FEBS Letters xxx (2015) xxx–xxx

journal homepage: www.FEBSLetters.org

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

John R. Gittins *

*Ocean and Earth Science, University of Southampton, Waterfront Campus, National Oceanography Centre, Southampton SO14 3ZH, UK*

ARTICLE INFO

*Article history:*
Received 13 November 2014
Revised 18 April 2015
Accepted 4 May 2015
Available online xxxx

Edited by Miguel De la Rosa

*Keywords:*
Cloning
Copper resistance gene cluster
Heterologous expression
Homologous recombination
Mutant complementation
Recombineering recovery

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 *Escherichia 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

*Author contributions:* J.R.G. devised the concept, designed and performed the experiments, analyzed the data and prepared the manuscript.

* Fax: +44 023 8059 3059.
*E-mail address:* jrgi@noc.soton.ac.uk

http://dx.doi.org/10.1016/j.febslet.2015.05.014
0014-5793/

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 the 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

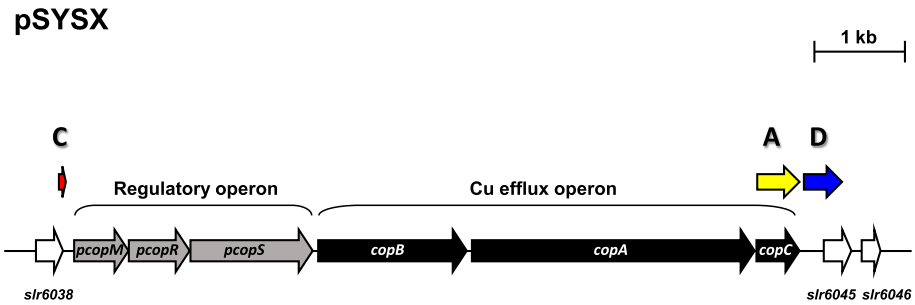


**Fig. 1.** Genetic organization of the copper resistance gene cluster within plasmid pSYSX of *Synechocystis* sp. PCC 6803. The genes of the *pcopMRS* and *copBAC* operons are differentiated by shading and their transcriptional orientation is indicated. Genes flanking the resistance gene cluster are 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.

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