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PII: S0014-5793(15)00390-7
DOI: http://dx.doi.org/10.1016/j.febslet.2015.05.014
Reference: FEBS 37170

To appear in: *FEBS Letters*

Received Date: 13 November 2014
Revised Date: 18 April 2015
Accepted Date: 4 May 2015

Please cite this article as: Gittins, J.R., Cloning of a copper resistance gene cluster from the cyanobacterium *Synechocystis* sp. PCC 6803 by recombineering recovery, *FEBS Letters* (2015), doi: http://dx.doi.org/10.1016/j.febslet.2015.05.014

# Cloning of a copper resistance gene cluster from the cyanobacterium *Synechocystis* sp. PCC 6803 by recombineering recovery

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Running title: Recombineering recovery cloning

**ABSTRACT**

A copper resistance gene cluster (6 genes, ~8.2 kb) was isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 by recombineering recovery (RR). Following integration of a narrow-host-range plasmid vector adjacent to the target region in the *Synechocystis* genome (pSYSX), DNA was isolated from transformed cells and the plasmid plus flanking sequence circularized by recombineering to precisely clone the gene cluster. Complementation of a copper-sensitive *Escherichia coli* mutant demonstrated the functionality of the *pcopM* gene encoding a copper-binding protein. RR provides a novel alternative method for cloning large DNA fragments from species that can be transformed by homologous recombination.

## 1. Introduction

Recent technological advances have led to a massive increase in the volume of DNA sequence data. The number of sequenced bacterial genomes is growing rapidly [1,2] and now a major research bottleneck is the functional analysis of genes. In order to study microbial metabolic pathways and utilize them for biotechnology, it is frequently necessary to clone and express multiple genes present in clusters that can be tens of kilobases in size. Traditional methods for the cloning of large DNA fragments such as cosmid, phage or BAC library construction, followed by screening and sub-cloning, are time consuming and costly. PCR amplification and assembly (e.g. Gibson assembly [3]) is quicker and cheaper, but the polymerases used have an inherent error rate and incorrect pairing of fragments can occur. Therefore, the assembled DNA fragment must be sequenced to confirm the absence of mutations. Total gene synthesis [4] can be rapid, but again sequence verification is necessary.

The *in vivo* cloning of DNA fragments by homologous recombination has been refined over the last two decades. Initially the *E. coli* recombination machinery was employed to join vector and fragment DNAs that share identical terminal regions [5,6]. Cloning was achieved by the recombination of homologous termini as short as 10 bp [5], and became more efficient as the extent of the homology was increased to between 67 and 83-bp in size [6]. This procedure utilizes RecBCD-deficient *E. coli* strains in which the recombination machinery is constitutively active [5,6], and so may result in DNA rearrangements and deletions. *In vivo* cloning can also be accomplished in RecA-deficient strains [5], but the underlying mechanism remains unclear and the efficiency, particularly for larger DNA fragments, may be low. More recently, the inducible expression of recombination protein pairs Redα/Redβ encoded by the phage lambda Red operon or RecE/RecT encoded by the Rac prophage has been used to promote cloning by homologous recombination. This strategy, named recombineering, increases the efficiency of recombination between homologous ends of ≥35 bp and facilitates the genetic engineering of chromosomal and episomal replicons [7,8]. A variant of recombineering called linear-linear homologous recombination (LLHR) has recently been used to clone large DNA fragments representing entire bacterial biosynthetic pathways [9,10].

*Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is a well characterized freshwater cyanobacterium that is widely used as a model [11]. Like other photosynthetic organisms it requires copper as an essential component of plastocyanin (photosynthetic electron transport) and cytochrome oxidase (respiration). Due to the harmful effects of an excess of copper ions, cellular levels are tightly controlled by homeostatic mechanisms affecting acquisition, sequestration and

efflux. In *Synechocystis* these include a copper efflux system and its associated regulatory system, respectively encoded by the genes *copBAC* and *pcopMRS* [12](Fig. 1). These two operons are adjacent in pSYSX, a 106-kb plasmid native to *Synechocystis* [13], and are transcribed in the same direction, while *copMRS* genes are also present on the chromosome. The *pcopMRS* operon encodes the CopR/CopS copper-responsive two-component regulatory system [12] and also CopM, a protein that has recently been shown to bind copper and contribute to copper resistance in *Synechocystis* [14]. The *copBAC* genes encode proteins that comprise a member of the heavy metal efflux-resistance, nodulation and division (HME-RND) family [15]. CopB and CopA represent the periplasmic membrane fusion protein (MFP) and inner membrane RND protein, respectively, while the role of CopC remains unknown. These three proteins are thought to assemble in the cell envelope to promote the efflux of copper ions and confer copper resistance. A similar HME-RND family complex encoded by the *cusCFBA* operon of *E. coli* mediates tolerance to copper and silver ions [16].

To examine the functioning of the *Synechocystis* copper resistance gene cluster, attempts were made to clone a fragment of pSYSX for heterologous expression. After a PCR-based strategy was thwarted by sequence errors introduced during amplification, an inexpensive and reasonably rapid alternative method was sought. *Synechocystis* is naturally transformable [17] and transgenes can be precisely targeted to a particular genomic location by homologous recombination. With this knowledge and an appreciation of developments in recombineering, a procedure was devised to clone the copper resistance gene cluster from a specially created *Synechocystis* transformant strain. This strategy was named recombineering recovery (RR) (Fig. 2). This report describes the application of RR to clone a gene cluster and discusses the features and possible uses of this method.

## 2. Materials and Methods

### *2.1. Strains, culture media and growth conditions*

The bacterial strains and plasmids used in this study are shown in Table 1. The culture media and growth conditions for *E. coli* and *Synechocystis* strains are described in **Supplementary methods 1**.

### *2.2. DNA isolation and manipulation*

The isolation of plasmid DNA from *E. coli* and genomic and plasmid DNA from *Synechocystis* is described in **Supplementary methods 2**. PCR amplicons and other DNA fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, Manchester, UK). Other standard molecular biology techniques were employed throughout [18]. Oligonucleotide primers were supplied by Eurofins Genomics (Ebersberg, Germany) and are shown in **Supplementary Table S1**. DNA sequencing was performed by Eurofins Genomics.

*2.3. PCR amplification and cloning of a Synechocystis copBAC gene fragment*

Primers SYSX_Cu_Xba_F and SYSX_Cu_Xba_R were used in a PCR with a *Synechocystis* plasmid preparation and Q5 polymerase (NEB, Hitchin, Herts. UK) to amplify a 5.5-kb DNA fragment from pSYSX. The gel-purified amplicon was digested with XbaI (NEB) to excise a 5197-bp fragment comprising the 5'-truncated *copB* gene plus the *copA* and *copC* genes. This fragment was cloned into XbaI-cleaved vector pBAD24 to produce construct pBAD24-SYSX-Cu-Xba.

*2.4. Preparation and integration of Synechocystis transformation construct*

The design of the construct used to transform *Synechocystis* is shown in Fig. 2. Three regions of plasmid pSYSX flanking the copper resistance gene cluster (fragments A, C and D; Fig. 1) were amplified and joined by PCR, then the composite fragment was cloned in the vector pSMARTGC LK (fragment B; Lucigen, Middleton, WI, USA), as described in **Supplementary methods 3**. The resulting construct pSMART-SYSX-Cu-REC was linearized by digestion with StuI (Thermo-Fisher Scientific, Loughborough, Leics. UK). Transformation of *Synechocystis* with this DNA fragment was performed using a standard procedure [19] and transformants selected by plating on medium containing kanamycin (2.5→25 μg/ml). The fully segregated nature of a single transformant was verified by colony PCR using primers SYSX_Cu_3F and SYSX_Cu_Xba_R. This *Synechocystis* strain was named SYSX-Cu-REC.

*2.5. Recombineering recovery cloning of the Synechocystis copper resistance gene cluster*

Genomic DNA prepared from *Synechocystis* SYSX-Cu-REC was digested with ScaI (Thermo-Fisher Scientific). The cleaved DNA and an undigested control sample were then purified by

butanol extraction [20], plus an additional wash with 70% ethanol. The DNA was quantified by comparison with bands of a size ladder following agarose gel electrophoresis.

An optimized protocol used to prepare electrocompetent *E. coli* cells for recombineering, is described in **Supplementary methods 4**. Two preparations of strain GL05 (pSC101-BAD-gbaA-tet) cells, one induced with L-arabinose and a non-induced control, were electroporated with approximately 20 and 100 ng of prepared *Synechocystis* SYSX-Cu-REC genomic DNA, either cleaved with ScaI or undigested. Transformants were selected on LB agar medium containing kanamycin (30 μg/ml). The desired construct containing the pSYSX copper resistance gene cluster was named pSMART-SYSX-Cu-pcopMRS+BAC. The cloned fragment was sequenced using primers M+Bseq_1–M+Bseq_22.

*2.6. Preparation of constructs for regulated expression of the Synechocystis copper resistance genes in E. coli*

The cloned copper resistance gene cluster was transferred from pSMART-SYSX-Cu-pcopMRS+BAC to the expression vector pBAD24 in a two-step process described in **Supplementary methods 5**. The resulting construct was named pBAD24-SYSX-Cu-pcopMRS+BAC. A deleted form of this plasmid containing the *pcopM* gene under the control of $P_{BAD}$ was prepared by restriction fragment excision and religation as described in **Supplementary methods 5**. This construct was named pBAD24-SYSX-Cu-pcopM.

*2.7. Creation of a copper-sensitive E. coli mutant*

*E. coli* mutant strain JG01 (Δ*copA* Δ*cueO* Δ*cusCFBA*:Cm) was created by modification of strain AY1053 [21] as described in **Supplementary methods 6**.

*2.8. E. coli copper sensitivity determination*

The copper sensitivity of *E. coli* JG01 strains was determined by examining their growth on $CuCl_2$ gradient plates, as described in **Supplementary methods 7**.

**3. Results**

*3.1 Cloning of the Synechocystis copper resistance gene cluster by recombineering recovery*

A PCR-amplified fragment of *Synechocystis* plasmid pSYSX containing the *copBAC* genes was treated with restriction endonuclease XbaI and cloned in vector pBAD24 to produce construct pBAD24-SYSX-Cu-Xba. Despite the use of a high-fidelity DNA polymerase in the PCR, sequencing of the termini of several clones revealed base changes in the fragment (data not shown). To avoid these unwanted sequence alterations, an alternative method for cloning the copper resistance gene cluster was sought.

The high efficiency of transformation of this cyanobacterium with naked DNA and the ability to precisely target transgene insertions by homologous recombination suggested a novel cloning method using recombineering, outlined in Fig. 2. The implementation of this strategy, named recombineering recovery (RR), required the transformation of *Synechocystis* with a DNA fragment comprised of 4 elements: A – left homology arm; B – linear plasmid vector (pSMARTGC LK); C – a short element representing a sequence outside the genes to be cloned; D – right homology arm.

The homology arms (~450 bp) used to target the transgene within pSYSX, matched most of the *copC* gene (left arm, A) and a sequence downstream of the copper resistance gene cluster including part of the gene *slr6045* (right arm, D), so that no host genes would be disrupted by the insertion (Fig. 1). The short sequence element (C, 70 bp) was located inside the homology arm furthest from the targeted gene cluster (D). This element was designed to be identical to a sequence upstream of *pcopM*, at the other end of the region to be cloned (Fig. 1). The length of this short sequence was close to the size required for efficient homologous recombination in *E. coli* [22] and also that used in the LLHR strategy [10]. Vector pSMARTGC LK (B) was chosen for (i) its narrow-host-range replicon (ColE1) that is non-functional in *Synechocystis* [23], (ii) kanamycin resistance determinant active in *Synechocystis*, (iii) small size (1993 bp) to minimize the size of the recovered transgene construct, (iv) relatively low copy number (~20 copies/cell) to reduce any deleterious effects of the cloned genes in multicopy, and (v) the presence of transcription terminators to prevent transcription into and out of the cloned DNA.

The three PCR-amplified fragments of pSYSX were joined by overlap extension and this composite fragment was cloned into vector pSMARTGC LK (B) and its sequence verified. The resulting construct, pSMART-SYSX-Cu-REC, was linearized by StuI digestion and used for transformation of *Synechocystis*. A fully-segregated transformant clone, named *Synechocystis* SYSX-Cu-REC, was obtained. Genomic DNA was isolated from this clone using a simple and rapid method, and a portion of the preparation was digested with ScaI, cleaving outside the

introduced plasmid vector and the copper resistance gene cluster, to reduce the size of the DNA fragments to be used as the substrate for recombineering.

Since the transformation of *E. coli* with linearized plasmid DNA is considerably less efficient than with intact circular molecules [24], an optimized protocol was employed for the preparation of electrocompetent cells of the recombineering strain, both with and without L-arabinose induction. The two cell preparations were electroporated with the *Synechocystis* SYSX-Cu-REC DNA, both cleaved with ScaI and undigested, and transformants were selected.

Few kanamycin-resistant colonies were produced unless the *E. coli* recombineering strain was treated with L-arabinose prior to electrocompetent cell production. This confirmed that induction of the factors encoded by plasmid pSC101-BAD-gbaA-tet (i.e. Redγ, Redβ, Redα, RecA) was necessary for efficient recombineering [25]. Furthermore, digestion of the *Synechocystis* transformant DNA with ScaI greatly enhanced the number of colonies, as did raising the amount of DNA used in the transformation (data not shown). Plasmid DNA isolated from 12 *E. coli* transformant clones was characterized by digestion with restriction endonucleases (Fig. 3). All produced the expected banding patterns, which confirmed circularization by homologous recombination between the directly repeated short sequence elements (separated by approx. 10.1 kb in the genome of the *Synechocystis* transformant) and recovery of the intervening DNA containing vector pSMARTGC LK plus the copper resistance gene cluster. A single clone selected for further study was named pSMART-SYSX-Cu-pcopMRS+BAC. The cloned copper tolerance gene cluster was fully sequenced (GenBank: KP676897) and found to be identical to the published sequence [13], which confirmed its authenticity and proved the efficacy of recombineering recovery (RR) cloning.

### *3.2 Complementation of a copper-sensitive E. coli mutant with the Synechocystis copper resistance gene cluster*

The functionality of the *Synechocystis* gene cluster isolated by RR was examined in *E. coli* mutant JG01, defective in three copper resistance mechanisms: Cu(I)-translocating P-type ATPase CopA, multi-copper oxidase CueO and the CusCFBA multi-component copper efflux system (a HME-RND system related to CopBAC). As previously reported for an equivalent mutant [26], strain JG01 was considerably more sensitive to copper than its wild-type parent strain (data not shown).

The entire copper resistance gene cluster was transferred from construct pSMART-SYSX-Cu-pcopMRS+BAC to the vector pBAD24 to place it under the control of the tightly regulated arabinose operon promoter $P_{BAD}$ and thus minimize leaky expression of

potentially toxic cell envelope proteins. Mutant JG01 was transformed with this construct, pBAD24-SYSX-Cu-pcopMRS+BAC, or the parent vector, and the copper sensitivity of these strains was examined using $CuCl_2$ gradient plates (Fig. 4). In the absence of inducer, both strains grew to the same extent across the copper gradient. With the addition of 0.5% (w/v) L-arabinose, the growth of both strains was weaker, possibly because of the increased metabolic load caused by induction [27]. More significantly, the strain carrying pBAD24-SYSX-Cu-pcopMRS+BAC grew at a higher concentration of $CuCl_2$ than the pBAD24 strain. This result demonstrated that the *Synechocystis* copper-resistance gene cluster was able to complement a copper-sensitive *E. coli* mutant.

During the course of this study it was reported that CopM, a hypothetical protein (196 aa; 22.9 kDa) of unknown function encoded by the first gene in the isolated gene cluster (*pcopM*) is in fact a novel periplasmic/extracellular copper-binding protein involved in copper resistance in *Synechocystis* [14]. This raised the possibility that expression of CopM was responsible for the increased copper tolerance of the complemented *E. coli* mutant. Therefore, a deletion derivative of the $P_{BAD}$ expression construct containing only the intact *pcopM* gene was prepared. Strain JG01 transformed with this plasmid, pBAD24-SYSX-Cu-pcopM, exhibited increased copper tolerance upon induction (Fig. 4), which showed that the expression of CopM and not the CopBAC HME-RND family complex produced the observed complementation. Interestingly, the strain carrying the *pcopM* construct appeared to tolerate a slightly higher concentration of $CuCl_2$ than that carrying pBAD24-SYSX-Cu-pcopMRS+BAC, but the reason for this is not known.

## 4. Discussion

Recombineering recovery (RR) was devised to facilitate the cloning of a copper resistance gene cluster from the cyanobacterium *Synechocystis* sp. PCC 6803. The efficacy of this method was verified by sequencing the recovered ~8.2-kb genomic DNA fragment. The cloned gene cluster was subsequently used to complement a copper-sensitive *E. coli* mutant, although only the *pcopM* gene was necessary for the increase in copper tolerance observed. Thus, the copper-binding protein CopM is active in a heterologous bacterial species through a mechanism that is likely to involve the sequestration of copper ions [14, 28].

Here, the principle of cloning by RR has been validated in a tractable model cyanobacterium, but this strategy is likely to be applicable for the isolation of large DNA fragments from species that meet the following criteria:

(1) Sequence data is available for the region to be cloned.

(2) The organism or its cells can be propagated or cultured and transformed with DNA that integrates into the genome by homologous recombination.

(3) Host cells do not support replication of the plasmid included within the transformation construct.

Advances in genome sequencing mean that the first pre-requisite is being fulfilled for a rapidly increasing number of species. Moreover, a growing panel of organisms fulfil the second requirement. The genomic integration of foreign DNA, often a selectable marker (e.g. drug resistance or nutrient autotrophy) and usually for the purposes of gene knockout, has been reported in numerous prokaryotes [29,30,31,32], yeast [33], filamentous fungi [34,35], *Dictyostelium* [36], single-celled protozoan parasites [37,38], cultured vertebrate cell lines [39,40,41] and the moss *Physcomitrella patens* [42]. The targeting construct (a plasmid suicide vector or linear DNA fragment) is introduced into the host cells and is unable to replicate. Transformants are then identified by appropriate selection and PCR screening confirms integration at the target locus. Several plasmid replicons, including ColE1 and p15a, have a narrow host range limited to *E. coli* and related bacteria [23], so the third pre-requisite is likely to be fulfilled for diverse species.

For RR to work, it is also vital that the selectable marker included in the transformation construct is functional in both the specified host species and *E. coli*. In this respect, resistance to bleomycin/phleomycin family antibiotics [43] or kanamycin/geneticin [44] may be used for selection in both prokaryotic and eukaryotic hosts. To permit expression of these resistance genes, promoters are available that function in both *E. coli* and eukaryotes [45,46,47,48], and polynucleotide sequences have been developed that drive transcription in bacteria, yeasts and fungi [49].

Two aspects of the RR method employed here are worthy of further comment. First, reduction of the size of the *Synechocystis* transformant genomic DNA fragments by digestion with ScaI prior to the electroporation of *E. coli* greatly enhanced the number of recovered clones. This is in agreement with the finding that appropriate cleavage of *Dictyostelium* genomic DNA containing an integrated shuttle vector resulted in a higher number of *E. coli* transformants carrying the recovered plasmid [50]. Furthermore, restriction endonuclease digestion was used to increase the efficiency of cloning by the LLHR strategy [10]. In the present study, a few transformants were still obtained using undigested genomic DNA, possibly because the fragment size was reduced to ~20 kb by shearing during DNA isolation (data not shown). Second, the efficiency of recovering the desired construct using the RR method appears to be 100% (Fig. 3). In comparison, the efficiency of cloning large genomic DNA fragments by LLHR is lower because the empty vector can recircularize, giving false positives [10]. In addition, the recovery of the

desired genomic fragment by LLHR depends on a double intermolecular homologous recombination event, so is likely to be less efficient than RR cloning, which requires only a single intramolecular recombination event.

It is envisaged that RR will be applicable for the targeted knockout and cloning of gene clusters specifying the production of useful secondary metabolites. Candidate clusters may be identified through bioinformatic analysis of a sequenced genome. These could then be systematically inactivated by the precise integration of a RR construct at one end of the cluster. Screening of these mutant strains to detect interference in the metabolic pathway would identify the relevant gene cluster, which could subsequently be isolated by RR for analysis. For larger gene clusters (10-50+ kb), use of an *E. coli* host expressing the full-length Rac prophage protein RecE and partner RecT may facilitate RR more efficiently than the strain expressing lambda phage Red operon proteins (α, β, γ) used here [10].

DNA cloned by RR is synthesized by the host DNA replication machinery and so should be free from errors, which might be present if PCR or *de novo* DNA synthesis were used to generate the same fragment. Furthermore, the DNA fragment could be tailored for future applications by rational design of the RR construct. A vector with a tightly repressed promoter would allow transcriptional control of the cloned genes to permit regulated expression of potentially harmful gene products. By the use of different plasmid replicons (bearing in mind pre-requisite 3, above), the copy number of the recovered fragment could be controlled or recovery performed in different bacterial species or yeast [51,52].

In conclusion, the concept of recombineering recovery has been proven by the cloning of a *Synechocystis* copper resistance gene cluster in *E. coli*. RR is less costly, quicker and more precise than traditional library construction and screening, is not dependent on error-prone PCR amplification and appears to be highly efficient. Therefore, this strategy represents an alternative to conventional methods for the isolation of large DNA fragments. Further research is required to determine the upper size limit of DNA fragments that can be isolated by RR and to examine its application in different species.

**Acknowledgements**

I acknowledge the receipt of financial support from Ocean and Earth Science, The University of Southampton. I am extremely grateful to Dr. Aixin Yan and Dr. Danny K. C. Fung of The University of Hong Kong for providing *E. coli* strains and helpful advice, and to Prof. A. Francis Stewart of TU Dresden for the gift of the recombineering strain. I would like to thank Hayley

Preston for her assistance in generating preliminary data and Dr. Andrew Hitchcock for performing the transformation of *Synechocystis* and for helpful discussions.

**References**

[1] Koonin EV, Wolf YI (2008) Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. Nucleic Acids Res. 36:6688-6719.

[2] Lagesen K, Ussery DW, Wassenaar TM (2010) Genome update: the 1000th genome--a cautionary tale. Microbiology 156:603-608.

[3] Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison III CA, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods 6:343-345.

[4] Kosuri S, Church GM (2014) Large-scale de novo DNA synthesis: technologies and applications. Nature Methods 11:499-507.

[5] Bubeck P, Winkler M, Bautsch W (1993) Rapid cloning by homologous recombination *in vivo*. Nucleic Acids Research 21:3601-3602.

[6] Oliner JD, Kinzler KW, Vogelstein B. (1993) *In vivo* cloning of PCR products in *E. coli.* Nucleic Acids Research 21:5192-5197.

[7] Zhang Y, Buchholz F, Muyrers JP, Stewart AF (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. Nature Genetics. 20:123-128.

[8] Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL (2000) An efficient recombination system for chromosome engineering in *Escherichia coli.* Proc. Natl. Acad. Sci. USA 97:5978-5983.

[9] Bian X, Huang F, Stewart FA, Xia L, Zhang Y, Müller R (2012) Direct cloning, genetic engineering, and heterologous expression of the syringolin biosynthetic gene cluster in *E. coli* through Red/ET recombineering. ChemBioChem 13:1946-1952.

[10] Fu J, Bian X, Hu S, Wang H, Huang F, Seibert PM, Plaza A, Xia L, Müller R, Stewart AF, Zhang Y (2012) Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. Nature Biotechnology 30:440-448.

[11] Ikeuchi M, Tabata S (2001) Synechocystis sp. PCC 6803 – a useful tool in the study of the genetics of cyanobacteria. Photosynthesis Research 70:73-83.

[12] Giner-Lamia J, Lopez-Maury L, Reyes JC, Florencio FJ (2012) The CopRS two-component system is responsible for resistance copper in the cyanobacterium Synechocystis sp. PCC6803. Plant Physiol. 159:1806-1818.

[13] Kaneko T, Nakamura Y, Sasamoto S, Watanabe A, Kohara M, Matsumoto M, Shimpo S, Yamada M, Tabata S (2003) Structural Analysis of Four Large Plasmids Harboring in a Unicellular Cyanobacterium, Synechocystis sp. PCC 6803. DNA Research 10:221-228.

[14] Giner-Lamia J, Lopez-Maury L, Florencio FJ (2014) CopM is a novel copper-binding protein involved in copper resistance in *Synechocystis* sp. PCC 6803. MicrobiologyOpen 4:167-185.

[15] Nies DH (2013) RND efflux pumps for metal cations. In Microbial Efflux Pumps: Current Research. Eds. Yu EW, Zhang Q, Brown MH, Ch. 5, pp. 79-121, Caister Academic Press.

[16] Franke S, Grass G, Rensing C, Nies DH (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J. Bacteriol. 185:3804-3812.

[17] Golden SS, Brusslan J, Haselkorn R (1987) Genetic engineering of the cyanobacterial chromosome. Methods Enzymol. 153:215-231.

[18] Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, Volume 1, 3rd edition, CSHL Press, New York.

[19] Eaton-Rye JJ (2011) Construction of gene interruptions and gene deletions in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Methods Mol Biol. 684:295-312.

[20] Thomas MR (1994) Simple, effective cleanup of DNA ligation reactions prior to the electro-transformation of *E. coli*. Biotechniques 16:988-990.

[21] Ka Chun Fung D, Yin Lau W, Tat Chan W, Yan A (2013) Copper efflux is induced during anaerobic amino acid limitation in *Escherichia coli* to protect iron-sulfur cluster enzymes and biogenesis. J. Bacteriol. 195:4556-4568.

[22] Watt VM, Ingles CJ, Urdea MS, Rutter WJ (1985) Homology requirements for recombination in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 82:4768-4772.

[23] Kües U, Stahl U (1989) Replication of plasmids in gram-negative bacteria. Microbiol. Rev. 53:491-516.

[24] Chua K-L, Oliver P (1992) Intramolecular homologous recombination of linearized plasmids in *Escherichia coli* K12. Mol. Gen. Genet. 232:199-205.

[25] Wang J, Sarov M, Rientjes J, Hu J, Hollak H, Kranz H, Xie Y, Stewart AF, Zhang Y (2006) An improved recombineering approach by adding RecA to λ Red recombination. Molecular Biotechnology 32:43-53.

[26] Grass G, Rensing C (2001) Genes involved in copper homeostasis in Escherichia coli. J Bacteriol. 183:2145-2147.

[27] Glick BR (1995) Metabolic load and heterologous gene expression. Biotechnology Advances 13:247-261.

[28] Abriata LA (2015) Homology- and coevolution-consistent structural models of bacterial copper-tolerance protein CopM support a 'metal sponge' function and suggest regions for metal-dependent protein-protein interactions. bioRxiv doi: http://dx.doi.org/10.1101/013581

[29] Shatalin KY, Neyfakh AA (2005) Efficient gene inactivation in *Bacillus anthracis*. FEMS Microbiology Letters 245:315-319.

[30] Kendall SL, Frita R (2009) Construction of targeted mycobacterial mutants by homologous recombination. Methods Mol. Biol. 465:297-310.

[31] van Dam V, Bos MP (2012) Generating Knock-Out and Complementation Strains of *Neisseria meningitidis*. Methods in Molecular Biology 799:55-72.

[32] Zhang C, Whitaker RJ (2012) A broadly applicable gene knockout system for the thermoacidophilic archaeon *Sulfolobus islandicus* based on simvastatin selection. Microbiology 158:1513-1522.

[33] Orr-Weaver TL, Szostak JW, Rothstein RJ (1981) Yeast transformation: A model system for the study of recombination. Proc. Nat. Acad. Sci. USA 78:6354-6358.

[34] Miller BL, Miller KY, Timberlake WE (1985) Direct and indirect gene replacements in *Aspergillus nidulans*. Mol Cell Biol, 5:1714-1721.

[35] Asch DK, Kinsey JA (1990) Relationship of vector insert size to homologous integration during transformation of *Neurospora crassa* with the cloned am (GDH) gene. Mol. Gen. Genet. 221:37-43.

[36] De Lozanne A, Spudich JA (1987) Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science 236:1086-1091.

[37] Cruz A, Beverley SM (1990) Gene replacement in parasitic protozoa. Nature. 348:171-173.

[38] ten Asbroek AL, Ouellette M, Borst P (1990) Targeted insertion of the neomycin phosphotransferase gene into the tubulin gene cluster of *Trypanosoma brucei*. Nature. 348:174-175.

[39] Capecchi M (1989) Altering the genome by homologous recombination. Science 244:1288-1292.

[40] Buerstedde JM, Takeda S (1991) Increased ratio of targeted to random integration after transfection of chicken B cell lines. Cell 67:179-188.

[41] Koller BH, Smithies O (1992) Altering genes in animals by gene targeting. Annu. Rev. Immunol. 10:705-730.

[42] Schaefer DG, Zrÿd JP (1997) Efficient gene targeting in the moss *Physcomitrella patens*. Plant J. 11:1195-206.

[43] Drocourt, D, Calmels TPG, Reynes JP, Baron M, Tiraby G (1990) Cassettes of the Streptoalloteichus hindustanus ble Gene for Transformation of Lower and Higher Eukaryotes to Phleomycin Resistance. Nucleic Acids Res. 18: 4009.

[44] Agaphonov M, Romanova N, Choi E-S, Ter-Avanesyan M (2010) A novel kanamycin/G418 resistance marker for direct selection of transformants in Escherichia coli and different yeast species. Yeast 27: 189-195.

[45] Kwak JW, Kim J, Yoo OJ, Han MH (1987) A method to isolate DNA sequences that are promoter-active in Escherichia coli and in yeast. Biochem Biophys Res Commun. 149:846-851.

[46] Antonucci TK, Wen P, Rutter WJ (1989) Eukaryotic promoters drive gene expression in Escherichia coli. J Biol Chem. 264:17656-17659.

[47] Jacob D, Lewin A, Meister B, Appel B (2002) Plant-specific promoter sequences carry elements that are recognised by the eubacterial transcription machinery. Transgenic Res. 11: 291-303.

[48] Lewin A, Mayer M, Chusainow J, Jacob D, Appel B (2005) Viral promoters can initiate expression of toxin genes introduced into Escherichia coli. BMC Biotechnol. 5:19

[49] Van Den Berg MA. Multi-Species Polynucleotide Control Sequences. International patent WO2009150195 (A1), December 17, 2009.

[50] Barth C, Wilczynska Z, Pontes L, Fraser DJ, Fisher PR (1996) Efficient circularization in *Escherichia coli* of linear plasmid multimers from *Dictyostelium discoideum* genomic DNA. Plasmid 36:86-94.

[51] Yin J, Zhu H, Xia L, Ding X, Hoffmann T, Hoffmann M, Bian X, Müller R, Fu J, Stewart AF, Zhang Y (2014) A new recombineering system for Photorhabdus and Xenorhabdus. Nucleic Acids Res. [Epub ahead of print]

[52] Oldenburg KR, Vo KT, Michaelis S, Paddon C (1997) Recombination-mediated PCR-directed plasmid construction in vivo in yeast. Nucleic Acids Res. 25: 451-452.

[53] Grant SG, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc Natl Acad Sci U S A. 87:4645-4649.

[54] Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12.Science 277:1453-1462.

[55] Williams JGK (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. Methods Enzymol. 167:766-778.

[56] Guzman L, Belin D, Carson MJ, Beckwith J (1995) Tight regulation and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. 177:4121-4130.

[57] Chang AC, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141-1156.

**Table and Figure Legends**

**Table 1** Strains and plasmids used in this study.

**Supplementary Table S1** Oligonucleotide primers used in this study.

**Fig. 1.** Genetic organization of the copper resistance gene cluster within plasmid pSYSX of *Synechocystis* sp. PCC 6803. The genes of the *pcopMRS* and *copBAC* operons are differentiated by shading and their transcriptional orientation is indicated. Genes flanking the resistance gene cluster are shown without shading. The positions of the short homology element (C, red arrow), and right (D, blue arrow) and left (A, yellow arrow) homology arms included in the RR cloning construct pSMART-SYSX-Cu-REC are indicated above the main diagram.

**Fig. 2.** Schematic summary of recombineering recovery cloning. The RR construct is a narrow-host-range plasmid vector (B) containing three elements with homology to sites flanking the target region to be cloned (e.g. a gene cluster): a short homology element (C, red arrow), and right (D, blue arrow) and left (A, yellow arrow) homology arms. The construct is cleaved at a unique restriction site located between the two homology arms (black arrowhead) to produce a linear DNA with the component elements in the order ABCD (**1**). This DNA fragment is introduced into cells of the desired species using a suitable transformation method (**2**) and the construct integrates adjacent to the target region in the host genome by homologous recombination mediated by the homology arms (**3**). A transformant is selected and characterized by PCR analysis. DNA is prepared from the transformed cells and digested with a restriction endonuclease to reduce the fragment size (**4**). The cleaved DNA is introduced into an *E. coli* strain expressing recombination proteins (Recombineering$^{+}$) by electroporation (**5**) and homologous recombination between the short direct repeats either side of the target region leads to plasmid circularization and completes the cloning (**6**).

**Fig. 3.** Characterization of plasmid clones produced by RR, containing the *Synechocystis* copper resistance gene cluster, by digestion with EcoRI (A) and HindIII (B). M – 1 kb DNA ladder.

**Fig. 4.** Growth of *E. coli* JG01 strains on $CuCl_2$ gradient plates. Cell suspensions of mutant JG01 carrying parent vector pBAD24 (24), pBAD24-SYSX-Cu-pcopMRS+BAC (MRS+BAC) or

pBAD24-SYSX-Cu-pcopM (M) were spotted on LB agar plates containing a concentration gradient of $CuCl_2$, with or without 0.5% (w/v) L-arabinose. The plates were photographed after 48-h incubation at 37°C. The same pattern of growth was seen for three other clones of each strain spotted on replicate plates.

**Supplementary Methods**

*1. Bacterial culture media and growth conditions*

*E. coli* strains were propagated in LB or SOB medium (Sambrook and Russell, 2001) supplemented with selective antibiotics and other compounds as required. Except where indicated, strains carrying pBAD24 constructs were grown in the presence of 0.5% (w/v) glucose to repress the $P_{BAD}$ promoter. Liquid cultures (shaken at 150-250 rpm) and plates were incubated at 37°C, apart from strains for recombineering, which were grown at 30°C prior to transformation.

A glucose-tolerant strain of the freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (Williams, 1988) was propagated in BG11 medium (Rippka et al. 1979) buffered with 10 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)-KOH pH 8.2 and supplemented with glucose (5 mM). BG11 plates contained 0.6% (w/v) agarose plus kanamycin to select for transformants. Liquid cultures (shaken at 150 rpm) and plates were incubated at 30°C with continuous illumination of ~50 µmol photons $m^{-2}$ $s^{-1}$.

*2. DNA isolation*

Plasmid DNA was isolated from *E. coli* strains using Wizard plus SV (Promega, Southampton, UK) and NucleoBond BAC100 (Macherey-Nagel, Düren, Germany) kits, and also by a rapid boiling method (Holmes and Quigley 1981). A method for extracting large plasmid DNA from bacteria (Heringa et al. 2007) modified by the use of a cell lysis buffer containing Triton X-100 (Rychlik 1996) was used to isolate *Synechocystis* plasmids. Genomic DNA was prepared from the *Synechocystis* transformant using a modification of a rapid method for the isolation of total nucleic acids from *E. coli* (Cheng et al. 2004). Cells harvested from 5 ml of liquid culture by centrifugation (2600 g, 20 min) were resuspended in modified STE buffer (200 mM NaCl; 10 mM Tris buffer, pH 7.0; 1 mM EDTA). An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the mixture vortexed vigorously for 2 minutes. After centrifugation

(13,000 g, 5 min), the aqueous phase was extracted twice with chloroform:isoamyl alcohol (24:1). Nucleic acids were then ethanol precipitated, the pellet washed twice in 70% ethanol, briefly dried and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA).

*3. Preparation of the Synechocystis transformation construct*

Three DNA fragments representing regions of *Synechocystis* plasmid pSYSX flanking the copper resistance gene cluster were generated by PCR using a *Synechocystis* plasmid preparation as template, Accuzyme polymerase (Bioline, London, UK) and primer pairs SYSX_Cu_3F/SYSX_Cu_3R (fragment A, 457 bp), SYSX_Cu_1F/SYSX_Cu_1R (fragment C, 93 bp) and SYSX_Cu_2F/SYSX_Cu_2R (fragment D, 461 bp). The gel-purified fragments were joined in the order CDA by splicing by overlap extension (SOE, Horton et al. 1989), using 5' phosphorylated primers SYSX_Cu_1F/SYSX_Cu_3R and EconoTaq DNA polymerase (Lucigen). The terminal sequences of the right (D) and left (A) homology arms were selected so that a unique StuI restriction endonuclease cleavage site was created when these fragments were joined. The composite fragment (968 bp) was cloned in the vector pSMARTGC LK (fragment B, 1993 bp) and its sequence verified. The resulting construct was named pSMART-SYSX-Cu-REC.

*4. Preparation of electrocompetent E. coli cells for recombineering*

Recommendations for high-efficiency electrotransformation with large BAC plasmids (Novakova et al. 2013) were incorporated in a method used to prepare electrocompetent *E. coli*. The strain carrying the recombineering plasmid pSC101-BAD-gbaA-tet was propagated in 250 ml SOB medium + tetracycline (4 µg/ml) at 30°C with shaking (150 rpm) and the $A_{600}$ of the culture monitored using a spectrophotometer (Jenway 7315, Bibby Scientific Limited, Stone, Staffs. UK). At early log-phase ($A_{600}$ ~0.2), the culture was supplemented with L-arabinose to 0.1% (w/v) and incubation continued for a further 30 min ($A_{600}$ ~0.4). The cells were then harvested by centrifugation (800 g, 45 min, 4°C). The cell pellet was resuspended in 50 ml of ice-cold 10% glycerol and the centrifugation repeated. This washing procedure was performed twice more and the pellet resuspended in ice-cold 10% (v/v) glycerol to give a cell density of approximately 1.25 to 2.5 x $10^{10}$ cells/ml ($A_{600}$ = 25-50; Wu et al. 2010). Aliquots of the cell suspension (50 µl) were used for electroporation (Gene Pulser, Bio-Rad Laboratories, Hercules, CA, USA). After shocking, 0.95 ml of ice-cold LB + 5 mM $MgCl_2$ was added to the cells before incubation at 37°C

with shaking (250 rpm) for 70 min. Transformants were selected by plating on LB medium containing the appropriate antibiotic.

5. *Preparation of constructs for regulated expression of the Synechocystis copper resistance genes in E. coli*

Complete gene cluster (*pcopMRS+copBAC*)

(i) A 5143-bp fragment comprising the *copBAC* genes was excised from plasmid pSMART-SYSX-Cu-pcopMRS+BAC using XbaI and EcoRV (NEB) and gel purified. In parallel, plasmid pBAD24-SYSX-Cu-Xba was digested with XhoI and BseRI (NEB), which removed most of the 5197-bp XbaI fragment, leaving just the error-free termini. This gel-purified vector DNA was used as the substrate for recombineering with the 5143-bp XbaI/EcoRV fragment to create construct pBAD24-SYSX-Cu-Xba-EF, containing an authentic copy of the 5197-bp XbaI *copBAC* fragment.

(ii) A 3176-bp EcoRV/XhoI fragment, carrying the *pcopMRS* genes and the 5' end of *copB*, excised from plasmid pSMART-SYSX-Cu-pcopMRS+BAC, and pBAD24-SYSX-Cu-Xba-EF digested with SmaI and XhoI (NEB), were both gel-purified and then ligated to reconstruct the entire pSYSX copper resistance gene cluster in construct pBAD24-SYSX-Cu-pcopMRS+BAC.

*pcopM* gene

Plasmid pBAD24-SYSX-Cu-pcopMRS+BAC was digested with XagI and HindIII (Thermo Scientific) to excise 5 fragments comprising the 5' truncated p*copR* gene, the *pcopS* gene and the *copBAC* operon. The deleted plasmid (5315 bp) was treated with T4 DNA polymerase to produce blunt ends and then recircularized by ligation. This construct, containing the *pcopM* gene plus the 5' end of *pcopR* (11 codons), was named pBAD24-SYSX-Cu-pcopM.

6. *Creation of copper-sensitive E. coli mutant JG01*

A method for the inactivation of chromosomal genes in *E. coli* using PCR products (Datsenko and Wanner 2000) was employed to create mutant JG01. The chloramphenicol acetyltransferase (*cat*) gene of plasmid pACYC184 was amplified by PCR using Q5 polymerase (NEB) with primers delcusCFBA-up/delcusCFBA-down. This fragment was treated with restriction enzymes and gel purified to remove the template DNA. Strain AY1053 (Δ*copA* Δ*cueO* Δ*cusC*::Kan) (Ka Chun Fung et al. 2013) carrying recombineering plasmid pSC101-BAD-gbaA-tet was cultured at 30°C

in the presence of 0.1% (w/v) L-arabinose and then transformed with the *cat* gene fragment by electroporation (electrocompetent cells prepared as in 4 above). A chloramphenicol resistant transformant was characterized by PCRs using primers cusCFBA-F, cusCFBA-R, Cat-F and Cat-R. This strain was named JG01 (Δ*copA* Δ*cueO* Δ*cusCFBA*:Cm).

*7. $CuCl_2$ gradient plate assay to determine the copper sensitivity of E. coli JG01 strains*

$CuCl_2$ gradient plates were prepared using selective LB agar (without glucose). A 15 ml bottom layer, supplemented with $CuCl_2$ to 4 mM [± 0.5% (w/v) L-arabinose], was poured into a 90 mm Petri dish slanted just sufficiently for it to cover the whole bottom. Once this had set, the plate was moved to a level surface and a 15 ml top layer [± 0.5% (w/v) L-arabinose] was poured onto the wedge. The plates were dried then held overnight at 4°C prior to use. Assuming that an $A_{600}$ of 1 is equivalent to approximately 5 x $10^8$ cells/ml for *E. coli* (Wu et al. 2010), overnight cultures of the required JG01 strains were diluted in LB medium (no glucose) to give a suspension of ~3.3 x $10^6$ cells/ml. Volumes of 3 µl of these cell suspensions (~10,000 cells) were spotted in a grid pattern across the $CuCl_2$ gradients and the plates incubated at 37°C for 48 h.

References

Cheng L, Li T-Y, Zhang Y (2004) Rapid preparation of total nucleic acids from *E. coli* for multi-purpose applications. J. Biochem. Mol. Biol. 37: 351-355.

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97:6640-6645.

Heringa SD, Monroe JD, Herrick JB (2007) A simple, rapid method for extracting large plasmid DNA from bacteria. Available from Nature Proceedings. http://dx.doi.org/10.1038/npre.2007.1249.1

Holmes DS, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114: 193-197.

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61-68.

Ka Chun Fung D, Yin Lau W, Tat Chan W, Yan A (2013) Copper efflux is induced during anaerobic amino acid limitation in *Escherichia coli* to protect iron-sulfur cluster enzymes and biogenesis. J. Bacteriol. 195:4556-4568.

Novakova J, Izsakova A, Grivalsky T, Ottmann C, Farkasovsky M (2013) Improved method for high-efficiency electrotransformation of *Escherichia coli* with the large BAC plasmids. Folia Microbiol (Praha). 59:53-61.

Rippka R, Deruelles J, Waterbury J, Herdman M, Stanier R (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111: 1-61.

Rychlik I (1996) Rapid method for isolation of high molecular weight plasmids suitable for digestion with restriction endonucleases. Veterinarni Medicina 41: 359-360.

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, Volume 1, 3rd edition, CSHL Press, New York.

Williams JGK (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. Methods Enzymol. 167: 766-778.

Wu N, Matand K, Kebede B, Acquaah G, Williams S (2010) Enhancing DNA electrotransformation efficiency in *Escherichia coli* DH10B electrocompetent cells. Electronic Journal of Biotechnology 13(5). (DOI: 10.2225/vol13-issue5-fulltext-11)

**Table 1**

Strains and plasmids used in this study.

| | Description | Reference or Source |
|---|---|---|
| *E. coli strains* | | |
| XL-1blue | endA1 gyrA96(nal$^R$) thi-1 recA1 relA1 lac glnV44 F'[ ::Tn10 proAB$^+$ lacI$^q$ Δ(lacZ)M15] hsdR17(rK$^-$ mK$^+$)<br>General cloning strain | Stratagene |
| E. cloni 10G | F$^-$ mcrA Δ(mrr-hsdRMS-mcrBC) endA1 recA1 Φ80dlacZΔM15 ΔlacX74 araD139 Δ(ara,leu)7697galU galK rpsL nupG λ- tonA (Str$^R$)<br>Cloning strain used in preparation of *Synechocystis* transformation construct | Lucigen |
| GB05 | DH10B (Δ*fhuA*, Δ*ybcC*, Δ*recET*)<br>Host strain for recombineering plasmid | [53]<br>[10] |
| AY1053 | MG1655 (Δ*copA* Δ*cueO* Δ*cusC*::Kan)<br>Copper-sensitive mutant | [54]<br>[21] |
| JG01 | MG1655 (Δ*copA* Δ*cueO* Δ*cusCFBA*::Cm)<br>Copper-sensitive mutant derived from AY1053 | This study |
| *Synechocystis strains* | | |
| *Synechocystis* PCC 6803 | Glucose tolerant form of the freshwater cyanobacterium *Synechocystis* | [55] |
| *Synechocystis* SYSX-Cu-REC | *Synechocystis* PCC 6803 transformant with construct pSMARTSYSX-Cu-REC integrated adjacent to the pSYSX copper resistance gene cluster | This study |
| *Plasmids* | | |
| pBAD24 | Ap$^R$, ColE1 ori, *araC* gene, arabinose $P_{BAD}$ promoter. Expression vector | [56] |
| pBAD24-SYSX-Cu-Xba | 5197-bp XbaI fragment of Synechocystis pSYSX comprising the 5' truncated *copB* gene plus the *copA* and *copC* genes, cloned into pBAD24 | This study |
| pSMARTGC LK | Km$^R$, ColE1 ori, transcription terminators. Direct cloning of PCR products | Lucigen |
| pSMART-SYSX-Cu-REC | Combined fragments A, C and D of pSYSX | This study |

| | | |
|---|---|---|
| | representing regions flanking the copper resistance gene cluster, cloned into pSMARTGC LK. *Synechocystis* transformation construct | |
| pSC101-BAD-gbaA-tet | $Tc^R$, pSC101 ori, *araC* gene, $P_{BAD}$ promoter regulating the expression of λ Redγ Redβ, Redα and *E.coli* RecA (gbaA). Recombineering plasmid | [25] |
| pSMART-SYSX-Cu-pcopMRS+BAC | Recovered copper tolerance gene cluster of pSYSX comprising operons *pcopMRS* and *copBAC* in pSMARTGC LK | This study |
| pBAD24-SYSX-Cu-Xba-EF | "Error-free" version of 5197-bp XbaI fragment of *Synechocystis* pSYSX in pBAD24 | This study |
| pBAD24-SYSX-Cu-pcopMRS+BAC | Copper resistance gene cluster of pSYSX reconstructed in pBAD24 | This study |
| pBAD24-SYSX-Cu-pcopM | Deletion derivative of pBAD24-SYSX-Cu-pcopMRS+BAC containing just the *pcopM* gene (and 5' end of *pcopR*) | This study |
| pACYC184 | $Cm^R$ , $Tc^R$, p15A ori, cloning vector. Source of *cat* gene | [57] |

**Supplementary Table S1**

Oligonucleotide primers used in this study.

| | Primer name | Sequence (5'–3') |
|---|---|---|
| Amplification of *Synechocystis copBAC* | SYSX_Cu_Xba_F | TGCTGGTAATGTCTCCCCAAGCGACCC |
| | SYSX_Cu_Xba_R | TGTTTCTGACTTTTCAAGCGTCACTGTTTG |
| Preparation of *Synechocystis* transformation construct | SYSX_Cu_1F | GGAATATAACACCAGGACAGCTAA |
| | SYSX_Cu_1R | TTGGACAAAGTCGAACTCAGATCTGCTACCAGTTCATGCTTTTGT |
| | SYSX_Cu_2F | TCTGAGTTCGACTTTGTCCAA |
| | SYSX_Cu_2R | CTATGAGTAGAAGGATAACAGGCCTTCACTTCCAGCTCCATG |
| | SYSX_Cu_3F | CCTGTTATCCTTCTACTCATAGTTTTTC |
| | SYSX_Cu_3R | GTCAAAAGCTAAAACTGAATCTTCCATTG |
| Sequencing of cloned copper resistance gene cluster | M+Bseq_1 | TACGCCCGGTAGTGATCTTATTTC |
| | M+Bseq_2 | CCCACCATCAAATGGCAGTG |
| | M+Bseq_3 | TCGGCGGAATCCACCAGTAATG |
| | M+Bseq_4 | GCCGGTTTATCGTTCCTACCAAC |
| | M+Bseq_5 | TTCCTGTGAAACACTAAGAACG |
| | M+Bseq_6 | AAACAGGTCAAATTGAGCGAGG |
| | M+Bseq_7 | AAACGCCAACAAAGTCAGCAG |
| | M+Bseq_8 | CCGCCTCAAAGGGTTTAATATTTC |
| | M+Bseq_9 | TTTCCACAGTCATCATCGTTG |
| | M+Bseq_10 | GTGCGTCAGGTACAAATTCAG |
| | M+Bseq_11 | TTTGATGACCGCCCTCACTTC |

| | | |
|---|---|---|
| | M+Bseq_12 | GTGAAGGTGAGCCAGTGAGTTG |
| | M+Bseq_13 | AATTTCCTTCCCGGCTCCAG |
| | M+Bseq_14 | CTACCATTCAATGCTGTTTCTACC |
| | M+Bseq_15 | AAATGGACAATAAATAGGCCACCC |
| | M+Bseq_16 | CCGCATTGACATTGGCCTCTTC |
| | M+Bseq_17 | TTTCAACTTCCAGGGATGGTTC |
| | M+Bseq_18 | GGGTCAACAACGATCCCATC |
| | M+Bseq_19 | GGGAGACATTACCAGCAACC |
| | M+Bseq_20 | GGTTCTGCATTTAATGTCGTTTCC |
| | M+Bseq_21 | AAACGATACCCTAAGCCATACAC |
| | M+Bseq_22 | GAGCGGATAATGTCATCCATC |
| *E. coli cusCFBA* deletion | delcusCFBA-up | GTAAAGTTGGCGGCATAAAATCACCAGAAATTATGAGCCTTTACGCCCCGCCCTGCCAC |
| | delcusCFBA-down | GCAAGACACAATCCACACGGTTAAACGGGGTATCCTGCTTTACCGGGAAGCCCTGGGCC |
| Verification of *cusCFBA* deletion | cusCFBA-F | TTCTAGAGTAGCGGGATCAG |
| | cusCFBA-R | TTGAGGGGCACCTTTTAACA |
| | Cat-F | TACCGGGAAGCCCTGGGCC |
| | Cat-R | TTACGCCCCGCCCTGCCAC |

Note: Nucleotides added or changed to introduce restriction sites are underlined.

# Fig. 1

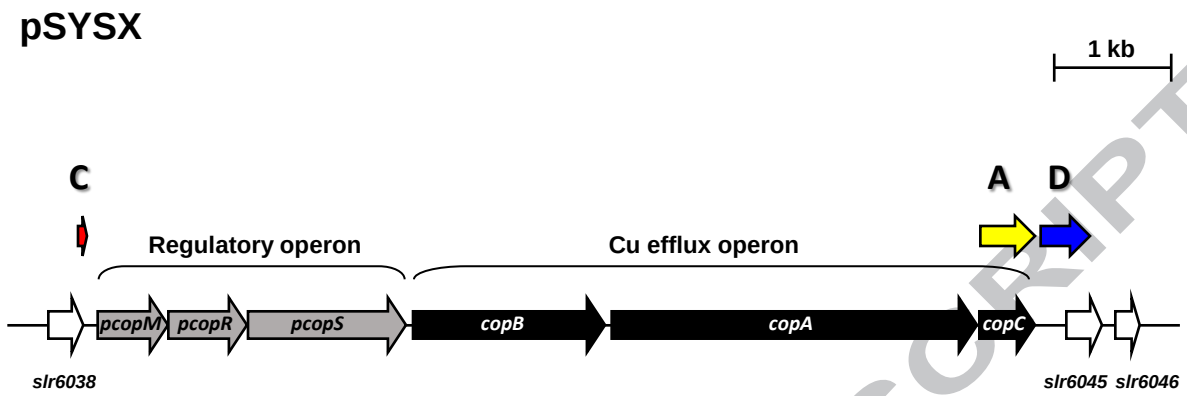

Fig. 2

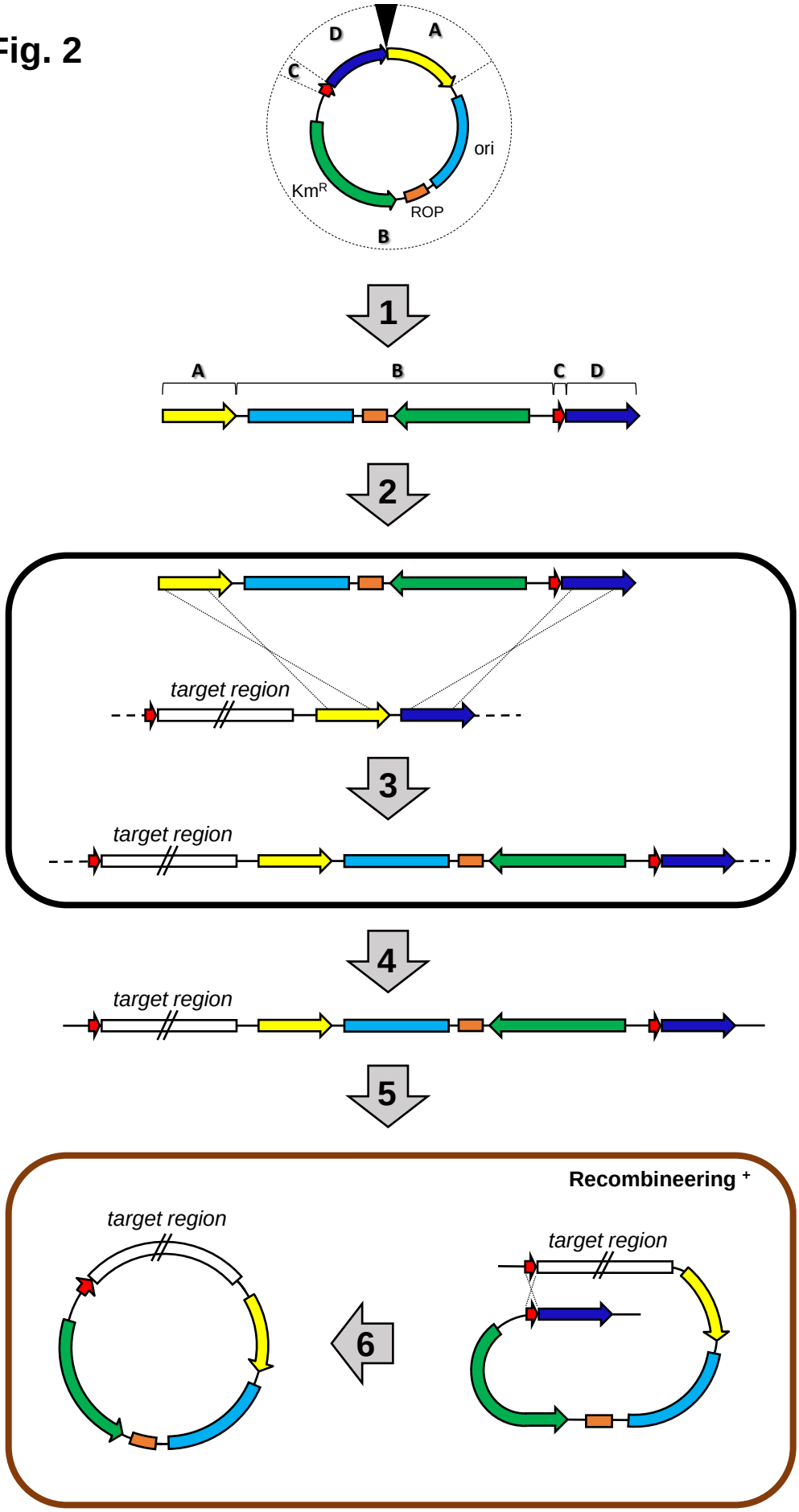
D
A
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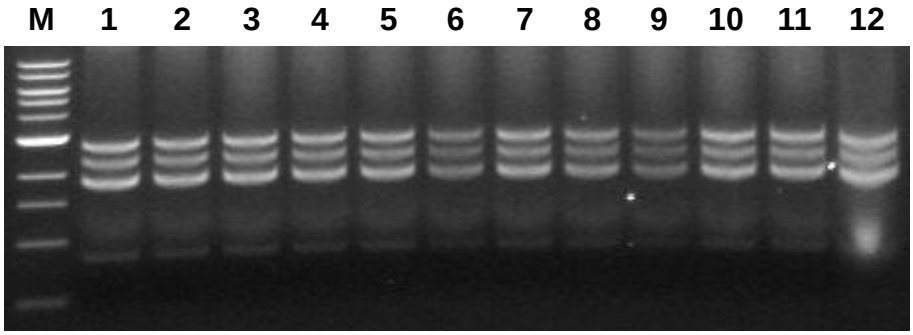
Fig. 3

A

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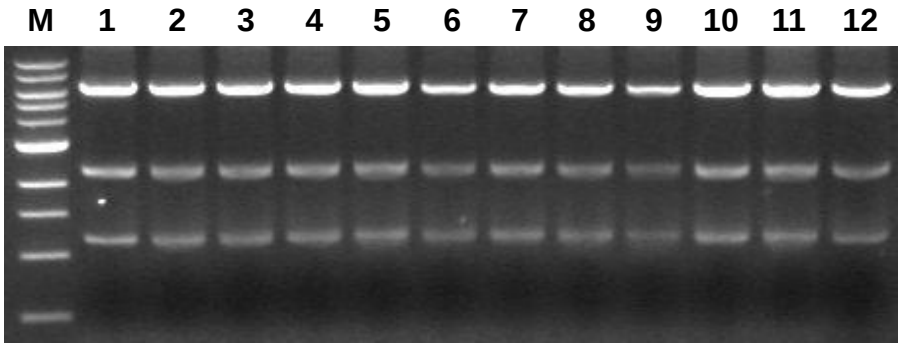

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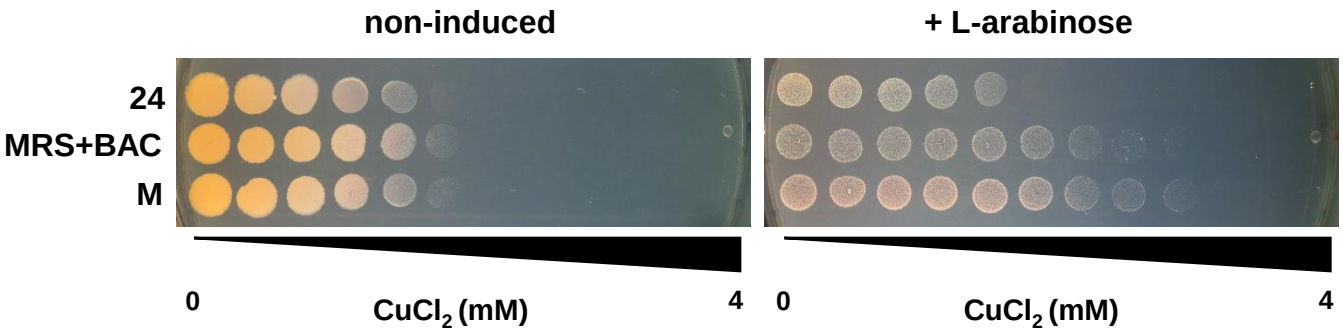

Highlights

- A *Synechocystis* copper resistance gene cluster was cloned by recombineering recovery (RR).
- Functionality of the *pcopM* gene in *E. coli* was confirmed by mutant complementation.
- RR represents an efficient way of isolating large DNA fragments.
- The method should be applicable to species transformed by homologous recombination.