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Efficient transfer of two large secondary metabolite pathway gene clusters into heterologous hosts by transposition

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ABSTRACT

Horizontal gene transfer by transposition has been widely used for transgenesis in prokaryotes. However, conjugation has been preferred for transfer of large transgenes, despite greater restrictions of host range. We examine the possibility that transposons can be used to deliver large transgenes to heterologous hosts. This possibility is particularly relevant to the expression of large secondary metabolite gene clusters in various heterologous hosts. Recently, we showed that the engineering of large gene clusters like type I polyketide/nonribosomal peptide pathways for heterologous expression is no longer a bottleneck. Here, we apply recombineering to engineer either the epothilone (*epo*) or myxochromide S (*mchS*) gene cluster for transpositional delivery and expression in heterologous hosts. The 58-kb *epo* gene cluster was fully reconstituted from two clones by stitching. Then, the *epo* promoter was exchanged for a promoter active in the heterologous host, followed by engineering into the MycoMar transposon. A similar process was applied to the *mchS* gene cluster. The engineered gene clusters were transferred and expressed in the heterologous hosts *Myxococcus xanthus* and *Pseudomonas putida*. We achieved the largest transposition yet reported for any system and suggest that delivery by transposon will become the method of choice for delivery of large transgenes, particularly not only for metabolic engineering but also for general transgenesis in prokaryotes and eukaryotes.

INTRODUCTION

Transposable elements were discovered by Barbara McClintock in maize (1) and subsequently in the vast majority of cells from prokaryotes to eukaryotes (2–4). The simplest transposon is a segment of DNA flanked by sequences present as inverted repeats that are recognized by a corresponding transposase, which catalyzes transposition via a cut and paste mechanism. DNA transposons have been applied for gene delivery to insert transgenes at new locations in the genome (5). Unlike homologous recombination, transposition only requires the inverted repeats and does not require homology between the transposon and its target site (6,7). Transposon technology is now widely used for several applications, including *in vitro* mutagenesis for DNA sequencing or protein structural–functional studies, or *in vivo* insertional mutagenesis for functional gene analysis and gene transfer.

Unlike the severe limitations of size for transgenes in retroviral vectors, size limits for transposon vectors remain poorly defined. Only a few studies have examined the issue (8–12). These studies all report reductions of transpositional insertion frequencies with increasing size, with suggestions that efficiency might be dramatically or even exponentially sensitive to insert size. *In vitro*, transposition of DNA as large as 86 kb has been reported (13), but to our knowledge, there is no report in any system of successful transposition of a transgene larger than 20 kb into a heterologous host.

In many bacterial strains, transferring and integrating large sized DNA molecules into the chromosome is difficult because both homologous and random recombination efficiencies are low. Therefore, transposition technology

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could be an attractive method for transgene introduction, unless inherent size limitations restrict its utility.

The *mariner* family of transposable elements was first discovered in *Drosophila mauritiana* (2) and is named because it has spread to virtually all eukaryotic and some prokaryotic organisms. Mariner transposons are usually small elements around 1300-bp long with 27 bp inverted terminal repeats, which contain a single open reading frame encoding a transposase of 345 amino acids (14). The *mariner* family is most closely related to the Tc1 family of transposons found in nematodes, *Drosophila* and fish (14,15). The MycoMar transposable element (16), a mariner transposon, has been frequently used in Gram-negative hosts for genetic modification.

DNA cloning and mutagenesis are important technologies for molecular biology and the biosciences. Conventional DNA engineering tools like restriction enzymes and DNA ligases have been successfully used for several decades (17,18). However, these technologies encounter difficulties for engineering and mutating DNA molecules >15–20 kb. We pioneered a technique for DNA manipulation, now called recombineering or Red/ET recombination, which overcomes these obstacles (19–25). Recombineering is mediated through homologous recombination, which allows the exchange of genetic information between two DNA molecules in a precise, specific and faithful manner. These qualities are optimal for DNA engineering regardless of size. Furthermore, recombineering technology requires only short regions of sequence identity (~40–50 bp) for efficient homologous recombination. These homology sequences are short enough to be easily integrated into synthetic oligonucleotide (oligo) primers for PCR reactions (19–25).

Bioactive natural products are often synthesized by pathways of enzymes encoded in large gene clusters, such as the genes encoding megasynthetases of the polyketide synthase (PKS) and the nonribosomal peptide synthetase (NRPS) types. These pathways present enormous potential for combinatorial biosynthesis (26–28). However, complete PKS/NRPS gene clusters are often >30 kb. Modifying these large gene clusters by using conventional DNA engineering technology is difficult and time-consuming. Because recombineering has no size and site limit, it is an ideal technique for engineering large DNA molecules like these gene clusters. The myxochromide (*mchS*, ~30 kb) and myxothiazol (*mta*, ~60 kb) gene clusters from the myxobacterium *Stigmatella aurantiaca* have been engineered by using recombineering for heterologous expression in *Pseudomonas putida* and *Myxococcus xanthus* (29–31). These gene clusters were introduced into the chromosome of the heterologous host by conjugation or transformation and homologous recombination (29,31,32). Here, we extend and improve this metabolic engineering technology by showing that transposons are more efficient than other methods for achieving transgenic delivery, even for large gene clusters.

We describe engineering of two gene clusters for transposition in this article, the 30-kb myxochromide S (*mchS*) gene cluster (29–31) and the ~60 kb epothilone gene cluster (33–35). Epothilones are produced by the myxobacterium *Sorangium cellulosum* (33) and the gene cluster (*epo*) has been introduced into *Streptomyces coelicolor* (35), *M. xanthus* (36) and *Escherichia coli* (37). In each case, the cluster was introduced in at least two smaller pieces using laborious protocols based on DNA transformation. Here, the full-length *epo* gene cluster was first stitched together into a single plasmid and then the promoter was exchanged, followed by addition of a transpositional cassette, conjugation origin and selection markers. Afterwards, the full size gene clusters with all the necessary components were introduced into heterologous hosts for expression.

Because many successful drugs come from prokaryotic secondary metabolites encoded in large operons, the combination of recombineering and transpositional delivery into heterologous hosts opens a new window for metabolic engineering, drug development and production.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

All recombineering was performed in *E. coli* strain GB2005, which is a derivative of DH10B, using Luria broth (LB) medium and antibiotics (kanamycin [K_m], 15 μg/ml; ampicillin [Amp], 100 μg/ml; blasticidin S [BSD], 50 μg/ml; gentamycin [Genta], 6 μg/ml; zeocin [Zeo], 15 μg/ml and tetracycline [Tet], 5 μg/ml). Heterologous hosts for PKS/NRPS gene cluster expression were *M. xanthus* DK1622 (38) and *P. putida* KT2440 (25). *Myxococcus xanthus* DK1622 was grown at 32°C in CTT medium (1% casitone, 8 mM MgSo4, 10 mM Tris-HCl, pH 7.6, and 1 mM potassium phosphate, pH 7.6) (38) with or without K_m (50 μg/ml) before or after introduction of epothilone (*epo*) gene cluster. *Pseudomonas putida* KT2440 or FG2005 (30) was maintained in LB medium and the recombinants were selected in Pseudomonas minimum medium (PMM) medium with K_m (50 μg/ml) (29) or Genta (10 μg/ml) after conjugation.

Recombineering

Recombineering, also called Red/ET recombination, was described previously (19–25,39). GB2005 cells harboring pSC101-BAD-gbaA with Tet resistance (39) were used for preparing recombineering proficient competent cells. Electrocompetent cells were electroporated with 0.3 μg of a linear DNA fragment (modification cassette), which was obtained by PCR. The selection of recombinants was carried out depending on the selection marker in the cassette. PCRs were performed with Phusion polymerase (New England Biolabs, GmbH, Frankfurt am Main, Germany) according to the manufacturer's protocol.

Engineering of the *mchS* gene cluster

The pUC-mchS was derived from a SuperCos 1 vector that contains most of the mchS pathway. The missing gene at the 3′ end was added, along with a cassette for conjugation as described (29). Two rounds of recombineering were used for engineering of the pUC-mchS plasmid. We used our recently developed technology named triple recombination for the first round.

By electroporation, PCR products of *IR-Tps* cassette (IR, inverted repeat; Tps, MycoMar transposase gene) and the ampicillin resistance gene (0.3 μg each in 2 μl) were cotransformed into recombineering proficient competent cells in which the pUC-mchS is resident. Recombinants were selected on LB plates containing 100 μg/ml of Amp. For second round recombineering, the *IR-Tn5-kan* cassette (Tn5, Tn5 promoter; kan, kanamycin resistance gene) flanked with homology arms was generated by PCR. The PCR product (0.3 μg) were used for recombineering and the recombinants were selected on LB plates with 15 μg/ml of K_m (Figure 1).

Oligos used for *IR-Tps-amp* insertion are listed below. Sequence as homologous arm for recombineering is in lowercase.

irtase3

5′ tcaaatccgctcccggcggatttgtcctactcaggagaTTATTCAA CATAGTTCCCTTC 3′ irtase5 (the underlined sequence is the IR)

5′ tccacaggtcattcaagcgcgcgctggggaaggcaggcaggatgggatc tgatcAGACCGGGGACTTATCAGCCAACCTGTTAT GTGG 3′

amptase3

5′ tccagactttacgaaacacggaaaccgaagaccattcatgttgttgctcag gtcgcagaTTACCAATGCTTAATCAGTGAG 3′

amptase5

5′ tcgttataatcgttgtatcgctcttgaagggaactatgttgaataaTCT CCTGAGTAGGACAAATCCG 3′

Oligos used for insertion of *IR-Tn5-kan* cassette were tn5neoIR3 and tn5neoIR5.

tn5neoIR3 (the underlined sequence contains the ribosomal binding site)

5′ tggacactaccccacggctagaaacttcggtcaatacagaatttcctatca tAATCTGTACCTCCTTAAGTCAGAAGAACTCGTCAAGAA G 3′

tn5neoIR5 (the underlined sequence is the IR)

5′ tgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagA CAGGTTGGCTGATAAGTCCCCGGTCTTCACGCT GCCGCAAGCACTCAG 3′

For expression of *mchS* in *P. putida*, an *oriT-tetR-tet* cassette was re-inserted into the backbone to be able to conjugate the gene cluster into *P. putida*. A PCR product of the *oriT-tetR-tet* cassette with homology arms was inserted into the pTps-mchS between *amp* and *pUC* origin by recombineering (Figure 1).

Oligos used for insertion of *oriT-tetR-tet* cassette were oriTOtps3 and oriTOtps5.

oriTOtps3

5′ cacggaaaccgaagaccattcatgttgttgctcaggtcgcagaTCAG CGATCGGCTCGTTGCCCTG 3′

oriTOtps5

5′ tagacagatcgctgagataggtgcctcactgattaagcattggtaATA ATGGTTTCTTAGAGCTTACGGCCAG 3′

Engineering the *epo* gene cluster

The cosmid library of *S. cellulosum* ce90 genomic DNA (40) was screened by using probes generated by PCR.

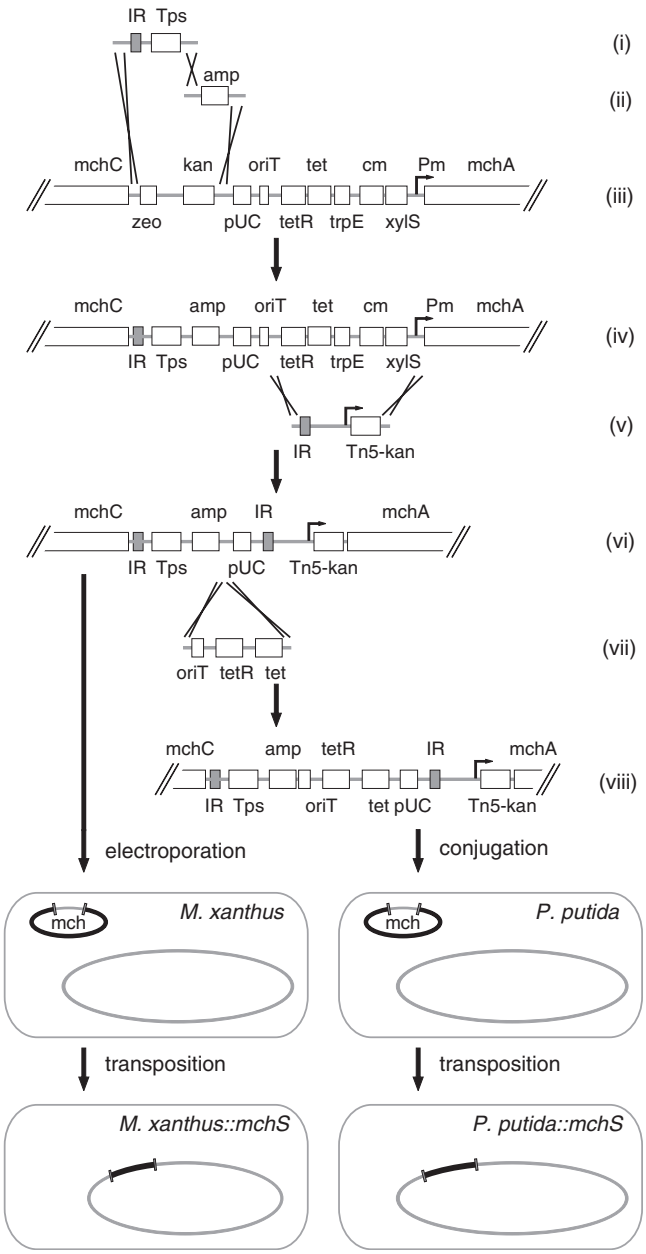

Figure 1. Diagram of myxochromide S gene cluster engineering. In the first step, the inverted repeat and MycoMar transposase gene (*IR-Tps*, i) plus *amp* (ii) were inserted into the *mchS* expression plasmid (iii) backbone by triple recombination to delete *zeo* and *kan* (iv). The MycoMar *Tps* plus *IR* fragment (i) was generated by PCR from the original MycoMar transposon (12). In the second step, the *oriT-tet-trpE-cm-xylS* section (13) in the backbone was replaced with a right *IR* and *Tn5-kan* cassette (v), which included a ribosomal binding site for the *mchS* gene cluster, by selection for kanamycin resistance. In the third step, the modified plasmid (vi) was electroporated into *M. xanthus* and kanamycin-resistant colonies were selected. To introduce the gene cluster into *P. putida*, an additional cassette containing *oriT* and the tetracycline-resistant gene (*oriT-tetR-tet*, vii) was inserted between amp and the pUC origin (*pUC*) to form the final construct (viii) for *P. putida* expression. See remarks in the Materials and Methods section.

The primers epoA-check5 (5′-TTACGCGCGCA TCTTTCCTGAG-3′) and epoA-check3 (5′-TTTCAGCA CGATTTCTTGGGAG-3′) were used to amplify a fragment in *epoA*. The primers epoK-check5 (5′-TATGAC

ACAGGAGCAAGCGAATC-3′) and epoK-check3 (5′-TAAGGTAGATCGTGGTATCGGTG-3′) were used to amplify a fragment in *epoK*. The cosmid end sequencing results show that pSuperCos-epo35 contains the part of *epoC* and full of *epoD* till the end of *epoK*, and pSuperCos-epo14 contains *epoA* to *epoC* and the part of *epoD*.

To stitch the epothilone gene cluster together (Figure 3A), *epoD-K* genes were subcloned into a p15A-Cm minimum vector that was generated by using linearized pACYC184 (41) as template to form p15A-epo35. The zeocin (*zeo*) resistance gene with a 5′ end homology arm to p15A-cm-epo35 and a 3′ end homology arm to a region in front of *epoA* in pSuperCos-epo14 was amplified by PCR. pSuperCos-epo14 was digested with ScaI to release a 26-kb fragment which carries the short homology arm to the *zeo* PCR product and a long homology arm (~9.45 kb) to p15A-cm-epo35. After mixing the *zeo* PCR product with the digested pSuperCos-epo14, the mixture was transformed into a recombineering proficient host containing p15A-cm-epo35. The stitched full-length epothilone gene cluster was selected by *zeo* and analyzed by restriction digestion (Figure 3B). The junction regions of the stitched cluster were verified by sequencing.

To generate the conjugation/transposition cassette, by recombineering the blasticidin S-resistant gene (*bsd*) was inserted between the MycoMar transposase gene (*Tps*) and *oriT* in pMycoMar-hyg plasmid (32) to form the *IR-Tps-bsd-oriT* cassette (Figure 4A). The PCR product of this cassette with homology arms to the stitched p15A-epo-cm-zeo plasmid was used to replace the chloramphenicol (*cm*) gene in the backbone to form p15A-epo-IR-Tps-bsd-oriT-zeo. A PCR fragment containing the second *IR* plus *Tn5-kan* plus a ribosomal binding site (*rbs*) after the *kan* stop codon flanked with two homology arms to the p15A-epo-IR-Tps-bsd-oriT-zeo was used to replace *zeo* to form the final epothilone expression plasmid p15A-epo-IR-Tps-bsd-oriT-IR-kan for expressing in *M. xanthus* (Figure 4A). Since the *Tn5* promoter is weak in *P. putida* (data not shown), after two rounds of recombineering the *Pm* promoter (toluic acid inducible) (29) plus its regulator gene (*xylS*) and the gentamycin-resistant gene (*genta*) were used to replace *Tn5-kan* to form p15A-epo-IR-Tps-bsd-oriT-IR-genta-xylS-Pm which is the final expression plasmid for *P. putida* expression. All of the engineering was done by recombineering.

To create the *IR-Tps-bsd-oriT* cassette, BSD PCR was generated by using oligonucleotides bsdINtps3 5′ tgacgccgttggatacaccaaggaaagtctacacgaacccttggcaTTTAAATCGGATCTGATCAGCACGTGTTG 3′ and bsdINtps5 5′ ttggaaggtcgttataatcgttgtatcgctcttgaagggaactatgttgaataaTTTAAATTAGCCCTCCCACACATAACCAG 3′.

A minimum linear vector containing p15A ori and cm for subcloning of epo gene cluster from pSuperCos-epo35 was generated by using oligo 15epo35a 5′ tgcgatcaactgcgtcagatcgtcctgcgacatctcctcgACTAGTACAACTTATATCGTATGGGGCTG 3′ and 15epo35b 5′ cgaacctcattccctcatgatacagctcgcgcgcgggtgcACTAGTTAACCGTTTTTATCAGGCTCTGG 3′.

One pair of oligos epo-tri-a and epo-tri-b was used for the generation of *zeo*. epo-tri-a is 5′ tatctcttcaaatgtagcacctgaagtcagccccatacgatataagttgtAGCACGTGTTGACAATTAATC 3′ and epo-tri-b is 5′ gggccgcgagatcggcggcggcgaaggagtTCAGTCCTGCTCCTCGGCCAC 3′.

The *bsd* gene for pMycoMar-hyg was generated by using oligonucleotides 5′-tcgttataatcgttgtatcgctcttgaagggaactatgttgaataatttaaaTTAGCCCTCCCACACATAAC-3′ and 5′-tgacgccgttggatacaccaaggaaagtctacacgaacccttggcaTTTAAATCGGATCTGATCAGCACGTGTTG-3′.

The oligonucleotides used for *IR-Tps-bsd-oriT* PCR were 5′-tagctcgcgggggtatcgcttcccgaacctcattccctcatgatacagAGACCGGGGACTTATCAGCCAACCTGTTATGTGGC-3′ (the underlined sequence is the IR) and 5′-tccggcggtgcttttgccgttacgcaccaccccgtcagtagctgaacaggagggacctagagTCGATCTTCGCCAGCAGGGCGAG-3′. *IR-tn5-kan* fragment was generated by using oligonucleotides 5′-tctcttcaaatgtagcacctgaagtcagccccatacgatataagttgtactagtACAGGTTGGCTGATAAGTCCCCGGTCTTCACGCTGCCGCAAGCACTCAGGG-3′ (the underlined sequence is the IR) and 5′-cgacgatcgcaatcggatcttcggctgcgcgctcgatgggacgatccgccatAATCTGTACCTCCTTAAGTCAGAAGAACTCGTCAAGAAGG-3′ (the underlined sequence contains the ribosomal binding site). *Cm-xylS-Pm* fragment was generated by using oligonucleotides 5′-tcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacgagttcttctgaTCCTGGTGTCCCTGTTGATACCG-3′ and 5′-tcgcaatcggatctcggctgcgcgctcgatgggacgatccgccatGTTCGTGACCTCCATTATTATTGTTTCTGTTGC-3′. The gentamycin gene was generated by using oligonucleotides 5′-tgccgcaagcactcagggcgcaagggctgctaaaggaagcggaacacgtagaaagccagtccgcTGAAGGCACGAACCCAGTTGAC-3′ and 5′-tgggttggcatcgcccggctttcttagacactctccaagctctgaaatagcgttttacaaaTCGGCTTGAACGAATTGTTAGGTG-3′.

Electroporation of *M. xanthus*

The engineered gene clusters were introduced into the chromosome of *M. xanthus* DK1622 by electroporation. Briefly, *M. xanthus* cells from 1.7 ml of overnight culture with $OD_{600} \sim 0.6$ were collected and electrocompetent cells were prepared after two times washing with ice-cold water. Fifty microliter of the cell suspension in cold water were mixed with 3 μg of DNA and electroporated (Electroporator 2500, Eppendorf AG, Hamburg, Germany) at 1200 V using 0.2 cm cuvette. After electroporation, 1.2 ml of CTT medium were used to resuspend cells, and the cells were incubated at 32°C in a 2 ml Eppendorf tube with a hole punched in the lid on a Thermomixer (Eppendorf) at 11 000 r.p.m. for 6 h. One milliliter of 1.5% CTT agar solution at 42°C was added to the tube and the cells were plated in soft agar for selection on CTT agar plates supplemented with K_m (50 μg/ml). K_m-resistant colonies appeared after 6 days and were checked by colony PCR as follows. Part of a single colony was washed once in 1 ml of H_2O and resuspended in 100 μl of H_2O. Then, 2 μl of the resulting suspension was used as a PCR template using *Taq* polymerase (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's protocol. The myxochromide-specific primers used to check the integration of *mchS* gene cluster into the *M. xanthus* chromosome were the same as used in previous study (29). Epothilone-specific primers were designed to detect *epoA, epoC* and *epoK* of the gene cluster to verify the integration

of the whole biosynthetic gene cluster into the chromosome. For amplification of *epoA* fragment, epoA-check5 (5′-TTACGCGCGCATCTTTCCTGAG-3′) and epoA-check3 (5′-TTTCAGCACGATTTCTTGGGAG-3′) were used; for amplification of *epoC* fragment, epoC-check5 (5′-GACTGCCGAGAGAAAATCGAG-3′) and epoC-check3 (5′-ATCTGCATAGATGTCCGTCTTG-3′) were used; and for amplification of *epoK* fragment, epoK-check5 (5′-TATGACACAGGAGCAAGCGAATC-3′) and epoK-check3 (5′-TAAGGTAGATCGTGGTATCG GTG-3′) were used.

Conjugation of *P. putida*

Engineered gene cluster expression constructs were introduced into the chromosome of *P. putida* FG2005 for *epo* and *P. putida* KT2440 for *mchS* (30) by triparental conjugation, as described previously (42). The selection of integrants was carried out on PMM agar plates containing K_m (50 μg/ml) for *P. putida* KT2440 for *mchS* and Genta (10 μg/ml) for *P. putida* FG2005. The obtained clones were tested by colony PCR (*Taq*-polymerase, Invitrogen) with the specific primers as used for checking the *mchS* and *epo* containing *M. xanthus* clones above.

Integration site detection

Single-primer (semi-random) PCR has been described previously (43,44). It is used to detect the integration site of transposition. Primer epo-Po-1 or epo-Po-2 was employed to amplify a PCR product containing a known region and the flanking unknown region of the epothilone gene cluster.

epo-Po-1
5′ TAGCCGAATAGCCTCTCCACC 3′

epo-Po-2
5′ GCGGGGCATCGATCAAGAAAG 3′
Primer epo-Pn-1 or epo-Pn-2 is for sequencing.

epo-Pn-1
5′ TCTTGATCCCCTGCGCCATC 3′

epo-Pn-2
5′ CTCCAGGCCAGACGTGTTTG 3′

The PCR was performed using an Eppendorf MasterCycler using either oligo epo-Po-1 or epo-Po-2. After 30 min at 94°C for denaturation, 20 cycles of 10 min at 94°C, 10 min at 55°C and 1 min at 72°C were run. Next 30 cycles with low stringency, programmed as 10 min at 94°C, 10 min at 40°C and 1 min at 72°C were employed. Finally, 30 cycles of 10 min at 94°C, 10 min at 55°C and 1 min at 72°C were used for further amplification.

Analysis of myxochromide S production in *M. xanthus*

The *M. xanthus* DK1622::pTpS-mchS mutant containing the mch S biosynthetic gene cluster was incubated in 250 ml shaking flasks containing 50 ml CTT medium (casitone 10 g/l, 1 M Tris pH 7.6 10 ml/l, 1 M K_2HPO_4 pH 7.6 1 ml/l, 0.8 M $MgSO_4$ 10 ml/l) amended with the adsorber resin XAD-16 (1%) and with K_m (50 μg/ml). The culture was inoculated with 500 μl of a well-grown preculture and incubated for 4 days at 30°C on a rotary shaker (160 rpm). The cell mass and the Amberlite XAD-16 adsorber resin from the culture broth were harvested by centrifugation and extracted with acetone/methanol (1:1), respectively. The extract was evaporated, redissolved in 1 ml of methanol and 5 μl of the concentrated extract was analyzed using a DIONEX HPLC system with a diode-array detector (PDA-100). Chromatographic separation was carried out on a RP column (Nucleodur C18, 125 × 2 mm, 3 μm partical size; Macherey & Nagel, GmbH & Co. KG, Düren, Germany) equipped with a precolumn (8 × 3 mm/5 μm). The mobile phase gradient (solvent A: water + 0.1% formic acid and solvent B: acetonitrile + 0.1% formic acid) was linear from 50% B at 2 min to 60% B at 22 min and from 60% B at 22 min to 95% B at 26 min, followed by 3 min with 95% B at flow rate of 0.4 ml/min; detection was carried out at 400 nm. Myxochromides S were identified by comparison to the retention times and UV spectra of authentic reference standards. For quantitative analysis peak integration was carried out utilizing the Chromeleon software package (Version 6.50). A calibration curve was established from serial dilutions of myxochromides S_1 and S_3. Samples under investigation were diluted as required to fit the dynamic range of the method.

Analysis of Myxochromide S production in *P. putida*

The *P. putida* strain containing the myxochromide S biosynthetic gene cluster (*P. putida*::pTps-mchS-oriT) was incubated in 250 ml shaking flasks containing 50 ml LB medium amended with K_m (50 μg/ml). The culture was inoculated with an overnight culture (1:100) and incubated for 2 days at 30°C on a rotary shaker (160 rpm). The cells were harvested by centrifugation and extracted with acetone. The extract was evaporated and redissolved in 500 μl of methanol and 5 μl of the concentrated extract was analyzed by high-pressure liquid chromatography–mass spectrometry (HPLC–MS); an Agilent 1100 series solvent delivery system coupled to Bruker HCTplus ion trap mass spectrometer was used. Chromatographic separation was carried out on an RP column Nucleodur C18 (125 by 2 mm, 3 μm particle size; Macherey and Nagel) equipped with a precolumn C18 (8 × 3 mm, 5 μm). The mobile-phase gradient (solvent A: water + 0.1% formic acid and solvent B: acetonitrile + 0.1% formic acid) was linear from 50% B at 2 min to 60% B at 22 min and from 60% B at 22 min to 95% B at 26 min, followed by 3 min with 95% B at flow rate of 0.4 ml/min. Detection was carried out in positive ionization mode. Myxochromide S_1 was identified by comparison to the retention time and the MS^2 pattern of the authentic reference standard (m/z $[M + H]^+ = 723$). For quantitative analysis, samples were separated on a gradient linear from 57% B at 2 min to 90% B at 7.50 min, followed by 1.50 min at 90% B. Quantitation was carried out in manual MS^2 mode. Ions of m/z $[M + H]^+ = 723$ were collected and subjected to fragmentation. Peak integration of the characteristic fragment ions m/z 322 was carried out utilizing the Bruker Quant-Analysis v1.6 software package. A calibration curve was established from serial dilutions of myxochromide S_1 down to 1 μg/ml.

Analysis of the heterologous production of epothilones

Myxococcus xanthus strains containing the gene cluster were inoculated from an overnight culture and incubated in 300-ml flasks containing 50 ml CTT medium supplemented with K_m (50 μg/ml) and containing 2% XAD 16 adsorber resin (Rohm und Haas, Frankfurt, Germany) for 5 days at 30°C (200 rpm). The cells and the resin were harvested by centrifugation and extracted with acetone and methanol. Solvents were removed *in vacuo*, and the residue was dissolved in 1 ml methanol. An aliquot of 5 μl was analyzed by HPLC–MS as described previously (31). Detection was carried out in positive ionization mode. Epothilones were identified by comparison to the retention time and the MS^2 pattern of the authentic reference standards.

The *P. putida* FG2005 clones carrying the epo gene cluster were cultured in LB medium with 10 μg/ml gentamycin. Induction of the culture with toluic acid was performed according to previous publications (29). Compound extraction and analysis were performed similar as described above for extraction of epothilones from *M. xanthus*.

RESULTS

Engineering the *mchS* gene cluster

The myxochromide S biosynthetic gene cluster is 29.6-kb long including three large genes. For the work here, the starting construct was the one which we previously used for heterologous expression in *P. putida* (29). As illustrated in Figure 1, we used triple recombination to insert the MycoMar transposase, right *IR* and the *ampicillin* resistance gene downstream of *mchS* gene cluster (Figure 1). The development of triple recombination for DNA engineering will be described elsewhere (J.F., A.F.S. and Y.Z.; manuscript in preparation). Briefly, we used two PCR products, with a 40 bp overlapping region to each other and one homology arm each to the targeting molecule. Further recombineering was performed to insert the left *IR* plus *Tn5-kan* in front of the *mchS* gene cluster. The *Tn5* promoter will drive both the *kan* gene and the *mchS* gene cluster expression (Figure 1). The final construct was verified by sequencing the regions generated by PCR, as well as the recombination junctions. The MycoMar transposase gene sequence is identical to the sequences in the Gene Bank (DQ236098 or AY672108). The left *IR* is 5′ ACAGGTTGGCTGATAAGTCCCCGGTCT 3′ and the right *IR* sequence is 5′ AGACCGGGGACTTATCAGC CAACCTGT 3′.

The engineered *mchS* gene cluster in the transposon (pTps-mchS) for *M. xanthus* expression was used to electroporate *P. putida* KT2440 directly without success. Therefore, an *oriT* cassette was integrated into the construct to be able to conjugate the gene cluster into *P. putida* (Figure 1). As discussed below, after conjugation of this construct into *P. putida*, successful transposition took place.

Production of myxochromides after introduction of the *mchS* pathway from *S. aurantiaca* into *M. xanthus* and *P. putida*

Myxochromide S compounds are characterized by their yellow–orange color and are easily observed in culture. Colonies from pTps-mchS transformation were reddish (Figure 2A) and the liquid cultures are reddish as well (data not shown). A methanol extract from *M. xanthus* DK1622::pTps-mchS was analyzed with HPLC and HPLC/MS for the production of myxochromides S. Myxochromides S_{1-3}, known from *S. aurantica*, could be identified in extracts of the *M. xanthus* mutant strains via HPLC [Figure 2B, peaks 1 (S_1), 2 (S_2), 3 (S_3)], which could also be verified via HPLC/MS analysis (data not shown). Due to the high production of myxochromides S in *M. xanthus* (~500 mg/l), minor myxochromide S derivatives could also be detected (peaks marked with an asterix, Figure 2B).

After integration of an additional *oriT* cassette, the transposon was conjugated and subsequently also integrated into *P. putida* (Figure 1). By using tri-parental conjugation, we detected more than 10^6 K_m-resistant colonies. Twelve of these were analyzed by PCR and found to be correct genotypically. These clones were cultured and compounds were extracted from cells. The engineered *mchS* gene cluster was indeed expressed in *P. putida* but the production level was found to be low (~100 μg/ml; data not shown).

Stitching of the epothilone gene cluster and engineering for transposition

Cosmid pSuperCos-epo35 is 53 kb in size and contains 45.3 kb of the epothilone gene cluster, whereas cosmid pSuperCos-epo14 is 42-kb large and contains 34.5 kb of the epothilone gene cluster. They share a 24-kb overlap. The stitching procedure to reconstitute the entire cluster is diagrammed in Figure 3A, and was based on two rounds of recombineering and the generation of two intermediates (*zeo* PCR product and linear *epoA-D* fragment). The first recombineering step to subclone the *epoD-K* region into the p15A-cm minimal vector was accomplished with high efficiency and $>5 \times 10^3$ cm-resistant colonies were obtained. When 24 colonies were analyzed by restriction digestion, 21 of 24 were correct (the other 3 were found to be pACYC without inserts; data not shown). In the second step, a *zeo* PCR product was used in a triple recombineering exercise to bring the 26-kb long linear *epoA-D* fragment into p15A-cm-epo35. To facilitate this step and reduce the recombineering background due to carryover of the PCR template, the zeocin resistance gene was amplified from an R6Kγ plasmid, because replication of R6Kγ plasmid needs the π protein encoded by the pir gene, however the pir gene is not present in the *E. coli* strain GB2005. We expected the second round of recombineering to be difficult because: (i) long linear fragments, here 26 kb, only inefficiently enter *E. coli* cells; (ii) after entry into *E. coli*, the identical repeats in the *epoA-D* region can promote intramolecular rearrangements; and (iii) in triple recombination, three molecules (p15A-cm-epo35, the *zeo* PCR product and the 26-kb long

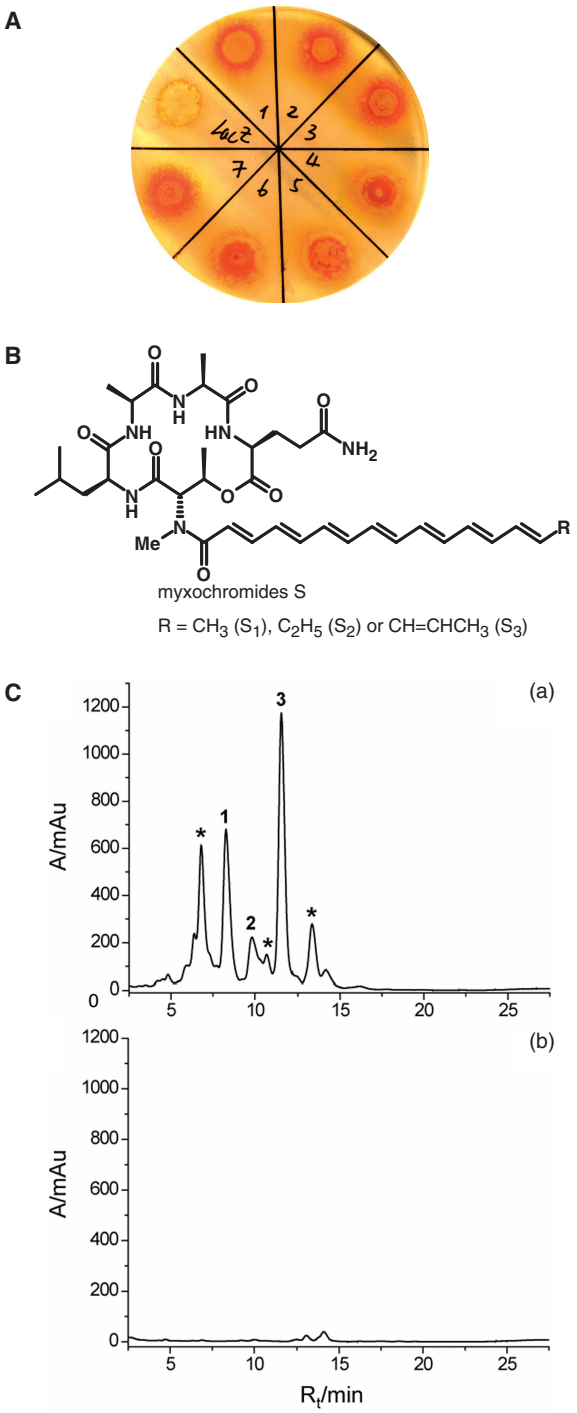

Figure 2. Detection of myxochromide S compounds in *M. xanthus*. (**A**) The color of *M. xanthus* colonies transformed with the *mchS* gene cluster is shown. Colonies from *M. xanthus*::pTps-mchS (1–7) and *M. xanthus*::pTps-lacZ (LacZ) were picked and replated on a kanamycin plate. The photo was taken after 2 days incubation. (**B**) the chemical structures of myxochromides S_{1-3}. (**C**) HPLC profiles from extracts of the *M. xanthus*::pTpS-mchS mutant strain (a) in comparison to *M. xanthus* wild-type (b); diode array detection at 400 nm. Numbers correspond to substances as follows: **1**, Myxochromide S_1; **2**, Myxochromide S_2; **3**, Myxochromide S_3. Peaks marked with an asterisk are assumed to be Myxochromide S derivatives as well.

linear *epoA-D* fragment) must meet each other in the same cell for productive recombination. After triple recombination, we found 100 colonies on zeocin plates. By checking with restriction digestion, we found that the majority of the recombinants (21 out of 24 clones) contained *zeo* plus only a short part of the *epoA-D* fragment because of intramolecular rearrangement. However, the other three were correct recombinants. After retransforming the correct plasmids to separate them from the unrecombined original plasmid, one plasmid was rechecked by NcoI digestion (Figure 3B) and the junction regions were verified by sequencing.

The transpositional cassette, *IR-Tps-bsd-oriT*, was constructed by inserting the *bsd* gene between the *Tps* and *oriT* in pMycoMar-hyg to form pMycoMar-bsd-hyg. A PCR product of *IR-Tps-bsd-oriT* (2.6 kb) flanked with homology arms was generated using high-fidelity Phusion polymerase and linearized pMycoMar-bsd-hyg as the template (Figure 4A). After electroporation into *E. coli* containing the stitched *epo* cluster, recombinants were selected by plating on BSD plus Zeo plates and incubated at 30°C overnight. This replaced the chloramphenicol (*cm*) resistance gene in p15A-cm-zeo-epo with the *IR-Tps-bsd-oriT* cassette to form the intermediate p15A-epo-IR-Tps-bsd-oriT-zeo. In the next round of recombineering, an *IR-Tn5-kan-rbs* PCR product flanked with homology arms was used to remove *zeo* and also place a ribosomal binding site in front of *epoA* to generate p15A-epo-IR-Tps-bsd-oriT-IR-kan. These final recombinants were selected by plating on BSD plus K_m plates. More than 50 colonies were pooled from the plate and plasmid DNA was prepared and retransformed into empty *E. coli* cells to separate unrecombined and recombined plasmids. Three pure recombinants were verified by sequencing of *IR-Tps-bsd-oriT* cassette and *IR-Tn5-kan-rbs-epoA* region and two were found without any mutation in the functional regions.

Production of epothilones after introduction of the *epo* pathway into *M. xanthus*

Plasmid DNA from verified engineered clones was used to transform *M. xanthus* (Figure 4A). After entering *M. xanthus*, transposase expression will integrate the engineered *epo* cluster randomly into the *M. xanthus* genome. Stable integrants were selected on K_m plates. For p15A-epo-IR-Tps-bsd-oriT-IR-kan, which is 60.5 kb in size, we obtained 90 colonies after 6–8 days incubation at 30°C. For pTps-oriT-mchS, which is 35 kb in size, we obtained >500 K_m-resistant colonies using the same protocol (Table 2). Hence, as expected, transposition efficiency is reduced for larger transposons. Nevertheless, we report transposition of a 60-kb transgene at a readily obtained frequency that was well beyond the rate required for successful transgenesis.

Twelve K_m-resistant colonies were checked by colony PCR reactions to verify full integration of the biosynthetic gene cluster. Ten were correct and eight of these were fermented for compound extraction. All eight produced epothilones B, C and D and detectable amounts of epothilone A (Figure 4B and C). Hence, we show that multiple

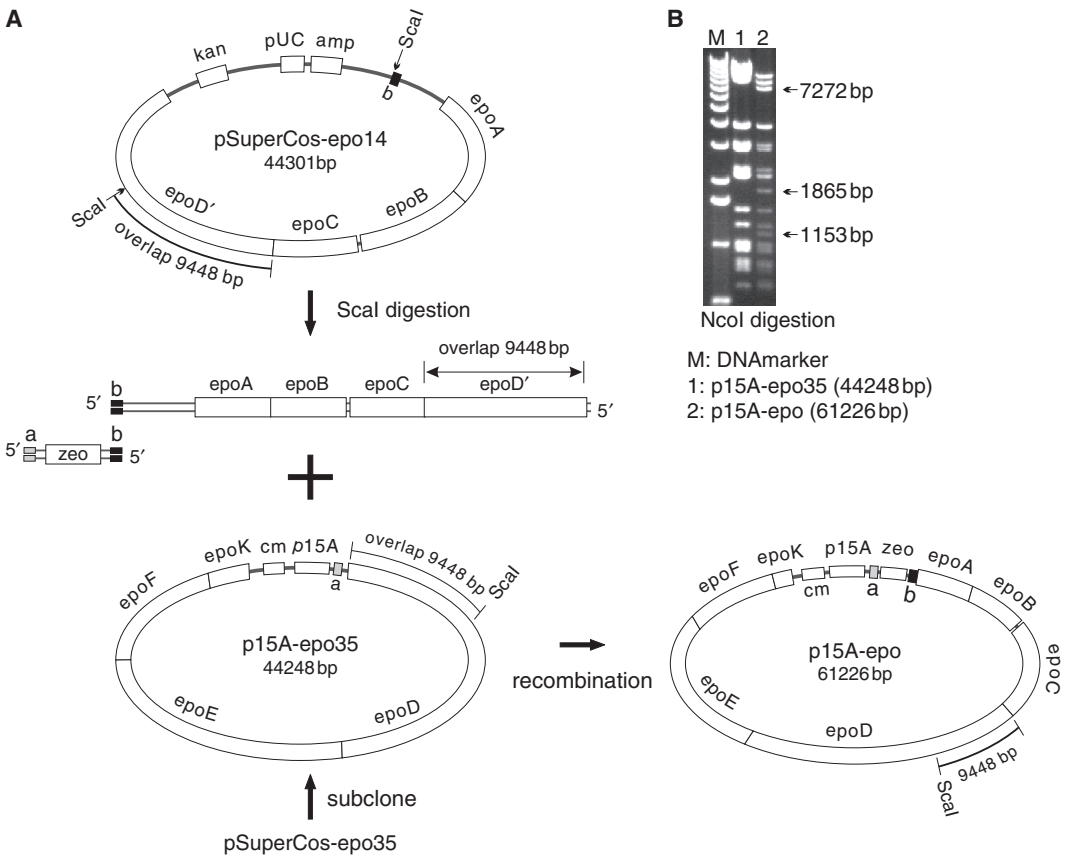

Figure 3. Engineering diagram for stitching of the epothilone gene cluster. pSuperCos-epo35 and pSuperCos-epo14 were the starting clones, which include an overlap in the *epoD* gene. pSuperCos-epo35 was retrofitted with the p15A origin and the chloramphenicol resistance gene from pACYC184 by subcloning to remove the pSuperCos backbone, introduce the short homology arm '(**A**)' and create p15A-epo35. pSuperCos-epo14 was digested with ScaI and the *epoA-D* genes were recombined into p15A-epo35 by triple recombination using a bridging *zeo*. In (**B**), the NcoI digest reveals the correct product. (the NcoI sites in p15A-epo35 and p15A-epo are shown in Appendix 1 in Supplementary Material).

rounds of recombineering to stitch and engineer the *epo* cluster, followed by transpositional integration, resulted in functional expression.

In contrast to myxochromide formation, epothilone production was low (average ~100 μg/l), but the difference between each clones was small (data not shown) indicating that the integration site in the chromosome had little effect on *epo* gene cluster expression driven by the *Tn5* promoter.

Conjugation and transposition to introduce the *epo* pathway into *P. putida*

The *epo* gene cluster is derived from myxobacterium *S. cellulosum* and it is not surprising that it can be used to produce epothilone compounds in *M. xanthus*. To test the gene cluster expression in a different bacterial species, *P. putida* was chosen. Wild-type *P. putida* has successfully been used for *mchS* gene cluster expression (29) and genetically engineered *P. putida* FG2005 producing methylmalonyl-CoA (*mm-CoA*) has been applied for *myxothiazol* gene cluster expression (30). During the *P. putida* engineering process, the *Tn5* promoter was used to drive the *lacZ* gene expression and it was found to be weak in this strain (unpublished data). Therefore, the construct used for *M. xanthus* is most likely not suitable for *epo* gene cluster expression in *P. putida*. When introducing the mutase-epimerase-*meaB* genes for production of methylmalonyl-CoA from *S. cellulosum* So ce56 into *P. putida*, the K_m resistance gene has been used for selection (30). Thus, a new selection marker gene had to be used for introducing the *epo* gene cluster into FG2005. Analyzing natural resistances of *P. putida*, we found this strain to be sensitive to gentamycin at 5 μg/ml. To test if a gentamycin resistance gene (*genta*, *aacC1*) functions in *P. putida*, *genta* was inserted into the *RK2* ori-based plasmid pJB866 (45) to build pJB866-genta which conferred gentamycin resistance after transformation of *P. putida*.

We next aimed to generate a derivative of the *epo* gene cluster for expression in *P. putida* by inserting the Pm promoter. To achieve this, a *cm-xylS-Pm* cassette with homology arms was amplified from the template plasmid pJB866-cm (in which *cm* was inserted behind the *Pm* regulator gene *xylS* in pJB866). The *cm-xylS-Pm* PCR product was inserted in front of the *epoA* gene in p15A-epo-IR-Tps-bsd-oriT-IR-kan to form the construct p15A-epo-IR-Tps-bsd-oriT-IR-kan-cm-xylS-Pm (Figure 5).

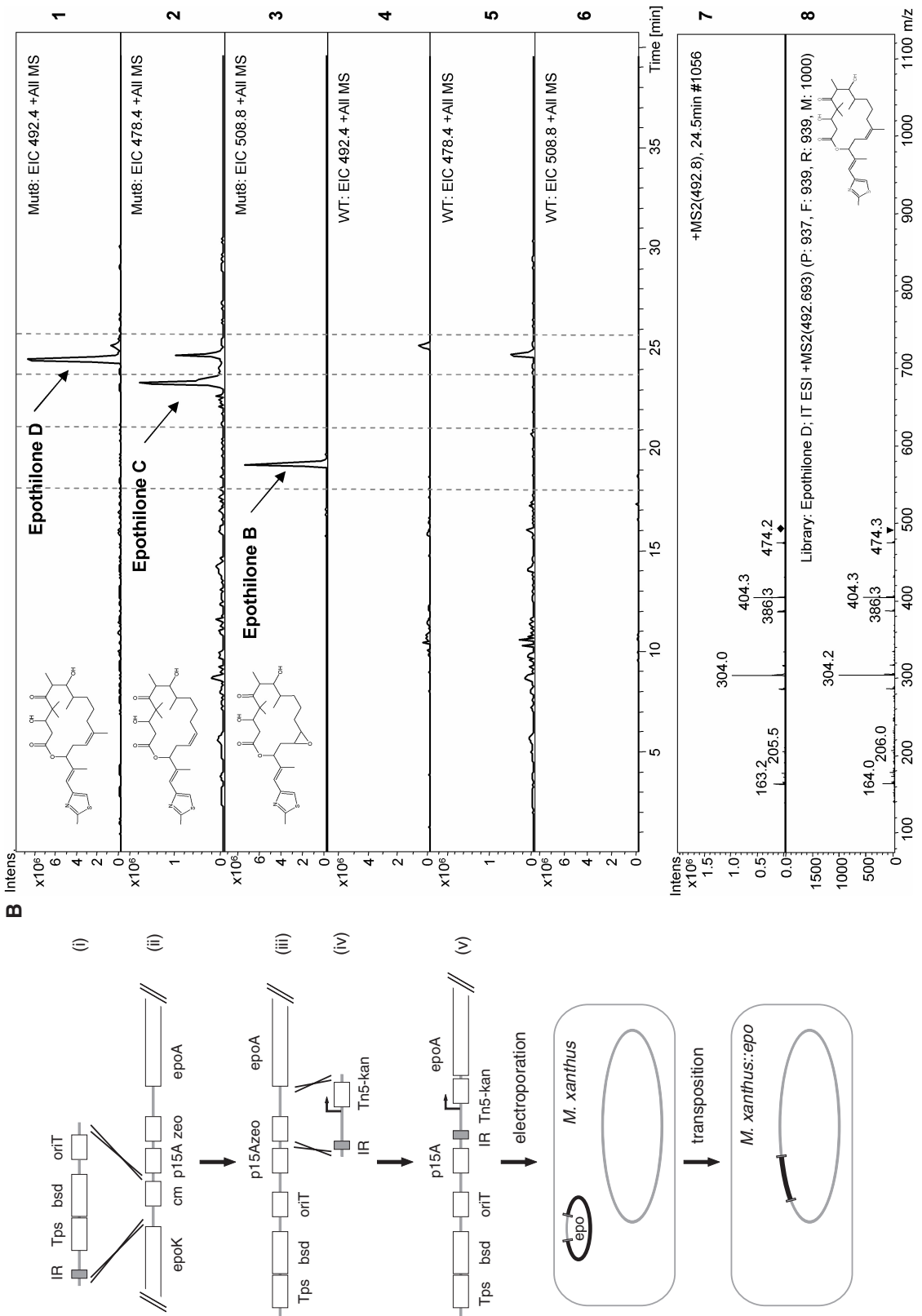

Figure 4. Construction of the *epo* gene cluster and its expression in *M. xanthus*. (**A**), *IR-Tps-bsd-oriT* cassette (i) in pMycoMar-bsd-hyg plasmid was used as template to generate the PCR product of *IR-Tps-bsd-oriT* with homology arms. After recombineering, *IR-Tps-bsd-oriT* was inserted into the plasmid backbone of the stitched *epo* gene cluster (ii) to form p15A-epo-IR-Tps-bsd-oriT-zeo (iii). Background free template *R6K-Tn5-kan* was then used to generate *IR-Tn5-kan* PCR product (iv) with homology arms. The second round of recombineering was performed to build the final expression construct p15A-epo-IR-Tps-bsd-oriT-IR-kan (v). The verified and purified expression construct was electroporated into *M. xanthus* and the DNA fragment between the two IRs was integrated into the *M. xanthus* chromosome. (**B**) Extracts from the stable integrant *M. xanthus* DK1622-Mut8 were analyzed by HPLC-MS. Rows 1–3 show extracted ion chromatograms of HPLC–MS runs demonstrating that the different epothilones are biosynthesized. Epothilone B, C and D are the major compounds produced in this clone. Rows 4–6 show the same analysis for the wild-type *M. xanthus* DK1622 (WT) without the *epo* gene cluster. The fragmentation pattern of epothilone D produced in *M. xanthus* (row 7) is compared to authentic epothilone D reference (row 8).

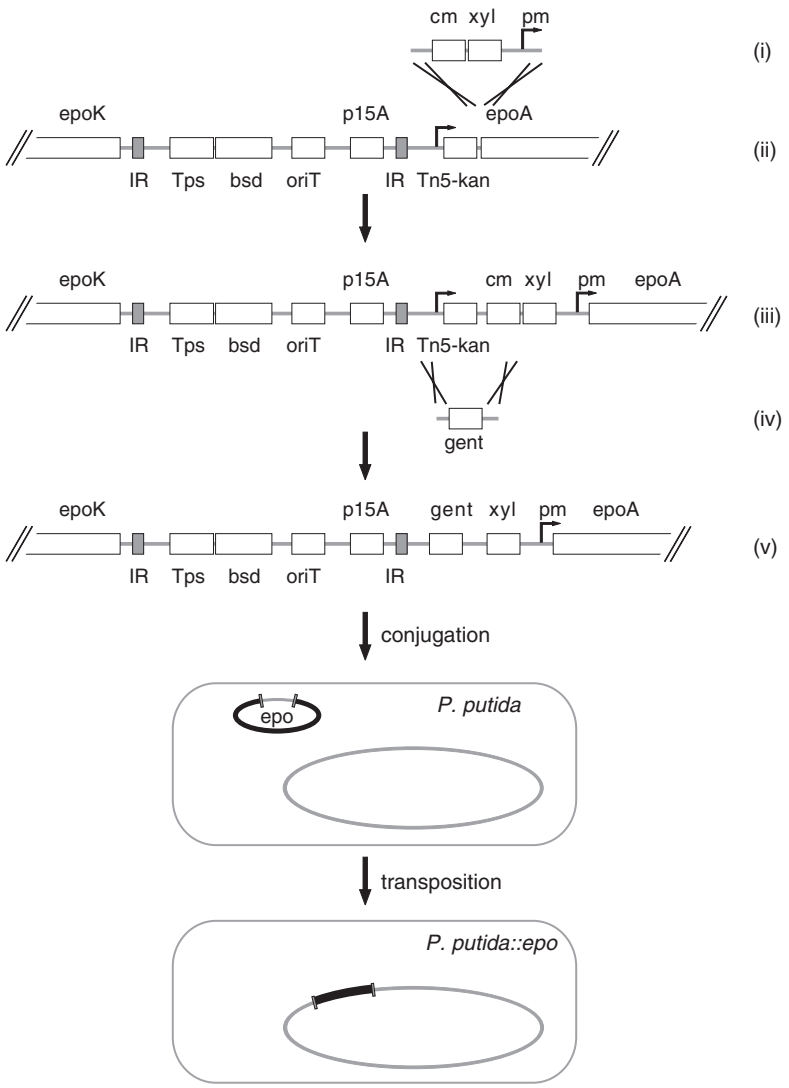

Figure 5. Engineering of *epo* gene cluster for *P. putida* FG2005 expression. PCR product of Cm resistance gene, Pm promoter and its regulator *xylS* gene (i) were inserted in front of *epoA* in the *epo* gene cluster (ii) to drive expression of the whole *epo* gene cluster (iii). PCR product of gentamycin resistance gene (iv) was used to replace *Tn5-kan* and *cm* to generate the final construct (v) for *P. putida* FG2005 expression. The verified final construct was integrated into the FG2005 genome by conjugation and transposition.

Afterwards, *genta* with homology arms was used to remove *Tn5-kan* and *cm* to form the final *epo* construct p15A-epo-IR-Tps-bsd-oriT-IR-genta-xylS-Pm for *P. putida* FG2005 expression.

Sequencing verified the correctness of the construct, which was conjugated into FG2005 by tri-parental conjugation and stable integrants of *epo* clones were selected by gentamycin on PMM plates. Conjugation/transposition is an efficient process and more than 10^4 Genta-resistant colonies were obtained. The conjugation/transposition efficiency of this large sized DNA molecule was found to be similar to small sized DNA molecules like mchS gene cluster (data not shown). The same primers used for colony PCR in *M. xanthus* were used again to verify the *P. putida* clones. Verified clones were induced by toluic acid during fermentation and compounds were extracted from culture medium and cells. No epothilone could be detected in any of these extracts.

Verification of gene integration by transposition

To verify that the plasmid, p15A-epo-IR-Tps-bsd-oriT-IR-kan, had integrated into *P. putida* FG2005 by transposition, we used single primer PCR (43,44) to determine the sequence of the integration site of three clones (Table 1). All three sites include a duplicated TA dinucleotide at the insertion site, which is diagnostic of transposition. The three insertion sites are in the 619th gene, 4266th gene and 5136th genes of *P. putida* KT2440 (GenBank AE015451). The flanking sequences of the insertion sites in the three genes were found to be exactly the right IR plus the left IR. This validates the successful transposition by the designed construct with the mariner transposable element.

Table 1. Integration site of epothilone in *P. putida* KT2440

Clone	Insertion locus and orientation (IS = IR-tn5-kan-epoA-epoB-epoC-epoD-epoE-epoF-epoK-IR)	Genome location	Name of the gene
1	AGTTGCTCGGCCACCCGCTCAAGTA**(IS)** TACAAAGTCATCGACACCGAAGGCA	725684	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein
2	TGTCGCGCAACGAAATCGGCAATTA **(IS)** TACCTTGGCCTGGCGGTAGAAACCG	4850861	Transcriptional regulator Anr
3	CCTGGTACTGGGCCTGGGCTACGTA **(IS)** TATTCTTCTTCAACCTGAACGGCAAC	5860472	ABC transporter, permease protein

Table 2. Transformation efficiency in *M. xanthus*

	p15A-epo-IR-Tps-bsd-oriT-IR-kan	pTps-mchS	pTps-lacZ	pOPB18
Size (kb)	61	35	15.6	6.7
Colonies	90	635	1020	9

Transformation versus transposition efficiency in *M. xanthus*

Myxococcus xanthus can be transformed by electroporation and transgenes can be integrated into the chromosome via homologous recombination. However, the efficiency of integration of large DNA molecules into the chromosome is low and candidate clones must be screened carefully to exclude spontaneous mutants which give K_m resistance. Transposition has been used frequently in myxobacteria for insertional mutagenesis and the efficiency of stable integration is much higher than homologous recombination (32).

To compare homologous and transpositional integration in *M. xanthus*, we used the validated *epo* and *mchS* expression constructs described above in a comparison with a homologous integration plasmid pOPB18 (total size 6.7 kb containing a 1.7-kb homology region to the myxovirescin gene cluster in *M. xanthus*) (31) and a small transposition plasmid, pTps-lacZ (derivative of pMycoMar-hyg, containing a 5.5-kb lacZ-kan-R6K cassette inside of *IRs*). Table 2 shows the number of transformants which were obtained. The numbers are averages of three transformations done for each plasmid on a molar basis. Interestingly, we found that the colonies which appeared on K_m plates before 7 days were all correct but the colonies which appeared after 8 days were spontaneous mutants containing no inserts.

pTps-mchS is 35 kb in size and the integration fragment inside of the two *IRs* is 31 kb large. p15A-epo-IR-Tps-bsd-oriT-IR-kan is 61 kb in size and the transposable element is 57.5 kb. Although the large constructs have lower efficiency than small integration fragments (pTps-lacZ), their integration process is much more efficient than the small homologous integration plasmid (pOPB18), regardless of the fact that, in general, the transformation efficiency drops dramatically with increasing size of the DNA (46).

DISCUSSION

Transposons for large transgenes and metabolic engineering

Transposons have been employed as efficient mutagens in both prokaryotes and eukaryotes. They have also been used as vectors for efficient and stable introduction of transgenes into recipient genomes. These applications have been invariably based on relatively small (<10 kb) transposons. As far as we can determine, the potential to use transposons as vectors for the integration of large transgenes has not been explored in any system, whether prokaryotic or eukaryotic. Although we do observe decreased efficiencies with increased transgene size as expected, transposon-mediated transgenesis with a 57.5-kb transgene was more efficient than transgenesis via homologous recombination using a small construct. Hence, we suggest that transposition could be a valuable tool for delivery of large transgenes in various prokaryotic and eukaryotic systems. The size of the transgene is particularly relevant for metabolic engineering where the new challenges lie with manipulation of complete pathways, not just single gene products.

For prokaryotes, there are two limiting factors for integrating transgenes into the host chromosome by conventional methods: transformation efficiency and homologous recombination efficiency. Many bacterial hosts, especially those which are known as prominent secondary metabolite producers (e.g. actinomycetes, cyanobacteria, myxobacteria), appear to possess limited capacity for homologous recombination. Consequently, chromosomal integration appears difficult even with small transgenes. Transposition *in vivo* is an active process and often thousands of colonies can be obtained after each transformation with small constructs. To evaluate transposition for delivery of large transgenes in *M. xanthus*, we compared a small (~6 kb), medium (~31 kb) and a large sized insert (over 57 kb) in transpositional constructs. As expected, the transformation and transpositional integration efficiencies depended on the size of the insert. When a medium size construct with a 1-kb homology arm for homologous integration was used to transform *M. xanthus*, very few (<10) colonies appeared. But more than 500 colonies were obtained when the same construct was introduced by transposition (Table 2). With the larger *epo* gene cluster (57.5 kb), transposition delivered fewer colonies. However, this number (90 clones) is still considerably larger than our experience with integration via homologous recombination

of smaller constructs. Hence, we suggest that transpositional delivery can be a useful tool for stable transgenesis, which is particularly relevant for large constructs.

Aspects of secondary metabolite expression in heterologous hosts

Other observations from this report are discussed below.

(i) The production of *mchS* gene cluster in *M. xanthus* was higher than 500 mg/l and the total myxochromide compounds in some isolates was >1 g/l. Interestingly, the same (*Tn5*) promoter-driven *mchS* gene cluster in *P. putida* produced only 100 μg/l of myxochromides at 30°C (and no compound was detected at 16°C). However, *mchS* production in *P. putida* from the *Pm* promoter reached 40 mg/l at 16°C (29). Together with other observations (unpublished data), we conclude that the *Tn5* promoter in *P. putida* is weak, or under unexpected repression.

(ii) More than 80% of the clones carrying the *epo* or *mchS* gene clusters were correct as ascertained by PCR. Together with other data presented here, this indicates that the transformation/transpositional integration strategy delivers a high proportion of full length, unmutated, large transgenes into heterologous hosts like *M. xanthus*. The transposition is mediated by a transient expression of MycoMar transposase gene. Therefore, after integration, the insert is stable in the chromosome. Since the type I PKS/NRPS gene clusters are composed by many repetitive sequences, we have tested the clones carrying the gene clusters by Southern blot and PCR. There was no rearrangement in six tested clones (data not shown). In our experience with various strains, we have not seen production or growth changes over many generations. We conclude that transpositional integration of type I PKS/NRPS gene clusters into heterologous host is stable, with few rearrangements or position effects.

(iii) Electroporation of large transgenes into *P. putida* is difficult. Small plasmids can be easily electroporated into *P. putida* but plasmids >30 kb construct can hardly be transformed (data not shown). Conjugation has been applied in *P. putida* to deliver large chromosomal sections (47,48). The combination of conjugation and transposition with small constructs has been reported some time ago (49–51). We integrated the ~60 kb *epo* gene cluster into the *P. putida* genome with similar efficiencies compared to a small construct and the majority of the recombinants were correct as confirmed by PCR tests. Hence, previous size problems with *P. putida* transformation appear to have been solved.

(iv) The epothilone gene cluster was engineered and introduced into the heterologous hosts, *M. xanthus* and *P. putida*. Epothilone production was low or undetectable, respectively. There are several possible reasons. First, the Tn5 promoter may be too weak to drive substantial expression in these heterologous hosts. Second, culture conditions like temperature or media components may not be optimal. Third, in *P. putida* FG2005, endogenous *mm-CoA* levels may be the limiting factor for epothilone production.

Advanced recombineering for metabolic engineering

Recombineering is a DNA engineering methodology based on homologous recombination in *E. coli* (19–25). Conventional DNA engineering technology relies on DNA restriction and ligation. However, it is very hard to find convenient restriction sites in the large DNA regions which encode secondary metabolite pathways. DNA ligation relies on purified linear DNA molecules, which are difficult to obtain in time-consuming processes that are inefficient especially for large sizes. Because there are no size limits for recombineering, and *in vitro* DNA handling steps are eliminated or reduced, it is well suited for the challenges presented by secondary metabolite pathways.

In addition to several routine applications, here we present an advanced recombineering exercise involving DNA stitching by triple recombination. DNA stitching mediated by recombineering has been previously applied for bacterial artificial chromosome (BAC) stitching (52,53) and a similar approach has been applied in yeast for more than a decade (54–56). Here, we were working with cosmids, so the genomic DNA insert average is 40 kb in size. Because most type I PKS/NRPS gene clusters are rarely contained within one cosmid and usually present in two or three, a convenient method for stitching cosmids together will be useful. We developed a strategy to stitch using a selection marker (*zeo*) as a bridge to bring two large molecules, which share a region of sequence overlap at one end, together in a triple recombination step. Triple recombination presents two advantages for stitching. First, it avoids PCR amplification of the cloned DNA, so it can be applied to large molecules and without fear of mutagenesis in functional regions. Second, it represents a simple and easy way to introduce the second region of homology. The first region is the overlap between the two parental cosmids. However for productive recombination, another region is required. This can be easily introduced into the reaction via the oligonucleotides used to PCR the zeocin cassette. Because the PCR product is small and encodes the zeocin resistance gene, the disadvantages of PCR mutagenesis do not compromise the recombination product.

Triple recombination for stitching presents the further advantage of speed and simplicity because it can save several DNA engineering steps. However, it is much less efficient than standard recombineering applications based on recombination of a single linear DNA substrate into a circular target. Furthermore, we observed a high unspecific recombination rate when stitching the epo gene cluster due to intramolecular rearrangement.

This work further extends our technology platform for the engineering of full-length gene clusters for heterologous expression. This platform includes DNA stitching by recombineering, transpositional cassette insertion, conjugation cassette insertion and inducible promoter insertion. Several technologies including recombineering, electro-transformation, conjugation and transposition have been explored for this platform. We aim to make further technical advances as well as to venture into the

unknown territory represented by silent secondary metabolite clusters discovered in genome sequencing and metagenomic programs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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