Genome integration and excision by a new Streptomyces bacteriophage, φJoe

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Abstract

Bacteriophages are the source of many valuable tools for molecular biology and genetic manipulation. In Streptomyces, most DNA cloning vectors are based on serine integrase site-specific DNA recombination systems derived from phage. Because of their efficiency and simplicity, serine integrases are also used for diverse synthetic biology applications. Here we present the genome of a new Streptomyces phage, ΦJoe, and investigate the conditions for integration and excision of the ΦJoe genome. ΦJoe belongs to the largest Streptomyces phage cluster (R4-like) and encodes a serine integrase. The attB site from S. venezuelae was used efficiently by an integrating plasmid, pCMF92, constructed using the ΦJoe int/attP locus. The attB site for ΦJoe integrase was occupied in several Streptomyces genomes, including S. coelicolor, by a mobile element that varies in gene content and size between host species. Serine integrases require a phage-encoded recombination directionality factor (RDF) to activate the excision reaction. The ΦJoe RDF was identified and its function was confirmed in vivo. Both the integrase and RDF were active in in vitro recombination assays. The ΦJoe site-specific recombination system is likely to be an important addition to the synthetic biology and genome engineering toolbox.

Importance

Streptomyces spp. are prolific producers of secondary metabolites including many clinically useful antibiotics. Bacteriophage-derived integrases are important tools for genetic engineering as they enable integration of heterologous DNA into the Streptomyces chromosome with ease and high efficiency. Recently researchers have been applying phage integrases for a variety of applications in synthetic biology, including rapid assembly of novel combinations of genes, biosensors and biocomputing. An important requirement for optimal experimental design and predictability when using integrases, however, is the need for multiple enzymes with different specificities for their integration sites. In order to provide a broad platform of integrases we identified and validated the integrase from a newly isolated Streptomyces phage, ΦJoe. ΦJoe integrase is active in vitro and in vivo. The specific
recognition site for integration is present in a wide range of different Actinobacteria, including Streptomyces venezuelae, an emerging model bacterium in Streptomyces research.
Introduction.

Over the past few decades, serine integrases have become widely established as tools for genome engineering and synthetic biology (1, 2). Serine integrases are phage-encoded, DNA site-specific recombinases that mediate recombination between two short (<50 bp) sequences. The integration reaction occurs during the establishment of lysogeny, during which the integrase causes a single crossover between the attB site on the bacterial chromosome and the attP site on the circularised phage genome leading to the integrated phage DNA flanked by the recombinant sites, attL and attR (1, 3). Integrase dimers bind to the two att sites and produce double-strand breaks with 2 bp overhangs (3, 4); the cut ends are then exchanged and the DNA backbone is re-ligated to produce the recombinant products (5). The attL and attR sites each contain reciprocal halves of the attP and attB sites (6). As integrases are unable to use attL and attR as substrates without an accessory protein, the recombination directionality factor (RDF), the integrated phage genome is stable until the RDF-encoding gene is expressed during prophage induction (3). Recombination between attL and attR is the excision reaction and is essentially the reverse of integration, releasing the phage genome and reforming attP and attB. Whilst only integrase is required to mediate integration, excision requires both integrase and the RDF. Genome engineers have exploited these systems to integrate genes of interest into a specific site on the chromosome, which can either be the endogenous attB or an introduced attB or attP used as a docking site (1). The simplicity of the serine integrase mediated site-specific recombination systems means that they are reliably portable to heterologous hosts where DNA can be integrated stably and in single copy.

The simple requirements of serine integrases make them amenable to a wide variety of applications. The earliest examples of this were to integrate an attP plasmid into a target genome containing the cognate attB (or vice versa) (7), allowing stable delivery of genes into diverse species, including bacteria (6, 8–10), mice (11), mosquitos (12) and humans (13). More complex genetic engineering approaches use integrases in in vitro ordered assembly of
multiple DNA fragments (14, 15). In vivo genome manipulations can also be achieved either by iterative rounds of recombination (16, 17) or multiplexing orthogonal integrases/att sites (18). Integrase mediated DNA rearrangements can also be used to provide permanent genetic memory in novel types of biosensors (19, 20). Some applications, such as post factum modifications (15) or biocomputing (19, 21), need controlled excision and this requires integrase and its cognate RDF. The RDF binds directly to the integrase protein and is thought to induce a conformational change that allows attL and attR to be used as recombination substrates whilst inhibiting recombination of attB and attP (22, 23).

A limiting factor for the use of serine integrases for complex, multiplexed applications is the number of well-characterized integrases and, perhaps more pressingly, RDFs. Only seven integrase/RDF pairs have been characterized to date (from phages TP901-1 (24), C31 (22), BT1 (25), Bxb1 (23), Rv1 (26) and SPBc (27), and from the excisive element of Anabaena and Nostoc cyanobacteria species (28)), but many more integrases have been studied without their RDFs (1, 2, 29–31). Integrase genes are easily identified by comparative sequence analysis and, when the integrase is prophage encoded, the attachment sites can also be predicted (31). RDFs, however, are far more difficult to predict because known examples share little sequence homology, vary markedly in size and also differ in gene location in phage genomes (1). Expansion of the available arsenal of serine integrases and RDFs is desirable to enable advanced synthetic biology applications.

Phages that encode serine integrases are prevalent in Gram-positive bacteria, and in particular in Actinobacteria. Here, we describe a newly isolated Streptomyces phage, Joe, and its serine integrase (Int) that is only distantly related to characterized integrases. Joe Int is active in vivo in Streptomyces and E. coli, the integrase protein is readily purified and is able to carry out efficient in vitro recombination. We also describe the Joe RDF, a 6.8 kDa protein that is able to promote excisive recombination and inhibit integration.
Materials and Methods

Growth media

Escherichia coli strains were generally grown in LB, except where otherwise noted. Antibiotics were added for selection where appropriate (apramycin: 50 μg/ml, chloramphenicol: 50 μg/ml, kanamycin: 50 μg/ml, ampicillin: 100 μg/ml). Preparation of competent cells and transformation of E. coli were performed as described in Sambrook et al., 2001 (32). Streptomyces strains were grown on Mannitol Soya agar (33) supplemented with 10 mM MgCl₂ for plating conjugation mixtures and antibiotics, where required (apramycin: 50 μg/ml, nalidixic acid: 25 μg/ml).

Phage Isolation. The procedures for isolation, plating and titre of phage with Streptomyces as the isolation host are described in detail in Kieser et al., 2000 (33). Raw soil samples were enriched for environmental phage using S. coelicolor M145 as a propagation host (34). Briefly, 3 g of soil was added to 9 ml Difco™ nutrient (DN) broth (BD Diagnostics, Oxford, UK) supplemented with 10 mM CaCl₂, 10 mM MgSO₄ and 0.5% glucose. Streptomyces spores were added to a concentration of 10⁶ colony forming units/ml (cfu/ml) and incubated at 30°C with agitation for 16 h. Soil and bacteria were removed by centrifugation and filtration through a 0.45 μm filter. A dilution series of the filtrate in SM buffer (100 mM NaCl, 8.5 mM MgSO₄, 50 mM Tris-HCl pH 7.5, 0.01% gelatin) was plated with S. coelicolor spores to isolate single plaques. Phage were recovered from single, well-isolated plaques by single plaque soak outs in DN broth and re-plated with the host strain for three rounds of plaque purification. A high titre phage preparation was generated from plates inoculated with sufficient plaque forming units (pfu) to generate almost confluent lysis (33). The phage suspensions were filtered, pelleted by ultracentrifugation and resuspended in 0.5 ml SM buffer (35). The concentrated phage were further purified by caesium chloride isopycnic density gradient centrifugation (36).

Next Generation Sequencing. Phage DNA was extracted by phenol:chloroform purification (32) and the presence of pure phage DNA was confirmed by restriction digest. Phage DNA
was sequenced and assembled in collaboration with Dr Darren Smith at NU-OMICS (Northumbria University). DNA was prepared for next generation sequencing on the Illumina MiSeq platform using the Nextera XT library preparation kit (Illumina, Saffron Waldon, UK). Samples were loaded and run using a 2 × 250 cycle V2 kit. DNA samples were diluted to 0.2 ng/μl, prior to normalization and pooling. Paired end sequencing reads were provided as FASTQ files (NU-OMICS, Northumbria University, Newcastle, UK) and subjected to downstream analysis. ORF prediction and annotations were assigned using DNA master (Lawrence lab, Pittsburgh, PA), Glimmer (37) and Genemark (38). The annotated genome sequence was submitted to GenBank (accession number: KX815338).

**Electron Microscopy.** Purified phage were negatively stained with uranyl acetate (39) and imaged in a FEI Tecnai 12 G2 transmission electron microscope fitted with a CCD camera.

**Mass Spectrometry.** Whole phage samples were run into a 7 cm NuPAGE Novex 10% Bis-Tris gel (Life Technologies) at 200 V for 6 mins. The total protein band was excised and digested in-gel with 0.5 μg trypsin, overnight at 37°C. Peptides were extracted, concentrated and loaded onto a nanoAcquity UPLC system (Waters) equipped with a nanoAcquity Symmetry C18, 5 μm trap (180 μm x 20 mm Waters) and a nanoAcquity HSS T3 1.8 μm C18 capillary column (75 μm x 250 mm, Waters). The nanoLC system was interfaced with a maXis HD LC-MS/MS system (Bruker Daltonics) with CaptiveSpray ionisation source (Bruker Daltonics). Positive ESI-MS and MS/MS spectra were acquired using AutoMSMS mode. Instrument control, data acquisition and processing were performed using Compass 1.7 software (microTOF control, Hystar and DataAnalysis, Bruker Daltonics. The collision energy and isolation width settings were automatically calculated using the AutoMSMS fragmentation table, absolute threshold 200 counts, preferred charge states: 2 – 4, singly charged ions excluded. A single MS/MS spectrum was acquired for each precursor and former target ions were excluded for 0.8 min unless the precursor intensity increased fourfold. Protein identification was performed by searching tandem mass spectra against the NCBInr database.
using the Mascot search program. Matches were filtered to accept only peptides with expect
scores of 0.05 or better.

**Plasmid Construction.** Plasmids used in this study are listed in Table 1 and oligonucleotides
in Table 2. General molecular biology techniques including plasmid DNA preparation, genomic
DNA preparation, restriction endonuclease digestion and agarose gel electrophoresis were
performed as described in Sambrook et al., 2001 (32). In-fusion cloning technology (Clontech)
was generally used for construction of plasmids. Polymerase chain reaction (PCR)-amplified
DNA was generated using primers with Infusion tags for insertion into plasmid vectors, which
had been cut with restriction endonucleases. The φJoe integrating plasmid, pCMF92, was
created by Infusion cloning of the φJoe int gene and attP region, obtained by PCR with Joe
Int-attP F/R primers and φJoe genomic DNA as a template, into the 3.1 kbp EcoRI-SphI
fragment from pSET152. Plasmid pCMF91 was generated by inserting the amplified attP site
prepared using φJoe genomic DNA as a template and primers Joe attP F/R into EcoRI
linearized pSP72. The integration sites in S. coelicolor were named attLsc and attRsc and
were amplified from S. coelicolor gDNA using Joe attB1 F/R and Joe attB2 F/R. The attB site
from S. venezuelae (attBsv) was amplified using S. venezuelae gDNA with Joe attB Sv F/Joe
attB R primers. All three attachment sites were inserted into EcoRI-linearized pGEM7 to
produce pCMF90, 94 and 95, respectively. The reconstituted S. coelicolor attB sequence
(attBsc) was prepared from two complementary oligonucleotides, Joe attB Recon F and Joe
attB Recon R (Ultrimer primers, IDT) that were annealed and inserted into EcoRI-linearized
pGEM7 to produce pCMF97. pCMF98 contains the φJoe attLsv and attRsv sites in head-to-
tail orientation and was isolated by transformation of an in vitro recombination reaction
between pCMF91 (containing φJoe attP) and pCMF95 (containing attBsv) into E. coli. The
attLsv and attRsv sites in pCMF98 were confirmed by Sanger sequencing (GATC Biotech Ltd,
London, UK). The recombination reporter plasmid pCMF116 was constructed by PCR
amplification of lacZα using E. coli MG1655 gDNA (40) as a template and Joe BzP forward
and reverse primers encoding the φJoe attBsv and φJoe attP, respectively, resulting in the
attBsv and attP sites flanking the lacZα gene in head-to-tail orientation. The amplified DNA was inserted into XmnI-linearized pACYC184. pCMF103 was constructed in the same way as pCMF116 except that Joe LzR F/R primers containing the ΦJoe attLsv and attRsv sites were used.

The integrase expression plasmid for protein purification, pCMF87, was constructed by insertion of a PCR fragment encoding the ΦJoe int gene, amplified from ΦJoe gDNA using primers Joe H6-Int F/R, into Ncol-linearized pEHISTEV expression vector. ΦJoe g52, encoding the RDF, was PCR-amplified from ΦJoe gDNA using primers Joe MBP-g52 F/R and inserted into pETFPP_2 MBP-tag expression vector linearized by PCR with CleF/R to create pCMF96.

For in vivo recombination assays the integrase expression plasmid, pCMF107, was constructed by insertion of a PCR fragment encoding the ΦJoe int gene, amplified from ΦJoe gDNA using primers Joe pBAD Int F/R, into Ncol-linearized pBAD-HisA expression vector. A ΦJoe gp52 and integrase co-expression plasmid, pCMF108, was created by amplification of each gene using Joe pBAD gp52 F/R and Joe pBAD Int Co-Ex F/Joe pBAD Int R primers, respectively, and insertion of both PCR products simultaneously into pBAD-HisA. The co-expression insert from pCMF108 was subsequently PCR-amplified using Joe H6-gp52 F/Joe H6-Int R primers and transferred to Ncol-linearized pEHISTEV to produce an alternative expression vector, pCMF117.

**Conjugation and integration of plasmids in Streptomyces.** Transfer of plasmids into Streptomyces strains was performed according to the procedures described by Kieser et al., (2000) (33). Conjugation donors were produced by introduction of plasmids into the non-methylating E. coli strain, ET12567, containing an RP4 derivative plasmid (pUZ8002), by transformation. Recipient Streptomyces spores were used at a concentration of 10^8/ml, mixed with the E. coli donors, plated onto mannitol soya agar supplemented with 10 mM MgCl₂ with no antibiotic selection and incubated at 30°C overnight. Plates containing the donor cells were overlaid with 1 ml water containing 0.5 mg nalidixic acid (for E. coli counterselection) and antibiotic for selection of exconjugants (apramycin) before further incubation of all plates at
30°C for three days. Integration efficiency was calculated as the number of apramycin-resistant colonies/10⁸ cfu (8).

**Protein Purification.** E. coli BL21(DE3) containing the relevant expression plasmid were grown (37°C with agitation) in 500 ml 2YT medium (1.6% w/v tryptone, 1.0 % w/v yeast extract, 0.5 w/v NaCl) to mid-exponential growth phase. The cultures were rapidly chilled on ice for 15 min, IPTG was added (final concentration 0.15 mM) and the cultures were further incubated (17°C, 16 h, with agitation). Cells were harvested by centrifugation, resuspended in 20 ml lysis buffer (1 M NaCl, 75 mM Tris pH 7.75, 0.2 mg/ml lysozyme, 500 U Basemuncher Endonuclease; Expedeon Ltd.) and incubated on ice (30 min). The cells were lysed by sonication and debris was removed by centrifugation (18,000 g, 5 min, 4°C). The supernatant was applied to a 5 ml HisTrap FF crude column that had been pre-equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) on an ÄKTA pure 25 chromatography system (GE Healthcare). Bound, his-tagged protein was eluted with a step gradient of binding buffer containing 125 mM and 250 mM imidazole. Imidazole was removed from the eluted fractions by pooling the fractions containing the desired protein and applying the pooled solutions to a HiPrep 26/10 desalting column (GE Healthcare) equilibrated with imidazole-free binding buffer. Finally, the protein extracts were subjected to size exclusion chromatography on a HiLoad 16/60 Superdex column. Purified protein fractions were concentrated in a Vivaspin sample concentrator (GE Healthcare) and quantified by absorbance at 280 nm on a Nanodrop spectrophotometer (Thermo Scientific). Protein analysis was performed by denaturing acrylamide gel electrophoresis using pre-made gels (4-12% gradient acrylamide; Expedeon Ltd.); gels were stained with InstantBlue (Expedeon, Ltd.). For storage, an equal volume of 100% glycerol was added to protein samples before freezing at -80°C.

**In vitro Assays.** Recombination reactions (final volume of 20 μl) were carried out in φC31 RxE buffer (10 mM Tris pH 7.5, 100 mM NaCl, 5 mM DTT, 5 mM spermidine, 4.5% glycerol, 0.5 mg/ml BSA) (41), Bxb1 RxE buffer (20 mM Tris pH 7.5, 25 mM NaCl, 1 mM DTT, 10 mM
Spermidine, 10 mM EDTA) (23) or TG1 RxE (as Bxb1 RxE plus 0.1 mg/ml BSA) (42).

Integrase and RDF proteins were added at the concentrations indicated for each experiment. Plasmids containing the recombination substrates were used at 100 ng per reaction. Reactions were either incubated at 30°C for 2 h (to reach steady state) or for specified times. Reactions were stopped by heat (10 min, 75°C), the buffer was adjusted to be compatible with restriction enzymes and the plasmids were digested with XhoI (NEB). The linearized reaction mixtures were run on a 0.8% agarose gel and the relative band intensities were measured to assess activity. Recombination efficiencies were calculated as intensity of product band(s)/sum intensity of all bands.

**Bioinformatics.** The φJoe genome was visualized using DNAplotter (43). The attB DNA alignment and logo consensus sequence were created with Jalview (44). Protein sequence alignments for visual presentation were produced using the Clustalw (45) program within the Bioedit suite (46). Protein alignments for phylogenetic analysis were produced using Clustal Omega (47) and maximum likelihood trees were created in Mega6 (48). The BLOSUM62 similarity matrix was used for protein alignment and annotation (49). Structural alignment of the small RDF proteins was carried out with Promals3D (50). Band densities for in vitro assays were measured using the FIJI GelAnalyzer module (51). Accession numbers for all sequences used here are provide in Table S2.
Results and Discussion.

**Isolation of actinophage φJo and genome sequence.** Raw soil samples were enriched for environmental phage using S. coelicolor strain M145 as a propagation host. The phage chosen for further analysis, φJo, is a siphovirus with a capsid diameter of 46.5 nm (SD 1.6 nm, n=9) and a long flexible tail of 199.5 nm (SD 12 nm, n = 8) with clear striations visible in most images (Fig. 1). φJo is able to plaque on a broad range of Streptomyces hosts, producing lytic infection of seven out of nine species tested (Table 3). Saccharopolyspora erythraea (formerly Streptomyces erythraeus) and Streptomyces venezuelae were resistant to infection.

Genomic DNA was extracted from high titre φJo suspensions (>10^{10} pfu/ml) and sequenced on the Illumina MiSeq platform with 2,542x coverage. The phage genome is 48,941 bases (Accession: KX815338) with a GC content slightly lower than the host bacteria; 65.5% compared to ~72% for most Streptomyces species. BLASTn was used to measure nucleotide identity for the closest relatives to φJo; the generalized transducing phage φCAM (52) and two newly sequenced Streptomyces phages, Amela and Verse (Fig. S2), are 73, 76 and 76% identical, respectively, in global alignments. The φJo genome contains 81 predicted open reading frames (Fig. 2), the majority of which have similar amino acid sequences to the three phages above and the well characterized R4 phage (53). Notably, similarity to φJo integrase (gp53) is absent from each of the closest genome matches but is instead present in several more distantly related phages (Fig. 3), indicative of phage mosaicism (54). Specifically, φJo integrase is homologous to the uncharacterized integrases from five complete phages - Lannister (78% amino acid identity), Zemyla (74%), Danzina (73%), Lika (73%) and Sujidade (73%) (Fig. 3). Comparison to known integrases suggests that the catalytic serine is likely to be at position 46 in the protein sequence (VRLSVFT).

Purified phage particles were submitted for shotgun LC-MS/MS analysis to determine the structural proteome. At least one peptide match was detected from fourteen φJo gene
products, five of which have predicted functions – Portal, Capsid, Tail Tape Measure, Scaffold, Head-Tail Adaptor (Figure 2, Table S1). The remaining nine gene products have no known function but all cluster close to the predicted structural genes within a region of the genome spanning ~21 kbp.

**Characterization of φJoe integrase and attachment sites.** For most phage-encoded integration systems, the attP site lies adjacent to the int gene encoding the integrase. The attP sites for serine integrases are characteristically about 45 to 50 bp in length and contain inverted repeat sequences flanking a spacer of approximately 20 bp (3, 55). Examination of the φJoe genome identified a candidate attP site located 18 bp upstream of the int gene. A plasmid, pCMF92, was constructed by replacing the φC31 int/attP locus from the widely used integrating vector pSET152, with the φJoe int/attP locus (Fig. S1). Integration of pCMF92 would confirm whether the integrase is functional, the nature of the attP site and, by rescuing the DNA flanking the integrated plasmid, the identity of the attB site could be deduced (Fig. 4).

pCMF92 was introduced into S. coelicolor J1929 and S. lividans TK24 by conjugation and apramycin resistant colonies were obtained, but the frequencies were low (10^{-4} to 10^{-5} exconjugants/cfu) compared to other integrating vectors (10^{-2} to 10^{-3} exconjugants/cfu) (9, 18). To test whether integration was site-specific, four S. coelicolor:pCMF92 cell lines were amplified from independent exconjugants and the genomic DNAs were analysed by Southern blotting using a probe derived from the φJoe int gene. In the four cell lines pCMF92 had integrated into one of two different integration sites, as revealed by hybridisation of the probe to two different restriction fragments (data not shown).

We then sought to characterize the two integration sites for pCMF92 in S. coelicolor by rescuing the integrated plasmids along with flanking DNA into E. coli. In pCMF92 there is 3.9 kbp of DNA between the φJoe attP site and the PstI cleavage site that contains the plasmid origin of replication and the apramycin resistance gene (Fig. S1). Genomic DNA from two S. coelicolor:pCMF92 cell lines, each containing pCMF92 integrated into one of the two different integration sites, was digested with PstI endonuclease, self-ligated and introduced into E. coli.
DH5α by transformation. The rescued plasmids were sequenced over the recombination sites to validate the nature of the ΦJoe attP site and to identify the chromosomal positions of the two S. coelicolor integration sites. The ΦJoe attP site was confirmed to be ≤ 50 bp and the 5’ GG dinucleotide at the centre of an imperfect inverted repeat is predicted to be where the crossover occurs (Fig. 4A).

The two S. coelicolor integration sites for pCMF92 are located 3.9 kbp apart, separated by an apparent mobile genetic element comprising sco2603, encoding a putative serine integrase with 68% identity to ΦJoe integrase and two further genes (Fig. 4B). Its product, SCO2603, is 68% identical to ΦJoe integrase. We hypothesized that the ΦJoe integrating plasmid is inefficient in S. coelicolor because an ancestral and optimal attB site is occupied by the SCO2603-encoding element. The two integration sites for pCMF92 in S. coelicolor were therefore called attLsc and attRsc to reflect the provenance of the sites containing the mobile element. To test this hypothesis, the sequence of the ancestral attB site, attBsc, was predicted by removing the sequence between attLsc and attRsc, including the attP moieties that would have originated from the inserted mobile element (Fig. 4C). The reconstituted attBsc was used to interrogate the GenBank Streptomyces database for closely related extant sequences. Three species were chosen from the top ten hits returned (S. avermitilis, S. albus and S. venezuelae, Fig. 4D) and assayed for in vivo integration efficiency. S. venezuelae was the only host to support highly efficient integration after conjugation with pCMF92, 160-fold greater frequency than S. coelicolor and 1,600-fold greater than S. lividans (Fig. 5A). The integration frequencies for pCMF92 into S. venezuelae are similar to those reported for other characterized serine integrases (9, 18) and we demonstrate below that the attB site from S. venezuelae, attBsv, is indeed used efficiently by ΦJoe integrase. Plasmid pCMF92 could therefore be used as a new integrating vector for use in this newly emerging model system for Streptomyces research.

The S. venezuelae attBsv site was used as a BLASTn query to estimate the prevalence of potential ΦJoe insertion sites in sequenced species. In many instances, each half of the
query sequence matched separate locations in the target genome, suggesting that \( \Phi \text{Joe-like} \) attB sites are frequently occupied by either a prophage or a similar mobile element to that observed in S. coelicolor J1929. Hits were subsequently filtered for matches of at least 80% coverage with an e-value of \(<1 \times 10^{-10}\) and a bit score \(>75\), which revealed numerous apparently unoccupied \( \Phi \text{Joe} \) attB sites in diverse Streptomyces, Kitasatospora and Dermacoccus species (Fig. 4D). Generally, the attB site for \( \Phi \text{Joe} \) and the SCO2603 integrase-encoding elements is located 74bp from the end of an ORF encoding a SCO2606-like predicted B12 binding domain-containing radical SAM protein. Insertions close to the end of an ORF may not necessarily cause loss of function of the gene product and this could explain the prolific number of mobile elements that use this locus as an insertion site. Other than the recombination genes, the genetic content of the mobile elements located here varies markedly in different bacterial species (Fig. S2). Some Streptomyces strains have an almost identical SCO2603-containing genetic element to S. coelicolor J1929 (e.g. WM6391), others have no genes other than the recombination genes (e.g. NRRLF-5123) and some contain up to 40 kbp between the predicted attL and attR sites (Fig. S2).

\( \Phi \text{Joe integrase} \) catalyses efficient in vivo and in vitro integration. In order for an integrase to have broad appeal as a bioengineering tool it must be functional in heterologous hosts. As a proof of principle, we tested the activity of \( \Phi \text{Joe} \) integrase in E. coli by cloning the integrase gene into an arabinose-inducible expression vector, pBAD-HisA, to produce pCMF107. Meanwhile, we constructed a reporter plasmid, pCMF116, containing the E. coli lacZ\( _{\alpha} \) gene flanked by \( \Phi \text{Joe} \) attBsv and attP sites in head to tail orientation (Fig. S3). Both plasmids were introduced into E. coli TOP10 cells (Invitrogen) by co-transformation and plated on selective agar plates containing 0.2% L-arabinose and 80 \( \mu \)g/ml X-Gal. pBAD-HisA lacking an insert was used as a negative control. All of the transformants were white in the presence of \( \Phi \text{Joe int} \), indicating efficient recombination between the attBsv and attP sites leading to loss of the lacZ\( _{\alpha} \) gene (Fig. 5B & S3). \( \Phi \text{Joe} \) integrase and its cognate attBsv and attP sites are, therefore, active in E. coli.
Another key application for serine integrases is for in vitro combinatorial assembly of genes for optimising expression of metabolic pathways (14, 15). In this application different integrases are used to join (by recombination) specific pairs of DNA fragments tagged with their cognate attachment sites. In theory this procedure can be multiplexed to assemble many DNA fragments together using different, orthogonally acting integrases. The aim is to generate artificial operons with defined or random order. To test the suitability of ΦJoe Int for in vitro recombination reactions, the integrase gene was cloned into the His-tag expression vector pEHISTEV and purified after overexpression in E. coli. In vitro recombination assays were carried out with ΦJoe attP (pCMF91) versus each of attBsc, attLsc, attRsc and attBsv (pCMF97, pCMF90, pCMF94 and pCMF95, respectively) and using a range of ΦJoe integrase concentrations. Successful recombination between attachment sites produces a co-integrant plasmid, which can be distinguished from the substrate plasmids by a restriction digest and agarose gel electrophoresis (Fig. S3). In this assay, recombination was undetectable when attLsc (pCMF90) or attRsc (pCMF94) were used with attP (pCMF91) as substrates. A small amount of recombination was observed (≤2%, Fig. S4) when the reconstituted attBsc (pCMF97) was used with attP (pCMF91). However, consistent with the observations in E. coli and in Streptomyces, the S. venezuelae attBsv site (pCMF95) was a highly efficient substrate for recombination with the ΦJoe attP site. ΦJoe integrase was effective over a broad range of concentrations (50 – 1000 nM) (Fig. 5C & S4). Using 200 nM integrase, detectable recombination product was produced after ~10-15 min, and after 2 h approximately 70% of the substrate molecules were converted to product (Fig. 5C & D).

There are only 6 bp that differ between attBsc and attBsv, and all the differences are on the left-hand arm of the attB sites (Fig. 4C). Previously, a mutational analysis of the ΦC31 attB site showed that mutationally sensitive bases occur 2, 15 and 16 bases to either side of the crossover dinucleotide (56). As two of the differences between attBsc and attBsv are also 2 and 16 bases from the putative crossover 5’GG (Fig. 4C), these base pair differences might account for the poor activity of attBsc in the in vitro assays.
Identification and validation of the φJoe RDF protein, gp52. Although there are dozens of serine integrases that have been described in the literature, there are only seven published RDFs for serine integrases (φC31 gp3 (22), φBT1 gp3 (25), Bxb1 gp47 (23), TP901 ORF7/Xis (24), Anabaena/Nostoc XisI (57), SPBc SprB (27), and φRv1 Rv1584c/Xis (26)). The Bxb1 and φC31 RDFs are amongst the largest of these RDF proteins (approximately 27.5 kDa, 250 amino acids) and their genes are located in proximity to the phage DNA replication genes. Both RDFs have functions during phage replication in addition to acting as RDFs but they are evolutionarily unrelated (25, 58). The RDFs from φBT1 and another φC31-like phage, TG1, are close relatives of the φC31 RDF at the sequence level (85% and 59% identical, respectively); furthermore, the φBT1-encoded RDF acts on φC31 integrase and vice versa (25). The φRv1 and SPBc RDFs are located within 1 or 2 ORFs of the int gene, a feature which is reminiscent of the xis genes that act with tyrosine integrases. φRv1, SPBc, TP901 and Anabaena/Nostoc RDFs are much smaller proteins than φC31 gp3 or Bxb1 gp47 (58 and 110 amino acids). Given the variation in RDF size, sequence and genomic location, there are no sound generalizations yet for identifying new RDFs in phage genomes.

A list of four candidate genes (g40, 43, 49 and 52) for the φJoe RDF was drawn up based on comparable size to known, small RDFs and genomic location (i.e. not located amongst the late/structural genes) (Fig. 2). One of the potential RDF genes (g52) is adjacent to int in the φJoe genome, but it is transcribed divergently, with the attP site situated between int and g52 (Fig. 2). Unlike the other candidate RDFs, gp52 homologues are only found in those phages with φJoe-like integrases (Fig. 3), and phylogenetic analysis of gp52 and the integrase indicated that both proteins have followed a parallel evolutionary path (Fig. S5).

Pairwise alignment of the 6.8 kDa (62 amino acids) gp52 protein with other known small RDFs revealed homology with φRv1 RDF (25.7% identity and 35.1% similarity; Fig. 6A). Also, examination of the mobile elements that have inserted into the attB sites in S. coelicolor and other Streptomyces spp, revealed that they also contain a gene encoding a gp52 homologue in a similar genetic context i.e. the int and g52 genes are adjacent to the attL and attR sites,
respectively, and would flank attP after excision (Fig. 4B & S2). The predicted secondary structure of ΦJoe gp52 contains an alpha-helix in the N-terminal region, a beta-sheet in the C-terminal region and an unstructured region in between (Fig. S6). Alignment of the ΦJoe-like RDFs found in intact phages and the RDFs found in the SCO2603-encoding mobile elements indicated that both of the structured regions are well conserved, particularly the putative alpha-helix, but the centre of the protein is variable (Fig. S6).

RDFs are able to influence integrase-catalysed recombination in two ways; they activate the attL x attR reaction to regenerate attP and attB (excision) and they inhibit the attB x attP integration reaction (22, 23). We were unable to produce sufficient soluble gp52 protein for in vitro assays when expressed with a simple histidine-tag; however, a maltose-binding protein MBP-gp52 fusion protein was more soluble. We tested the ability of MBP-gp52 to inhibit integration by titrating the protein against a fixed concentration of integrase at MBP-gp52:Int ratios of 1:2 to 22.5:1. When the MBP-gp52 was in excess integration was repressed to less than 10%; however, at less than equimolar concentrations, recombination was equivalent to the control in which no MBP-gp52 was added (Fig. 6B). These results are similar to observations for ΦC31 and Bxb1 integrases and their cognate RDFs, gp3 and gp47 (22, 23).

To test the ability of gp52 to activate an excision reaction, a plasmid containing the cognate attLsv and attRsv sites was produced, pCMF98 (Fig. S3). The MBP-gp52 protein was unable to promote efficient excision under any conditions tested (not shown). Removal of the MBP-tag, using 3c protease, increased excision activity but the reaction was still inefficient after 2 h incubation (Fig. 6C). Longer incubations of 5 – 20 h further increased the amount of substrates converted to product up to 45%, but also led to significant amounts of excision products (10-20%) by the integrase alone. Thus, in comparison to the activity of other RDFs, gp52 has rather poor activity; ΦC31 gp3 activates approximately 60 to 80% conversion of the attL x attR substrates to products (22) and similar results are obtained with other RDFs (23, 25, 26).
To test the excision ability of ΦJoe gp52 in vivo, a g52 and int co-expression operon
was designed in which int and g52 were located directly downstream of the T7 promoter and
ribosome binding site (RBS) in the expression vector pEHISTEV to produce pCMF117. A
reporter plasmid, pCMF103, was produced containing the lacZα gene flanked by ΦJoe attLsv
and attRsv sites (Fig. S3). pCMF117 and pCMF103 were introduced into E. coli BL21(DE3)
cells by co-transformation and plated onto LB agar supplemented with 0.5 mM IPTG to induce
expression of the g52-int operon (30). The reporter plasmid was then extracted from the
BL21(DE3) transformants and introduced into E. coli DH5α to determine the percentage of
plasmids that had undergone attLsv x attRsv recombination and had lost the lacZα gene. As
controls, plasmids expressing either only integrase (pCMF87) or only gp52 (pCMF100) were
also introduced together with the reporter (pCMF103) into BL21(DE3) and the assay was
repeated using the same procedure. When ΦJoe integrase alone was expressed, excision
occurred at a frequency of 37.6% (SD=5.1%, n=5) but when co-expressed with gp52 the
frequency rose to 96.8% (SD=1.3%, n=5) (Fig. 6D). Expression of gp52 without integrase led
to no detectable excision events (Fig. 6D). Although overall recombination in vivo was higher
than in vitro, the relative levels of attLsv x attRsv recombination by ΦJoe integrase alone and
ΦJoe integrase with gp52 were comparable. Taken together, the in vivo and in vitro data
indicate that ΦJoe gp52 has RDF activity.

The observation that ΦJoe integrase has a basal level of excision activity in the absence of its
RDF is highly unusual for a phage-encoded integrase and further study may provide novel
insights into the mechanism and evolution of the serine integrases. Streptomyces phage ΦBT1
integrase was shown to catalyse bidirectional recombination, albeit at extremely low levels
(59). The archetypal ΦC31 integrase is only able to mediate attL x attR recombination in the
absence of gp3 when certain mutations are introduced just upstream or within a motif, the
coiled coil motif, required for subunit-subunit interactions during synapsis of DNA substrates
(60). The coiled coil motifs are also thought to play a role in inhibiting recombination between
attL and attR in the absence of the RDF; the ΦC31 IntE449K mutation or its RDF, gp3, relieves
this inhibition (55, 60–62). Three independent structural predictions indicate the presence of
a coiled coil domain in the ΦJoe Int C-terminal domain (A395-T453, Fig. S7). The high basal
excision activity of ΦJoe integrase could be due to incomplete inhibition of synapsis by the
coiled coil motif when integrase is bound to attL and attR, reminiscent of the hyperactive ΦC31
mutant IntE449K (60). Natural bidirectional, large serine recombinases include the
transposases TnpX (63) and TndX (64) from clostridial integrated conjugative elements
(ICEs); ΦJoe integrase could be an evolutionary intermediate between these bi-directional
recombinases and the highly directional recombinases such as ΦC31 and Bxb1 integrases.
Our data show that, under the in vitro conditions used, gp52 was highly effective at inhibiting
integration by ΦJoe integrase but only weakly activated excision. It remains to be seen whether
this system, with its unusual properties, is sufficiently robust to regulate phage genome
integration and excision according to the developmental choices of ΦJoe.

The properties of the ΦJoe integrase and gp52 are compatible with some of the existing
applications for serine integrases, but they could also present opportunities for new
applications. ΦJoe integrase is highly efficient in integration assays in vivo and in vitro, and in
vivo excision when the RDF is present. In attB x attP integration assays, the yield of products
by ΦJoe integrase was comparable to well established integrases such as those of ΦC31 or
Bxb1. Furthermore, ΦJoe integrase is active in buffers compatible with other characterized
integrases indicating that it could be used in DNA assembly procedures in combination with
other integrases. Although yet to be tested, assemblies generated with ΦJoe integrase could
later be used as substrates for modification by ΦJoe integrase in a single step. The innate
excision activity of ΦJoe integrase could excise a fragment flanked by attLsv or attRsv sites
and, in the same reaction, replace it via an integration reaction. ΦJoe integrase could therefore
provide a more streamlined tool than the existing requirement for two steps by the more
directional integrases such as those from ΦC31 and Bxb1 (15). Furthermore, given that ΦJoe
Int can mediate basal levels of excision in the absence of RDF, integrating plasmids based on
ΦJoe int/attP may display a degree of instability. Selection for the plasmid marker would ensure
plasmid maintenance when desired but, if the plasmid is easily lost without selection, this trait
could be desirable if there is a need to cure the strain of the plasmid or during studies on
synthetic lethality.

Conclusions. On the basis of sequence and genome organisation, phage Joe is a member
of a large cluster of R4-like Streptomyces phages. Its closest relatives at the nucleotide level
are Streptomyces phages Amila and Verse with very high levels of nucleotide identity in the
regions encoding essential early and structural genes. However, Joe integrase is more closely
related to the integrases from five other R4-like cluster phages - Lannister, Danzina, Zemlya,
Lika and Sujidade. At the present time the majority of Streptomyces phages belong to the R4-
like cluster phages, but there is a continuum of relatedness throughout the cluster; for example
R4 is a more distant relative to \( \Phi \text{Joe} \) than any of the other phages mentioned above.

We identified the RDF for Joe integrase on the basis of its gene location, small size and distant
similarity to another known RDF, Rv1584c. Although this identification was relatively
straightforward, it is not clear yet how general such an approach might be. The activity of \( \Phi \text{Joe} \)
inTEGRASE and RDF contributes to the growing number of complete serine integrase site-
specific recombination systems that are available for use in synthetic biology applications. The
\( \Phi \text{Joe int/attP} \) plasmid, pCMF92, also adds to the number of useful integrating vectors for use
in Streptomyces species. However, and unusually for a phage integrase, \( \Phi \text{Joe Int} \) displays a
significant level of excisive recombination in the absence of its RDF while still being efficient
at mediating integration. This bi-directional property could be applied in new ways in future
applications of serine integrases.

Acknowledgements

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and electron microscopy. This research was performed with funding from the Biotechnology and Biological Sciences Research Council (project grant BB/K003356/1) and the Microbiology Society (formerly Society for General Microbiology) with a Harry Smith Vacation Scholarship for JH.
References.


Figure 1: A φJoe virion imaged by transmission electron microscopy.
Figure 2: Schematic of the φJoe genome.
Figure 3: Circos plot of the φJoe genome versus nine related phages.
Figure 4: \(\phi\)Joe attachment sites and integration sites.
Figure 5: Activity of φJoe integrase in vivo and in vitro.
Figure 6: Identification of the φJoe RDF, gp52.
**Figure Legends.**

**Figure 1:** A φJoe virion imaged by transmission electron microscopy. Viral particles were negatively stained with uranyl acetate and this image was taken at 220,000x magnification. The scale bar represents 100 nm.

**Figure 2:** Schematic of the φJoe genome. The genome is 48,941 bp in length. ORFs were predicted using GeneMark and Glimmer then manually curated. ORFs are labelled and colour-coded based on their predicted function. Orange = recombination; cyan = metabolism and DNA processing/replication; green = structural proteins; purple = lysis; black = regulatory; grey = hypothetical proteins with no known function; red = candidate RDF genes. Genes marked with an asterisk encode structural proteins that were detected by tandem MS:MS. The histogram below the genome contains purple bars to indicate below-average GC content (65.5%) and green bars to indicate above-average GC content (1000 nt window size, 20 nt step).

**Figure 3:** Circos plot of the φJoe genome versus nine related phages. A Blastn comparison was carried out for φJoe, the five sequenced phage with a φJoe-like integrase, the three genome matches and the well-characterized R4 phage. The E-value cut-off was set to $1 \times 10^{-100}$ and the HSPs to 100, ribbons are coloured by genomic regions as defined in Figure 1 and depicted above the Circos plot. The histograms above each genome are coloured to reflect relative homology to the φJoe sequence based on Blast score (Red>Orange>Green>Blue).

**Figure 4:** φJoe attachment sites and integration sites. A. Diagram of φJoe attP showing the central dinucleotides (Purple) and imperfect inverted repeats (Orange and arrows). B. Schematic of the genomic context of the two S. coelicolor integration sites (attLsc and attRsc, red boxes) used by the φJoe integrating plasmid pCMF92. The location of the PstI sites used for identification of the att sites are shown. The DNA between the attLsc and attRsc sites is an apparent mobile genetic element with homologous integrase and RDF genes (orange arrows) to those of φJoe. C. Alignment of S. venezuelae attB (attBsv) with the two S. coelicolor att sites (attRsc and attLsc) and the reconstituted attB site (attBsc) that would be produced by excision of the DNA between attRsc and attLsc. D. Alignment of closely related attB sites identified by a Blastn search against the non-redundant Genbank database. Hits were first filtered for matches of at least 80% and then for an e-value of $<1 \times 10^{-10}$ and a bit score $>75$. Nucleotide positions in C and D are shown as distance from the crossover dinucleotides (XX).

**Figure 5:** Activity of φJoe integrase in vivo and in vitro. A. Conjugation efficiency of an integrating vector, containing φJoe int and attP, into five recipient species - Streptomyces coelicolor (Sc), S. lividans (Sl), S. venezuelae (Sv), S. albus (Sal) and S. avermitilis (Sav). Levels of significance for S. venezuelae versus all other species in a one-way ANOVA was $p = <0.001$ (3 asterisks), all other comparisons were non-significant (n.s.). Error bars are standard deviation (Sc $n=5$, Sv and Sl $n=3$, Sal and Sav $n=2$). B. Representative image of an in vivo integration assay to assess attBsv/attP recombination by φJoe integrase (pCMF107) and a negative control (pBAD-HisA). Recombination leads to deletion of an intervening lacZα gene and white colonies, inactivity produces blue colonies. Integration efficiency is shown in brackets (n=3). C. Representative image of in vitro recombination of two substrate plasmids, attP (pCMF91) and attBsv (pCMF95), to produce the co-integrant plasmid pCMF98. The
concentration of φ Joe Integrase and incubation time for each reaction is indicated above the gel. D. Time-course for the integration reaction shown in part C.

Figure 6: Identification of the φ Joe RDF, gp52. A. Alignment of φ Joe and RV1 RDFs, coloured using the BLOSUM62 scheme. B. Representative agarose gel showing in vitro inhibition of integration by φ Joe RDF. The concentration of φ Joe Integrase and RDF for each reaction is indicated above the image. Reactions were stopped after 2 h and linearized using Xhol. C. Representative agarose gel showing in vitro excision reactions catalysed by φ Joe Integrase and RDF. The concentration of φ Joe Integrase and RDF for each reaction is indicated above the image. Reactions were stopped after 2 h and linearized using Xhol. D. In vivo excision assay to assess attLsv x attRsv recombination by φ Joe integrase alone, φ Joe RDF alone and φ Joe integrase co-expressed with the RDF. Recombination leads to deletion of an intervening lacZα gene and white colonies, inactivity produces blue colonies. Expression from the T7 promoter successfully achieved almost complete excision activity for φ Joe Int + RDF.
Table 1. Plasmids used in this study

<table>
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<th>Resistance</th>
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<td>$\phi$C31 int + attP integrating vector</td>
<td>Apra</td>
<td>(65)</td>
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<td>Expression vector, T7 promoter, C-terminal HIS6, TEV cleavage site</td>
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<td>Expression vector, araBAD inducible promoter</td>
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<td>Invitrogen</td>
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<td>pCMF87</td>
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<td>pCMF90</td>
<td>pGEM7 + S. coelicolor attRsc (274 bp)</td>
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<td>pCMF92</td>
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### Table 2. Primers used in this study

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36
Table 2. φJoe Host Range.

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</tbody>
</table>

^ Pfu/ml values quoted are illustrative of the relative plaquing efficiencies when challenged with the same phage stock propagated on S. coelicolor J1929