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**Title**
Rapid metabolic pathway assembly and modification using serine integrase site-specific recombination.

**Permalink**
https://escholarship.org/uc/item/6v09v9xf

**Journal**
Nucleic acids research, 42(4)

**ISSN**
1362-4962

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**Publication Date**
2014-02-01

**DOI**
10.1093/nar/gkt1101

Peer reviewed
Rapid metabolic pathway assembly and modification using serine integrase site-specific recombination

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Received September 26, 2013; Revised October 17, 2013; Accepted October 18, 2013

ABSTRACT

Synthetic biology requires effective methods to assemble DNA parts into devices and to modify these devices once made. Here we demonstrate a convenient rapid procedure for DNA fragment assembly using site-specific recombination by ϕC31 integrase. Using six orthogonal attP/attB recombination site pairs with different overlap sequences, we can assemble up to five DNA fragments in a defined order and insert them into a plasmid vector in a single recombination reaction. ϕC31 integrase-mediated assembly is highly efficient, allowing production of large libraries suitable for combinatorial gene assembly strategies. The resultant assemblies contain arrays of DNA cassettes separated by recombination sites, which can be used to manipulate the assembly by further recombination. We illustrate the utility of these procedures to (i) assemble functional metabolic pathways containing three, four or five genes; (ii) optimize productivity of two model metabolic pathways by combinatorial assembly with randomization of gene order or ribosome binding site strength; and (iii) modify an assembled metabolic pathway by gene replacement or addition.

INTRODUCTION

Synthetic biology seeks to engineer new function in biological systems for a variety of different applications. A key limitation in synthetic biology is the time taken to assemble genes or other DNA parts into new devices, pathways and systems, and to alter these assemblies once made. The current methods for gene assembly and systematic variation of DNA parts are far from ideal (1). Existing assembly methods use restriction enzymes and DNA ligases to join DNA segments together, either sequentially (2,3) or in a single reaction (4,5), or use homology-dependent assembly in vitro (6–9) or in vivo (10–13). The junction sequences left by these methods make it difficult to change individual parts of the assembly without starting the assembly process from the beginning again. Furthermore, most of these methods cannot produce large libraries of variant assemblies from large numbers of DNA parts for use in combinatorial assembly strategies. Here we report the development of a highly efficient, rapid and easy recombinational assembly method (SIRA, serine integrase recombinational assembly) that uses ϕC31 integrase to assemble pre-designed pathways and devices from DNA parts. We illustrate the use of SIRA to assemble functional metabolic pathways from individual gene cassettes. Once assembled, genes or other DNA elements can be replaced, deleted or added at will. The assembly process can easily incorporate multiple variants at all gene positions, randomize gene order and vary expression levels, allowing rapid pathway optimization.

MATERIALS AND METHODS

Bacterial strains and growth

Routine bacterial growth was in LB medium (14), with agar (15 g/l) and antibiotics (ampicillin 100 μg/ml, chloramphenicol 25 μg/ml, kanamycin 50 μg/ml) added as
required. *Escherichia coli* TOP10 (Life Technologies) was used for routine transformation and maintenance of plasmids. *E. coli* DB3.1 carrying the gyrA462 mutation (15) was used for propagation of plasmids containing the *ccdB* gene. *E. coli* DS941-Z1, carrying the lacI and *tetR* genes from DH5αZ1 (16) P1 transduced into DS941 (17), was used for expression of the violacein biosynthetic pathway.

*E. coli* cells were made chemically competent with 50 mM calcium chloride and transformed using standard methods (14). Transformation routinely yielded between $2 \times 10^6$ and $6 \times 10^6$ transformants per μg pUC19 plasmid DNA. Electrocumpetent TOP10 *E. coli* were prepared by repeated washes in ice-cold 10% glycerol (14) and electroporated using a Biorad micro pulser apparatus set on EC2 (2.5 kV, 5.9 ms time-constant), in a 2 mm cuvette according to the manufacturer’s instructions. Electroporation routinely yielded $\sim 1 \times 10^8$ transformants per μg pUC19 plasmid DNA.

*Pantoea ananatis* (DSM number 30080; formerly *Erwinia uredovora*), containing the carotenoid biosynthetic gene cluster (Genbank accession number D90087) and *Chromobacterium violaceum* (DSM number 30191), containing the violacein biosynthetic gene cluster (Genbank accession number NC_005085 coordinates 3558964-3566288) were obtained from the DSMZ German collection of microorganisms and cell cultures. Genomic DNA was purified from these organisms using the QIAGEN Gentra Puregene kit according to the manufacturer’s instructions for Gram-negative bacteria.

**Plasmid construction**

The plasmids used in this study are shown in Table 1. Oligonucleotides used in their constructions are shown in Supplementary Table S1.1. Biobricks were obtained from the registry of standard biological parts (http://parts.igem.org). Full plasmid sequences are available on request. pSIRA1 vectors were based on pSBIA3, an ampicillin-resistant pMB1 origin vector containing the P1010 *ccdB* biobrick (3), pSIRA1 was constructed by inserting *attB*-RX-TT top and bottom strands between *PstI* and *SpeI*. pSIRA2 was constructed in the same way with *attP*-RX-TT and *attP*-PS-TC oligo-nucleotides, whereas pSIRA3 used *attB*-RX-TT and *attP*-SP-TC oligonucleotides. *pL*-laco and *pL*-tetO promoters (16) were made by annealing *pL*-Eco top and bottom strands with either *pL*-tet or *pL*-lac top and bottom strands and inserted into the EcoRI site of pSIRA1 to produce pSIRA4 and pSIRA5, respectively. The *crtB*, *crtE*, *crtI* and *crtY* genes were amplified together with designed ribosome binding sites (RBS) by PCR using Phusion polymerase (New England Biolabs) and the primers shown in Supplementary Table S1.1. Biobricks were obtained from the registry of standard biological parts (3) and inserted into pCR2.1-TOPO by TOPO-TA cloning (Life Technologies) to create pCM1, pCM2, pCM3 and pCM4, respectively. p-ccdB-CmR was constructed by inserting the P1004 chloramphenicol resistance biobrick as an XbaI-ccdB biobrick as an XbaI-ccdB biobrick as an XbaI-ccdB biobrick as an XbaI-ccdB biobrick.

**PCR**

Fragments for gene assembly were amplified by PCR using Phusion high-fidelity DNA polymerase (New England Biolabs) with the primers and templates shown in Supplementary Table S1.3. Reactions (50 μl) generally contained 1 μl HF Phusion buffer with an additional 0.5 mM MgCl$_2$, 200 μM of each deoxynucleotide triphosphate, 3% dimethyl sulfoxide, 500 nM of each primer, ~1 ng of plasmid template or 50 ng genomic template and 0.5 μl Phusion polymerase. Reactions were subjected to an initial denaturation step of 98°C for 1 min, followed by 30 cycles of 98°C for 20 s, 55°C for 30 s and 72°C for 60–120 s. After a final step of 72°C for 10 min, products were separated on 1% agarose gels in tris-acetate buffer [40 mM Tris base, 20 mM acetic acid, 1 mM ethyleneaminetetraacetic acid (EDTA)], stained with ethidium bromide, excised under long-wave ultraviolet illumination and purified using a QIAquick gel extraction kit (QIAGEN) according to the manufacturer’s instructions, with an extra wash step with 0.5 ml buffer PE. Purified PCR products were quantified by agarose gel electrophoresis with known DNA standards or by measuring UV absorbance at 260 nm.

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**Table 1. Plasmids used in this study**

| Plasmid name | Description | Resistance | Reference |
|--------------|-------------|------------|-----------|
| pCR*².1-TOPO | TOPO TA cloning vector | AmpE KanR | Life technologies |
| pSBIA3 | pMB1 ori biobrick cloning vector containing the P1010 *ccdB* cell death biobrick | KanR | Registry of biological parts (3) |
| pSW23 | suicide cloning vector oriV(R6Kgamma) | CmR | Demarre et al. (18) |
| pSW29 | suicide cloning vector oriV(R6Kgamma) | KanR | Demarre et al. (18) |
| pCM1 | *P. ananatis* *crtB* PCR product in pCR*².1-TOPO* | AmpR KanR | This work |
| pCM2 | *P. ananatis* *crtE* PCR product in pCR*².1-TOPO* | AmpR KanR | This work |
| pCM3 | *P. ananatis* *crtI* PCR product in pCR*².1-TOPO* | CmR | This work |
| pCM4 | *P. ananatis* *crtY* PCR product in pCR*².1-TOPO* | AmpR | This work |
| p-ccdB©-CmR | pSBIA3 with P1010 (*ccdB*) and P1004 (*CmR*) | CmR | This work |
| pSIRA1 | *attB*-TT *attB*-TC SIRA vector based on pSBIA3 | AmpR | This work |
| pSIRA2 | *attP*-TT *attP*-TC SIRA vector based on pSBIA3 | AmpR | This work |
| pSIRA3 | *attB*-TT *attP*-TC SIRA vector based on pSBIA3 | AmpR | This work |
| pSIRA4 | pSIRA1 with *pL*-tet upstream of *attP*-TT | AmpE | This work |
| pSIRA5 | pSIRA1 with *pL*-lac upstream of *attP*-TT | AmpE | This work |
Proteins

The φC31 integrase and the gp3-encoded recombination directionality factor (RDF) were purified as described by Olorunnijii et al (19). Proteins were diluted in a buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM DTT, 1 M NaCl and 50% (vol/vol) glycerol and stored at −20°C.

Assembly reactions

For SIRA reactions, each PCR fragment (final concentration ~5 nM) and vector (final concentration ~3 nM) were mixed together in a 20 μl reaction containing 10 mM Tris–HCl (pH 7.5), 5 mM spermidine-3HCl, 2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin and between 0 and 20% (vol/vol) ethylene glycol. Recombination was initiated by the addition of 2 μl of 2 μM φC31 integrase in protein dilution buffer. For attL × attR recombination, equal volumes of 4 μM integrase and 8 μM RDF were pre-mixed in protein dilution buffer and incubated on ice for 10 min, and reactions were started by adding 2 μl of this mixture. Reactions were incubated at 30°C for the indicated time (5 min–24 h) and then stopped by heating to 75°C for 10 min. For chemical transformation, 2 μl of heat-treated assembly reaction was added directly to 100 μl of competent E. coli cells. For electroporation, DNA was first precipitated by the addition of 2.2 volumes of 100% ethanol in the presence of 0.3 M sodium acetate and 1 μg of yeast tRNA (Sigma-Aldrich). Precipitated DNA was redissolved in 10 μl of distilled H2O, and 2 μl of this DNA was used to transform 30 μl of electropotent cells.

RESULTS

Recombinational cloning using φC31 integrase

Bacteriophage φC31 integrase inserts the phage genome into its host’s DNA by recombining specific ~46 bp attP and attB DNA sequences found in the phage and host genomes, respectively (20,21). Integrase makes staggered cuts on either side of a central two base-pair ‘overlap’ sequence in both attP and attB (22), exchanges the resulting half-sites and rejoins them to form two new sites, attL and attR (Figure 1A). Recombination between attP and attB is highly efficient both in vitro and in vivo. In the presence of integrase alone, the products of recombination between attP and attB (attL and attR) do not recombine further, and the reaction is highly directional (23). However, the addition of the phage-encoded recombination directionality protein (RDF) allows efficient recombination between attL and attR and inhibits recombination between attP and attB (24).

The central 2 bp overlap sequences of two sites must match perfectly for efficient recombination, but their precise sequence has little effect on the efficiency of recombination (25,26). Throughout this article, we refer to attP with the 2 bp overlap sequence 5′-TT on the top strand (and 5′-AA on the bottom strand) as attP TT and so forth. Thus, attP TT recombines efficiently only with attB TT, attP TC with attB TC and so on. The 2 bp overlap sequence also determines the polarity of attP and attB sites, so that for instance attP TT will recombine with a reversed attB AA site, because the 5′-TT on the top strand of attP TT matches the 5′-TT on the bottom strand of attB AA (25). Furthermore, att sites with symmetrical central 2 bp overlap sequences (e.g. attP AA and attB TA) will recombine in both possible relative orientations. Therefore, different matched attP/attB pairs that recombine only with each other in a defined orientation can be made using the non-symmetrical overlap sequences (top strand/bottom strand) 5′-TT/5′-AA, 5′-CT/5′-AG, 5′-GT/5′-AC, 5′-CA/5′-TG, 5′-CC/5′-GG and 5′-TC/5′-GA (Figure 1B, Supplementary Figure S1A). Because each attP site recombines with its matching attB site but not with any other, we refer to these matched att site pairs as ‘orthogonal’.

![Figure 1. The SIRA process.](image-url)
Table 2. Efficiency of SIRA reactions

| Number of fragments assembled | Pathway | Reaction time | Transforms per 20 µl reaction (CaCl2) | Transforms per 20 µl reaction (electrocompetent) | Transforms per µg vector DNA | Percent correctly assembled (%) |
|------------------------------|---------|---------------|--------------------------------------|-------------------------------------------------|-----------------------------|--------------------------------|
| 1                            | KanR    | 1 h           | \(2 \times 10^4\)                     | \(9 \times 10^5\)                               | \(8 \times 10^7\)            | 100                            |
| 3                            | crtBEI  | 4 h           | \(4 \times 10^4\)                     | \(8 \times 10^5\)                               | \(7 \times 10^6\)            | 59                             |
| 3                            | crtBEI  | 22 h          | \(1 \times 10^4\)                     | \(1 \times 10^5\)                               | \(9 \times 10^6\)            | 87                             |
| 4                            | crtBEIZY| 24 h          | \(9 \times 10^4\)                     | \(1 \times 10^5\)                               | \(9 \times 10^6\)            | 37                             |
| 5                            | crtBEIZY| 22 h          | \(9 \times 10^4\)                     | \(7 \times 10^5\)                               | \(6 \times 10^6\)            | 18                             |
| 5                            | violABCDE| 22 h         | \(2 \times 10^3\)                     | \(4 \times 10^3\)                               | \(4 \times 10^4\)            | 18                             |
| Replacement                  | crtBEI/ZY| 22 h         | \(9 \times 10^4\)                     | \(4 \times 10^3\)                               | \(1 \times 10^6\)            | 94                             |
| Addition                    | crtI-idi-dxs| 16 h        | \(7 \times 10^2\)                     | \(1 \times 10^5\)                               | \(3 \times 10^5\)            | 48                             |

\(^a\)Number of transformants obtained from a 20 µl assembly reaction introduced into CaCl2 TOP10 competent cells (~10^6 transformants per µg pUC19).

\(^b\)Number of transformants obtained from a 20 µl assembly reaction introduced into electrocompetent TOP10 cells (~10^8 transformants per µg pUC19).

\(^c\)Number of transformants obtained per µg of vector DNA using electrocompetent TOP10 cells (~10^8 transformants per µg pUC19).

\(^d\)Replacement of the crtI gene in the 5-gene crtBEIZY assembly with a Phusion-amplified crtI cassette, after first replacing crtI with the ccdB-Cm cassette.

\(^e\)Replacement of the crtI gene in the 5-gene crtBEIZY assembly with crtI, idi and dxs, after first replacing crtI with the ccdB-Cm cassette.

Using these six orthogonal recombination site pairs, we have developed a recombinational assembly and cloning strategy. This SIRA strategy uses plasmid vectors containing two att sites with different central dinucleotides, flanking a conditionally lethal ccdB gene. Recombination between the sites on the plasmid and matching sites at either end of a linear DNA fragment replaces ccdB with the linear fragment (Figure 1D). The product can easily be isolated by selection for loss of ccdB.

We have generated a set of pSIRA vectors containing different att sites and regulated promoters to express the inserted genes (Table 1).

We first demonstrated that purified ϕC31 integrase can be used for efficient insertion of a single linear DNA fragment into a plasmid vector by cassette exchange. A kanamycin resistance gene flanked by attP sites with two different central dinucleotides (attP<sup>TT</sup> and attP<sup>TC</sup>) was generated by PCR (Figure 1C) and mixed with pSIRA1, which carries attB<sup>TT</sup> and attB<sup>TC</sup> sites flanking ccdB (Figure 1D). After reacting for 1 h with ϕC31 integrase, the reaction products were electroporated into TOP10 <i>E. coli</i> cells and transformants were selected for resistance to ampicillin and then tested for resistance to kanamycin. All transformants tested (100/100) were resistant to kanamycin (Table 2; Supplementary Figure S2), confirming that the linear DNA fragment was efficiently exchanged into pSIRA1. This single-gene SIRA reaction routinely gave 8 × 10<sup>7</sup> colonies per µg of vector DNA, corresponding to 9 × 10<sup>8</sup> colonies from a single SIRA cloning reaction (Table 2). Analysis of plasmid DNA from a sample of colonies confirmed that they contained the kanamycin resistance gene from the PCR product in single copy with a defined orientation, as expected if each attP can recombine only with its matching attB site (data not shown). The reaction was efficient enough that calcium chloride transformation could be used instead of electroporation (Table 2), and a five-minute SIRA reaction was sufficient to clone a single DNA fragment (Supplementary Figure S2). Similar results were obtained with kanamycin or chloramphenicol resistance cassettes flanked by two attB sites or one attP and one attB site, with pSIRA2 and pSIRA3, respectively (Supplementary Figure S2).

A small number of colonies lacking the expected antibiotic resistance cassette were detected with pSIRA3 (containing attB<sup>TT</sup> and attP<sup>TC</sup>), suggesting a low level of intramolecular recombination between the mismatched attP and attB sites in the vector, leading to deletion of ccdB (Supplementary Figure S2).

Metabolic pathway assembly by SIRA

We designed SIRA schemes to assemble three, four or five DNA fragments (‘cassettes’) into a plasmid vector in a single recombination reaction (Figure 2B). Each cassette is flanked by either two attP sites or two attB sites, except in the four-gene strategy, where one cassette has one attP and one attB. The central overlap sequences of the att sites are chosen such that each site can recombine with only one other type of site in the mixture. Recombination between all matching attP-attB pairs joins the cassettes together end-to-end in a specific order and inserts the resulting linear assembly into the pSIRA vector, replacing ccdB (Figure 2B).

To test these SIRA schemes, we assembled functional metabolic pathways from multiple genes. The carotenoid biosynthetic pathway of <i>Pantoea ananass [E. uredovora]</i> produces zeaxanthin (yellow) via lycopene (red) and β-carotene (yellow–orange) (27). Three genes from <i>P. ananass</i> (crtE, crtB and crtI) are sufficient to produce lycopene in <i>E. coli</i>, four genes (crtE, crtB, crtI and crtY) produce β-carotene and five genes (crtE, crtB, crtI, crtY and crtZ) produce zeaxanthin (Figure 2A).

The five carotenoid biosynthetic open reading frames were each amplified by PCR, with designed RBS upstream of their ATG start codons and terminal attP or attB incorporated in the PCR primers. The combinations of DNA fragments required for three-, four- or five-gene assemblies were mixed together with pSIRA1. Following incubation with ϕC31 integrase, the reactions were electroporated into <i>E. coli</i> TOP10. Plasmids that
had lost the lethal **ccdB** gene, and therefore should contain the desired pathway, were selected by growth of transformants on medium containing ampicillin. The efficiency of assembly was measured as the percentage of coloured colonies obtained. As the number of fragments in the assembly was increased from three to five, both the number of colonies obtained and the efficiency of assembly decreased (Table 2; Figure 2). This decrease in efficiency was overcome by increasing the incubation time and adding ethylene glycol to the assembly reaction (Table 2; Supplementary Figure S3). A 22-h three-gene assembly reaction in the presence of 5% ethylene glycol yielded $1 \times 10^6$ colonies (7 $\times 10^6$ colonies/μg vector DNA), nearly 90% of which produced lycopene (red) and contained plasmid with the expected structure, as confirmed by restriction analysis and DNA sequencing of plasmid DNA. Four- and five-gene assembly reactions had similar colours, but restriction analysis confirmed that plasmids from these reactions all had the expected 4- and 5-gene structures.

![Figure 2](image_url)

**Figure 2.** Assembly of 3-, 4- and 5-gene carotenoid biosynthetic pathways. (A) The *E. coli* host genes *idi* and *dks* encode enzymes necessary for the production of IPP and DMAPP. IPP and DMAPP are converted to geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) by further *E. coli* enzymes. The products of the *crtE*, *crtB*, *crtI*, *crtY* and *crtZ* genes from *P. ananas* convert FPP via geranylgeranyl pyrophosphate (GGPP) and phytoene to the coloured carotenoids lycopene (red), β-carotene (yellow-orange) and zeaxanthin (yellow). (B) Assembly of the three-gene lycopene, four-gene β-carotene and five-gene zeaxanthin biosynthetic pathways by SIRA. The diagrams show the arrangement of **att** sites used on each fragment for ordered assembly of genes into pSIRA1. The photographs show cell pellets from one correctly assembled construct (coloured) and one incorrectly assembled construct (white) for each pathway. The percentages of transformant colonies that were coloured or white are shown below each pellet. Cell pellets from 4- and 5-gene assemblies had similar colours, but restriction analysis confirmed that plasmids from these reactions all had the expected 4- and 5-gene structures.

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**Substituting and adding genes at specific positions within the assembly**

The SIRA strategies described earlier in the text produce arrays of gene cassettes separated by **attL** sites, each with a different overlap sequence (Figure 3A). In the presence of integrase and RDF (φC31 Gp3) (24), each **attL** will recombine with a matching **attR** site. This recombination can potentially be used to replace any chosen DNA segment in the array. In practice, we found that direct replacement of a gene in the array by **attL**/**attR** recombination was less efficient than the original **attP**/**attB** SIRA reactions. We have observed DNA cleavage at **attL** sites in assemblies in the presence of integrase and RDF, presumably due to abortive recombination between mismatched **attB**TT and **attP**TC sites can lead directly to deletion of **ccdB** (Supplementary Figures S3).
attB \times attR reaction with integrase alone. Under these conditions, the remaining attL sites in the array are essentially inert and do not interfere with the reaction.

To illustrate how this procedure can be used to replace one gene with a library of gene variants, we replaced the wild-type crtI gene in the crtBEIZY zeaxanthin biosynthetic pathway with a mutagenized library of crtI genes. The crtI gene was first replaced with the ccdB-CmR cassette flanked by appropriate attR sites (Figure 3A), using \( \phi C31 \) integrase and RDF. Selection for chloramphenicol-resistant colonies in a ccdB-tolerant E. coli strain yielded hundreds of colonies. Ten of ten transformants examined carried the expected plasmid with crtI replaced by the ccdB-CmR cassette flanked by attB (Figure 3B). The ccdB-CmR cassette was then replaced by a Phusion- or Taq-polymerase amplified crtI cassette carrying appropriate attP sites in a standard SIRA reaction (Figure 3B), yielding \( >10^3 \) colonies for both Phusion and Taq (Table 2). In all, 94% of colonies obtained with the high-fidelity Phusion polymerase had a functional zeaxanthin biosynthetic pathway as detected by production of yellow-coloured colonies, whereas only 65% of colonies from the mutagenic Taq reaction were coloured. DNA sequencing revealed that the colourless colonies contained plasmids with the expected (crtBEIZY) structure, but with mutations in crtI consistent with error-prone PCR.

The attB sites produced by replacing one gene in an assembly with the ccdB-CmR cassette could also be used to incorporate a further three, four, or five genes, yielding seven-, eight- or nine-gene assemblies (Figure 3C). To demonstrate this, we took the zeaxanthin metabolic pathway in which crtI had already been replaced with the ccdB-CmR cassette (Figure 3B) and added three linear PCR products, containing the E. coli idi and dxs genes and a new copy of crtI (Figure 3C). The idi and dxs genes are required for the synthesis of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), precursors in the synthesis of carotenoids (Figure 2). Overexpression of these enzymes in E. coli is reported to increase the available pools of these intermediates and thus increase carotenoid yields (28–31). SIRA reactions with \( \phi C31 \) integrase efficiently assembled these three genes into the zeaxanthin biosynthetic pathway, yielding plasmids with eight genes in the expected order, with an insert size of 7.8 kb (Figure 3C; Table 2). E. coli cells containing the full eight-gene assembly grew more vigorously than those containing just crtBEIZY, and were more intensely coloured, suggesting an increased yield of zeaxanthin (Figure 3D).

Varying the order of genes in the assembly

The assemblies described here have an artificial operon structure, with all genes in the same orientation, transcribed from a single upstream promoter. In this structure, genes closest to the promoter will typically be expressed at a higher level than those further away (32). Therefore, it should be possible to adjust the expression levels of the individual genes in an assembly by changing the order of genes in the operon. To illustrate this we used SIRA to assemble the three-gene lycopene biosynthetic pathway in all possible gene orders. Versions of each lycopene biosynthetic gene (crtB, crtE and crtI) were produced by PCR, with different terminal att sites to specify position one, position two or position three in a three-gene assembly (Figure 4A). By reacting one crtB fragment, one crtE fragment and one crtI fragment with pSIRA1, we easily assembled all six possible orders of these three genes (Figure 4B). Overexpression of the lycopene
biosynthetic pathway has been reported to be toxic to *E. coli*, possibly because lycopene production diverts intermediates from essential metabolic pathways (33). Carotenoid yield and toxicity have also been reported to vary with temperature, with optimal lycopene production and lowest toxicity at 28–30°C (33–35). Assembly reactions for each gene order were transformed into *E. coli* TOP10, and transformants were incubated at either 28°C or 37°C. Assembly reactions for five of the six possible gene orders transformed *E. coli* efficiently at 37°C (Figure 4C), and 60–80% of transformant colonies were red (Figure 4D). One gene order (*crtIEB*) gave few colonies and a much lower frequency of red colonies when transformants were selected at 37°C (Figure 4C; D) suggesting that this gene order is toxic to *E. coli* at 37°C. To confirm this, the plating efficiency of cells containing each gene order in pSIRA1 was measured at 30 and 37°C. Gene orders *crtBIE* and *crtIEB* plated poorly at 37°C and gave noticeably smaller colonies than the other gene orders at 30°C (Figure 4E). There were also clear differences in lycopene production levels for the different gene orders; for example, *crtBIE* gave noticeably paler colonies than *crtEBI* at 30°C. Thus, changing the order of lycopene biosynthetic genes in pSIRA1 had clear phenotypic effects, and varying gene order could be a useful way to optimize production from biosynthetic pathways.

To select an optimal gene order, it might be advantageous to produce a library containing all possible gene orders. To do this, we reacted all nine *crt* gene cassettes (as described earlier in the text) with pSIRA1 in a single reaction. This reaction should produce assemblies of three genes in pSIRA1, with any one of the three *crt* genes at each position (27 combinations in total, of which 6 combinations will contain one copy of each gene; Figure 4A). When the reaction products were transformed into *E. coli*, ~28% of transformants formed red colonies at 28°C, roughly as expected (6/27 = 22%) (Figure 4D). We verified that plasmids isolated from red colonies contained all three lycopene genes in all possible orders, whereas plasmids from white colonies contained other combinations of three cassettes (e.g. *crtB*-crt*B*-crt*I*; data not shown).

**Optimization of biosynthetic pathways by assembly with degenerate RBS**

Another way to optimize expression levels of the different enzymes in a metabolic pathway is to vary the RBS of the
genes in the pathway. Because we had observed toxic effects with lycopene overproduction, we used a different pathway to illustrate this approach. The violacein biosynthetic pathway from the Gram-negative bacterium *C. violaceum* produces a purple compound derived from tryptophan in a pathway requiring the activity of five enzymes encoded by *vioA*, *vioB*, *vioC*, *vioD* and *vioE* (36,37). These genes range in size from 576 to 2997 bp, and the total pathway is encoded on 7.5 kb of DNA. A prototype violacein biosynthetic pathway was built by amplifying the five *vio* genes directly from *C. violaceum* genomic DNA, using PCR primers incorporating designed RBS sequences and the required *attP* or *attB* sites. The resulting gene cassettes were assembled directly into pSIRA1 using *C31* integrase and the total pathway is encoded on 7.5 kb of DNA.

Figure 5. Assembly of the violacein biosynthetic pathway with degenerate RBS sequences. (A) Degenerate RBS sequence used for each gene in the violacein biosynthetic pathway. To obtain an appropriate range of predicted RBS strengths (37), the RBS sequences for *vioA* and *vioD* had AAAAT and ACAAA, respectively (changed sequences are shown boxed), rather than AAAAA upstream of the ATG. (B) Structure of the assembled synthetic *vio* operon, transcribed from an IPTG-regulated *pL-lacO* promoter in pSIRA5. (C) Phenotype of colonies containing *vio* assemblies with different RBS sequences at each gene, in the absence or presence of 0.1 mM IPTG. The table shows the predicted RBS strengths (arbitrary units) (37) for each gene in the operon.

| RBS Strength | *vioA* | *vioB* | *vioC* | *vioD* | *vioE* |
|--------------|--------|--------|--------|--------|--------|
| 1223         | 3430   | 6157   | 1394   | 7372   |
| 105          | 1394   | 8313   | 161    | 7372   |
| 65           | 6157   | 432    | 158    | 850    |
| 131          | 1051   | 918    | 158    | 6737   |
| 1223         | 1452   | 217    | 390    | 14922  |
| 469          | 452    | 8313   | 15     | 2960   |
| 131          | 827    | 916    | 65     | 2960   |

DISCUSSION

We have shown here that *φC31* integrase can be used to rapidly insert one, three, four or five genes or other DNA fragments in any chosen arrangement directly into a SIRA plasmid vector. We note that other serine integrases could substitute for *φC31* integrase in all of the DNA manipulations shown here. Use of >2 integrases would allow the introduction of further orthogonal *attP*/*attB* pairs and would potentially allow the assembly of larger numbers of DNA segments in a single reaction.

The speed with which assemblies can be designed and produced by SIRA allows for rapid prototyping of metabolic pathways. Unlike other assembly methods, there is no need to subclone fragments into plasmid vectors before assembly or to remove internal restriction sites from within the DNA fragments. Assemblies can be easily modified by substituting ≥1 DNA segments with natural or artificial variants or by adding regulatory sequences or more genes at chosen locations.

The efficiency of SIRA facilitates the production of large combinatorial libraries with multiple variants of each DNA cassette at each position. One-fragment SIRA reactions routinely yield 8 × 10⁷ transformants per μg of vector DNA by electroporation (Table 2), higher than reported for homology-based cloning strategies such as Gibson assembly (7), SLiCE (8) and SLIC (6). Furthermore, the efficiency of SIRA holds up well compared with these methods as the number of DNA fragments is increased (Table 2).

The *attL* sites flanking the components of SIRA assemblies, each with different overlap sequences, could be used to move the complete assembly, or sub-assemblies, from one location to another. Site-specific recombination could shuttle assemblies between vectors with different antibiotic resistances, host ranges, or regulated promoters, or deliver metabolic pathways and devices to genomic ‘landing pads’ in chassis organisms.

Site-specific recombination has been used previously in a number of different approaches to insert DNA fragments into plasmid cloning vectors (39–43). However, to the best of our knowledge, this study is the first time that site-specific recombination has been used to assemble...
multiple genes into fully functional metabolic pathways. SIRA could easily be adapted for the assembly of synthetic genetic circuits or to generate translational fusions from multiple gene segments (e.g. to produce novel polyketide synthases from multiple enzymatic modules). Furthermore, SIRA is a highly flexible technology, which should be adaptable to many other applications in metabolic engineering and synthetic biology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Zhao Jia for constructing the DS941 Z1 strain and Liz O’Donnell for her expert technical assistance.

FUNDING

UK Engineering and Physical Sciences Research Council [EP/H019154/1 to S.J.R.]; the UK Biotechnology and Biological Sciences Research Council [BB/K003356/1 to W.M.S., BB/H005277/1 to M.C.M.S. and BB/J004561/1 to A.O.]; the National Science Foundation [0943392 to J.K.]; and the John Innes Foundation (to W.M.S., BB/H005277/1 to M.C.M.S. and BB/EP/H019154/1 to S.J.R.).

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