Construction of *Rhodococcus* random mutagenesis libraries using Tn5 transposition complexes

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The ability to generate tagged mutants of *Rhodococcus* spp. will facilitate a deeper understanding of this medically and commercially important genus. The absence of efficient transposon systems in these organisms has here been overcome by the use of Tn5-based DNA–protein transposition complexes which can transpose at high efficiency. To achieve this, electroporation efficiencies and antibiotic selection were optimized. A *Rhodococcus rhodochrous* CW25 Tn5 insertion library of 1500 mutants was created. Southern blotting of 23 representative mutants demonstrated random insertion. A number of auxotrophic mutants were isolated and the disrupted regions involved were identified by inverse PCR and subsequent sequencing. Transposition of Tn5 was confirmed by the presence of 9 bp direct repeats of *Rhodococcus* DNA flanking the transposon insertion site. To further test this system, a Tn5 insertion library was constructed in a wild-type soil isolate of *Rhodococcus* spp. This is the first viable transposon knockout system reported for *Rhodococcus*.

Keywords: transposon, kanamycin, Gram-positive, *Mycobacterium*, *Nocardia*

INTRODUCTION

The genus *Rhodococcus* encompasses a multi-faceted group of bacteria of clinical, industrial and environmental significance. *Rhodococcus* belongs to a supra-generic group which includes *Mycobacterium* and *Corynebacterium*. This group is characterized by the presence of mycolic acids incorporated into the outer cell wall structure. Members are found in soil and ocean environments, and also cause disease in humans and animals (Colquhoun *et al.*, 1998; Goodfellow & Alderson, 1977).

Originally primarily of veterinary significance, *Rhodococcus* has become clinically important in the immunocompromised, particularly in patients with AIDS (Mosser & Hondalus, 1996). Rhodococci and members of related genera such as the nocardiae have been shown to cause a number of complaints, including brain abscesses, sub-acute endocarditis and pulmonary disease (Berkowitz, 1994).

Rhodococcus appears specialized to recycle complex plant-derived compounds in the soil, including a wide

range of aromatic hydrocarbons. As a result, *Rhodococcus* isolates have been shown to catalyse a wide range of biotransformations of industrial relevance, such as xenobiotic degradation and surfactant synthesis (Goodfellow & Alderson, 1977; Larkin *et al.*, 1998; Warhurst & Fewson, 1994), and the manufacture of other commercially important products (Allen *et al.*, 1997; Larkin *et al.*, 1998).

Currently, *Rhodococcus* genetics lacks a transposon system which functions efficiently enough to permit the generation of comprehensive random insertion libraries. Such systems have been widely used in the characterization of genes in *Escherichia coli* and many other genera (Kleckner, 1981). The development of these systems for many Gram-positive bacteria (in particular the recalcitrant actinomycetes) has, until recently, been hampered by a general lack of genetic tools. In a strain of *Rhodococcus fascians*, tagged mutagenesis has been achieved by the random illegitimate insertion into the chromosome of suicide vectors delivered by electroporation (Crespi *et al.*, 1994). However, high frequencies of illegitimate recombination have not been observed upon introduction of various non-replicating vectors into other strains of *Rhodococcus*; consequently this technique is not generally applicable (Powell & Archer, 1998; Shao & Behki, 1995; Yao *et al.*, 1994a).

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Abbreviation: iPCR, inverse PCR.

In vivo transposition systems based on suicide delivery suffer from the combined effects of low transformation efficiency in wild-type strains and low transposition frequency, precluding the generation of insertion libraries (Guilhot *et al.*, 1994; Pelicic *et al.*, 1997). Replicative delivery systems avoid this problem but require both a functioning replicon and a means of selecting for transposition events. Selection can be achieved with a counter-selectable marker such as *sacB*, a temperature-sensitive replicon, or by insertional activation of a promoterless antibiotic resistance gene carried on the transposable element. In *Mycobacterium tuberculosis*, it has proven necessary to use both *sacB* counter-selection and temperature-sensitive plasmid replicons simultaneously to ensure clean selection (Pelicic *et al.*, 1997). The use of replicative vectors runs a risk of generating a large number of sibling colonies due to the sustained period of growth prior to selection. Although a number of *Rhodococcus* IS elements have been isolated, none have been engineered into a usable mutagenesis system (Denome & Young, 1995; Jager *et al.*, 1995; Lessard *et al.*, 1999; Nagy *et al.*, 1997; Yao *et al.*, 1994a).

In vitro mutagenesis of DNA using the Tn552 system followed by integration back into the host chromosome has proven effective in *Mycobacterium smegmatis*. This approach depends on the frequency of homologous recombination, which has been shown to be low in strains of *Rhodococcus* (Barnes *et al.*, 1997; Powell & Archer, 1998) and its utility in this genus has yet to be demonstrated.

The recent development of the Tn5-based 'Transposome' system eliminates a number of these problems. Tn5 is one of the simplest and best characterized transposons. *In vitro* transposition requires only Tn5 transposase, transposon DNA flanked by Tn5 inverted repeats, and target DNA. The transposome system consists of Tn5 transposase bound to transposon DNA *in vitro*. This bound complex is prevented from completion of the transposition reaction by the absence of magnesium ions. Following electroporation of the protein-DNA complex into a bacterial cell, the reaction proceeds due to the presence of magnesium ions, resulting in insertion of the transposon DNA at random into the genome. For a poorly characterized host like *Rhodococcus* this approach has a number of advantages. Firstly, the expression of transposase in the target strain is not required. Secondly, the transposition frequency once the complex has been transformed into the cell is very high. There is therefore no requirement for replicative delivery and the concomitant problems of selecting for transposition events. Transposome insertion frequencies are limited only by the rate of DNA transformation. The lack of requirement for host-encoded factors means that the system has the potential to operate in a very wide range of organisms (Goryshin *et al.*, 2000).

The use of this system in the genus *Rhodococcus* or the related genera *Corynebacterium*, *Nocardia* or *Mycobacterium* has not previously been investigated. In this

paper we describe the use of the EZ::TN(KAN-2)Tnp Transposome to create random transposon libraries in *Rhodococcus rhodochrous* CW25 and in a wild-type *Rhodococcus* soil isolate. The transposable element in this case consists of the Tn903 kanamycin resistance gene flanked by two 19 bp Tn5 inverted repeats. During the course of this work the use of the kanamycin-resistance transposome to create a transposon library in *M. smegmatis* was reported (Derbyshire *et al.*, 2000).

METHODS

Bacterial strains and plasmids. *R. rhodochrous* CW25 was a gift from E. Dabbs, University of the Witwatersrand, South Africa. *Rhodococcus* sp. RC89, isolated from a polluted gasworks site in Newcastle, was a gift from R. Cain, University of Newcastle. The broad-host-range plasmid pCY104 (Yao *et al.*, 1994b) containing the Tn903 kanamycin resistance gene and the thiostrepton resistance gene from *Streptomyces azureus* was used to test electroporation efficiencies and antibiotic selection. pCY104 DNA was isolated from *E. coli* XL1-Blue (Stratagene). *Rhodococcus* spp. were routinely grown on Luria-Bertani (LB) medium (Difco) with Bacto Agar (16 g l⁻¹) added for plates (LA). Auxotrophic and hydrocarbon utilization screens were carried out on minimal media (Dabbs, 1987) supplemented with the relevant carbon source (25 mM succinate or 200 µl toluene or benzene provided in an Eppendorf tube inside the agar plate). Growth of *R. rhodochrous* CW25 on minimal media required the addition of 100 µg phenylalanine ml⁻¹, 40 µg glutamic acid ml⁻¹ and 5 µg thiamin ml⁻¹; *Rhodococcus* sp. RC89 did not require any additional supplements. Cultures grown in complex media were incubated at 30 °C for 2–3 d and liquid cultures were shaken. Cultures grown in minimal media were incubated at room temperature for 3–7 d.

Transformation of *Rhodococcus* spp. Ten millilitres of a stationary-phase culture was used to inoculate a 500 ml flask containing 200 ml LB supplemented with 2% (w/v) glycine for *R. rhodochrous* CW25 and 1% glycine plus 0.5% (w/v) glucose for *Rhodococcus* sp. RC89. The flask was incubated at 30 °C with shaking until the culture reached late exponential phase or an OD₅₅₀ of no more than 0.9 units using a Beckman DU 650 spectrophotometer. Cells were then incubated on ice for 20 min and pelleted by centrifugation (Sorvall RC-5B) at 4000 r.p.m. for 20 min at 4 °C. The cell pellet was washed twice in 100 ml sterile 10% (v/v) glycerol. Washes were carried out at 4 °C and cells were stored on ice at all other times. Aliquots (3 µl) of cells were diluted in 1 ml LB and the OD₅₅₀ was measured. The concentration of cells for electroporation was adjusted to give an OD₅₅₀ of 0.4 in the diluted sample.

Mutagenesis. Electrocompetent *Rhodococcus* cells (40 µl) were placed on ice in a 0.1 cm electrode gap Bio-Rad electroporation cuvette and mixed with 20 ng (1 µl) EZ::TN(KAN-2)Tnp Transposome (Epicentre Technologies). Electroporation was carried out at 1.6 kV, 25 µF, 200 Ω. Cells were resuspended in 1 ml SMME (0.7 M sucrose, 50 mM maleic acid, 10 mM MgCl₂·6H₂O and 10 mM CaCl₂, pH 6.5) (Haynes & Britz, 1990) and immediately plated in 100 µl aliquots onto LA plates containing 1% glucose. Plates were incubated for 12 h at 30 °C, then overlaid with soft agar containing sufficient kanamycin to give a final concentration of antibiotic in the plate (taking the volume of LA into account) of 400 µg ml⁻¹ for *R. rhodochrous* CW25 and 100 µg ml⁻¹ for *Rhodococcus* sp. RC89. The soft agar was

allowed to set and plates incubated at 30 °C. Transposants appeared within 5 d.

DNA extraction and manipulation. DNA extractions were based on the salting out method of Pospiech & Neumann (1995). *Rhodococcus* cultures were grown in 10 ml LB containing the appropriate concentration of kanamycin and pelleted (Sorvall RC-5B) at 4500 r.p.m. for 12 min. Samples were resuspended in 5 ml SET buffer (75 mM NaCl, 25 mM EDTA pH 8, 20 mM Tris pH 7.5). Lysozyme (5 mg ml⁻¹) was added and samples were incubated at 37 °C for 1 h with shaking. Proteinase K (1 mg ml⁻¹ final concentration) plus 2% (w/v) SDS (final concentration) were then added and samples were incubated at 60 °C for 2 h. NaCl (5 M; 2 ml) was added; the samples were mixed and allowed to cool to less than 37 °C. Chloroform (5 ml) was then added and the samples were incubated at 4 °C for 30 min with shaking. Cell debris was pelleted by centrifugation at 4500 r.p.m. at 4 °C and supernatants transferred to a 15 ml Corex tube. DNA was precipitated with 0.6 vol. 2-propanol at -20 °C for 1 h and recovered by centrifugation at 8000 r.p.m. for 15 min. DNA pellets were left to dry and were then resuspended in 100 µl warm sterile distilled water.

Restriction enzymes and T4 DNA ligase were from New England Biolabs and were used according to the manufacturer's instructions. Ligations were carried out at room temperature for 2 h.

Southern hybridization. Probes were labelled by the incorporation of digoxigenin-11-dUTP by PCR using the Roche PCR DIG Probe Synthesis Kit, used according to the manufacturer's instructions. All hybridizations were carried out using the DIG system for filter hybridization (Roche) according to the manufacturer's instructions. DNA was transferred onto positively charged nylon membranes (Boehringer Mannheim) and fixed by UV cross-linking. Hybridization and post-hybridization washes were carried out at 68 °C in a Hybaid 'shake'n'stack' hybridization oven.

Inverse PCR (iPCR). To sequence tagged genome insertions, total DNA was extracted and 1–2 µg samples were digested with the appropriate restriction enzymes (*Mlu*I, *Nco*I and *Pst*I were used). Digested DNA was cleaned up by extraction with phenol/chloroform (1:1) and precipitated with 2-propanol. Digested samples for each transposant were pooled in 17 µl sterile distilled water and ligation carried out at room temperature for 2 h. Ligations were heat-killed and then drop-dialysed for 1 h on 0.025 µm Millipore filters before proceeding to iPCR. After drop dialysis, iPCR was carried out as follows using GibcoBRL reagents: 1× PCR buffer, 1.5 mM MgCl₂, 200 µM of each dNTP (Promega), 0.2 µM of forward primer (KAN-2 FP-1, 5'-ACCTACAACAAAGCTCTCATCAACC-3') and of reverse primer (KAN-2 RP-1, 5'-GCAATGTAA-CATCAGAGATTTTGAG-3'), 2.5 U *Taq* DNA polymerase and 1 µl template DNA (from ligation). Sterile distilled water was added to a final volume of 50 µl. PCR reaction conditions were as follows: initial denaturation at 96 °C for 5 min; followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min. Following iPCR, samples were run on 0.85% agarose gels, products were cut out and DNA extracted using the Concert rapid gel extraction system (GibcoBRL). Finally, salt was removed by drop dialysis of samples prior to sequencing.

DNA sequencing and analysis. Sequencing reactions were carried out using ABI PRISM BigDye sequencing kits on an ABI PRISM 377 DNA sequencer. KAN-2 FP-1 and KAN-2 RP-

1 primers used for iPCR were also used for sequencing. Sequences were assembled using Staden software programs PREGAP and GAP4 (Bonfield *et al.*, 1995) on a Silicon Graphics Unix computer. DNA and protein similarity searches were carried out with BLAST 2.0 using the WWW interface at the NCBI (National Center for Biotechnology Information) (Altschul *et al.*, 1997). The Prosite (Bairoch *et al.*, 1997) and Pfam (Sonnhammer *et al.*, 1998) profile databases were searched using the server of the Bioinformatics group at the ISREC (Swiss Institute for Experimental Cancer Research) and the ExPASy WWW molecular biology server of the Geneva University Hospital and the University of Geneva. Other analyses were carried out using the GCG (Genetics Computer Group, Madison, Wisconsin) package, version 9.1.

RESULTS

Optimization of *R. rhodochrous* CW25 electroporation

R. rhodochrous CW25 was used to test the transposome mutagenesis system as this strain has been widely used by researchers (Bresler *et al.*, 2000) and has a high electroporation efficiency.

The generation of significant numbers of insertion mutants using the transposome system is critically dependent on the electroporation efficiency of the strain, particularly with heterologously derived DNA, as is the case for both pCY104 and the transposome system. Electroporation efficiencies are most easily measured using a replicative vector. Highly electrocompetent *E. coli* will generate 10⁹ c.f.u. µg⁻¹ of replicative, homologously derived plasmid DNA and 10⁵ c.f.u. (20 ng)⁻¹ of transposome preparation. Such high efficiencies with replicative plasmid DNA are not readily achievable using wild-type strains of *Rhodococcus* and it would therefore be expected that the number of transposome insertions generated would also be lower.

It has been shown that recovery after electroporation on solid media prior to selection results in a higher frequency of transformants than recovery in liquid media (Powell, 1998). Although selection can be carried out by resuspension of the cells from non-selective solid media and subsequent replating on selective media, this generates a significant number of sibling colonies, thus obscuring the true number of independent transformants. To overcome this problem, the use of soft agar overlays was investigated. Kanamycin levels ranged from 50 to 450 µg ml⁻¹. Using *R. rhodochrous* CW25 it was found that at levels below 400 µg ml⁻¹ a significant number of spontaneous resistant mutants arose, whereas at levels above 500 µg ml⁻¹ the number of transformants was reduced. A soft agar overlay supplemented with kanamycin to give a final concentration of 400 µg ml⁻¹ in each plate was therefore used to select transformants for the remainder of the work.

To determine the optimum recovery time after electroporation, 100 µl of each sample was plated onto LAG (Luria agar with 1% glucose) and incubated at 30 °C for 0, 6 and 12 h prior to addition of selective overlay. Selection immediately after plating cells on LA with

Fig. 1. Efficient transposition of a Tn5 transposome in *Rhodococcus*. Lanes 1–21 and 24–25 contain total DNA from putative *R. rhodochrous* CW25 transposants probed with the DIG-labelled Tn903 kanamycin gene. Lane 26 contains *R. rhodochrous* CW25 DNA not subjected to transposon mutagenesis. Lane 27 contains the Tn903 kanamycin-carrying plasmid pVJT131 as a positive control. This blot shows that each of the kanamycin-resistant colonies chosen at random contained an individual transposition event. Furthermore, of all 23 transposants chosen from the overlay selection, none shared the same insertion site. Lanes 22 and 23 contain DNA from sibling mutants from an earlier experiment in which the overlay technique was not used. They show bands in common as the siblings arose due to the nature of transposant recovery. Positions of markers (*Hind*III-digested λ DNA) are shown on the left.

glucose greatly reduced efficiency, 5×10^3 c.f.u. $(0.5 \mu\text{g DNA})^{-1}$, as compared with those allowed to recover for longer periods post-electroporation. The longer recovery times of 6 and 12 h gave rise to 2×10^4 and 7×10^4 c.f.u. $(0.5 \mu\text{g DNA})^{-1}$, respectively. After 12 h of recovery, the number of transformants was increased 14-fold compared with immediate plating onto selective media. If cells were left to recover for longer periods (more than 12 h), the amount of background growth no longer allowed for selection of transformants.

Growth on glycine and/or isoniazid has been shown to enhance the electroporation efficiencies of strains of *Corynebacterium glutamicum* (Haynes & Britz, 1990), *Mycobacterium bovis* BCG (Cho *et al.*, 1998) and *Rhodococcus equi* (Sekizaki *et al.*, 1998), most likely by affecting growth of the cell wall. *R. rhodochrous* CW25 grown in the presence of glycine (2%), or glycine (2%) plus isoniazid ($100 \mu\text{g ml}^{-1}$) gave rise to a 10-fold increase [7×10^5 c.f.u. $(0.5 \mu\text{g DNA})^{-1}$] in electroporation efficiency (using the replicative vector pCY104) as compared to cells grown in unsupplemented media. These high-efficiency cells were stored at -70°C and subsequently used for electroporation with transposome.

Electroporation with transposome

A preliminary experiment was carried out in which *R. rhodochrous* CW25 (grown without media supplementation) was electroporated with 20 ng of the trans-

posome preparation and spread onto non-selective LA plates. Following overnight incubation, cells were resuspended and plated onto LA supplemented with $400 \mu\text{g kanamycin ml}^{-1}$. A number of transposants were isolated and chromosomal insertions confirmed by Southern blotting, but electroporation/transposition frequencies could not be determined as the nature of the recovery gave rise to large numbers of siblings (data not shown). To confirm these results, the experiment was repeated using the glycine-grown electrocompetent cells described above. Transposants were selected using the kanamycin overlay technique to prevent isolation of sibling colonies. One thousand five hundred kanamycin-resistant colonies were obtained from 20 ng transposome complex.

Confirmation of insertion by Southern blotting

To confirm insertion of the transposome, chromosomal DNA was prepared from 23 transposant colonies chosen at random. The genomic DNA was digested to completion with *Pst*I and Southern blot analysis was performed using the Tn903 kanamycin resistance gene as a probe. The Southern blot shown in Fig. 1 demonstrates that all insertions were unique and random insertion of the transposon occurred, in all cases at different places on the chromosome with the exception of the colonies in lanes 22 and 23, which were siblings from the preliminary experiment described earlier in which the overlay technique was not used. These results

were confirmed by an additional blot in which samples were digested with *EcoRI* (data not shown).

Confirmation of Tn5 transposition by sequencing

When transformed into strains of *Rhodococcus* and *Mycobacteria*, circular and linear DNA molecules have been shown in some instances to undergo illegitimate recombination with the chromosome (Desomer *et al.*, 1991; Kalpana *et al.*, 1991; Larkin *et al.*, 1998). Transposase-mediated Tn5 insertions are characterized by the generation of 9 bp direct repeats (Berg *et al.*, 1988) in the host DNA flanking the insertion. To establish whether the transposome DNA had been inserted into the *Rhodococcus* genome by illegitimate recombination or by transposition, the sequence of the DNA flanking the insertions was determined by sequencing the iPCR products generated from 10 representative mutants. The sequence data results (six mutants displayed) show the presence of 9 bp direct repeats in the host DNA flanking the transposable element (Fig. 2), thus proving that transposition has occurred.

Auxotrophy screen

To test the comprehensiveness of this library, 800 *R. rhodochrous* CW25 mutants were patched onto minimal media with and without Casamino acids (880 µg ml⁻¹). Of the 800 mutants, 20 (2.5%) failed to grow on minimal media without Casamino acids, as expected from earlier publications which reported similar results (Desomer *et al.*, 1991). Auxotrophic phenotypes of seven mutants were deduced by supplementation with amino acids (Davis *et al.*, 1982) and transposon insertion sites sequenced. Table 1 summarizes the phenotypic and genotypic data on auxotrophic mutants. In each case the phenotype corresponds to that expected based on the disrupted gene.

Library construction in *Rhodococcus* sp. RC89

The wild-type soil isolate *Rhodococcus* sp. RC89 was selected for its ability to catabolize a wide range of aromatic hydrocarbons. Unlike *R. rhodochrous* CW25, *Rhodococcus* sp. RC89 has not been subjected to laboratory manipulation.

Rhodococcus sp. RC89 was transformed by electroporation with pCY104 and allowed to recover on LA with 1% glucose for 12 h at 30 °C. Soft agar overlays were added to give a range of final kanamycin concentrations from 100 to 400 µg ml⁻¹. A concentration of 100 µg kanamycin ml⁻¹ was found to be optimal. The pCY104 transformation efficiency of *Rhodococcus* sp. RC89 was 3 × 10⁴ c.f.u. (0.5 µg DNA)⁻¹.

Electroporation with 20 ng (1 µl) transposome gave rise to 500 *Rhodococcus* sp. RC89 transposants. Four hundred transposants were patched onto minimal media

Fig. 2. Confirmation of Tn5 transposition by sequencing transposon/*Rhodococcus* junctions. Sequences of six putative transposants are shown here, mutants B, C and E picked at random and auxotrophic mutants F, P and R. Sequencing reactions using the forward and reverse primers (KAN-2 FP-1 and KAN-2 RP-1) were carried out. Five base pairs (gacag) of the Tn5 inverted repeats are shown flanking the Tn903 kanamycin resistance gene (KAN>). Nine base pair repeats of *Rhodococcus* DNA flanking the transposon are shown in bold (arrows indicate repeat sequence orientation). Sequencing of 10 out of 10 (additional 4 not shown) *R. rhodochrous* CW25 transposants revealed 9 bp repeats in the *Rhodococcus* genome at the insertion site of Tn5.

with succinate and 24 (6.5%) failed to grow, indicating efficient library coverage. Around 200 mutants were screened for their inability to utilize toluene and benzene as sole sources of carbon and energy. Two mutants incapable of utilizing these compounds and five further mutants that grew slowly on them were isolated. The insertion site of one of the mutants was sequenced and transposition confirmed by the presence of 9 bp repeats. The disrupted gene was 57% identical to a diamino-butyric acid aminotransferase from *Bacillus halodurans* (NCBI accession no. BAB04638). As there is no evidence to suggest that this gene product is involved in the

Table 1. Summary of auxotrophic requirements and associated genes of *R. rhodochrous* CW25 auxotrophic mutants

Auxotroph	Auxotrophic requirement	Associated genes	NCBI accession no.
A	Isoleucine/valine	70 % identical to acetolactate synthase (<i>ilvB</i>) from <i>M. tuberculosis</i>	O53250
D	Isoleucine (weak growth in the presence of other amino acids)	80 % identical to dihydroxy-acid dehydratase (<i>ilvD</i>) from <i>C. glutamicum</i>	CAB57218
I	Methionine	77 % identical to 5-methyltetrahydrofolate-homocysteine methyltransferase (<i>metH</i>) from <i>M. tuberculosis</i>	O33259
L	Isoleucine	64 % identical to threonine dehydratase (<i>ilvA</i>) from <i>C. glutamicum</i>	Q04513
P	Methionine/cysteine	55 % identical to a probable uroporphyrin-III methyltransferase (<i>cysG2</i>) from <i>M. tuberculosis</i>	B70589
R	Leucine	74 % identical to 3-isopropylmalate dehydrogenase (<i>leuB</i>) from <i>C. glutamicum</i>	P94631
S	Isoleucine/valine	77 % identical to acetolactate synthase (<i>ilvB</i>) from <i>M. avium</i>	Q59498

degradation of monoaromatic hydrocarbons, it is likely that the associated phenotype is due to a polar effect. A number of *Rhodococcus* sp. RC89 aromatic hydrocarbon degradation mutants are currently under investigation.

DISCUSSION

Using electrocompetent *E. coli* [10^9 transformants (μg replicative DNA) $^{-1}$], 10^5 transposants can be obtained from 20 ng transposome. In comparison, 1.5×10^3 transposants were obtained from 20 ng transposome electroporated into *R. rhodochrous* CW25 [electroporation efficiency of 10^5 (μg replicative DNA) $^{-1}$]. Thus, when normalized against replicative plasmid transformation efficiencies, the transposition frequency appears to be 100-fold higher than *E. coli*. These figures may, however, be misleading as electroporation of *E. coli*-derived plasmid DNA into *Rhodococcus* is often subject to restriction, which can reduce electroporation efficiencies up to 10^4 -fold when compared with *Rhodococcus*-derived plasmid DNA (Powell, 1998). Restriction is dependent on the presence of the enzyme recognition sequence in the introduced DNA. The short 1 kb transposome DNA is less likely to contain such recognition sequences than the 8 kb pCY104 vector used to derive the replicative vector efficiencies. Restriction of pCY104 could therefore increase apparent normalized transposition frequency in *Rhodococcus*. Thus the short transposome DNA is at a distinct advantage compared with larger plasmid-based suicide vectors when dealing with restriction competent strains.

Because of polar effects, it is difficult to calculate exactly the number of insertions required for a high confidence library. However, given a plausible genome complement of 4000 genes arranged in 2500 transcriptional units, this number should lie between 3000 and 10000 insertions (Blattner *et al.*, 1997). This number is readily achievable by simple scale-up with the system described in this paper for both *R. rhodochrous* CW25 and the wild-type strain *Rhodococcus* sp. RC89. Library construction would be facilitated by optimization of the electroporation efficiency for each strain. We have found that pCY104 has a particularly broad host range amongst the rhodococci and is therefore an ideal test vector. This is the first report of transposon mutagenesis on this scale in *Rhodococcus*.

The advantages of this system over the standard *in vivo* transposase expression approach are the high efficiency, the lack of requirement for complex replicative vectors or native inducible promoters to drive transposase expression, and the generation of unique insertions without risk of isolating sibling colonies (using the overlay technique described here). Transposome electroporation is likely to become the method of choice for generating transposon insertion libraries in *Rhodococcus*.

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REFERENCES

- Allen, C. C. R., Boyd, D. R., Larkin, M. J., Reid, K. A., Sharma, N. D. & Wilson, K. (1997). Metabolism of naphthalene, 1-naphthol, indene, and indole by *Rhodococcus* sp. strain NCIMB 12038. *Appl Environ Microbiol* **63**, 151–155.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Bairoch, A., Butcher, P. & Hofmann, K. (1997). The PROSITE database, its status in 1997. *Nucleic Acids Res* **25**, 217–221.
- Barnes, M. R., Duetz, W. A. & Williams, P. A. (1997). A 3-(3-hydroxyphenyl)propionic acid catabolic pathway in *Rhodococcus globerulus* PWD1: cloning and characterization of the *hpp* operon. *J Bacteriol* **179**, 6145–6153.
- Berg, D. E., Kazic, T., Phadnis, S. H., Dodson, K. W. & Lodge, J. K. (1988). Mechanism and regulation of transposition. In *Transposition* (Society for General Microbiology Symposium **43**), pp. 107–129. Edited by A. J. Kingsman, K. F. Chater & S. M. Kingsman. Cambridge: Cambridge University Press.
- Berkowitz, F. E. (1994). The gram-positive bacilli – a review of the microbiology, clinical aspects, and antimicrobial susceptibilities of a heterogeneous group of bacteria. *Pediatr Infect Dis J* **13**, 1126–1138.
- Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A. & 14 other authors (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
- Bonfield, J. K., Smith, K. F. & Staden, R. (1995). A new DNA sequence assembly program. *Nucleic Acids Res* **23**, 4992–4999.
- Bresler, M. M., Rosser, S. J., Basran, A. & Bruce, N. C. (2000). Gene cloning and nucleotide sequencing and properties of a cocaine esterase from *Rhodococcus* sp. strain MB1. *Appl Environ Microbiol* **66**, 904–908.
- Cho, S. N., Hwang, J. H., Park, S., Chong, Y. S., Kim, S. K., Song, C. Y. & Kim, J. D. (1998). Factors affecting transformation efficiency of BCG with a *Mycobacterium-Escherichia coli* shuttle vector pYUB18 by electroporation. *Yonsei Med J* **39**, 141–147.
- Colquhoun, J. A., Mexson, J., Goodfellow, M., Ward, A. C., Horikoshi, K. & Bull, A. T. (1998). Novel rhodococci and other mycolate actinomycetes from the deep sea. *Antonie Leeuwenhoek* **74**, 27–40.
- Crespi, M., Vereecke, D., Temmerman, W., Vanmontagu, M. & Desomer, J. (1994). The Fas operon of *Rhodococcus fascians* encodes new genes required for efficient fasciation of host plants. *J Bacteriol* **176**, 2492–2501.
- Dabbs, E. R. (1987). A generalized transducing bacteriophage for *Rhodococcus erythropolis*. *Mol Gen Genet* **206**, 116–120.
- Davis, R. W., Botstein, D. & Roth, J. R. (1982). *A Manual for Genetic Engineering: Advanced Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Denome, S. A. & Young, K. D. (1995). Identification and activity of two insertion sequence elements in *Rhodococcus* sp. strain IGTS8. *Gene* **161**, 33–38.
- Derbyshire, K. M., Takacs, C. & Huang, J. (2000). Using the EZ::Tn[®] – Transposome[®] – for transposon mutagenesis in *Mycobacterium smegmatis*. *Epicentre Forum* **7**, 1–4.
- Desomer, J., Crespi, M. & Van Montagu, M. (1991). Illegitimate integration of non-replicative vectors in the genome of *Rhodococcus fascians* upon electrotransformation as an insertional mutagenesis system. *Mol Microbiol* **5**, 2115–2124.
- Goodfellow, M. & Alderson, G. (1977). The actinomycete genus *Rhodococcus*: a home for the “rhodochrous” complex. *J Gen Microbiol* **100**, 99–122.
- Goryshin, I. Y., Jendrisak, J., Hoffman, L. M., Meis, R. & Reznikoff, W. S. (2000). Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. *Nature Biotechnol* **18**, 97–100.
- Guilhot, C., Otal, I., Vanrompaey, I., Martin, C. & Gicquel, B. (1994). Efficient transposition in mycobacteria – construction of *Mycobacterium smegmatis* insertional mutant libraries. *J Bacteriol* **176**, 535–539.
- Haynes, J. A. & Britz, M. L. (1990). The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation. *J Gen Microbiol* **136**, 255–263.
- Jager, W., Schafer, A., Kalinowski, J. & Puhler, A. (1995). Isolation of insertion elements from Gram-positive *Brevibacterium*, *Corynebacterium* and *Rhodococcus* strains using the *Bacillus subtilis* *sacB* gene as a positive selection marker. *FEMS Microbiol Lett* **126**, 1–6.
- Kalpana, G. V., Bloom, B. R. & Jacobs, W. R. (1991). Insertional mutagenesis and illegitimate recombination in mycobacteria. *Proc Natl Acad Sci USA* **88**, 5433–5437.
- Kleckner, N. (1981). Transposable elements in prokaryotes. *Annu Rev Genet* **15**, 341–404.
- Larkin, M. J., DeMot, R., Kulakov, L. A. & Nagy, I. (1998). Applied aspects of *Rhodococcus* genetics. *Antonie Leeuwenhoek* **74**, 133–153.
- Lessard, P. A., O'Brien, X. M., Ahlgren, N. A., Ribich, S. A. & Sinskey, A. J. (1999). Characterization of IS1676 from *Rhodococcus erythropolis* SQ1. *Appl Microbiol Biotechnol* **52**, 811–819.
- Mosser, D. M. & Hondalus, M. K. (1996). *Rhodococcus equi*: an emerging opportunistic pathogen. *Trends Microbiol* **4**, 29–33.
- Nagy, I., Schoofs, G., Vanderleyden, J. & DeMot, R. R. (1997). Transposition of the IS21-related element IS1415 in *Rhodococcus erythropolis*. *J Bacteriol* **179**, 4635–4638.
- Pelacic, V., Jackson, M., Reyat, J. M., Jacobs, W. R., Gicquel, B. & Guilhot, C. (1997). Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **94**, 10955–10960.
- Pospiech, A. & Neumann, B. (1995). A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Genet* **11**, 217–218.
- Powell, J. A. C. (1998). *Genetic analysis in Rhodococcus sp. V49: the ohp gene cluster*, pp. 264–289. PhD thesis, University of Cambridge.
- Powell, J. A. C. & Archer, J. A. C. (1998). Molecular characterisation of a *Rhodococcus ohp* operon. *Antonie Leeuwenhoek* **74**, 175–188.
- Sekizaki, T., Tanoue, T., Osaki, M., Shimoji, Y., Tsubaki, S. & Takai, S. (1998). Improved electroporation of *Rhodococcus equi*. *J Vet Med Sci* **60**, 277–279.
- Shao, Z. O. & Behki, R. (1995). Cloning of genes for the degradation of the herbicides EPTC (S-ethyl dipropylthiocarbamate) and atrazine from *Rhodococcus* sp. strain TE1. *Appl Environ Microbiol* **61**, 2061–2065.
- Sonnhammer, E. L. L., Eddy, S. R., Birney, E., Bateman, A. & Durbin, R. (1998). Pfam: multiple sequence alignments and HMM-profiles of protein domains. *Nucleic Acids Res* **26**, 320–322.
- Warhurst, A. M. & Fewson, C. A. (1994). Biotransformations

catalysed by the genus *Rhodococcus*. *Crit Rev Biotechnol* **14**, 29–73.

Yao, W. S., Yang, Y. L. & Chiao, J. S. (1994a). IS204 – an insertion sequence from *Nocardia asteroides (mexicana)* yp21. *Plasmid* **32**, 262–269.

Yao, W., Yang, Y. & Chiao, J. (1994b). Cloning vector system for *Nocardia* spp. *Curr Microbiol* **29**, 223–227.

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