COMMENTARY

Uncovering novel mobile genetic elements and their dynamics through an extra-chromosomal sequencing approach

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ABSTRACT

Staphylococcus aureus is a major clinically important pathogen with well-studied phage contributions to its virulence potential. In this commentary, we describe our method to enrich and sequence stealth extra-chromosomal DNA elements in the bacterial cell, allowing the identification of novel extra-chromosomal prophages in *S. aureus* clinical strains. Extra-chromosomal sequencing is a useful and broadly applicable tool to study bacterial genomics, giving a temporal glance at the extra-chromosomal compartment of the cell and allowing researchers to uncover lower-copy plasmidial elements (e.g., prophages) as well as gain a greater understanding of mobile genetic elements that shuffle on and off the chromosome. Here, we describe how episomal and plasmidial DNA elements can have profound downstream effects on the host cell and surrounding bacterial population, and discuss specific examples of their importance in Gram-positive bacteria. We also offer potential avenues of future research where extra-chromosomal sequencing may play a key role in our understanding of the complete virulence potential of infectious bacteria.

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The development of next-generation sequencing (NGS) technologies has allowed the expanded use of DNA sequencing in novel approaches to study microbial biology. In our study, *Beyond the Chromosome: The Prevalence of Unique Extra-chromosomal Bacteriophages with Integrated Virulence Genes in Pathogenic Staphylococcus aureus*, we directed NGS toward DNA elements in the extra-chromosomal compartment of *Staphylococcus aureus*, to identify and better understand mobile genetic elements (MGEs) present off the chromosome of various clinically relevant strains. As the extra-chromosomal compartment often contains a diverse array of MGEs encoding virulence factors and resistance genes important in infection, we developed a method to screen for such elements and in particular, prophages, by enriching extra-chromosomal DNA prior to NGS. More specifically, we grew *S. aureus* clinical strains to mid-logarithmic phase before lysing cultures with a combination of lysostaphin, lysozyme, and the bacteriophage endolysin PlySs2. A combination of lytic enzymes allowed for

more controlled and gentle lysis upon addition of lysis buffer, potentially limiting unwanted DNase activity. Following controlled lysis, a modified QIAGEN HiSpeed Plasmid Midi Kit protocol was followed. To clear the lysate, a centrifugation and filtering step was substituted in place of the commercial filter cartridge to prevent shearing of larger DNA elements. In addition, all DNA elutions were carried out using an elution buffer or nuclease-free water heated to 65°C to increase yields of larger DNA elements. Extra-chromosomal DNA-enriched whole samples were then sent for NGS using a Roche-454 platform. Our approach allowed a temporal glimpse of the extra-chromosomal landscape and revealed the presence of a number of likely clinically relevant MGEs, including prophages and plasmids.¹

While our screening allowed easy identification of plasmids, directing NGS toward enriched extra-chromosomal DNA also uncovered likely lower-copy plasmidial prophage elements in the cell, as well as more “active” episomal phages mobilizing from the

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chromosome. For example, extra-chromosomal DNA sequencing of vancomycin-intermediate *S. aureus* (VISA) strain NRS19 yielded an extra-chromosomal prophage, ϕ BU01, encoding multiple virulence genes and with high homology to β -hemolysin (*hly*)-converting phages (those that integrate within and disrupt the β -hemolysin gene) that appeared plasmidially-maintained under our conditions.¹ Traditional chromosome-focused sequencing approaches would likely miss the extra-chromosomal nature of such prophages, and we believe that an extra-chromosomal sequencing approach such as ours, in parallel with chromosomal sequencing should help identify more MGEs and virulence determinants present in the cell and thus reveal a more complete virulence potential of an organism during characterization of clinical strains.

In addition to identifying lower copy MGEs, our extra-chromosomal NGS approach can lend insights into the temporal dynamics of mobile genetic elements within the cell. Episomal prophages enriched in our extra-chromosomal DNA preparations are those mobilizing at higher rates from the chromosome, and in poly-lysogenized strains, this approach allows identification of more “active” phages and provides a greater understanding of excision/integration dynamics. Such phage dynamics in Gram-positive bacteria can have major downstream effects on the host. For example, in *Streptococcus pyogenes* SF370, the attB site of SpyCIM1 (previously termed ϕ 370.4) is located within the mismatch repair (MMR) operon, and the phage-like element displays episomal activity; excising from the chromosome at low cell densities (logarithmic growth) and reintegrating in stationary phase. Excision yields a functional MMR operon, while phage integration disrupts transcription of the operon, and consequently increases the mutation rate of the host cell nearly 100-fold.^{2,3} Extra-chromosomal sequencing could serve as a useful approach to uncover such dynamics in the cell. For example, we uncovered an episomal prophage present in VISA NRS26,¹ and indeed, preliminary extra-chromosomal sequencing of *S. pyogenes* SF370 shows the predicted enrichment of the episomal element SpyCIM1 (data not published).

In *S. aureus*, excision/integration dynamics have shown important roles in infection. Goerke *et al.* examined *hly*-converting phages in *S. aureus* strains recovered from the lungs of cystic fibrosis and bacteremic patients, finding that they often integrated in off-target locations yielding a functional β -hemolysin

gene presumed to be advantageous for infection in the lung.⁴ Plasmidially-maintained rather than atypically integrated *hly*-converting phages would also allow for uninterrupted transcription of the β -hemolysin gene, creating a comparable subtype, but through a different mechanism. Similar to off-target phage integration, plasmidial prophage maintenance—in contrast to typical phage integration—can preclude disruption of host-encoded virulence factors (e.g., β -hemolysin), yet still allow the phage to positively-convert a cell with its own arsenal of virulence factors, after which the environment can select for cell subpopulations best suited for infection.

Importantly, we believe that the prophages uncovered using our extra-chromosomal DNA sequencing approach represent phages in a lysogenic rather than purely lytic state, and are excising from the chromosome and/or are plasmidially maintained in a manner distinct from spontaneous induction. In *S. aureus* NRS19, the clinical strain where ϕ BU01 was detected, culture supernatants did not produce clearing zones on a *S. aureus* RN4220 soft-agar lawn in the absence of mitomycin C treatment, while supernatants of mitomycin C-treated cultures produced plaques on the same reporter lawn.¹ Enrichment of prophage elements in the extra-chromosomal compartment solely due to lytic induction events suggests the production of phage particles in culture supernatants that would plaque a reporter lawn in the absence of external inducing agent. The lack of phage plaques from uninduced cultures in our assay indicates that this prophage is in a more lysogenic-like state. While we cannot fully rule out rare spontaneous induction into the lytic cycle as a mechanism for uncovering prophage elements in the extra-chromosomal compartment with our approach, we believe results thus far suggest the phage represented in our sequencing approach to be lysogenic rather than lytic. Future experiments to address this question could employ a quantitative PCR approach to examine extra-chromosomal phage copy-number and replication in such strains as well as relative expression of lytic genes or cro-like repressors to more fully ascertain whether the extra-chromosomal prophages we uncover are indeed lysogenic or lytic.

Regardless, our approach allows a distinction among strains in their “mobilization capacity.” Approximately half of the staphylococcal strains we examined did not appear to carry prophage elements in their extra-chromosomal compartment, however a

number of such strains produced viable phage after mitomycin C treatment, as examined by plaque formation and/or transmission electron microscopy.¹ While these strains did contain phage, they were undetected in the cytoplasm using our approach, likely because their prophages were firmly integrated and not maintained in the extra-chromosomal compartment. In some strains, prophage elements are excising from the chromosome at high rates, which can have important downstream biological effects, and in others, phage mobilization does not appear to occur to the same degree; directing NGS specifically to the extra-chromosomal compartment of the cell allows us to see these events in certain strains, and shows a relative lack of phage mobilization in others. In a recent review, Feiner *et al.* described various highly-excising prophages in a number of bacterial species whose excision occurs without spontaneous induction, terming such a phage-state as “active lysogeny.”⁵ We agree with the Authors’ perspective, and interpret the prophage elements identified in our extra-chromosomal sequencing of *S. aureus* clinical strains as likely “active lysogenic” prophages. One outstanding question for such extra-chromosomal prophage elements is how they ensure their own segregation into daughter cells, if maintained off the host chromosome. In our study, ϕ BU01 was shown to possess a putative DnaD protein-encoding gene,¹ and although its role in *S. aureus* phage replication is unclear, DnaD was shown to play an essential role in replication initiation in *Bacillus subtilis*.⁶ Future research should investigate how extra-chromosomal prophage elements successfully replicate and partition their genomes to secure dissemination throughout a population.

While our approach did enrich prophage and other DNA elements present in the extra-chromosomal compartment of the cell, utilizing the QIAGEN HiSpeed Midi Plasmid Kit does impart size limits of elements enriched (up to 50 kb). The majority of prophages infecting *S. aureus* are *Siphoviridae*, possessing a genome size of ~38–45 kb.⁷ It remains possible however, that a number of larger elements (e.g., large plasmids and large-genome *Myoviridae* phages) could have been missed using our approach, or may have been uncovered using alternate DNA-enrichment methods. The QIAGEN Large-Construct Kit offers purification of plasmids up to 250 kb; researchers interested in uncovering larger extra-chromosomal elements may prefer to

use this or comparable kits in combination with our technique, or alternatively, employ a cesium chloride (CsCl) gradient-based approach to isolate and enrich such DNA elements.

As previously described in this commentary, and covered more extensively in Feiner *et al.*,⁵ active lysogenic phages can have profound effects on the bacterial host cell, warranting their further study in both clinically-relevant and non-pathogenic species alike. For example, *Bacillus anthracis* isolates worldwide show almost identical chromosomal sequences containing 4 defective prophages, but many harbor a diverse array of inducible, functional phage by mitomycin C or fosfomycin treatment, suggesting the presence of plasmidially-maintained prophage elements exclusively in the cell’s extra-chromosomal compartment.^{8–11} We believe our extra-chromosomal DNA enrichment and sequencing approach is an extremely useful tool to identify a cell’s “complete” genomic profile that can be easily applied with minor modifications to species beyond *S. aureus*. Future avenues of research should also direct extra-chromosomal sequencing to compare single species’ MGE dynamics under different growth states and host environments, and their possible downstream effects both on the cellular and population levels. Prophages in particular could change between plasmidial, integrated and episomal states, leading to dramatic phenotypic changes and cell subtype selection dependent upon the host environment. Extra-chromosomal sequencing should serve to uncover a world of diverse lower-copy DNA elements—previously undetected but likely hidden in plain sight off the bacterial chromosome—enhancing our knowledge and appreciation of the roles phage play in bacterial survival, adaptation, and pathogenesis.

Abbreviations

<i>hly</i>	β -hemolysin
MGEs	mobile genetic elements
MMR	mismatch repair
NGS	next-generation sequencing
SpyCIM1	<i>Streptococcus pyogenes</i> chromosomal island M1
VISA	vancomycin-intermediate <i>Staphylococcus aureus</i>

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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