Random transposon mutagenesis of the *Saccharopolyspora erythraea* genome reveals additional genes influencing erythromycin biosynthesis.

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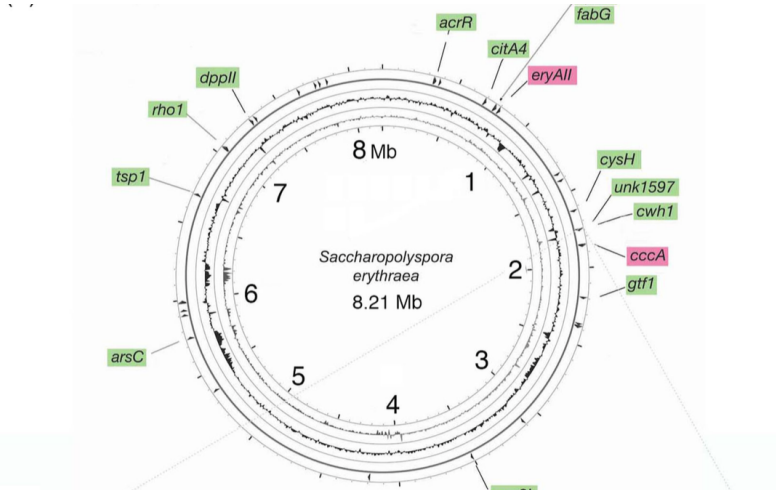

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Abstract

A single cycle of strain improvement was performed in *Saccharopolyspora erythraea mutB* and 15 genotypes influencing erythromycin production were found. Genotypes generated by transposon mutagenesis appeared in the screen at a frequency of ~3%. Mutations affecting central metabolism and regulatory genes were found, as well as hydrolases, peptidases, glycosyl transferases and unknown genes. Only one mutant retained high erythromycin production when scaled-up from micro-agar plug fermentations to shake flasks. This mutant had a knockout of the cwh1 gene (SACE_1598), encoding a cell-wall-associated hydrolase. The *cwh1* knockout produced visible growth and morphological defects on solid medium. This study demonstrated that random transposon mutagenesis uncovers strain improvement-related genes potentially useful for strain engineering.

Keywords: *transposon; strain improvement; Actinomyces; directed evolution; erythromycin*

Chapter One

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 ingre- dients (Be´rdy 2005). When microorganisms from the soil are transferred to the laboratory, they tend to produce only trace amounts of the active compound. Therefore, strain improve- ment is often a necessary step of the commercialization process (Vinci and Byng 1999; Demain and Adrio 2008).

Traditional strain improvement that leads to improved pro- duction levels is an empirical stepwise process performed through multiple labor-intensive cycles of random mutation and screening using higher antibiotic production as the selective cri- terion. It requires only basic microbiological methods (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 in- creases in yield (Barrick and Lenski 2013). The underlying ge- netics of strain improvement were never characterized during the 'golden age' when the first commercial antibiotic-producing strains were generated, but genomic technology today allows for the reverse engineering of the process, opening it up to sci- entific study. Thus, high-producing strains resulting from mul- tiple cycles of mutation and selection have been compared to wild-type reference strains (Peano *et al.* 2012; Li *et al.* 2013). And randomly generated yield improvement mutations have been reverse engineered, advancing knowledge-based engineering of yield (Gehring *et al.* 2000; Santos and Stephanopoulos 2008).

For this project, *in vitro* transposition (Goryshin and Reznikoff 1998) was used to generate random mutations. Transposition creates single mutations that can be identified and mapped by plasmid rescue and DNA sequencing, generating a direct link be- tween the higher yield (phenotype) and the transposon inser- tion (genotype). Once the genotype is known, it can be incor- porated into different genetic backgrounds. Experiments were performed directly in a *Saccharopolyspora erythraea mutB* strain (FL2302), circumventing the need to use *Aeromicrobium erythreum* as a model host as was done in the past (Reeves *et al.* 2004). A single round of random transposon mutagenesis was used on a *mutB* strain of *S. erythraea* (FL2302) and a sample was knocked out comprising ~7% of the genes in the chromosome. The mu- tants were screened by static micro-agar plug fermentations for changes in erythromycin yield and 15 mutants of interest were found. DNA sequence analysis revealed the site of insertions of the transposon. The function of the mutant genes was

queried using the BLASTP algorithm. One of the mutant strains was scaled-up to flask fermentations making it a strong candidate for further study.

Chapter Two

Materials and Methods

Bacterial strains and growth conditions

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 deriva- tive 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. For more detail, see Supplementary Materials and Methods.

Construction of plasmid pFL2073

A previously described cloning vector, pFL8 (Reeves *et al.* 2002; NCBI sequence accession: BankIt1812822 KR061299), was dou- bly digested with endonucleases *BglII* and *BssHII;* the short single-stranded ends were blunted and ligated to delete the *neo* (neomycin/kanamycin resistance) gene thus creating pFL2073 (Fig. 1). This plasmid has both *Streptomyces* and *Escherichia coli* origins of replication (pIJ101 and pBR322, respectively), the thiostrepton (*tsr*) and ampicillin (*amp*) resistance genes and the *lacZ* gene with a multicloning site for blue/white screening on X-gal-containing media. Plasmid pFL2073 was used as the par- ent plasmid for the generation of Library 1. Despite the fact that pFL2073 is a high copy autonomously replicating plasmid in *Streptomyces lividans*, it functions as an integrative plasmid in *S. erythraea*. When pFL2073 contains *S. erythraea* DNA clones, it integrates into the *S. erythraea* chromosome by homologous re- combination (if the fragment is >0.4 kb) and can be readily used for single-crossover integration to knock out genes, or it can be used for double-crossover gene replacements (Weber *et al.* 1990) which is how it was used in this study to deliver transposon mutations into the chromosome.

Library 1: DNA cloning

Saccharopolyspora erythraea chromosomal DNA was partially di- gested into ~10–15 Kb fragments and ligated into pFL2073 (Fig. 1). Ligation mixtures were transformed into *E. coli* DH5α by electroporation. A high-purity DNA preparation was generated from transformants. Plasmids carrying fragments larger than 9 kb and with similar copy numbers

were combined in pools of approximately 20 plasmids each. Eleven different plasmid pools were used in the *in vitro* transposition reactions to create Library 2. For more detail, see Supplementary Materials and Methods.

Library 2: transposon mutagenesis

Eleven plasmid pools from Library 1 were mutagenized *in vitro* using the EZ-Tn5 <R6Kγ ori/Kan-2> kit from Epicentre Biotech- nologies (Madison, WI). The mutagenized plasmid reaction mix- ture was used to transform competent *E. coli* cells, the cells were plated on LB and selected for ampicillin and kanamycin- resistant transformants. Primary transformants were harvested and high-purity DNA was prepared from them to create Library 3. For more detail, see Supplementary Materials and Methods.

Library 3: *S. erythraea* transformation and DNA replacement

Library 3 was generated by transforming plasmid DNA from Library 2 into protoplasts of *S. erythraea mutB* FL2302 using a modification of the method of Weber and Losick (1988). Eight highly transformable protoplast preparations were transformed with DNA from different pools of Library 2. Selection was for thiostepton resistance. Spores were harvested from confluent lawns of *S. erythraea*, diluted and plated for single colonies then replica plated onto agar media to determine their kanamycin- and thiostrepton-resistance phenotypes. Kanamycin-resistant and thiostrepton-sensitive colonies were chosen. Mutants were analyzed by plasmid rescue, taking advantage of the R6Kγ ori- gin of replication present in the transposon, followed by DNA sequence analysis to confirm the randomness of the library. In *S. erythraea*, pIJ101-derived plasmids such as pFL2073 integrate into the chromosome via homologous recombination when they carry an *S. erythraea* genomic fragment >0.4 kb. The transpo- son with neighboring *S. erythraea* DNA is twice delivered into the genome by double-crossover gene replacement; no plasmid DNA is permanently incorporated into the genome by this procedure (Weber *et al.* 1990).

Transposon insertions made by double crossovers in the chromosome (kan^r and $thio^s$) were found in ~5–10% of the spores from these confluent lawns. This created a library of *S. erythraea* mutant strains (Library 3) which was then screened for increased or decreased erythromycin production. The remain- ing spores were made up of single-crossover plasmid insertions (~50%, $thio^r$ and kan^r) or spores with no plasmid or transposon DNA (~45%, $thio^s$ and kan^s). These were not used further.

Library 3 (composed of 1048 mutant strains made by double- crossover recombination of the mutagenized plasmids from Li- brary 2) was screened by micro-agar fermentation twice. Since half of the mutants in Library 3 were estimated to be mutants that shared a common parent, ~524 genes (or 7% of the total in the genome) were knocked out in this study.

Identification of *S. erythraea* sequences flanking transposon insertions

This method has been described previously (Reeves and We- ber 2012). Briefly, chromosomal DNA was prepared from the high-producing strain and digested with a frequent cutting en- donuclease enzyme that did not cut within the transposon. The digested DNA was ligated to create circular DNA from individual fragments and electroporated with *E. coli* and plated on LB agar with kanamycin (40 μg ml^{-1}) and the *oriV* inducer. The resulting Kan^r colonies were grown for isolation of plasmid DNA which was subjected to DNA sequence analysis using the primers at the ends of the transposon.

Micro-agar fermentations and erythromycin titer determinations

The wells of a flat-bottomed 96-well microtiter plate were par- tially filled with 250 μl of E20A agar. Spores from mutant and control strains were inoculated into the center of the micro- agar plugs with sterile toothpicks. Each mutant was screened in duplicate. The plates were incubated for 5 days at 32°C un- der controlled humidity. After 5 days, the micro-agar plugs were transferred to the top of 20 cm agar plates seeded with the sensi- tive indicator organism, *Bacillus subtilis*. After overnight incuba- tion at 32° C, the erythromycin titer determination plates showed zones of growth inhibition around the base of the micro-agar plugs in proportion to the amount of erythromycin being made. The inhibition zone diameters were measured and the amount of erythromycin produced was calculated compared to an ery- thromycin A reference standard. Methods for the determination of erythromycin yields in liquid culture broths have been de- scribed previously (Reeves *et al.* 2006).

Shake flask fermentation screen

Mutant *cwh1 was* tested in 250-ml shake flask cultures contain- ing 25 ml of OFM1 medium (Reeves *et al.* 2006). Mutant num- ber S6.07–03 and control FL2302 were compared in triplicate cul- tures; mutant S6.07–06 and control FL2302 were compared in du- plicate fermentations (Fig. 2C).

Chapter Three

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 mu- tants 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 mu- tations in the same gene accounted for the duplicate genotypes. Of the 15 knockout mutant strains found, 13 showed a >25% im- proved yield and 1 genotype had reduced yield and 1 genotype was neutral but showed reduced yield upon later scale-up anal- ysis (Fig. 2). The mutants from the first screen showed mean in- creases in erythromycin yield of 34%–109%.

DNA sequence analysis revealed 15 gene targets affecting erythromycin production with transposons falling into coding regions in 13 cases, and promoter regions in 2 cases; nucleotide numbers of the transposon insertion sites are given (Table 1). Nine mutants showed sporulation or pigmentation defects in addition to influencing erythromycin production. The 15 tar- geted genes fell into six general functional categories deter- mined by BLASTP analysis: transcriptional regulators (*acrR* and *rho1)*, cell wall biogenesis (*cps2I, cwh1 and possibly gtf1*), hydro- lases (*tsp1*, *dppII and cwh1*), metabolism, (*citA4*, *hpcH*, *fabG*, *cysH*, *arsC*), antibiotic biosynthesis (*eryAII* and possibly *gtf1*) and un- known (*unk1597*) (Table 1). Tn5 insertion into the *eryAII* gene pro- duced the expected complete blockage in erythromycin produc- tion. A neutral phenotype was produced by insertion into the *cccA* gene coding for a cytochrome-c related protein involved in energy production; however, in shake flasks this mutant showed reduced erythromycin yield (data not shown).

The transposon insertions were mapped broadly around the *S. erythraea* chromosome (Fig. 3A). Insertions influencing ery- thromycin production were most frequently found in the upper- half 'core-metabolism' region of the genome (0–2.5 and 5.5–8.0 Mb) and less frequently in the lower half 'non-core' region (2.5– 5.5 Mb) (Oliynyk *et al.* 2007). Sibling mutants were found for six of the genes (*acrR, fabG, eryAII, cwh1, gtf1 and rho1*) and three in- sertion events occurred into the cell wall hydolase gene *cwh1* (Fig. 3B). For 11 of the 15 genes in the collection, the closest NCBI GenBank homologs were found in *S. spinosa*.

Interaction between genotype and environment

The fermentation growth environment determines which phe- notypes can be scaled-up and which cannot. Genotypes influ- encing increased erythromycin production are potentially

com- mercially viable. In this study, only one of the genotypes, mutant *cwh1*, showed increased erythromycin production after scale-up in shake flasks (Fig. 2C). Two independent *cwh1* insertion mu- tants were tested: mutant S6.07–03 whose insertion was near the middle of the gene and mutant S6.07–06 whose insertion was near the 5′ end of the gene (Fig. 3B). Both mutant strains showed statistically significant increases in erythromycin production (Fig. 2C). Upstream of the *cwh1* gene one insertion ('72', Fig. 3B) showed increased erythromycin production simi- lar to that with insertions in *cwh1*; downstream of *cwh1* one in- sertion ('14') was found that had a neutral phenotype—evidence that the Cwh1 phenotype is not due to an effect on the down- stream gene. Based on DNA sequence data, Cwh1 is predicted to be cell-wall associated and to hydrolyze cell walls (see Sup- plementary Results and Fig. S1, Supporting Information). There- fore, it was not surprising that the Cwh1 phenotype included a growth defect (Fig. 4A) and non-sporulation (Fig. 4B) on solid medium.

Chapter Four

Discussion

Improvement of antibiotic production, driven by multiple rounds of random mutagenesis and screening, is an empirical process that has been an integral part of fermentation manu- facturing since the beginning of the penicillin era and is widely practiced to this day. This study used targeted mutagens to ex- plore the genetics behind this process.

Traditional 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 ra- tionally incorporated into other desired genetic backgrounds.

Another limitation of traditional mutagenic screening is that no information is generated concerning the frequency of unique strain improvement mutations. However, by us- ing transposon mutagenesis it was possible to measure this frequency at ~3%; mutations being both yield related and capable of scale-up occurred at ~0.2%. In a typical actino- mycete 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, 14 of which might have potential for scale-up under a certain set of conditions.

The large number of mutations yielding increases in ery- thromycin production may reflect the fact that the strain used had not previously been subjected to any rounds of mutations for improvement (except for the *mutB* knockout). As more cycles of the transposon-based procedure are performed, the diversity and number of additional genes that might be mutated to cause improved yield should gradually diminish (Barrick and Lenski 2013).

The transposition approach has additional value in that all mutations influencing erythromycin production are useful, even yield-reducing mutations because they can also be ex- ploited for gain. Only one locus, *cwh1,* was found that could be scaled-up to a 100-fold increase in fermentation volume (0.25– 25 ml). Nevertheless, mutations that could not be scaled-up under our desired conditions might scale-up under different conditions.

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 improve- ment phenotype could result from the diversion of cell wall precursors such as NDP-rhamnose, from cell wall biosynthe- sis into erythromycin biosynthesis (Mikusová *et al.* 1996; Crick, Mahapatra and Brennan 2001). This could explain the increase in erythromycin production particularly when NDP-rhamnose is the limiting metabolite (Summers *et al.* 1997). This diversion of precursors would be aided in *S. erythraea* by the fact that cell wall biogenesis and erythromycin biosynthesis occur simultaneously (McDermott, Lethbridge and Bushell 1993; Wardell *et al.* 2002) unlike most other antibiotic producers that produce antibiotic only after rapid growth is completed (Chater 2006). Consistent with this hypothesis, the rhamnose biosynthetic pathway has been previously shown to supply precursors to both primary and secondary metabolism in the closely related organism *S. spinosa* (Madduri, Waldron and Merlo 2001).

Interestingly, neither *cwh1* nor any of the other homologous hydrolase genes mentioned above were found to be mutated in either of the two high-producing *S. erythraea* genomes re- ported so far (Peano *et al.* 2012; Li *et al.* 2013). However, tran- scriptome analysis in these higher producing strains did show an under representation of cell wall/membrane/ envelope bio- genesis transcripts (Peano *et al.* 2012). One other result in com- mon between our mutations and the mutations found in high- producing strains was for mutations in citrate synthase. In our study, a *citA* (SACE 0632) knockout led to increased erythromycin production; in the two high-producing strains reported thus far, *citA* knockouts were also found (in SACE 0633; Peano *et al.* 2012; Li *et al.* 2013). The fact that most of the genotypes from this study were not found in the genome sequences of high-producing strains, which in turn do not closely match one another (Peano *et al.* 2012; Li *et al.* 2013), may be due to the nature of strain im- provement as a convergent evolutionary process (Wagner 2009; Kryazhimskiy *et al.* 2014).

Chapter Five

Figures

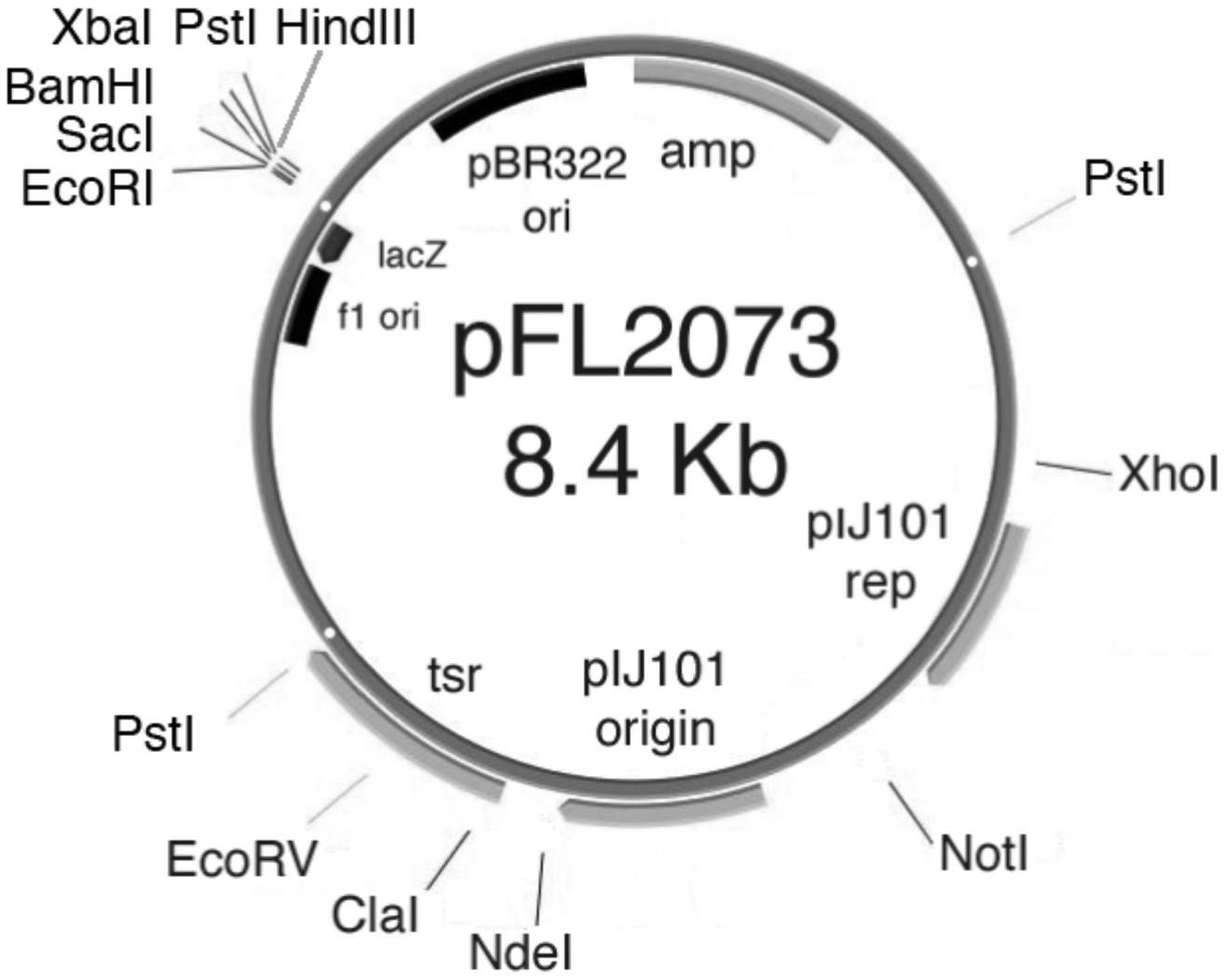

Figure 1. Map of plasmid pFL2073. Enzymes with unique restriction endonuclease sites are shown (except for *PstI* whose three sites are shown). Abbreviations: *amp*, ampicillin resistance gene; *ori*, origin of replication; *lacZ*, beta-galactosidase gene; f1, f1 phage; *tsr*, thiostrepton resistance gene; pIJ101, *Streptomyces* plasmid; rep, replicon.

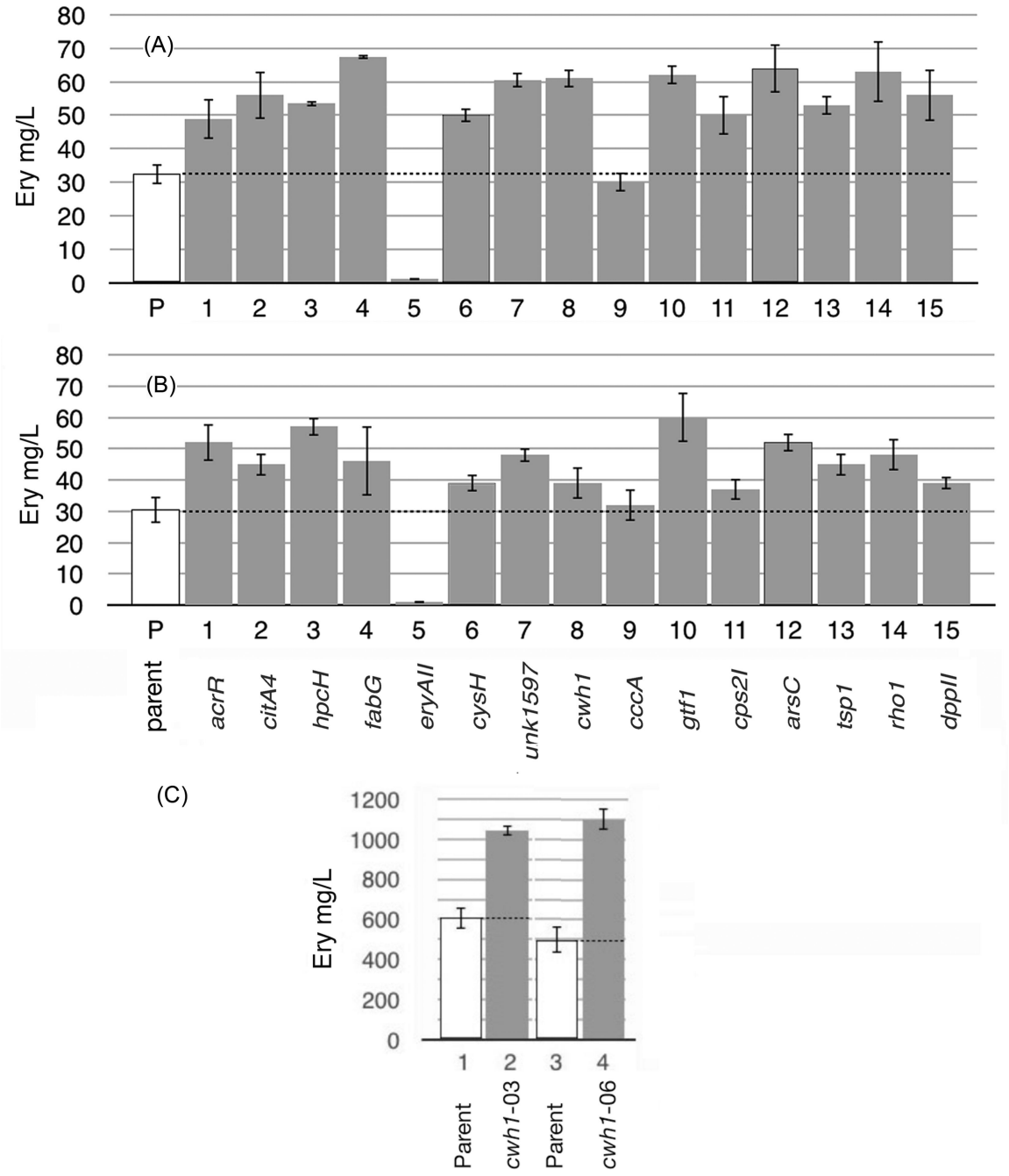

Figure 2. (**A**) Micro-agar plug fermentation screen. Erythromycin production levels of 15 transposon-insertion mutants are compared to the parent strain FL2302 (P). The dotted lines indicate the base-line level of erythromycin production by the parental strain; brackets represent standard deviation, *n* = 2. (**B**) The repeated microgel fermentation screen; brackets represent standard deviation, *n* = 2. (**C**) Scale-up from micro-agar plug to shake flask fermentation results for *cwh1* (SACE 1598) mutants. Cultures were grown in 25-ml of OFM1 (oil-based medium) in 250-ml shake flasks. Bar numbers 1 and 3 represent parental strain FL2302; bar numbers 2 and 4 represent *cwh1* mutant strains, S6.07–03 and S6.07–06, respectively.

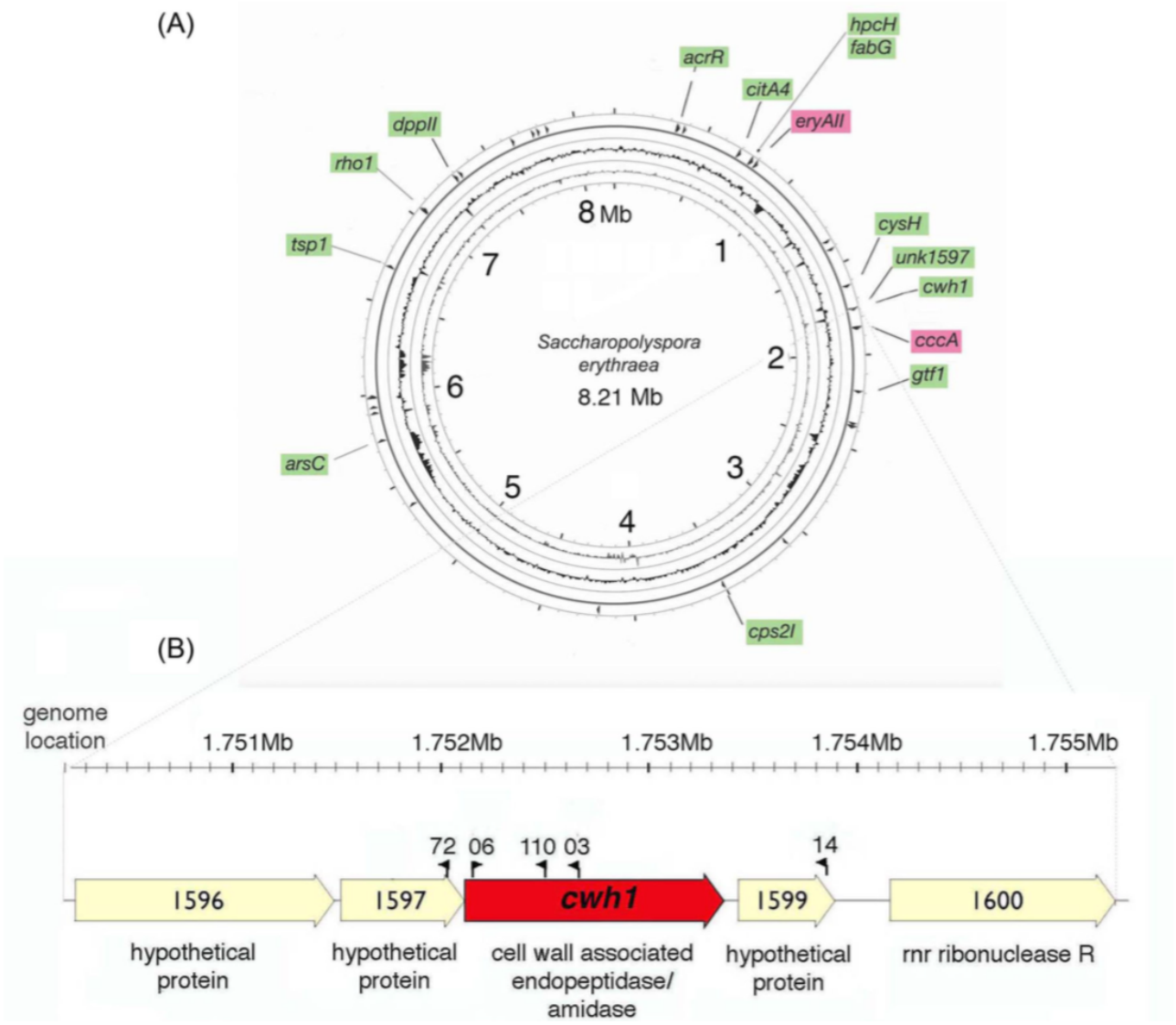

Figure 3. (**A**) Genome map of *S. erythraea* showing transposon insertion sites of Library 3 mutants. Map is based on data generated by Oliynyk *et al*. (2007). Knockouts of genes highlighted in green gave increases in yield, and red shading is for decreases in yield. Map positions are shown in megabase 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.

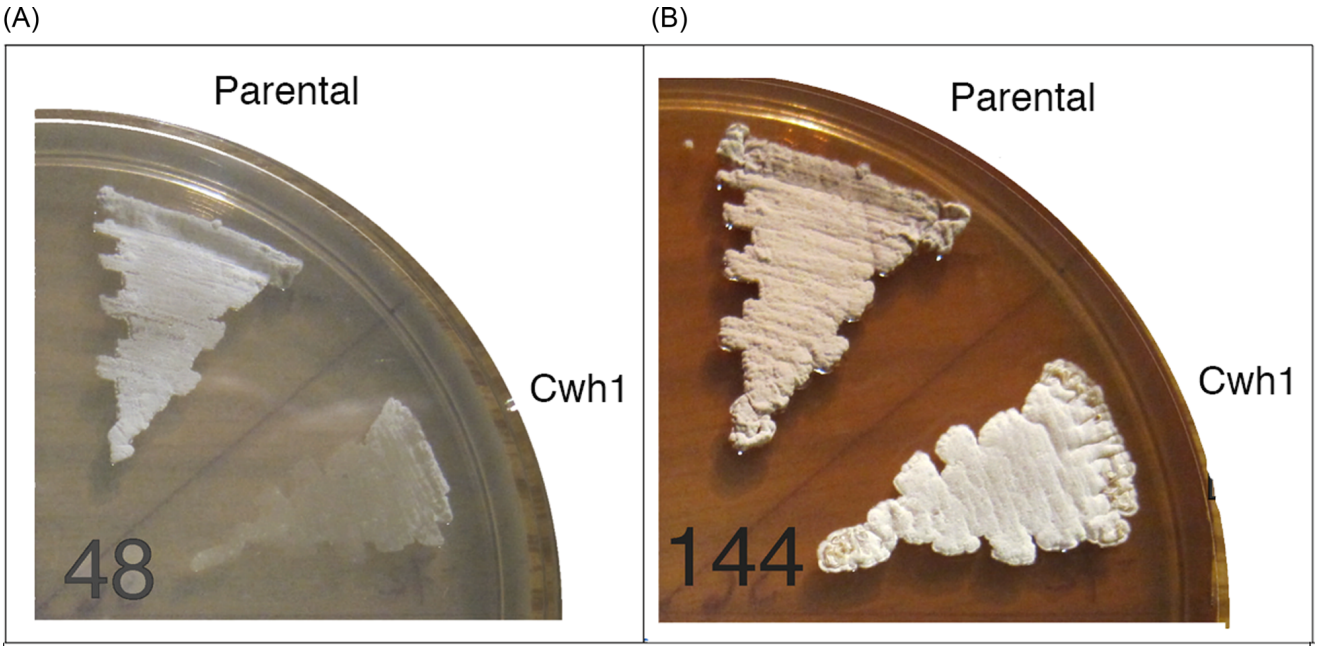

Figure 4. The parental and Cwh1 phenotypes are shown at 48 and 144 h (32°C, E20A agar). The parental phenotype of normal growth and sporulation was displayed by FL2302 as well as by mutants with insertions downstream of cwh1 (mutant S6.07–14 is shown). The Cwh1 phenotype of slower growth and no sporulation was displayed by mutants with insertions in *cwh1* (S6.07–03 and –06), as well as by mutants with insertions upstream of cwh1 (S6.07–72 shown).

Chapter Six

Table

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)
citA4	S6.18–32	In SACE_0632; 696,384	+	+	+	citA4 citrate synthase (Viollier *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*, hypotheical 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)
cccA	S5.17–12	In SACE_1685; 1,852,856	–	–	–	cccA - cytochrome c mono-and diheme variants
gtf1	S7.05–53	Upstream of SACE_2010; 2,197,132	+	–	–	*gtf1*, glycosyltransferase- GT1_Gtf_like family (Liang and Qiao 2007)
cps2I	S6.15–149	In SACE_3177; 3,507,747	+	bld	+	*cps2I*, nucleotide-sugar-dependent glycosyltransferase, group 1 (Lakkitjaroen *et al.* 2014)
arsC	S6.18–12	In SACE_5143; 5,750,836	+	–	–	*arsC*, arsenical resistance protein, arsenate reductase, arsenic transporter (EC 1.20.4.1)
tsp1	S6.18–17	In SACE_5967; 6,700,519	+	+	+	*tsp1*, 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_6505; 7,295,927	+	+	+	*dppII*, X-Pro dipeptidyl-peptidase (Maes, 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.

Chapter Seven

Supplementary Data

Supplementary Materials and Methods

Bacterial strains and growth conditions.

General materials and methods for Actinomycetes are described in (Kieser *et al*., 2000); and for *Escherichia coli* as described in (Sambrook *et al*.,1989). The erythromycin-producing strain for transformations was *Saccharopolyspora erythraea mutB* FL2302 (Reeves *et al*., 2006) a derivative of *S. erythraea* FL2267 having a 126-bp in-frame deletion in *mutB* replaced by a 6-bp *BamHI* site. The deletion was in-frame so it inactivated the function of the *mutB* gene but did not affect downstream transcription. The lyophilized vial of *S. erythraea* ATCC 11635 obtained by Fermalogic contained a mixture of "white" strains and red-pigmented variant strains when grown on E20A agar. The strains in this study were derived from a purified "white" colony. White strains including FL2302 still make red pigment on E20A agar, but significantly less than the red varient. ATCC 11635 is the equivalent strain to NRRL 2338 and presumably to NRRL 23338 (Oliynyk *et al*., 2007). *S. erythraea* FL2267 (the parent to FL2302) was the source of the genomic DNA used in the formation of Library 1 (see below). Recipes for OFM1 medium, CFM1 medium (Reeves *et al*., 2006), SCM (Reeves *et al*., 2004) and E20A agar (Reeves *et al*., 2002) have been reported. The composition of SGGP broth is: 4.0 g Bacto peptone (Difco), 4.0 g Bacto Yeast extract (Difco), 0.5 g magnesium sulfate, heptahydrate (Sigma), and 960 ml distilled water. The mixture is adjusted to pH 7, autoclaved, then the following sterile solutions were added: 20.0 ml 50% D-(+)-glucose (Sigma) in distilled water, and 20.0 ml 0.5M potassium phosphate monobasic (Fisher).

Library 1: DNA cloning

S. erythraea chromosomal DNA was prepared according to methods for *Streptomyces lividans* (Kieser *et al*., 2000). Chromosomal DNA was partially digested using a combination of *BamHI* and *EcoRI*; *HindIII* and *SacI*; or *BamHI* and *SacI.* After electrophoresis and purification from a 0.6% agarose gel, the chromosomal fragments (~10-15 kb) were ligated to similarly digested pFL2073 (Fig. 1). Ligation mixtures were transformed into *E. coli* DH5α by electroporation. Transformants were plated on LB agar containing ampicillin (100 μg/ml), X-gal, and IPTG. A total of 620 small white ampicillin-resistant transformant colonies were transferred by sterile toothpicks to test tubes containing 4 ml of LB with 100 μg/ml ampicillin. A high-purity DNA preparation was generated from each overnight test tube culture using GeneJet Kits (Fermentas, Vilnius, Lithuania). Plasmid DNA was digested with *PstI* and analyzed on agarose gels to determine the size of the cloned DNA fragment and the relative

copy number of the plasmid. Library 1 plasmids were examined by DNA sequence analysis to confirm that DNA was being randomly cloned from throughout the genome. Plasmids carrying fragments larger than 9 kb and with similar copy numbers were combined in pools of approximately 20 plasmids each. Eleven different plasmid pools were prepared in this manner from the different ligation mixtures and were used in the *in vitro* transposition reactions to create Library 2. Approximately 2.2 MB of cloned chromosomal DNA was represented in Library 2.

Library 2: transposon mutagenesis

Eleven plasmid pools from Library 1 were mutagenized *in vitro* using the EZ-Tn5 <R6Kγori/Kan-2> kit from Epicentre Biotechnologies (Madison, WI). EZ-Tn5 <R6Kγori/Kan-2> is a 2 kb DNA fragment containing the R6Kγ origin of replication, the kanamycin-resistance gene, forward and reverse primer sequences. To perform *in vitro* transposition, DNA from each plasmid pool from Library 1 (16 μl) was combined with buffer (2 μl), transposon DNA (1 μl), and transposase enzyme (1 μl) supplied by the kit. The mixtures were incubated at 37°C for 2 hours, at which point stop buffer (2 μl) was added. One μl of the final reaction mixture was added to transformation competent *E. coli* cells (50 μl) after a 1 hr outgrowth, the cells were plated on LB and selected for ampicillin and kanamycin-resistant transformants. Individual randomly chosen plasmids from Library 2 were inspected by DNA sequencing to confirm random insertion of the transposon. Densely grown (~6,000 per plate) primary transformants were harvested off agar plates and a high-purity DNA preparation was created (Qiagen Midi plasmid purification kit), one from each of the 11 plasmid pools.

Supplementary Results 1

Bioinformatic analysis of the *cwh1* gene

From DNA sequence data it was deduced that the *cwh1* gene codes for a protein comprised of 413 amino acids. Cwh1 is predicted to be membrane bound due to a single transmembrane alpha helix (amino acids 5 – 27, Fig. S1); residues 28-413 are outside the membrane and contain an NlpC/P60 endopeptidase domain as predicted by InterProScan 5 (Jones *et al*., 2014) and Protter (Omasits *et al*., 2014). NlpC/P60 proteins define a family of cell wall peptidases that are extensively found in bacteria (Anantharaman and Aravind, 2003).

The C-terminal region of the protein is proline-rich with 27% of the residues from amino acids 319 - 413 being proline (Fig. S1). The proline-rich domain of Cwh1 contains a total of seven PXXP motifs which adopt the PPII conformation and bind to SH3 domains which are found in proteins of signaling pathways regulating the cytoskeleton (Zarrinpar *et*

al., 2003). The accessibility of the seven PXXP motifs (A-G, Fig. 4C), is enhanced by their being at the C-terminus of the protein; possibly making this region able to function as a "sticky-arm" (Kay *et al*., 2000; Chandra *et al*., 2004).

BLASTP analysis revealed over 100 homologs for the *S. erythraea* Cwh1 protein having 37-82% identity. The proline-rich C-terminal region of the protein, however, was unique to *S. erythraea*. For *Saccharopolyspora spinosa,* the *cwh1* homolog (Sequence ID**:** WP_010695429.1) was present in only *one* of the three genome sequences that have been reported thus far for this organism (*S. spinosa,* taxid 60894; Pan *et al*., 2011).

Phylogenomic profiling using Gene Ortholog Neighborhoods analysis (Bowers *et al*., 2004; Fong *et al*., 2008) revealed that *Saccharopolyspora erythraea* shares significant genomic context with *Saccharopolyspora spinosa* NRRL 18395 and *Saccharopolyspora flava* DSM 44771 in a 15 gene region from SACE_1593 to _1609. Many other actinomycetes share genomic context with *S. erythraea* in a 6-gene region between SACE_1593 and _1599 but the specific function of this group of genes is currently unknown.

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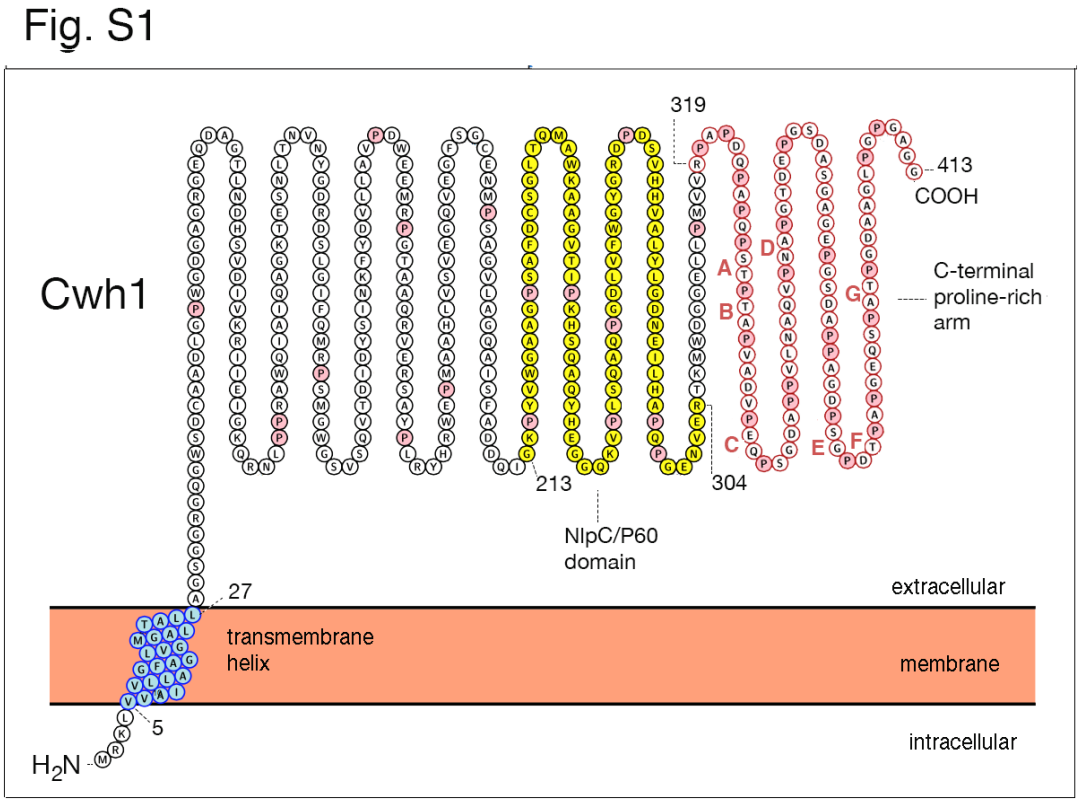
Fig. S1

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).



Chapter Eight

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Chapter Nine

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