Random transposon mutagenesis of the *Saccharopolyspora erythraea* genome reveals additional

Genes influencing erythromycin biosynthesis

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SUMMARY

A gene influencing the production level of the antibiotic erythromycin was uncovered using a random transposon mutagenesis procedure.

INTRODUCTION

Commercial production of antimicrobial products by large-scale submerged fermentation began with penicillin in the 1940s. Since then, hundreds of natural products have been produced for medicine and agriculture including antibiotics, anti-cancer agents, immunosuppressants and active pharmaceutical ingredients (Bérdy 2005). Traditional strain improvement that leads to improved production levels is an empirical stepwise process performed through multiple labor-intensive cycles of random mutation and screening using higher antibiotic production as the screening criterion (Demain and Adrio 2008). Typically the strongest positive mutation steps are found during the first few cycles of the process followed by cycles that, despite a larger screening effort, produce smaller increases in yield (Barrick and Lenski 2013). For this project, *in vitro* transposition (Goryshin and Reznikoff 1998) was used to generate random mutations. Experiments were performed directly in a *Saccharopolyspora erythraea mutB* strain (FL2302) and a sample of genes was knocked out comprising ~7% of the genes in the chromosome. One of the mutations, in the cwh1 gene, had the ability to be scaled-up making it of special interest for further study.

METHODS

General materials and methods are described in Kieser *et al*. (2000) and Sambrook, Fritsch and Maniatis (1989). *Saccharopolyspora erythraea mutB* FL2302 (Reeves *et al*. 2006) is a derivative of *S. erythraea* FL2267 which was obtained from a lyophilized vial of *S. erythraea* ATCC 11635. *Saccharopolyspora erythraea* FL2267 was the source of the genomic DNA used in the formation of Library 1..

- Construction of plasmid pFL2073
- .Library 1: DNA cloning
- Library 2: transposon mutagenesis
- Library 3: S. erythraea transformation and DNA replacement
- Micro-agar fermentations and erythromycin titer determinations.
- Shake flask fermentation screen.
- Recover plasmids from blue transformants and identify the activator gene.

RESULTS

A collection of mutants from a single cycle of strain improvement

Thirty-five mutants influencing erythromycin production were obtained from the screening of 1048 transposon-generated mutants of *S. erythraea* representing ~7% of the genes in the genome. DNA sequence analysis of the transposon insertion sites revealed 15 unique genotypes; siblings and multiple mutations in the same gene accounted for the duplicate genotypes. Of the 15 knockout mutant strains found, 13 showed a >25% improved yield and 1 genotype had reduced yield and 1 genotype was neutral but showed reduced yield upon later scale-up analysis (Fig. 1). The mutants from the first screen showed mean increases in erythromycin yield of 34%–109%.

Fig. 1A&B. Micro fermentation screen.

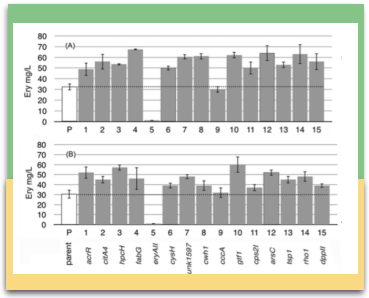


Figure 1. (**A**) Micro-agar plug fermentation screen. Erythromycin production levels of 15 transposon-insertion mutants are compared to the parent strain FL2302 (P). The dotted lines indicate the base-line level of erythromycin production by the parental strain; brackets represent standard deviation, *n* = 2. (**B**) The repeated microgel fermentation screen; brackets represent standard deviation, *n* = 2.

Fig 2A. Genome map of S. erythraea showing transposon insertions.

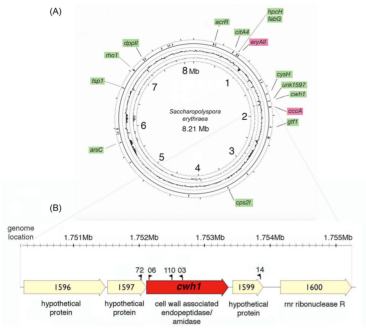


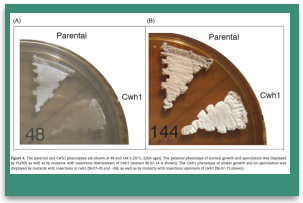
Table 1. Transposon mutations from this study influencing erythromycin biosynthesis in *S. erythraea*.

Gene	Mutant #	Transposon nucleotide (nt) insertion site	Ery[1]	Spo[2]	Pig[3]	Predicted Function (reference where known)
	FL2302		Control	+	+	Parental strain control
acrR	S6.18-36	In SACE_0303; 339,794	+	+	+	acrR, regulator of multidrug efflux pump (Wu et al. 2014)
ctaA4	S6.18-32	In SACE_0632; 696,384	+	+	+	ctaA4 citrate synthase (Moiller et al. 2001)
hpcH	S7.11-58	In SACE_0699; 769,528	+	–	–	hpcH 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase
fabG	S6.07–125	In SACE_0700; 770,333	+	–	–	fabG, short-chain dehydrogenase/reductase family
eryAII	S5.17–06	In SACE_0723; 802,912	–	–	–	eryAII erythromycin polyketide synthase
cysH	S6.18-04	In SACE_1474; 1,626,645	+	+	+	cysH, sulfate adenylyltransferase subunit 2
unk1597	S6.07–72	In SACE_1597; 1,752,052	+	–	+/-	unk1597, hypothetical protein, polar effects expected on SACE_1598
cwh1	S6.07–03	In SACE_1598; 1,752,509	+	–	+/-	cwh1, cell-wall-associated hydrolase, NlpC/P60 superfamily (Anantharaman and Aravind 2003)
cocA	S5.17–12	In SACE_1685; 1,852,856	–	–	–	cocA – cytochrome c mono-and diheme variants
gtf1	S7.05–55	Upstream of SACE_2010; 2,197,132	+	–	+	gtf1, glycosyltransferase- GT1.GtfJ-like family (Liang and Qiao 2007)
cpa2t	S6.15–149	In SACE_3177; 3,507,747	+	bld	+	cpa2t, nucleotide-sugar-dependent glycosyltransferase, group 1 (Lukduti Jansen et al. 2014)
arsC	S6.18–12	In SACE_5163; 5,750,836	+	–	–	arsC, arsenical resistance protein, arsenate reductase, arsenic transporter (EC 1.20.4.1)
bip1	S6.18–17	In SACE_5967; 6,700,519	+	+	+	bip1, secreted trypsin-like serine protease
rho1	S7.12–136	In SACE_6295; 7,045,657	+	+	+	rho1, rho1-like transcription terminator (Cardinale et al. 2008)
dppII	S6.15–143	Upstream of SACE_8505; 7,295,927	+	+	+	dppII, X-Pro dipeptidyl-peptidase (Mann, Scharpé and De Meester 2007)

Erythromycin production phenotype; increased production +, reduced production –, compared to parent strain FL2302.
Sporulation phenotype: +, wild type sporulation; –, makes aerial mycelium but no spores; bld, makes no aerial mycelium or spores.
Pigmentation, +, normal red pigmentation; –, no red pigmentation; +/-, reduced red pigmentation.

Figure 2. (**A**) Genome map of *S. erythraea* showing transposon insertion sites of Library 3 mutants. Map is based on data generated by Oliynyk *et al*. (2007)**.** Knockouts of genes highlighted in green gave increases in yield, and red shading is for decreases in yield. Map positions are shown in megabase pairs. (**B**) Map of the *cwh1* (SACE 1598) region of the *S. erythraea* genome (Oliynyk *et al*. 2007). Transposon insertion and orientation are indicated by directional flags. Numbers above the flags refer to mutant numbers; all mutant numbers shown are from the S6.07 pool, for example, '03' indicates mutant number S6.07–03. Map positions are shown in megabase pairs.

Fig. 3. *Sac. erythraea* Cwh1 phenotype



Mutations in *cwh1* produced visible changes in growth on solid media, which is consistent with the predicted function of Cwh1 in cell wall biogenesis.

Fig. S1. Cwh1 a.a. sequence

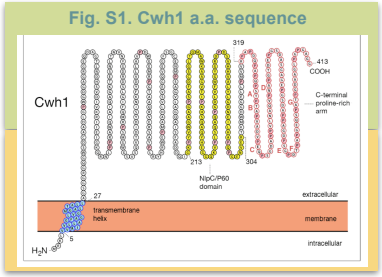


Fig. S1. Amino acid sequence of cell wall associated hydrolase, Cwh1 (SACE_1598), cell wall- associated hydrolase with embedded transmembrane helix (blue-filled circles), NlpC/P60 endopeptidase domain (yellow-filled circles), and a proline-rich domain likely to function as a "sticky-arm" that attaches to other proteins (proline residues are pink-filled circles). The seven PxxP motifs are labelled with the larger bold red letters A-G. Original figure generated by Protter (Omasits et al., 2014).

Discussion

This study used targeted mutagens to explore the genetics behind the process of strain improvement. Traditionally, strain improvement mutations are created by chance and chosen on phenotypic performance alone, with no knowledge of genotype needed; however, no benefit is passed to future strain improvement programs. The transposon-based strategy used in this study allows the characterization of the high-performing genotypes so that these mutations might be rationally incorporated into other desired genetic backgrounds. By using transposon mutagenesis it was possible to see that ~3%; mutations generated had an effect on erythromycin yield. In a typical actinomycete such as *S. erythraea* with over 7000 genes, this might mean that a saturating random mutagenesis could yield as many as 210 unique strain improvement mutations. Only one locus, *cwh1*, was found that could be scaled-up to a 100-fold increase in fermentation volume (0.25–25 ml). Mutations in *cwh1* produced visible changes in growth on solid media, which is consistent with the predicted function of Cwh1 in cell wall biogenesis. If the *cwh1* mutation affects the early stages of cell wall biosynthesis, then the yield improvement phenotype could result from the diversion of cell wall precursors such as NDP-rhamnose, from cell wall biosynthesis into erythromycin biosynthesis.

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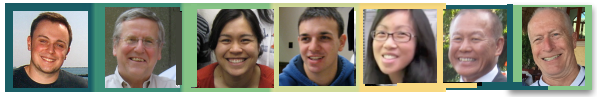

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