'genomic islands' with specific signatures of MGEs and is key to bacterial evolution as it leads to 'evolution in quantum leaps' (Hacker and Carniel, 2001). The horizontal gene transfer contributes to the

diversification and adaptation of microorganisms, thus impacting the genome flexibility and plasticity (Juhas et al., 2009). Mobile genetic elements that increase bacterial fitness, either directly or indirectly, most likely will be positively selected owing to their fitness advantage to the host (Dimitriu et al., 2016).

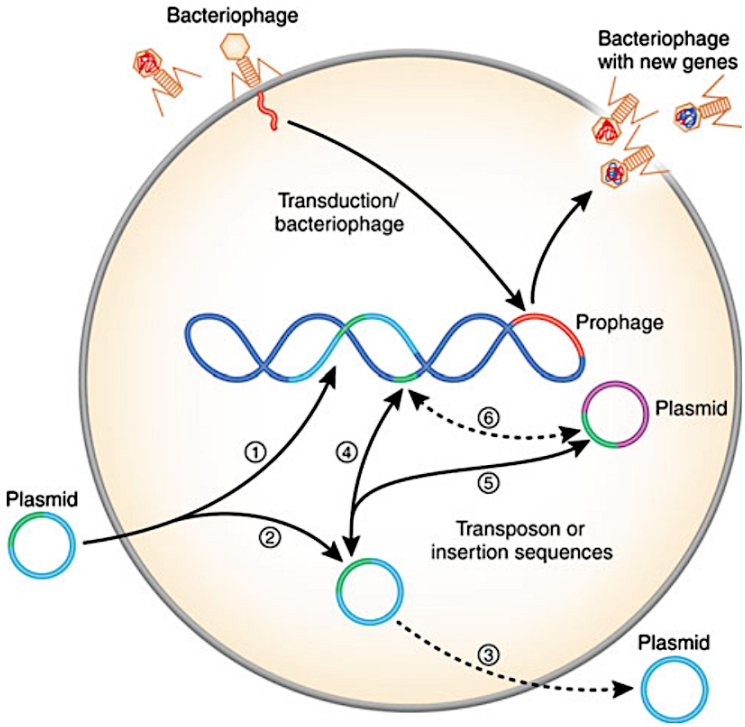

Figure 2.3 | Acquisition of mobile genetic elements (MGEs) by *S. aureus*. (1) Incorporation of plasmids or plasmid elements into genomic DNA. (2) Plasmids can be maintained as free circular DNA. (3) Suicide plasmid. (4) Transfer of a transposon or an insertion sequence between plasmid and genomic DNA. (5) Transfer of a transposon or an insertion sequence between plasmids within the cell. (6) Transfer of a transposon or an insertion sequence from genomic DNA to another plasmid. #Reproduced from Malachowa and DeLeo (2010)

The genomic plasticity and pathogenicity of *S. aureus* are primarily attributed to mobile genetic elements (MGEs) such as plasmids, prophages, staphylococcal pathogenicity islands (SaPIs), genomic islands, staphylococcal cassette chromosomes (SCCs), arginine catabolic mobile element (ACME) and other transposable elements like insertion sequences (IS) and transposons (Tn) which encode an array of virulence factors,

antibiotic resistance determinants and genes involved in other contingency functions (Malachowa and DeLeo, 2010). This is particularly relevant to *S. aureus* because MGEs represent around 22% of the *S. aureus* genome (Fitzgerald et al., 2001; Jamrozy et al., 2017). *S. aureus* can accommodate multiple prophages in its genome, a condition called poly-lysogeny, and accounts for up to 8.0% of the *S. aureus* genome (Nepal et al., 2021). Prophages can modify their host's fitness, virulence, and evolution in numerous ways. The lysogenic conversion involving potent toxins is prophages' most important contribution to their host. However, as we study phage-host interactions in different bacterial species and humans, we find novel ways by which prophages can influence their host. Lately, it is increasingly evident that the co-existence of bacteria and prophage is associated with multifaceted bacterial fitness elevating risk to human health as many virulence factors and AMR genes are prophage-encoded. However, in clinical settings, the role of prophage domestication and their productive induction that may lead to microbiome alteration and an anti-viral immune response is poorly understood.

Among several MGEs, prophages are crucial in *S. aureus* because evolutionary and population genomic studies have shown distinct prophage-encoded genetic signatures (particularly immune-evasion factors) for host-switching (Chaguza et al., 2022). While the core genome of *S. aureus* usually encodes for basic cellular functions (e.g. translation, metabolism, architecture) and exhibit rather homogeneous G+C contents and codon usage, the flexible prophages (or prophage-like elements) harbor distinct virulence factor-encoding genes (VFGs) such as Panton-Valentine leukocidin (*lukFS-PV*), the immune evasion cluster (IEC) associated with human immune evasion (*chp, sak,* and *scn*), exfoliative toxins (*eta* and *etb*) and enterotoxins (*sea, see, seg, sek*, and *sep*) that

play a crucial role in immune evasion, tissue evasion, toxins, adherence, and iron uptake or may code for toxins among human and animal infection (Naorem et al., 2021).

2.4 PROPHAGE

The term 'prophage' was coined by André Lwoff and Antoinette Gutmann in 1950 and is derived from a French word *probactériophage* (Lwoff and Gutmann, 1950), who along with Francois Jacob and Jacques Monod, also discovered that phages could use lysogeny to replicate their genome thereby contributing to vertical gene transfer (Wang et al., 2010). Prophages are quiescent temperate bacteriophage (phage) genomes integrated into the bacterial genome. Prophage can be cytoplasmic or nuclear. If cytoplasmic, it could be either free in the endoplasm, like some other cytoplasmic particles, or bound to a specific bacterial plasmagene endowed with genetic continuity (Lwoff, 1953). Prophages are a prevalent feature of bacteria and can constitute as much as 10-20% of the bacterial genome and thus play a key role in introducing diversity in bacteria (Casjens, 2003). Bacteria usually contain several putative prophages implying infection by multiple temperate phages during evolution. Many prophages carry genes that improve the fitness of their bacterial hosts, including genes which control biofilm formation as well as factors determining microbial pathogenicity.

A prophage is a quantitatively important genetic element of the bacterial chromosome and a mobile DNA element that acts as a vector for lateral gene transfer between bacteria. Indeed, numerous virulence factors from bacterial pathogens are prophage-encoded. It was postulated that this role of prophages is not limited to pathogenic bacteria but that prophage genomes might also mediate some adaptations of

nonpathogenic bacterial strains to their ecological niche. Furthermore, prophages account for a substantial amount of interstrain genetic variability in several bacterial species (e.g., *S. aureus* and *S. pyogenes)*. Prophage reactivation was first reported by Jacob and Wollman (1953) who discovered that the survival of UV-irradiated phage was higher on hosts containing a homoimmune prophage than on either non-lysogenic bacteria or on lysogenic bacteria containing a prophage with different immunity (Defais et al., 1983).

2.4.1 Fates of a prophage

Prophages are not always productive and may behave differently under different environmental stress or need. As such, prophages may have the following lifestyles (Figure 2.4):

a. **Lytic lifestyle:** Excision of prophage immediately follows DNA replication and assembly with the release of virion particles leading to host cell lysis. Each virion is free to infect another cell and lysogenize them, leading to the clonal expansion of lysogens. Thus, the lytic lifestyle of prophage results in the release of phage particles (or pyocins/tailocins) that may be able to transduce to other susceptible cells present in the same biome and disseminate genes they encode potentially contributing to the evolution of the bacteria arming them, with an arsenal of prophage-encoded accessory genes (piggyback-the-winner model) (Silveira and Rohwer, 2016). Also, the released phage may infect and kill competing colonizers (kill-the-winner model) thereby establishing the lysogen (Maslov and Sneppen, 2017).

b. **Lysogenic lifestyle:** A prophage is stably integrated into the host genome and replicates with the host chromosome as a prophage unless the lytic cycle is triggered. All of the prophage-encoded genes are thus vertically transferred to the progeny cells.

c. **Pseudo-lysogenic lifestyle:** The prophage genome exists in an episomal state within the host cell. The prophage is excised from the host genome but does not undergo a productive lytic cycle leading to the episomal existence of the prophage. Episomal prophage usually segregates asymmetrically during cell division. While a small fraction undergoes productive lysis, the other fraction of host cells is phage resistant as they are already exposed to the prophage genome.

d. **Chronic lifestyle:** Chronic lifestyle is characteristic of a prophage from filamentous phage (family *Inoviridae*). These prophages continuously produce progeny that is released by extrusion without cell death/lysis.

Either way, prophages arm bacteria with multiple functions as prophages may encode auxiliary metabolic genes (AMGs), virulence factors (VFs), antimicrobial resistance genes (ARGs) and toxins that can be disseminated either vertically or horizontally. Lysogenic conversion through transduction is one of the most efficient mechanisms through which bacteria acquire accessory functions. Although it is evident that the bacterial pathogenicity is combinatorial, requiring both core genes and auxiliary prophage-

encoded genes, clinical impact of prophage and associated VFs is poorly understood in human health and diseases.

2.4.2 Lysogenic conversion

Lysogenic conversion (historically called 'phage-conversion') was observed in many bacteria around the 1950s and the production of some bacterial toxins was speculated to be the result of the lysogenic conversion by specific phages (Inoue and Iida, 1970). Lysogenic conversion is a phenomenon where a bacterial host acquires a new trait as a direct result of the expression of a gene encoded in a prophage DNA. A regulator gene produces a repressor protein (e.g., CI repressor protein) that suppresses the lytic lifestyle of the prophage, but various environmental factors, such as ultraviolet irradiation may prevent the synthesis of the repressor, leading to normal prophage induction and lysis of the host bacterium. Often prophages compensate for their detrimental effects on their host by providing them with beneficial traits that augment their fitness and can confer completely novel phenotypes (Figure 2.5). These new phenotypes known as 'lysogens' comprise immunity to phage super-infection, resistance to other phages, tolerance to various stresses, pathogenicity, and, very rarely, antibiotic resistance (Sausset et al., 2020). Importantly, the rate of lysogenization of temperate phages is variable, since it is dependent on, amongst others, the cell volume (St-Pierre and Endy, 2008) and phage-phage interactions (Trinh et al., 2017), at the time of infection.

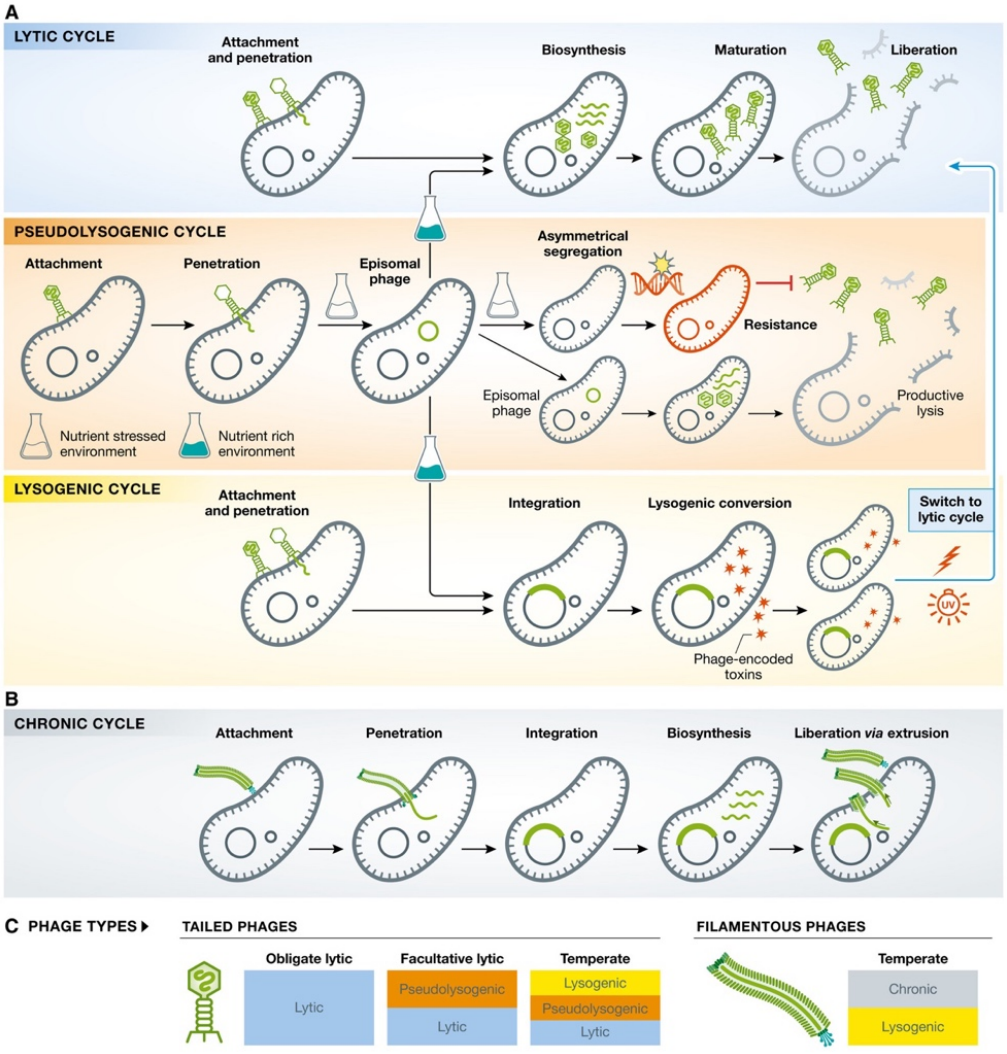

Figure 2.4 | Various modes of phage infection. (A) Phage infection characteristic of tailed phages (i) Lytic cycle: phage infection immediately follows with assembly and release of virions leading to cell lysis. Each virion starts a new lytic cycle leading to a burst of 'productive' infections; (ii) Lysogenic cycle: injected phage DNA integrate into the bacterial chromosome and replicate with it as prophages, until a lytic cycle is triggered; and (iii) Pseudolysogeny: phage DNA persist in an episomal state within the host cell before resolving into a lytic or lysogenic cycle. Episomal phages typically segregate asymmetrically during cell division depending on the environmental stress. A small fraction may undergo a productive lytic cycle (scavenger response) favouring development of phage-resistant bacterial subpopulations. (B) Chronic infection cycles that are characteristic of 'filamentous phages' (family = *Inoviridae*) that continuously produce progeny released by extrusion without cell death or lysis. (C) Phage types based on replication cycle: tailed phages that always lyse bacteria upon infection are 'virulent' or 'obligate lytic', while 'facultative lytic' phages may also undergo pseudolysogeny. 'Temperate' phages may have a lysogenic or pseudolysogenic lifestyle until triggered to enter a lytic cycle, typically when the host bacteria experience stress conditions. Filamentous phages usually follow a chronic productive cycle, though some have the capacity to also enter a lysogenic cycle. #Reproduced from Venturini et al. (2022)

2.4.3 Lysogeny and the discovery of prophage-encoded toxigenicity

In 1926 D'Herelle noted that the toxigenicity of *Corynebacterium diphtheriae* was transmissible and assumed that productive prophage induction resulting in host lysis liberates the toxin (Lwoff, 1953). A year later, in 1927, the toxigenicity of productive prophage was first documented in the streptococci by Frobisher and Brown (Frobisher and Brown, 1927). By growing non-toxigenic haemolytic streptococci with a filtrate from a toxigenic scarlatinal strain of the same species, the authors were able to transmit the toxigenicity to an inherently non-toxigenic strain. The acquired toxigenicity was lost after a few subcultures indicating the loss of prophage. Later, in 1951, Freeman discovered that the non-toxigenic strain of *C. diphtheriae* becomes toxinogenic when lysogenized by a certain 'phage B' isolated from a toxinogenic strain (Freeman, 1951). A year later, Hewitt confirmed that the property to produce toxins can be conferred to a non-toxinogenic strain of *C. diphtheriae* through the lysogenization (Hewitt, 1952) which was further supported by the observations made by Groman (1953). In following years, the production of some bacterial toxins was reported to be associated with lysogenization by specific phages (Groman, 1953; Matsuda and Barksdale, 1967; Zabriskie, 1964) in a phenomenon known today as 'lysogenic conversion'. The mechanism involved was extensively studied in the production of somatic antigens of the *Salmonella* (Losick and Robbins, 1967; Uetake et al., 1958). Around the same time, Dolman (1964) suggested a relationship between the toxigenicity of *Clostridium botulinum* type E and its prophage. Inoue and Iida later verified the prophage imparted toxigenicity of *C. botulinum* (Inoue and Iida, 1970). Also, in 1982, Nida and Ferretti

reported secretion of exotoxin type A and B by group A streptococci were prophage-encoded (Nida and Ferretti, 1982).

2.4.4 Prophage-mediated pathogenicity and its clinical significance

Today, many hallmark examples of prophage-encoded toxins causing deadly outbreaks are known. It is well established that toxicity in *C. diphtheriae* (diphtheria toxin), *C. botulinum* (botulinum toxin), *Vibrio cholera* (cholera toxin), *Escherichia coli* O157:H7 (shiga-toxin), and *Salmonella enterica* (*SopE* effector protein) is prophage mediated (Feiner et al., 2015). Duerkop et al. found that the opportunistic pathogen *Enterococcus faecalis* induces prophage to gain dominance over competing harmless strains in a mouse model (Duerkop et al., 2012). Prophage excision in *Listeria monocytogens* activates the expression of the competence gene (*comK*), promoting phagosomal escape, intracellular growth and virulence, which leads to listeriosis (Pasechnek et al., 2020). Phage-associated dynamic control of major DNA repair has been observed in *Streptococcus pyogenes* that allow the organism to rapidly respond to changing environments crucial for persistence. Further, Javan et al. noted that prophages are widespread in *Streptococcus* and play a significant role in pneumococcal pathogenies (Rezaei Javan et al., 2019).

Prophage-mediated pathogenicity of bacteria has received wider attention lately as more prophage-encoded pathogenic determinants are discovered in complete genomes of clinical strains. Despite the bioenergetic cost of prophage DNA integration and replication, domestication of a prophage is known to be beneficial to bacteria in multiple

aspects (Marshall et al., 2021). Recent studies have shown more evidence that commensals or non-virulent strains harboring a prophage (a lysogen) are relatively more pathogenic as prophages encode multiple virulence factors, toxins, superantigens, effectors translocated by a type III secretion system, intracellular survival/host cell attachment proteins, and antimicrobial resistance genes (Boyd, 2012; Busby et al., 2013).

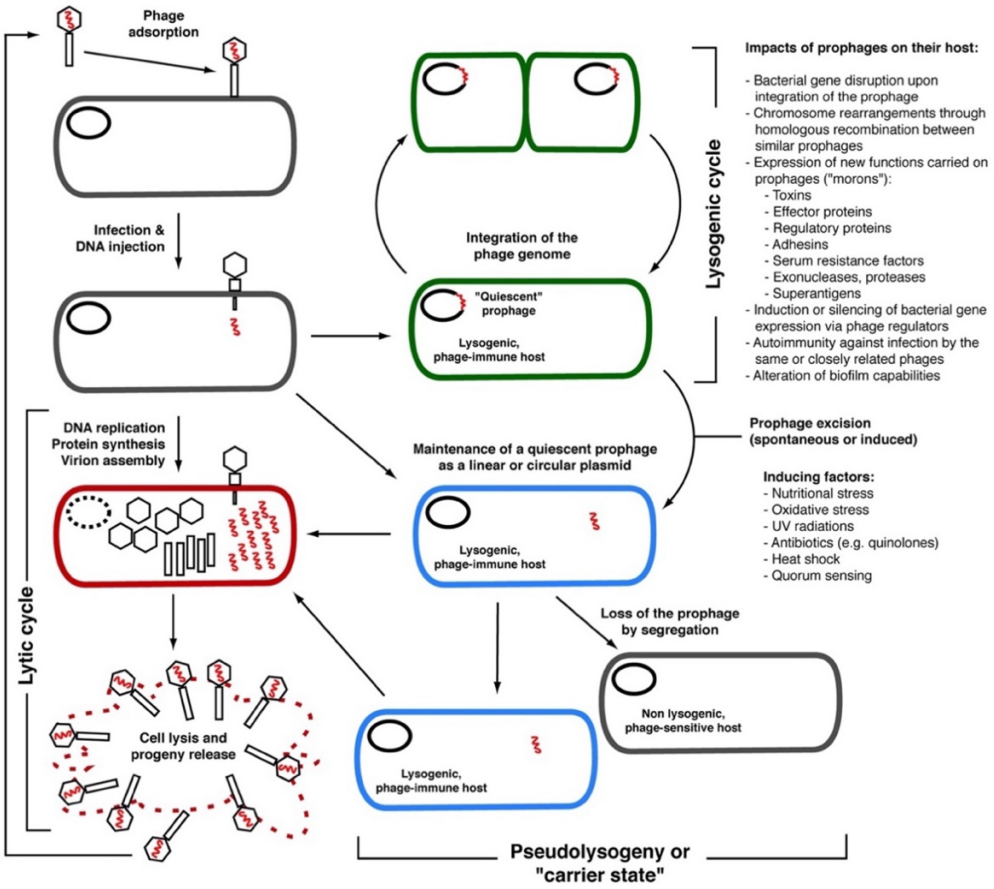

Figure 2.5 | Different lifestyles adopted by phages and their implications. Virulent phages will only follow the lytic cycle for their replication and will lead to the lysis of the host cell at the end of the cycle. Temperate phages have the choice to replicate through either the lytic cycle like virulent phages or undergo lysogenic cycle. In most cases of lysogeny, the phage integrates its genome into the host bacterial chromosome and remains quiescent. The prophage DNA is replicated along with the bacterial chromosome and is transmitted to daughter cells. Lysogeny can sometimes have significant impacts on the host (lysogenic conversion). Under certain conditions, including various stresses causing DNA damages, the prophage is excised and initiates a lytic cycle. Some phages also adopt a pseudolysogenic lifestyle. #Reproduced from Fortier and Sekulovic (2013)

Prophages influence their host pathogenicity either by directly encoding virulence factors or indirectly by enhancing bacterial fitness via evolution during the infection (Hacker and Carniel, 2001). Prophages of *Pseudomonas aeruginosa* are often associated with chronic wound infections, cystic fibrosis (CF), bronchiectasis and adaptation during infection. Filamentous Pf phages released from colonizing *P. aeruginosa* help bacteria escape macrophage engulfment, enhance antibiotic tolerance and increase the biofilm adhesion (Secor et al., 2015). Induction of filamentous Pf phage from lysogenic *P. aeruginosa* in a wound environment results in the production of type I interferons (IFNs), inhibiting TNF production subsequently leading to reduced phagocytosis of *P. aeruginosa* resulting in persistent and chronic wound infections (Sweere et al., 2019). In a cohort of 92 *Pseudomonas*-positive CF patients, Pf phages released by colonizing *P. aeruginosa* were not only associated with the virulence of the bacteria but also with increased antibiotic resistance, chronicity and reduced lung function (Burgener et al., 2019). In a separate study, Gloag et al. reported that *Pseudomonas aeruginosa* mutants with hyper-biofilm phenotype, termed as rugose small colony variants (RSCVs), were selected among six different strains in a porcine full-thickness chronic burn wound model (Gloag et al., 2019). Later, the same group reported that an adaptive single nucleotide polymorphism in *wspF* and *retS* genes, and, more importantly, lysogenization by a prophage released from co-inoculated strain resulting in disruption of *dipA* caused the hyper-biofilm RSCV colony phenotype (Marshall et al., 2021). Further, the RSCV mutants were immune to phage reinfection (resist superinfection), had elevated fitness relative to its ancestral PA01 and outcompeted the competing strains within 48 hours, suggesting extreme competitive advantage. In other similar studies on *P. aeruginosa,*

researchers have found a temperate Pf (filamentous phage) in human wound infections that delay the wound healing (Bach et al., 2022; Burgener et al., 2019; Secor et al., 2015; Sweere et al., 2019). Also, the stimulation of mammalian cells with Pf phages triggered an antiviral immunity, thereby impairing the bacterial clearance (Secor et al., 2020; Sweere et al., 2019). Further, Høyland-Kroghsbo and Bassler reported that lysogenization of *P. aeruginosa* (PA14) by a specific temperate phage (JBD44) restores bacterial quorum sensing, thereby enhancing the growth (Hoyland-Kroghsbo and Bassler, 2022).

Similarly, phages released from lysogenic *Salmonella* have also been associated with inflammatory bowel disease. A similar effect has also been reported in *Salmonella,* causing diarrhoea where the lysogenic *Salmonella* strain elicits intestinal inflammation. The inflammatory by-product then triggers the SOS response in bacteria leading to increased prophage induction and lysogenic conversion and progression of the enteric disease (Wahida et al., 2021). Further, Nepal et al. reported enrichment of *hlb*-converting *S. aureus* prophages in chronic rhinosinusitis patients developing nasal polyposis (Nepal et al., 2021). These prophages are known to confer human immune evasion capability to the bacteria. Additionally, several toxins and regulatory genes encoded in a prophage enhance virulence, adhesion, colonization and persistence. Further, Kondo et al. analysed 1,623 complete genomes of seven different bacteria representing ESKAPE pathogens and found multiple prophage-encoded virulence factors encoded in the genomes of nosocomial pathogens like *Enterobacter cloacae, Escherichia coli, Enterococcus faecium, Klebsiella pneumoniae, Pseudomonas aeruginosa, and S. aureus.* Among these, AMR genes were more abundant in *K.*

pneumoniae (20.9%), followed by *P. aeruginosa* (12.7%) and *S. aureus* (12.6%) (Kondo et al., 2021)

Table 2.1 | Bacteria harbouring toxigenic prophage and its associated pathogenesis.

Organism	Toxigenic prophage	Genes/toxins	Disease/effect on bacterial virulence
Bordetella bronchiseptica	PHB09	Entire phage	Reduced twitching motility due to disruption *pilB*
Clostridium botulinum	CEβ, DEβ	C1, D	Botulism/Botulinum toxin production
Clostridium difficile	Ancient prophage	tcdA, tcdB	TcdA and TcdB toxin production
	PhiSemix9P1	Cdt	CDTa and CDTb toxin production
	φCD38–2	cwpV	Increased bacterial cell aggregation
	PhiDHM1	agrB, agrC, agrD	Interfering with the quorum sensing system
	φCD119	repR	Decreased β-toxin expression
	φCD27	Unknown	Decreased β-toxin expression
	φCD38–2	Unknown	Increased β-toxin expression
	φCD2, φCD6, φCD8	Unknown	Increased β-toxin expression
Corynebacterium diphtheriae	β-phage	cdtA, cdtB	Diphtheria/Diphtheria toxin production
Corynebacterium ulcerans **(Sekizuka et al., 2012)**	φCULC0102-I	tox	Cutaneous infection and diphtheria-like illness
Enterococcus faecalis	pp1, pp4, pp6	EF0348, EF2001, EF2811, EF2813	Mediates bacterial adherence to platelets
Escherichia coli	933W, H-19B	stx1, stx2	Enterohaemorrhagic diarrhoea, haemolytic uraemic syndromes, Shiga toxin production
Shiga-toxigenic *E. coli* (STEC)	Sp5	Unknown	Downregulation flagellin
including *E. coli* O157:H7	Phi4	EC958_1546	Increased flagellin expression
	-	Cro	Activation transcription type three secretion system
	SpIE1	esr41	Increase in cell motility and resistance against colicin, attenuation of iron uptake
	Sp5	agxR	Increased iron uptake
Listeria monocytogenes	φ10403S	Entire phage	Interruption *comK* gene
Neisseria meningitidis	φMDA	Entire phage	Promotion adhesion of bacterial cells to epithelial cells
Pasteurella multocida	-	-	Cellulitis and other wound infections
Pseudomonas aeruginosa	D3	Lap	Reduction O-chain length by inhibition of the host cell alpha polymerase
	JBD26	Unknown	Inhibition working mechanism Type IV pili
	Pf4	Entire phage	Promotion adhesion of bacterial cells to mucin
	Pf	Entire phage	Reduced inflammation and phagocytosis
	PaP3	70.1	Inhibition stress regulator *RpoS*
Salmonella enterica **serovar**	ε15	22	Reduction O-chain length by inhibition of the host cell alpha polymerase
Typhimurium	P22	Gtr	Addition of glucose units to the O-chain
	Fels-2	STM2699	Crosslinks bacterial cells to the eukaryotic spectrin receptor
	BTP1	STnc6030	Provides superinfection exclusion
Salmonella enterica	Gifsy-1	gogB	Increased inflammatory response, enhanced tissue damage and increased bacterial colonization
	Gifsy-1	sarA	Lowering production reactive oxygen species by macrophages

Staphylococcus aureus	80α	Tst/Tsst	Toxic-shock syndrome toxin-1 production, staphylococcal scalded skin syndrome, food poisoning
	φSPβ	sasX	Expression of a surface protein promoting bacterial adherence to epithelial cells
	CC398	Unknown factors associated with biofilm	Increased expression of fibronectin-binding proteins
	φ11, φ80α	cI	Induction *SigB* regulon, impacting the development of the bacterial infection
	φ13, NN3, Sa3int	Entire phage encoding superantigens (SAg), PVL toxin, enterotoxins, *sak, chp, scn*	Loss of β-toxin production due to disruption *hlb,* and other diseases like *c*ellulitis, impetigo, osteomyelitis, pneumonia, endocarditis, speticaemis
Staphylococcus mitis	φSM1	PblA, PblB	Mediation bacterial interaction with platelets
***Streptococcus pyogens* (group A) and some group G and C *Streptococcus* species**	-	-	Scarlet fever, necrotizing fasciitis, streptococcal toxic shock syndrome, septicaemia, myositis
Vibrio cholera	CTXφ	Ctx, Tcp	Cholera/cholera toxin production; expression of toxin-coregulated pili
	VP882	vqmA	Interfering with the quorum sensing system

Note: Not all diseases are proven to be directly associated with a prophage-encoded virulence factor. #Adapted and reproduced from Kuhl et al. (2012); Schroven et al. (2021); Sekizuka et al. (2012)

2.4.5 Prophages as antibiotic gene carriers and its mobilization

Antibiotic resistance developed by bacteria is a significant threat to global health and can occur in bacteria through multiple dissemination routes, including horizontal gene transfer (HGT) mediated by temperate phages (Rodriguez-Rubio et al., 2020). Phage particles have emerged as elements with the potential to mobilize antibiotic resistance genes (ARGs) in different environments, including the intestinal habitat (Brown-Jaque, Calero-Caceres, et al., 2018). Antibiotic-resistance genes (ARGs) encoded in a phage genome show considerable persistence under disinfection treatment (i.e., chlorination, ozonation, and UV irradiation) and natural attenuation due to their protection inside the protein capsid (Calero-Caceres and Muniesa, 2016). Prophages have the potential to transfer genetic material between hosts using either generalized or specialized transduction (Balcázar, 2020). Recent studies on bacterial genomes and environmental metagenomes have discovered ARGs in the prophages (Brown-Jaque et al., 2015; Moon et al., 2020). Antibiotic-resistance genes encoded in prophages are considered especially threatening due to their prolonged persistence in the host, fast replication rates, and ability to switch their lifestyle into lytic and infect diverse bacterial hosts through induction (Moon et al., 2020). These characteristics make prophages suitable vehicles for acquiring, maintaining and spreading antibiotic resistance determinants and increasing HGT events among bacteria. Also, as some induced prophages (or temperate phages) are known to have a wide host range, even within different bacterial orders, and efficient integration mechanisms, prophage-encoded ARGs are considered significant due to their possibility for a wider dissemination (Yu et al., 2016).

Recent reports indicate that prophage implication in the transfer of ARGs could be more important than previously thought (Brown-Jaque et al., 2015). A BLAST search against public databases (e.g., GenBank, EMBL, and DDBJ) revealed that many prophage genomes contain ARGs (Balcázar, 2020) . Further, research on the gut microbiome of the murine model has also shown that antibiotic treatment leads to the enrichment of prophage-encoded genes that confer resistance via disparate mechanisms to the administered drug, as well as genes that confer resistance to antibiotics unrelated to the administered drug (Modi et al., 2013). The presence of ARGs in transducing phage particles has also been reported in contaminated water bodies (Balcázar, 2014) , wastewater treatment plants (Colomer-Lluch et al., 2014; Muniesa et al., 2013), meat products and chicken feces (Blanco-Picazo et al., 2022; Gomez-Gomez et al., 2019) as well as environments with high bacterial density, such as the human/animal gut and lungs (Brown-Jaque, Rodriguez Oyarzun, et al., 2018). Further, Kondo et al. have reported prophage-encoded AMR genes in multiple bacterial strains like *Acinetobacter baumannii, E. coli, Enterobacter faecium, Klebsiella pneumoniae, P. aeruginosa* and *S. aureus* (Kondo et al., 2021).

2.4.6 Prophage induction and microbiome modulation

Prophage induction plays a crucial role in modulating bacterial populations in various environments like human/animal gut, soil, and marine environment (Bruce et al., 2021; Jiang and Paul, 1996; X. Liang et al., 2020). In soil bacteria, prophages may act as gene transfer agents and mobilize pesticide catabolic genes and AMR genes (Ghosh et al., 2008; Pratama and van Elsas, 2017). Prophage induction and its role in a marine

ecosystem have been extensively studied. Several studies have shown that lysogenic conversion and prophage induction is an important part of the marine microbial community and helps in metabolic economization that enables the host to survive unfavourable conditions (McDaniel and Paul, 2005; Paul, 2008).

Prophages and their induction are critical in modulating the microbiome from early life and are considered as gut virome pioneers (Liang et al., 2020; Shamash and Maurice, 2022; York, 2020). Temperate phages (induced from gut colonized bacteria) are the most abundant entity within the first week of life till 3 years (Gregory et al., 2020; Lim et al., 2015). Recently, prophages have been found to be a key component of infant gut virome that may have far-reaching influences on the composition and functions of the infant gut microbiome (Redgwell et al., 2021; Vatanen et al., 2022). A pioneering large cohort work by Jason et al. on inflammatory bowel disease (IBD) indicated the role of prophage induction in IBD as increased phage richness (*Caudovirales*) and decreased bacterial richness in IBD compared to healthy controls (Norman et al., 2015). Indeed, individuals with IBD harbored increased richness and abundances of *Caudovirales* and decreased abundances of Microviridae, indicating the disease is associated with an expansion of *Caudovirales* phages. They further observed a disease-specific phageome change in Crohn's disease and ulcerative colitis, suggesting the role of prophage induction in modulating the gut microbiome. Temperate phages uniquely associated with Crohn's disease were closely related to *Lactobacillus*, *Clostridium*, *Enterococcus*, and *Streptococcus,* while these phages were not observed in individuals with ulcerative colitis, highlighting that specific phages were associated with distinct diseases (Norman et al., 2015). These findings were consistent with other researchers' observations suggesting prophage induction plays a substantial role in functional distortion of the gut

microbiome, thereby landscaping the gut health and disease pathogenesis (Clooney et al., 2019; Cornuault et al., 2018; Zuo et al., 2019). This is all the more relevant because around 80% of intestinal bacteria are lysogens and various dietary compounds, as well as common oral medications, exhibit species-specific growth inhibitions by inducing prophages (Boling et al., 2020; Kim and Bae, 2018; Sutcliffe et al., 2021).

Prophages can bring advantages to their bacterial host by either encoding auxiliary toxins or eliminating the competing sensitive bacteria by producing the infective phages or a threat to the host as enhanced prophage induction may lead to rapid lysogen depletion (De Paepe et al., 2016). Thus a prophage can landscape the microbiota balance (Caroline Henrot and Marie‐Agnès Petit, 2022). As a growing field of research is now focused on the contribution of dysbiosis-related pathologies, including CRS (Chalermwatanachai et al., 2018), unravelling the role of lysogeny and prophage induction is critical.

2.4.7 Prophages of Staphylococcus aureus and its role in virulence

Among various mobile genetic elements that confer pathological fitness to *S. aureus*, prophages are a crucial element for the acquisition and spread of virulence and occasionally antibiotic-resistant determinants. In 1952, Hewitt made a quite unexpected observation that some lysogens of *S. aureus* produced an infecting phage able to induce the transformation of non-toxinogenic *C. diphtheriae* into a toxigenic strain (Hewitt, 1952), suggesting inter-species prophage mobility and virulence dissemination. Since then, multiple prophage-encoded virulence factors have been discovered in *S. aureus*. Advancement in genomic sequencing and its application has revealed insights regarding

the genetic basis of virulence, immune evasion, antimicrobial resistance, pathogenicity, colonization, healthcare adaptation, non-communicable disease risk, host adaptation, and transmission of *S. aureus* and related species (Chaguza et al., 2022). Today, prophages are so strongly associated with *S. aureus*'s evolution that it is rare to find an *S. aureus* without a prophage (Deghorain and Van Melderen, 2012).

In *S. aureus*, prophages are considered an accessory flexible gene pool and are a major determinant for *S. aureus* evolution that leads to significant changes in the composition of its genome over a relatively short time period (Goyal et al., 2019; McCarthy et al., 2012; Xia and Wolz, 2014). Prophages increase the genome plasticity of *S. aureus* during infection, facilitating the pathogen's adaptation to various host conditions (Goerke et al., 2007; Goerke et al., 2006). In particular, prophages play an important role in the pathogenicity of *S. aureus* either by carrying accessory virulence factors such as Panton-Valentine leukocidin (PVL) (*luk-PV* operon), staphylokinase (*sak*), enterotoxin A (*sea*), and exfoliative toxin A (*eta*) or by interrupting chromosomal virulence genes such as those for β-hemolysin (*hlb*) and lipase (*geh*) upon insertion (Goerke et al., 2009; Wirtz et al., 2010). Additionally, phages are the primary vehicle of lateral gene transfer between *S. aureus* strains, providing the species with the potential for broad genetic variation.

The multiple alignments of *S. aureus* prophages reveal a chimeric and mosaic structure that may have resulted from lateral gene transfer of interchangeable genetic elements (modules) from different prophages (Goerke et al., 2009). Due to this, *S. aureus* (pro)phage classification is still debatable and numerous approaches (integrase type, serogroup, holin group) have been proposed. Among these, classification based on

integrase polymorphism proposed by Goerke et al. is most widely accepted because (1) the integrase gene (*int*) is well conserved and also has good discriminatory power reflecting the diversity of *S. aureus* prophages, making it ideal for PCR amplification, (2) allows the prediction of the chromosomal location of the prophage, and (3) the *int* typing has a strong correlation with prophage-encoded virulence factors and thus can predict the pathogenic potential of the lysogenized bacteria which is clinically important (Goerke et al., 2009). Based on integrase type, prophages of *S. aureus* can be clustered into 12 groups (7 major and 5 minor) (Figure 2.6). Based on amino acid sequence homology and catalytic residues, most integrases of *S. aureus* prophages belong to the tyrosine recombinase type family and the rest to serine recombinase type family. Further, most *S. aureus* prophages belong to one of seven major groups (Sa1int to Sa7int) (Goerke et al., 2006).

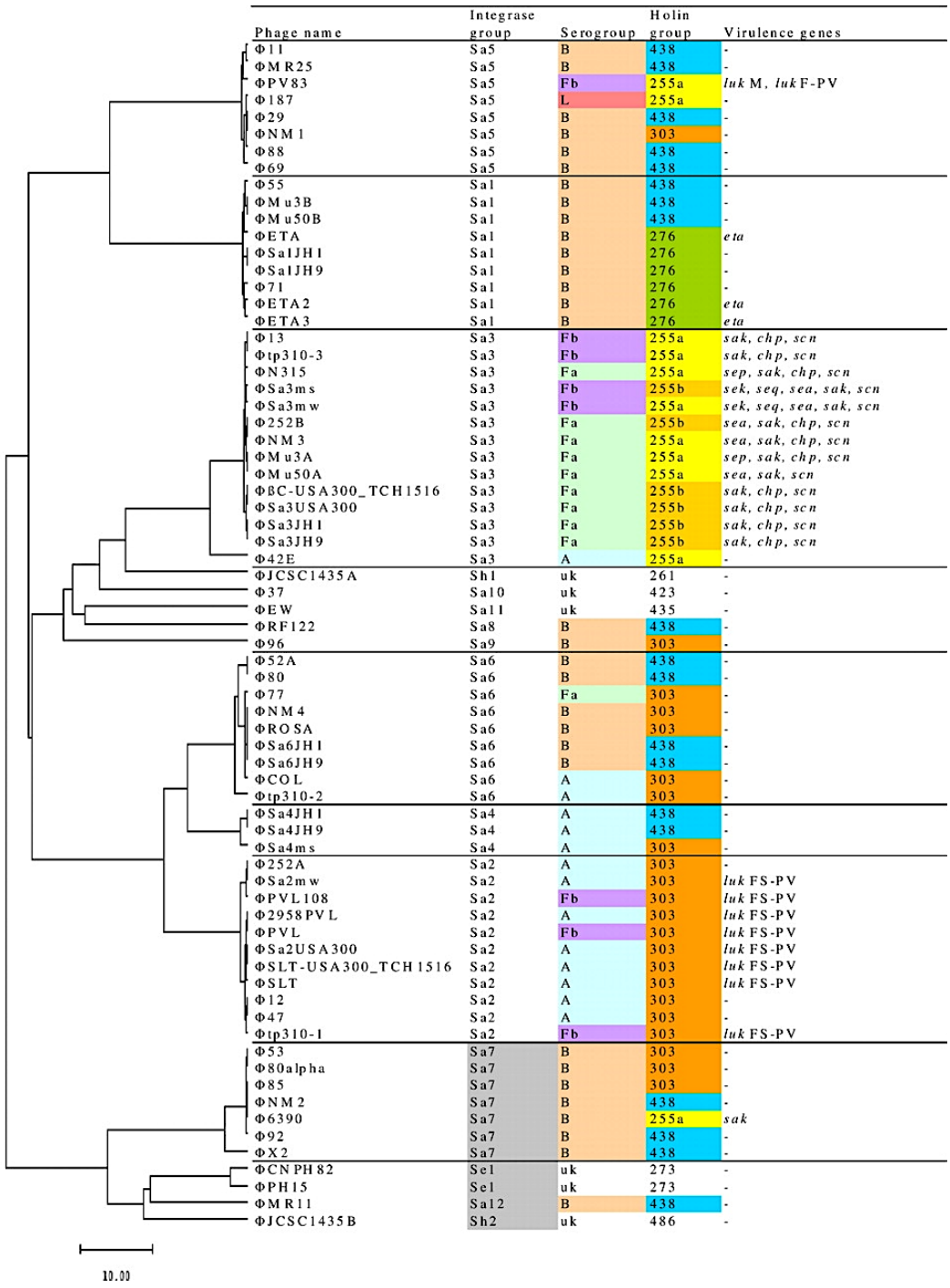

Figure 2.6 | Classification of *Staphylococcus aureus* prophages (Class = Siphoviridae) based on integrase type, serogroup and holin group. Identical serogroups and holin groups are color coded. Integrases of the serine recombinase-type family are shaded in gray. #Reproduced from Goerke et al. (2009)

2.4.8 Sa2int and Sa3int prophages in S. aureus virulence and human adaptation

The prophages of *S. aureus* associated with human and animal infections harbor a distinct set of virulence factors that may play a role in immune evasion, tissue evasion, toxins, adherence, and iron uptake or may code for toxins (Chaguza et al., 2022; Naorem et al., 2021). Epidemiological data strongly suggest that Sa2int and Sa3int prophages have co-evolved with *S. aureus* to facilitate its human adaptation (Chaguza et al., 2022; Laumay et al., 2021). These prophages usually encode a distinct set of virulence factors. Sa2int prophages often encode pore-forming toxins like Panton-Valentine leukocidin (PVL), a bi-component pore-forming leukotoxin encoded by various genes (*lukSF*, *lukED, lukAB/HG)* predominantly causing skin and soft-tissue infections (Kaneko et al., 1998; Oliveira et al., 2018). The leukotoxin lukSF targets and lyses human macrophages, polymorphonuclear leukocytes and monocytes and also incites the human inflammatory immune responses (Yoong and Pier, 2012). LukED promotes *S. aureus* replication by directly killing phagocytes and thus is considered critical for bloodstream infection. Similarly, LukAB/HG promotes the survival of *S. aureus* in human whole blood, restricts neutrophil-mediated killing and promotes *S. aureus* pathogenesis (Alonzo et al., 2012). On the other hand, Sa3int prophages lack leukotoxins but encode a distinct set of genes known as human immune evasion cluster (IEC) genes and enterotoxin A (*eta/sea*). The IEC genes of Sa3int prophages are strongly associated with human hosts as they are rarely found in animal-associated *S. aureus* (Chaguza et al., 2022).

Multiple studies have shown that more than 95% of *S. aureus* colonizing human nasopharynx carry Sa3int prophages integrated into the *hlb* gene, whereas invasive *S. aureus* isolates tend to lose these prophages (Goerke et al., 2009; Goerke et al., 2006; Rohmer and Wolz, 2021). The integration of Sa3int prophage disrupts the expression of

the sphingomyelinase beta-haemolysin, an important virulence factor, under specific infection conditions. The Sa3int prophages remain highly mobile (inducible) in the bacterial chromosome and thus can restore the interrupted *hlb* gene function when needed. The activation of *hlb* function may be achieved via active lysogeny, temporal re-localization of the prophage or prophage curing in a fraction of the bacterial population (Rohmer and Wolz, 2021).

Virulence factors encoded by genes carried by Sa3int phages include staphylokinase, enterotoxins, chemotaxis-inhibitory protein, and staphylococcal complement inhibitor, all of which are highly human specific and probably essential for bacterial survival in the human host. The transmission of *S. aureus* from humans to animals strongly correlates with the loss of Sa3int phages, whereas phages are regained once a strain is transmitted from animals to humans. Thus, both the insertion and excision of prophages may confer a fitness advantage to this bacterium. There is also growing evidence that Sa3int phages may perform 'active lysogeny', a process during which prophages are temporally excised from the chromosome without forming intact phage particles. The molecular mechanisms controlling the peculiar life cycle of Sa3int phages remain largely unclear. Nevertheless, their regulation is likely fine-tuned to ensure bacterial survival within different hosts (Rohmer and Wolz, 2021). Further, as Sa3int prophage is strictly human host-adapted and preferentially integrates into the *hlb* gene, researchers have proposed *hlb* as a molecular biomarker for pathogenicity of *S. aureus* (Miruka et al., 2022)

Table 2.2 | *Staphylococcus aureus* virulence determinants encoded on prophages.

Toxin/virulent determinant	Genes	GO* Molecular function	GO* Biological process	Function/mechanism of action	Prophage
Chemotaxis inhibitory protein of *S. aureus* (CHIPS)	chp, chs	-	Virulence	Blocks C5a and fMLP-induced neutrophil activation and chemotaxis; blocks C5a and formylated peptide receptor	ϕ13, ϕtp310-3, ϕN315, ϕ252B, ϕNM3, ϕMu3A, ϕSa3USA300, ϕSa3JH1, ϕSa3mw, ϕSa3ms, ϕSa3JH9, ϕβC-USA300_TCH1516
Exfoliative toxin A	eta	Hydrolase, Protease, Serine protease, Toxin	Virulence, Metal binding (calcium)	Has serine protease-like properties and binds to the skin protein profilaggrin. Cleaves substrates after acidic residues. Exfoliative toxins cause impetigous diseases commonly referred as staphylococcal scalded skin syndrome (SSSS), Ritter disease, and bulbous impetigo in neonates	ϕETA, ϕETA2, ϕETA3
Enterotoxin A	entA, sea	Enterotoxin, Superantigen, Toxin	Metal-binding (zinc)	Super antigen (SAg), activates the host immune system by binding as unprocessed molecules to MHC complex class II and TCR molecules	ϕSa3ms, ϕSa3, ϕSa3mw, ϕ252B, ϕNM3, ϕMu50A,
Enterotoxin G	entG, seg	Enterotoxin, Superantigen, Toxin	Virulence	SAg, known to cause food poisoning	ϕSa3
Enterotoxin K	entK, sek	Enterotoxin, Superantigen, Toxin	-	SAg, known to cause food poisoning	ϕSa3ms, ϕSa3mw
Enterotoxin K2	entK2, sek2	Enterotoxin, Superantigen, Toxin	-	SAg, known to cause food poisoning	ϕSa3
Enterotoxin P	entP, sep	Enterotoxin, Superantigen, Toxin	-	SAg, known to cause food poisoning	ϕN315, ϕMu50A
Enterotoxin Q	entQ, seq	Enterotoxin, Superantigen, Toxin	-	SAg, known to cause food poisoning	ϕSa3ms, ϕSa3mw
Leukocidin	lukM, lukF	Toxin	Cytolysis Haemolysis Virulence	Pore-forming leukocyte toxin that causes cytotoxic changes in polymorphonuclear leukocytes.	ϕPV83

Pantone-Valentine leukocidin	lukF-PV, hlgA, hlg2, lukS-PV	Toxin	Cytolysis Haemolysis Virulence	Pore-forming leukocyte toxin, linked by epidemiology to necrotic infections. Gamma-hemolysin causes haemolysis in red blood cells. Toxicity requires sequential binding and synergistic association of a class S and a class F component which form heterooligomeric complexes. HlgA (class S) associates with HlgB (class F) thus forming an AB toxin in strains producing both gamma-hemolysins and leukocidins. HlgA and LukF-PV can also form a complex.	ϕSa2mw, ϕPVL108, ϕSa2, ϕSa2USA300, ϕSLT, ϕPVL, ϕSLT-USA300_TCH1516, ϕtp310-1, ϕ2958PVL
Staphylococcal inhibitor of complement (SCIN)	scn	-	Virulence	Inhibits phagocytosis of *S. aureus* by human neutrophils; blocks formation of C3b. Involved in countering the first line of host defense mechanisms. Efficiently inhibits opsonization, phagocytosis and killing of *S. aureus* by human neutrophils. Acts by binding and stabilizing human C3 convertases (C4b2a and C3bBb), leading to their inactivation. The convertases are no longer able to cleave complement C3, therefore preventing further C3b deposition on the bacterial surface and phagocytosis of the bacterium. Also prevents C5a-induced neutrophil responses	ϕ13, ϕtp310-3, ϕN315, ϕSa3mw, ϕ252B, ϕNM3, ϕMu50A, ϕSa3JH1, ϕSa3 ms, ϕSa3JH9, ϕMu3A, ϕSa3USA300, ϕβC-USA300_TCH1516
Staphylokinase	sak	Kinase	Plasminogen activation	Proteolytic destruction of host tissue; activates conversion of plasminogen to plasmin; inhibits opsonization by degradation of IgG and C3b, promotes resistance to defensins	ϕN315, ϕMu50A, ϕSa2, ϕSa3mw, ϕ6390, ϕ13, ϕ252B, ϕNM3, ϕMu3A, ϕSa3 ms, ϕtp310-3, ϕβC-USA300_TCH1516, ϕSa3USA300 ϕSa3JH1, ϕSa3JH9

*GO = Gene Ontology based on UniProt database search, MHC = major histocompatibility, TCR = T-cell receptor, #Adapted from Malachowa and DeLeo (2010)

2.5 PROPHAGE INDUCTION AND ITS ANTIBIOTIC-DEPENDENT MOBILITY

Based on the completeness and integrity of their sequences, prophages can be induced either spontaneously or under stress by external factors. A lytic prophage induction leads to the release of infecting phage particles lysing the host bacteria while a chronic induction of prophage leads to the excision of prophage from the bacterial chromosome and release of phage particles without lysis of the host bacteria, usually observed in 'filamentous' phages (Secor et al., 2020; Secor et al., 2015; Venturini et al., 2022). Most prophages do not produce phage particles, as only a small percentage of lysogens undergo productive induction (Lwoff, 1953). Inducible prophages have been shown to alter the fitness of bacteria as well as modulate the microbiome in the environment as well as animals, through various mechanisms.

Under appropriate conditions, a prophage can be induced, which initiates the lytic life cycle and ultimately releases phage particles (St-Pierre and Endy, 2008). Prophage induction can be spontaneous or can be initiated by changes in the external environment leading to cellular stress. Factors such as antibiotics, certain nutrients, and changes in pH and temperature can trigger prophage induction (Allen et al., 2011; Goerke, Koller, et al., 2006; Oh et al., 2019). The induced phages may then alter the microbiome composition (Mills et al., 2013). A fructose-enriched diet and short-chain fatty acids (SCFA) were recently added to the list of prophage inducers in lactobacilli (Oh et al. 2019a).

In addition to expression of prophage encoded toxins, induced prophage also expands the phage community that aggravates disease under certain physiological stress like antimicrobial exposure. Treatment of shigatoxigenic *E. coli* infection with quinolones

adversely affects the clinical consequences because shigatoxigenic prophage induction is accelerated under antibiotic pressure. This behaviour favours clonal expansion of the lysogenic strains because induced phages can lysogenize other closely related strains. Recent discoveries in genome and microbiome sequencing have further proven that inducible prophages carrying key virulent factors are abundant in clinical strains. As inappropriate antibiotic use is common globally, and prophage induction is enhanced under antibiotic stress, it is believed that prophage-mediated fitness not only shapes the microbial ecology but also has a significant impact on modulating the microbiome in human health. Altogether, these findings suggest that prophage domestication and/or induction play a considerable role in patho-adaptation and provide key survival benefits for their bacterial hosts, thereby significantly increasing the disease burden through various mechanisms. Furthermore, released phages can also stimulate the mammalian immune system to activate antiviral inflammatory responses that abrogate bacterial phagocytosis (Wahida et al., 2021). Further, prophages may gradually degrade into an incomplete non-inducible sequence and undergo rapid diversification during bacterial evolution.

2.6 S. AUREUS PROPHAGE-ENCODED VIRULENCE AND ITS SIGNIFICANCE IN CRS

Chronic rhinosinusitis is a highly heterogeneous inflammatory disease of nasal and paranasal sinuses that is exacerbated by long-term exposure to various etiological agents, including *S. aureus*. Epithelial barrier dysfunction, aberrant eicosanoid metabolism, poor wound healing, and dysfunctional host-bacteria interactions usually lead to recalcitrant disease and worse surgical outcomes in CRS (Vickery et al., 2019). *S. aureus* is well known to produce many prophage-encoded virulence factors and

enterotoxins that promote inflammation (Kuhl et al., 2012). The toxins of *S. aureus* are potent T-cell activators which result in excessive and uncoordinated T-cell responses resulting in eosinophil activation, one of the important inflammatory pathways of CRS (Schubert, 2001). The inflammatory responses found in CRS are predominantly T-helper 1 (Th1) (predominant in CRSsNP) and T-helper 2 (Th2) (predominant in CRSwNP) type according to the cytokines they produce, which can be regulated by *S. aureus*. Biofilm is another factor that leads to poor surgical outcomes in CRS (Psaltis et al., 2008). Antibiotics and host immune responses are largely ineffective against *S. aureus* bacteria within biofilms leading to persistent infection.

Lysogenic conversion of *S. aureus* not only arms *S. aureus* with toxins and virulence factors responsible for human immune evasion and adaptation, but also alters the biofilm formation (Fernandez et al., 2018). Prophage-encoded extracellular proteins secreted by *S. aureus* like IEC (*sak, scn, chp*), enterotoxin B (*entB/seb*), and various leukocidins have been directly associated with CRS exacerbations (Chegini et al., 2022). Co-ordinated prophage induction is known to landscape the microbiome by killing the susceptible competitors thereby establishing persistent colonization by a virulent strain. Since CRS is an inflammatory disease that is thought to be modified by *S. aureus*, all factors that promote *S. aureus* colonization directly or indirectly impact CRS pathophysiology skewing it towards a more severe form.

2.7 SUMMARY AND SCOPE

In summary, it is now established that prophages constitute 10-20% of the *S. aureus* bacterial genome, encoding many putative virulence factors, antibiotic resistance determinants and regulatory proteins. Active lysogeny is known to fuel rapid, parallel adaptations within an infection to establish chronic infection either by expressing the auxiliary genes or by disrupting the function of the gene of its insert. Further, the productive induction of the prophages in small populations during the stress of competition results in the killing of the susceptible competing population but not the sibling clone that already has a dormant prophage leading to clonal expansion of the lysogen. These recombination events and competition ultimately lead to the generation of persistent hypervirulent phenotypes associated with poor clinical outcomes.

As more prophages are reported in clinical strains of bacteria and associated with chronic infections, it is important to study their prevalence and elucidate their association with disease including CRS. Further, as prophage induction impacts bacterial pathogenesis and fitness, including but not limited to biofilm, metabolic activity, growth kinetics, nutrient competition, and, more importantly, antiviral immune response in humans, it is important to understand how antibiotics and/or other drugs affect prophage activity in inflammatory diseases like CRS. Emerging evidence has also suggested that lysogenic conversion significantly impacts microbiome equilibrium, either lysogenizing the susceptible strain or lysing the competing strain, which ultimately modulates disease development. Further, the expression of prophage-encoded toxins can arm commensal bacteria with deadly toxins and cause an outbreak of virulent clones. As domestication of prophages modulates bacterial fitness and

augments pathogenicity, it is time we look deeper into bacterial genomes for prophage and its role in disease development.

Taken together, there is no doubt that bacteria-phage interactions represent a complex synergistic relationship that drives evolutionary processes in the microbial world, often benefitting the microbe, suggesting profound and direct effects on human health and physiology. In CRS, although it has been established that chronic infection by high biofilm-forming *S. aureus* leads to poor CRS pathophysiology and recovery, factors leading to such outcomes are not completely understood. Prophages of *S. aureus* are particularly important because it has already been established that most of the disease-causing virulent clones of *S. aureus* are prophage-rich and express multiple prophage-encoded toxins and virulence factors that equip the bacteria with robust immune evasion capability. However, the study of prophage-encoded virulence and developing them as biomarker tools is challenging because, prophages are extremely diverse, mosaic, flexible and transient MGEs. We currently grasp only a small fraction of the total *S. aureus* prophage diversity, both in terms of genetic content and novel molecular mechanisms enabling modulation of bacterial phenotypes and lifestyles, including virulence. As such, elucidating their role in CRS disease progression and/or severity remains elusive and is not well studied in clinical settings.

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Executive summary of reviewed literatures

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Prophage: a crucial catalyst in infectious disease modulation

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Prophage: a crucial catalyst in infectious disease modulation

Lysogenic conversion, in which a temperate bacteriophage sequence integrates into the bacterial genome and forms a prophage, is one of the most efficient mechanisms bacteria use to acquire accessory traits. The contribution of prophage genetic material to the bacterial DNA might constitute up to 20% of the bacterial genome, with variation between species and strains. Furthermore, it has been suggested that approximately 25% of all the bacteriophage genomes on the Earth exist as a prophage (Bondy-Denomy and Davidson, 2014). These prophage sequences propagate vertically to progeny with bacterial cell division, excise and replicate separately as a plasmid, or induce and enter the lytic cycle to form new bacteriophage particles spontaneously or under the influence of various inducers. Prophages are capable of efficiently transferring genes vertically and horizontally. Such extrachromosomal plasmids and induced prophages can transduce to susceptible cells present in the same biome, thereby disseminating their genetic material and contributing to the adaptive evolution of bacteria (Silveira and Rohwer, 2016). Induced prophages can also infect and kill competing colonisers (Maslov and Sneppen, 2017).

Lysogeny generates diversity among strains and allows bacteria to fine-tune their econiche adaptation and, at the same time, confers immunity against secondary bacteriophage attacks (Figure 2.7). Both are crucially important for the survival and dominance of the lysogen within its habitat. As an example, Duerkop and colleagues have shown that a prophage induction from *Enterococcus faecalis* was necessary and

sufficient for the lysogen to gain dominance over competing strains in a mouse model (Duerkop et al., 2012). The inducibility of a prophage is likely to be of crucial importance, at least to some pathogens, because it allows prophages to become more fit to dominate the econiche, potentially contributing to disease pathophysiology. However, prophages might gradually degrade into incomplete sequences. Although incomplete prophages cannot enter the lytic cycle anymore, potentially making their host susceptible to competition for space and nutrients from related strains, they can still contribute important remnant prophage genetic material to the host. Polylysogeny, in which a single bacterial strain carries more than one prophage sequence, is common and prophage remnants have been shown to contribute their genetic material to form hybrid novel bacteriophage particles once induced into the lytic cycle (Duerkop et al., 2012). Such hybrid bacteriophage particles are used by their host as a weapon, infecting and lysing related strains during colonization (Duerkop et al., 2012).

Prophages are also known to contribute to the virulence potential of their host bacteria by encoding toxins that can cause deadly outbreaks. These include prophage-mediated toxicity in *Corynebacterium diphtheriae* (diphtheria toxin), *Clostridium botulinum* (botulinum toxin), *Vibrio cholera* (cholera toxin), *Escherichia coli* O157:H7 (Shiga toxin), and *Salmonella enterica* (SopE effector protein) (Feiner et al., 2015). Apart from toxins, prophages can supply bacteria with multiple functions because they might also encode auxiliary metabolic genes, virulence factors, antimicrobial resistance genes, and immune evasion genes, which are often present in clusters. β-haemolysin-converting

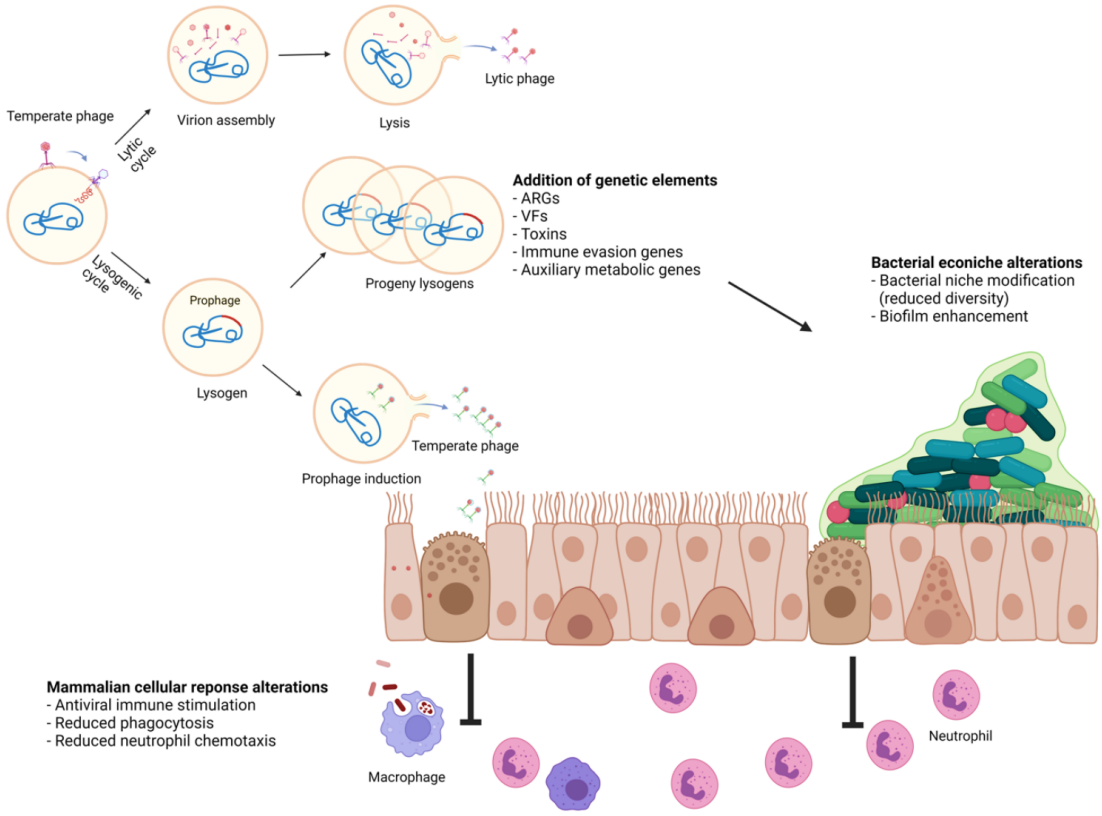

Figure 2.7 | Various mechanisms how prophage impacts bacterial fitness. Prophages may carry various antimicrobial and virulence genes that may lead to better survival of bacteria in different econiche. Prophage induction also may lead to antiviral response in mammalian cells including humans thereby avoiding the phagocytosis and reducing the bacterial clearance. #Reproduced from Nepal et al. (2022)

bacteriophages (βC-ϕs) typically encode immune evasion cluster (IEC) genes in *Staphylococcus aureus.* Although the presence of IEC genes does not assist with initial colonisation, they are associated with disease severity in chronic inflammatory diseases, such as chronic rhinosinusitis (Verkaik et al., 2011, Nepal et al., 2021). Immune evasion cluster genes encode various proteins that counteract the innate and adaptive immune systems in a multifaceted way, which includes inhibition of neutrophil-dependent phagocytosis and killing the βC-ϕ lysogen by blocking complement activation and reducing neutrophil chemotaxis. Immune evasion cluster genes also include the staphylococcal enterotoxin A gene, notorious for promoting a massive but inefficient polyclonal activation of the adaptive immune system, which is skewed away from a

protective response against *S. aureus* to benefit its own survival (Tuffs et al., 2018). Whereas bacteriophages are generally considered to target only bacteria in a highly specific way, in the past decade research has shown the possibility of bacteriophage uptake and subsequent synthesis of bacteriophage mRNA by mammalian cells, which resulted in the induction of antiviral inflammatory responses that reduce the efficiency of bacterial elimination by phagocytosis leading to chronic infection and inflammation (Gogokhia et al., 2019; Sweere et al., 2019). Targeting those prophages using active or passive immunisation could protect against lysogenic infections (Sweere et al., 2019).

In conclusion, there is increasing evidence that the coexistence of bacteria and prophages is associated with multifaceted bacterial fitness elevating the risk to human health. However, the role of lysogeny and prophage induction in immune evasion, supporting the survival and dominance of lysogens within their econiche, are poorly understood in clinical settings. Because prophages are very diverse, mosaic, and transient, they are likely to be important drivers shaping microbial ecosystems and a promising area for further investigation.

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CHAPTER THREE

PROPHAGES ENCODING HUMAN IMMUNE EVASION CLUSTER GENES ARE ENRICHED IN *Staphylococcus aureus* ISOLATED FROM CHRONIC RHINOSINUSITIS PATIENTS WITH NASAL POLYPS

Original research article

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Prophages encoding human immune evasion cluster genes are enriched in *Staphylococcus aureus* isolated from chronic rhinosinusitis patients with nasal polyps

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Abstract

Prophages affect bacterial fitness on multiple levels. These include bacterial infectivity, toxin secretion, virulence regulation, surface modification, immune stimulation and evasion and microbiome competition. Lysogenic conversion arms bacteria with novel accessory functions thereby increasing bacterial fitness, host adaptation and persistence, and antibiotic resistance. These properties allow the bacteria to occupy a niche long-term and can contribute to chronic infections and inflammation such as chronic rhinosinusitis (CRS). In this study, we aimed to identify and characterize prophages present in *Staphylococcus aureus* from patients suffering from CRS in relation to CRS disease phenotype and severity.

Prophage regions were identified using PHASTER. Various *in silico* tools like ResFinder and VF Analyzer were used to detect virulence genes and antibiotic resistance genes respectively. Progressive MAUVE and maximum likelihood were used for multiple sequence alignment and phylogenetics of prophages respectively. The disease severity of CRS patients was measured using computed tomography Lund Mackay scores.

Fifty-eight *S. aureus* clinical isolates (CIs) were obtained from 28 CRS patients without nasal polyp (CRSsNP) and 30 CRS patients with nasal polyp (CRSwNP) patients. All CIs carried at least 1 prophage (average = 3.6) and prophages contributed up to 7.7% of the bacterial genome. Phage integrase genes were found in 55/58 (~95%) *S. aureus* strains and 97/211 (~46%) prophages. Prophages belonging to Sa3int integrase group (phiNM3, JS01, phiN315) (39/97, 40%) and Sa2int (phi2958PVL) (14/97, 14%) were the most prevalent prophages and harboured multiple virulence genes such as *sak, scn, chp,*

*luk*E/D, *sea*. Intact prophages were more frequently identified in CRSwNP than in CRSsNP (p = 0.0021). Intact prophages belonging to the Sa3int group were more frequent in CRSwNP than in CRSsNP (p = 0.0008) and intact phiNM3 were exclusively found in CRSwNP patients (p = 0.007).

Our results expand the knowledge of prophages in *S. aureus* isolated from CRS patients and their possible role in disease development. These findings provide a platform for future investigations into potential tripartite associations between the bacteria-prophage-human immune system, *S. aureus* evolution and CRS disease pathophysiology.

Highlights

- 211 prophage regions were identified in 58 *S. aureus* genomes isolated from CRS patients suggesting widespread distribution of prophage elements in clinical strains colonizing nasal niche.
- Sa2int and Sa3int group prophages belonging to family *Siphoviridae* and genus *Biseptimavirus* were most frequently found in *S. aureus* from CRS patients.
- *S. aureus* isolated from CRS patients with nasal polyps predominantly harboured intact Sa3int group prophages encoding human immune evasion cluster (IEC) genes.
- Prophages in *S. aureus* did not encode any antibiotic resistant genes (ARGs).

Keywords:
phage; bacteriophage; S. aureus; phage-encoded virulence factors (PEVF); CRS

3.1 INTRODUCTION

Chronic rhinosinusitis (CRS) is a multifactorial inflammatory disease of the sinonasal mucosa associated with relapsing infections (Fokkens et al., 2020). Phenotypically, CRS is broadly differentiated into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). Development of polyp tissue results in reduced nasal airflow and anatomical obstruction of the sinus drainage pathways which exacerbates CRS symptoms and is often mirrored by elevated levels of inflammation seen on computed tomography (CT) (Garneau et al., 2015). The pathophysiology of CRS remains unclear and no single genetic and/or environmental factor has been solely linked to the development of this disorder. In the last decade, there has been increasing evidence that bacterial virulence, the presence of microbial mucosal biofilms and microbiome dysbiosis can affect the persistence of symptoms, disease severity and post-operative recovery (Copeland et al., 2018; Foreman et al., 2010; Nayak et al., 2016; Psaltis et al., 2008). Although *Staphylococcus aureus* is considered a commensal capable of colonizing diverse ecological niches within human and animals and is carried by ~30% of the human population asymptomatically (Chambers and Deleo, 2009; Tong et al., 2015), it is also one of the most invasive, highly pathoadaptive, opportunistic pathogens and etiological agent of diverse human and animal maladies including CRS. An increased colonization of *S. aureus* was demonstrated in patients with CRSwNP (64%) but not in patients with CRSsNP (33%) versus control (20%) patients suggesting contribution of *S. aureus* in CRS (Van Zele et al., 2004; Vickery et al., 2019). Of further concern is the emergence and spread of methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA). The successful pathoadaptive evolution of virulent *S. aureus* is largely due to

acquisition of large mobile genetic elements (MGEs) carrying virulence, toxin and resistance genes (Beceiro et al., 2013). Such MGEs include plasmids, transposons (Tn), insertion sequences (IS), *S. aureus* pathogenicity islands (SaPIs), staphylococcal cassette chromosomes (SCCs) and (pro)phages. They can be exchanged between strains by horizontal gene transfer (HGT) and/or transferred to progeny through vertical gene transfer (VGT) (Malachowa and Deleo, 2010; Hiramatsu et al., 2013; Lebeurre et al., 2019). Among multiple MGEs contributing to virulence and pathogenicity of *S. aureus*, active prophages are one of the most efficient elements, that can mobilize 'clusters' of genes between genetically related clones (Davies et al., 2016; Calero-Cáceres et al., 2019; Balcázar, 2020).

In contrast to virulent (lytic) phages that are unable to insert their DNA into the bacterial host genome, temperate (lysogenic) phages can integrate their DNA into the bacterial host genome or occasionally exist as extrachromosomal DNA. Once stably integrated, the phage DNA is named 'prophage' and the host bacteria becomes 'lysogenic'. By doing so, temperate phages can introduce and mobilize resistance genes, toxins and phage-associated virulence factors (VFs) via phage-mediated transduction (Loh et al., 2020), thereby altering bacterial genomic information and phenotype (Harrison and Brockhurst, 2017). Such prophages can switch to the lytic cycle through a variety of mechanisms, producing infectious phage particles provided they have all the functional and structural genes required for genome excision, replication and phage particle assembly. One mechanism by which this lysogenic to lytic switch can occur is because of biotic and/or abiotic stresses which gives rise to DNA damage (UV exposure, antibiotics, chlorine, H_2O_2) (Kim and Little, 1993; Shearwin et al., 1998; Jin et al., 2020).

In other phage, the switch to lytic development can be a stochastic decision, influenced by the density of phages in the environment (Selva et al., 2009; Sousa and Rocha, 2019).

As more genomic sequences of clinical isolates become available, a considerable number of prophages have been discovered recently and they have been shown to account for as much as 20% of the host genome (Khan and Wahl, 2020). Lysogens can release phages as weapons against other invading bacterial strains, accelerate clonal expansion of virulent bacteria through lateral transduction and/or trigger the immune system to produce specific antibodies that may worsen inflammatory disease (Wahida et al., 2021). Further, Li et al. (2002) demonstrated that integration of specific prophage φSA169 in methicillin-resistant *S. aureus* increased biofilm formation, enhanced δ-hemolysin activity and reduced vancomycin sensitivity.

There is growing evidence that accessory genes carried by prophages of *S. aureus* significantly modulate bacterial fitness as they carry multiple VFs. These VFs include human immune evasion cluster (IEC) comprising the genes *sak, chp, scn* and *sea/sep* which encodes staphylokinase, chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal complement inhibitor (SCIN) and enterotoxin A/P (SEA or SEP) respectively in different combinations (Van Wamel et al., 2006). In addition, they also comprise a bi-component cytotoxin Panton-Valentine leukocidin (PVL, *luk* F/S) and related leukocidins (*luk* M/F) involved in necrotic infections; and exfoliative toxin A (*eta*) involved in skin infections (Deghorain and Van Melderen, 2012; Shearwin and Truong, 2021). Furthermore, phage-associated virulence is strongly associated with the phage 'integrase' (*int* types) type in *S. aureus,* Sa3int type being the most abundant among nasal colonizers (Goerke et al., 2009). Further, expression of prophage-associated VFs

varies according to the infection site and external stimulus. Despite the widespread presence of prophages in *S. aureus* clinical isolates and their role in pathoadaptive gene acquisition, mobility, virulence and pathogenicity, prophages are one of the most understudied elements. Knowledge of prophage presence and organisation in *S. aureus* clinical isolates and their potential role in CRS disease pathophysiology is not known. Previous research by our team on *S. aureus* core genome (n = 58) found even distribution of virulence genes in CRS sub-groups (CRSsNP vs CRSwNP) and their origin, status and/or evolutionary association was elusive. Further, no significant difference in pathogenic gene abundance was observed between CRSsNP and CRSwNP (Bardy et al., 2018).

Here, we implement an *in-silico* approach to re-analyse the data focussing primarily on accessory genes (particularly prophages) in the genomes of 58 *S. aureus* clinical isolates from CRS patients. We report the discovery of 211 prophage-like regions and provide detailed insight into prophage types, genomics and their phylogenetics. We further explore the contribution of these prophages to the bacterial genome, major VFs they encode and investigate a possible contribution of prophage-rich lysogens in CRS disease status and severity.

3.2 MATERIALS AND METHODS

3.2.1 Ethics, bacterial isolates and measure of disease severity

S. aureus clinical isolates (CIs) were obtained from patients with CRS and non-CRS control patients at the time of endoscopic sinus surgery, isolated by an independent laboratory (Adelaide Pathology Partners, South Australia) and stored at -80°C in glycerol stocks (20%). CRS patients fulfilled the CRS diagnostic criteria according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS2020) (Fokkens et al., 2020). Control patients did not have symptoms of CRS with no evidence of mucosal inflammation on endoscopic evaluation of the nasal and paranasal sinuses. CRS type (CRSsNP or CRSwNP) was determined based on presence/absence of nasal polyp tissue and disease severity was scored based on Lund-Mackay (LMK) staging system (Hopkins et al., 2007) by the surgeon (PJW and AJP) at the time of clinical isolate collection. Ethics approval and written informed consents from each patient was obtained prior to the study for the use of *S. aureus* clinical isolates (HREC/18/CALHN/69).

3.2.2 Prophage prediction and characterization within S. aureus genomes

Integrated prophage regions were predicted using PHASTER (Phage Search Tool – Enhanced Release) (https://phaster.ca/) with default settings (Supplementary text S1) and the regions were classified as intact, questionable and incomplete (Arndt et al., 2016) which roughly translates to active (intact) and inactive (questionable and incomplete). Further, prophage sequences, putative prophage attachment sites (*attL/attR*), the GC percentage, size, protein hit (total ORFs), most similar phage and details of protein family were manually identified and extracted from the output. Most

similar prophage was further queried against viral nr/nt NCBI database (taxid:10239) and Virus-Host DB (https://genome.jp/virushostdb/) to predict the prophage family and genus based on their maximum homology. All visualizations were performed using GraphPad Prism 9 (Ver 9.1), R (Ver 4.0.0) in RStudio (Ver 1.3.1093) using the R package 'ggplot2' (Ver 3.3.2) unless stated otherwise.

3.2.3 In silico detection of virulent and AMR genes within prophages

A concatenated DNA sequence file (FASTA) of prophage sequences was created. Antimicrobial resistance genes (ARGs) and virulence factors (VFs) associated with *S. aureus* were scanned within the prophage sequences using ResFinder 4.1 (Bortolaia et al., 2020) and VFanalyzer (Liu et al., 2019) respectively. The biological (pathogenesis) and/or molecular function for major VFs associated with prophage was assigned according to the gene ontology (GO) knowledgebase through UniProtKB (https://uniprot.org/).

3.2.4 Multiple sequence alignment and prediction of major phage-associated VF clusters

The complete sequences of predicted prophages were extracted and concatenated in a separate file (FASTA) with most similar phage hit as a reference. Groups with more than four intact prophage hits were considered. The prophage sequences of all intact and questionable prophages were aligned with the reference sequence (extracted from NCBI) using progressive Mauve in R package 'genoPlotR' (Guy et al., 2010). Only major

pathogenic genes were visualised in MSA analysis. Incomplete prophages (scores < 70) were excluded. To determine the IEC clusters, a customised BLAST database was created with the amino acid sequences of the 5 possible genes (*sea, sep, chp, sak, scn*). Each intact Sa3int group prophage genome was then compared against the database using BLAST, specially using the blastx algorithm. A threshold of 95% identity was chosen as the cut-off for presence of the genes. The prophages were then assigned into the clusters based on the classification by Wamel et. al. (2006).

3.2.5 Genome assembly and phylogenetics

For *S. aureus*, genomes were assembled using Unicycler (v 0.4.8) and annotated with Prokka (v 1.14.6). Assemblies were quality controlled using QUAST (v 5.0.2) (Gurevich et al., 2013; Seemann, 2014; Wick et al., 2017). CIs were grouped into clonal complexes (CC) by assigning Multi-Locus Sequence Typing using the program MLST (Jolley et al., 2018). The core genome of *S. aureus* isolates was inferred with Roary (v 3.7.0) with the Prokka annotations as input (Page et al., 2015). This core genome alignment was used to create a maximum likelihood phylogenetic tree using IQtree (v 2.0.3) (Minh et al., 2020). Specifically, the resulting maximum likelihood tree was created using 1000 ultrafast bootstrap replicates, applying the SH-like approximate likelihood ratio test (Guindon et al., 2010). For prophage phylogenetics, DNA sequences of all putative prophage were aligned using MAFFT7 (Multiple Alignment using Fast Fourier Transform, ver 7) (Katoh, 2002) and a maximum likelihood tree was created with FastTree 2.1 (Price et al., 2010). Further, amino acid (aa) sequences for integrase genes were extracted from PHASTER annotations. Representative integrase sequences (Sa1int-Sa12int) were

retrieved from NCBI (Goerke et al., 2009; Kahánková et al., 2010; Ene et al., 2021), aligned along with query sequences using MAFFT and phylogenetic diversity was inferred using FastTree 2.1 in Geneious Prime 11.09 (ver 21.1, Biomatters Ltd. Auckland, New Zealand). All trees, unless specified, were visualized using iTOL V5 (https://itol.embl.de) (Letunic and Bork, 2019). The percentage identity heat-map matrix was also exported from Geneious Prime 11.09. Further, integrase sequences of unassigned phages were retrieved from Virus-Host DB (https://genome.jp/virushostdb/) and homology was inferred using similar approach as mentioned above.

3.2.6 Statistical analysis

Descriptive statistical methods were used to determine the frequency, percentage, and means while one-way ANOVA was used to compare between groups. Fisher's exact test (two-tailed) was used to determine the significance of each prophage (intact) between CRSsNP/CRSwNP and lower/higher LMK severity groups. Unless mentioned, all statistical analyses were performed using GraphPad Prism 9 (ver 9.1) and $p < 0.05$ was considered statistically significant. No statistical methods were used for the predetermination of sample size and experiments were not randomized.

3.3 RESULTS

3.3.1 Prophages are significant components of S. aureus clinical isolates

Although *S. aureus* has often been associated with CRS, phylogenetics analysis has failed to correlate any specific sequence type (ST) or clonal complex (CC) with CRS disease severity and/or phenotype including methicillin resistance. We analysed genomes of *S. aureus* clinical strains isolated from CRS patients (n = 58) and control (n = 9). All CIs were predicted to be lysogenic as they carried at least 1 recognizable prophage (range = 1-10, average = 3.63 prophages/strain) (Figure 3.1A, Figure 3.1B). All *S. aureus* from control patients had at least 1 intact prophage. Among 58 strains isolated from CRS patients, 53 (91%) were poly-lysogenic (Figure 3.1A), 47 (81%) harboured at least one 'intact' prophage, 4 (7%) had only 'incomplete' prophages whereas 7 (12%) had a combination of questionable and incomplete prophages (Figure 3.1B). Altogether, 211 prophage-like sequences were predicted from 58 *S. aureus* genomes (Figure 3.1A and 3.1C). Out of those, 64 (30%, average = 1.1/strain) were intact, 33 (16%, average = 0.57/strain) were questionable and, 114 (54%, average = 1.96/strain) were incomplete. The mean genome size of intact, questionable and incomplete prophage was 44.30, 27.83 and 17.83 kb respectively (Figure 3.1C) Prophages accounted for a maximum of 220.8 kb which amounts to 7.7% (average = 3.57%) of the total bacterial genome (Figure 3.1D). Although there was no significant difference in average prophage percentage between CRSsNP and CRSwNP groups, the density of intact prophages was significantly higher in CRSwNP group (Figure 3.1E) and most of them belonged to the size range of 20-70 kb (Figure 3.1F). The average GC% of the bacterial genome was 32.7% (range = 32.6-32.8), whereas

the average GC% of intact and incomplete prophages was 33.54% (range = 31.93-36.31) and 30.92 (range = 25.56-34.93) respectively (Figure 3.1G).

From the 58 CRS patients, 28 were classified as CRSsNP and 30 as CRSwNP. Although the average number of prophage regions was similar between CRSsNP (3.64/strain) and CRSwNP (3.63/strain), intact prophages were more frequently identified in CRSwNP (29/30, 96.6%, average = 1.3/strain) than in CRSsNP (18/28 (64.28%, average = 0.89/strain) ($p = 0.0021$, Fisher's exact test) (Table 3.1). Similarly, intact prophages were more frequent (29/32, 91%, average = 1.21/strain) in *S. aureus* strains isolated from CRS patients with more severe disease (LMK score > 12) compared to those with less severe disease (LMK score < 12) (16/22, 72%, average = 1.04/strain) even though statistical significance was not reached ($p = 0.1363$, Fisher's exact test, Table 3.1). Similar analysis of *S. aureus* isolated from 'control' group (n = 9) revealed that at least one intact prophage was present in all strains (9/9, 100%, average = 1.33/strain), indicating prophage associated adaptation is common in human nasal colonization and prophage retention and/or gain may occur at the later stage.

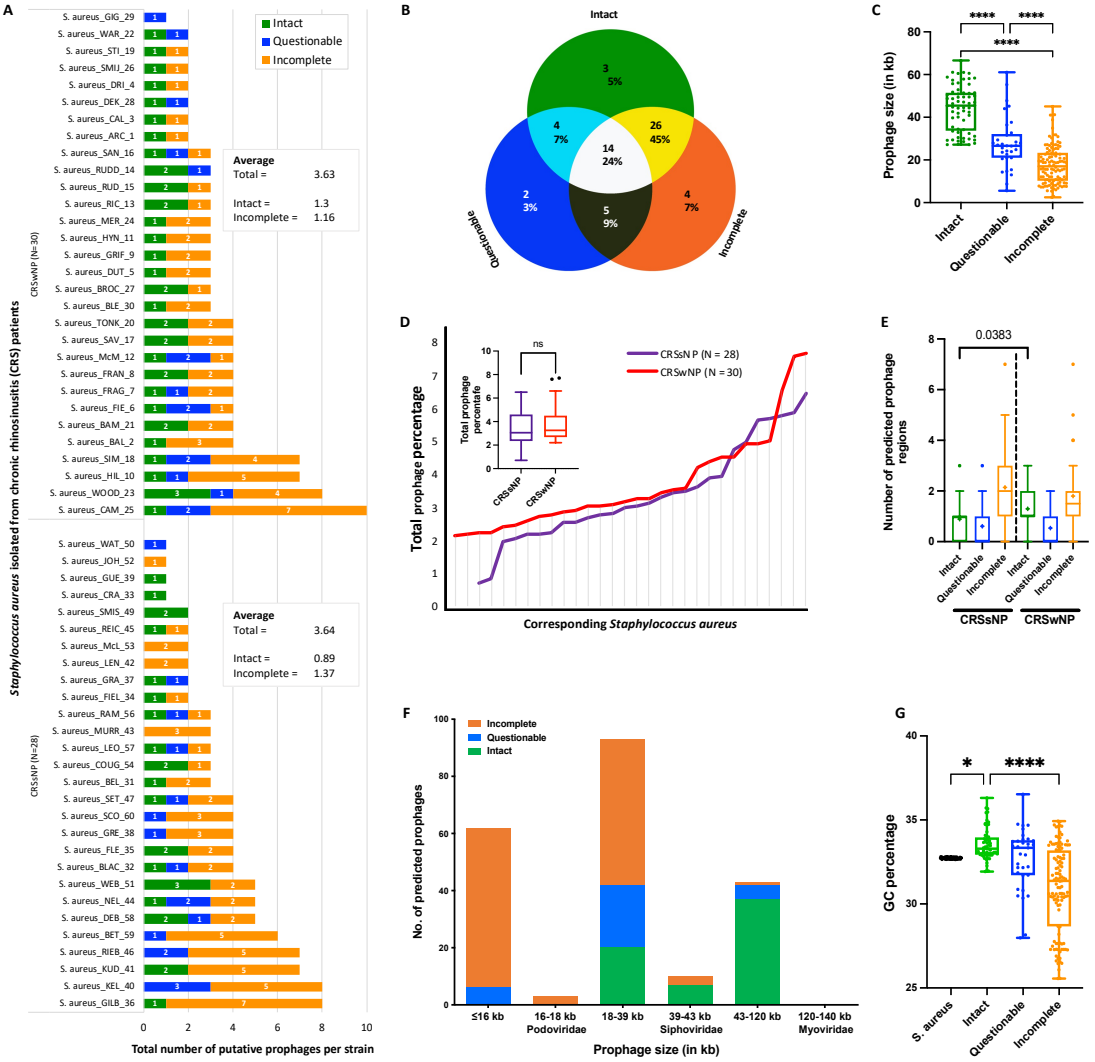

Figure 3.1 | Prediction and Distribution of prophages from *S. aureus* genome. (A) Among 58 clinical strains, 53 (~91%) were poly-lysogenic, while only 5 strains had a single prophage. Out of total 211 (3.6 prophages/strain) predicted prophages, 64 (30.33%) were intact, 33 (15.64%) were questionable and 114 (54.03%) were incomplete. The numbers inside the bar represents number of prophages. (B) Venn-diagram representing distribution of prophages. Out of 58 strains, 47 harbored at least one intact prophage, 4 had only incomplete prophage while 7 had mix of questionable and incomplete prophages but lacked intact prophages. (C) Distribution of predicted prophages according to their size. The average size of prophages decreased from intact to incomplete. The solid red line represents median. (D) The genome shares of prophages on the host genome ranged from 0.7% to 7.7% (average = 3.6%). The box plot on the inset shows difference in prophage genome between CRSsNP and CRSwNP. Although prophage content in CRSwNP was relatively higher, the difference was not statistically significant. (E) Distribution of prophages between CRSsNP and CRSwNP. The number of intact prophages was significantly higher in CRSwNP (p = 0.038, Welch's t-test). (F) Distribution of candidate prophage regions based on their predicted size and reference genome size. All intact prophages fell in size range closer to Siphoviridae (39-43 kb). (G) Comparison of GC% across host genome, combined prophage, and different types of prophages. The average GC% of the host (*S. aureus*) was 32.72% compared to 31.98% of the combined prophages. Further, the average GC% of intact, questionable and incomplete prophages were 33.5%, 32.7% and 30.9% respectively.

Table 3.1 | Correlation between CRS disease status/severity and presence of prophages in *S. aureus* recovered from CRS patients.

CRS disease type/severity		Intact prophage			p-value (Fisher's exact test)
		Average density	Present	Absent	
Disease phenotype	CRSsNP (N = 28)	0.89	18	10	0.0021 (significant)
	CRSwNP (N = 30)	1.30	29	1	
Disease severity (LMK)[b]	LMK ≤ 12 (N = 22)	1.04	16	6	0.1363
	LMK > 12 (N = 32)	1.21	29	3	
Control (N = 9)		1.33	9	0	

a Only intact prophages considered as they are likely functional and comprise complete sets of genes (including virulence genes), have ability to switch between lytic-lysogenic cycle and pass virulence to other strains. CRSsNP = Chronic rhinosinusitis without nasal polyposis, CRSwNP = Chronic rhinosinusitis with nasal polyposis. LMK = Lund-Mackay score (0-24). Density = Number of intact prophages/S. aureus strain. b LMK scores only available for 54 patients. Refer to supplementary figure S4/data.

3.3.2 Prophage genomes significantly contribute to S. aureus strain variability

We then compared the distribution and abundance of phage-hit genes across intact, questionable and incomplete prophages through a heat-map according to their corresponding structural and/or functional gene families assigned by PHASTER. Among 211 predicted prophages, only 118 (56%) were flanked by at least one pair of attachment sites *(attL/attR)* (Supplementary data S2). Similarly, head-like protein genes were found in 125/211 (59%) followed by tail in 92/211 (44%) and capsid in 51/211 (24%). Integrase genes were found in 97/211 (46%) followed by portal in 86/211 (41%), terminase in 75/211 (36%) prophages. Lysin, protease, transposase and recombinase were less frequent and found only in 28/211 (13%), 25/211 (12%), 19/211 (9%) and 2/211 (1%) prophages, respectively (Figure 3.2A-B). Compared to intact prophages, incomplete prophages often lacked tail, capsid, portal, terminase, lysin and protease genes. Further, transposases were relatively more frequent in incomplete prophages (15/114, 13%) than in intact prophages (2/64, 3%) whilst recombinase genes were found exclusively in incomplete prophages (Figure 3.2C). However, as genomes are spliced at these regions during short-read sequencing, this may be underestimated and thus

carefully reported. Altogether, 7523 open reading frame hits (ORF-hits) (average = 35.65 ORFs/prophage, including hypothetical proteins) were predicted from 211 prophage regions (Supplementary data S2). Out of those, 3655 (48%), 1177 (16%) and 2691 (36%) were in intact, questionable and incomplete prophages respectively (Table S). Further, 6693 (89%) had known functions, mainly involved in phage structure, transcription, replication, and lytic/lysogenic regulation, while 830 (11%) were 'hypothetical' with unknown function. The total number of phage-hit proteins (including hypothetical) and the total prophage genome size significantly correlated with the size of *S. aureus* genome ($p < 0.0001$, linear regression fit, Figure 3.2D).

3.3.3 Gene density in a prophage is inversely proportional to its genome size

The number of phage-hit proteins in prophage genomes positively correlated with the size of the prophage ($r^2 = 0.86$, $p < 0.0001$) (Figure 3.3A) and the GC% was higher in larger prophage genomes (Figure 3.3A). In addition, prophage sequences had a high gene density (average = 1.43 genes/kb) (Figure 3.3B) which was highest in smaller prophage sequences (genome size < 10 kb) and those had relatively low GC% (Figure 3.3B).

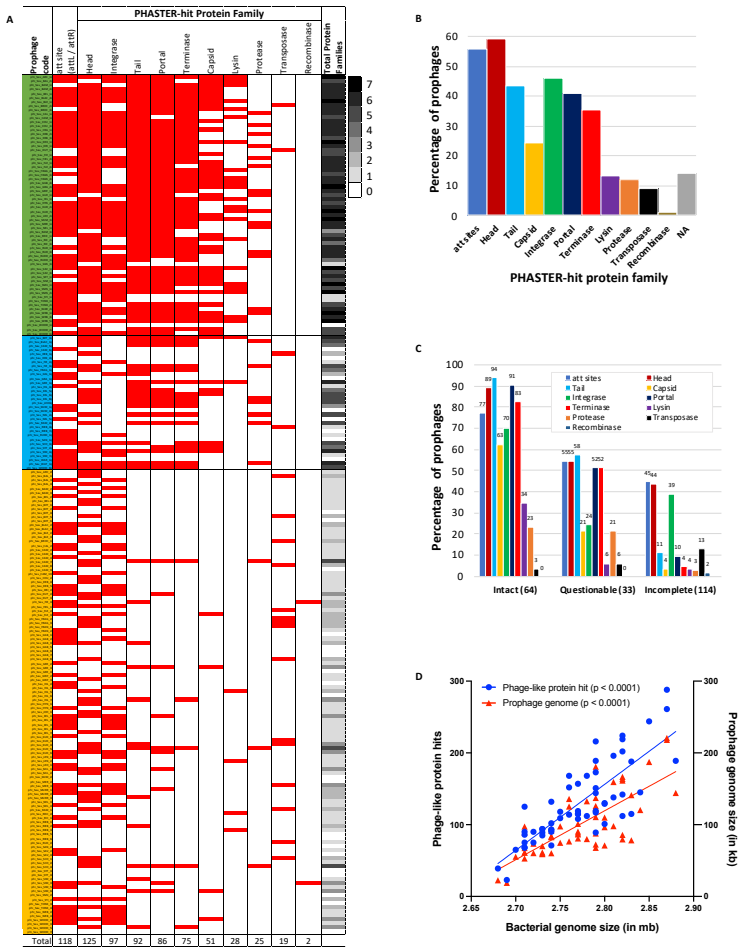

Figure 3.2 | Distribution of phage-like proteins (PLP) across different types of prophages. (A) Heat map of prophages and phage associated proteins in all *S. aureus* strains. Prophages (y-axis) are plotted in alphabetical order grouped according to their status (green = intact, blue = questionable, yellow = incomplete) against each protein hit (x-axis). Red boxes indicate the presence of the indicated protein. White spaces indicate the lack thereof. The numbers in the last column indicate total number of PHASTER-hit protein families and is also represented by gradient of black-colour. The number in last row indicates total number of prophages with corresponding protein-family hit. Please refer to the PDF of the figure and use the zoom function to identify names of prophages and proteins. (B) Among 211 prophages, at least one attachment site (attL/attR) was present in 118 (56%), while the most abundant structural protein was associated with head (125) followed by tail (92) and capsid (51). Similarly, the most abundant functional protein was integrase (97) followed by portal (86) and terminase (75). Lysin, protease, transposase and recombinase were found only in 28, 25, 19 and 2 prophages, respectively. (C) Comparison of phage-associated protein distribution between intact, questionable and incomplete prophages revealed that intact and questionable prophages completely lacked recombinase genes, and transposases were significantly enriched in incomplete prophage (compared to present only in 2 each in intact (3%) and questionable (6%) prophages. Arrows represent enriched proteins in incomplete prophages compared to the complete ones. (D) Correlation between host genome (*S. aureus*) vs number of phage-like proteins (PLPs) ($p < 0.0001$, linear regression) and prophage genome ($p < 0.0001$, linear regression). The gain of genome size is significantly contributed by prophage as the prophage content increases with increase in genome of the host.

3.3.4 Most prevalent S. aureus prophages belonged to Biseptimavirus genus

Based on nucleotide homology, among 211 prophages, 196 (93%) were Staphylococcus prophage whereas 15 (7%) were non-Staphylococcus prophage. Altogether 44 different phage strains were found, mostly belonging to the *Siphoviridae* family (41/44, 93%) (Figure 3.3C, 3.3D), out of which 36 were Staphylococcus phages while 8 resembled non-Staphylococcus phages (Figure 3.3C, indicated by star). Among the 44 prophage strains, 5 (PT1028, phiNM3, JS01, phiN315 and phi2958PVL) accounted for almost 51% (108/211) of the prophages and at least one of those was present in 54/58 (93%) isolates.

Further, 22/44 prophage strains (50%) (a total of 64 prophages) were found in intact form and none of those were non-Staphylococcal phages. The most abundant intact prophage was similar to Staphylococcus phage JS01 (14/64, 21.8%) followed by Staphylococcus phage phiNM3 (10/64, 15.6%), Staphylococcus phage phi2958PVL (9/64, 14.0%) and Staphylococcus phage phiN315 (4/64, 6.25%) (Figure 3.3C). Further, among 196 Staphylococcal like prophages, most of them belonged to the genus *Biseptimavirus* (75/196, 38%) followed by *Phietavirus* (44/196, 22.4%) and *Triavirus* (22/196, 11.22%) (Table 3.2). Based on the most similar phage-hit, among 197 *S. aureus* prophages, most of the prophages were like Sa3int group (68, 35%) phages, followed by Sa2int (27,14%) and Sa1int (11, 6%) (Table 3.2).

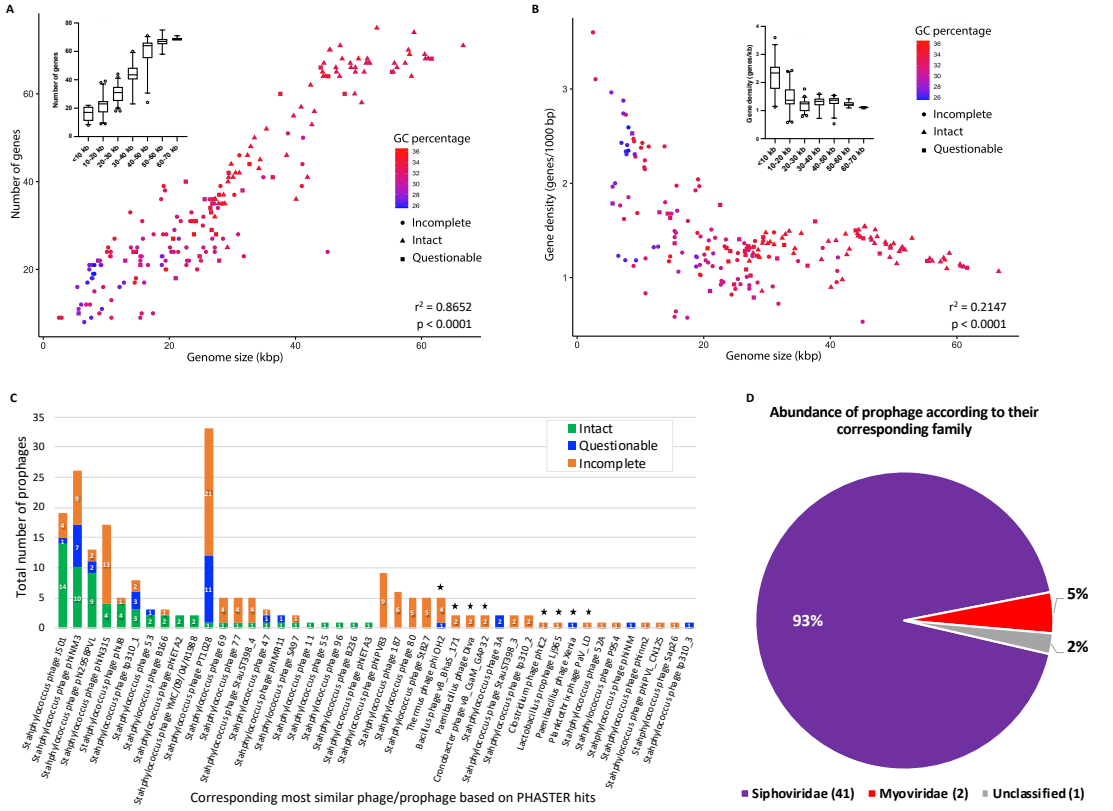

Figure 3.3 | Identification and characteristics of predicted prophages. (A) Correlation between number of genes, prophage genome size and GC%. The number of genes and GC% increases with increase in size of prophage genome indicating bigger prophages have more coding sites and high GC. (B) Correlation between gene density, prophage genome size and GC%. The gene density (genes/kb) is relatively high in smaller prophages accompanied by lower GC (higher AT), suggesting that they efficiently pack more genes within their small genome as compared to intact prophages. (C) Distribution of predicted prophages based on their most similar hit. Although 211 prophages were predicted by PHASTER, they all were most similar to 44 different phages available in the PHASTER database. Among 211, 108 (~51%) belonged to 5 most common temperate phages (Staphylococcus phage PT1028, Staphylococcus phage phiNM3, Staphylococcus phage JS01, Staphylococcus phage phiN315, Staphylococcus phage phi2958PVL) and almost 83% (175/211) of prophages were represented by 18 different strains of prophages. (Stars represent non-Staphylococcus phage-hits, and numbers inside bar represents total prophages of that type). (D) Among 44 (pro)phage hits, most of them (41, 93%) belonged to Siphoviridae family, 2 were from Myoviridae family (non-Staphylococcal) whereas 1 phage (PT1028) was unclassified till date. (Note: The Myoviridae, Siphoviridae and Podoviridae family are currently defunct as of 2023)

Table 3.2 | Predicted Staphylococcal prophage, associated integrase group, major virulence factors (VFs), corresponding phage genus and family based on maximum homology (as assigned by PHASTER).

Most similar phage hit	Integrase group^	Associated VFs[b]	No. of prophages			Prophage genus Total (IN, Q, IC)	Predicted family
			IN	Q	IC		
Staphylococcus phage PT1028	NA	NA	1	11	21	NA 33 (1, 11, 21)	Unclassified
Staphylococcus phage StB27	NA	NA	0	0	5	NA	*Siphoviridae*
Staphylococcus prophage phiN315	Sa3int	*sak, chp, scn, sep*	4	0	13	22 (4, 0, 18)	
Staphylococcus phage JS01	Sa3int[c]	*sak, chp, scn, sep*[c]	14	1	4	*Biseptimavirus* 75 (29, 12, 34)	
Staphylococcus phage phiNM3	Sa3int	*sak, chp, scn, sea*	10	7	9		
Staphylococcus phage StauST398-4	Sa3int[c]	-	1	0	4		
Staphylococcus phage tp310-3	Sa3int	*sak, chp, scn*	0	1	0		
Staphylococcus phage tp310-1	Sa2int	*luk* S/F-PV	3	3	2		
Staphylococcus phage phiPVL-CN125	Sa2int	*luk* S/F-PV	0	0	1		
Staphylococcus phage 77	Sa6int	-	1	0	4		
Staphylococcus prophage phiPV83	Sa5int	*luk* M, *luk* F-PV	0	0	9		
Staphylococcus phage P954	Sa7int	-	0	0	1		
Staphylococcus phage phi2958PVL	Sa2int	*luk* S/F-PV	9	2	2	*Triavirus* 22 (12, 5, 5)	
Staphylococcus phage YMC/09/04/R1988	Sa2int[c]	-	2	0	0		
Staphylococcus phage 47	Sa2int	-	1	1	1		
Staphylococcus phage 3A	NT	-	0	2	0		
Staphylococcus phage tp310-2	Sa6int	-	0	0	2		
Staphylococcus phage phiJB	Sa6int	-	4	0	1	*Phietavirus* 44 (18, 3, 23)	
Staphylococcus phage B166	Sa1int[c]	-	2	0	1		
Staphylococcus phage phiETA2	Sa1int	*eta*	2	0	0		
Staphylococcus phage SA97	Sa1int[c]	-	1	0	1		
Staphylococcus phage 55	Sa1int	-	1	0	0		
Staphylococcus phage B236	Sa1int[c]	-	1	0	0		
Staphylococcus phage phiETA3	Sa1int	*eta*	1	0	0		
Staphylococcus phage Sap26	Sa1int[c]	-	0	0	1		
Staphylococcus phage 69	Sa5int	-	1	0	4		
Staphylococcus phage 11	Sa5int	-	1	0	0		
Staphylococcus phage 187	Sa5int	-	0	0	6		
Staphylococcus phage phiNM1	Sa5int	-	0	1	0		
Staphylococcus phage 53	Sa7int	-	2	1	0		
Staphylococcus phage phiNM2	Sa7int	-	0	0	1		
Staphylococcus phage 96	Sa9int	-	1	0	0		
Staphylococcus phage StauST398-3	Sa9int[c]	-	0	0	2		
Staphylococcus phage 80	Sa6int	-	0	0	5		
Staphylococcus phage 52A	Sa6int	-	0	0	1		
Staphylococcus phage phiMR11	Sa12int	-	1	1	0		
	Total (Staphylococcal prophages)		**64**	**31**	**101**		

[a] Based on most-similar phage-hit in PHASTER. Non-Staphylococcus prophage hits excluded. [b] Reference: Goerke et al. (2009), Kahánková et al. (2010), Varga et al. (2016). Colour coded according to *S. aureus* phage integrase group. [c] Predicted from this study based on integrase gene homology and phylogeny of the reference sequence with reference sequences of integrase gene. IN = Intact (or complete), Q = Questionable, IC = Incomplete, NA = Not assigned, NT = Non-typeable. (Note: Different colours represent different group of integrases found based on most similar hit)

3.3.5 CRS patients with nasal polyps often carried Sa3int type S. aureus prophages

We then performed phylogenetics analysis to identify integrase groups based on previously characterized representative sequences based on Goerke's classification (Goerke et al., 2009). Amino acid (aa) sequences of all 97 integrase genes identified in prophage regions were considered (intact = 45/64, 70%, questionable = 8/33, 24%, incomplete = 44/114, 38%). Phage integrases were found in 55/58 (~95%) *S. aureus* strains and were always accompanied by the presence of attachment sites (data not shown). The most prevalent prophage type based on integrase gene polymorphism was Sa3int followed by Sa2int and Sa1int (Table 3.2 and Figure 3.4A-B). We further report an unassigned integrase group (~390 aa) in 16 incomplete prophages that did not relate with any of the major Sa1int-Sa12int groups but had 100% identity with tyrosine-type recombinase/integrase (NCBI Ref. Seq: WP_048667711.1, non-redundant protein sequences (nr) database) in *S. aureus.* Limiting the BLAST search within NCBI virus database (taxid:10239) showed 88.24% identity (query coverage = 100%) with putative integrase from uncultured Caudovirales phage (GenBank: ASN72555.1). Similar dot-matrix and phylogenetic analysis of lysin and tail-fibre genes showed limited polymorphism in *S. aureus* prophages. Details and amino acid sequences of representative integrase proteins are available as Supplementary data S2.

Table 3.3 | Distribution of prophage, integrase typing among various groups of patients based on polyp status and Lund-Mackay severity score (LMK)

Disease status	Prophage groups/strains	No. of strains having intact prophages[a]			p-value (Fisher's exact test between CRSsNP and CRSwNP)
Control / CRSsNP / CRSwNP	**Prophage strains based on integrase group**	**Control (N = 9)**	**CRSsNP (N = 28)**	**CRSwNP (N = 30)**	
	Sa3int	3 (33%)	7 (25%)	21 (70%)	0.0008 (significant)
	Sa2int	4 (44%)	10 (36%)	4 (13%)	0.0667
	Sa1int	2 (22%)	3 (11%)	5 (17%)	0.7073
	Individual phage strain				
	Staphylococcus phage JS01 (Sa3int)[b]	0	3 (11%)	11 (36%)	0.0331 (significant)
	Staphylococcus phage phiNM3 (Sa3int)	0	4 (14%)	6 (20%)	0.7316
	Staphylococcus phage phi2958PVL (Sa2int)	1 (11%)	6 (21%)	3 (10%)	0.2904
LMK ≤ 12 / LMK > 12	**Prophage strains based on integrase group**	**Control (N = 9)**	**LMK ≤ 12 (N = 22)**	**LMK > 12 (N = 32)**	
	Sa3int	na	9 (41%)	17 (53%)	0.4180
	Sa2int	na	6 (27%)	8 (25%)	1.0000
	Sa1int	na	6 (27%)	2 (6%)	0.0512
	Individual phage strain				
	Staphylococcus phage JS01 (Sa3int)[b]	na	8 (25%)	5 (16%)	0.1092
	Staphylococcus phage phiNM3 (Sa3int)	na	0	9 (28%)	0.0073 (significant)
	Staphylococcus phage phi2958PVL (Sa2int)	na	3 (14%)	6 (19%)	0.7230

[a] Only intact prophages considered. The integrase group is based on corresponding integrase group of phage identified as most similar hit by PHASTER through maximum homology. [b] Identified from this study. LMK = Lund-Mackay score. na = Not available because 'control' groups are not scored for LMK.

Further, 'intact' Sa3int prophages were significantly more prevalent in clinical isolates from CRSwNP patients than CRSsNP patients (Table 3.3, Figure 3.4A). Specific Staphylococcus prophage phiNM3 (also belonging to Sa3int prophages) was significantly more prevalent in patients within high disease severity compared to those with low disease severity (LMK ≥ 12 vs LMK < 12, p = 0.0073, Fisher's exact test) (Table 3.3).

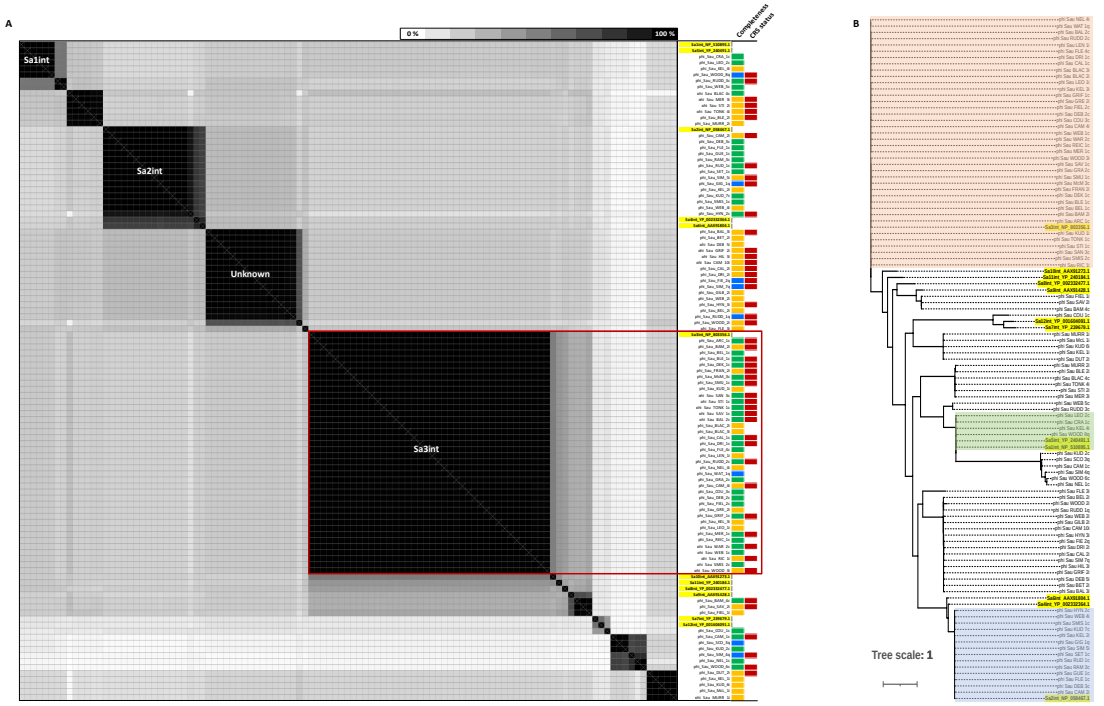

Figure 3.4 | Percentage identity dot-matrix and phylogenetics of integrase. (A) Percentage identity dot-matrix of integrase (N = 97) gene. The gradient bar at the top-right represents percentage identity, darkest being 100%. The green, blue and orange bar represents completeness (intact, questionable and incomplete respectively) of the corresponding prophage. The red bar represents positive polyp status (CRSwNP) of the corresponding *S. aureus*. (B) Phylogenetics of integrase (N = 97) gene. Together, these findings reveal that Sa3int group of phage infection (as prophage) is the most widely distributed in *S. aureus* clinical isolates isolated from chronic rhinosinusitis patients followed by Sa2int and Sa1int.

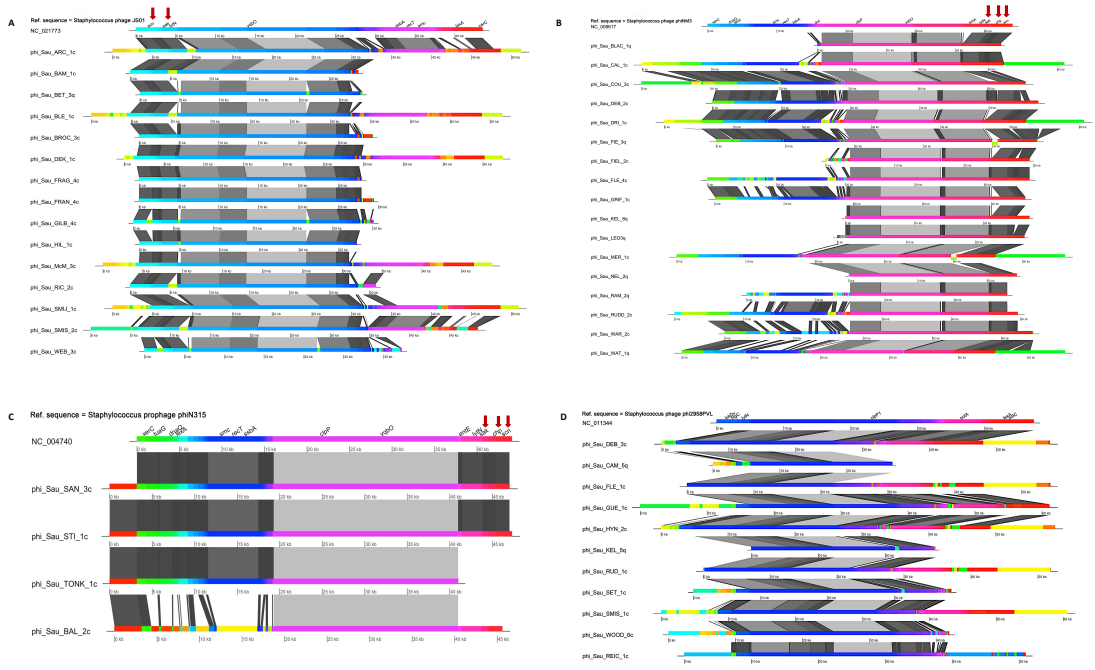

Figure 3.5 | Multiple sequence alignment (MSA) of predicted prophages (intact and questionable) using progressive MAUVE against most similar phage-hit as a reference sequence. (A) Sequence alignment of prophages with reference sequence Staphylococcus phage JS01 (Sa3int). (B) Sequence alignment of prophages with reference sequence Staphylococcus phage phiNM3 (Sa3int). (C) Sequence alignment of prophages with reference sequence Staphylococcus phage phiN315 (Sa3int). (D) Sequence alignment of prophages with reference sequence Staphylococcus phage phi2958PVL (Sa2int). The downward pointing red-arrow represents the immune evasion cluster (IEC) genes, the same colour between different prophage sequence indicates homology between prophages and the dark-grey band below every sequence represents percentage identity with the previous sequence. Please use zoom function from the PDF image for other individual genes.

3.3.6 Prophages of S. aureus carry virulence factors but not AMR genes

Prophages carried multiple phage-associated virulence factors. These included *sak, scn, chp, hlb, luk*G/H, *seg, seln, selu, sei, selm, selo, spl*C, *eap/map, sea* (Table 4). The most abundant phage associated VFs were *sak, scn, hlb, entA,* and *chp* found in 45, 40, 37, 36 and 22 prophages respectively. All 7 types of serine protease-like proteins (*slp*A/B/C/D/E/G/H) were found within prophage sequences suggesting them to be phage associated. VFs that are known to be human immune evasion factors such as *scn, chp* and *sak* were mostly present in prophages belonging to Sa3int or Sa3int homologues

(JS01, phiNM3, phiN315) while prophages similar to Sa2int group (phi2958PVL) lacked those genes (Figure 3.5A-D). IEC typing of all *S. aureus* strains and intact Sa3int prophages did not correlate with any specific type with CRS disease presentation (Table 5). Further, antimicrobial resistance genes (ARGs) were not identified within any of the prophage genomes in any of the *S. aureus* strains although 15/67 (22% including control group) were MRSA. A complete list of common VFs and other phage associated accessory genes is shown in Table 3.4 and IEC type of *S. aureus* and intact Sa3int prophages is elaborated in Table 3.5. Further, multiple sequence alignment (MSA) of prophages with the most similar phage-hit as a reference sequence confirmed that Sa3int group prophages (JS01, phiNM3, phiN315) consistently carried pathogenic IEC genes (*sak, chp, scn*) which were more conserved and uniformly distributed across intact prophages (Figure 3.5A-C). In contrast, Sa2int group prophage (phi2958PVL) lacked IEC genes (Figure 3.5D).

3.3.7 Prophage phylogenetics

Phylogenetic analysis based on maximum likelihood revealed three distinct evolutionary lineages of prophages with more diversified sub-clusters (Figure 3.6). There was a heterogeneous distribution of intact, questionable and incomplete prophages across the 3 major clusters. Further, within clusters, there were many highly unrelated sub-clusters and singletons representing both intact and incomplete prophages. No intact prophages found in the same strain were found to be phylogenetically related (clustered) (Figure 3.6).

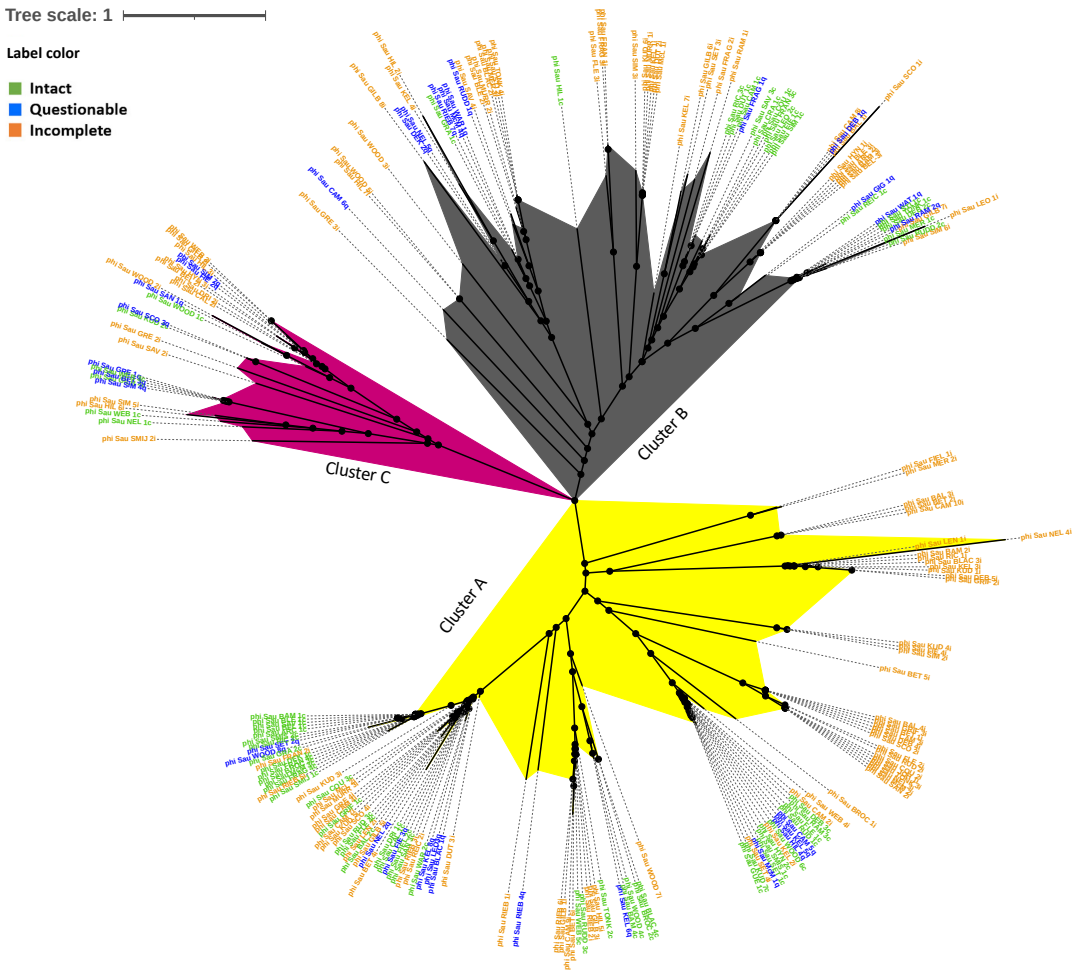

Figure 3.6| Phylogenetic tree of 211 prophages from 58 *S. aureus* genomes isolated from patients having chronic rhinosinusitis. Multiple sequence alignment of the prophage sequences was created with MAFFT (ver 7) and maximum likelihood tree was created with FastTree 2.1 through Geneious Prime 2021.1. The tree was further edited using iTol (ver 6). The tree signified that intact (green label), questionable (blue label) and incomplete (red label) prophages are not separate entities but related to each other in mosaic distribution. Please refer to the PDF of the figure and use the zoom function to identify label names of prophages.

Table 3.4| Major virulence factors and their GO* annotation encoded by *Staphylococcus aureus* prophages.

VF class	Virulence factor	Related genes	No. of Prophages	GO[a] annotation (biological process)
Immune evasion cluster (IEC)	Staphylococcal complement inhibitor (SCIN)	*scn*	40	pathogenesis
	Chemotaxis inhibitory protein (CHIPS)	*chp*	22	pathogenesis
	Staphylokinase	*sak*	45	pathogenesis
Enzyme	Serine protease	*sspA*	0	
	Serine protease-like proteins	*splA*	8	hydrolase and protease[b]
		splB	15	
		splC	7	
		splD	1	
		splE	9	
		splF	3	
Toxins	Delta-hemolysin	*hld*	7	pathogenesis
	Leukocidin	*luk* E/D	10	pathogenesis
	Enterotoxins (SEs)	*entA (sea)*	36	pathogenesis
		entB (seb)	8	pathogenesis
		entC	17	biosynthetic process
		entD (sed)	20	pathogenesis
		entE (see)	7	pathogenesis
		entG (seg)	13	pathogenesis
		entH (seh)	1	pathogenesis
		seln, selu, selu2	16 each	NA
		yent2	16	pathogenesis
		sei	15	pathogenesis
		selm, selo	15 each	NA
	Exfoliative toxin A	*eta*	3	pathogenesis
	Toxic shock syndrome toxin	*tst (tsst)*	5	pathogenesis
Other (non-virulent, prophage associated, responsible for successful prophage excision and induction)	Cell wall hydrolase	*lytN*	63	cell wall organization
	Tyrosine recombinase	*xerC*	39	cell division, transposition
	ssDNA-binding protein A	*ssbA*	38	DNA repair, replication, recombination
	Chromosome partition protein	*smc*	35	chromosome condensation, DNA replication, sister chromatid cohesion
	ATP-dependent *clp* protease proteolytic subunit	*clpP*	33	serine-type endopeptidase activity[b]
	DNA recombination protein	*recT*	33	NA
	60 kDa chaperonin	*groL*	22	protein refolding
	DNA replication protein	*dnaC*	21	DNA replication, synthesis of RNA primer
	10 kDa chaperonin	*groS*	21	protein folding

[a] Gene ontology, [b] GO Molecular function, NA = Not categorized according to GO knowledgebase. Pease refer to supplementary data for complete list of virulent and non-virulent gene hits in prophage sequence. Note: The gene name in parenthesis indicates the alternative name.

Table 3.5| Prevalence of different immune evasion cluster (IEC) types* in *S. aureus* and intact Sa3int (IEC) prophages.

Sample	Immune Evasion Cluster (IEC) Types							
	-	A	B	C	D	E	F	G
		sea, sak, chp, scn	*sak, chp, scn*	*chp, scn*	*sea, sak, scn*	*sak, scn*	*sep, sak, chp, scn*	*sep, sak, scn*
Control (N = 9)	0 (0%)	0 (0%)	3 (33%)	1 (11%)	1 (11%)	4 (44%)	0 (0%)	0 (0%)
CRSsNP (N = 28)	2 (7%)	3 (11%)	13 (46%)	2 (7%)	3 (11%)	3 (11%)	0 (0%)	2 (7%)
CRSwNP (N = 30)	1 (3%)	2 (7%)	8 (27%)	1 (3%)	6 (20%)	8 (27%)	3 (10%)	1 (3%)
Intact Sa3int prophages (N = 28)	7 (25%)	1 (4%)	5 (18%)	1 (4%)	5 (18%)	4 (14%)	2 (7%)	3 (11%)

* IEC typing is based on presence/absence of IEC genes (sak, chp, scn, sea/sep) based on van Wamel et. al. (2006)

3.4 DISCUSSION

This study demonstrated that all 58 *S. aureus* CIs from CRS patients carried at least 1 recognizable prophage, with a total of 211 prophage-like regions identified from the cohort. The majority of those were similar to temperate phages belonging to the *Siphoviridae* family, more specifically, the *Biseptimavirus* genus. The ubiquitous presence of prophages in *S. aureus* clinical isolates and strong positive correlation of prophage size and phage-hit proteins with the bacterial genome size indicate that the acquisition of prophage-encoded genetic material in *S. aureus* is common and likely an important driver of *S. aureus* evolution and host adaptation. This further implies that genome plasticity between *S. aureus* strains is likely to be driven in part by variability in temperate phage infection and integration. This process may improve the bacterial fitness and adaptation to the host environment potentially long term as these integrated phage DNA can pass to progeny. Further, a significant correlation between the prevalence of intact Sa3int group prophages carrying IEC genes including enterotoxins in *S. aureus* CIs from CRSwNP indicate that prophage associated VFs may contribute to the CRS disease severity and phenotype. Also, *S. aureus* prophages lacked AMR genes indicating phage-mediated spread of AMR genes is unlikely to be a major driver of antimicrobial resistance in the *S. aureus* population in this region (South Australia).

Clinical strains are usually laden with prophages (Tan et al., 2020) and multiple prophages encoded genes impacting the ability of S. aureus to colonize and persist in the human nasal niche have been reported (Mccarthy et al., 2012). Our results are in line with those observations and indicate that all S. aureus clinical isolates from CRS patients and non-control patients carried at least 1 prophage and prophages could

contribute up to 7.7% of accessory genomic data to the core S. aureus genome. As different prophages are known to carry different VFs, poly-lysogeny, that is the presence of various prophages within an individual strain, significantly contributes to pathoadaptive genome variation in clinical strains. Lysogeny furthermore provides a selective advantage to the bacterial strain as the prophage provides immunity against secondary phage attack (Ramisetty and Sudhakari, 2019). This is supported by our findings where no two intact prophages found in the same strain were phylogenetically related or clustered.

Our results on GC content of the whole *S. aureus* genome (32.7%) that is lower than intact prophage (33.5%) is in line with the GC content observed by Kwan et al. (2005) [*S. aureus* (32.9%) and *S. aureus* phage (33.7%)]. This is contrary to the tendency of a higher GC content in core genomes, compared to the corresponding accessory genomes in the majority of pathogens (Bohlin et al., 2017). The retention of such relatively stable but energetically expensive GC nucleotides of intact prophages within the bacterial core genomes suggests that selective pressures are at work and that those intact prophages are likely important components of host adaptation with a potential involvement in the disease process. This is further supported by our finding that the presence of intact prophages (particularly Sa3int group) significantly correlated with the CRSwNP phenotype, suggesting a role of intact prophages and/or associated accessory VFs in CRS disease pathophysiology. Similar correlations may be observed in other diseases associated with persistence of *S. aureus* as these active prophage elements are proven to increase bacterial fitness and mobilize VFs among competing populations. Unlike incomplete prophage regions that are considered non-inducible because they lack genes

essential for production of new phage particles, intact prophages may be induced into infectious phage particles. Prophage induction can occur spontaneously or can be promoted in the context of bacterial stress such as antibiotic pressure (Nanda et al., 2015). This can in term facilitate horizontal gene transfer (HGT) and support the distribution of prophage-encoded virulence factors within the community promoting host adaptation and colonization of the niche. In this study, most of these intact prophages belonged to Sa3int group phages which encode the immune evasion cluster (IEC) genes (*sak, scn, chp*, *sea/sep*). Furthermore, intact phiNM3 prophages (belonging to the Sa3int group, also carrying IEC) were more abundant in CRS patients that had high severity scores compared to those that had low disease severity scores. *S. aureus* is well known to deploy an arsenal of immune evasive strategies and the IEC genes are well known factors that interfere with host complement and immunoglobulins (*sak* and *scn*) and neutrophil and monocyte chemotaxis (CHIPS) (Canfield and Duerkop, 2020). Sak also neutralizes host antimicrobial peptides (Nguyen and Vogel, 2016) and promotes *S. aureus* invasion (Bergmann and Hammerschmidt, 2007). Interestingly, *S. aureus* invasion within sinonasal mucosa is also seen in the context of CRSwNP (Tan et al., 2013; Tan et al., 2014) and the potential involvement of Sa3int prophages and *sak* in that process requires further investigation. Comparison of prophage abundance and prophage type in CRS with control group also revealed that prophage acquisition in CIs is common and the higher prevalence of Sa3int prophage in CRSwNP compared to CRSsNP could be due to the gain of Sa3int prophage in CRSwNP or the loss of Sa3int prophage in CRSsNP. The gain or loss of specific prophage and associated VFs may impact the persistence of given bacteria, their role in chronic infections and

development of nasal polyp. As CRS is known to be associated with dysbiosis with an increased prevalence of *S. aureus*, we speculate that the gain of Sa3int group prophage in CRSwNP may contribute to CRS severity and chronicity as CIs carrying IEC genes are better equipped to persist. Activation and mobilization of those genes would therefore likely assist *S. aureus* in escaping immune surveillance in those patients. Interestingly, the Sa3int prophages also encode enterotoxins that can cross-link the T-cell receptor (TCR) and class-II major histocompatibility complex non-specifically and trigger a massive polyclonal T-cell activation and cytokine release. Through the production of cytokines and chemokines, a type-2 immune response is favoured which is common in the context of CRSwNP (Wise et al., 2018). This type-2-biased immune response promotes the differentiation of immunotolerant M2 macrophages which demonstrate decreased phagocytosis of *S. aureus* and may contribute to its persistence in CRSwNP (Martinez et al., 2009). Despite strong immune activation, *S. aureus* superantigen driven inflammation can skew adaptive immune responses of the host away from a protective response against *S. aureus* to the benefit of its own survival (Tuffs et al., 2018). Further, as it has been established that Sa3int prophages insert themselves into the beta-haemolysis (*hlb*) gene locus rendering it inactive, we postulate that beta-hemolysin activity is not required for nasal colonization by *S. aureus*. However, more research is required to evaluate the role of prophage-encoded VFs and the relevance of active prophages in *S. aureus* persistence in nasal microenvironment. Also, further studies are required to establish the potential causal relationships between the integrity of prophage in *S. aureus* and the formation or presence of nasal polyps.

In contrast to intact prophages, incomplete prophages had lower GC% (30.92%) than *S. aureus* core genome. It is well known that endosymbionts like prophages are often AT-biased, as AT-rich regions are metabolically cheaper to maintain (Dietel et al., 2018). Such relatively high AT contents can also result from increased levels of genetic drift and mutational bias, and it has been shown that increased AT content increases the bacterial fitness of the host (Dietel et al., 2018). Furthermore, prophage regions showed higher gene density compared to its host *S. aureus* genome (1.43 vs 0.97 genes/kb) (Rogozin, 2002). This result is similar to that of temperate *S. aureus* phages (1.67 genes/kb) reported by Kwan et al. (2005) which implies that intact prophage regions have similar gene densities as temperate *S. aureus* phages, and they are most likely recently integrated phage regions and are inducible. Gene density in prophage regions is expected to be higher as non-coding DNA segments (introns and intergenic regions) are continuously under selection pressure to manage the metabolic burden imposed by the addition of genomic material and by the limitations imposed by the amount of DNA able to be packaged into phage heads. Although gene density and prophage size are inversely correlated, phage-associated genes (including those necessary for viral replication) were less frequent in incomplete prophages. Loss of phage-associated genes like portal (critical roles in head assembly, genome packaging, neck/tail attachment, genome ejection), terminase (catalyse site-specific endo-nucleolytic cleavage of DNA and its packaging into phage proheads), lysin (cleave host's cell wall), proteases (encapsulation of viral DNA into capsid) lead to permanent domestication of a prophage and yet still confers a selective advantage (Dragoš et al., 2020; Kondo et al., 2020; Laumay et al., 2019; Popescu et al., 2021). As most of the virulent phages of *S. aureus* belong to

Myoviridae and almost all temperate phages to *Siphoviridae* family (now defunct) and, to our best knowledge, there are no known *Siphoviridae* phage < 20 kb (16-18 kb phages belong to *Podoviridae*) (Deghorain and Van Melderen, 2012; Ingmer et al., 2019), we can infer that prophage regions smaller than 20 kb in *S. aureus* may represent gene remnants of a *S. aureus* temperate phage that still confers evolutionary benefits to the progeny through vertical gene transfer or are remnants that are still in the process of being lost and confer relatively less fitness as they cannot get induced and offer competitive advantage to the host.

In this study, multiple clusters of phage integrases that do not belong to any of the reference (Sa1int-Sa12int) groups within prophage regions were identified. Protein blasting of one of the most prevalent 'unknown' integrases (a 390 aa long) against the NCBI database showed 100% homology with an integrase present in *S. aureus* which was also reported by Bui and Kidd (2015) in small colony variants (SCVs) of *S. aureus*. As this integrase type has been reported in unculturable phage and SCVs can underly chronic infections, it may be interesting to see if such association is clinically important and integrase typing can further predict transformation into SCVs. However, supporting experimental evidence is required to associate prophage with the SCV and its association with disease.

Prophage-encoded ARGs are sparsely reported in clinically important bacteria like *Acinetobacter*, *Pseudomonas*, *Escherichia* (Marti et al., 2014; Calero-Cáceres et al., 2019; Balcázar, 2020; Kondo et al., 2020; Loh et al., 2020). Also, ARGs have been occasionally reported in *S. aureus* prophages (Ene et al., 2021), especially in regions where inappropriate use of antibiotics is highly prevalent. Our results could not identify

complete ARGs in any of the prophage regions although ARGs like *tet-38, norA, blaZ, fosB* were highly prevalent in these isolates (Bardy et al., 2018). This indicates phage-mediated spread of AMR genes may not a major driver of antimicrobial resistance in the *S. aureus* population in South Australia.

Although Javan et. al (2009) suggests that complete and incomplete (satellite) prophages have separate evolutionary lineage and must be considered a separate entity, our results contradict those findings. Despite incomplete prophages having significantly lower GC%, higher gene density and lower prevalence of phage-hit genes compared to intact (complete) prophages, the heterogenous distribution of intact, questionable and incomplete prophages across major clusters in the phylogenetic tree indicate that incomplete prophages do not belong to separate evolutionary lineages. Rather, they may be truncated remnants of past infection suggesting an AT-biased endosymbiont-like co-evolution in *S. aureus* prophages that may have important roles in co-evolution of bacteria (Bobay et al., 2014; Wang et al., 2014; Ramisetty and Sudhakari, 2019). This is further supported by the MSA with the reference sequence and the fact that such cryptic entities encode multiple phage-associated structural as well as functional genes. Further, comparison between sub-clusters representing intact and incomplete prophages within a cluster indicates that, the evolution of intact prophages into incomplete is possibly non-specific resulting in highly unrelated sub-clusters and singletons. However, this may be because of the different programs used as the authors use their own algorithms to categorize prophage.

3.5 CONCLUSION

In summary, our findings expand the knowledge of prophages in *S. aureus* isolated from CRS patients, and their possible role in disease development. Discovery of 22 diverse strains of intact prophages in *S. aureus* within a restricted geographic region and from a well-defined population (CRS disease) reveals circulation of diverse temperate phages contributing genotypic and phenotypic plasticity as well as virulence. Of further concern is poly-lysogeny which aids in accumulation of additional phage encoded VFs. We also report prophages belonging to Sa3int (phiNM3, JS01, phiN315) and Sa2int (phi2958PVL) group phages most dominant in *S. aureus* from CRS patients that consistently harboured multiple pathogenic genes such as *sak, scn, chp, sea/sep, luk*E/D. We further speculate that *S. aureus* carrying Sa3int type prophage might impact CRS disease severity and phenotype as they are better equipped to evade the immune system as well as increase the pathogenicity of the strain. However, the potential role of Sa3int prophage in CRS severity and the development of nasal polyps requires further study.

We believe that our findings reveal a novel area for future investigations which will not only increase our understanding of prophage biology but also uncover undiscovered tripartite associations between the prophage-bacteria-human immune system, *S. aureus* evolution and CRS disease epidemiology.

Future directions

Our study was designed to understand the distribution of prophages in *S. aureus*, potential prophage-encoded virulence factors and its possible correlation with disease phenotype and severity in a very defined population, CRS. As our results showed a significant correlation between the presence of Sa3int group prophages in *S. aureus* and the presence of nasal polyps in CRS disease, it may be important to see if these prophages release any protein(s) that impacts disease development and severity. Also, as phage released from lysogens are known to directly stimulate/induce/worsen the mammalian immune response, thus impacting inflammation and disease outcomes, it will be important to see if these intact prophages can be induced either spontaneously and/or under stress conditions.

Limitations of the study

We acknowledge that experimental verification of prophage induction is required in addition to *in-silico* population genomics to claim that intact prophages are inducible and specific prophage impact CRS disease phenotype, progression, and severity. We also acknowledge that genetic makeup and prior environmental predisposition has a profound impact on the inflammatory response to any external stimulus, and overall CRS pathogenesis and prophage is unlikely to be the sole factor affecting CRS disease pathogenesis. We further note that the sample size is not large enough for robust statistical correlation and similar-sized control (non-CRS) groups must be included in future research.

Data summary

Genomes of previously sequenced *S. aureus* (n = 58) were retrieved from the local database. The sequences are also publicly available in NCBI Genome Depository under BioProject Accession Number: PRJNA436815. The additional sequences from control group are included as a Supplementary data S1 and complete information on prophage (analysed: November 2020) is available as Supplementary data S2. All supplementary material is publicly available for download at figshare (https://doi.org/10.6084/m9.figshare.16590359).

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CHAPTER FOUR

EXPOSURE TO SUB-INHIBITORY ANTIBIOTICS ENHANCE PROPHAGE INDUCTION AND RELEASE OF INFECTIVE PHAGES IN PATIENT-DERIVED *Staphylococcus aureus*

Exposure to sub-inhibitory antibiotics enhance prophage induction and release of infective phages in patient-derived *Staphylococcus aureus*

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Abstract

Genomic studies have revealed that prophages, temperate phages that are embedded in the genome of *Staphylococcus aureus*, have a significant impact on the distribution and spread of virulent factors, whether through vertical transmission during bacterial cell division or horizontal transmission through prophage induction. Induction of prophages is often triggered by stress, such as exposure to UV radiation, antibiotics, or other chemical agents. In *Staphylococcus aureus*, fluoroquinolones, trimethoprim, and β-lactams have been found to trigger prophage induction. Antibiotic-mediated prophage induction leads to a high frequency of transfer of staphylococcal pathogenicity islands, suggesting that antibiotics may have unintended consequences in certain clinical strains carrying specific prophages. In our previous study, we computationally discovered that nearly 85% of *S. aureus* isolated from chronic rhinosinusitis (CRS) patients had at least one intact prophage DNA integrated into their chromosomes that were likely inducible. The objective of this study was to determine if these prophages could be induced under sub-lethal concentrations of routinely used antibiotics. Additionally, we investigated whether there was a difference in biofilm and metabolic properties between *S. aureus* isolates that had inducible prophages and those that did not have inducible prophages.

To gain an understanding, we first determined the antibiotic resistance pattern of *S. aureus* (N = 66) primarily isolated from sinonasal cavities of CRS patients retrospectively using the broth microdilution method. We then used the sub-lethal concentrations of various antibiotics and steroids for prophage induction including mitomycin C as a positive control. We then assessed their infectivity in an RN4220 indicator strain using a

spot assay. Additionally, we studied the biofilm and metabolic activities of *S. aureus* concerning the inducibility of prophage by using crystal violet and Alamar blue assays, respectively. We further observed the beta-hemolysis activity of the isolates on sheep blood agar to understand the prevalence of beta-hemolysin activity as beta-hemolysin gene is likely truncated in human-adapted *S. aureus* by a specific group of prophages commonly known as hlb-converting prophages.

Spontaneous prophage induction (SPI) was observed in around 26% of the *S. aureus* clinical isolates while mitomycin C dependent prophage induction was observed in almost 52% of the clinical isolates. Most of the isolates showing prophage induction harboured at least one intact prophage(s). Exposure of exponentially growing bacteria to sub-inhibitory concentrations of antibiotics enhanced the release of prophage compared to spontaneous prophage induction (SPI) in almost 50% of active lysogens. Among antibiotics tested, ciprofloxacin was the most potent prophage inducer inducing prophages from 51% of the isolates followed by amoxicillin, doxycycline, mupirocin, clindamycin and azithromycin, all of which enhanced the release of prophage in > 40% of the isolates. There was no correlation between *S. aureus* harbouring active prophages and inactive prophages with their biofilm biomass and metabolic activity. However, the disease severity score of patients harbouring inducible prophage within *S. aureus* was significantly lower implying the role of active lysogeny in CRS disease. In addition, beta-hemolysin activity was absent in almost 92% of *S. aureus* isolated from the sinonasal cavities of chronic rhinosinusitis patients.

In conclusion, our findings expand on the distribution of active lysogeny in *S. aureus* clinical isolates and the effects of commonly used antibiotics in prophage mobilization.

As prophage mobilization directly correlates with virulence and antimicrobial resistance dissemination, our research contributes to expanding our understanding of the unwanted use of antibiotics and how exposure of bacteria to sub-lethal antibiotics may also play a role in the development of virulent clones in addition to antimicrobial resistance.

Highlights

- Spontaneous prophage induction (SPI) was observed in around 26% of the *S. aureus* clinical isolates while mitomycin C dependent prophage induction was observed in almost 52% of the clinical isolates.
- Sub-inhibitory concentrations of antibiotics enhanced the release of prophage compared to spontaneous prophage induction (SPI).
- Ciprofloxacin was the most potent prophage inducer (51%) followed by amoxicillin, doxycycline, mupirocin, clindamycin and azithromycin, all of which enhanced the release of prophage in > 40% of the isolates.
- Beta-hemolysin activity was not observed in most of the *S. aureus* isolates recovered from sinonasal cavities of chronic rhinosinusitis patients.

4.1 INTRODUCTION

Genomic studies have revealed that temperate phages are a significant component in human biomes, strongly influencing the distribution and dynamics of the microbiota (Navarro and Muniesa, 2017). Such temperate phages find their origin in various bacteria where they exist as prophages making their host lysogenic (Ganeshan and Hosseinidoust, 2019). Prophage DNA can be embedded into a bacterial chromosome or in an extrachromosomal plasmid. Prophages may be released by secretion without causing cell lysis (chronic life cycle) (Hobbs and Abedon, 2016; Venturini et al., 2022) or are induced and released as infective phage particles causing cell lysis (lytic life cycle). Prophage induction can occur either spontaneously or by environmental factors such as diets high in sugar, fatty acids, and drinks like kombucha (Boling et al., 2020). Prophage induction may also occur by various host-related stresses such as nutritional deprivation, temperature upshifts, low/high pH, UV irradiation, exposure to sub-lethal concentrations of certain antibiotics such as mitomycin C (MMC) and chemicals like hydrogen peroxide (H_2O_2) (Boling et al., 2020; Cortes et al., 2019; Ibarra-Chávez et al., 2021; Oh et al., 2019). As prophages often carry genes encoding various virulence factors (VF) and toxins and antimicrobial resistance genes (ARGs), lysogenic bacteria are thought to be more pathogenic compared to prophage-free clones of the same species or strain (Davies et al., 2016; Kondo et al., 2021). Additionally, induced prophages can infect and either lysogenize or lyse susceptible competing strains thereby providing competitive survival fitness to the parent strain. Further, although prophage induction kills a small number of bacterial cells (< 1%), the released phage particles can lysogenize other genetically similar strains in the niche thereby transferring the phage-associated

pathogenic traits horizontally. It is believed that bacterial biofilm benefits from the lysogeny (Silveira and Rohwer, 2016). When prophages are released from a few lysogens, the lysing cells release extracellular DNA (eDNA) and nutrients locally, which may strengthen biofilm structure. Further, the lysis of a few lysogens may also break the biofilm bonds allowing for an enhanced cell dispersal (Obeng et al., 2016). Also, eDNA and other molecules released during prophage induction function as quorum-sensing molecules to send stress signals and mount bacteria with additional survival traits (Høyland-Kroghsbo et al., 2013). Induction of prophages and release of phage particles (particularly Pf phages) may also activate the anti-viral immune response in mammalian cells, which further protects bacteria from phagocytosis (Sweere et al., 2019, Wahida et al., 2021). Further, integration of prophage is also associated with increased biofilm (Carrolo et al., 2010; Liu et al., 2020). Because of all these factors, prophages are proposed as one of the crucial catalysts in disease modulation (Nepal et al., 2022).

Lately, prophage induction and its role in gut health has been well studied as exposure to antibiotics enhance movement of toxins in gut bacteria (Boling et al., 2020). Antibiotic-dependent prophage-associated adverse effects have been observed in Shiga-toxin producing *E. coli* O157:H7 where treatment with ciprofloxacin or norfloxacin causes an increase in Shiga toxin release (Wang and Wood, 2016). Similar effect was observed in *Streptococcus canis* where fluoroquinolone enrofloxacin induced transcription of the *scm* gene by 58-fold which is responsible for streptococcal toxic-shock syndrome and necrotizing fasciitis (Ingrey et al., 2003). Similarly, prophages of *Enterococcus faecalis* V583 excise in the presence of fluoroquinolones, while hydrogen-peroxide and metronidazole can induce prophages in *Salmonella* (Wang and Wood,

2016). In *Staphylococcus aureus*, in addition to fluoroquinolones and trimethoprim, β-lactams are also capable of triggering prophage induction (Goerke et al., 2006). β-lactam mediated prophage induction further resulted in high-frequency transfer of staphylococcal pathogenicity islands, implying that antibiotics may have unintended consequences in certain clinical strains carrying specific prophages (Úbeda et al., 2005; Wang and Wood, 2016). Recent studies have highlighted the great diversity of prophages in clinical isolates of *S. aureus* (Bae et al., 2006; Goerke et al., 2009; Naorem et al., 2021; Nepal et al., 2021; Xia and Wolz, 2014). In chronic rhinosinusitis (CRS), *S. aureus* is often isolated from the sinuses of patients and is believed to play a major role in disease exacerbations (Vickery et al., 2019). Earlier, using *in-silico* approaches, we reported that CRS patients developing nasal polyps (CRSwNP) often harboured *S. aureus* with beta-hemolysin (*hlb*) converting prophages (hereafter Sa3int prophage) that downregulate the production of beta-hemolysin (Nepal et al., 2021). Beta-hemolysin is a sphingomyelinase hemolysin that significantly contributes to *S. aureus* pathogenesis (Salgado-Pabon et al., 2014; Tran et al., 2019) and may also reduce ciliary activity of nasal epithelial cells and induce sinusitis (Kim et al., 2000). However, there are controversial reports about beta-hemolysin in *S. aureus* as a virulence factor, many arguing that beta-hemolysin is required for successfully colonizing the nasal niche while others negating beta-hemolysin as a major virulence factor in humans due to negative conversion by the Sa3int prophage (Salgado-Pabon et al., 2014).

Lately, it has been shown that prophage induction plays a significant role in landscaping the gut microbiome (Henrot and Petit, 2022; Hu et al., 2021; Redgwell et al., 2021). Prophages are abundant in clinical strains and their presence and induction can have

important clinical implications; nonetheless, we lack knowledge on the effects of routinely used medications such as various antibiotics and steroids on prophage induction of clinically relevant strains. Further, as infections are usually treated with antibiotics, identification of factors and drugs that enhance or suppress prophage excision and induction is cardinal in clinical science because prophage behavior can both upregulate or downregulate bacterial virulence. While the effects of sub-lethal antibiotics in the development of antibiotic resistance, production of VF and enhancing bacterial invasion have been well studied, the impact of sub-lethal drugs on prophage behavior is largely unknown.

The aim of this research was to investigate the effects of routinely used drugs (antibiotics and steroids) on prophage induction. We further wanted to know if prophage inducibility correlates with CRS type and/or disease severity and biofilm production. To do this, we retrospectively analyzed *S. aureus* isolated from CRS patients for prophage induction using commonly used drugs for their condition.

4.2 MATERIALS AND METHODS

4.2.1 Study design and ethics

Ethics approval for the use of clinical isolates was obtained from the Human Research Ethics Committee of the Central Adelaide Local Health Network (HREC/18/CALHN/69). All clinical isolates used in this study were isolated from the nasal cavity of chronic rhinosinusitis patients with nasal polyps (CRSwNP), CRS without nasal polyps (CRSsNP) and controls and were stored in glycerol stocks at -80^{o}C until use. The classification of patients into CRSwNP and CRsNP was according to EPOS 2020 guidelines (Fokkens et al., 2020) and disease severity was graded using Lund Mackay scoring (Hopkins et al., 2007). The genomic and prophage characteristics of *S. aureus* used in this study were described previously (Bardy et al., 2018; Nepal et al., 2021).

4.2.2 Bacterial strains, media and growth conditions

S. aureus ATCC25923 (American Type Culture Collection, ATCC, Manassas, USA), a prophage-free *S. aureus* RN4220 (German Collection of Microorganisms and Cell Cultures, DSMZ, GmbH, Germany) and all clinical isolates (N = 66) were retrieved from glycerol stocks and cultured on tryptone soy agar (TSA, 1%, Oxoid Ltd., Hampshire, UK) at 37^{o}C overnight. A single colony was propagated in 15 ml of nutrient broth (NB) or tryptic soy broth (TSB) (Oxoid Ltd., Hampshire, UK) in a shaking incubator (180 rpm) at 37°C unless specified. Isolates were classified as methicillin resistant *S. aureus* (MRSA) or methicillin sensitive *S. aureus* (MSSA) based on the presence or absence of the *mecA* gene on whole genome sequencing.

4.2.3 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of 7 different antibiotics (Table 4.1) was determined by the microtiter broth dilution (MBD) method as described by Wiegand et al (Wiegand et al., 2008). Briefly, the overnight culture was adjusted to 0.5 McFarland on sterile saline (0.9% NaCl) and diluted 100-fold in cation-adjusted Mueller Hinton Broth (MHB, Oxoid Ltd., Hampshire, UK). One hundred microliters of the diluted culture were transferred to the microtiter plate and an equal volume of desired antibiotics prepared in MHB was added across rows with the final antibiotic concentration decreasing by half (32.0 µg/ml to 0.06 µg/ml) on successive wells. The penultimate column was used as positive 'growth control' and the last column as negative 'broth control'. The MIC value was determined according to CLSI guidelines and half of MIC (sub-MIC) was used for prophage induction experiments. All the experiments were performed in triplicates.

4.2.4 Prophage identification, induction, purification and spot assay

Prophage in all *S. aureus* isolates was predicted and categorized into intact, questionable or incomplete using the PHASTER tool (Arndt et al., 2016) as described earlier (Nepal et al., 2021). Mitomycin C (MMC) was used as a positive control for prophage induction. Briefly, MMC (final concentration = 1.0 µg/ml) (Sigma-Aldrich, Missouri, USA. Lot# SLBX4310) was added to exponentially growing cells (OD_{600} = 0.3) in nutrient broth and further incubated for 6 hrs at 37°C in a rotating incubator (180 rpm). Optical density at 600 (OD_{600}) was measured every hour. After 6 hrs, the culture was centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was filtered through 0.2 µm pore size

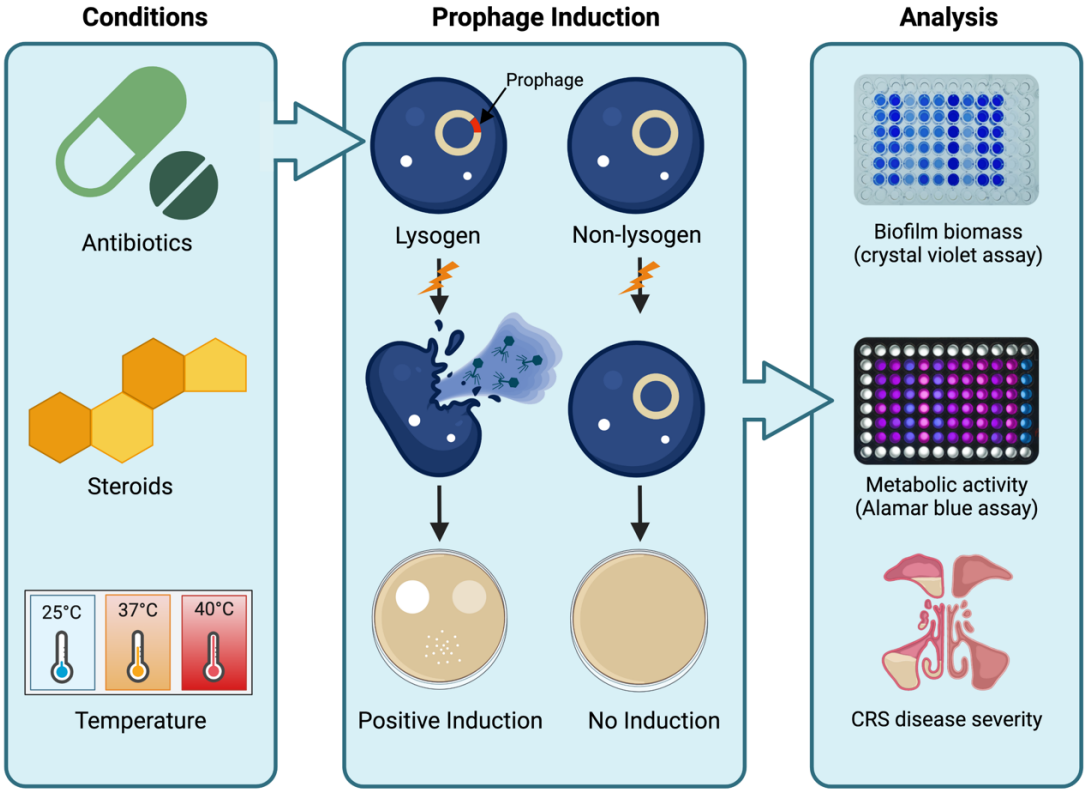

Figure 4.1 | Schematic representation of the experimental design. Prophages were induced using sub-lethal antibiotics, commonly used steroids in their working concentrations and under varying temperatures. The clinical isolates were then divided into active lysogens and inactive lysogens to compare their correlation with overall biofilm, metabolic activity and chronic rhinosinusitis severity score (Lund-Mackay score).

(13 mm, Acordisc®, Pall International, Fribourg, Switzerland) when required. A similar method was used for antibiotics and corticosteroids for prophage induction (Table 4.1). Antibiotic-free bacterial culture was used as a negative control. For effect of temperature on prophage induction, the microtiter plates were incubated in different temperatures (35°C , 37°C and 40°C) and induced prophage were estimated by spot assay. The successful induction of a prophage was screened by spot assay on indicator strains using double-layer agar overlay (DLAO) technique as described previously (Hockett and Baltrus, 2017). Briefly, previously prepared bottom agar (TSA, agar = 1%) was overlaid with 3 ml of semi-solid TSA (agar = 0.4%, T = 50°C) containing 100 µl of exponentially growing bacterial strains (*S. aureus* RN4220 or *S. aureus* ATCC25923).

After solidification, 20 µl of filter-sterilized supernatant (serially diluted if required) was spotted on the agar plates in duplicates. The plates were air-dried and incubated at 37°C overnight at an upright position. The next day, the plates were visually inspected for the presence of plaque-like lysis in the spotted area. The presence of lysis spots (complete or partial) at the spotted area was regarded as positive (induction of re-infective phage) whereas all others (no lysis and faint lysis akin to antibiotic killing) were recorded as no induction (Figure 4.2a, 4.2b).

Table 4.1| Sub-inhibitory concentration of drugs used in this study.

Drugs used for prophage induction	Concentration
Amoxicillin (penicillin)	1.0 µg/ml
Azithromycin (macrolide)	0.5 µg/ml
Ciprofloxacin (quinolone)	0.5 µg/ml
Clindamycin (macrolide)	0.06 µg/ml
Doxycycline (tetracycline)	0.125 µg/ml
Mupirocin (carboxylic acid)	0.125 µg/ml
Rifampicin/rifampin (antimycobacterials)	0.03 µg/ml
Hydrocortisone 21-hemisuccinate sodium salt (HCHS)	1.4 mg/ml, 22.8 mg/ml
Budesonide	2.0 µg/ml

4.2.5 Estimation of biofilm biomass and biofilm metabolic activity

Biofilm biomass and biofilm metabolic activity (cell viability) assay were performed using crystal violet (CV) assay (Shukla and Rao, 2017) and alamarBlue® cell viability assay as per manufacturer's guidelines (Life Technologies, Oregon, USA) in biofilms established for 48 hours in 96-well flat bottomed (Costar, Corning Incorporated, USA. #Ref. 3599) and 96-well flat and clear bottom black assay plate (Costar, Corning Incorporated, USA. #Ref. 3603) respectively. Briefly, overnight broth culture of *S. aureus* was adjusted to 1.0 McFarland standard and diluted 1:15 in nutrient broth with or without antibiotics. One-hundred-fifty microliters of each diluted culture was pipetted in inner wells of

respective 96-well plates. Appropriate concentration of antibiotics and drugs was added to the wells accordingly. The peripheral wells were filled with sterile water, sealed with aluminum foil and incubated at 37°C in an orbital shaker (80 rpm). After 48 hours of incubation, supernatants were carefully aspirated, and the plates were washed twice with phosphate buffer saline (PBS, 1X) followed by the CV or alamarBlue® assay.

For CV, plates were air-dried and 200 µl of 0.1% CV solution was added to each inner well and left at room temperature for staining. After 10 minutes, the excess CV was aspirated, washed twice with PBS (1X) and air-dried. Finally, the biomass-bound crystal violet was solubilized in 150 µl of 30% acetic acid and the biomass was estimated in terms of absorbance (OD_{600}) at 600 nm using FLUOstar Optima (BMG Labtech, Germany). Similarly, for metabolic activity, 200 µl working solution of alamarBlue® (1X) was added to each inner well, covered with aluminum foil and incubated at 37°C in an orbital shaker (80 rpm). Resorufin fluorescence was measured at the 1-hr interval for 6 hrs using the FLUOstar OPTIMA (BMG Labtech, Germany) microplate reader (excitation = 530 nm, emission = 590 nm). The metabolic activity (expressed as fluorescence units) at 150 minutes time point was considered for further analysis as it showed the broadest distribution. All experiments were performed as 6 biological replicates and 2 technical replicates with nutrient broth as a sterility control.

4.2.6 β-hemolysin activity

The CAMP test (Christie-Atkins-Munch-Peterson) on 5% sheep blood agar (SBA, ThermoFisher, Australia. #Cat: R01202) was used to determine the alpha (α), beta (β),

and delta (δ) hemolysin activity. *S. aureus* RN4220 (a known beta-hemolysin producer) was used as a reference strain (vertical streak). Overnight culture from nutrient agar plates were streaked horizontally (~3 mm apart) from the vertical streak of RN4220 and incubated overnight at 37°C followed by incubation at 4°C for 2 days. The alpha, beta, and delta hemolysis were determined based on the visual inspection of the sheep blood agar plates. The interruption of beta-hemolysis gene (*hlb*) was also confirmed by *in silico* analysis of the genome sequences with primer sets *hlb*-F (5'-TATGTTATCGACCGTGTTGTATCC-3') and *hlb*-R (5'-ATCCCATGGCTTAGGTTTTTCAGT-3'). The analysis parameters are elaborated in Supplementary Text ST1.

4.2.7 Statistical and bioinformatics analysis

All statistical analyses were performed using GraphPad Prism 9 (ver 9.1). Fisher's exact test was used to evaluate the significance of induction, and an unpaired t-test was used to evaluate the statistical significance between groups. $P < 0.05$ was considered statistically significant. PHASTER was used to characterize the completeness of prophages using the default settings (Arndt et al., 2016). No statistical methods were used for predetermination of sample size and experiments were not randomized.

4.3 RESULTS

4.3.1 Antibiotic resistance pattern of clinical isolates

Sixty-six *S. aureus* clinical isolates were used, all isolated from the sinonasal cavity of patients (CRSsNP=27, CRSwNP=30, Control=9). The highest observed resistance was against amoxycillin (18%, 12/66) followed by azithromycin (14%, 9/66) and ciprofloxacin (11%, 7/66) (Supplementary data). None of the isolates were resistant to clindamycin, doxycycline, rifampin and mupirocin. Whole genome sequences were analysed for the presence of the *mecA* gene showing 3/27 CRSsNP, 7/30 CRSwNP and 0/9 control isolates had the *mecA* gene present and were classified as MRSA (Table 4.2).

Table 4.2 | Genetic and phenotypic characteristics of *Staphylococcus aureus* isolated from chronic rhinosinusitis patients.

Genetic background of *Staphylococcus aureus*	CRSsNP (n=27)	CRSwNP (n=30)	Control (n=9)	Total (n=66)
MSSA	24	23	9	56
MRSA	3	7	0	10

CRSsNP = chronic rhinosinusitis sans nasal polyp, CRSwNP = chronic rhinosinusitis with nasal polyp, MSSA = methicillin sensitive *Staphylococcus aureus*, MRSA = methicillin resistant *S. aureus*. Note: Patient demographics and their antibiotics use are already publicly available as supplementary data through our previous study at https://doi.org/10.6084/m9.figshare.16590359.

4.3.2 Prevalence of inducible prophage in patient-derived S. aureus

Spontaneous prophage induction (SPI) and release of infective phage particles were observed in almost 26% (17/66) of the *S. aureus* clinical isolates (Figure 4.2a, 4.2b). All of these CIs harboured at least one intact prophage(s) identified by PHASTER. Upon MMC treatment, prophages were induced and released as infective phage particles from 52.5% (35/66) of isolates tested (Figure 4.2b) termed as 'active lysogens' hereafter. From those, all released phages could infect *S. aureus* RN4220 whilst only around 48%

(17/35) of the released phage particles could weakly infect *S. aureus* ATCC25923 (data not shown). However, the SPI was weaker and only a few plaques were observed in plates compared to MMC-dependent prophage induction, as represented in Figure 4.2a. Out of 35 isolates from which infective prophages were induced upon MMC treatment, 91% (32/35) had at least one identified intact prophage whereas 9% (3/35) had questionable prophage as per the PHASTER analysis (data not shown). Further, although 85% CIs (56/66) had intact prophages, infective phage particles were released from only 57% (32/56) of the isolates either spontaneously or under MMC treatment (Figure 4.2b). Infective phage particles were not released from any strains harboring only incomplete prophages (data not shown). MMC-dependent prophage induction was highest in isolates from control patients (69%) followed by CRSsNP isolates (56%) and were lowest in CRSwNP isolates (50%) (Figure 4.2c).

4.3.3 Sub-inhibitory antibiotics enhanced prophage induction from active lysogens

Overall, enhanced release of reinfecting phages was observed in ~25% of clinical isolates using various sub-inhibitory concentrations of antibiotics compared to control at 37°C (Figure 4.3a). Out of 7 antibiotics tested, ciprofloxacin was the most potent prophage inducer. Among 35 strains releasing infecting phage particles, ciprofloxacin activated prophages in 51% (18) of isolates followed by amoxicillin (48%, 17), doxycycline (43%, 15), mupirocin (43%, 15) clindamycin (40%, 14), azithromycin (40%, 14) and rifampin (37%, 13) (Figure 4.3b). Further, two commonly used steroid drugs (HCHS and budesonide) also enhanced prophage release in almost half of the active lysogens (Figure 4.3b). A change in incubation temperature to 25°C and 40°C (from 37°C) was

tested and did not enhance prophage induction compared to the control but suppressed the prophage release (Figure 4.3c). However, this may be due to slower growth of bacteria rather than suppression. Further, the infectivity of released phages in host RN4220 was higher than in ATCC25923 (data not shown) suggesting that RN4220 is a better host for spot assays and indicating that the released phages can infect multiple hosts.

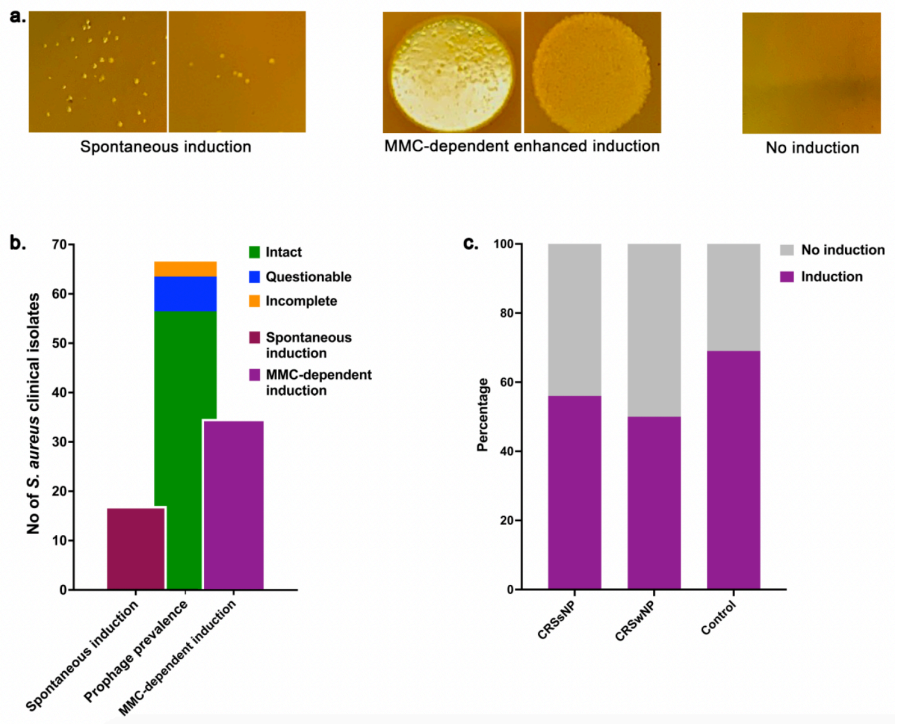

Figure 4.2 | Relative induction of prophage from *S. aureus* clinical isolates. (a) Representative lysis spots produced by spontaneously induced prophages, the negative control and mitomycin-dependent enhanced prophage induction in indicator strain RN4220. Clear spots indicate strong lysis (presence of higher number of phage particles) while individual plaques indicate lower concentration of induced phage particles. 1 µg/ml (final concentration) of mitomycin C (MMC) was used to induce prophage from exponentially growing broth culture of all (N = 66) clinical strains. (b) Out of 66 clinical strains, spontaneous prophage induction (SPI) was observed in only 17 isolates although 56 isolates had at least one intact prophage. Under MMC treatment, prophage induction was observed in 35 isolates including the ones that showed SPI. However, the prophage induction was relatively stronger after MMC treatment compared to the SPI. (c) There was no significant difference in frequency of prophage induction between CRSwNP, CRSsNP and control groups under MMC treatment as prophage induction was observed in almost 50% of isolates from each group. CRSsNP = chronic rhinosinusitis without nasal polyps, CRSwNP = chronic rhinosinusitis with nasal polyps

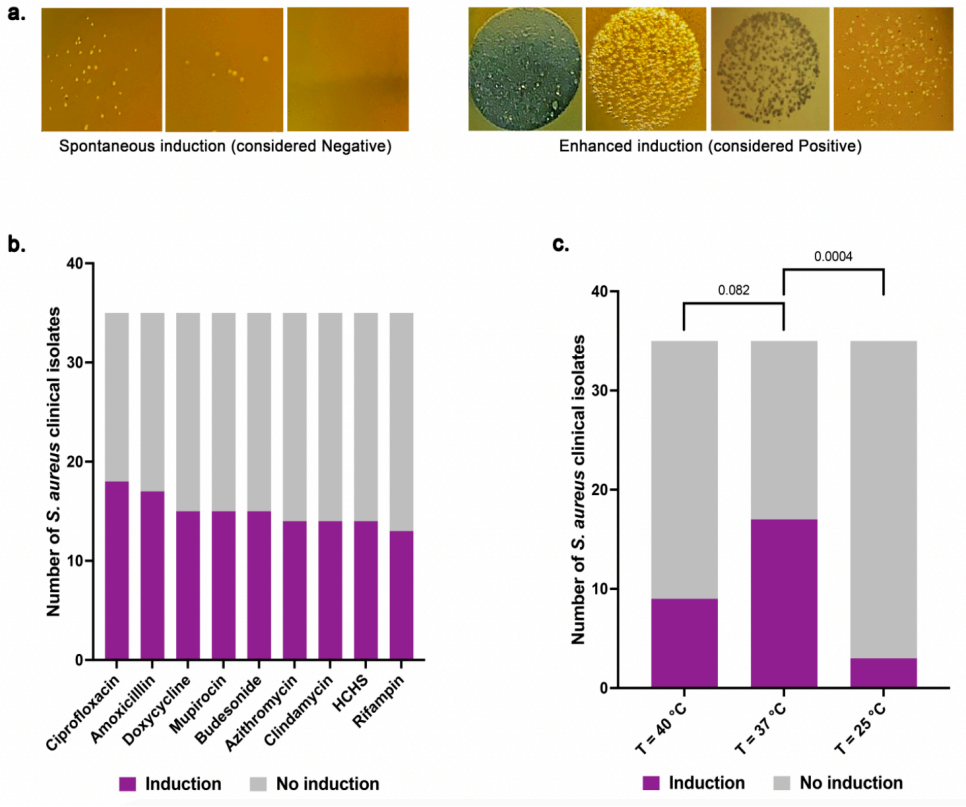

Figure 4.3 | Antibiotics-dependent prophage induction from *S. aureus* clinical isolates. (a) Representative lysis spots produced by spontaneously induced prophages and no-lysis (control group, considered negative in this analysis), and antibiotic-dependent enhanced prophage induction in indicator strain RN4220, which were considered positive. (b) Out of all the antibiotics tested, ciprofloxacin was the most potent prophage inducer which induced prophages in almost 51% (18/35) of the active lysogens followed by amoxicillin (48%). (c) Prophage induction in *S. aureus* was significantly suppressed at 25°C and 40°C compared to 37°C (Fischer's exact test) under normal circumstances. However, this may be due to the slower growth of bacteria rather than suppression.

4.3.4 Active lysogeny did not alter mature S. aureus biofilm formation and biofilm metabolic activity but correlated inversely with chronic rhinosinusitis disease severity

Despite few evidence supporting the role of prophage induction in the formation of a biofilm, we did not observe significantly high biofilm formation in *S. aureus* CIs with inducible (active) prophages compared to isolates harbouring non-inducible prophages.

Also, there was no significant variation in biofilm metabolic activity and biofilm biomass between clinical isolates harbouring active inducible prophage and non-inducible quiescent prophages (Unpaired student t-test, $p > 0.05$) (Figure 4.4a). Further, we did not observe a statistically significant difference in biofilm biomass between strains isolated from CRSsNP and CRSwNP patients (Figure 4.4b) compared to the control group. However, Lund-Mackay severity score of CRS patients harbouring active *S. aureus* lysogens was significantly lower ($p < 0.05$, student t-test) compared to the group harbouring *S. aureus* with non-inducible prophages (Figure 4.4c).

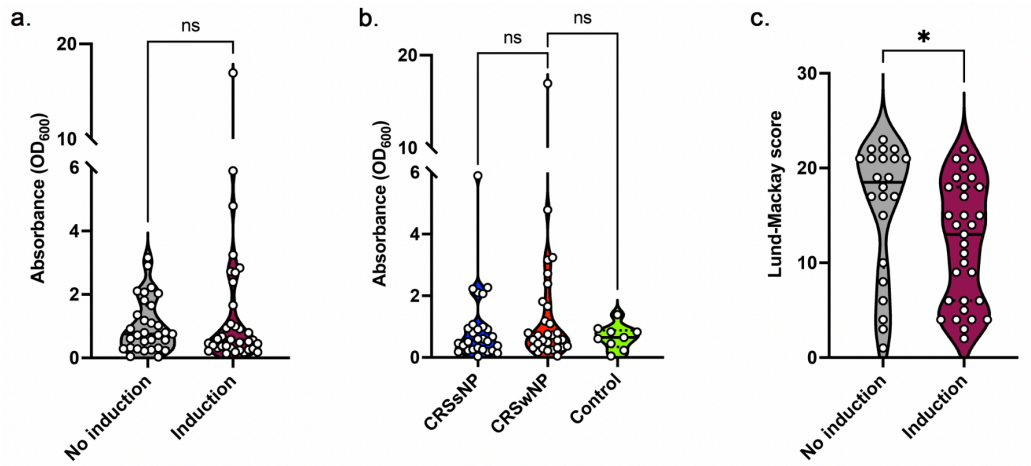

Figure 4.4 Correlation of biofilm biomass with prophage activity and disease type. (a) No significant increase in biofilm biomass was observed between clinical strains harbouring active inducible prophage and quiescent non-inducible prophage. (b) Similarly, there was no significant difference in biofilm biomass between CRSwNP and CRSsNP groups compared to the control. (c) However, Lund-Mackay score (severity score of chronic rhinosinusitis) was significantly lower in patients harbouring *S. aureus* with inducible prophage compared to non-inducible prophages. Note: the solid black line within the violin plot represents the mean while the upper and lower dashed lines indicate the upper and lower quartiles. CRSsNP = chronic rhinosinusitis without nasal polyps, CRSwNP = chronic rhinosinusitis with nasal polyps, * $p < 0.05$, ns = not significant

There was significant variation in the overall metabolic activity of *S. aureus* in a biofilm between clinical isolates (Figure 4.5a) and the metabolic activity of *S. aureus* clinical isolates in a biofilm positively correlated with the biofilm biomass ($p = 0.008$, $R^2 = 0.118$,

simple linear regression) (Figure 4.5b). However, we did not observe any difference in the metabolic activity between *S. aureus* isolated from CRSsNP, CRSwNP and control groups (Figure 4.5c). Although statistical significance was not reached in these cases, it may be clinically relevant. Further, although biofilm biomass was significantly lower in *S. aureus* clinical isolates harbouring active inducible prophages (Figure 4.4c), the metabolic activity of the same biofilm was not significantly different (Figure 4.5d).

4.3.5 Beta-hemolysin was absent in most of the S. aureus nasal colonizers

Beta-haemolytic activity on blood agar was observed only in 8% (5/66) *S. aureus* CIs isolated from the nasal cavity of CRS and control patients (Figure 4.6a, 4.6b, 4.6c) although (39.4%) 26/66 CIs did not harbour identifiable Sa3int prophages that disrupts the beta-haemolysis gene. Further, delta- and alpha-haemolysis were observed in 26% (17/66) and 50% (33/66) of the clinical isolates while 17% (11/66) isolates did not show alpha, beta or delta haemolysis in CAMP test on sheep blood agar.

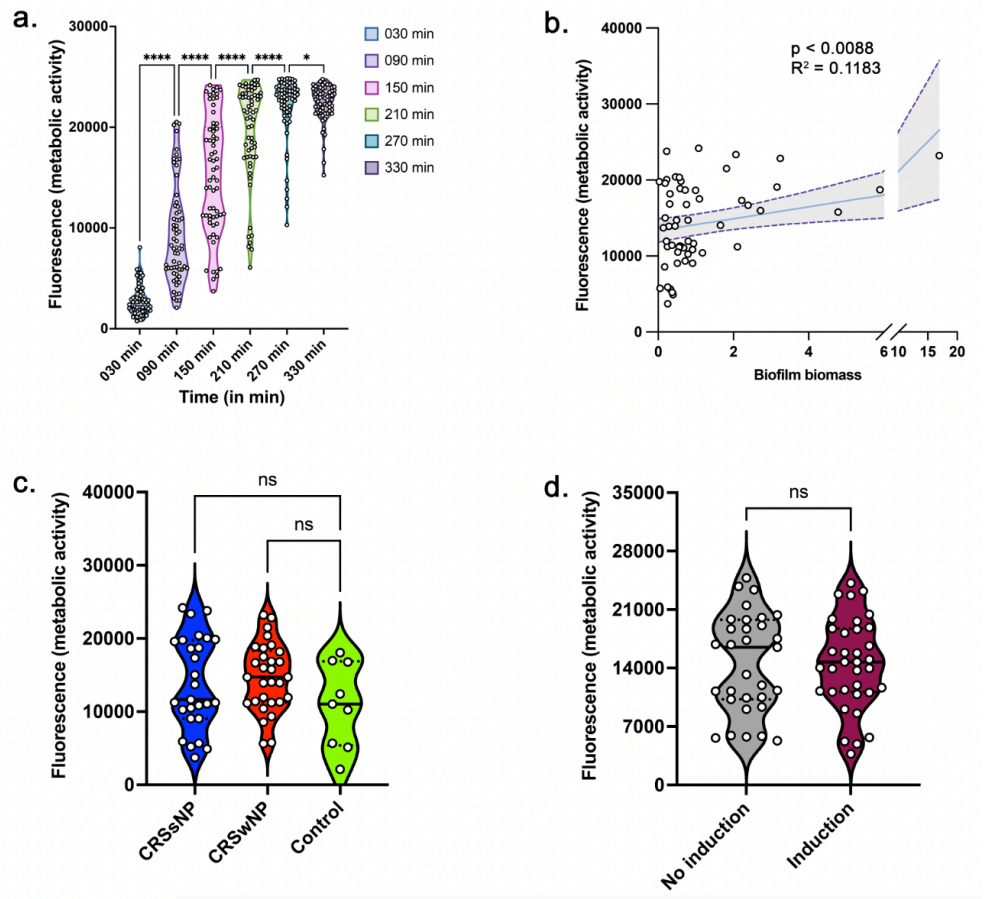

Figure 4.5 | Correlation of metabolic activity of the biofilm with CRS disease type and prophage activity. (a) The highest biofilm metabolic activity variation was observed at 150 minutes, so this time point was considered for further analysis. (b) The biofilm biomass and biofilm metabolic activity were significantly correlated ($p < 0.05$, simple linear regression) in *S. aureus* clinical isolates. (c) There was no significant difference in biofilm metabolic activity between CRSwNP and CRSsNP groups compared to the control group. (d) The metabolic activity of biofilm harbouring active prophage was slightly lower than strains without active prophage, but this was not statistically significant. Note: the solid black line within the violin plot represents the mean while the upper and lower dashed lines indicate the upper and lower quartiles. CRSsNP = chronic rhinosinusitis without nasal polyps, CRSwNP = chronic rhinosinusitis with nasal polyps, * $p < 0.05$, **** $p < 0.0001$, ns = not significant

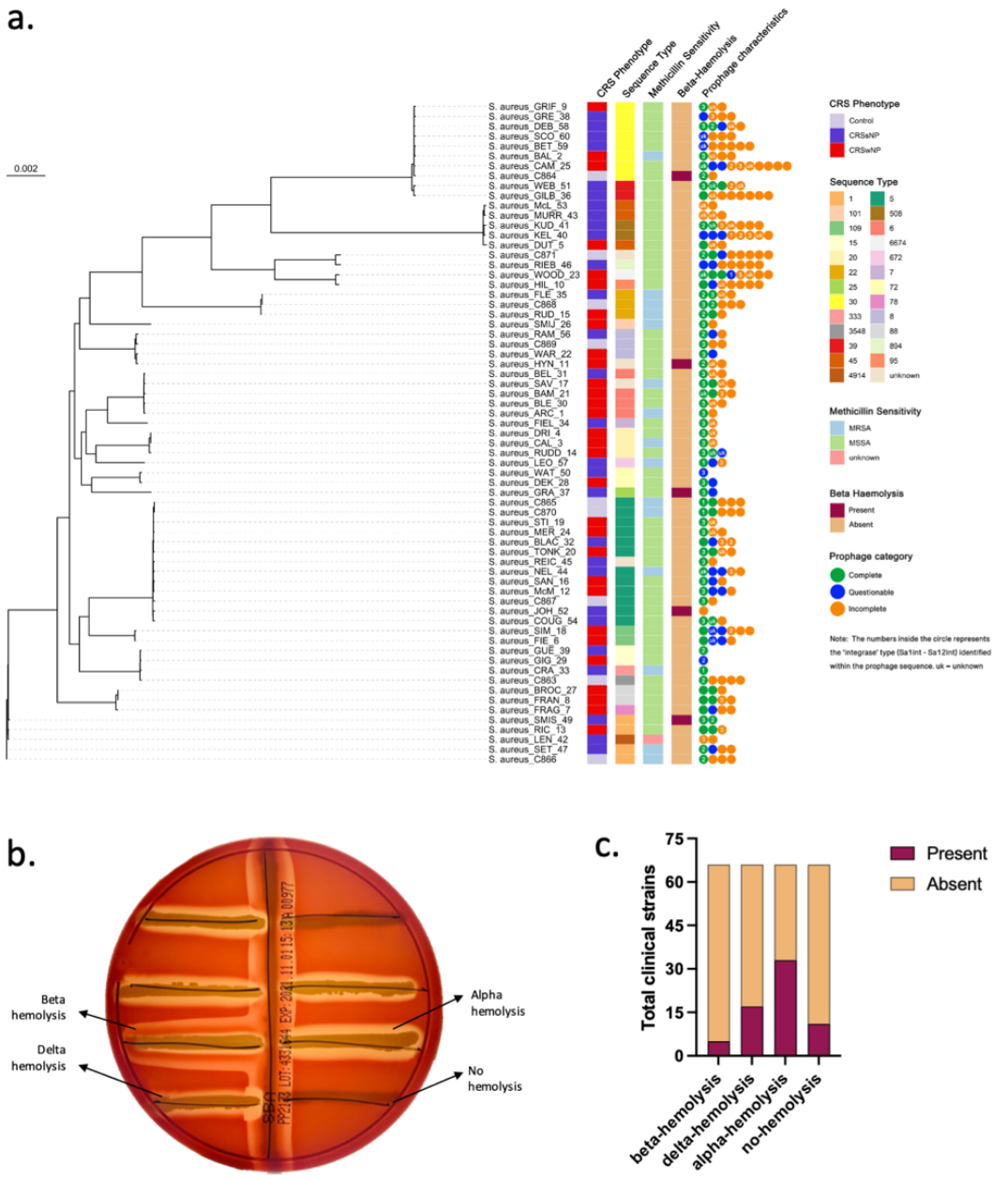

Figure 4.6 | Prophage characteristics and different hemolysis observed in sheep blood agar plates. (a) Phylogenetic tree of all the *Staphylococcus aureus* clinical isolates and their prophage characteristics. (b) Representative sheep blood agar plate showing different types of hemolysis activity by *S. aureus* clinical isolates. (c) Beta-hemolysis was only observed in 8% of the total clinical isolates, while alpha-hemolysis and delta-hemolysis were more prevalent. Note: The phylogenetic tree is adapted from Nepal et al. (2021)

4.4 DISCUSSION

Prophages are widespread in clinical strains, and it has been established that *S. aureus* prophages carry multiple virulence factors that alter bacterial pathogenicity (Chaguza et al., 2022; Naorem et al., 2021). However, not all prophages carry identical genes and behave uniformly in various stressed conditions. Prophage-mediated horizontal gene transfer is one of the most efficient ways of gene acquisition and virulence dissemination in nature (Chen et al., 2018; Penades et al., 2015; Schneider, 2017). Further, it is now established that clinical isolates often carry prophages and release them in the presence of antibiotics and other drugs that may modulate disease progression (Goerke et al., 2006; Mccarthy et al., 2012; Tyler et al., 2013). Our results indicated that although most of the *S. aureus* clinical isolates from CRS patients harbored intact (or complete) prophages, only about half of them were inducible with MMC. In contrast, spontaneous induction of prophage was rare and was only observed in a few clinical isolates after 24 hours of culture implying induction is accelerated in response to antibiotic stress. Routinely used antibiotics in the treatment of CRS enhanced prophage induction in a small fraction of isolates uniformly across control patients and CRS patients. Further, beta-hemolysin which is considered an important pathogenic trait in *S. aureus* was absent in most of the isolates, possibly truncated by prophages that are known to insert themselves into the *hlb* gene thereby abrogating the production of beta-hemolysin.

Various studies have shown that *S. aureus* prophages are released when bacteria are exposed to sub-inhibitory concentrations of antibiotics and other enzymes (Boling et al., 2020; Diene et al., 2017; Jancheva and Bottcher, 2021; Laumay et al., 2019; Nanda et al.,

2015; Rasigade et al., 2016). Our observation agrees to the published literature as we observe release of infective phages in almost 52% isolates using MMC and in almost in 25% using sub-inhibitory antibiotics. Further, infective phage particles were also released from isolates carrying questionable (non-intact) prophage. Such induction of questionable prophage has also been observed by De Sousa et al. in *Klebsiella pneumoniae* (de Sousa et al., 2020). Thus, we agree with De Sousa and co-workers that *in silico* completeness score is not reflective of the inducibility and it is imperative to experimentally validate the lytic/lysogenic lifestyle switching as it modulates the bacterial fitness (Carrolo et al., 2010; Wang et al., 2010; Nanda et al., 2015; Tan et al., 2020). Also, identifying the status of prophage in a lysogen is of paramount importance because a higher transduction rate has been observed in the presence of antibiotics (Meessen-Pinard et al., 2012). Thus, inappropriate use of antibiotics not only assists in development of AMR clones but also may promote the horizontal transfer of prophage encoded toxic/virulence genes between different strains mediated by induced temperate phages and assist in clonal expansion of virulent strains (Roberts et al., 2014). Further, the high abundance of such non-inducible cryptic sequences suggests that bacterial strains are continuously infected with temperate phages and turn into cryptic remnants during evolution.

Few studies have suggested a role of lysogenic-to-lytic switching in disease presentation. Indeed, released phage particles have been suggested to stimulate antiviral immunity thereby influencing inflammation (Sweere et al., 2019). Interestingly, in this study, inducible prophages were more prevalent in isolates from non-CRS control patients than in those from CRSwNP patients and appeared to be inversely related to CRS disease

severity scores. Further studies are required to evaluate whether this inducibility impacts the inflammatory process in the sinus mucosa and might have a protective role against overactivation of immune response pathways contributing to CRS. Previous in silico analysis by our team, however, has shown that certain types of intact phages (Saint3) were more prevalent in CRSwNP than controls (Nepal et al., 2021). This apparent discrepancy with the current findings may indeed be because of the variability in prophage type carried and induced by *S. aureus* isolated from CRSsNP and CRSwNP. Indeed, most *S. aureus* studied were poly-lysogenic and, without further investigation, it is uncertain which prophage is induced upon stimulation with MMC or antibiotics. Furthermore, this study also indicated that not all intact prophages are inducible with MMT or antibiotics. Further in-depth analysis is required to understand the identity and specific role of intact and inducible prophages in the disease process. Further, there are reports that lysogenization and prophage induction in *S. aureus* enhances biofilm development through quorum sensing (Fernandez et al., 2018). Despite high prevalence of infective prophages in *S. aureus*, we did not find significant differences between active lysogen and a passive lysogen in terms of biofilm development. This may be because we were comparing the biofilm development between different clinical isolates and role of prophage release in biofilm is minimal compared to other factors. Further studies are required to establish direct correlations between prophage integrity, prophage types (genes they harbor) and their impact on bacterial behavior at various stressed conditions. Further, no significant decrease in bacterial growth was observed between control and sub-MIC treated bacteria although a fraction of cells switched to lytic lifestyle and phage was more abundant after treatment with sub-inhibitory

antibiotics. This phenomenon clearly explains that prophage activation and phage release occur only in a fraction of actively growing cells that do not impact bacterial growth but increases bacterial fitness. Our results further suggest that inter strain variability in phenotypic characters such as metabolic activity and biomass of a biofilm is not significantly dependent on the type and status (inducible/non-inducible) of the prophage. This suggests minimal role of lytic-lysogenic switching in biofilm development that is clinically important but may hold significance in evolutionary adaptation.

Despite research suggesting a role of *hlb* in reducing the ciliary activity of nasal epithelium (Kim et al., 2000) and *S. aureus* pathogenicity (Burnside et al., 2010; Hayashida et al., 2009; Motamedi et al., 2018; O'Callaghan et al., 1997), we found that the majority of *S. aureus* isolated from CRS patients (~92%) lacked beta-hemolytic activity. This agrees to the report by Roetzer *et al*. who reported that the majority of *S. aureus* isolates (80.4%) from the nose and blood harboured a disrupted beta-hemolysin gene (Roetzer et al., 2016). This may be because Sa3int prophages disrupt the beta-hemolysin gene equipping bacteria with immune evasion cluster (IEC) genes, that help bacteria evade the mammalian immune system through various mechanisms like neutralization of neutrophils, enhanced biofilm formation, stimulation of antiviral responses. This may explain the higher prevalence of Sa3int prophages with disrupted *hlb* gene in severe forms of CRS. However, not all the isolates had Sa3int prophages and further research on role of the role of Sa3int prophages and *hlb* in *S. aureus* is warranted.

Active prophages have a high potential for horizontal dissemination of virulence genes and other genetic traits under stressed conditions, and stress activates pathogenicity. Importantly, antibiotics can induce the production of phages and phage-encoded

products, disseminating these viruses and virulence factors that increases disease severity. These unwanted side-effects of antibiotics cast doubt on the suitability of some antimicrobial treatments and may require new strategies to prevent and limit the selection of antibiotics that actively induce prophages. Understanding the lytic/lysogenic switch would help us design a treatment approach where we could force bacteria to go into the lytic cycle, which would help in suppressing the bacterial load and thereby decreasing the concentration of exotoxins they produce. As exotoxin has been one of the cardinal factors in various diseases, this would significantly decrease the disease severity or may also be useful in food industries where lysogens could be counterproductive as they sometimes can produce life-threatening toxins.

4.5 CONCLUSION

Our results suggest that inducible prophages are common in *S. aureus* clinical strains isolated from the nasal cavity of CRS patients and demonstrate that sub-inhibitory concentrations of clinically used antibiotics increase phage mobilization through prophage induction. Such induced phage particles could infect other isolates and switch to a temperate lifestyle. As such it can be inferred that low antibiotic concentration enhances horizontal gene transfer through transduction. However, there was no significant change in growth rate and biofilm biomass when lysogens were exposed to sub-MIC concentrations of drugs. Further, beta-hemolysis was absent in almost all clinical strains isolated from CRS patients, indicating a minimal role of beta-hemolysin for long-term nasal colonization.

Limitations and future direction

The estimation of biofilm between *S. aureus* having active and passive prophage is broadly categorized not considering other factors associated with biofilm development. A more narrow-downed approach must be considered as clinical isolates vary significantly in other virulence genes that impact biofilm development. The infectivity of released phage must be examined in a broad host range to verify they can transfer and establish lysogeny in multiple clinical isolates. Further, the controlled induction of prophages may also be explored as a therapeutic approach, and it is important to study its prevalence, type, and association with disease.

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CRediT author statement

Conceptualization: SV, RN; Methodology, formal analysis and investigation: RN, GH, GS; Data curation: RN, GH, GB; Visualization and formal analysis: RN, GH; Writing – original draft: RN; Writing – review and editing: RN, GH, GS, SF, SV; Supervision: SV, PJW, AJP; Funding acquisition: SV, PJW.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial and/or financial relationships that could be construed as a potential conflict of interests.

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CHAPTER FIVE

PROPHAGE ACQUISITION BY *Staphylococcus aureus* CONTRIBUTES TO THE EXPANSION OF STAPHYLOCOCCAL IMMUNE EVASION

Prophage acquisition by *Staphylococcus aureus* contributes to the expansion of Staphylococcal immune evasion

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Abstract

Staphylococcus aureus colonizes 30% of the human population, but only a few clones cause severe infections. *S. aureus'* virulence varies and partly depends on the presence of prophages, viral DNA embedded in the *S. aureus* core genome, such as hlb-converting prophage (φSa3int). Human-adapted *S. aureus* often harbours a φSa3int group of prophages preferentially integrated into their β-hemolysin (*hlb*) gene that encodes human immune evasion cluster (IEC) genes. Exotoxins and immune modulatory molecules encoded by this prophage can inhibit human innate immunity increasing *S. aureus* pathogenicity. This study aims to investigate the genomic and phenotypic plasticity of *S. aureus* and changes in its extracellular proteome after the acquisition of φSa3int prophage.

To achieve this, we used *S. aureus* strains isolated from the sinus cavities of a patient with severe chronic rhinosinusitis (CRS) at two different time points (*S. aureus* SA222 and *S. aureus* SA333) and hybrid sequenced the strains using short-read Illumina and long-read Oxford nanopore technology. *In silico* analysis showed the presence of a φSa3int prophage in the later isolate but not in the earlier isolate while most of the core genes remained identical. Using mitomycin C, we induced the φSa3int prophage, and transduced it into the Sa3int-prophage-free SA222 isolate to obtain a laboratory generated 'double lysogen'. We confirmed the successful lysogenisation with culture methods (spot assay, blood agar) and by sequencing. We compared growth kinetics, biofilm biomass and metabolic activity between parent and the lysogen by establishing growth curves, crystal violet and resazurin assays. Exoproteins were identified and quantified using mass spectrophotometry.

Integration of φSa3int prophage in SA222 down-regulated the beta-hemolysin expression of the lysogen. *In silico* analysis of the *S. aureus* genome confirmed the insertion of a ~43.8 kb φSa3int prophage into *hlb* gene. Insertion of prophage DNA did not alter the growth kinetics, biofilm formation, adhesion to primary human nasal epithelial cells and the metabolic activity in a biofilm. However, the acquisition of φSa3int prophage significantly changed the expression of various secreted proteins, both bacterial and prophage-encoded. Altogether, thirty-eight exoproteins were significantly differentially regulated in the laboratory-created lysogen, compared to its recipient strain SA222. Among these proteins, there was significant upregulation of 21 exoproteins (55.3%) including staphylokinase (sak), SCIN (scn), and intercellular adhesion protein B (icaB) and downregulation of 17 exoproteins (44.7%), including β-hemolysin (hlb/sph) and outer membrane porin (phoE). Most of the upregulated proteins were involved in immunomodulation that help *S. aureus* escape human innate immunity and help cause chronic infection. These findings may contribute to the development of novel approaches to render S. *aureus* susceptible to the immune response by blocking prophage-associated defence mechanisms.

Highlights

- A φSa3int prophage preferentially integrates into the β-haemolysin gene (*hlb*) gene thereby disrupting the beta-hemolysin function.
- A ~43.8 kb φSa3int prophage acquisition by *S. aureus* has no impact on its growth kinetics, biofilm formation and adhesion to primary human nasal epithelial cells (HNECs).
- The presence of a φSa3int group prophage likely enhances *Staphylococcus aureus'* human immune evasion capability as the prophage encodes a complete set of immune evasion cluster (IEC) genes.
- Targeted identification of virulence factors in addition to species and strain identification may lead to better-personalized therapy as not all *S. aureus* carry the same virulence genes.

5.1 INTRODUCTION

Bacteria often harbour dormant phage DNA (prophages) embedded within their chromosome. These prophage sequences can confer auxiliary functions, frequently increasing bacterial fitness. However, prophages can also carry various virulence factors (VF), toxins and antimicrobial resistance genes (ARGs), such that lysogens (bacteria carrying a prophage) are often considered more virulent than the corresponding prophage-free strain (Davies et al., 2016; Kondo et al., 2021). Prophages can be induced spontaneously or in response to various extrinsic factors like UV, sub-lethal antibiotics and chemicals (Henrot and Petit, 2022). Although spontaneous prophage induction (SPI) usually occurs at a low level and kills a small fraction of bacterial cells within a population, the released phage particles lysogenize other susceptible strains in the niche, thereby transducing the prophage-encoded virulence factors (PVFs) horizontally. Further, the induction of prophages into phage particles can also activate an anti-viral immune response in mammalian cells that protects bacteria from phagocytosis (Gogokhia et al., 2019; Popescu et al., 2021). It is well established that prophage induction is enhanced under sub-lethal concentrations of various antibiotics and chemicals (Allen et al., 2011; Boling et al., 2020; Goerke, J. Koller, et al., 2006). Moreover, some studies have also found that prophage domestication and induction enhance biofilm formation, further increasing bacterial survival and fitness (Carrolo et al., 2010; Nanda et al., 2015). As bacteria often acquire virulent traits under inadequate or inappropriate antimicrobial treatment regimens despite the DNA replication cost involved, it is essential to understand prophage diversity, prophage dispersal and their role in virulence dissemination. Most bacterial infections are caused by a small number of successful clones that are virulent and pathogenic compared to the commensal

(Beceiro et al., 2013). Therefore, understanding the dynamics of prophage-mediated dissemination of prophage-encoded virulence may provide information about the origin and spread of virulent clones.

Staphylococcus aureus is a genetically and metabolically diverse, highly successful opportunistic bacterial pathogen colonizing the mucosal surfaces of approximately 30% of humans (Tong et al., 2015). *S. aureus* is often isolated from the sinuses of chronic rhinosinusitis (CRS) patients, more often in CRS with nasal polyposis (CRSwNP) (Vickery et al., 2019). Factors related to virulence in S. aureus are often associated with mobile genetic elements (MGEs) like plasmids, insertion sequences and prophages, suggesting that horizontal acquisition of MGEs plays a significant role in the development of the virulence (Howden et al., 2023). A well-known example is beta-hemolysin-converting prophages (hereafter φSa3int) carrying various immune evasion cluster (IEC) genes (*sak, chp, scn* and *sea/sep)* that protect bacteria from neutrophil-dependent phagocytosis (Nepal et al., 2022; van Wamel et al., 2006). Earlier, we reported that *S. aureus* isolated from CRSwNP patients often harboured φSa3int prophages that disrupt the production of β-hemolysin (Nepal et al., 2021). β-hemolysin is a sphingomyelinase hemolysin that significantly contributes to *S. aureus* pathogenesis (Tran et al., 2019), reduces the ciliary activity of nasal epithelial cells and induces sinusitis (Kim et al., 2000). However, the pathogenesis of β-hemolysin toxin in humans is argued due to negative conversion by the φSa3int prophage which is present in most *S. aureus* colonising humans (Salgado-Pabon et al., 2014). Widespread distribution φSa3int prophages among *S. aureus* isolated from the nasal cavity of humans suggests that the induction and re-integration of the released phages drive the dissemination of virulence genes,

particularly IEC, contributing to the genetic diversification and functional adaptations of *S. aureus* (Chaguza et al., 2022). However, the presence of prophage DNA does not imply the functionality of the virulence genes it carries.

This study aimed to investigate φSa3int prophage-mediated phenotypic alteration and virulence expression in *S. aureus* isolated from CRS patients. By transducing an intact φSa3int prophage, induced from *S. aureus* (SA333) isolated from a severe CRS patient, into another genetically close φSa3int-free clinical strain isolated from the same patient at a different time point (SA222), we demonstrated that φSa3int prophages integrate into *hlb* gene and significantly upregulates expression of various exotoxins responsible for human immune evasion. However, the domestication of the prophage did not alter the lysogen's growth kinetics and biofilm properties. Hence, φSa3int prophages are crucial factors in the dissemination of IEC genes and virulence in *S. aureus* that may contribute to the chronic colonization in the nasal cavity of CRS patients.

5.2 MATERIALS AND METHODS

5.2.1 Ethics, bacterial strains, cells and growth conditions

Ethics approval for the use of clinical isolates (CIs) and primary human nasal epithelial cells (HNECs) was obtained from the Human Research Ethics Committee of the Central Adelaide Local Health Network (HREC/18/CALHN/69). All the *S. aureus* clinical isolates used in this study were retrieved from glycerol stocks and cultured at 37°C overnight on nutrient agar (NA, Oxoid Ltd, Hampshire, UK). *S. aureus* RN4220 and *S. aureus* ATCC25923 were from the German Collection of Microorganisms and Cell Cultures (DSMZ, GmbH) and American Type Culture Collection (ATCC, Manassas, USA) respectively. An isolated colony was propagated in 15.0 ml tryptic soy broth (TSB, 1X, Oxoid Ltd, Hampshire, UK) overnight in a shaking incubator (180 rpm) at 37°C unless stated otherwise. The HNECs used for the adhesion assay were collected from a non-CRS (control) patient at the time of surgery.

5.2.2 Genomic DNA extraction, sequencing and genome assembly

The genomic DNA (gDNA) of all *S. aureus* CIs were extracted using DNeasy Blood and Tissue Kit (Qiagen Pty. Ltd, Australia. Cat. #69504) according to the manufacturer's guidelines with slight modifications. Briefly, 700 µl of overnight broth culture in TSB was centrifuged (4000 x g) in a 1.5 ml Eppendorf tube for 10 minutes. The pellet was suspended in 180 µl of enzymatic lysis buffer (20 mM Tris-Cl, pH8; 2mM sodium EDTA; 1.2% Triton X-100, 200 µg/ml final concentration lysostaphin, filter sterilized) and incubated at 37°C for 30 minutes. Then 25 µl of proteinase K and 200 µl of Buffer AL (undiluted, provided with extraction kits) were added and mixed by vortexing followed

by incubation at 56°C. After 30 minutes, 200 µl of 99% ethanol (chilled) was added and mixed by vortexing. The mixture was then transferred to DNeasy Mini Spin column (Qiagen Pty. Ltd, Australia, Cat. #69504), and DNA was extracted following the manufacturer's guidelines.

The extracted gDNA was sequenced using the short-read Illumina platform (Illumina Inc, San Diego, USA) and in-house long-read Oxford Nanopore Technology (ONT) using the MinION Mk1C device (Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's instructions and in-house established protocol (Shaghayegh et al., 2023). Briefly, the short-read sequencing was done on Illumina NextSeq 550 platform using NextSeq 500/550 Mid-Output kit v2.5 (Illumina Inc, San Diego, USA) at a commercial sequencing facility (SA Pathology, Adelaide, SA, Australia). Briefly, gDNA was isolated using the NucleoSpin Microbial DNA kit (Machery-Nagel GmbH, Duren, Germany). Sequencing libraries were prepared using a modified protocol for the Nextera XT DNA library preparation kit (Illumina Inc, San Diego, USA). The gDNA was fragmented and amplified using a low-cycle PCR reaction. After the manual purification and normalisation of the amplicon library, 150 bp reads were obtained. Long-read whole genome sequencing was performed using MinION flowcells (R9.4.1) with the Rapid Barcoding Kit (Oxford Nanopore Technology, UK, #Cat: SQK-RBK 110.96) according to the manufacturer's instructions. In brief, 50 ng of gDNA from each CIs was used for sequencing. Base-calling was conducted with Guppy v6.2.11 (mode = super accuracy) using the 'dna_r9.4.1_450bps_sup.cfg' configuration (Oxford Nanopore Technology, UK).

Complete chromosomal *S. aureus* assemblies were created using a customised Snakemake pipeline (Mölder et al., 2021) available at https://github.com/gbouras13/hybracter via the Snaketool (Roach et al., 2022) powered command line tool hybracter following the protocols outlined in Wick et al. (2023). The complete pipeline can be found as supplementary text ST1. Chromosome assemblies were annotated with Bakta v1.6.1 (Schwengers et al., 2021). All CIs were typed to determine sequence type (ST) and clonal complex (CC) according to the PubMLST database using MLST (Jolley et al., 2018; Seemann, mlst). We used Snippy v4.6.0 to detect single nucleotide polymorphisms (SNPs) between CIs (Seemann, 2015) and Abricate v1.0.1 to screen for anti-microbial resistance and virulence factor genes (Seemann, 2020).

5.2.3 In silico identification of prophage and prophage annotation

Prophage regions in both *S. aureus* CIs (SA222 and SA333, isolated from the same patient at different time points) were first identified using PHASTEST (https://phastest.ca) and PhiSpy (https://github.com/linsalrob/PhiSpy) with default settings (Akhter et al., 2012; Arndt et al., 2016; Wishart, 2023). The exact genome of the φSa3int prophage was then curated with our in-house program hlbroken (https://github.com/gbouras13/hlbroken), which extracts the sequences between *hlb* gene only. The identified φSa2int and φSa3int prophage sequences were also annotated and visualized with Pharokka v1.2.0 (Bouras et al., 2023).

5.2.4 Prophage induction and multi-host range assay of induced prophages

Prophages from both CIs were induced using mitomycin C (MMC), purified and spotted on previously studied *S. aureus* CIs (N = 66) using the soft-agar overlay technique as described earlier (Nepal et al. 2023) to study its multiple-host range. Briefly, MMC (final concentration = 1.0 µg/ml) (Sigma-Aldrich, Missouri, USA, #Lot: SLBX4310) was added to exponentially growing cells (OD_{600} = 0.3) in TSB and further incubated for 6 hrs at 37°C in a rotating incubator (180 rpm). Optical density at 600 nm (OD_{600}) was measured every hour. After 6 hrs, the culture was centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was filtered through 0.2 µm pore size syringe filter (13 mm, Acordisc®, Pall International, Fribourg, Switzerland, #Cat: 4612) to obtain pure phage lysate. Briefly, host bacteria were overlayed in double-layered TSA and 10.0 µl of purified lysate was spotted on the top agar in triplicates.

5.2.5 Construction and verification of lysogens

Ten microliters of purified phage lysate induced from SA333 were spotted on a soft agar overlaid with recipient bacteria SA222. The plates were dried and incubated overnight at 37°C . The next day, a loopful of bacteria from the lysis spots were streaked on sheep blood agar (SBA, ThermoFisher, Australia, #Cat: R01202) and incubated overnight at 37°C . Colonies without beta-hemolysis (possibly lysogens) were picked and sub-cultured in TSA. The stability of these constructs possibly harboring both φSa2int and φSa3int prophages was confirmed through multiple sub-cultures in SBA. Two constructs devoid of beta-hemolysin (hereafter SA-L1 and SA-L2) were picked for further analysis. The integrity and re-inducibility of integrated prophage were then confirmed by re-

induction from the constructs using MMC as described earlier and spot assayed on SA222, SA333 and RN4220. The integration of φSa3int prophage into the *hlb* gene was verified by inspecting the *hlb* gene in assembled genomes using the Integrative Genomics Viewer (IGV) (Robinson et al., 2011).

5.2.6 Growth curve, biofilm biomass and biofilm metabolic activity

Bacterial growth kinetics were determined by measuring the optical density of broth culture at 600 nm (OD_{600}). Briefly, 100 µl of 1.0 McFarland standard unit (MFU in saline, prepared from overnight cultured colonies on NA plates) was added to 15.0 ml of TSB in a 50 ml Falcon tube. The tubes were incubated at 37°C in a shaking incubator (180 rpm). Every hour, 100 µl of culture was removed and mixed with 900 µl of sterile TSB in a cuvette. The OD_{600} was then measured using a SmartSpec™ 3000 UV/Vis spectrophotometer (Bio-Rad Laboratories Inc, California, USA).

The biofilm variation between the clinical isolates and lysogens was qualitatively assessed by culturing the bacteria on modified Congo red agar (CRA) (37 gm/l brain heart infusion broth supplemented with 50 g/l sucrose, 0.8 g/l Congo red stain and 1.0% agar) according to Freeman et al. (1989). Colony morphology of SA222, SA333 and SA-L1/SA-L2 on CRA was assessed after 48 hours of incubation at 37°C . Further, biofilm biomass and biofilm metabolic activity (cell viability) was performed using crystal violet (CV) assay and alamarBlue® cell viability assay as per manufacturer's guidelines (Life Technologies, Oregon, USA) in biofilms established for 48 hours in 96-well flat-bottomed (Costar, Corning Incorporated, USA. #Ref: 3599) and 96-well flat and clear bottom black assay plate (Costar, Corning Incorporated, USA. #Ref: 3603) respectively,

as described earlier (Nepal et al. 2023). Briefly, the overnight NA culture of *S. aureus* was adjusted to 1.0 McFarland standard and diluted 1:15 in tryptic soy broth. One-hundred-fifty microliters of each diluted culture were pipetted into the inner wells of the respective 96-well plates. The peripheral wells were filled with sterile water, sealed with aluminium foil and incubated at 37°C in an orbital shaker (80 rpm). After 48 hours of incubation, planktonic cells were carefully aspirated, and the plates were washed twice with phosphate buffered saline (PBS, 1X) followed by the crystal violet or alamarBlue® assay. For CV, plates were air-dried and 180 µl of 0.01% CV solution was added to each inner well and left at room temperature for staining. After 10 minutes, the excess CV was aspirated, washed twice with PBS (1X) and air-dried. Finally, the biomass-bound crystal violet was solubilized in 200 µl of 30% acetic acid. The biomass was measured in terms of absorbance (OD_{600}) at 600 nm using CLARIOstar Plus (BMG Labtech, Ortenberg, Germany). Similarly, for biofilm metabolic activity, 200 µl working solution of alamarBlue® (1X) was added to each inner well, covered with aluminium foil and incubated at 37°C in an orbital shaker (80 rpm). Resorufin fluorescence was monitored at the 1-hr interval for 6 hours using the CLARIOstar Plus (BMG Labtech, Ortenberg, Germany) microplate reader (excitation = 530 nm, emission = 590 nm). The metabolic activity (expressed as fluorescence units) at a 2-hour timepoint was considered for further analysis. All experiments were performed in triplicate with six technical replicates.

5.2.7 Adhesion assay of S. aureus to primary human nasal epithelial cells

The adhesion of *S. aureus* clinical strains and lysogens to primary human nasal epithelial cells (HNEC) was studied following the protocol by Yang and Ji with slight modifications (Yang and Ji, 2014). Briefly, primary HNECs were cultured in RPMI 1640 working media (supplemented with 10% FBS and 1% antibiotic-antimycotic, hereafter RPMI 1640-WM) to 70% confluency in a tissue culture flask (T-75, Sarstedt, Nümbrecht, Germany) at 37°C, 5% CO_2 incubator and transferred (1.0 ml/well) into a 24-well tissue culture plate (Sarstedt, Nümbrecht Germany). An overnight broth culture of bacteria was made in 5.0 ml of TSB, pelleted and resuspended and the bacterial density was then adjusted to ~0.3 (OD_{600}). In a separate tube, 5.0 ml of RPMI 1640+10% FBS (without antibiotics) was aliquoted, and 150 µl of the diluted bacteria (OD_{600} = 0.3) was added to prepare the working bacterial culture. The cell culture media was replaced with RPMI 1640+10% FBS media with and without bacterial suspensions and cells incubated for 2 hours at 37°C , 5% CO_2 incubator. The wells were again washed and then incubated with 400 µl of 0.025% Triton X-100 by pipetting, transferred into corresponding Eppendorf tubes and mixed by vortexing for 30 sec. The recovered bacteria were serially diluted in sterile PBS (up to 10^{-4}), and 20 µl was spotted in TSA for CFU estimation. The plates were dried and incubated at 37°C along with previous plates containing serially diluted working bacterial culture spots. The next day, the colonies in each plate were counted. The relative adhesion was calculated using the following formulae:

$$\text{Relative adhesion} = \frac{Adhesion\ of\ the\ lysogen}{Adhesion\ of\ the\ parent\ strain}\ x\ 100\%$$

5.2.8 Proteomics of the secretome

The proteomics of the secretome was analyzed using a data-independent acquisition mass spectrometry (DIA-MS) using Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, USA) with Dionex UltiMate™ 3000 UHPLC system (Thermo Fisher Scientific, USA) at The Flinders Omics Facility, Flinders University according to established protocol (Supplementary text ST2). Briefly, a few colonies from overnight cultured NA plates were dissolved in sterile saline to obtain 1.0 MFU standard. One hundred microliters of the solution were added to 15.0 ml of TSB in a 50.0 ml Falcon tube and incubated at 37°C in a shaking incubator (180 rpm, 45° angle). After ~7 hours, the tube was briefly vortexed and centrifuged at 4000 x g for 10 min. The secretome was sterilized by passing it through a 0.2 µm syringe filter (25 mm, Acordisc®, Pall International, Fribourg, Switzerland) and concentrated using a Pierce™ Protein Concentrator PES (3K MWCO, Thermo Scientific, USA. #Cat: 88525) by centrifuging the sample down to approximately 2-3 ml. The protein concentration was determined using NanoOrange™ Protein Quantitation Kit (Invitrogen, USA. #Cat: N6666) as per the manufacturer's instruction. The proteins were then processed at the Flinders Omics Facility, Flinders University. The complete set-up and protocols are elaborated in Supplementary text ST2.

The DIA spectra were then processed and quantified using Spectronaut v15 (Biognosis AG, Schlieren, Switzerland) with default settings. A *S. aureus* proteome database created from all genes identified in SA333 was used as a reference. Gene annotations were assigned based on SA333 strain using Bakta v1.6.1 (Schwengers et al., 2021). Differential protein expression analysis was performed in R v4.2.0 (https://www.R-

project.org/) using the DEP package v1.20.0 to calculate differentially expressed proteins (DEP) (Zhang et al., 2018). The threshold for identifying differentially expressed proteins was set at a false discovery rate (FDR) of less than 0.05.

5.2.9 Bioinformatics and statistical analysis

Pairwise alignment and multiple-sequence alignment between the genomes were performed using MAFFT, and the phylogenetic tree was inferred using PhyML tree in Geneious Prime 2022.2.2 (http://www.geneious.com). The average nucleotide identity (ANI) of assembled genomes was calculated using a FastANI web tool available at https://proksee.ca (Jain et al., 2018). Statistical analysis was performed using Prism v9.4 (GraphPad Software, USA), and graphs were made in R v4.2.0 (https://www.R-project.org/). The difference in means of biofilm and metabolic activity was tested using the student t-test. $P < 0.05$ was considered statistically significant unless stated otherwise.

5.2.10 Data availability

The genomic data (reads and assemblies) can be found in the Sequence Read Archive (SRA) under project number PRJNA914892, specifically with sample numbers SAMN32360844 (SA222) and SAMN32360890 (SA333).

5.3 RESULTS

5.3.1 Genomic features of SA222, SA333, lysogens and the prophages

Sequencing and genomic analysis revealed that there were minimal genetic variations between SA222 and SA333, which were isolated from the same CRS patient 567 days apart. The strains had the same 32.8% GC content, clonal complex (CC22) and sequence type (ST22) (Figure 5.1A; Table 5.1). The average nucleotide identity (ANI) between SA222 and SA333 was 99.99%, and the average aligned length between the two genomes was 2,390,601 bp (Figure 5.1B). The alignment also identified an additional φSa3int-group prophage (hereafter φSa3int prophage) in SA333. Similarly, the ANI between SA222 (the recipient strain) and lysogenized SA222 which had been genetically modified by lysogenization with φSa3int prophage (SA-L1, SA-L2) was ~100%, with an average aligned length of 2,746,692 bp and the ANI between donor SA333 and lysogens (SA-L1, SA-L2) was 99.99%, with an average aligned length of 2,385,728 bp (Figure 5.1B). The key difference between the clinical isolates was that while SA222 harbored one intact prophage (φSa2int, 52,500 bp), SA333 had two intact prophages (φSa2int; 50,792 bp and φSa3int; 43,795 bp) (Table 5.1). Upon gene annotation, we could see that although the φSa2int prophage in SA333 lost some DNA compared to SA222, SA333 had gained two transposases (length = 1236 bp and 768 bp) (Figure S5.1-A-D). The identified φSa2int prophage in SA222 and SA333 was most closely related to Staphylococcus phage phi2958PVL (NC_011344, length = 47,342 bp), while the additional prophage in SA333 was most closely related to Staphylococcus phage IME1361_01 (NC_048657, length = 43,516 bp) (Table 5.1). There was only 60,421 bp of non-identical nucleotide bases between SA222 and SA333, including the 43,795 bp φSa3int prophage (Figure 5.1D), confirming that SA222 and SA333 were the same strain, but had gained the

flexible prophage (φSa3int) at some point in time. Phylogenetic analysis and non-identical nucleotide differences between clinical isolates and the lysogens implied, as expected, that the laboratory-generated double lysogens were closer to SA333 than SA222 (Figure 5.1C-D). Surprisingly, the non-identical nucleotide bases between the recipient SA222 and SA-L1/L2 were 47,747 bp, which was 3,952 bp larger than the exact prophage identified suggesting some auxiliary cargo DNA and rearrangements in the lysogens during prophage integration (Figure 5.1D).

Table 5.1 | Details of the *Staphylococcus aureus* clinical isolates used in this study

Bacterial isolate	Date of collection	Genome size (bp)	Genotypic and phenotypic characters	No of prophage[a]	Prophage type[b] (size) / Proteins	Closest prophage (NCBI reference)	Virulence genes[c]
SA222	08/01/2014	2,788,579	CC22/ST22, MRSA, high biofilm, hlb (+), non-mucoid	1	φSa2int (52.5 kb) / 68	Staphylococcus phage phi2958PVL (NC_011344)	*luk* SF-PV
SA333	29/07/2015	2,837,533	CC22/ST22, MRSA, hyper biofilm, hlb (-), mucoid	2	φSa2int (50.8 kb) / 66	Staphylococcus phage phi2958PVL (NC_011344)	*luk* SF-PV
					φSa3int (43.8 kb) / 69	Staphylococcus phage IME1361_01 (NC_048657)	*sak, chp, scn*
SA-L1, SA-L2	This study	2,832,387	CC22/ST22, MRSA, high-biofilm, hlb (-), non-mucoid	2	φSa2int (52.5 kb) / 68	Staphylococcus phage phi2958PVL (NC_011344)	*luk* SF-PV
					φSa3int (43.8 kb) / 69	Staphylococcus phage IME1361_01 (NC_048657)	*sak, chp, scn*

a = only intact prophages considered for the analysis, however a 10,194 bp incomplete prophage encoding 17 proteins was also present in SA222 and SA333, b and c = based on *Staphylococcus aureus* integrase typing (Goerke et al., 2009), hlb(-) = beta-hemolysin absent, hlb(+) = beta-hemolysin present, MRSA = methicillin-resistant *S. aureus, luk* FS-PV = leukocidin (Panton-Valentine FS), *sak* = staphylokinase*, chp* = chemotaxis inhibitory protein*, scn* = Staphylococcal complement inhibitor, SA-L1 and SA-L2 (SA222+φSa3int) are independent lysogens created by infecting SA222 with prophage induced from SA333.

5.3.2 Prophages induced from clinical isolates displayed a multiple host-range

The prophage genome annotation revealed that φSa2int prophage mostly encoded phage-related genes and hypothetical genes (Figure 5.2A). Similarly, the φSa3int prophage encoded a complete set of IEC genes (*sak, chp, scn*) and phage-related genes (Figure 5.2B). Neither prophage encoded any antibiotic-resistance genes (ARGs) within their genomes. However, both prophages had a *clpP* gene encoding Clp protease

involved in lysogenic-lytic switching (Thabet et al., 2022), indicating that both prophages should be capable of productive prophage induction (Figure 5.2A-B).

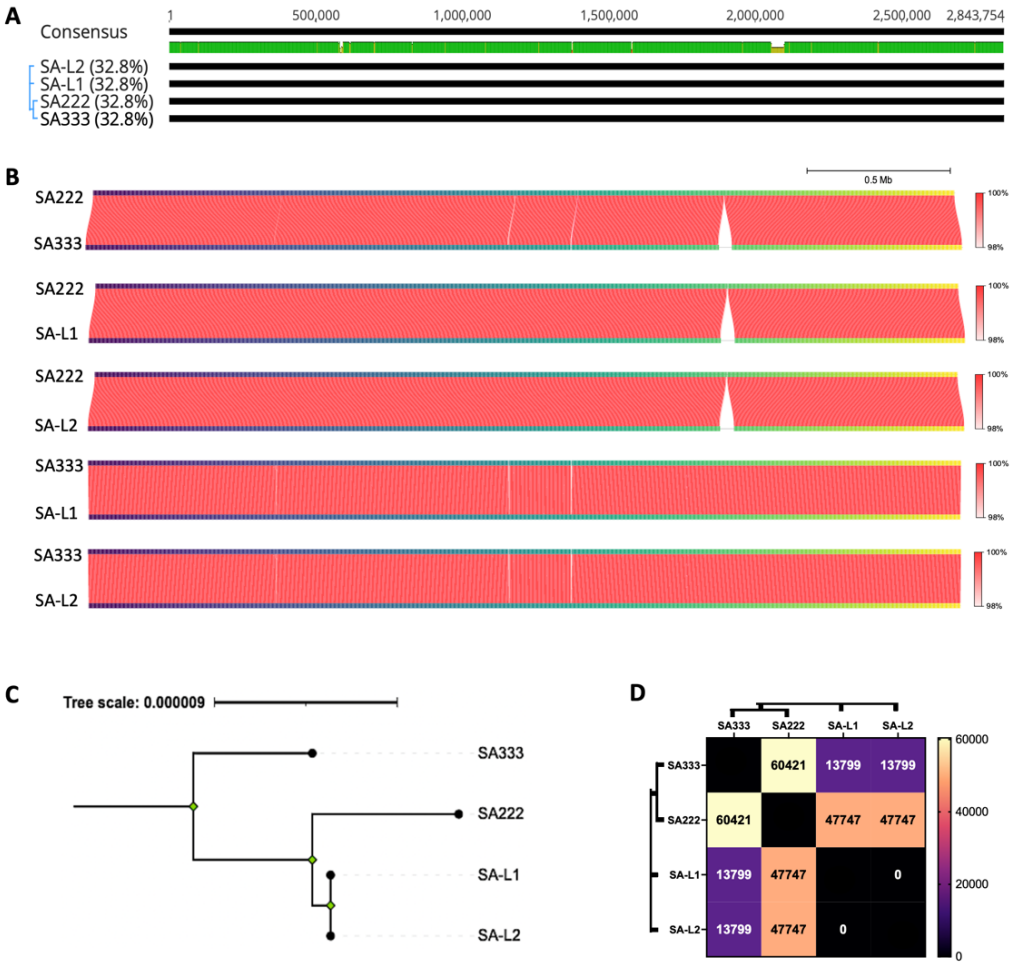

Figure 5.1 | Multiple sequence alignments of clinical isolates and the lysogens. (A) Multiple sequence alignments of SA222, SA333 and laboratory generated double lysogens (two isolates; SA-L1 and SA-L2) show almost identical genome maps except for the prophage insert around 2.1 Mbp. (B) The average nucleotide identity (ANI) score between the clinical isolates and the lysogens further confirms that the prophage inserts on SA333 are the only significant genome change that has happened over time. The red colour gradient indicates the ANI percentage. (C) A rooted phylogenetic analysis between clinical strains and laboratory generated double lysogens reveals that as expected the double lysogens are genetically closer to the recipient strain SA222. (D) Distance matrix indicating the number of bases which are not identical.

Productive prophage induction was observed in both clinical strains, as lysis zones (plaques) were observed in the spot assay. A clear lysis zone was observed on indicator strain RN4220, indicating an intact lysogenic-to-lytic switch in the prophages, a productive induction, and a re-infectivity of φSa2int and φSa2int and/or φSa3int

prophages induced from SA222 and SA333, respectively (Figure 5.2C). While induced prophage from SA333 (φSa2int and/or φSa3int) was also able to infect and lyse SA222 (lacks φSa3int prophage), induced prophage from SA222 (φSa2int) could not infect and lyse SA333 (which already has both φSa2int and φSa3int prophages), likely because of superinfection exclusion (Figure 5.2C). Similarly, induced prophage from lysogens (SA-L1 and SA-L2) could infect the parent strain SA222, indicating productive induction of transduced φSa3int prophage.

Further, released phages from both clinical isolates (SA222 and SA333) produced a partial or complete lysis spot on multiple clinical isolates using a spot assay, indicating a broad-host-range of the induced prophages and their ability to transduce the prophage-encoded virulence factors to multiple clinical isolates. Representative negative and positive lysis spots are shown in (Figure 5.2D). Prophages induced from SA222 (φSa2int) could infect 39.4% (26/66) of clinical strains, while productive prophages from SA333 (φSa2int and φSa3int) could infect 25.8% (17/66) of clinical strains (Figure 5.2D). Phages released from both clinical isolates did not produce either a partial or a complete lysis spot on ATCC25923 (Figure 5.2C).

5.3.3 Integration of φSa3int prophage inhibited the production of beta-hemolysin

Whole genome sequencing of the lysogens (SA-L1 and SA-L2) confirmed that a ~43.8 kb φSa3int prophage DNA induced from SA333 was integrated into the SA222 chromosome within the *hlb* gene (start = 2,041,825 bp, end = 2,088,955 bp) (Figure S5.1), generating a double-lysogen with quadruple conversion (negatively converted *hlb* gene and incorporation of all three IEC genes: *sak, scn* and *chp*) (Figure 5.2E). The *hlb*

gene was truncated near the 5' end resulting in two incomplete genes: 201 bp (66 aa, molecular weight = 7321.09 amu) and 825 bp (274 aa, molecular weight = 31257.36 amu) (Figure S5.2). As such, the lysogens lacked β-hemolytic activity in sheep blood agar (Figure 5.2F). Genomic analysis also revealed that the φSa3int prophage in this study lacked *sea/sep* genes, which are commonly found prophage-encoded virulence factors in Sa3int-group prophages (Figure 5.2B).

5.3.4 Domestication of a φSa3int prophage had no impact on bacterial growth, biofilm biomass, metabolic activity and adhesion to primary human nasal epithelial cells

The domestication of the additional ~43.8 kb φSa3int prophage DNA did not alter the growth kinetics of the lysogens compared to the recipient host SA222 (Figure 5.3A). Also, there was no significant change in biofilm biomass between the lysogens and SA222 (Figure 5.3B). The colony morphology of SA222 and SA-L1/SA-L2 on Congo red agar were similar. In contrast, the colony morphology of donor SA333 on Congo red media was wrinkled, suggesting strong mucoid production (Figure 5.3C). Further, the metabolic activity of the biofilm was similar between lysogens and recipient host SA222 (Figure 5.3D). However, the biofilm biomass and metabolic activity of SA333 (donor) were significantly higher than the laboratory-generated double lysogens, despite having a non-aligned nucleotide difference of 13,797 bp only (Figure 5.1B, 5.1D). Further, there was no significant difference in adhesion properties between the recipient SA222 and the double lysogens in primary HNECs. The relative adhesion of the lysogen to the HNEC compared to the recipient host SA222 was 94%, implying a slight reduction in the

adhesion of *S. aureus* after infection with φSa3int prophage but did not reach statistical significance.

5.3.5 φSa3int prophage domestication equips S. aureus with human immune evasion factors

The proteomics of the secretome collected from SA222, SA333 and one of the laboratory-generated double lysogens (SA-L1) indicated that acquisition of the φSa3int prophage arms *S. aureus* bacteria with multiple prophage-associated human immune evasion factors. These include staphylokinase, SCIN, CHIPS and recombinase protein (recT) that is secreted as an exoprotein (Figure 5.4A, cluster 1). Altogether, the lysogen significantly regulated thirty-eight exoproteins that were differentially expressed in the lysogen compared to its recipient strain SA222. Among them, twenty-one (55.3%) were up-regulated including staphylokinase (sak), SCIN (scn), and intercellular adhesion protein B (icaB). In contrast, seventeen proteins (44.7%) were down-regulated including β-hemolysin (hlb/sph) and outer membrane porin (phoE) (Figure 5.4B-C). Among these genes, 60.5% (23/38) were of unknown function. Similarly, despite having few genetic variations between lysogen and its donor host SA333 (Figure 5.1B, 1D), forty proteins were significantly differentially regulated (Figure 5.4A, clusters 2 and 3). Among them, twenty-seven proteins (67.5%), including enterotoxins SEO and SEG were up-regulated. In contrast, thirteen proteins (32.5%), including elastin binding protein (ebp) were down-regulated (Figure 5.4D-E). As predicted, most of these proteins were of bacterial origin, and the hypothetical proteins, predominantly prophage encoded, constituted

only 15.0% (6/40) of proteins because both strains (SA333 and SA-L1) carry identical mobile genetic elements (Figure 5.1B).

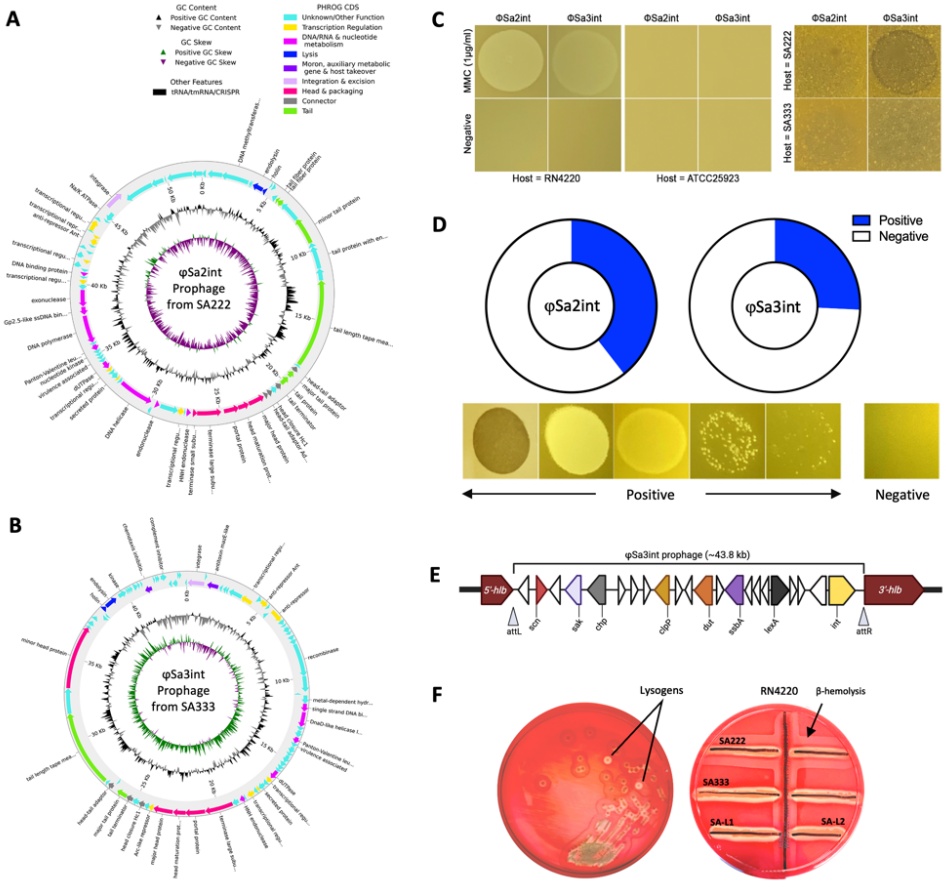

Figure 5.2 | Annotation of identified prophages in SA222 and SA333 and their inducibility. (A) The φSa2int prophage is present in both clinical strains SA222 and SA333. The 52.5 kb prophage region primarily consists of hypothetical genes of bacterial as well as phage origin whose functions are yet unknown. (B) The φSa3int prophage was present in clinical isolate SA333 in addition to φSa2int. The ~43.8 kb prophage encoded major human immune evasion genes (*sak, scn, chp*). These genes are clustered together near the integrase (*xerC*). (C) Productive induction of prophage was observed from both clinical isolates (SA222 and SA333) using mitomycin (1.0 μg/ml). The induced phages showed clear lysis on indicator host RN4220 but did not show lysis on *S. aureus* ATCC25923. Further, the induced phage from SA333 (φSa3int) was able to lyse SA222, but the induced phage from SA222 (φSa2int) was unable to infect SA333 or SA222, possibly because of superinfection immunity. (D) The multiple host-range of induced phages from SA222 (φSa2int) and SA333 (φSa3int) indicated that the phages can infect and kill multiple clinical isolates. The φSa2int prophage from SA222 was able to infect almost 40% (26/66) clinical strains while φSa3int induced phage from SA333 could infect almost 26% (17/66) clinical isolates. The lower figure panels indicate the spots that were considered positive and negative. (E) Schematic chromosomal location of transduced φSa3int prophage and identified genes in SA-L1. The 43.8 kb insert was integrated within the *hlb* gene thereby truncating it. (F) Assessment of beta-hemolytic activity on sheep blood agar. The modified lysogens (SA-L1 and SA-L2) lost their β-hemolytic property.

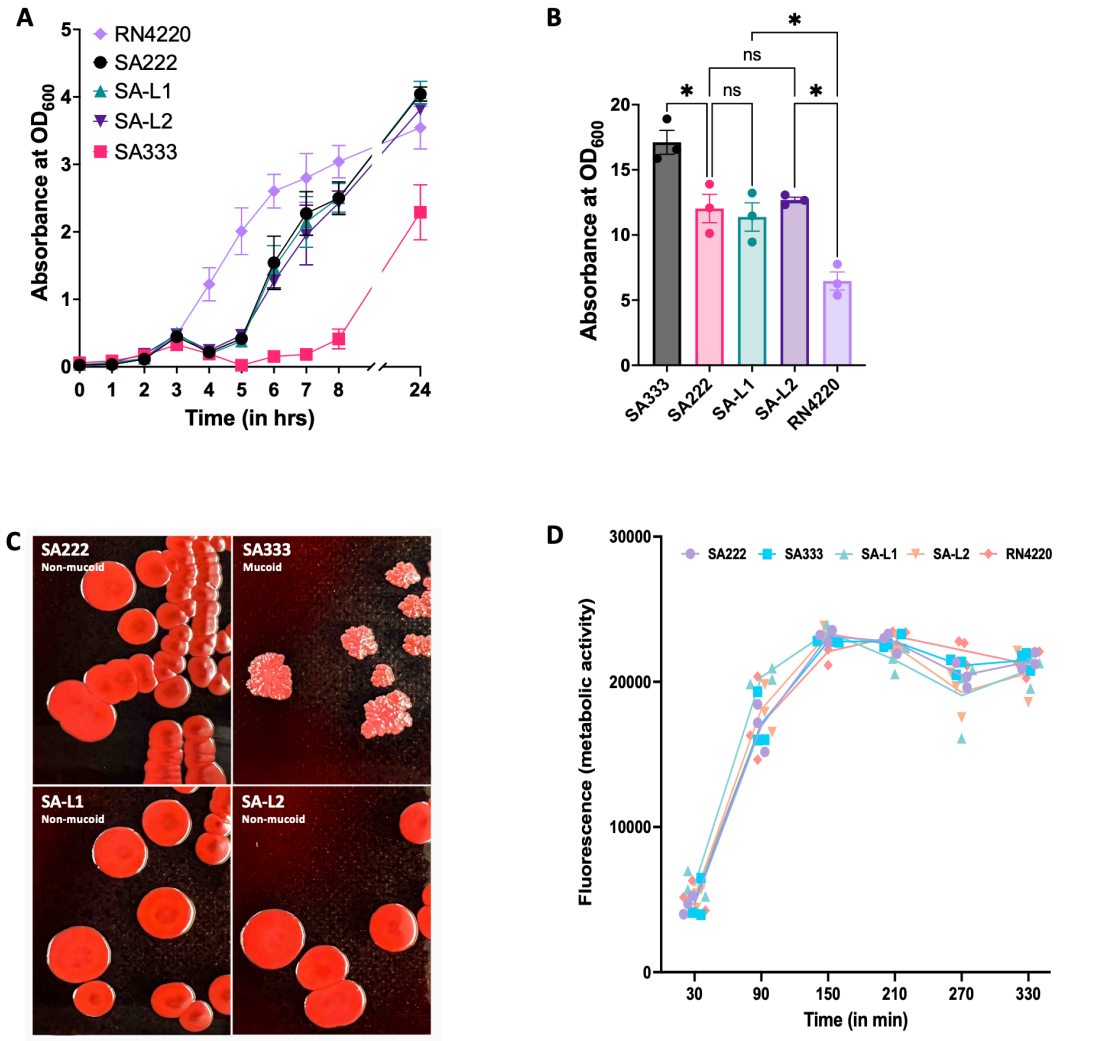

Figure 5.3 | Comparisons of *in vitro* characteristics between SA222, SA333 and laboratory generated double lysogens (SA-L1 and SA-L2). (A) Bacterial growth curve in tryptic soy broth did not show any significant change in growth kinetics between recipient SA222 and double lysogens (SA-L1 and SA-L2), indicating domestication of an additional ~43.8 kb φSa3int prophage did not impact the growth of the recipient strain SA222. (B) Biofilm estimation by crystal violet assay also indicated that the domestication of ~43.8 kb φSa3int prophage did not impact biofilm formation. (C) Overnight culture of *S. aureus* strains in Congo red for qualitative estimation of mucoid phenotype further indicated that the φSa3int prophage and the genes it carried had no impact on phenotypic differentiation between mucoid and non-mucoid phenotype. (D) Study of biofilm metabolic activity by Alamar Blue assay indicated that there was no significant change in metabolic activity between recipient isolate (SA222) and double lysogens (SA-L1 and SA-L2).

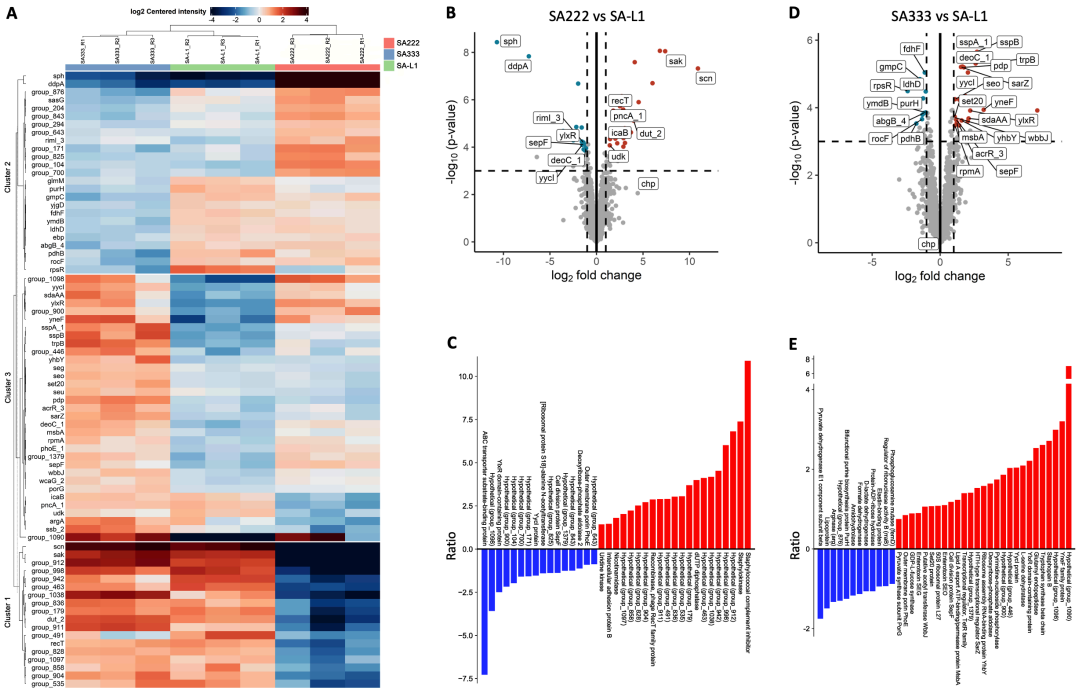

Figure 5.4 | Proteomics of the secretome from SA222, SA333 and SA-L1. (A) The heatmap showing the differential expression between donor SA333, recipient SA222 and laboratory-generated double lysogen (SA-L1). The proteins in cluster 1 (including *sak, scn, recT, dut*) were significantly upregulated in SA-L1 compared to recipient isolate SA222. The expression of genes in this group was similar between the lysogen and the donor isolate SA333. (B) Volcano plot showing the differential expression of proteins between recipient host SA222 and lysogen (SA-L1). (C) A bar plot showing the relative ratio of significantly up- and down-regulated proteins between SA222 and SA-L1. The virulence factors responsible for human immune evasion (sak and SCIN) are significantly upregulated along with the intercellular adhesion protein B (icaB). (D) Volcano plot showing the differential expression of proteins between donor host SA333 and lysogen (SA-L1). (E) A bar plot showing the relative ratio of significantly up- and down-regulated proteins between SA333 and SA-L1. There was no significant change in virulence factors encoded by prophage φSa3int, indicating the difference in phenotypes is not associated with prophage-associated genes or functions. $P < 0.05$ was considered significant in all the above analyses.

5.4 DISCUSSION

The productive induction of prophages from a lysogen leads to the release of phage particles that can either kill the competing strains or lysogenize the susceptible strains (Matos et al., 2013). It is now established that various drugs, antibiotics and even dietary compounds enhance prophage induction and promote the lysogenic conversion (Boling et al., 2020; C. Goerke et al., 2006; Sutcliffe et al., 2021). The prophages can also be induced spontaneously in response to nutrient availability and/or other environmental conditions, including niche variation. As over 90% of human clinical isolates carry Sa3int-group prophages, predominantly integrated into the *hlb* gene (van Wamel et al., 2006), we aimed to elucidate the contribution of φSa3int prophage domestication in *S. aureus,* particularly in the secretome of the bacteria. To achieve this, we introduced a φSa3int prophage (also called β-hemolysis-converting prophage) induced from a patient-derived double-lysogen (SA333), into a genetically similar single (φSa2int)-lysogen (SA222), isolated from the same patient almost 2 years earlier, to create a laboratory-generated double-lysogen. We then studied its phenotypic characteristics as well as proteomics. We chose these clinical isolates for our experiments because, our preliminary study suggested that although there were significant phenotypic variations between SA222 (high-biofilm, hlb(+)) and SA333 (hyper-biofilm, hlb(-)), the two CIs were genetically similar except for the gain of φSa3int prophage by SA333 and a few SNPs (data not shown) and also were collected from the same patient who had severe CRS.

Lysogenic conversion in *S. aureus* arms bacteria with many survival fitness traits like virulence factors, toxins, and biofilm upregulation and is common in clinical strains (Bae et al., 2006; Fernandez et al., 2018; Naorem et al., 2021). Prophage-mediated

enhancement of biofilm has been observed in various bacteria like *Enterococcus faecalis* (Rossmann et al., 2015), *Streptococcus pneumoniae* (Carrolo et al., 2010), *Escherichia coli* (Li et al., 2022) and *S. aureus* (Fernandez et al., 2018). Further, multiple studies have described prophage-mediated phenotypic alteration, host adaptation and pathogenesis (Bobay et al., 2013; Liu et al., 2020; Resch et al., 2013). Our findings support the notion that lysogenic conversion in clinical isolates is common (Chaguza et al., 2022), as almost 40% of the CIs tested could be infected with a productive prophage derived from another clinical isolate, meaning they could gain prophage-associated virulence and pathogenicity via phage infection. Further, the variations in the strength of lysis spots in multiple host-range assays indicated that the induced temperate phages had varying degrees of susceptibility among CIs, suggesting variable lysogenic conversion of the host.

Several studies on *S. aureus* clinical isolates have suspected Hlb as an important non-pore-forming toxin promoting colonization and impacting ciliary clearance of bacteria in an animal model (Katayama et al., 2013; Kim et al., 2000). A similar function of Hlb has thus been speculated in humans. However, most human-associated *S. aureus* lack β-hemolysin because of the integration of the Sa3int-group prophage in the *hlb* gene (Nepal et al., 2021; van Wamel et al., 2006). A similar truncation of the *hlb* gene in this study is seen after integrating φSa3int prophage DNA, thereby completely disrupting the production of the Hlb protein in the secretome. This seems counterintuitive as Hlb is a strong sphingomyelinase that promotes sphingomyelin degradation and stimulates biofilm formation in the presence of eDNA (Huseby et al., 2007; Huseby et al., 2010). Thus, we speculate that nasal colonizers might trade-off the Hlb function by gaining the

immune evasion factors that are encoded by the φSa3int prophage and are known to protect bacteria from phagocytosis. However, re-expression of Hlb has been noted in *S. aureus* isolated from cystic fibrosis patients upon antibiotic (ciprofloxacin or trimethoprim) treatment and increased frequency of genomic alterations have been associated with prophage mobilization (Goerke et al., 2004). Other studies have shown the conditional excision of φSa3int prophage in a sub-set of the population in *in vivo* conditions and *S. aureus* thriving as a heterogeneous population that aggravates the infection (Guan et al., 2021). As the laboratory generated φSa3int prophage in our study could be re-induced, we support the notion that φSa3int prophage integration and excision are conditional and largely depend on external factors. However, more *in vivo* study is required to confirm the hypothesis and understand the conditional switching of *S. aureus* from Hlb positive to negative or vice versa.

S. aureus biofilm and its adhesion to various surfaces are important features that aid bacteria in colonizing and establishing themselves in various niches. Fernandez et al. suggested the role of *S. aureus* prophages in biofilm development and adhesion (Fernandez et al., 2018). Other researchers have suggested that the eDNA released during spontaneous prophage induction acts as a quorum sensor leading to an enhanced biofilm formation (Carrolo et al., 2010). However, we did not observe significant enhancement of biofilm production or adhesion to HNECs when comparing SA222 and SA222 following introduction of the φSa3int prophage, although biofilm-associated intercellular adhesion protein (icaB) was upregulated in the double lysogen. The contradictory findings may be due to the different prophage type used for lysogenization because Fernandez et al. used φ11 (Sa5int-group) and φ80 (Sa6int-

group) to lysogenize the lab strain RN4220, while we used a Sa3int-group prophage to lysogenize another clinical isolate. Further, the possible explanation for non-significant biofilm change may be because the recipient host SA222 was already a high-biofilm forming isolate, and the contribution of integrated prophage was minimal. Although the result was unexpected, the observation ruled out a major contribution of φSa3int prophage including all the hypothetical genes it carries in biofilm development and adhesion of *S. aureus*. Since *S. aureus* SA333 and lysogens were genetically almost identical (99.51% similar) except for a few SNPs and insertion sequences (data not shown) but had different biofilm, adhesion and mucoid phenotypes, the result has helped us narrow down on other possible bacterial genes responsible for hyper-biofilm characteristics. However, further experiments with knock-out mutants are required to confirm the role of these candidate genes in biofilm development.

Further, our results suggest that although integration of a Sa3int-group prophage seems expensive in terms of replication energy cost and also disrupts the β-hemolysin expression, the prophage equips the host bacteria with a multitude of accessory virulence factors like *sak, scn, chp, icaB*. The high incidence of human-specific anti-innate immunity factors in *S. aureus* isolated from humans is well known (Rohmer and Wolz, 2021). It is also established that φSa3int prophages carry and disseminate IEC genes (*sak, chp, scn, sea/entA*), either partial or a complete set (Rohmer and Wolz, 2021). *In vitro* secretome profiling of the SA222, SA333 and lysogen (SA-L1) revealed major changes in virulence, particularly human immune evasion modulation of *S. aureus* associated with the domestication of the prophages. Our results confirm and extend the existing knowledge that φSa3int-group prophages carry IEC genes in a cluster that

are significantly upregulated and secreted as exoproteins. Staphylokinase protein (Sak) encoded by *sak* gene is a potent plasminogen activator that converts plasminogen into plasmin. Sak-mediated plasmin activity increases the local invasiveness of *S. aureus* leading to skin disruption and reduced clearance of bacteria by the host (Peetermans et al., 2014). Chemotaxis inhibitory protein (CHIPS) encoded by *chp* gene counters the first line of host defence, specifically inhibiting the response of human neutrophils and monocytes to complement anaphylatoxin C5a and formylated peptides. It directly binds to the C5a and formylated peptide receptors, preventing phagocytosis of the bacterium (Postma et al., 2004). Similarly, Staphylococcal complement inhibitor protein (SCIN) encoded by *scn* gene also counters the first line of host defence. It efficiently inhibits opsonization, phagocytosis and the killing of *S. aureus* by human neutrophils (de Jong et al., 2018). All of these genes were significantly upregulated in the lysogen secretome along with downregulation of hemolysin, indicating a quadruple conversion of SA222 by φSa3int prophage induced from SA333. These results also hint that the patient isolate SA222 gained a φSa3int prophage to establish itself in the nasal niche, as multiple CIs isolated after this time point from the same patient were also double-lysogens with IEC genes (data not shown).

All these observations together indicate that despite an increase in the genome size of the double-lysogen by almost 43.8 kb of Sa3int-group prophage DNA, there was no significant change in growth kinetics, biofilm and adhesion properties of *S. aureus*. However, the proteomics analysis of the secretome clearly indicated that lysogenization by Sa3int-group prophage DNA armed the bacterial host with additional flexible virulence features likely to help bacteria evade the human immune system by avoiding

phagocytosis. Further, as induced phages could infect other clinical isolates but not their own parental host, we can assume that prophage domestication not only increases the virulence of the lysogenized host but also can landscape the microbiome, which may lead to bacterial dysbiosis and ultimately pathological conditions. Temperate phages (which are formed by productive prophage induction) have been observed in various environments like human gut, chronic wounds, and cystic fibrosis patients aggravating the disease. As recent advances in genomic sequencing have revealed that most *S. aureus* clinical isolates adapted to humans' harbour prophages in their genome, and not all of the clinical isolates carry similar mobile genetic elements, verification of prophage-encoded toxigenic trait is of paramount importance in inflammatory diseases like CRS.

5.5 CONCLUSION

We conclude that lysogenic conversion of *S. aureus* by φSa3int prophage alters the bacterium's virulence by upregulating the human immune evasion factors like sak, scn and chp and downregulating beta-hemolysin, a sphingomyelinase. Our research further confirmed that the growth, biofilm and adhesion of *S. aureus* are not associated with Sa3int-group prophage domestication. These findings demonstrate the need to consider mobile genetic elements like prophages while developing a treatment strategy in chronic diseases like CRS, as strains with/or without prophages have different virulence properties and risks of chronic colonization, despite having almost identical core genomes.

Limitations and future direction

Although we could affirm the origin of human immune evasion cluster genes in *S. aureus* and predict the location of φSa3int-group prophage insertion with accuracy, we could not identify the genes responsible for increased biofilm and adhesion in SA333. However, the successful introduction of φSa3int prophage into SA222 ruled out the role of prophage and prophage-associated genes in the high biofilm and mucoid phenotype of *S. aureus* SA333, redirecting future research to the limited number of SNPs and insertion sequences that are present on SA333 but not on the lysogens. Also, the hypothesis can be further expanded to other types of prophages which carry different set of virulence factors.

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Data availability

All the supplementary data, protocol and materials pertaining to this research is available in a public database under the following doi address: http://10.6084/m9.figshare.22696627

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CHAPTER SIX

CONCLUSION

6.1 CONCLUDING REMARKS

There is mounting evidence that the coexistence of bacteria and prophages is linked to multifaceted bacterial fitness elevating risks to human health. However, the precise role of lysogeny and prophage induction in disease development including chronic rhinosinusitis, and how they support the survival and dominance of lysogens within their ecological niche in clinical settings is not well understood. In chronic rhinosinusitis (CRS), *Staphylococcus aureus* is believed to play a modulatory role in disease pathogenesis, severity, and post-operative recovery. *S. aureus* is an opportunistic pathogen, and its virulence, including but not limited to toxin secretion, biofilm formation, and niche adaptation, is profoundly altered by prophages. As such, these prophage-associated virulence factors could also be predictive of the pathogenesis of the bacteria and CRS disease development and severity which may ultimately assist in identifying appropriate therapeutic interventions to quench clonal expansion and survivability of more virulent *S. aureus* clones.

Therefore, the present study was designed to retrospectively identify and categorize prophages and associated virulence factors in *S. aureus*, which were predominantly circulating in chronic rhinosinusitis patients. Further, our aim was to understand the inducibility of prophages under various conditions like elevated temperature, sub-lethal concentrations of antibiotics, and exposure to steroids often used in ear, nose and throat (ENT) clinics. Finally, our goal was to understand the impact of Sa3int-type prophage domestication in human-adapted *S. aureus*.

Our findings report a widespread presence of intact (complete) prophages in *S. aureus* recovered from CRS patients. Among 12 different types of prophages, Sa3int-type prophages (prophages with type-3-integrase) were predominant in *S. aureus* recovered from chronic rhinosinusitis patients with nasal polyps (a more severe form of CRS). These prophages encoded a set of human immune modulatory genes, known as immune evasion cluster (IEC) genes. Thus, we speculated that prophage-associated virulence has a profound role in the pathophysiology of inflammatory diseases like chronic rhinosinusitis.

Further, our results suggest that inducible prophages are common in clinical strains of *S. aureus* isolated from the nasal cavities of patients with chronic rhinosinusitis (CRS). Additionally, our research demonstrated that sub-inhibitory concentrations of clinically important antibiotics increased phage mobilization via prophage induction. These induced phage particles could infect other isolates and switch to a temperate lifestyle, thereby enhancing horizontal gene transfer through transduction. Furthermore, almost all clinical strains isolated from CRS patients lacked beta-hemolysis, indicating a minimal role of beta-hemolysin in long-term nasal colonization.

Our research further concluded that lysogenic conversion of *S. aureus* by Sa3int prophage alters the bacterium's virulence by upregulating human immune modulatory toxins like sak, scn, and chp, while downregulating beta-hemolysin, a sphingomyelinase. Additionally, our research confirms that the bacterial growth, biofilm biomass, and adhesion of *S. aureus* are not associated with Sa3int-type prophage domestication. These findings demonstrate the need to consider mobile genetic elements like prophages when developing a treatment strategy for chronic diseases like CRS, as strains

with prophages have altered virulence properties and risks of chronic colonization, despite having almost identical core genomes. These findings provide a platform for investigating the contribution of prophages in determining the pathogenicity of bacteria and their potential use as diagnostic, prognostic, and therapeutic targets.

To sum up, our discovery of a diverse range of prophages in *S. aureus* within a restricted geographic region and from a well-defined population with CRS disease reveals the circulation of diverse temperate phages in human-adapted *S. aureus* that contribute to genotypic and phenotypic plasticity as well as the virulence of the bacterium. Furthermore, our findings enhance our understanding of the distribution of active lysogeny in clinical *S. aureus* isolates and the effects of commonly used antibiotics on prophage induction and mobilization, which directly correlates with virulence and antimicrobial resistance dissemination. As such, our research underscores the implications of the inappropriate use of antibiotics and the potential role of sub-lethal antibiotics in the development of virulent clones in addition to antimicrobial resistance. Further, we are also concerned about poly-lysogeny, which may aid in the accumulation of auxiliary prophage-encoded virulence factors as most of the *S. aureus* had more than one prophage in them. Further, we speculate that *S. aureus* carrying Sa3int-type prophage may impact inflammatory diseases like chronic rhinosinusitis by evading the human immune system and increasing the pathogenicity of the strain releasing various virulent factors. Further, as we prove that prophages can be mobilized without any phenotypical changes, our research emphasizes the development of personalized diagnostic tools that incorporates mobile genetic elements like prophages and the genes they encode to distinguish between virulent and avirulent clones. In addition, our research sheds light on novel approaches that can be implemented to render *S. aureus*

susceptible to the immune response by blocking prophage-associated defence mechanisms. Finally, our findings reveal a novel area for future investigations that will not only increase our understanding of prophage biology but also uncover undiscovered tripartite associations between the prophage-bacteria-human immune system, *S. aureus* evolution and CRS disease pathophysiology. Given their diversity, mosaicism, and transient presence, prophages are likely to be important drivers that shape microbial ecosystems, making them a promising area for further investigation in clinical settings and its correlation with diseases like CRS.

6.2 KEY FINDINGS OF THIS RESEARCH

Our research expands our knowledge of prophages in *Staphylococcus aureus* isolated from chronic rhinosinusitis (CRS) patients and their possible role in disease development. Our research concludes that *S. aureus* isolates recovered from the sinuses of chronic rhinosinusitis patients frequently harbor inducible prophages, particularly Sa3int-type which often encodes human immune evasion cluster (IEC) genes.

Altogether, 250 prophages were identified from 66 *S. aureus* clinical isolates. Out of which, 211 prophages were identified in *S. aureus* genomes recovered from chronic rhinosinusitis (CRS) patients (N = 58), and 39 prophages from isolates recovered from control samples (N = 9) suggesting the widespread distribution of prophages in clinical isolates colonizing human nasal niches. Sa2int and Sa3int-type prophages were most frequently found in *S. aureus* from CRS patients. Among them, Sa3in-type was predominant in chronic rhinosinusitis patients with nasal polyps (CRSwNP), a more severe form of CRS. Most of the prophages encoded unique sets of virulence genes like human immune evasion cluster (IEC) genes by Sa3int, and leukocidins by Sa2int prophages. None of the prophages harboured any antibiotic-resistant genes (ARGs).

Spontaneous prophage induction (SPI), often weak, was observed in around 26% of the *S. aureus* clinical isolates while mitomycin C dependent prophage induction was observed in almost 52% of the isolates. Sub-inhibitory concentrations of antibiotics enhanced the release of prophage compared to spontaneous prophage induction (SPI). Among the tested antibiotics, ciprofloxacin was the most potent prophage inducer

(51%) followed by amoxicillin, doxycycline, mupirocin, clindamycin and azithromycin, all of which enhanced the release of prophage in > 40% of the isolates. There was no correlation of prophage inducibility with biofilm biomass and the metabolic activity of *S. aureus*. However, the severity score of patients harbouring inducible prophage within *S. aureus* was significantly lower compared to the patients harbouring *S. aureus* with non-inducible prophage(s).

Integration of a ~43.8 kb φSa3int prophage into the beta-haemolysin gene (*hlb*) gene disrupted the beta-hemolysin function in human-adapted clinical isolates. The prophage domestication had no impact on bacteria growth, biofilm formation and adhesion to primary human nasal epithelial cells (HNECs). However, *S. aureus* lysogenized with the φSa3int prophage expressed various human-specific immune modulators known as human immune evasion cluster (IEC) like sak, chp and scn as extracellular components. These proteins are known to enhance *S. aureus'* human immune evasion capability thereby stabilizing the persistent colonization.

To sum up, our research expands our pre-existing knowledge in prophage diversity and its mobilization, particularly Sa3int-type prophages in human-adapted *S. aureus*. We believe our findings collectively reinforce researchers and clinicians to consider prophage-mediated consequences in the pathogenicity of human-adapted pathogenic bacteria including *S. aureus*.

6.3 LIMITATIONS, RECOMMENDATIONS AND FUTURE DIRECTION

We acknowledge that our research had a few limitations that shall be addressed in future research. One of the most striking limitations of the research was the low sample size for robust statistical correlation and the lack of matched control (non-CRS) isolates. Our study was designed retrospectively to understand the distribution of prophages in *Staphylococcus aureus*, potential prophage-encoded virulence factors and its possible correlation with disease phenotype and severity in a very defined population suffering from chronic rhinosinusitis. Our study also lacks on considering the combined role of other bacterial species and their prophages that are present in the nasal niche. Recently, phage particles released via prophage induction from lysogens are known to directly stimulate the mammalian immune response and worsen inflammatory diseases like chronic rhinosinusitis. Thus, it is important to uncover if induced prophages have any role in the inflammation of the nasal tissues. We further acknowledge that genetic makeup and predisposition to different environmental stimulants have a profound impact on the inflammatory stimulation and overall CRS pathogenesis and prophage is unlikely to be the sole factor responsible for the pathophysiology of CRS disease.

The estimation of biofilm between *S. aureus* having active prophage and passive prophage is broadly categorized not considering other factors associated with the biofilm development. A more narrow-downed approach must be considered in future research as biofilm development in a particular strain is not always associated with genetics but also environmental factors. Another, limitation of the study was to categorize the active- and passive lysogen based on re-infectivity of induced prophage

rather than microscopy. This may have underestimated the prophage induction in clinical isolates as the released phage particle may not always be able to infect and lyse RN4220. Further, the controlled induction of prophages may also be explored as a therapeutic approach, and it is important to study its prevalence, type, and association with disease. Further, the immune evasion capability of the laboratory-generated isolate was inferential from proteomics data, and we strongly recommend a cell-line (macrophage) or primary human nasal epithelial cell culture study to prove the hypothesis.

Despite a few limitations, we believe that our research has contributed significantly to exploring prophage diversity in human-adapted *S. aureus* and how targeted identification of mobile virulence factors like those encoded by prophages in addition to species and strain identification. We further propose that screening and identification of key auxiliary elements of virulence in addition to core genes may be predictive of the clinical outcome of the disease and its chronicity. Thus, our research sets the stage for more research on prophage and/or prophage-associated auxiliary genes as preclinical biomarkers. Further research in prophage may also provide insights into how the combinatorial co-existence of prophage and bacteria leads to increased fitness in adverse niches that could eventually lead to new therapeutic targets that disrupt the co-existence and combat the epidemically persistent strain. This may ultimately lead to better-personalized therapy as not all *S. aureus* (and other bacteria) are equipped with the same virulence gene pool.

APPENDICES

A. First author publication during the candidature not included as a thesis chapter

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ORIGINAL PAPER

Genomic characterization of three bacteriophages targeting multidrug resistant clinical isolates of *Escherichia, Klebsiella* and *Salmonella*

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Abstract
Application of bacteriophages (phages) to treat complex multidrug-resistant bacterial infection is gaining traction because of its efficacy and universal availability. However, as phages are specific to their host, a diverse collection of locally isolated phage from various geographical locations is required to formulate a wide host range phage cocktail. Here, we report morphological and genomic features of three newly isolated phages from river water of the urban region in Kathmandu, Nepal, targeting three different bacteria (*Escherichia coli, Klebsiella pneumoniae* and *Salmonella enterica.*) from the *Enterobacteriaceae* family. Morphological identification and genome analysis indicated that two phages (Escherichia phage vB_EcoM_TU01 and Klebsiella phage vB_KpnP_TU02) were strictly lytic and free from integrases, virulence factors, toxins and known antimicrobial resistance genes, whereas Salmonella phage vB_SalS_TU03 was possibly a temperate phage. The genomic features of these phages indicate that natural phages are capable of lysing pathogenic bacteria and may have potential in bacterial biocontrol.

Keywords Bacteriophage · Phage · Genomics · Phage therapy · *Enterobacteriaceae*

Abbreviations

MDR	Multidrug resistance
DLAA	Double layer agar assay
PCR	Polymerase chain reaction
CDS	Coding DNA sequence
tRNA	Transfer RNA
ARG	Antibiotic resistant gene
PATRIC	Pathosystems resource integration center
NCBI	National center for biotechnology information
TEM	Transmission electron microscopy
dsDNA	Double-strained DNA
GO	Gene ontology
G+C	Guanine and cytosine

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Introduction

Enterobacteriaceae is a large family of Gram-negative rod-shaped facultatively anaerobic bacteria comprising a wide range of pathogens such as *Escherichia, Klebsiella*, *Salmonella*, *Enterobacter*, *Citrobacter*, *Shigella* and more. These pathogens are associated with considerable morbidity and mortality on compromised hosts and can cause life-threatening illnesses like septicaemia, haemolytic uremic syndrome, gastroenteritis, meningitis and pneumonia in healthy individuals (Donnenberg et al. 2015). These infections are usually treated with antibiotics, but lately, most human-associated pathogens are becoming increasingly resistant to antibiotics, thereby limiting the effectiveness of the antibiotic treatment. Furthermore, the emergence of carbapenem-resistant *Enterobacteriaceae* is a concern as there is no therapy or vaccines available to prevent acquisition of infection with multidrug resistant (MDR) strains. As current antibiotic therapies are ineffective to treat such infections or

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eliminate once infected, alternative approaches are highly sought in the management of MDR infections.

Bacteriophage (phage) is a virus that infects bacterial cells but leaves eukaryotes unscathed. Because of its host specificity, phages can be used to kill bacteria without harming untargeted cells. In the past decade, therapeutic application of phage has been gaining widespread attention because of its specificity and efficacy against MDR bacterial pathogens (Pirnay 2020). Further it is also regarded as 'dynamic' solution to continuously emerging MDR strains because of its co-evolving lifestyle with the bacteria. Phage therapy uses 'strictly' lytic phages or its derivatives to kill pathogenic bacteria. Although phage therapy is not novel and had been employed shortly after the discovery of phages around 1920s (d'Herelle 1931), invention of antibiotics curbed the widespread usage of phages therapeutically as antibiotics were more effective against a broad spectrum of bacteria. However, emergence of multidrug-resistant 'superbugs' has rekindled the interest in phage therapy. Studies have shown that phage therapy can be used as an alternative biocontrol agent or adjuvant therapy to antibiotics in human and animals (Petrovic Fabijan et al. 2020; Schooley et al. 2017; Ooi et al. 2019; Waters et al. 2017; Greene et al. 2021).

However, the efficacy of phage therapy targeting the pathogen of interest still has room for improvement. As phages are highly specific in regard to infecting their host, extending up to the level of bacterial strains, phages isolated from geographically same region as the bacterial host would have a higher probability of infecting the bacterial strain of interest due to the co-evolutionary adaptations (Hampton et al. 2020). Therefore, a local 'phage bank' comprising various phages isolated in the same region as bacterial pathogens of interest would facilitate a more effective strategy for the use of phages. Further, since most of the genes in phage genome is yet 'hypothetical', a comprehensive database reporting phage genome from different geolocations and clinical isolates is essential to study the co-evolution between phage and bacteria. As such, genome report provides invaluable information that can be useful in elucidating 'conserved and unknown' functions in phage genomes. Furthermore, the use of genomics and phenotyping of phages and their host could improve the efficacy of phage therapy in the future regarding the choice of phage for the pathogen of interest. In line with the aim of expanding phage research, previously, we reported phages exhibiting lytic activity against multidrug resistant *Pseudomonas* and *Klebsiella* (Dhungana et al. 2021a; Maharjan et al. 2022) and also studied pharmacokinetics and pharmacodynamics of our Klebsiella phage Kp_Pokalde_002 in a mouse model (Dhungana et al. 2021b). Here, we report the isolation, genome analysis and taxonomic position of three newly isolated phages targeting MDR human pathogens: *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica* from *Enterobacteriaceae* family.

Materials and method

Bacterial strain

Three multidrug-resistant clinical isolates of *E. coli* ($N=1$), *K. pneumoniae* ($N=1$) and *S. enterica* ($N=1$) were collected from the Microbiology Laboratory, Tribhuvan University Teaching Hospital, Kathmandu, Nepal. The clinical isolates were confirmed to be MDR by AMR testing in the microbiology department of the hospital and used as hosts for isolation and amplification of phages. The MDR status was also validated evaluating the strains against 11 different antibiotics (Supplementary table S1) using Kirby–Bauer disc-diffusion method (Hudzicki 2009). Nutrient agar (NA, agar = 1.5%, HiMedia, India) was used to grow fresh overnight culture (at 37 °C) from glycerol stock and Luria–Bertani broth (HiMedia, India) was used to propagate the host bacterium for phage isolation and amplification.

Phage manipulation: isolation, purification and amplification

A water sample was collected from the Bagmati river, Kathmandu, Nepal flowing through the urban region of the city which is heavily polluted by untreated sewers and industrial waste (Mishra et al. 2017). Phages were isolated using Double Layer Agar Assay (DLAA) as described previously with some modifications (Dhungana et al. 2021a). Briefly, the water sample was centrifuged at 3220*g* (Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 10 min to pellet down the debris and subsequently the supernatant was filtered through a 0.45-µm and 0.22-µm pore-size Whatman™ syringe filter (Sigma-Aldrich, Missouri, United States). One millilitre filtrate was mixed with 100 µl exponentially growing host bacteria (OD_{600} 0.5) and left at room temperature (10 min) for phage adsorption. Three millilitre semisolid top agar (Tryptic Soya Agar (TSA), agar = 0.4%, stored at = 50 °C) (HiMedia, India) was added to the mixture, mixed well by swirling and poured on to the surface of previously prepared bottom agar (TSA, agar = 1.0%, HiMedia, India). After overnight incubation at 37 °C, the plates were examined for the presence of phages in the form of plaques. A single isolated clear plaque was cut and dissolved in 1.0 mL of Sodium chloride-Magnesium sulfate (SM) buffer (10 mM Tris–HCl, 10 mM $MgSO_4.7H_2O$, 2% gelatin and 100 mM NaCl, pH 7.5). Subsequently, the phage was purified by performing three rounds of DLAA from a single isolated plaque.

Phage characterization

Transmission electron microscopy

High titre purified phage lysates were fixed with fixative (2.5% glutaraldehyde and 2% paraformaldehyde prepared in 0.1 M sodium phosphate buffer (pH 7.2)). For fixation, equal volume of phage lysate and fixative were added, mixed and left overnight. The next day, the fixed phages were subjected to high-speed centrifugation (35,000*g*) for 3 h. Per sample 10.0 µL fixed phage lysate was deposited on a separate 300 mesh carbon-coated copper grid. The copper grid was then flooded with 2% (w/v) uranyl acetate (pH 4.5) for 2 min. The copper grid was dried and examined in JEM-2100F Transmission Electron Microscope (JEOL, USA) at 200 kV under different magnifications. TEM micrographs were processed using ImageJ 1.50i (https://imagej.nih.gov/ij) (Schneider et al. 2012).

Genomic DNA extraction, sequencing and annotation

Phage genomic DNA (gDNA) was isolated using Phage DNA Isolation Kit (Norgen Biotek Corp., Ontario, Canada. Cat. #46,800) per manufacturer's instructions. Qualitative and quantitative control were performed using conventional electrophoresis and Qubit® 2.0 Fluorometer (ThermoFisher Scientific, USA), respectively. Five microliter gDNA of each sample was loaded on 1% agarose gel and run for 30 min at 110 Volt. Also, 1.0 µl of each sample was loaded in NanoDrop 8000 (ThermoFisher Scientific, USA) for determining A260/280 ratio and Qubit® 2.0 for determining concentration of gDNA.

The paired-end sequencing library was prepared using TruSeq® Nano DNA HT Library Preparation Kit (Illumina, USA). Two hundred nanograms of gDNA was fragmented by Covaris shearing that generated dsDNA fragments with 3' or 5' overhangs. The fragments were then subjected to end-repair. The ligated products were purified using SP beads supplied in the kit. The size-selected product was PCR amplified as described in the protocol. The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies, USA) using High Sensitivity (HS) DNA chip as per manufacturer's instructions. After obtaining the Qubit® concentration for the library and the mean peak size from Bio-analyser profile (Fig. S1A–C), the library was loaded onto Illumina HiSeq 2000/2500 (Illumina, USA) for cluster generation and sequencing. The cluster generated was assembled using CLC Genomics Workbench 6.0 (Qiagen, USA) at default parameters (Minimum contig length: 200, Automatic word size: Yes, Perform scaffolding: Yes, Mismatch cost: 2, Insertion cost: 3, Deletion cost: 3, Length fraction: 0.5, Similarity fraction: 0.8). Phage genomes were annotated for coding DNA sequences (CDS), tRNA, virulence factors, toxins, antimicrobial resistance genes (ARGs) and drug targets using the Pathosystems Resource Integration Center (PATRIC 3.6.12) webtool (https://www.patricbrc.org/) (Wattam et al. 2013; Brettin et al. 2015) using viruses (taxid = 10,239) as the reference database. A circular map of the phage genome was generated using CGview server (http://cgview.ca/) (Stothard and Wishart 2004), and a phylogenetic tree was constructed BLASTing the query sequence against NCBI database using neighbor-joining method. Only the ten most common phages were included in the phylogenetic analysis. The tree was further visualized using ggtree package in R 4.1.1 (https://www.R-project.org/). The lifestyle, order, family and host of the phages were computationally predicted through PhageAI (https://phage.ai/) (Tynecki et al. 2020).

Results and discussion

Three following phages, viz: Escherichia phage vB_EcoM_TU01 (hereafter vB_EcoM_TU01), Klebsiella phage vB_KpnM_TU02 (hereafter vB_KpnM_TU02) and Salmonella phage vB_SalS_TU03 (hereafter vB_SalS_TU03) targeting multidrug resistant clinical isolates of *E. coli*, *K. pneumoniae* and *S. enterica*. were isolated from the water sample collected from the Bagmati river (Fig. 1A, C, E). TEM revealed that among three phages, two (vB_EcoM_TU01, vB_KpnM_TU02) were from the *Myoviridae* family whereas vB_SalS_TU03 belonged to *Siphoviridae* family (Fig. 1B, D, F and Table 1). All phages were tailed phages (Order = *Caudovirales*) and consist of a linear double-stranded DNA (dsDNA) genome with gene density of approximately 1.7 genes/kilo-basepairs which is much

Table 1 Classification of phages according to ICTV* guidelines (ICTV 9th report) based on transmission electron micrograph

Phage	Capsid (in nm^)	Tail (W × L, in nm^)	Shape	Family (Morphotype#)
vB_EcoM_TU01,	82 × 108	19 × 111	Elongated	Myoviridae (A2)
vB_KpnM_TU02	82 × 99	25 × 109	Elongated	Myoviridae (A2)
vB_SalS_TU03	63	9 × 106	Icosahedral	Siphoviridae (B1)

*ICTV = The International Committee on Taxonomy of Viruses. ^ nm = nanometre. The capsid and tail lengths are an average of three measurements of a phage electron micrograph from a purified stock.

#Morphotypes are based on classification by Ackermann (2001)

higher that of the bacterial host (0.5–1.0 genes/kilo-base-pairs) (Norwood and Sands 1997). The CDS coverage of all the phages was higher than 95% whereas the average gene length ranged between 540 and 567 basepairs (Table 2).

The genome of vB_EcoM_TU01 was 169,046 bp with a G + C content of 37.42% [lower than that of its host *E. coli* (~ 50.6%)] encoding 286 proteins (Fig. 2). The average length of genes was 566 bp with a CDS coverage of 95.9%. Furthermore, vB_EcoM_TU01encoded 2 transfer-RNAs (tRNA) (tRNA-Met-CAT and tRNA-Arg-TCT). Regarding the gene function, 83.2% (238/286), were functional of which 5.6% (16/286) had a Gene Ontology (GO) assigned function, and the remaining 16.8% (48/286) were hypothetical. Similarly, the genome of vB_KpnM_TU02 was 166,230 bp with a G + C content of 38.34% [lower than that of its host *K. pneumoniae* (~ 57%)] and encoded 294 proteins (Fig. 3). The average gene size in vB_KpnM_TU02 was 540 bp with a CDS coverage of 95.6%. The phage vB_KpnM_TU02 also encoded 15 tRNAs (tRNA-Thr-TGT, tRNA-Leu-TAA, tRNA-Arg-TCT, tRNA-Met-CAT, tRNA-Pro-TGG, tRNA-Gly-TCC, tRNA-Trp-CCA, tRNA-Ile-GAT, tRNA-Ser-TGA, tRNA-His-GTG, tRNA-Gln-TTG, tRNA-Met-CAT, tRNA-Asn-GTT, tRNA-Lys-TTT and tRNA-Tyr-GTA). Out of 294 encoded proteins, 110 (37.4%) were functional, and 184 (62.6%) were hypothetical, whereas only 11 (3.7%) encoded proteins had GO assigned function. Further, the genome of vB_SalS_TU03 was 41,756 bp with a G + C content of 47.06% [slightly lower than that of its host *Salmonella* (~ 52.2%)] and encoded 71 proteins (Fig. 4). The average gene size in vB_SalS_TU03 was 562 bp with a CDS coverage of 95.7%. Out of 71 encoded proteins, 45 (63.4%) aligned with the functional protein whereas 26 (36.6%) were hypothetical. Only 2 out of 71 (2.8%) encoded proteins had GO assigned function.

Although the functions of tRNA in phages remain elusive, it is believed that more tRNA corresponds to increased virulence of the phage as it facilitates a more robust integration of the phages (Bailly-Bechet et al. 2007; Almeida et al. 2022). Since two of our phages encoded multiple tRNAs, it is more likely that these phages are virulent (lytic) and thus more suitable for therapeutic purposes. The 'functional' proteins include proteins involved in DNA packaging, transcription, replication, regulation, lysis and structural proteins whereas 'hypothetical' proteins are coding DNA sequences (CDS) with unknown functions. All the three phage genomes were free from genes encoding known toxins, antibiotic resistant genes (ARGs), virulent factors (VFs) of bacterial origin and lysogenic markers such as integrase, recombinase, repressor/anti-repressor protein, and excisionase. However, the in silico tool we used (phageAI) only categorized vB_EcoM_TU01 and vB_KpnM_02 as virulent/lytic with high confidence (96.34% and 99.27%, respectively), whereas

Table 2 Genomic and protein features of three novel phages targeting multidrug resistant *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica* clinical isolates

Features	Escherichia phage vB_EcoM_TU01	Klebsiella phage vB_KpnM_TU02	Salmonella phage vB_SalS_TU03
NCBI accession	**MZ560701**	**MZ560702**	**MZ560703**
Genomic features			
Length (in base pairs)	169,046 bp	166,230 bp	41,756 bp
Guanine-cytosine (G + C) content	37.42%	38.34%	47.06%
Total CDS	286	294	71
tRNAs	2	15	0
Gene density (per kbp)	1.69	1.77	1.70
Average gene size (in bp)	566	540	562
CDS coverage	95.9%	95.6%	95.7%
Protein feature			
Hypothetical proteins	48 (16.78%)	184 (62.59%)	26 (36.62%)
Functional proteins	238 (83.22%)	110 (37.41%)	45 (63.38%)
Proteins with GO assignments	16 (5.60%)	11 (3.74%)	2 (2.82%)
Other features/genes			
Transporter genes (Ref = TCDB)	5	0	0
Drug target genes (Ref = DrugBank)	3	0	0
Order	Caudovirales	Caudovirales	Caudovirales
Family	Myoviridae	Myoviridae	Siphoviridae
Genus (Ref = PhageAI, NCBI)	Mosigvirus	Jiaodavirus	Jerseyvirus
Lifestyle (Ref = PhageAI)	Virulent (C = 96%)	Virulent (C = 99%)	Temperate (C = 57%)

NCBI National Center for Biotechnology Information, *CDS* Coding DNA sequences, *tRNA* transfer RNA, *kbp* kilo basepairs, *GO* Gene ontology (http://geneontology.org/), *TCDB* Transporter classification database (https://www.tcdb.org/), *C* Confidence

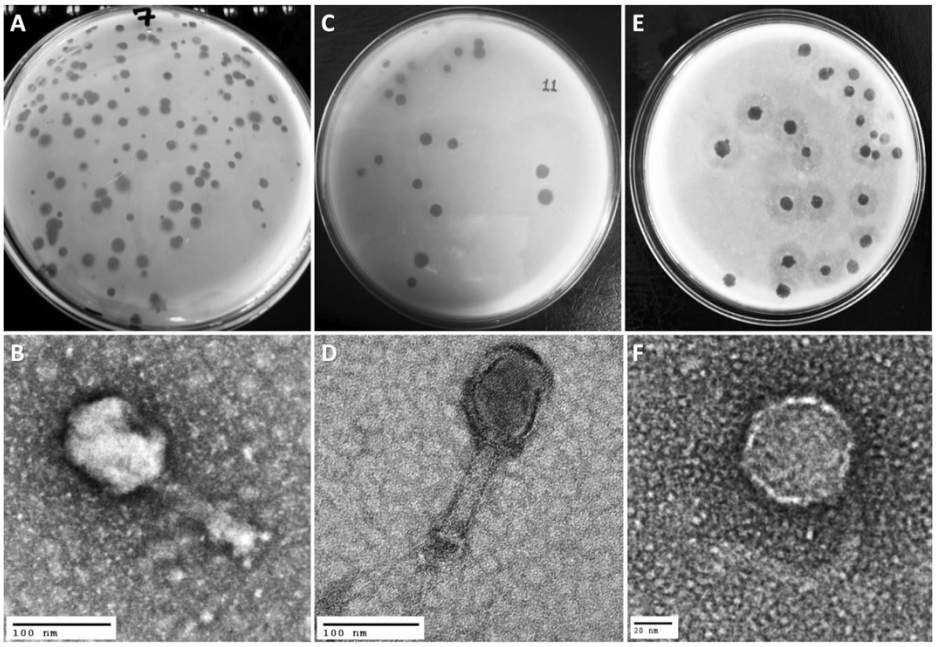

Fig. 1 Phage isolation using double layer agar assay and their transmission electron micrograph (TEM). **A**, **C**, **E** Three double layered agar plates showing different types of phage plaque morphologies isolated directly from river water. **B** TEM of Escherichia phage vB_EcoM_TU01 (scale bar = 100 nm), **D** TEM of Klebsiella phage vB_KpnM_TU02 (scale bar = 100 nm), **F** TEM of Salmonella phage vB_SalS_TU03 (scale bar = 20 nm)

vB_SalS_TU03 was tagged as temperate/lysogenic with a low confidence of 57%. The substantial number of hypothetical proteins in all phages clearly indicates that phages carry numerous genes that are yet to be characterized, and whose function is yet to be understood. The detailed information about the genomes of all three phages and their respective lifestyle is summarized in Table 2. These results suggest that vB_EcoM_TU01 and vB_KpnM_02 could potentially be used as therapeutic phages against multidrug resistant *E. coli* and *K. pneumoniae,* whereas vB_SalS_TU03 would less likely succeed in lysing its host as it may switch to lysogenic lifestyle and incorporate in the host genome as a prophage. Since prophages play a catalytic role in disease modulation (Nepal et al. 2022) and are known to carry genes increasing bacterial fitness which could be detrimental to humans (Balcazar 2014; Helbin et al. 2012; Khalil et al. 2016; Kondo et al. 2021; Nepal et al. 2021), such phages are not suitable for phage therapy.

Further, comparing the phage genome in the NCBI database using nucleotide BLAST (nBLAST) revealed that the phage vB_EcoM_TU01 was closely related to a T4-like lytic Escherichia phage vB_EcoM_JS09 (NCBI accession = KF582788, query coverage = 99%, per cent identity = 98.04%) isolated in China from the sewage of a swine factory. Similarly, phage vB_KpnM_TU01 was similar to a lytic Klebsiella phage JD18 (NCBI accession = KT239446, query coverage = 96%, per cent identity = 97.89%) isolated in China. Further, phage vB_SalS_TU03 was closest to lytic Salmonella phage LSPA1 (NCBI accession = KM272358, query coverage = 93%, per cent identity = 99.17%) isolated in China from a hospital sewage (Zeng et al. 2015). These analyses indicate that our phages were novel, but highly similar to the phages isolated in neighbouring China around the same time and might have a very similar host range. Phylogenetic relatedness of all three phages against ten most common phages and their per cent identity is elaborated in Fig. 5. It is noted that, among ten most common hits, phylogenetics reveal that vB_EcoM_TU01 is also closely related to *Shigella* phages (also an *Enterobacteriaceae*). Although more

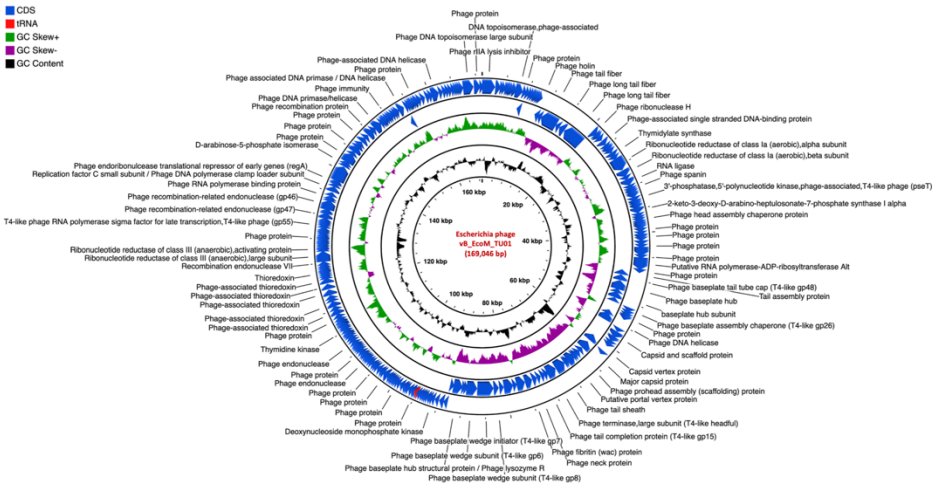

Fig. 2 Genome organization of Escherichia phage vB_EcoM_TU01 targeting multidrug resistant *Escherichia coli* clinical isolate. Predicted coding regions are shown by arrows indicating the direction of the transcription

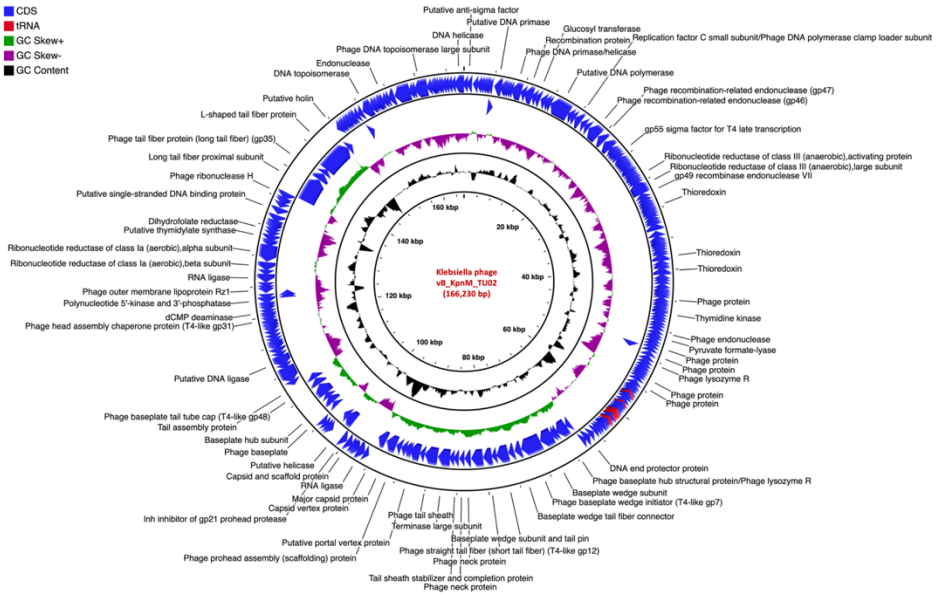

Fig. 3 Genome organization of Klebsiella phage vB_KpnM_TU02 targeting multidrug resistant *Klebsiella pneumoniae* clinical isolate. Predicted coding regions are shown by arrows indicating the direction of the transcription

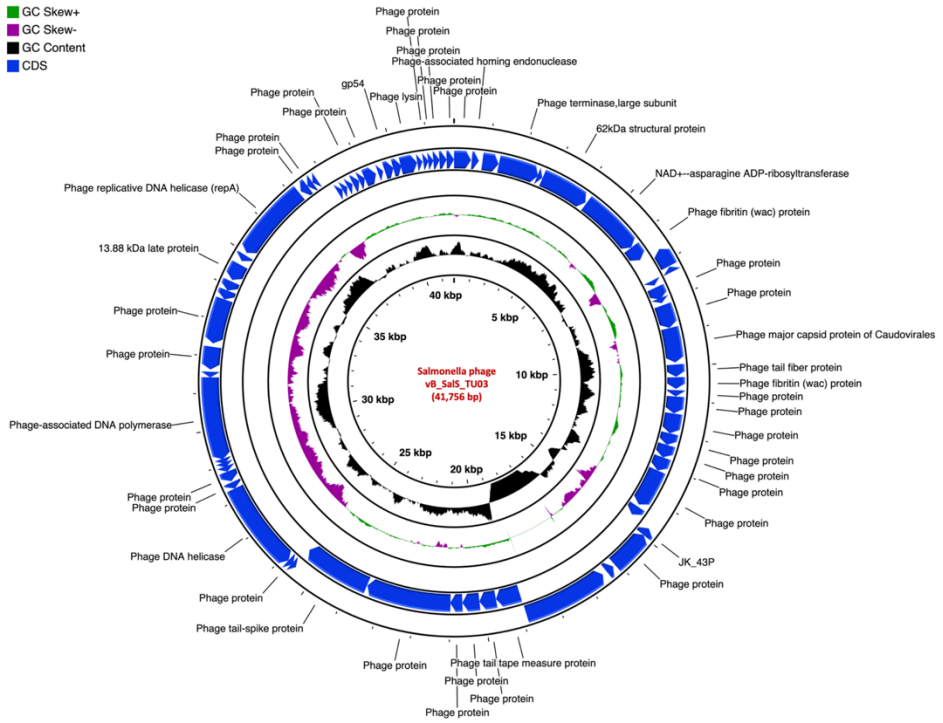

Fig. 4 Genome organization of Salmonella phage vB_SalS_TU03 targeting multidrug resistant *Salmonella enterica*. clinical isolate. Predicted coding regions are shown by arrows indicating the direction of the transcription

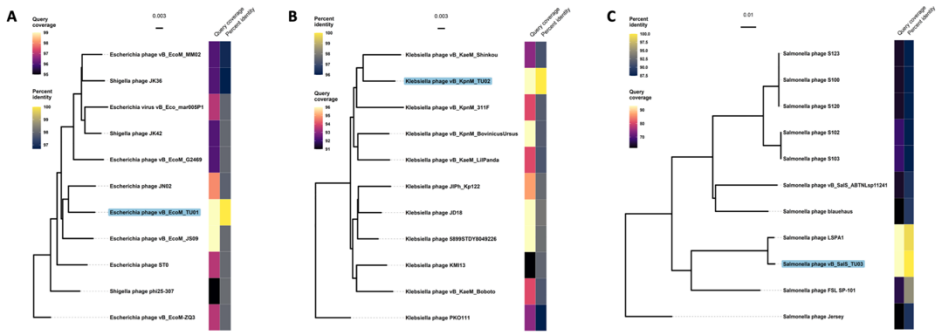

Fig. 5 Phylogenetic relatedness of Escherichia phage vB_EcoM_TU01 (**A**), Klebsiella phage vB_KpnM_TU02 (**B**) and Salmonella phage vB_SalS_TU03 (**C**) against most common phage hits ($N = 10$) in the NCBI database. The phylogenetic tree was constructed using neighbour-joining method

study is required, we can arbitrarily predict that phages isolated against different genus of bacteria have higher degree of similarity between them. This may explain (although not studied in this research) why some phages are polyvalent (showing inter-genus or even inter-order infectivity) and show expansive host spectrum (Gambino et al. 2020; Hamdi et al. 2017; Sui et al. 2021; Yu et al. 2016). This property thus holds immense applicability if further study is performed to determine the mechanism of phage infection and identify the factors/proteins/enzymes that determine phage-bacteria specificity.

Conclusion

Three phages infecting multidrug-resistant *E. coli, K. pneumoniae and S. enterica* were isolated, sequenced and banked. Genome analysis indicated that two of them (Escherichia phage vB_EcoM_TU01 and Klebsiella phage vB_KpnP_TU02) were strictly lytic and free from integrases, virulence factors, toxins, and antimicrobial resistance genes. Although additional studies are required, the genomic features of these phages provide valuable insights into the possibility of using natural phages as biocontrol agents against multidrug resistant human pathogens.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-02948-0.

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Author contributions RM, RN: Conceptualization and funding acquisition. RN, SK: Methodology, investigation. RN, SK, GH: Analysis and visualization. RM: Supervision. RN, SK, GH: Writing – original draft. RN, GD, GH, SV: Writing – review & editing.

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Data availability The annotated genome assembly of Escherichia phage vB_EcoM_TU01, Klebsiella phage vB_KpnP_TU02, Salmonella virus vB_SalS_TU03 is available through GenBank accession MZ560701, MZ560702 and MZ560703, respectively. In addition, *fastq* file pertaining to raw sequence data is deposited at NCBI and is available through BioProject accession PRJNA383466 and Sequence Read Archive (SRA) identifiers SRR5460626, SRR5460625, SRR5460624, respectively.

Declarations

Conflict of interest We declare no conflicts of interest.

Ethical approval The study does not involve any human and/or animal subjects. The clinical isolates obtained from hospital was deidentified, and no personally identifiable patient information was disclosed to the researchers.

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B. Supplementary figures (Chapter 3)

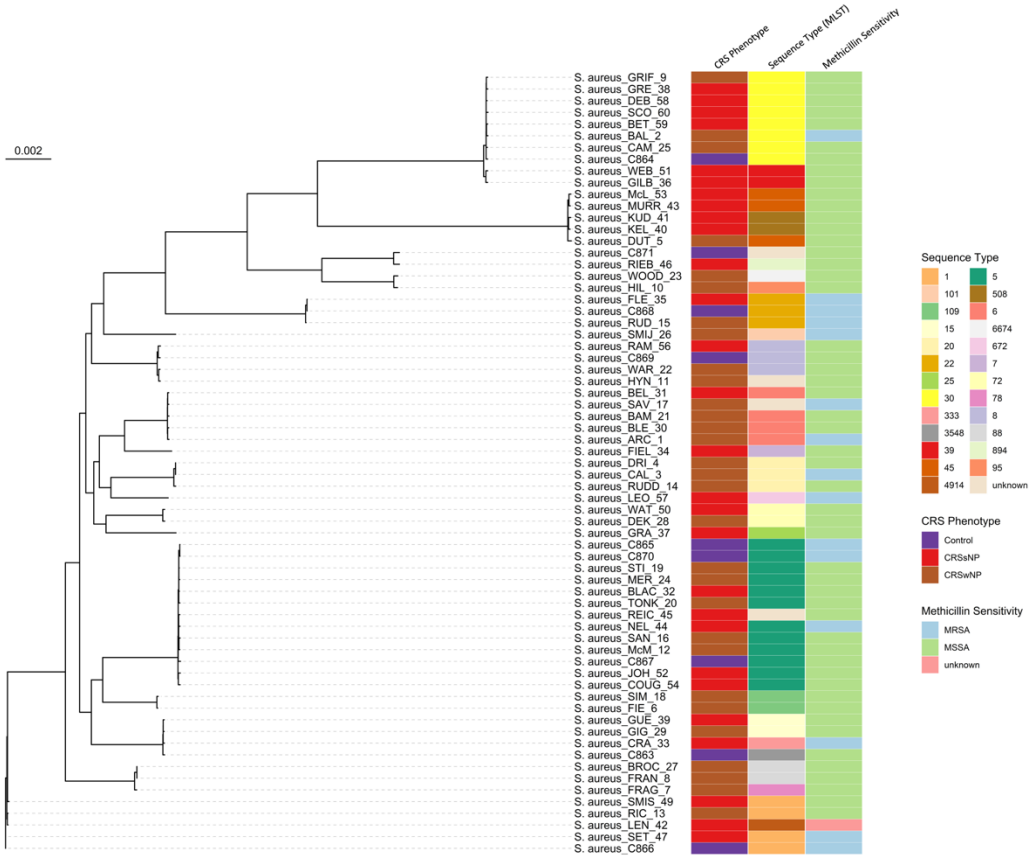

Figure S3.1 | Phylogenetic tree and heat-map showing the phylogenetic relationships and chronic rhinosinusitis phenotypes. From left to right: the phylogenetic tree constructed with IQtree using the core genome, heat map showing the CRS phenotype of the host, the multilocus sequence type (MLST) of clinical isolates and the methicillin sensitivity. The corresponding strain number is shown next to the heat map.

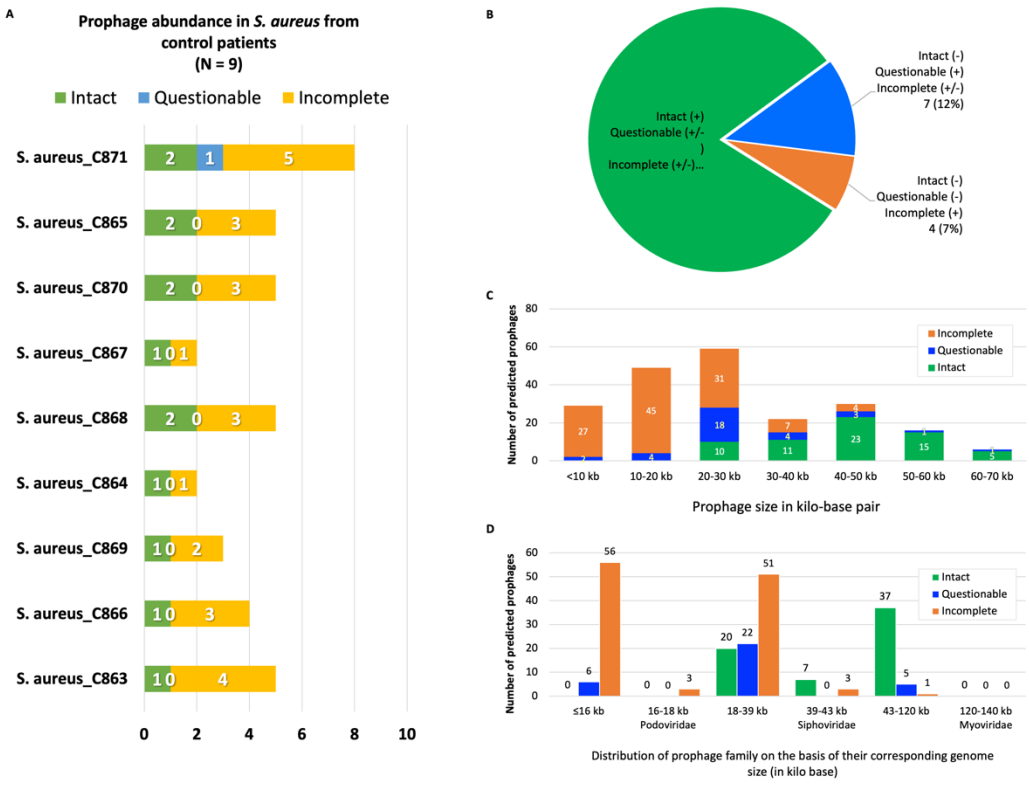

Figure S3.2 | Prophage distribution and characterization. (A) Prophage abundance in *S. aureus* isolated non-CRS patients (control). (B) Distribution of prophage (intact, questionable and incomplete) in 58 S. aureus strains isolated from chronic rhinosinusitis patients. (C) Distribution of prophages based on genome size and their completeness. (D) Distribution of prophage according to genome size based on phage size range of *Podoviridae*, *Siphoviridae* and *Myoviridae*.

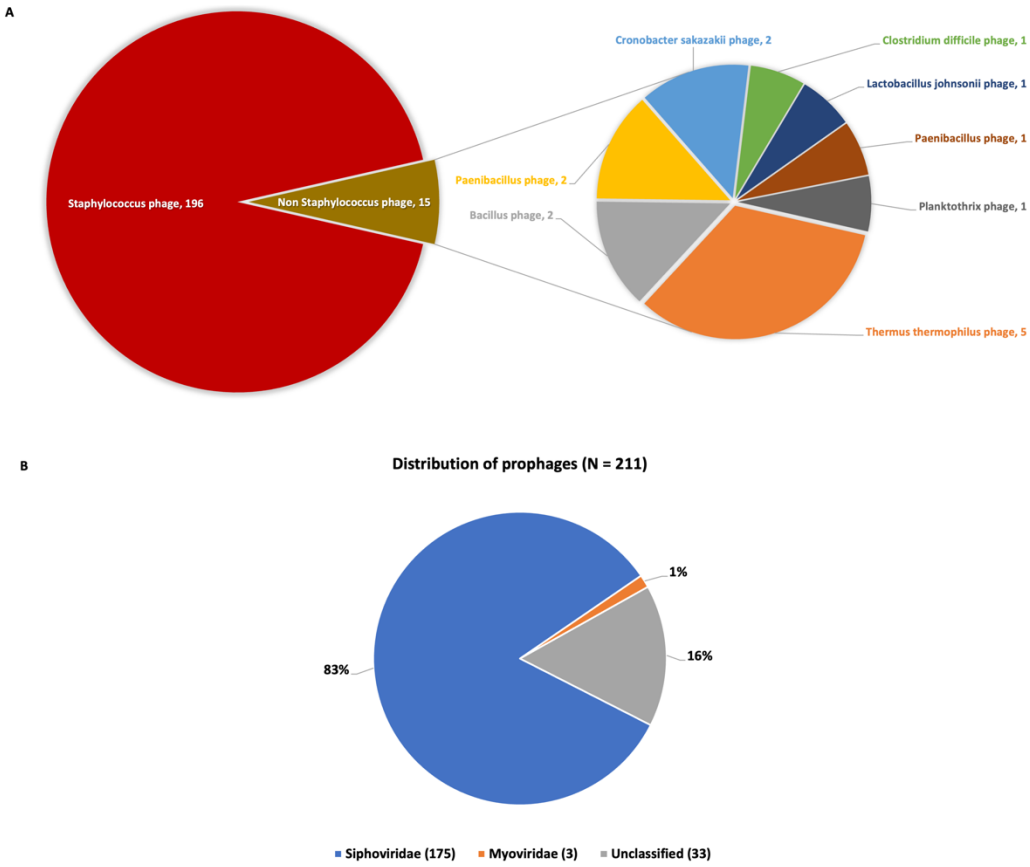

Figure S3.3 | Distribution of prophages based on maximum homology as predicted by PHASTER. (A) Among 211 prophages, 196 were most similar to Staphylococcus phage whereas 15 of them resembled phages from another bacterial genus. (B) Among 211 predicted prophages, based on their most- similar hit relative, 175 (83%) were Siphoviridae phages while 3 (1%) resembled Myoviridae and 33 (16%) resembled unclassified phage.

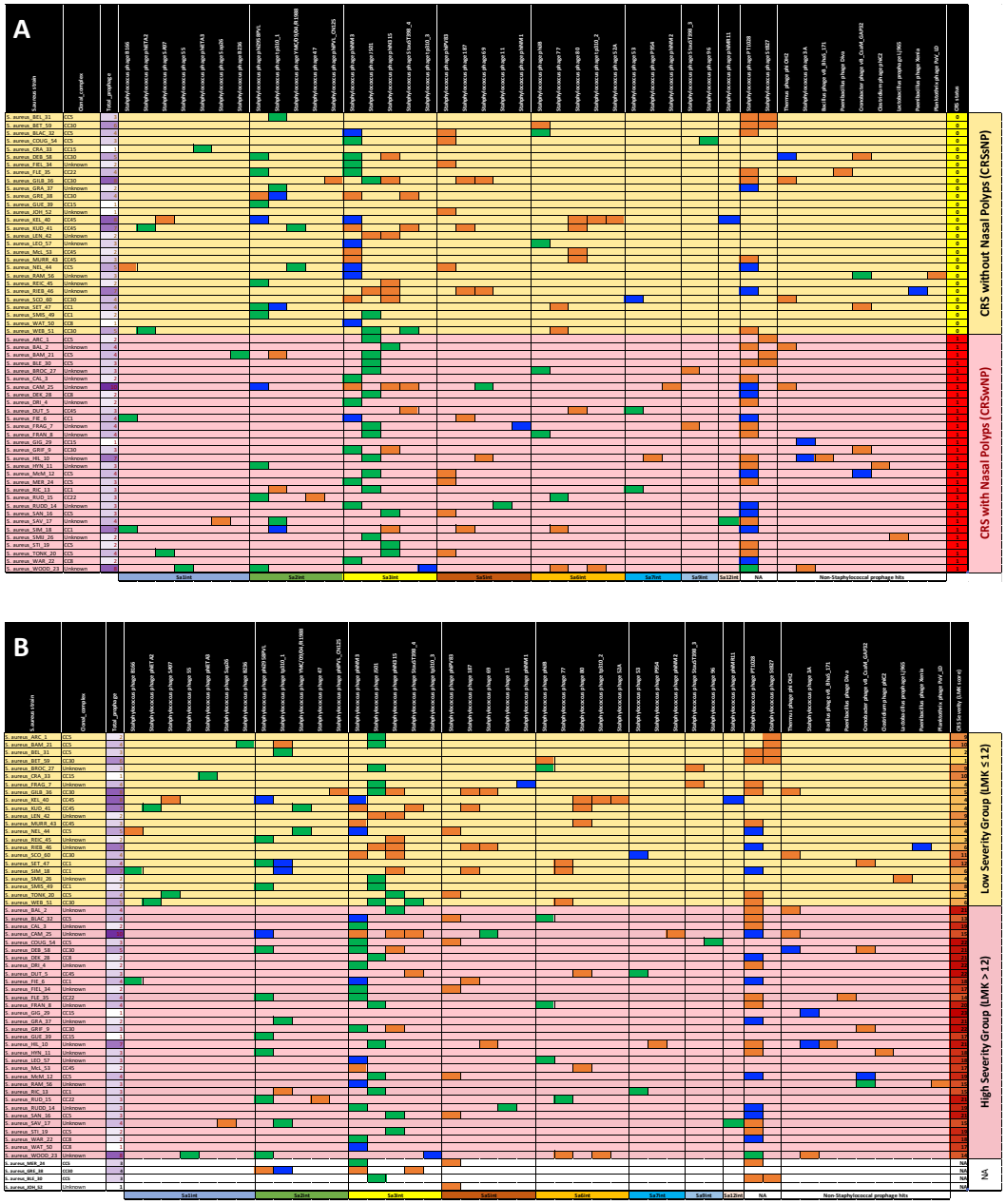

Figure S3.4 | Absence/presence heat-map showing distribution of prophages in *S. aureus* (N = 58) isolated from CRS patients. (A) Distribution of S. aureus prophages across CRS patients with nasal polyps ((CRSwNP) and without nasal polyps (CRSsNP). Presence of 'intact' prophages is more abundant in *S. aureus* isolates from CRS patients with nasal polyps (CRSwNP) compared to CRS patients without nasal polyps (CRSsNP). (B) Distribution of *S. aureus* prophages across CRS patients with low/high Lund-Mackay score (LMK > 12 and LMK > 12).

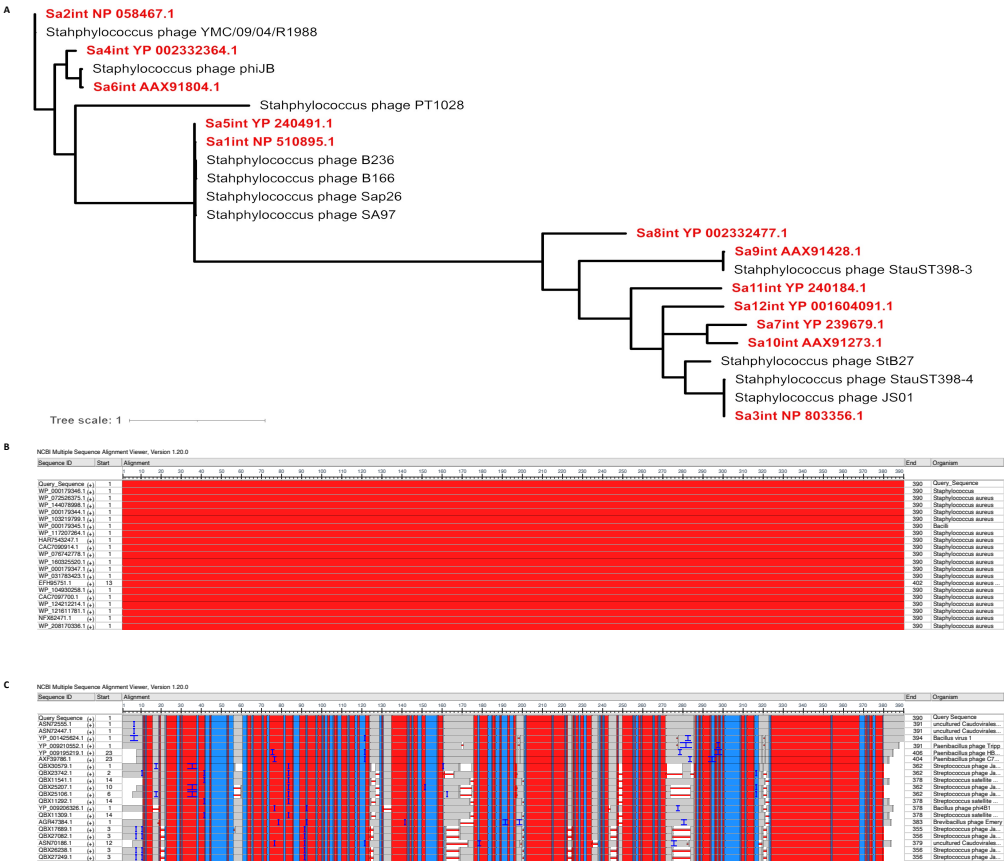

Figure S3.5 | Multiple sequence alignment and phylogenetics of integrase gene. (A) Phylogenetic relationship of representative phage based on integrase genes they encode from unassigned phage-hits with reference to Sa1int-Sa12int. (B) NCBI BLAST (nucleotide) analysis of unassigned integrase (unknown, Figure 4A) that is usually found in incomplete prophages. The BLAST analysis showed that it completely (100%) matches with tyrosine-type integrase from *S. aureus.* (C) NCBI BLAST (viruses: taxid 10239) analysis of unassigned integrase (unknown, Figure 4A) that is usually found in incomplete prophages. The BLAST analysis showed that it partially matches with tyrosine-type integrase reported from an uncultured Caudovirales.

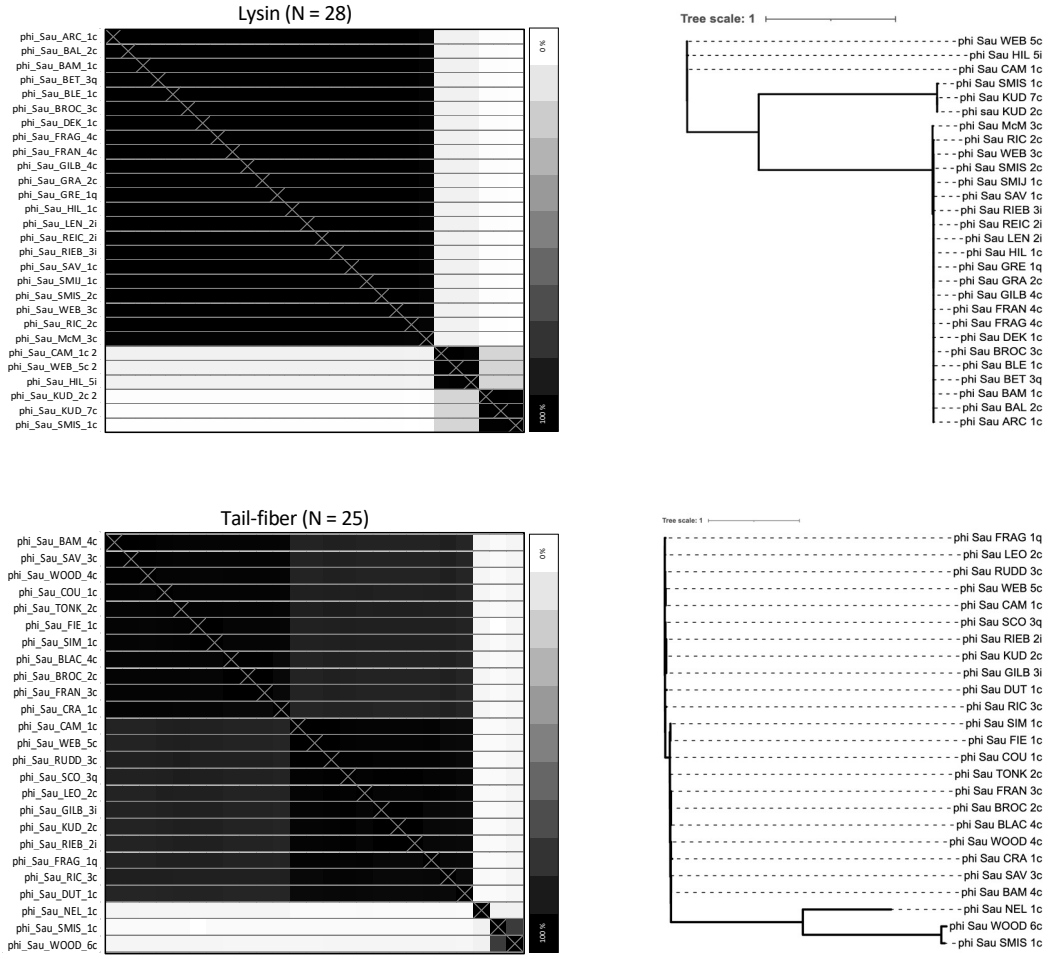

Figure S3.6 | Percentage identity dot-matrix and phylogenetics of lysin (N = 28) and tail-fiber (N = 25). (A) Percentage identity dot-matrix of lysin (N = 28) gene. The gradient bar at the right represents percentage identity, darkest being 100%. (B) Phylogenetics of lysin (N = 28) gene. (C) Percentage identity dot-matrix of Tail-fiber (N = 25) gene. The gradient bar at the right represents percentage identity, darkest being 100%. (D) Phylogenetics of tail-fiber (N = 25) gene.

C. Supplementary figures (Chapter 5)

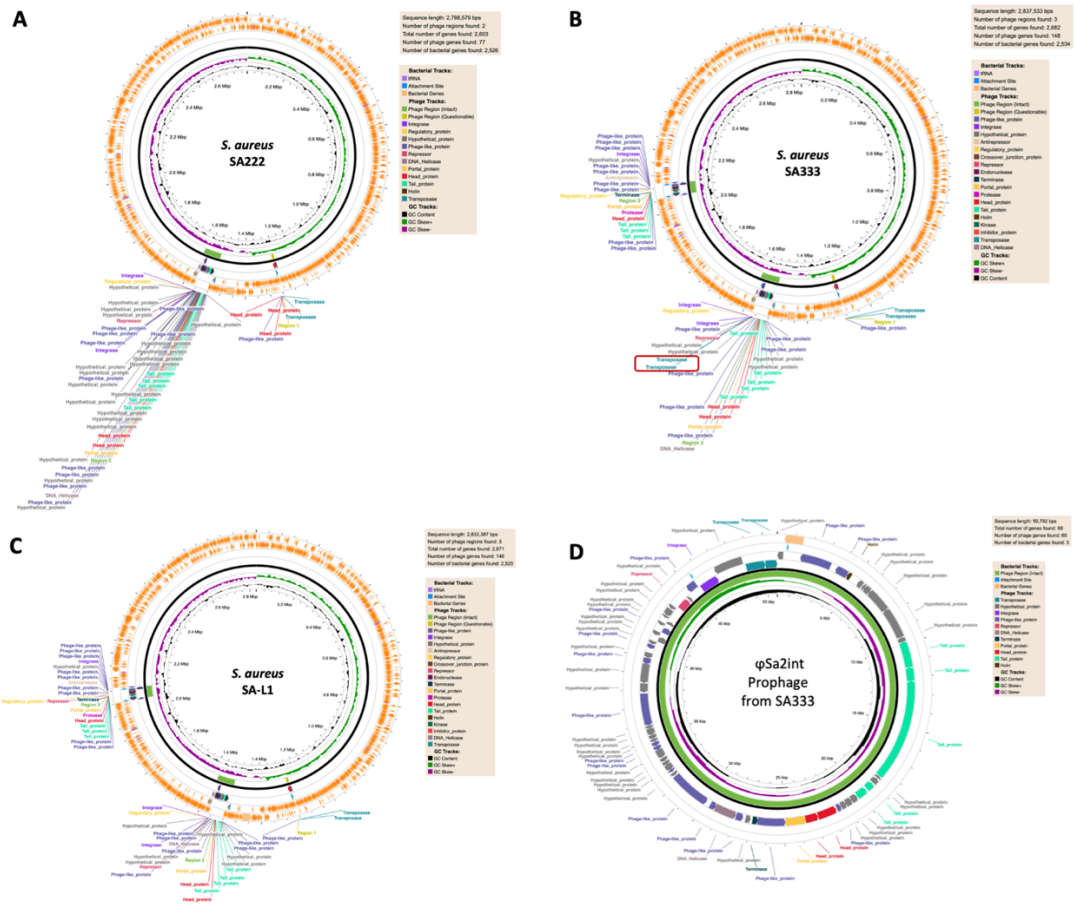

Figure S5.1 | Identification of prophage region of SA222, SA333 and SA-L1 by PHASTEST. (A) *S. aureus* SA222 only harbours one intact prophage region (52.5 kb). (B) *S. aureus* SA333 harbours two intact prophage regions (50.8 kb and 43.8 kb). It is noted that the first prophage in *S. aureus* SA333 is almost similar to the one from *S. aureus* SA222 but has two transposases integrated into the prophage region (red box). (C) Laboratory-generated *S. aureus* SA-L1 harbours two intact prophages, one from *S. aureus* SA222 and one from *S. aureus* SA333. The second prophage was induced from *S. aureus* SA333 and inserted into *S. aureus* SA222. (D) The genetic mapping of φSa2int prophage from *S. aureus* SA333. Note the gain of two transposase enzymes compared to the same prophage present in *S. aureus* SA222.

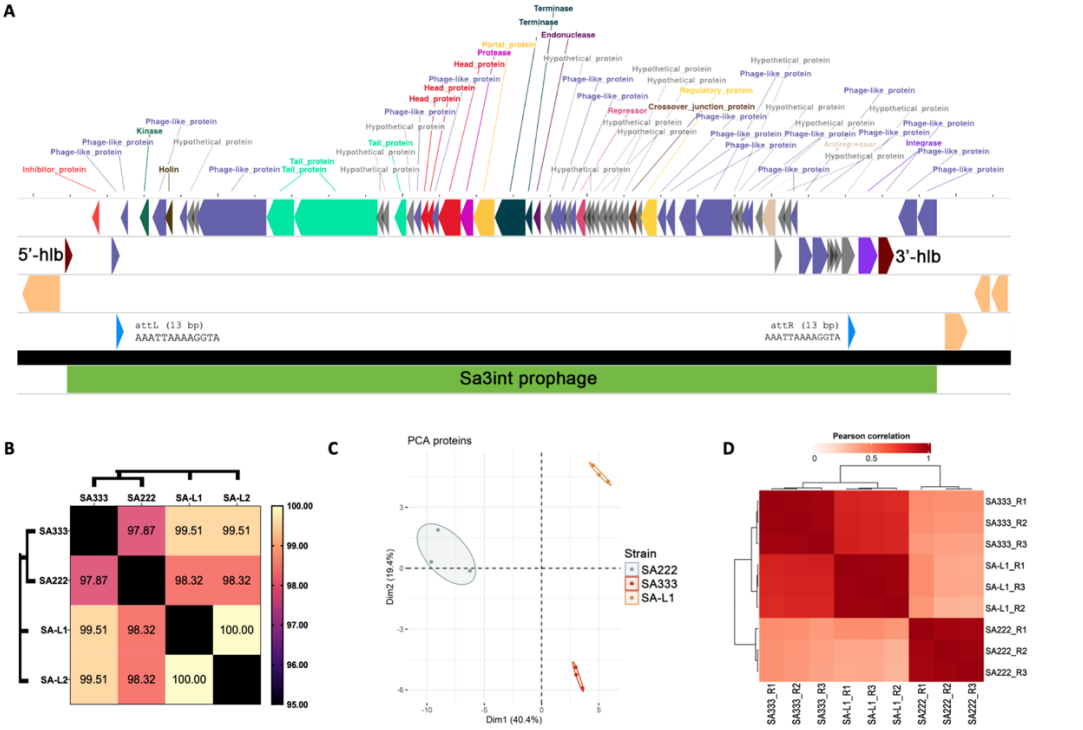

Figure S5.2 | Chromosomal location φSa3int prophage and correlations between SA222, SA333 and SA-L1. (A) Chromosomal location of the φSa3int prophage insert in SA-L1. (B) A heatmap representing the genomic similarities between donor (SA333), recipient (SA222) and laboratory-generated double lysogen (SA-L1 and SA-L2). The numbers inside the square represent aligned identical bases/nucleotides between the strains in percentage. (C) Principal component analysis (PCA) of proteomics (triplicates) between SA222, SA333 and SA-L1. The analysis shows that the proteomics of triplicates clustered together, indicating consistency of the secretome. (D) Pearson's correlation of proteomics (triplicates) between SA222, SA333 and SA-L1 also represents consistency in release factors in the secretome.

THE END

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