Construction of a new integrating vector from actinophage ϕOZJ and its use in multiplex *Streptomyces* transformation

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Keywords: multiplex transformation, ϕOZJ, *Streptomyces*, intergeneric conjugation, site-integrating vectors

Abstract

Streptomyces and other closely-related actinobacteria are important sources of bioactive molecules. *Streptomyces* synthetic biology and genetics empower therapeutic and agrichemical development through strain improvement and biosynthetic understanding. Such efforts rely on the availability of developed molecular toolsets. Among these tools, vectors that enable combinatorial chromosomal manipulations are particularly desirable. Towards developing tools for facile multiplex engineering, we herein describe the development of new integrating vectors derived from BD1 subgroup actinophage OzzyJ (ϕOZJ). By demonstrating the transformation of several *Streptomyces* spp. using ϕOZJ-derived vectors, we reveal their potential for strain engineering. We further report the development of new ϕC31 and ϕBT1-based vectors having orthogonal resistance, replication and integration features for concomitant transformation with our ϕOZJ-derived vectors. Importantly, the resulting compatible vector panel enabled us to demonstrate the transfer of up to three plasmids each into *Streptomyces venezualae, Streptomyces roseosporus* and *Streptomyces pristinaespiralis* during a single conjugation

experiment. To our knowledge this is the first documentation of conjugation-mediated multiplex plasmid transformation, a useful approach for rapid combinatorial strain development.

Introduction

Streptomyces and related filamentous actinomycetes are widely recognized for their biotechnological value. Producing a plethora of medicinally and agriculturally important metabolites[30,7,3], they also serve as biocontrol agents[8,31,24], encode industrial enzymes[26], and degrade recalcitrant biomass[15]. Because of this, these organisms are frequently targeted for metabolic engineering and synthetic biology[18].

Modern bioengineering efforts require robust and versatile genetic toolsets. Among the most extensively-utilized vectors for actinomycete engineering are phage-derived chromosomally-integrating plasmids[1]. These vectors integrate into bacterial chromosomes by the action of DNA recombinases that recognize and cause crossover between host-encoded *attB* sites and phage-derived *attP* sites. In wild phage, this type of recombination is used to enter lysogeny[11]. When exploited for plasmid construction, *attP*-recombinase (*int*) pairs form functional cassettes that enable stable, site-specific vector integration in hosts with cognate *attB*s. Due to inherent desirable traits that include recombination directionality, stability, and independence from host-encoded factors, most actinobacterial integrating vectors specifically incorporate recombinases of the large-serine integrase family[1,11].

The most commonly used actinomycete chromosomal-integrating vectors carry *attP-int* cassettes cloned from ϕC31 and ϕBT1 phage[1]. Several additional actinophage *attP-int* pairs have been exploited for strain manipulation and other biotechnological uses, including TG1[22], R4[21], SV1[9], and more recently, ϕJoe[12]. Despite the increasing availability of diverse actinophage-derived integrating vectors, there is robust demand for new toolsets for actinomycete bioengineering. Novel integrating vectors are desirable because strain-to-strain differences in genome architecture can limit the spectrum of integrating vector(s) that will function in a given actinomycete. Relatively few actinomycete strains are compatible with the full complement of existing tools[1], which stymies combinatorial engineering efforts.

Towards expanding the actinobacterial integrating vector toolset, we herein describe a series of new *E. coli-Streptomyces* shuttle vectors that incorporate an *attP-int* recombination cassette cloned from actinophage OzzyJ (ϕOZJ). ϕOZJ is a member of the BD1 subcluster of actinophage (https://phagesdb.org/phages/OzzyJ/) isolated from St Louis, MO soils previous to this work. We used a synthetic biology approach to functionally assess how BD1 subcluster phage *attP* nucleotide variation effects transformation efficiency in several *Streptomyces* spp. Further, we assessed the relative integration efficiencies of a panel of ϕC31, ϕBT1 and ϕOZJ – derived plasmids in a panel of 30 *Streptomyces* and related actinobacterial strains. Finally, we constructed a complementary set of plasmids having compatible *Escherichia coli (E.coli)* replicons, resistance determinants, and orthogonal ϕC31, ϕBT1 or ϕOZJ integrating functions (pJMD5, pJMD13, and pJMD14, respectively). Importantly, using these site-specific integrating vectors, we showed that intergeneric conjugation is capable of transforming some *Streptomyces* recipients with up to three different plasmids during a single experiment. Conjugation-mediated multiplex plasmid transformation, as demonstrated here for the first time, thus represents a new method for accelerating combinatorial engineering in biotechnologically valuable Streptomycetes.

Materials and methods

Microorganisms, plasmids, and molecular biology

All strains used in this work and their relevant characteristics are listed in Table S1. *E. coli* strains were routinely propagated on LB-Miller agar supplemented with one or more of the following antibiotics as appropriate for maintenance of genetic elements: ampicillin (amp), 100 µg/mL; apramycin (apr), 50 µg/mL; chloramphenicol (cm), 25 µg/mL; kanamycin (kan), 200 µg/mL. Actinomycete strains were routinely propagated on either ISP2 (Difco) or ATCC172 agar (www.atcc.org). All chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Intergeneric conjugations were performed using DNA nonmethylating *E. coli* JV36[6] or JV156 (Table S1). Strain construction details for both JV36, absent from its original publication[6], and derivative JV156 are provided in the Supplementary Methods. Control data establishing similar conjugation efficacies for ET12567/pUZ8002, JV36 and JV156 when transforming multiple

Streptomyces spp are included in Fig. S1. *E. coli* ET12567/pUZ8002 was otherwise not used herein due to multiple antibiotic resistance markers that make it incompatible with our multiplex conjugation strategy.

All molecular cloning was performed according to standard methods[27] unless otherwise noted. All restriction enzymes were purchased from New England Biolabs (NEB). Gibson assembly was performed using NEBuilder HiFi DNA Assembly Master Mix according to manufacturer's instructions. Routine diagnostic PCR was performed using NEB Taq polymerase and proofreading KOD Hot Start Polymerase (Novagen) was used for cloning. All PCR reactions were carried out using Epicentre Failsafe PCR premixes (Lucigen) according to manufacturer's instructions. Oligonucleotides and synthetic gBLOCK DNAs were purchased from Integrated DNA Technologies. The sequences of all DNA oligonucleotides used in this study are in Table S2, and plasmids are listed in Table S3. Detailed plasmid construction notes and relevant cloning schemes are noted in the online Supplementary Materials. Both characterized and newly-isolated environmental actinomycete strains were used to assess conjugal transformation with site-specific integrating plasmids. To ensure diversity among the tested environmental isolates, strain uniqueness was confirmed by amplifying and sequencing an internal portion of the 16S rDNA locus of each, as reported elsewhere[28]. The obtained 16S rDNA amplicon sequences, after trimming to remove primer binding sites, are in Table S4. Sanger DNA sequencing was performed at Genewiz and analyzed using CLC Genomics Workbench or SnapGene software.

***Escherichia coli* - actinomycete intergeneric conjugation**

For intergeneric conjugation, germinating actinomycete spores were used as plasmid recipients. Spores were obtained after growth on one the following media at 28°C, where optimal production was determined empirically: ISP2 (BD Difco), ISP4 (BD Difco), ISP-S (L^{-1}; 15 g malt extract, 5 g yeast extract, 5 g soluble potato starch, 3 g $CaCO_3$, 20 g Bacto Agar, pH 7.2-7.5), SFM[14], SMMS[14], or ATCC 172. Spore stocks were prepared by adding 3 mL TX buffer[13] to the surface of sporulating confluent lawns and spores were dislodged by rubbing with a sterile nonabsorbent cotton swab. Spore suspensions were recovered into 15 mL conical tubes, vortexed, and sonicated for 10 seconds in an ultrasonic bath. Aliquots were created to avoid

sample freeze-thaw and single-use tubes were frozen at -20°C until use. Just prior to conjugation, spore aliquots were thawed on ice, briefly vortexed and then heat shocked for 10 minutes at 50°C.

E. coli donors carrying desired shuttle vectors were prepared for conjugation by inoculating an isolated colony into 3 mL of LB-Miller broth with appropriate antibiotics for plasmid maintenance. After incubation with shaking (250 rpm, 1' throw) for 18 hours at 37˚C, donors were stored at 4°C for up to 4 hours until conjugation. The resultant cultures were diluted as necessary and optical density at 600 nm was recorded using a Shimadzu UV-1800 spectrophotometer. Prior to use in conjugations, donor stocks were adjusted to ~1.6x10^9/ml, equivalent to OD_{600} =2.0. During this adjustment, donors were exchanged into fresh media by centrifugation (3 min @8000 x *g*) and resuspension in antibiotic-free LB broth.

Conjugations were performed by spotting a mixture of donor and recipient onto R2NS agar, as described[6]. Specifically, 2 µL of the donor *E. coli* was combined with 20 µL of recipient spores in a sterile PCR strip and spotted to plates in quadrants. Negative controls lacked the addition of any donor strain. Resulting conjugation plates were placed in a 30˚C incubator for 18-21 hours, then overlaid with 3 mL of soft nutrient agar[14] containing 1.5 mg colistin to suppress donor growth plus appropriate antibiotics for selection of exconjugants. Where required, each plate received: apr, 1.5 mg; kan, 6 mg; and/or thiostrepton (thio), 450 µg. Conjugations were outgrown 3-4 days at 28˚C until exconjugants could be manually counted. All conjugation experiments were performed in at least biological triplicate (n>3), and error bars denote standard deviation. In experiments where plasmid transfer frequencies were high, normalized donor *E. coli* cultures were diluted 10^{-1} - 10^{-3} prior to conjugation to obtain countable exconjugant numbers.

Exconjugants were streaked to ISP2 agar containing 50 □g/L colistin, and the appropriate selective agent(s) for plasmid maintenance (15 □g/L thio, 50 □g/L apr, 200 □g/L kan) and grown for 3-4 days at 28˚C. Where verification of transformation was required (using oligonucleotide primers listed in Table S5), individual colonies were ground in DMSO and directly used as PCR templates as in Van Dessel et al[29].

Conjugation efficiency calculations

Prior to use as conjugation recipients, *Streptomyces* spore concentrations (in CFU/mL) were determined by spread plating spore dilutions onto ATCC172 agar media and counting resultant colonies after 5 days of growth at 28 ˚C. Standard enumeration calculations were used (CFU/mL= # colonies/ vol. plated × dilution factor). Counting was performed using plates with 30-300 colonies. Varying by strain, spore stocks used gave ~10^7-10^9 CFU/mL. Conjugation efficiency was calculated using the following equation as a ratio of transformed actinomycetes against the total (or absolute number) of spore CFUs used in the conjugation attempt:

$$Conjugation\ efficiency = \frac{Exconjugants}{Spore\ CFUs}$$

Intergeneric conjugation depends on both donor *E. coli* numbers and available recipient numbers. Thus, for each conjugation experiment we calculated donor-normalized conjugation efficiencies by the following relationship, where *Absolute donor count* is the actual number of *E. coli* cells in a conjugation experiment and *Conjugation efficiency* was obtained as above:

$$Donor-normalized\ conjugation\ efficiency = \frac{Conjugation\ efficiency}{Absolute\ donor\ count}$$

Finally, to make our numbers comparable with previous works (ie [10]) and because certain experiments required donor dilutions to make exconjugants countable, we report all donor-normalized conjugations adjusted to 10^8 theoretical donor cells for all experiments.

ϕOZJ insertion site mapping

To elucidate the integration site of ϕOZJ, pAdB02 was first conjugated into *S. roseosporus.* A 15 mL TSB culture was prepared from a single exconjugant, the cells were harvested via centrifugation, and genomic DNA was extracted using a Qiagen® DNEasy Blood and Tissue Kit. Resulting genomic DNA containing the integrated plasmid was digested with AatII, which lacks recognition sites in pAdB02. The digested DNA was then self-ligated using T4 DNA ligase and transformed into WM3618. A recovered plasmid harboring the cloned *attL* and *attR* sites was sequenced using primers AdB19 and AdB20, which flank the predicted *attP* site of pAdB02. The resulting Sanger reads were mapped to the *S. roseosporus* NRRL 15998 genome by BLAST to deduce ϕOZJ *attB.*

Plasmid Stability Tests

PCR-confirmed *S. venezualae* transformants were re-streaked twice onto selective ISP2 agar medium. Single resulting colonies were then used to inoculate 50 ml Falcon tubes containing 5 ml TSB without selective antibiotic and incubated at 30°C in a shaker at 250 RPM with 1-inch throw for 2 days. This culture was serially diluted and plated on ISP2 agar plates without selective antibiotic in 100 ul aliquots and grown for 4 days at 30°C. Twelve resulting colonies originally harboring pJMD5, pJMD13, pJMD14 or pBYK01 were randomly chosen for PCR re-verification of plasmid maintenance. Specific methods for exconjugant PCR verification are listed in the Supplementary Methods online.

Results and Discussion

Bioinformatic identification of putative DNA recombinases and *attP* sites in phage ϕOZJ and ϕWTR

Several large-serine DNA recombinases sourced from actinophage have been exploited for molecular biology[19,1]. Towards identifying additional enzymes of this type for molecular toolset development, we engaged in a genome-mining approach. We analyzed the unannotated genome sequences of phage OzzyJ **(**ϕOZJ**)** and Whatever **(**ϕWTR**)** via BLASTX for the presence of large serine recombinases using ϕC31 and ϕBT1 homologs as queries. Isolated from local soils by Washington University in St Louis undergraduate students, the ϕOZJ and ϕWTR genomes were found to each harbor a single recombinase-encoding *int* gene. The translated *int* sequences from ϕOZJ and ϕWTR were further interrogated by BLAST and pairwise comparisons, revealing both translation products are highly similar to each other and several uncharacterized integrases found in actinophage of the BD1 subcluster (Fig. 1a). As expected for large serine integrases, each translation product was found to encode a conserved catalytic serine residue, identified by alignment against several apparent BD1 phage orthologs (Fig. 1b). Although the molecular biology of BD1 phage remains largely unexplored, an *int* locus from BD1 member ϕJoe was recently described by Fogg et al [2]. Further, this locus was exploited to create the new chromosomally-integrating vector pCFM92. While this supported the idea that the *int*-encoded enzymes of ϕOZJ and ϕWTR could be potentially exploitable DNA recombinases, it raised the question if they were functionally

distinct from ϕJoe. Protein alignments indicate the integrases of ϕOZJ and ϕWTR are more similar to each other (83% amino acid identity) than either is to ϕJoe (73/74% identity, respectively).

Leveraging the known *attP* of ϕJoe, we identified putative *attP* sites for ϕOZJ and ϕWTR by sequence similarity. From this, it appears the *attP* sites of all three phage occupy the same relative chromosomal position directly upstream of each phage's *int* gene. Each of the examined *attP*s are characterized by similar inverted sequence repeats (Fig. S2). We extended these observations to other genome-sequenced members of the BD1 phage group (ϕBrataylor, ϕLika, ϕRana, and ϕToma), finding similar sequence and repeat structures in their predicted *attP* regions (Fig. S2). Notably, for a number of the examined phage, subtle nucleotide differences mapping across their *attP-RDF* regions were observed (Figs. S2 and S3). How these observed differences might affect recombination activity, host range, and target *attB* specificity remained to be examined.

ϕOZJ and ϕWTR recombinase activity, *in vivo attP/attB* characterization, and host range

To investigate how *attP-int* region nucleotide heterogeneity in BD1 phage affect recombination and *attB* sequence tropism, we cloned the putative *attP-int* regions of ϕOZJ and ϕWTR into the conjugative suicide vector pOJ260[4]. Resulting in plasmids pAdB02 and pAdB04, respectively (Table S3), both vectors were tested for chromosomal integration in six *Streptomyces* hosts: *S. albus, S. coelicolor, S. venezuelae, S. pristinaespiralis, S. roseosporus* and *S. viridochromogenes* (Fig. 2a). After transforming these hosts via intergeneric conjugation, we found the two plasmids to have strikingly different host ranges and conjugal efficiencies (Fig. 2a). Specifically, pAdB02 (ϕOZJ-derived) transformed all six of the tested recipients while pAdB04 (ϕWTR-derived) only transformed two hosts (*S. venezualae* and *S. albus)*. Because unmodified pOJ260 cannot stably transform *Streptomyces*, these findings suggested both of the tested *attP-int* regions are functional, yet clearly distinct.

To investigate the apparent integrative disparity between pAdB02 and pAdB04, first we mapped the *attB* site of each plasmid. For pAdB02, the apparent ϕOZJ *attB* site was determined in *S. roseosporus* via plasmid rescue (described in the Methods). Sequencing the resulting cloned *attL* and *attR* sequences revealed that pAdB02 inserts into SSIG_RS23460, a gene encoding a putative B-12-binding radical SAM enzyme (Fig. S4). In the genome of *S. roseosporus* NRRL 11379 (~7.85 Mbp total length), ϕOZJ *attB*

maps near position 2165400. This orients it on the chromosome upstream of both ϕC31 *attB* (~1.56 Mbp downstream from ϕOZJ *attB*) and ϕBT1 *attB* (~2.74 Mbp downstream from ϕOZJ *attB*).

Because *S. roseosporus* was unable to be transformed by pAdB04, we obtained exconjugants for both pAdB02 and pAdB04 in the permissive host *S. venezualae* and monitored for plasmid insertion into SVEN_2383, a putative ortholog of SSIG_RS23460. We found both pAdB02 and pAdB04 target the same apparent *attB* site within SVEN_2383, which is also the known target of ϕJoe-derived pCMF92 insertion[12]. Together, this indicates a common *attB* tropism for ϕJoe, ϕOZJ and ϕWTR despite each phage having *attP* region sequence polymorphisms. We extended this investigation to explore pAdB02 in the amenable hosts *S. albus*, *S. viridochromogenes* and *S. pristinaespiralis,* confirming it also inserts into homologous *attB*s within orthologous host genes AOK06_RS13485, ADK35_RS12140 and SSDG_02763, respectively, via targeted PCR and amplicon sequencing (Fig. S5, also Supplementary Methods).

Encouraged by ϕOZJ *attP-int* directed pAdB02 insertion in a variety of *Streptomyces*, we created a new shuttle vector, pJMD9, based on these phage functions. Useful features of this plasmid include an apramycin resistance gene (*aac*(3)IV) for *E. coli* and *Streptomyces* selection, *oriT*^{RP4} for intergeneric conjugation, and a P*ermE** promoter fused to multiple cloning site flanked by transcriptional terminators for recombinant gene expression. Our investigations above confirmed ϕJoe, ϕOZJ and ϕWTR share the same *attB* site but were unable to explain the basis of our initial pAdB02 vs pAdb04 (Fig. 2a) host preference observations. Therefore, we created several pJMD9-derivative vectors that differ from the parent plasmid only in their cloned BD1 *attP* regions (pJMD9-Brataylor, pJMD9-Joe, pJMD9-Lika, pJMD9-Rana, pJMD9-Toma, pJMD9-WTR; Table S3) because we surmised subtle nucleotide heterogeneity in the *attP* region of each plasmid might influence host compatibility. We tested the ability of all of these pJMD9 variants to transform *S. coelicolor, S. roseosporus, S. venezuelae, S. pristinaespiralis, S. viridochromogenes, and S. albus* via conjugation. We found that each pJMD9 *attP* derivative successfully transformed each recipient at similar frequencies (Fig. 2c). This result was somewhat unexpected because we predicted pJMD9-WTR, having the same *attP* as pAdB04, would fail to transform most of these hosts as noted in Fig. 2a. Thus, instead of supporting *attP* heterogeneity as host compatibility

determinant, our data instead implicate yet-uncharacterized differences between ϕOZJ and ϕWTR integrases as the most parsimonious explanation.

During conjugation experiments using pAdB04 and the pJMD9-derived vectors, we noted a multifold decrease in exconjugant recovery in *S. coelicolor* compared to other tested hosts (Fig. 2a and 2c). Previous ϕJoe studies revealed *S. coelicolor*'s BD1 *attB* site is occupied with an ancestral (SCO2603-encoding) phage fragment[12]. The putative $attL^{Sc}$ and $attR^{Sc}$ sequences of this phage-derived element are permissive for ϕJoe *attP* recombination, albeit at a far lower frequencies than *S. venezualae attB*[12]. Because we deduced that ϕJoe, ϕWTR and ϕOZJ target the same *attB* site in multiple strains, we surmised the lowered conjugation efficiency in *S. colicolor* for plasmids constructed from the latter phage would be similarly explained. Using PCR and sequencing we confirmed each of our pJMD9-family vectors indeed target $attR^{Sc}$ (Fig. S6), but no integration was detected at $attL^{Sc}$. The reason why no integration was detected at the latter site remains unknown, but only 2-3 exconjugants for each plasmid variant were analyzed, perhaps overlooking rarer events.

Construction of versatile integrating vector pJMD14, RDF-less pBYK01, and comparative survey of integrating plasmid host range

To enable multiplex conjugation, the simultaneous transformation of multiple plasmids into a single host (see text further below), we created plasmids pJMD14 and pBYK01. Specifically, plasmid pJMD14 was created from pJMD9 by replacing the *E.coli* $oriR^{colE1}$ with that of p15A, and the *aac*(3)IV marker was replaced with neo^{Tn5} for kanamycin resistance (Fig. S7). Plasmid pBYK01 is a derivative of pJMD14 that lacks a short ORF proximal to *attP* that encodes a putative recombination directionality factor (RDF) protein (Fig. 2d, Fig. S8). The latter plasmid was made because prior ϕJoe *in vitro* work indicates RDF (in stoichiometric excess) interacts with recombinase to drive *attL/attR* recombination towards excision[12].

Preliminary stability tests comparing both pJMD14 and pBYK01 were performed by dilutive growth of ~20 individual *S. venezualae* exconjugants in the absence of antibiotic pressure. Subsequent PCR tests to confirm *attL* and *attR* status in each were performed (Fig. S9) and no discernable instability for either plasmid was observed at this scale. Conjugation frequency tests into multiple *Streptomyces* recipients (Fig. S10) also confirm the two plasmids have similar rates of inheritance. Thus the presence or

absence of RDF in pJMD14 and pBYK01 did not seem to effect stability or transformation during initial characterization.

Excision control for BD1 phage derived plasmids *in vivo* remains enigmatic, and mechanistic understanding of recombination directionality is important for the biotechnological use of phage-derived vectors. Our *in silico* analyses suggest RDF transcription is likely controlled by the promoter of the gene harboring the phage's *attB* site (Fig. 3a and 3b). Multiple lines of evidence support this idea. First, we noted that each examined BD1 subgroup phage apparently encodes a sequence-conserved alternate carboxyl-terminus of the ORF disrupted by *attP-attB* recombination (Fig. 3c) and these short ORF fragments are fused in-frame with *attP*. Thus, we predict two important consequences of *attP-attB* recombination. The first is the creation of a two-gene operon, where the *attB*-encoding gene is arranged directly upstream of RDF. Evidence for operon formation comes from the fact that the stop codon of the first gene overlaps with the start codon of RDF. The second consequence is that the *attB*-encoding gene loses its native carboxyl terminus, which could theoretically impair native function. However, due to the arrangement of the *attP*-overlapping ORF terminus inherited from the phage chromosome, the *attB*-inserted gene gains a new phage-encoded terminus, resulting in a short chimera on the 3' end (Fig. 3c). The observation that phage sometimes encode gene fragments that are spliced into host chromosomes upon *attB-attP* recombination is well documented, but this possibility for BD1 group phage had remained undiscovered. Our data also strongly suggest that BD1 group phage lysogeny/ lysis cycles may be directly impacted by the regulation of their cognate *attB*-harboring host genes.

Following the successful transformation of *S. venezualae, S. pristinaespiralis and S. roseosporus* with pJMD14 (Fig. 4b), we examined the plasmid's host range in 30 additional *Streptomyces* and related actinomycetes (Tables S1, S4-5). For this, we employed a diverse assemblage of strains obtained from both public culture collections (n=19) and environmental isolations (n=11). Plasmid pJMD14 conjugal transformation of these strains was carried out in parallel with two other plasmids, pJMD13 and pJMD5, that incorporate well-characterized integration functions from ϕC31 and ϕBT1, respectively (Table S3, see Fig. S11 and S12 for cloning schemes). This was done to understand the relative utility of ϕOZJ *attP-int* for future strain engineering against widely established toolsets. Overall, we found ϕC31-derived pJMD13 transformed the most strains (29/30), followed by pJMD13 (ϕBT1, 22/30). Plasmid pJMD14 transformed

the least strains (ϕOZJ, 14/30; Table S5). A few of the transformation failures for pJMD13 and pJMD14 resulted from endogenous antibiotic resistance for the selective agents used. These infrequent (4 total) instances aside, our overall findings suggest ϕOZJ tools are unlikely to supplant ϕBT1 or ϕC31 analogs in first-line actinomycete bioengineering efforts. However, several tested strains (9/30) could accept all three tested vectors.

Counted among the strains transformed by ϕOZJ- derived plasmids are several important producers of clinical antibiotics and commonly used heterologous hosts. Specifically, we showed pJMD9 transforms *S. albus* J1074, an important synthetic biology chassis capable of accepting ϕBT1 and ϕC31 -based plasmids[23,32]. Further, pJMD5, pJMD13, and pJMD14 all transformed *S. venezualae*[25], another important synthetic biology host, as well as drug-producing *S. roseosporus*, *S. pristinaespiralis,* and *S. nodosus.* The latter strains are noted producers of daptomycin and related analogs[20,2]), pristinamycin[16]), and amphotericin[33], respectively. Our summed transformation data thus reveal clear potential for ϕOZJ- derived plasmids during multiplex engineering of select industrially- important *Streptomyces*, as well as a number of environmental isolates.

Multiplex Conjugation

RP4-mediated intergeneric conjugation is a powerful method for transforming DNA into diverse bacterial lineages[5,17]. Typically, this process exploits a specialized *E. coli tra*$^{+}$ donor to conjugally transfer a single vector per experiment into a desired recipient strain (Fig 4a, top). To our knowledge, the simultaneous transfer of multiple plasmids in a single experiment via conjugal transfer has never been demonstrated. However, we surmised this might be possible if a single *E. coli* donor, carrying multiple plasmids with orthogonal selection markers, origins of replication, and phage-derived integration systems, was used (Fig 4a, bottom). Plasmids pJMD5, pJMD13 and pJMD14 meet these criteria, and the conjugal donors (*E. coli* strains JV36 and JV156) are able to host all three vectors concomitantly. Thus, we attempted the conjugal transformation of these three plasmids individually, in pairs, or all three at once into *S. pristinaespiralis, S. venezuelae,* and *S. roseosporus.* These recipients were chosen because they are efficiently transformed during single-conjugation experiments by each of our ϕC31, ϕBT1, and ϕOZJ-derived plasmids.

Remarkably, we found all attempted combinations of plasmids successfully transformed each of the test recipients (Fig. 4b). Broadly, we found that the conjugal transfer of a single plasmid is most efficient, but pairwise and triple plasmid multiplex transformation was possible with a notable loss in transformation efficiency (10-100X). Despite this, desired double or triple plasmid transformants were reproducibly recovered through these experiments. After single-colony purification in the presence of apr, kan and thio, the integration of each plasmid in all three recipients was confirmed by PCR detection of expected *attL/attR* formation (8/8 colonies tested have expected plasmid inheritance for recipient strain tested).

The mechanism by which multiplex plasmid transformation of recipient cells is achieved remains unknown. An attractive hypothesis is that the conjugation apparatus from a single donor cell is used to repeatedly transfer multiple plasmids. An alternate mechanism could involve multiple independent conjugation events, implicating several donor cells, crossing with a single recipient. Two experiments were performed to test these ideas. One assessed the ability of a mixed donor population (involving three JV36 parents independently carrying pJMD5, pJMD13 or pJMD14, mixed in equal proportion, and normalized to the total donor count used in the multiplex experiments above) to transform *S. venezualae*. This tetra-parental mating attempt failed to yield multiplex transformants, as did a parallel experiment that was identical in all parameters except the individual donor strains were three times concentrated before use. Because these mixes of individual donors, even at elevated concentration, could not replicate multiplex donor transformation, our summed data seemingly favor the multiple-transfer hypothesis mentioned above (illustrated in Fig. 4a). Further investigation is warranted to reveal absolute donor multiplicity and mechanism.

In summary, we assessed the integration functions cloned from two closely related actinophage, ϕOZJ and ϕWTR, for their potential in site-specific vector design. A series of integration experiments testing the native *attP-int* loci of these phages, as well as a panel of *attP*-refactored derivatives, revealed ϕOZJ *attP- int* to most efficiently transform several tested *Streptomyces.* While we found our plasmids based on these functions to transform fewer total strains than comparable plasmids having ϕC31 or ϕBT1 functions, several biotechnologically important strains were able to accept ϕOZJ- based plasmids. Importantly, we found our newly developed ϕOZJ- based plasmids to be useful for demonstrating that

multiplex conjugation-based plasmid transformation is indeed possible, a technique that transfers multiple plasmids into a recipient cell during a single mating experiment. Together, this work contributes new site-integrating plasmids for *Streptomyces* chromosomal insertion that function in the presence or absence of orthogonal integrating plasmids. Critical for developing multiplex conjugal transformation as a new method for rapid strain construction, the new vectors and methods described here should prove useful for significantly shortening strain construction timelines, easing combinatorial gene expression, and enabling other multi-plasmid applications in *Streptomyces* biotechnology.

Acknowledgements

We are indebted to WUSTL faculty C. Schafer and K. Hafer for generously providing ϕWTV and ϕOZJ lysates and assembled genome sequences prior to public release. We are grateful to W. Metcalf (U-Illinois, Urbana) for pBTC034 and R. Baltz (Cognogen Bioconsulting) for *Streptomyces griseofuscus*. We thank former WUSTL Biol3493 students K. Lou, J. Alex and B. Makhdoom for designing the multiplex screening primers for ϕBT1-dependent plasmid integration and assisting in the isolation of the environmental strains used in this work. This material is based upon work supported by the National Science Foundation under NSF-CAREER 1846005 to J Blodgett.

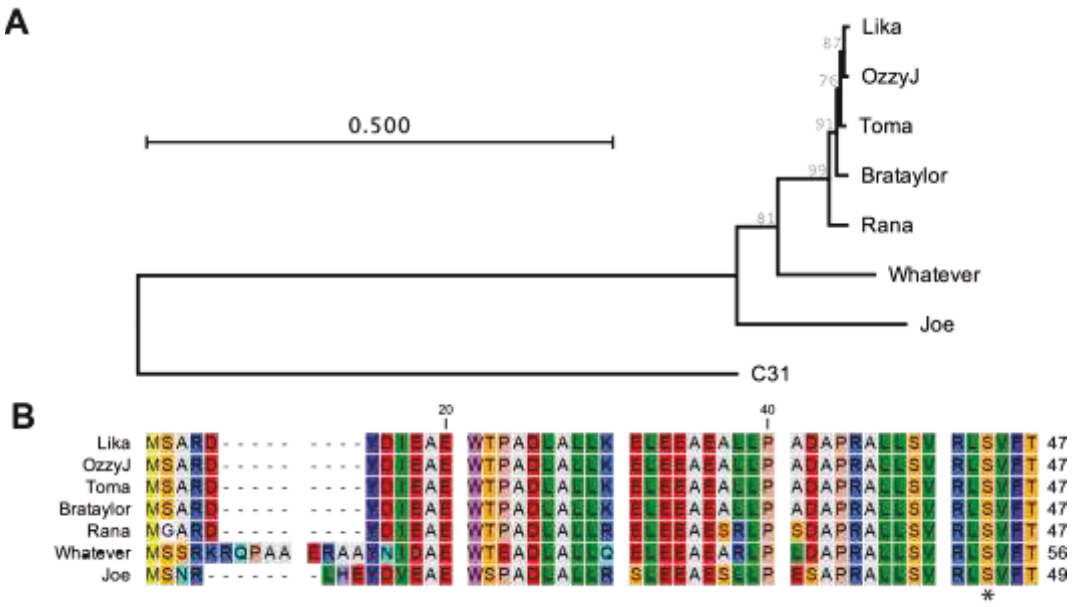

Fig. 1 Characterization of BD1 clade integrases. **a** Phylogram of BD1 clade phage large serine integrases. The large serine integrase of phage ϕC31 serves as an outgroup. A ClustalO alignment of BD1 family phage integrase sequences was imported into CLC Main Workbench 8.1 and the resulting tree was constructed by the Neighbor-Joining method. Bootstrap values resulting from the construction of 500 trees label each node. **b** Partial alignment of BD1 clade phage integrases, identifying the putative catalytic serine (marked with *) previously identified for ϕJoe[12].

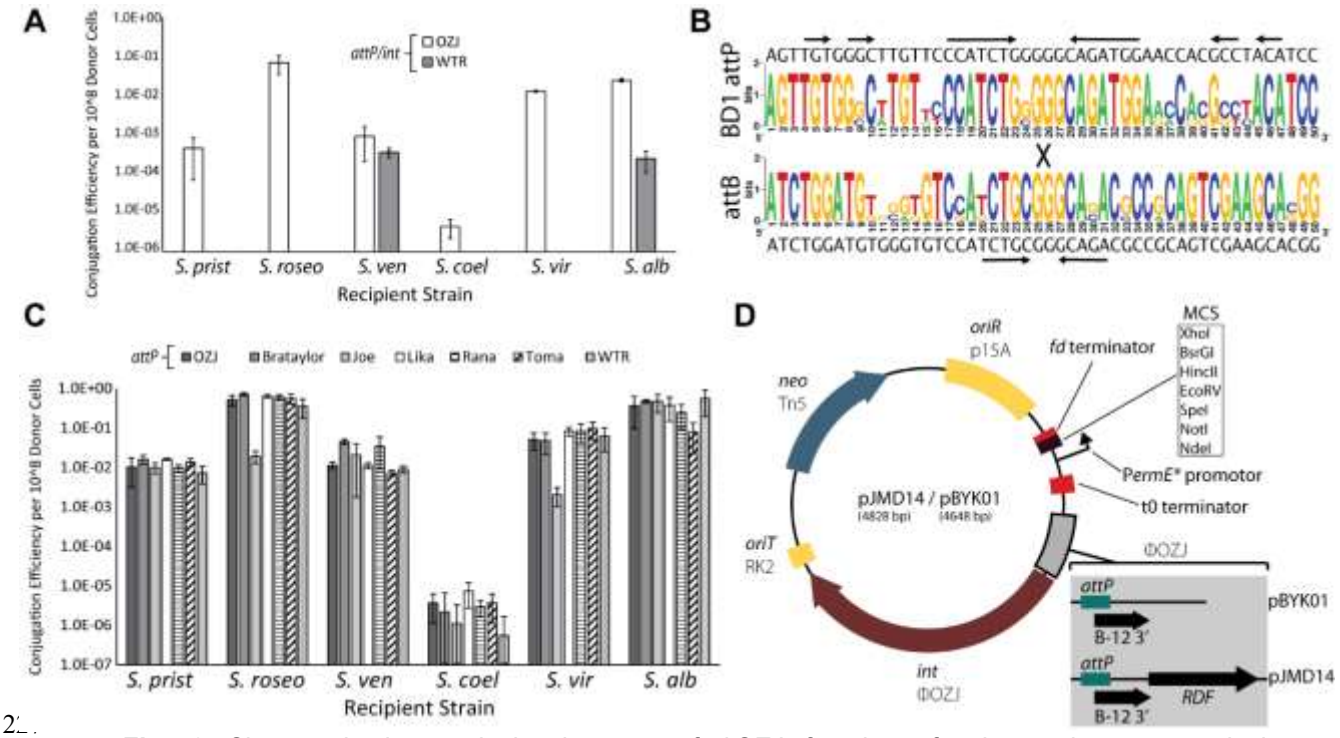

Fig. 2 Characterization and development of ϕOZJ functions for integrating vector design. **a** *Streptomyces* conjugal transformation with plasmids harboring *attP/int* pairs cloned from BD1 clade phage ϕOZJ (pAdBO2) and ϕWTR (pAdB04), expressed as donor-normalized conjugation efficiencies; **b** Consensus sequence of *attP*s from BD1 phage group members ϕOZJ, ϕBrataylor, ϕJoe, ϕLika, ϕRana, ϕToma, and ϕWTR aligned with corresponding consensus of *attB's* from *S. pristinaespiralis, S. roseosporus, S. venezuelae, S. viridochromogenes* and *S. albus*. The arrows mark inverse repeats and the **X** marks the crossover point (O site) previously predicted for ϕJoe[12]. Alignments used to generate the logo plots use here are in Figs. S2 and S5. **c** Donor-normalized conjugation efficiencies of plasmids harboring ϕOZJ *int* paired with various BD1 actinophage *attP* sequences; **d** Plasmid map of ϕOZJ *attP-int* dependent expression vectors pJMD14 and pBYK01. For panels **a** and **c**, error bars represent the standard deviation of three biological replicates.

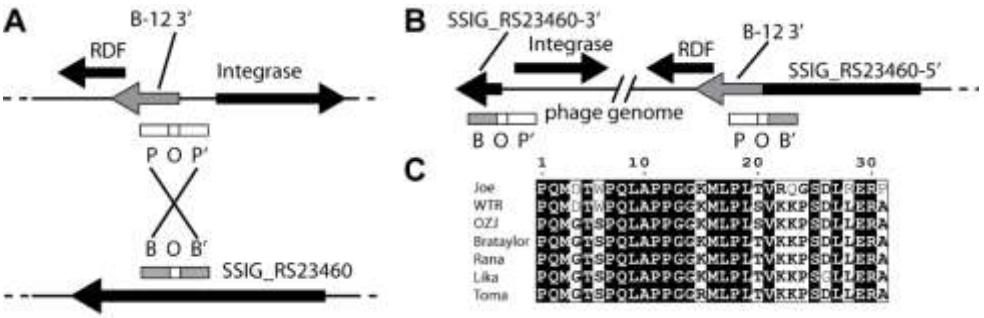

Fig. 3 Scheme for BD1 clade phage insertion into *S. rososporus attB* and resulting operon formation with RDF. **a** Chromosome of BD1 clade phage *attP* (POP', top strand) into *S. roseosporus attB* within SSIG_RS23460 (bottom strand). Strand exchange takes place at the O site; note the location of the phage-encoded 3' gene fragment (B-12 3') that encodes an alternate carboxyl-terminus for the ORF disrupted by phage insertion that overlaps *attP*. Only the *attP-int* phage chromosomal region is shown, the rest is omitted for clarity. **b** The genomic architecture of *S. roseosporus* following *attB/attP* recombination in **a**. The phage encoded partial orf (B-12 3') is now fused in-frame with the majority of the amino-terminus of SSIG_RS23460 resulting in a new chimeric protein. The gene encoding this protein has start/stop codon overlap with RDF, implying coupled transcriptional control from the SSIG_RS23460 promoter. **c** Aligned peptide sequences of the sequenced-conserved phage-encoded B-12 3' fragment encoded adjacent to several BD1 clade actinophage *attP* sites. These sequences correspond with grey-shaded carboxyl termini of chimeric SSIG_RS23460 in **a** and **b**.

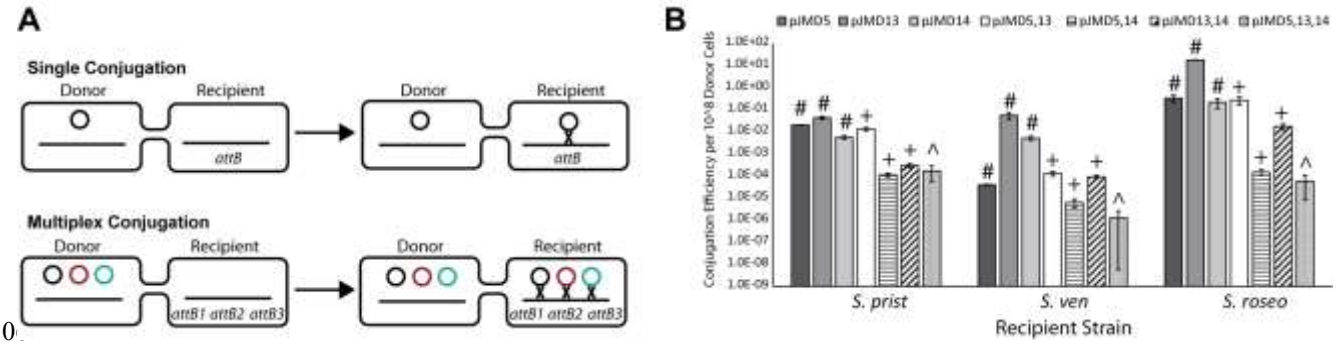

Fig. 4 *Streptomyces* multiplex transformation via intergeneric conjugation. **a** Conceptual depiction of a (top) standard conjugation with a generic phage derived plasmid vs (bottom) a multiplex conjugation (simultaneous conjugation of two or more functionally orthogonal phage derived plasmids); **b** Donor normalized conjugation efficiencies of single and multiplex conjugations with *E. coli* carrying one, two, or three plasmids, ϕBT1 (pJMD5), ϕC31 (pJMD13), ϕOZJ (pJMD14). Conjugation experiments testing transfer of one (#), two (+), or three (^) plasmids from a single parental donor strain are annotated above with appropriate symbols for clarity. Error bars represent the standard deviation of three biological replicates

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