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REVIEW ARTICLE

Phage satellites and their emerging applications in biotechnology

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One sentence summary: Phage satellites are genetic elements with a modus vivendi linked to specific helper phages, which they hijack and exploit to propagate into new bacterial hosts. Their unique lifestyle and ability to exploit phages make them an attractive option for developing new biotechnologies, including gene delivery strategies and creating alternatives to antibiotics and phage therapy.

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ABSTRACT

The arms race between (bacterio)phages and their hosts is a recognised hot spot for genome evolution. Indeed, phages and their components have historically paved the way for many molecular biology techniques and biotech applications. Further exploration into their complex lifestyles has revealed that phages are often parasitised by distinct types of hyperparasitic mobile genetic elements. These so-called phage satellites exploit phages to ensure their own propagation and horizontal transfer into new bacterial hosts, and their prevalence and peculiar lifestyle has caught the attention of many researchers. Here, we review the parasite–host dynamics of the known phage satellites, their genomic organisation and their hijacking mechanisms. Finally, we discuss how these elements can be repurposed for diverse biotech applications, kindling a new catalogue of exciting tools for microbiology and synthetic biology.

Keywords: phage satellites; SynBio; mobile genetic elements; phage-inducible chromosomal islands; virulence; molecular piracy

PHAGE SATELLITES—WHAT MAKES THEM ORBIT?

Bacteriophages (phages) are key drivers of bacterial evolution due to their function as predators and vectors of horizontal gene transfer (HGT) between cells in a process known as transduction. They hijack their bacterial host's transcriptional and translational machinery to replicate, produce phage particles and either release their progeny by secretion (chronic life cycle) or by cell lysis (lytic life cycle). A group of phages, known as temperate phages, can follow an alternative life cycle, integrating and remaining quiescent in the chromosome instead

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of lysing their host. Once integrated, the phage is termed a prophage, and its reproductive success becomes tightly linked to that of its host because its genome is vertically inherited by host daughter cells (Clokie *et al.* 2011; Fortier and Sekulovic 2013).

Interestingly, some genomic islands share a similar architecture and encode genes with overlapping functions to those found on phages. A common feature of these integrative mobile genetic elements (MGEs) is that they often encode mobility genes of phage origin that enable them to adapt and disseminate (Frost *et al.* 2005). A subclass of these is termed phage satellites due to their intimate relationship with certain phages whose life cycle they parasitise for mobilisation (Fig. 1) (Nilssen *et al.* 1996; Tormo-Más *et al.* 2008; Novick, Christie and Penadés 2010). To date, their origin remains unknown, and different hypotheses have been proposed and debated (Christie and Dokland 2012; Martínez-Rubio *et al.* 2017; Dokland 2019; Sousa and Rocha 2021):

(i) The *de novo* evolution view suggests that a distinct type of MGE specialised to co-opt phage components to transfer horizontally.
(ii) The notion that primordial genomic islands evolved by acquiring genes that enabled their mobility and specialised hijacking of phages.
(iii) The regressive or reduction hypothesis suggests that these elements are the evolutionary result of cryptic phages that have lost genes encoding for structural proteins required for HGT mobilisation.

The commonality that brings the diverse group of phage satellites together is their reliance on piracy of a 'helper' phage to unwillingly pack the satellite DNA instead of phage DNA (Christie and Dokland 2012). These phage-like particles are typically recognised as native helper phage particles and frequently have capsids that are smaller in size (Dokland 2019).

Phage satellites likely provide an advantage for the bacterial host in some environments, given their ubiquity and selection during long-term evolution (Frígols *et al.* 2015; Cervera-Alamar *et al.* 2018). For example, many satellites encode virulence genes, toxins and other accessory genes that enhance their host's adaptation potential (Cucarella *et al.* 2001; Penadés *et al.* 2015). In addition, phage satellites can limit the propagation of their helper phage, granting some level of phage immunity at the population level (Ram *et al.* 2012; McKitterick *et al.* 2019a). This is comparable to abortive infection (Abi) response, and in some instances, satellites also encode Abi-like systems as part of their accessory genes (Fillol-Salom *et al.* 2020). Furthermore, phage satellites also mediate high transfer rates of chromosomal and satellite DNA (Úbeda *et al.* 2005; Ram *et al.* 2012). Compared with other transducing particles, phage satellites produce more particles and increase the rate of successful transduction events into subsequent viable host cells (Chiang, Penadés and Chen 2019).

The identification and understanding of phage satellites and their host range is rapidly expanding. Here, we review the known diversity, hijacking strategies and how they affect bacterial evolution, with the particular interest in highlighting their emerging potential in biotechnology.

THE DYNAMICS OF P4-LIKE SATELLITES

One of the first phage satellites ever described was identified in *Escherichia coli*, termed P4. The SOS response does not induce this satellite, and its piracy lifestyle depends on its helper phage P2 (Six and Klug 1973; Shore *et al.* 1978; Christie and Dokland 2012). P4 and P2 mutually induce each other through a strictly timed crosstalk between repressor and transcriptional activators. P2 exploitation can happen under different instances: (i) infection by P2 of a cell harbouring P4 that is either integrated or episomal (Briani *et al.* 2001), (ii) coinfection of a cell by both P2 and P4 (Diana *et al.* 1978) and (iii) P4 infection of a P2 lysogen (Liu, Renberg and Haggård-Ljungquist 1997).

In all circumstances, it is evident that P4 can manipulate P2 to optimise the production of viral particles containing the P4 DNA. The phage-encoded Cox protein binds to the P_{LL} region of P4, resulting in the expression of protein E, which then blocks the P2 master repressor C and increases the production of Cox (Saha, Haggård-Ljungquist and Nordström 1989). Activation of P_{LL} leads to the excision of P4 by a transcriptional regulator named Vis, which negatively regulates the *int* promoter and promotes the formation of the excision complex (Calì *et al.* 2004) (Fig. 1A). Remarkably, when the P2 helper is present as a prophage, P4 can derepress it to activate its piracy cycle during its circular form by expressing the E protein (Liu, Renberg and Haggård-Ljungquist 1998). P4 can redirect the assembly of the capsid by a second late promoter, which regulates transcription of Sid (size determination) and Psu (stabilising decoration protein), resulting in the constriction and hijacking of P2 capsids (Agarwal *et al.* 1990; Nilssen *et al.* 1996; Kizziah, Rodenburg and Dokland 2020). While Sid generates smaller-sized P4-containing particles to interfere with the production of P2 progeny, Psu only binds to the small capsids formed by Sid but does not impact their viability (Isaksen *et al.* 1992; Kizziah, Rodenburg and Dokland 2020). However, Psu and Sid share a strong similarity, suggesting that Sid's redirection function could have preceded the ability of Psu to bind to the capsids during the evolution of P4 (Isaksen *et al.* 1992; Kizziah, Rodenburg and Dokland 2020). P4 DNA packaging benefits from having the same *cos* signal found on P2. The *cos* site from P4 is recognised by the small terminase subunit (gpM) and then cleaved by the large (gpP) subunit of P2, while the formation of small capsids interferes with the packaging of P2 DNA (Bowden and Modrich 1985; Ziermann and Calendar 1990; Six *et al.* 1991).

P4-like elements constitute a widespread family of phage satellites distributed amongst *E. coli* strains and in various Gammaproteobacteria, including *Shigella* and *Salmonella* (Nilsson, Karlsson and Haggård-Ljungquist 2004; Rousset *et al.* 2021; Sousa and Rocha 2021). Although it was previously accepted that P4-like elements were unique due to their plasmid form, a recent study showed that these elements are not plasmids under normal physiological conditions and have a distinct lineage with little homology to phages and plasmids (Sousa and Rocha 2021). Interestingly, P4-like elements constitute a significant reservoir of antiphage defence systems that can target nonhelper phages, in contrast to satellite interference with helper phages (Rousset *et al.* 2021). In both P2-like and P4-like elements, the hot spots are adjacent to the *cos* site and located between *psu* and *int*. In P4, these typically appear shorter than those on P2 phages, showing evidence of the evolutionary pressure faced by P4 to maintain its smaller size to hijack the smaller capsids (Rousset *et al.* 2021). Overall, these systems influence the ecology and evolution of bacteria, phages and these satellites. Additionally, the diversification of the integrases and the defence systems carried by P4-like elements are likely due to frequent gene exchanges with other MGEs promoted by their horizontal mobility.

PHAGE-INDUCIBLE CHROMOSOMAL ISLANDS (PICIs)

Phage-inducible chromosomal islands (PICIs) are a widespread family of phage satellites initially investigated for their implications in disseminating virulence and toxin genes in bacterial populations (Penadés and Christie 2015; Martínez-Rubio *et al.*

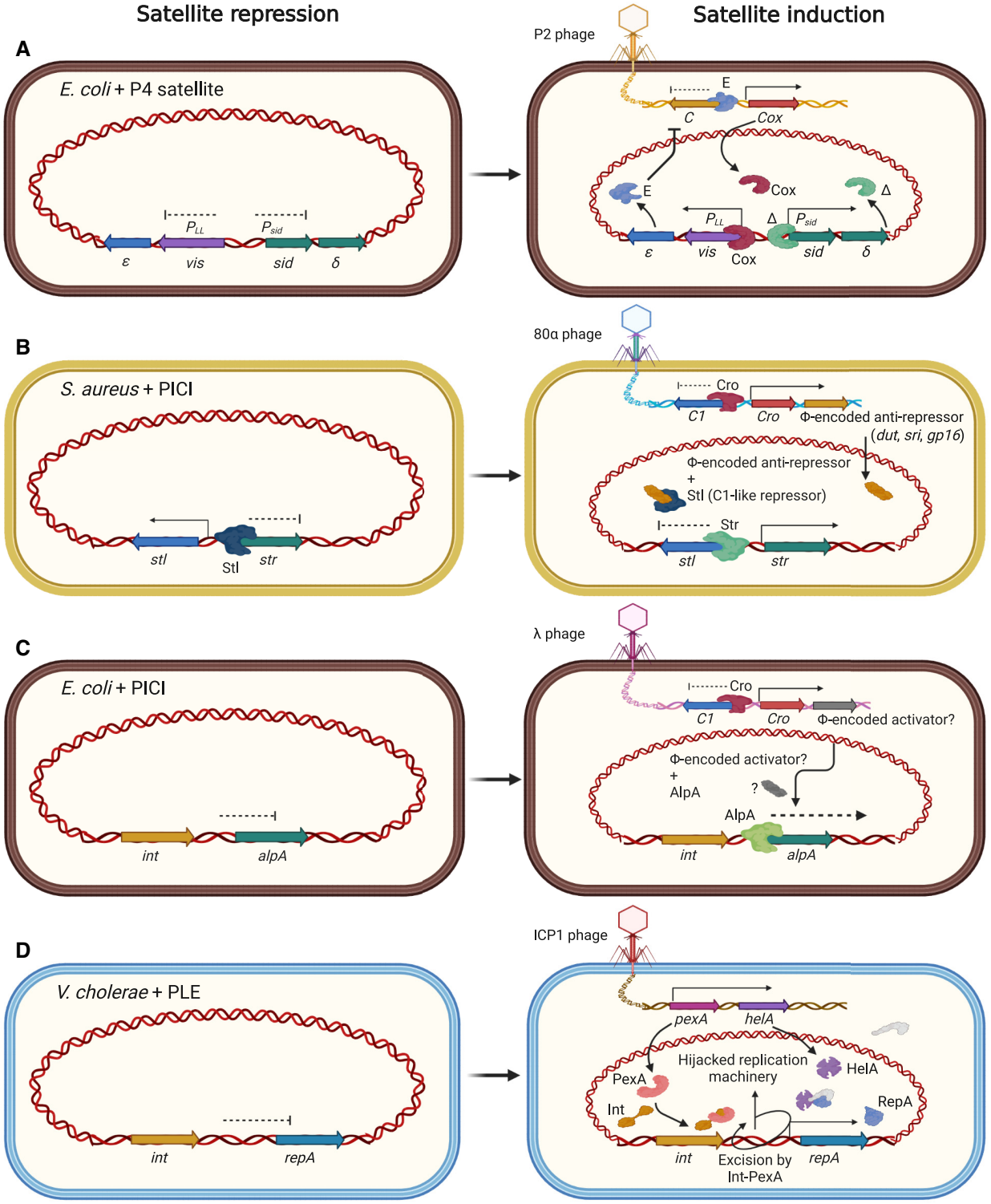

Figure 1. Parallel worlds, induction of phage satellites by helper phages. After infection or induction of a helper phage, a phage-encoded protein induces transcription of the satellite excision–replication–packaging cycle (ERP). **(A)** Transcription of the P4 satellite replication genes requires activation of P_{LL} by the P2 Cox protein. Vis and protein E are transcribed to excise P4 and block replication of P2. **(B)** For Gram-positive (G+) PICI (e.g. SaPI family), interaction with a ϕ-encoded anti-repressor (e.g. *dut*, *sri*, *gp16*) relieves StI-mediated repression of the *str* promoter, which then induces the PICI ERP cycle. **(C)** For the characterised Gram-negative (G−) PICIs (e.g. EcCI and PmCI families), induction requires the production of AlpA, which could be enhanced by a ϕ-encoded activator. **(D)** For the PLE family, infection by ICP1 leads to excision of PLE by interacting with the ϕ-encoded PexA as a recombination directionality factor with its cognate integrase. Excision of the PLE then promotes sequestering of the phage replication machinery by the initiation factor named RepA.

2017; Fillol-Salom *et al.* 2018). Many pathogenic strains harbour PICIs (Groisman and Ochman 1996). For instance, clinical isolates of *Staphylococcus aureus* are common carriers of PICIs encoding various biofilm and virulence genes (Fitzgerald *et al.* 2001; Dearborn and Dokland 2012; Cervera-Alamar *et al.* 2018). These genomic regions in *S. aureus*, denominated *S. aureus* pathogenicity islands (SaPIs), can be induced to replicate and encapsidated by different staphylococcal phages. The first discovered member of this family was a carrier of the toxic shock syndrome toxin-1 and initially termed *S. aureus* pathogenicity islands 1 (SaPI1) (Musser *et al.* 1990; Lindsay *et al.* 1998).

Today we understand the complex reproductive cycle of several PICIs, how they are linked to their helper phages and their ecological impact. This knowledge, provided by the study of SaPIs, has been the foundation for the subsequent search for SaPI-like elements across prokaryotic genomes. Together with SaPIs, the identification of these elements in other Gram-positive (G+) (Martínez-Rubio *et al.* 2017) and Gram-negative (G−) bacteria (Fillol-Salom *et al.* 2018) gave place to their redesignation as PICIs, given that not all encode known virulence factors and their taxonomic distribution extends beyond *S. aureus*.

In strains harbouring PICIs, infection by a helper phage or SOS-mediated induction of an endogenous helper prophage promotes excision and extensive replication of the PICI genome. Most PICIs follow a similar pattern for replication and packaging, but G− PICIs use a unique strategy to initiate their replication following induction by a phage-derived activator (Fillol-Salom *et al.* 2018). PICIs can coordinate their induction and parasitise conserved phage mechanisms, which allow them to respond to a range of different phage-encoded proteins. Once induced, they replicate autonomously and employ different strategies to manipulate the packaging machinery of the helper phage. In the process, the reproduction of the helper phage is severely impaired (Tormo-Más *et al.* 2008; Damle *et al.* 2012; Ram *et al.* 2014; Fillol-Salom *et al.* 2019). At this stage, phage-encoded capsid proteins are hijacked by the PICI to form phage-like particles. Conveniently, cell lysis is mediated by phage proteins, allowing the PICI infectious particles to burst out of the cell and transfer into new hosts. Noticeably, apart from the accessory PICI genes, their core genes are streamlined and almost exclusively encode proteins that ensure integration, induction, replication and the ability to hijack the morphogenetic program of helper phages. Thus, PICI piracy is efficient and results in high transfer frequencies and hence rapid dissemination of beneficial genes associated with pathogenicity, symbiosis, antibiotic resistance or phage defence (Schmidt and Hensel 2004; Frígols *et al.* 2015).

Genomic organisation and induction of PICIs

SaPIs are the best-characterised members of the PICI family. In general, their genetic architecture is similar to that of phages, with a core genome formed by induction, integration-excision, replication, packaging and accessory modules. They harbour homologues to phage genes such as the integrase (*int*), excisionase (*xis*), primase (*pri*), replication initiator (*rep*), origin of replication (*ori*) and, for some types, small terminase subunit (*terS*). Their genomes are approximately one-third of the size of their cognate helper phage genome (~15 kb), carrying genes involved in virulence, host adaptation, antibiotic resistance, and biofilm formation (Penadés and Christie 2015; Martínez-Rubio *et al.* 2017; Novick and Ram 2017; Fillol-Salom *et al.* 2018). Broadly, the genomic organisation of other PICIs also follows a modular structure found in SaPIs, where each module is involved in a particular function of their life cycle. Moreover, SaPI genomes are typically flanked by *attL* and *attR* sites involved in site-specific integration at the matching *attC* site on the *S. aureus* chromosome. Previously, these sequences were thought to be exclusive to phages, but it is now known that each MGE has unique *attC* sites, and these are distinct from those used by phages to integrate into the bacterial genome (Maiques *et al.* 2007; Chen *et al.* 2014). SaPIs are found at five different attachment sites; those in the same site are often more closely related than those in other sites (Chen *et al.* 2014; Novick and Ram 2016). They can be induced by diverse phage-derived anti-repressors (Bowring *et al.* 2017). Once the helper phage is induced and the phage-derived anti-repressor has sequestered the SaPI master repressor, the excision–replication–packaging (ERP) cycle is activated. Furthermore, studies have shown that SaPIs/PICIs are present within a wide range of staphylococcal species and that these elements transfer intra- and inter-specifically, an aspect that we discuss later in this review. In *S. epidermidis*, pathogenicity and resistance islands (SePI and SeRI, respectively) resemble the genomic organisation of SaPIs (Chen *et al.* 2013), and the transfer of SeCISE48 by a *S. epidermidis* temperate phages was recently reported (Fišarová *et al.* 2021).

The regulation of SaPI gene transcription is controlled by the *stl* and *str* gene products encoded in opposite directions flanking a divergent promoter region. Stl acts as the master regulator, repressing the transcription of genes involved in excision and replication of the satellite DNA and maintaining its stable integration by binding to the promoter regions between *stl* and *str*; thus, preventing transcription of *str* and downstream genes (Fig. 1B) (Úbeda *et al.* 2008; Tormo-Más *et al.* 2010). Although the SaPI Stl functions as an analogue of the C1 repressor found in *E. coli* phage λ and *S. aureus* phage 80α (Benson and Youderian 1989; Christie *et al.* 2010), it is SOS-insensitive and requires interaction with a phage-derived protein. Genomic analysis of *Enterococcus faecalis* strain V583 provided evidence that not only do Stl-homologues exist in other G+ species but they also share a similar regulatory module to SaPIs, where a PICI-encoded homologue of Stl (Rpr) is SOS-insensitive and can be derepressed by, e.g. the phage-encoded *xis* on *E. faecalis* phage ϕ1 (Matos *et al.* 2013; Martínez-Rubio *et al.* 2017). Further analysis led to the discovery of a variety of G+ PICIs in *Streptococcus pyogenes* (SpyCIM1) (Scott *et al.* 2012) *S. suis* (SsuCI-TL13), *S. oligofermentans* (SolCIAS1.3089-0.6), *S. pneumoniae* (SpnCIST556) (Nguyen and McShan 2014) and *Lactococcus lactis* (LlCIbIL311, LlCIbIL312, LlCIbIL313) (Chopin *et al.* 2001; Martínez-Rubio *et al.* 2017).

Recently, it was reported that PICI elements are also present in several G− bacteria (Fillol-Salom *et al.* 2018). Their identification was facilitated by the unique features they share, such as exclusive integration *attC* sites, replication origins, genes involved in capsid remodelling, interference with prophages and accessory fitness traits. Interestingly, the majority of these elements partially share the *cos* packaging sites (specifically the *cos*Q and *cos*N) of their helper phages compared with the P4 satellite and SaPIbov5 that have the same *cos* site as their helper phages (Viana *et al.* 2010; Quiles-Puchalt *et al.* 2014). Using the *E. coli* PICI EcCICFT073 and *Pasteurella multocida* PICI PmCI172, it was determined that G− PICIs use a different activation strategy to exploit the life cycle of their helper phage. They use a DNA-binding transcriptional activator (AlpA) whose activity is promoted by helper phage induction. In contrast to the P4 satellite that encodes the AlpA-homologue, Vis, AlpA does not control the expression of *int*. Furthermore, the G− PICIs studied to date cannot induce their helper phage as the P4 does. Once induced, their transduction appears to be exclusively unidirectional and AlpA drives the ERP cycle of the PICI independent of

the SOS-response (Fig. 1C) (Fillol-Salom *et al.* 2018). They do not contain an interference packaging module like the SaPI Operon I, but use a different strategy to hijack the phage machinery. It is intriguing as to why most G− PICIs would employ a different induction strategy, and one could argue that this is due to the evolutionary pressure of hijacking the higher number of *cos*-type phages found in G− bacteria. In principle, the PICI transcriptional organisation suggests that their unique features have undergone strong selection to the point that they represent coherent clades that have diverged from and evolved in parallel with phages and are spread widely among diverse bacterial genera. Despite similarities with the P4-like element, their divergent piracy strategy suggests that they could represent a separate evolutionary branch in which P4-like elements evolved from an ancestral element with modules for capsid redirection, and later acquired phage exploitation and antiphage defence mechanisms (Briani *et al.* 2001; Calì *et al.* 2004; Dokland 2019), while G− PICIs generated *de novo* or evolved from horizontal transfer of helper phages.

Helper phage piracy and packaging

The replication module is composed of the *ori*, *pri* and *rep* genes that are highly conserved between the different PICI families (Christie and Dokland 2012; Penadés and Christie 2015; Dokland 2019). Noticeably, the *ori* has a unique structure flanked by AT- rich regions, which differs from the *ori* of phages. These regions are specific binding sites for the PICI-encoded replicase/helicase, which initiate replication of the PICI DNA. The coordinated expression of *int* and *xis* enables the cleavage of *attL* and *attR* sites in a manner that ensures circularisation of the excised PICI DNA followed by its replication (Mir-Sanchis *et al.* 2012; Martínez-Rubio *et al.* 2017; Fillol-Salom *et al.* 2018).

Studies have revealed that different phage-encoded inducers influence numerous functions of the PICI development, such as signalling pathway modulation and induction (Tormo-Más *et al.* 2010). A diverse set of master repressors controls the life cycle of SaPIs and each of these interact with (a) specific phage-encoded anti-repressor(s). Many of these anti-repressors are categorised as moonlight proteins, where they have one function for the phage and a separate function in the context of PICI induction. For example, phage 80α contains four different moonlight proteins, which serve as individual SaPI anti-repressors (Ram *et al.* 2014). The Sri protein induces SaPI1 and SaPIpT1028 (Tormo-Más *et al.* 2010), the ORF15 gene product induces SaPIbov2 (Tormo-Más *et al.* 2010), and the phage-encoded recombinase Sak4 induces SaPI2 (Neamah *et al.* 2017). The phage trimeric dUTPase in ϕ11 and dimeric dUTPase in ϕNM1 can induce SaPIbov1 and SaPIbov5 (Hill and Dokland 2016; Donderis *et al.* 2017). These genes are encoded by several phages such as ϕNM1 (inducing SaPI1, SaPIbov1 and SaPIbov5), ϕ80 (inducing SaPIbov1 and SaPI2), among others (Penadés and Christie 2015).

Most SaPIs contain an operon, denoted as operon I, composed of interference genes and morphogenesis genes that redirect the assembly of the helper phage procapsids to produce smaller capsids, which can only accommodate the smaller SaPI genome (Poliakov *et al.* 2008; Damle *et al.* 2012). Given that operon I is repressed by LexA, expression of this gene cluster can be initiated by the SOS-response, in contrast to SaPI induction (Úbeda *et al.* 2007). Although there is variation in this operon among SaPIs, most of them have a conserved interference mechanism. Lastly, SaPIs often encode accessory genes at the extreme ends (Penadés *et al.* 2015; Novick and Ram 2017). Similar to operon I, clusters encoding Abi-like systems and virulence factors possess their own promoters and do not require induction of the island, which opens many possibilities to adapt new mechanisms to evolve against harsh environments and enable defence against nonhelper phages (Frígols *et al.* 2015; Fillol-Salom *et al.* 2020).

In G− bacteria, PICIs like the EcCICFT073 can be induced by their resident prophages in the *E. coli* strain CFT073. Interestingly, infection by *E. coli* lambdoid phage ϕ80 and phage λ can induce this PICI, and the production of PICI particles is higher than for induction by the native prophages (Fillol-Salom *et al.* 2018). The lower efficiency in prophage induction suggests that the variation between phage-encoded inducers is likely a result of the constant evolutionary pressure from the phage to overcome PICI induction since it inherently reduces phage transfer (Tormo-Más *et al.* 2008; Bowring *et al.* 2017). How G− PICIs drive the expression of AlpA and which phage-encoded proteins they employ remain to be elucidated.

A remarkable piracy strategy employed by PICIs is their ability to modulate the phage-encoded packaging machinery. Once induced, PICIs redirect the size of the capsid, interfering with the packaging of the phage (Poliakov *et al.* 2008; Damle *et al.* 2012; Ram *et al.* 2012) and even modulating phage late gene expression (Ram *et al.* 2014). Like a Trojan horse, PICIs are disguised in the phage capsids to spread horizontally into new host cells. Like their helper phages, PICIs employ one of two mechanisms, *pac* or *cos*, to pack their concatemeric dsDNA. With *pac* phages, this occurs in a headful manner, whilst *cos* phages are packaged in unit lengths. Normally, the phage-encoded terminase TerS-TerL complex (TerSφ and TerLφ) is used to recognise a specific *pac* or *cos* sequence in the phage genome. This recognition is mediated by TerSφ, which binds to the site required and allows TerLφ to cleave and translocate the phage DNA into the capsid. In PICIs, the packaging is mediated differently in both *cos* and *pac* types (Penadés *et al.* 2015). G+ PICIs with *pac* sites possess a different *pac* sequence from their helper phages and therefore encode their own small terminase subunit (TerS$_{SP}$), which can interact with TerLφ to form a TerS$_{SP}$-TerLφ complex that preferentially packs the concatemeric dsDNA of the PICI. The formation of this complex is promoted by the phage packaging interference protein (Ppi), which binds to the TerSφ and blocks its DNA recognition site (Fig. 2A) (Tormo-Más *et al.* 2008). This strategy seems highly conserved amongst G+ PICIs since homologues of Ppi have also been identified in other G+ bacteria (Ram *et al.* 2012). Alternatively, some SaPIs favour packaging into smaller capsids by using capsid morphogenesis proteins (CpmA and CpmB) (Fig. 2A) (Dearborn *et al.* 2011, 2017; Damle *et al.* 2012). SaPI1 and SaPIbov1 carry *cpmA* and *cpmB*, whilst SaPIbov2 and SaPIpT1028 only carry homologues to *cpmB* and SaPIbov2 does not produce small capsids. Despite this, such SaPIs can be transferred at high frequencies by utilising phage-sized particles (Ram *et al.* 2012; Carpena *et al.* 2016). PICIs found in *P. multocida* are also known to produce functional capsid size remodelling proteins, which strongly suggests that this strategy is widespread and utilised by satellites found in G− bacteria as well (Fillol-Salom *et al.* 2018). Therefore, capsid size redirection is not just a feature of SaPI biology, but a mechanism of phage interference employed by all phage satellite families described to date. However, the proteins responsible for capsid redirection and morphogenesis share no detectable homology, underscoring that this strategy is a convergent evolutionary process characteristic of most PICI elements, which in turn defines the size of these elements and contributes towards their highly successful lifestyle.

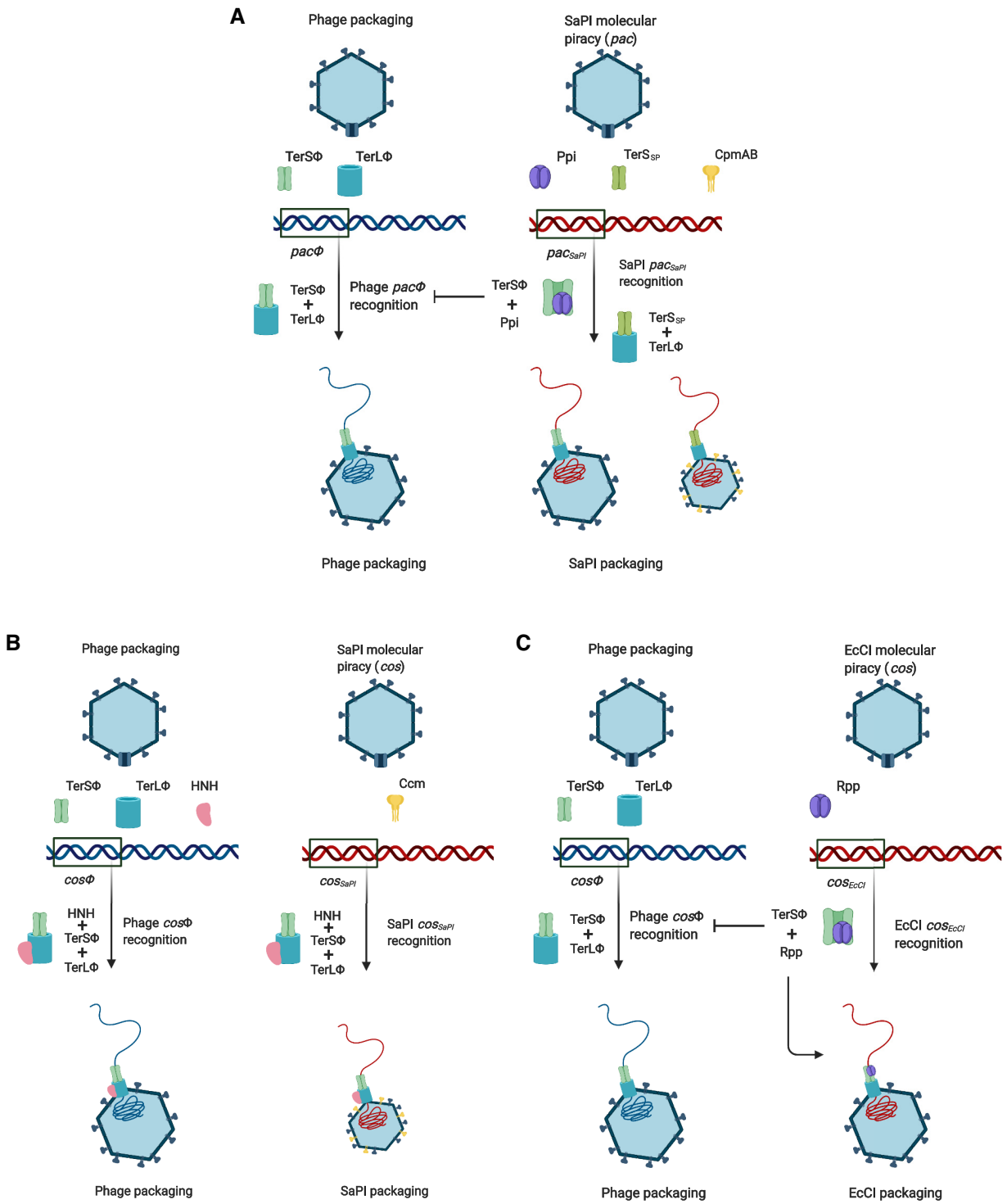

Figure 2. Packaging and piracy strategies of PICIs. **(A)** SaPI helper exploitation and piracy have been established as the model for *pac* type G+ PICIs. The SaPI-encoded Ppi binds to the TerSφ, directly interfering with the packaging of phage DNA mediated by the formation of TerSφ-TerLφ complex to recognise the phage *pac* site. The TerS$_{SP}$ hijacks the TerLφ to generate a hybrid terminase enzyme that recognises the SaPI *pac* site. The CpmAB proteins redirect the capsid assembly, resulting in the formation of smaller viral particles to pack the smaller SaPI DNA and contribute to the interference of phage packaging. **(B)** Likewise, PICIs can employ a *cos* type packaging as their helper phages. The phage-encoded HNH-TerSφ-TerLφ endonuclease complex is used to pack both phage and SaPI DNA into preformed capsids by cleavage of their respective *cos* sites. The Ccm protein redirects capsid assembly, interfering with phage DNA packaging. **(C)** The G- PICIs, like the EcCI family, employ a *cos* type packaging utilising the Rpp protein to block phage DNA packaging. The Rpp binds to TerSφ, forming a heterocomplex that hijacks the packaging machinery to recognise the PICI *cos* site and DNA encapsidation.

In the case of *cos* PICIs in G+ bacteria, the phage-encoded HNH nuclease-TerS-TerL complex promotes the recognition of the PICI *cos* site. In SaPIs, packaging of their dsDNA is promoted by capsid size modulation using a *cos* capsid morphogenesis protein (Ccm) (Fig. 2B) (Quiles-Puchalt *et al.* 2014; Carpena *et al.* 2016). In the prototype G− PICI, EcCICFT073, this seems unlikely since the PICI does not possess proteins with similar function to Ccm nor produces smaller capsids. Instead, EcCICFT073 possesses a protein with similar functionality to Ppi, named Rpp (redirecting phage packaging), which interacts with the phage TerS to form a heterocomplex. This heterocomplex is redirected to the *cos*B site located on the PICI genome and thus promotes PICI packaging while at the same time preventing the phage DNA from packaging. (Fig. 2C). Different *rpp* homologues have been found in other *E. coli* PICIs and those from different G− bacterial species (Fillol-Salom *et al.* 2019). Interactions between the *E. coli* Rpp (from EcCIEC2733.1) and a *Pluralibacter gergoviae* phage-encoded terminase, and *P. gergoviae* Rpp with the λ TerS revealed that these proteins have a conserved and widespread strategy for hijacking the phage packaging machinery. In contrast to SaPIs and their Ppi and TerS proteins, these PICIs only require Rpp to achieve interference and redirection of DNA packaging (Fillol-Salom *et al.* 2019). Although these types of G− PICIs lack the capsid morphogenesis genes carried by most SaPIs, there is a major capsid gene present, suggesting that EcCICFT073 might produce smaller capsids with another helper phage (Fillol-Salom *et al.* 2018, 2019).

Remarkably, some G+ PICIs, such as SaPIbov5, can be packaged by both *pac*- and *cos*-type phages (Viana *et al.* 2010; Quiles-Puchalt *et al.* 2014). SaPIbov5 can exploit *pac*-type phages but cannot remodel their capsids since Ccm only remodels *cos*-type helper phages (Carpena *et al.* 2016; Dokland 2019). It is evident that they possess a Ppi homologue and *pac* sites; however, the mechanism of how *pac*-type phages support transduction of SaPIbov5 remains unsolved (Carpena *et al.* 2016). Since both types of packaging are employed by G− and G+ phages, it would not be surprising to also discover these dual-hijacking PICIs in the G− genera. While some G− PICIs possessing a *ter*S have been identified in *E. coli* (Fillol-Salom *et al.* 2018), the molecular mechanism for hijacking the phage packaging machinery needs further characterisation.

Intra- and interspecies transduction of PICIs

The host range and reproductive success of a phage depend on several steps. In brief, the phage needs to adsorb to an appropriate receptor, inject its nucleic acid, hijack the transcriptional and translational machinery, replicate, produce and package capsids and finally lyse the host. Since these steps occur sequentially, the host range narrows stepwise and that the earlier steps of infection have a higher success rate than the latter. The detection of transfer has relied on the replication of the phage as seen by plaque formation; however, phage-mediated transfer has proven to not be limited by this and results have shown that DNA can be transduced silently to other genera without generating plaques (Chen and Novick 2009; Chen *et al.* 2015a).

Although the successful transfer of PICIs relies on helper phages as vehicles, the host range is not inextricably linked to the phage host range (Chen and Novick 2009; Chen *et al.* 2015a). Numerous host defence mechanisms [e.g. restriction-modification (RM) systems or CRISPR-Cas] have evolved to hinder the acquisition of foreign nucleic acids (Bernheim and Sorek 2020). Thus, successful transfer depends on (i) not being targeted by—or the ability to evade or repress—host defences, which tend to vary even between closely related strains (Howard-Varona *et al.* 2018) and (ii) whether another MGE already occupies the attachment site or excludes integration (Maiques *et al.* 2007). In addition, to persist in a given population, PICIs are required to either parasitise the host or provide a benefit in a given environment, thereby lowering the fitness cost they may impose (Koonin *et al.* 2020). In general, PICIs transfer to a wider range of hosts than their cognate helper phage is able to propagate within. A clear example are SaPIs, which transfer to other Staphylococci with high efficiency (Maiques *et al.* 2007; Chen *et al.* 2013, 2015b), but also cross the genus barrier and successfully integrate into the genome of *Listeria monocytogenes* (Chen and Novick 2009; Chen *et al.* 2015b). This indicates that phages frequently inject genetic material into species distant from their native host, which they are not able to integrate or reproduce, but where the PICI succeeds to integrate and is unharmed by the host defences (Chen and Novick 2009; Chen *et al.* 2015a).

Transfer of phage satellites also takes place through generalised and lateral transduction. While generalised transduction can occur at lower frequencies through random packaging of the satellite DNA, it can also take place by hijacking phages that do not encode for any PICI anti-repressors and hence does not initiate the ERP cycle of the satellite. In particular, SaPIs can still be transferred after activation of the SOS response, where the operon I is derepressed in parallel to the induction of a prophage, expressing the SaPI-encoded TerS ($TerS_{SP}$) and genes involved in interference. These proteins mediate mispackaging of the phage capsid, recognising *pac* sequences that are transferred without induction of the SaPI itself (Maiques *et al.* 2007; Mir-Sanchis *et al.* 2012; Bento *et al.* 2014). This further contributes to generalised transduction of chromosomal host DNA as $TerS_{SP}$ recognises *pseudo pac* sites (*ppac*) distributed across the chromosome as well (Chen *et al.* 2015b). Consequently, $TerS_{SP}$ binds to *pac* and *ppac* sites to direct packaging and cleavage of these regions when forming the $TerS_{SP}$-$TerL_{\varphi}$ complex, resulting in the production of SaPI-sized particles that contain bacterial DNA fragments (Frígols *et al.* 2015). Interestingly, several SaPIs can be identified downstream of prophages that undergo late excision in their induction process. This has led to the assumption that lateral transduction might also play a major role in the transfer of these chromosomal islands (Chen *et al.* 2018). Accordingly, it can be hypothesised that SaPI packaging could drive bacterial DNA to be packaged into transducing particles formed by the lateral transduction mechanisms (Chiang, Penadés and Chen 2019).

Given the successful lifestyle of SaPIs and their ability to integrate into alternative sites to their cognate *attC* site in other species, it would not be surprising to find evolutionary strategies for other PICIs to transfer into closely related bacteria and potentially exploit the HGT driven by their new host phages to adapt and mobilise accessory genes.

THE PICI-LIKE ELEMENTS (PLEs)

A family of genomic islands found in *V. cholerae* are induced by infection of the ICP1 phage, a virulent myophage isolated from the faecal samples of cholera patients (Seed *et al.* 2011). Similar to PICIs, these ∼18 kb integrated genetic elements interfere with and exploit the helper phage's life cycle to promote their own reproduction and horizontal dissemination. These phage satellites are referred to as PICI-like elements (PLEs) because certain aspects of the PLE life cycle differ substantially from that of PICIs (Seed *et al.* 2013; McKitterick and Seed 2018; Barth *et al.* 2020a).

In contrast to the temperate nature of the large majority of the phages exploited by known phage satellites, ICP1 is strictly

lytic (Seed *et al*. 2011). Furthermore, while P4 and PICIs only interfere partially with the infectivity/reproduction of their helper phages (Diana *et al*. 1978; Úbeda *et al*. 2007; Ram *et al*. 2012), PLEs completely abrogate the production of ICP1 progeny (O'Hara *et al*. 2017). Another distinctive characteristic of PLEs is that they do not perform their own replication and are thus thought to redirect the required machinery from ICP1 using an encoded initiation factor (RepA) and sequestering a phage-encoded helicase (HelA) (Fig. 1D) (Barth *et al*. 2020b). PLEs parasitise ICP1 particles for transduction by constructing smaller and apparently fewer stable capsids (~30 nm smaller than ICP1), and encode a novel DNA binding protein, CapR, that represses ICP1's capsid morphogenesis operon (Netter *et al*. 2021). Lastly, horizontal mobilisation of PLEs via helper phage transduction appears to be a comparatively infrequent phenomenon (<1 effective transduction event per ICP1-infected cell) (Barth *et al*. 2020b). The low transduction frequency appears to stem from the function of the PLE-encoded gene *lidI*, an inhibitor of the ICP1-programmed delay of host cell lysis that triggers an accelerated killing of infected cells before viral particles can be accumulated and released in the population. A similar strategy has been reported for prophages of diverse Enterobacteria, which confer protection against other phages via the expression of BstA, a protein that triggers an abortive infection response (Owen *et al*. 2020). The abortive infection phenotype provoked by PLEs and their low transduction frequencies has led to the hypothesis that the role of PLEs is primarily associated with phage defence. This is consistent with the notion that, while PLEs may once have emerged as phage parasites, in the face of ICP1 they have been evolutionarily selected to form part of the host's antiviral defence arsenal (Seed *et al*. 2013; O'Hara *et al*. 2017).

In response to the strong selective pressure exerted by PLEs, around half of all ICP1 isolates are found to carry a type I-F CRISPR-Cas system programmed for sequence-specific destruction of the host PLE (Seed *et al*. 2013; McKitterick and Seed 2018). Armed with such a weapon, ICP1 phages can not only overcome the antiviral activity of PLEs but also update their anti-PLE CRISPR memory bank during infection (McKitterick *et al*. 2019b). Interestingly, the targeting of chromosomal regions proximal to integrated PLEs are advantageous to ICP1 due to the high processivity of the Cas2-3f helicase-exonuclease that can translocate and degrade the PLE genome before its excision (McKitterick *et al*. 2019b).

Some ICP1(+) isolates that lack the CRISPR-Cas system encode an endonuclease in the same locus, providing ICP1 with immunity to a subset of PLEs (Barth, Nguyen and Seed 2021). This ICP1-encoded nuclease (Odn) targets the PLE origin of replication and cleaves the DNA proximal to that site. Interestingly, Odn equips ICP1 phages with extra DNA, which is otherwise taken up by the bulkier I-F CRISPR-Cas system, to adopt other auxiliary genes encoding strategies to overcome PLE interference. Mutations in the *ori* can render PLE resistant to Odn, suggesting that this anti-PLE strategy lacks the flexibility provided by CRISPR adaptation but provides more reliable interference against a subset of PLE variants (McKitterick *et al*. 2019b; Barth, Nguyen and Seed 2021). Evidently, this satellite and its virus are engaged in a coevolutionary arms race where the infection outcomes differ depending on the pairings of PLEs and ICP1. Also, PLEs occur in isolation within *V. cholerae*, where typically one dominates for a time before being succeeded by a new PLE. Therefore, suggesting a degree of competition between PLEs or that perhaps their selection is host driven, depending on whether they confer benefit in a given environment.

CURRENT AND POTENTIAL APPLICATIONS

Many techniques and advancements in molecular biology have emerged from repurposing biological parts of phage genomes, and in some situations, researchers have opted to use phages as a genetic engineering tool for bacteria (Lemire, Yehl and Lu 2018; Lammens, Nikel and Lavigne 2020; Marino *et al*. 2020). Restriction enzymes, recombinases and transcriptional regulators have become the basis of many molecular cloning techniques widely used in laboratories to date. Phage display is used to screen, select and produce large libraries of proteins (such as antibodies) by encoding a specific peptide fused to a phage coat protein on the surface of the phage (Pande, Szewczyk and Grover 2010; Anand *et al*. 2021). Moreover, phages are used as vehicles for delivering both DNA and proteins, for detecting pathogenic bacterial strains, and as a therapy to treat infections by specifically killing a particular species of bacteria (Meile *et al*. 2020; Fage, Lemire and Moineau 2021). Here we discuss the current and potential use of the described phage satellites for applications in molecular biotechnology, summarised in Table 1.

Genetic switches and recombination tools

In synthetic biology, the engineering of novel signalling systems able to sense, process and transmit information is a major challenge. Most research is based on the use of known protein domains with specific interactions; however, to precisely program a biological system, novel parts and complex logical gates are needed to avoid cross-reactivity with other molecules (Lammens, Nikel and Lavigne 2020).

Some phage satellites share high similarities with the transcription regulatory modules of their helper phages. The most well-described example, phage λ, employs a genetic switch where most of its genes, except that encoding the C1 repressor, are turned off. The C1-Cro switch has been widely used in synthetic biology due to its well-characterised function (Ptashne 2004; Hochschild and Lewis 2009). This genetic switch has, for example, been employed as an oscillator and has been mutagenised to create artificial orthogonal repressor/promoter pairs (Elowitz and Leibier 2000; Brödel, Jaramillo and Isalan 2016), functioning as a kill switch (Stirling *et al*. 2017) or enabling a pulse-detecting circuit (Kotula *et al*. 2014). As one of the few phage regulatory circuits exploited, researchers have proposed employing a larger range from distinct phage families and from different bacterial species for other model organisms (Lammens, Nikel and Lavigne 2020).

Indeed, phage transcriptional regulators offer a vast collection for synthetic gene circuits. However, PICI transcriptional regulators offer a distinct variety that are independent of SOS-response and can use numerous nonessential phage proteins to derepress and tightly regulate expression. Analogous to phages, the stable integration of most G+ PICIs in the chromosome is maintained by a master repressor (Stl in the case of SaPIs) (Fig. 1B). Similar to C1-Cro switches, the Stl-anti-repressor switch could be used to monitor cellular signals connected to a Str promoter which upon sensing can derepress Stl for the activation of a reporter encoding, for example, a fluorescent protein. An additional usage of this switch is the moonlight property for some of its anti-repressors (Fig. 3A). For example, a Stl-based switch from SaPIbov1 could be employed to study and monitor the role of dUTPases (Dut) in DNA uracilation (Vértessy and Tóth 2009) and virus infectivity in mammalian cells (Ariza, Glaser and Williams 2014). Similarly, the Stl switch of SaPI2 could also be used to monitor recombination events involving *sak* and

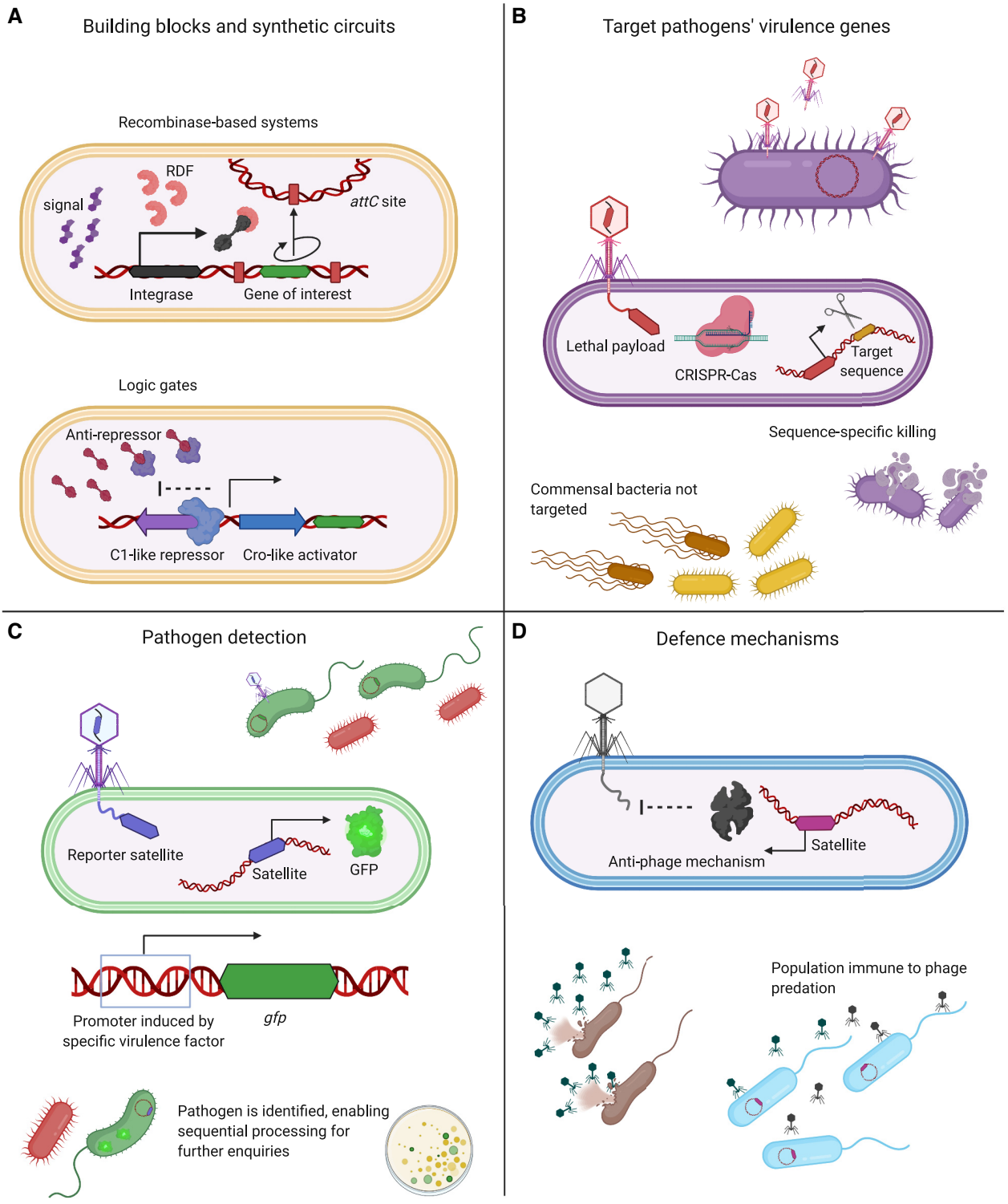

Figure 3. Biotechnology applications of phage satellites. Phage satellites show potential to be used as: **(A)** building blocks and synthetic gene circuits, where their components can be repurposed for genome engineering and translational processes; **(B)** targeted therapy, where satellites enable the delivery of lethal payloads such as lysins or CRISPR-Cas to kill a pathogen containing a specific virulence genotype without harming the surrounding microbiota; **(C)** pathogen detection, exploiting their ability to disseminate rapidly and across different species to detect bacterial species and monitor the upregulation/acquisition of virulence traits; **(D)** defence mechanisms, repurposing their interference components to stop predation from phages and ensure the protection of bacterial cultures used in several industries. Their antiphage modules could also be employed in phage therapy to minimise the impact on the microbiota and avoid dissemination/activation of virulent genes.

Table 1. Applications of phage satellites.

Application	Description	Key advantages	Key limitations
Genome rebooting	Design/manipulation *in vitro* or in yeast enables easy insertions, deletions and/or modifications of synthetic DNA that can be reactivated in a host	Assembly in different platforms employing selection markers	Transfer efficiency varies between taxa
		Smaller genomes facilitate rebooting	Helper phage needed for propagation in the rebooting host
Gene insertion or expansion	Addition of genes into the bacterial chromosome by satellite integration	Can carry large segments (>20 kb) for synthetic gene delivery	Dependent on capsid size, the packaging mechanism and size of helper phage
Host expansion	Adaptation and designs that alter the span of host range of the infecting phage	Tail fibre exchange and domain shuffling of receptor binding proteins (RBPs) through complementation on satellites Can employ multiple phage capsids that can be engineered or identified separately	Helper phage tail fibres, RBPs and receptors need to be well characterised to facilitate manipulation
Program host specificity	Alter specific factors for host-specific injection and satellite integration	Achievable by high-throughput RBP diversification on helper phage Monitor evolution of host specificity by transduction of markers with specific promoters to the host	Unexplored; however, satellites are known to transfer intra- and interspecifically
Antiphage defence	Ability to interfere with the reproduction of phages, mediated by different immunity and interference mechanisms	Satellites can be combined with encoded defence systems such as CRISPR-Cas for wide range protection	Phages could counter-evolve to circumvent the protection
Antimicrobials and antivirulence	Cell death and/or inhibition of virulence to prevent further spread of infection and pathogenicity	Payload delivery specifically targets virulence factors. Use of CRISPR-Cas systems would be considered nucleic-acid-based antimicrobials and not biological entities Delivery by element or phagemid Proteins are produced continuously upon integration Can be designed to lyse or manipulate host properties	Single dose since it requires a helper phage to reproduce and mobilise to other cells
Biosensors	The satellite is used to generate a signal that can be accumulated and read by an interphase	Detection of host by host-produced signal without lysis	Bacterial growth is required to use this approach
		Recovery of the host cell allows sequential interrogation	Signals rely on promoter strength since it cannot amplify by typical phage reproduction

ssb recombinases, an interesting approach to detect phage infection without using the plaque formation method (Neamah *et al.* 2017).

DNA modification is an essential part of the phage life cycle and an attractive application for synthetic biology. The capacity to generate a change in DNA sequences without DNA degradation, synthesis or relying on repairing mechanisms is a particular feature of site-specific recombinases. Both temperate phages and phage satellites often integrate at specific *attC* sites and rely on efficient integrases. Thus, they can be reprogrammed for integration, displacement, and deletion events in the host bacterial chromosome (Fogg *et al.* 2014; Menouni *et al.* 2015; Krishnamurthy *et al.* 2016). In synthetic biology, serine integrases have been established as versatile tools for *in vitro* and *in vivo* modifications in various simple and complex organisms due to their directionality and simple requirement to catalyse

recombination between an *attC* site on linear or circular DNA templates (Merrick, Zhao and Rosser 2018). This can be reversed using an accessory protein that functions as a recombination directionality factor (RDF) to excise the DNA from the allocated *attC* site. Other types of integrases, such as tyrosine integrases, have been employed effectively for gene editing (Gateway cloning method), but these have major limitations compared with serine integrases, which do not need a host-encoded integration factor and large *attC* sites to catalyse their basic function (Fogg *et al.* 2014). Most phage satellites employ tyrosine integrases, but PLEs possess an exemplar mechanism where they exploit a phage-encoded protein (PexA) as the RDF of their serine integrase to catalyse the excision of their DNA (Fig. 1D) (McKitterick and Seed 2018). This is the first instance where a constitutively expressed integrase can react upon a foreign RDF, since serine integrases such as the ϕC31 and Bxb1 phage-encoded integrases require their phage-encoded RDFs (gp3 and gp47 respectively) to carry the excision rearrangement (Ghosh, Wasil and Hatfull 2006).

Further characterisation of satellite integrases and *attC* sites could speed up DNA assembly tasks, enable the use of multiple recombination systems to scale up genome engineering and expand the specificity of homologous recombination in other hosts. Phage satellites have different attachment sites, which increases the catalogue of desired delivery *loci* to integrate synthetic gene circuits. Additionally, intra- and inter-specific transfer could contribute vastly to designing and implementing broader genetic circuits in other bacterial species. Complex circuits can be engineered by combining integrases and switches to monitor temporal recombination events, coupled with robust sensors to transform an analogue signal into a digital framework (Fig. 3A) (Siuti, Yazbek and Lu 2013; Weinberg *et al.* 2017). As with lytic/lysogeny switches and conditional recombination machineries, novel gates employing the interactive domains of the repressor and anti-repressors could be rationally designed and fine-tuned using direct evolution to not only expand the regulatory functions of a desired process but also use these gates to study interactions between bacteria–bacteria, bacteria–phage and phage–phage (Brödel *et al.* 2020).

Gene delivery and therapy

In recent years, phage-derived engineering tools have been optimised to improve genetic manipulation of bacterial genomes. These applications include both lytic and temperate phages; however, there has been a preference for the use of filamentous phages, such as M13, with smaller genomes that are easier to edit *in vitro* (Citorik, Mimee and Lu 2014; Krom *et al.* 2015). DNA editing is often enabled by homologous recombination and integration of desired DNA into the targeted host or by delivery of a circularised chromosome (Menouni *et al.* 2015; Krishnamurthy *et al.* 2016). Phage satellites can be used to develop new gene delivery vehicles. Their intrinsic *modus vivendi* of phage exploitation, phage interference and high transfer frequency makes them suitable candidates for engineering more complex and dynamic applications when combined with phages. Furthermore, rational design of packaging exploitation systems can ensure the stable retention of the element in the bacterial genome without promoting the spread of other MGEs carrying AMR genes.

In view of their Trojan horse-like strategy, SaPIs and more recently characterised PICIs have been repurposed to deliver sequence-specific antimicrobials by exchanging their virulence modules with CRISPR-Cas proteins (Fig. 3B). Ram *et al.* (2018) repurposed SaPI2 to deliver CRISPR-Cas9 and dCas9 to treat *S. aureus* and *L. monocytogenes* infection in a murine subcutaneous abscess model. Using two separate systems targeting a highly conserved virulence response regulator (*agr*) in staphylococci and a haemolysin-encoding gene (*hly*) that causes listeriosis, the SaPI particles were produced at high yields and able to deliver the lethal payload without interference to either *S. aureus* or *L. monocytogenes*, respectively. Similarly, two systems with SaPIbov2 have been developed to deliver CRISPR-Cas9 targeting the methicillin resistance gene *mec*A and the conserved small regulatory RNA *rsa*E (Ibarra-Chávez *et al.* 2020). The guided *rsa*E system employs an inducible promoter that can be used as prophylaxis with immobilised PICI-particles in a catheter, which can be used in conjunction with antibiotics to trigger the CRISPR-Cas9 expression against *S. aureus*. Besides targeting virulence regulators, creating guided systems against antimicrobial resistance genes could treat infections and prevent dissemination of resistance genes. For example, the RNA cleavage ability of CRISPR-Cas13a has been shown to eradicate populations of methicillin-resistant *S. aureus* and carbapenem-resistant *E. coli* (Kiga *et al.* 2020). In contrast to Cas9, this system was able to eliminate the bacteria regardless of whether the location of the targeted gene was episomal or chromosomal.

In all instances of these applications, the packaging machinery of phages were exploited by using the piracy modules of the PICIs. For the SaPIs, the *ter*Sφ was deleted to prevent phage DNA from being packaged (Fig. 4A) (Ram *et al.* 2018; Ibarra-Chávez *et al.* 2020; Kiga *et al.* 2020). To exploit the G− PICI packaging, a plasmid containing CRISPR-Cas13a was delivered by expressing the *rpp*A gene *in trans* with the *cos*N site of the EcCICFT073 PICI incorporated into a plasmid (Fig. 4B). The phage cognate *cos*N site was deleted to prevent phage DNA packaging, allowing the production of transducing EC-CapsidCas13a particles (Ibarra-Chávez *et al.* 2020; Kiga *et al.* 2020). In particular, this grants the efficient delivery of episomal DNA using the packaging redirecting genes from the PICI to exploit the helper phage. A similar iteration of this approach replaced the P2 packaging signal with the interference genes δ and ε from P4 to produce transducing particles containing a lysin-encoding plasmid (Tridgett *et al.* 2021). The expression of these late genes under an inducible promoter abolished the packaging of the P2 DNA and rendered higher yields of viral particles than the lytic variant P2*vir1*. Employing different satellite genes to exploit different types of helper phages and engineering defective phage genomes to expand the host range of the delivery system could significantly impact phage therapy and develop technologies to sensitise bacteria to antibiotics or deliver DNA for the production of *in situ* antimicrobials.

As seen with phages, phage satellites can be engineered by yeast gap assembly and rebooting their genome in the desired host (Ando *et al.* 2015; Kilcher *et al.* 2018; Pires *et al.* 2021). However, phage satellites cannot produce infecting particles independently; therefore, a modified strategy is required for their rational design. Rebooting of PICIs has been done by mirroring their structure from their genome to facilitate and promote their integration (Ibarra-Chávez *et al.* 2020). In contrast to phagemids, phage satellites exhibit higher transfer frequencies and can adapt larger synthetic gene modules than phages. PICIs can potentially be used as delivery vehicles where up to two-thirds of their size is manipulated. These assemblies can be performed in strains containing a packaging-defective phage, which is then exploited to package the synthetic satellite DNA. Besides increasing the host range by utilising different phage backgrounds, phage transfer and dissemination of MGEs can be

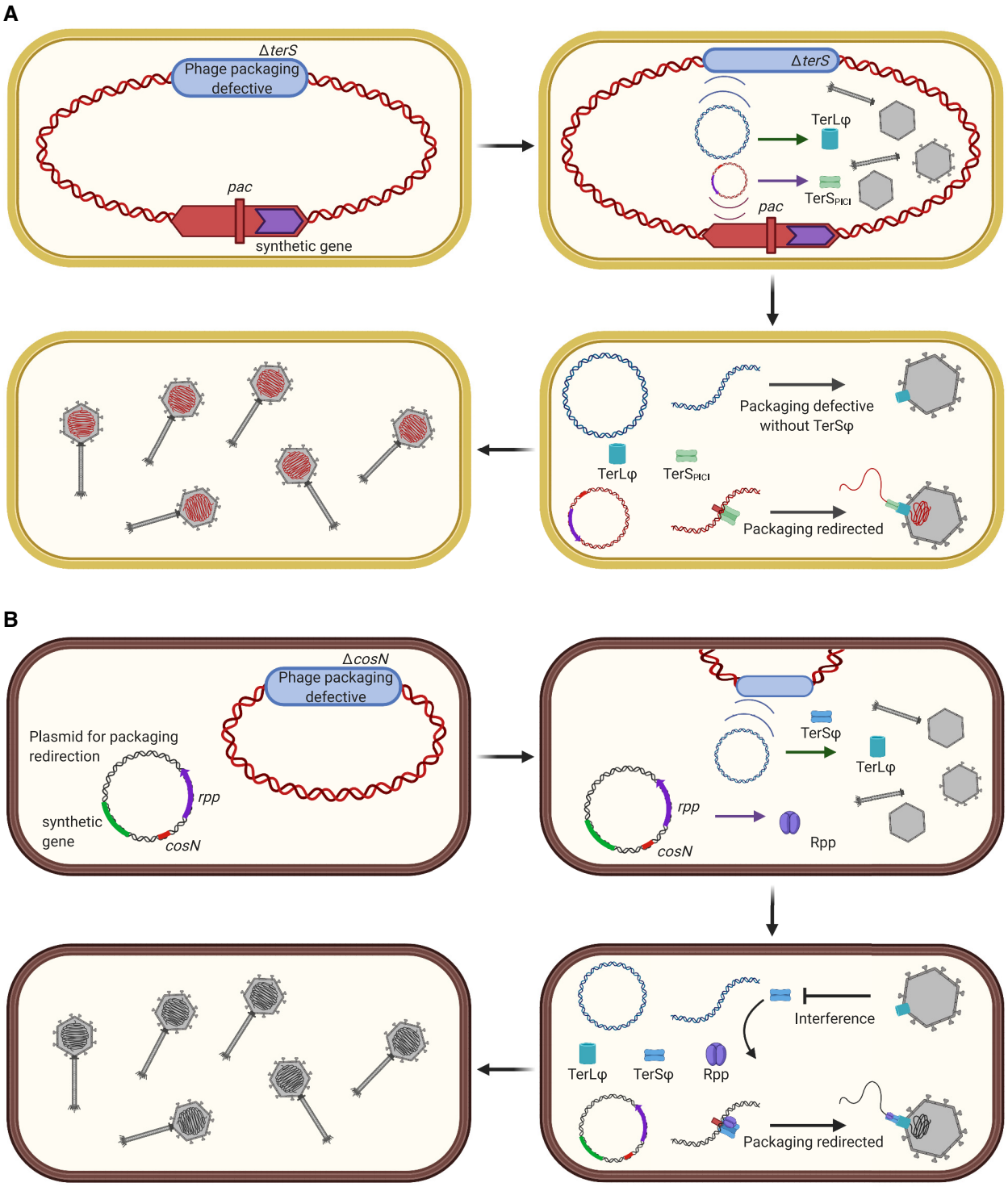

Figure 4. Packaging exploitation for gene delivery. **(A)** Synthetic satellites, such as PICIs, can act as delivery systems for genomic integration. Employing a packaging-defective helper phage (Δ*terSφ* for *pac* and Δ*cosN* for *cos* packaging phages), the satellite can complement and redirect the encapsidation of their DNA carrying synthetic genes (e.g. CRISPR-Cas systems, reporters, lysins, antivirulence factors). **(B)** Exploitation of packaging redirection genes to aid the delivery of episomal vectors can be achieved by cloning the packaging signal (*cos* or *pac*) and the hijacking components (e.g. Rpp) of the phage satellite into the plasmid. These approaches can produce phage-free transducing particles with user-defined synthetic genes for different applications without the need to strictly use a lytic phage to kill bacterial cells or identify a cognate phage for traditional therapy.

blocked by disrupting either their TerSφ or the *pac/cos* site, avoiding phage DNA packaging (Fig. 4).

To circumvent the limitation of phage-based delivery systems and disseminate the payload without employing phage reproduction after applying the first dose, conjugative delivery could be fused with phage satellite delivery (Citorik, Mimee and Lu 2014). Conjugative machinery, such as the IncP RK2 system, has been used to transfer plasmids into complex microbial communities (Klümper *et al.* 2015) and deliver a CRISPR-Cas9 system from *E. coli* to *Salmonella enterica* targeting different essential and nonessential genes (Hamilton *et al.* 2019). In *E. faecalis*, a pheromone-responsive plasmid (PRP) was used to deliver a CRISPR-Cas9 system targeting antibiotic resistance genes (Rodrigues *et al.* 2019). These well-studied conjugative machineries allowed high conjugation rates and the stable maintenance of the plasmids without selection, ensuring the removal of the antimicrobial resistance trait, and allowing the dissemination of the payload after several days. These applications offer an important advantage over common antibiotics and phage therapy by expanding the toolbox and enabling strain-specific elimination of individual strains among diverse bacterial communities to ensure eradication of multi-resistant bacteria without collateral damage of the surrounding commensal microbiome (Ronda *et al.* 2019; Reuter *et al.* 2020; Vo *et al.* 2020).

In addition to employing phage particles to deliver cargos, there is a growing interest in applying lytic phages as therapy and modulation of the microbiome (Abedon *et al.* 2011; Rasmussen *et al.* 2020). For example, ICP1 (the helper phage of PLEs) is currently under consideration as a promising therapeutic agent against *V. cholerae* (Yen, Cairns and Camilli 2017). Myriad phages are also being evaluated as an alternative to antibiotics or adjuvants to antibiotic treatment, particularly in the cases where the development of antibiotic resistance is common (e.g. ESKAPE pathogens) (Gordillo Altamirano and Barr 2019; Gordillo Altamirano *et al.* 2020). Importantly, the success of such interventions depends on the ability of the therapeutic phages to bypass the multiple defence mechanisms encoded by the targeted cells and the presence of phage satellites. Therefore, understanding the interplay between phages and the potential barriers to their propagation is paramount to design effective phage therapy strategies, including characterising the dynamics between phages and their satellites.

Host expansion

A long-standing challenge in phage engineering is expanding the effective host range, as it is typically desired to deliver genetic cargo into multiple bacterial strains. Conveniently, phage satellites exhibit a broader host range than their helper phages as evidenced by their intra- and inter-specific transfer capabilities (Chen *et al.* 2015b; Ram *et al.* 2018). Similar to engineering phages with different tail fibre proteins to expand the absorption of viral particles, satellite phages could be employed to expand the diversity of their helper tail fibres in their adaptable modules. Yosef *et al.* (2017) combined rational engineering with tail fibre evolution using phages lacking the tail fibre gene and employed a plasmid library encoding variants of tail fibres with known host range. Others have used phage rebooting to generate a library of mutants and select for the desired host range (Dunne *et al.* 2019; Yehl *et al.* 2019). This strategy could be taken forward by developing satellites that encode the tail variants and select for the desired host range mutations that allow the phage-like particle to infect. For example, the induction and recombination of a PICI carrying the tail fibre (gpJ) of the well-studied phage λ (Berkane *et al.* 2006; Chatterjee and Rothenberg 2012) could be constructed to encode and complement a new J tail fibre with the desired mutation that enables transduction of the PICI to the otherwise λ-resistant strains. One could also study different phages, as some satellites parasitise more than one type of helper phages, and subsequently make iterations with respective tail fibres to broaden the host range delivery.

Surveillance of communication and virulence systems

Phage satellites could be utilised to carry and deliver sensing systems to monitor different cell responses. Functioning as biosensors, engineered satellites carrying reporter circuits can be used as inexpensive epidemiological tools to identify specific bacteria. A notable difference between phages and satellites is that phages spontaneously induce and destroy the host of interest, hindering further investigation (Fig. 3C). Providing a phage satellite-based system where detection enables sequential processing for further enquiries, such as screening for upregulation of virulence factors or acquisition of antibiotic resistance through HGT, could play a crucial role in studying bacterial infections. Additionally, more complex synthetic circuits could be functional across different species and promote communication amongst switches allocated in different bacteria.

For example, many pathogens use quorum sensing (QS) signals to coordinate the production of extracellular factors as part of their virulence strategy (Van Delden and Iglewski 1998; Rumbaugh *et al.* 2009, 2012). The N-3-oxo-dodecanoyl-l-homoserine lactone (3O-C12-HSL) is a key QS signal secreted and sensed by *Pseudomonas aeruginosa* during infection. In concert with 3O-C12-HSL, the LasR protein functions as a transcriptional activator of virulence factors such as elastase, alkaline protease and pyocyanin, which aid *P. aeruginosa* in disruption of eukaryotic cell junctions and immune evasion (Gambello, Kaye and Iglewski 1993; Köhler, Buckling and Van Delden 2009; Rumbaugh *et al.* 2012). Therefore, we can deliver engineered satellites that respond to user-defined cues (e.g. species-specific signals) and can monitor the upregulation of QS-dependent virulence traits (Dziewit and Radlinska 2016).

In addition, QS signals and virulence factors can also function as costly 'public good' molecules, which are secreted into the shared environment and provide a collective benefit to neighbouring cells (West *et al.* 2006). The coordinated production of exoproducts within a bacterial population is an excellent example of how pathogens can cooperate during infection. However, the cooperative secretion of public goods is potentially susceptible to exploitation by 'cheats' that do not produce the exoproducts themselves but can benefit from those produced by others. Microbial social behaviour is well documented in the lab, but this framework is not applied routinely in natural settings. Given the ability of satellites to rapidly disseminate into bacterial populations and remain integrated, they can be utilised to monitor the frequency at which cheats invade and the population dynamics thereafter. This would allow us to study how specialised bacterial variants arise and the strong interactive effects on cells that cannot be understood by studying genotypes in labs.

Alternatively, the engineered satellites can be used to monitor the effects of strain-specific virulence traits in bacterial communities. For example, *P. aeruginosa* isolates display a striking variability in virulence, ranging from very moderate to highly virulent strains. This is somewhat dependent on the presence of pathogenicity islands bearing genes implicated in virulence

such as *exoU*, which encodes an effector of the type III secretion system, and the Rcs/Pvr two-component systems that control the *cupD* fimbrial cluster (He *et al*. 2004; Mikkelsen *et al*. 2009; Nicastro *et al*. 2009; Harrison *et al*. 2010). In addition, frame shifts in highly conserved genomic regions can lead to certain strains displaying hypervirulence and elevated cytotoxicity that are otherwise uncharacteristic of that species (Mikkelsen, McMullan and Filloux 2011). Such shifts in expression profiles could have important implications for an overall bacterial community, especially since bacteria commonly mix with other strains of their species. Therefore, we can use satellites to help provide information on how expression profiles affect the level of intermixing, regional genetic diversification, and the consequences of regional evolution.

Defence mechanisms

Many industrial processes depend on healthy bacterial cultures, for example, the production of fermented foods, beverages, pharmaceuticals, etc. In these industries, phage predation is a major concern contributing to substantial financial burdens (Samson and Moineau 2013). Notably, advances in understanding bacterial defence systems have received much attention, given their promising biotechnological applications to combat phages and plasmids (Doron *et al*. 2018; Pinilla-Redondo *et al*. 2020a). A timely example involves using CRISPR-Cas to develop phage-resistant strains in the dairy industry (Stout, Klaenhammer and Barrangou 2017; Donohoue, Barrangou and May 2018). However, given the fitness cost they impose on their bacterial host, defence systems tend to be lost due to autoimmunity when parasite pressure is low (Koonin *et al*. 2020).

As an inherent aspect of their parasitic lifestyle, phage satellites undermine the reproductive program of their helper phages (Seed *et al*. 2013; Fillol-Salom *et al*. 2020). Like other hyperparasites (parasites of parasites), phage satellites benefit the host population by effectively acting as a host defence strategy (Koonin *et al*. 2020). Furthermore, certain phage satellites (e.g. P4-like elements) encode clusters of antiphage defence systems (Rousset *et al*. 2021), suggesting that these elements may constitute a reservoir of bacterial immune systems. Following this rationale, the protective role of phage satellites could be harnessed to develop antiphage biotechnologies with high industrial relevance (Fig. 3D). Phage satellites could thus be used to combat phages encoding undesired traits, such as Stx prophages encoding Shiga toxin or *S. aureus* phages carrying staphylokinase (*sak*) and immune evasion gene clusters. As an advantage over conventional antibiotic therapies, this strategy would minimise the disruption of the surrounding microbiota. Similar approaches have been employed with temperate phages carrying C1-like repressors against toxin-encoding phages (Hsu, Way and Silver 2020). Moving forward, it will be crucial to evaluate the impact and potential risks associated with the biotechnological repurposing of phage satellites on a case-by-case basis. For example, rigorous investigation of phage satellite host range will be necessary to avoid the unintended fitness enhancement of pathogenic bacterial strains.

PLEs are particularly attractive for developing phage-refractive biotechnologies, given their complete interference with their helper phage reproduction (compared with the other described phage satellites) (O'Hara *et al*. 2017). However, PLEs have been only shown to parasitise ICP1 specifically, limiting their application scope. Future work is thus required to identify phage satellites across diverse taxa that completely restrict their helper phages, as well as research aimed at rationally expanding the range of helper phages restricted by PLEs.

Prospects and concluding remarks

Phage research has been essential in molecular biology and the development of novel genetic manipulation tools (Salmond and Fineran 2015). As the catalogue and characterisation of phages (Djurhuus *et al*. 2020; Hylling *et al*. 2020; Olsen *et al*. 2021) and their phage satellites (Dziewit and Radlinska 2016; Fillol-Salom *et al*. 2018) keep expanding, so does our understanding of phage diversity, ecology, and evolution. Historically, such studies have revealed how some phages modify genomic DNA and deploy anti-CRISPR and anti-RM proteins to avoid targeting by host defences (Bondy-Denomy *et al*. 2013; Bryson *et al*. 2015; Hynes *et al*. 2017; Hutinet *et al*. 2019; Pinilla-Redondo *et al*. 2020b). The recent identification of the *Autolykiviridae* family also emphasises that with the development of new methods of isolation and cultivation of phages, we explore intriguing new properties, interactions and potential HGT regimes (Kauffman *et al*. 2018).

In addition to their well-conserved gene organisation, both G+ and G− phage satellites identified to date have common features: (i) unique *attC* sites different than those occupied by prophages; (ii) induction of the ERP cycle independent from SOS response; (iii) the absence of lytic genes and inability to autonomously produce a functional viral particle; (iv) their core genome size is smaller than that of their helper phage, typically around 15 kb; and (v) they possess sophisticated strategies to usurp helper phage reproduction. The fact that phage satellites are scattered across distantly related taxa yet share a common genomic organisation suggests that their lifestyle has a strong selective value representing a novel strategy for conferring fitness and promoting genomic variability among bacteria. Alternatively, it may very well represent one that has evolved *de novo* that allows satellites to rapidly disseminate and integrate into bacterial chromosomes to ensure their survival and selection in the long run, thereby maximising their overall success. To date, no bioinformatic programs have been specifically designed to detect phage satellites and manual inspection is commonly required as the only strategy for *in silico* description of phage satellites (Crestani, Forde and Zadoks 2020; Durrant *et al*. 2020).

In combination with an advanced understanding of the interaction between phages and satellites and tools for identification and typing, this knowledge will likely form the basis of future therapeutic approaches and novel ways to manipulate genetic material. With this review, we aimed to introduce the potential of phage satellites and inspire the next generation of applications in synthetic and applied biology. The number of examples of their use will continue to rise and further reinforce their highly effective nature, and we anticipate that future investigations will enable efficient and broadly applicable molecular systems.

GLOSSARY BOX

1. **Mobile genetic elements (MGEs)**

An umbrella term that covers the collection of semiautonomous genetic entities that require a host cell to survive and can move position across genetic material in a cell (intracellular mobility) and/or between cells (intercellular mobility). Examples of such genetic agents include plasmids, transposons, phages, genomic islands, phage satellites, integrative conjugative elements and integrons.

2. **Genomic island**

Cluster of genes within a bacterial genome (usually between 10 and 200 kb) that appear to have been acquired by horizontal

gene transfer. These DNA segments, most of which are thought to be mobile, encode for genes involved in pathogenesis and adaptation of their host.

3. **Temperate phage and prophage**

Phages with the ability to follow the lysogenic life cycle and integrate in the bacterial chromosome. During lysogeny, the phage is termed a prophage but can exit this state (a process termed induction) spontaneously or as a response to certain triggers.

4. **Cryptic prophage**

A prophage without the genes required for lytic development that have a permanent lysogenic relationship with its host.

5. **Horizontal gene transfer (HGT)**

Transfer of DNA between cells that are not offsprings within and between species.

6. **Transduction**

The process when a phage transfers host-derived genetic material from one bacterium to another. Is divided into general- or specialized transduction depending on whether the phage is packaging host DNA exclusively or with its own DNA, respectively.

7. **Lateral transduction**

A distinct transduction mechanism where a prophage replicates before excision. In this process, adjacent DNA is included in the encapsidation process resulting in a high fraction of particles with bacterial DNA.

8. **Capsid**

The part of the proteinaceous phage particle wherein genetic material is packed and stored between transfer.

9. **Receptor binding proteins (RBPs)**

Proteins distributed on phage tail fibres, tail spikes and the central spike, which structures constitute the adsorption apparatus and are determinants for host specificity and infection.

10. **Phage satellite**

A genomic island, acquired through horizontal gene transfer, with the ability to transfer to new hosts by hijacking phage particles.

11. **Helper phage**

A phage that provides the necessary gene products for particle formation and is the subject of molecular piracy by phage satellites, which pack and mobilise their DNA instead of the phage DNA. Satellites depend on their helpers for their propagation.

12. **Molecular piracy**

The ability of one MGE to hijack and exploit the proteins encoded by another MGE to propagate and disseminate.

13. **Trojan horse**

The delivery of something hidden in something it is not. Referring to the legacy on how the Greek entered the city of Troy during the Trojan war by disguising inside a wooden horse.

14. **Pac packaging**

Phage-mediated cleavage and packaging of dsDNA where the terminase complex recognises and cuts a *pac* sequence to fill capsids in a headful manner.

15. **Cos packaging**

Phage-mediated cleavage and packaging of dsDNA where the terminase complex recognises and cuts a *cos* sequence generating a cohesive overhang at both initial and final termini to fill capsids in a unit-length manner.

16. **Phage interference**

Inhibition of phage reproduction caused by repression of their lytic modules, inhibition of packaging, down-modulation of replication, DNA degradation, competition for genome integration, and blockage of absorption or injection of the DNA.

17. **Abortive infection (Abi) system**

A defence strategy in which phage-infected bacterial cells self-destruct before the phage can complete its replication cycle to disrupt phage propagation.

18. **SOS response**

Upon DNA damage, RecA facilitates derepression of a range of LexA-regulated repair genes to promote survival.

19. **Phage therapy**

The use of phages for therapeutic applications such as treatment of bacterial infections.

20. **CRISPR-Cas**

Short for Clustered Regularly Interspaced Short Palindromic Repeats and their CRISPR-associated genes. A diverse family of adaptive immune systems that allow many prokaryotes to remember, recognise and destroy foreign nucleic acids. Their easy programmability has prompted their repurposing for diverse biotechnologies, with genome engineering being the most revolutionary application.

21. **Cas9 and Cas13a**

CRISPR-associated nucleases pertaining to type II (Cas9) and type VI (Cas13a) CRISPR-Cas systems. While Cas9 is most popular for its application in RNA-guided dsDNA-targeting biotechnologies, Cas13a is mainly employed for its RNA-guided and collateral ssRNA cleavage activities.

22. **Rebooting**

Reactivation of synthetic phage or phage satellite DNA.

23. **QS signalling**

Production, release and detection of signal molecules termed autoinducers, which enable orchestration of gene expression according to population density and composition.

24. **ESKAPE pathogens**

A collective term for six common nosocomial pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.

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