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Self-replicating shuttle vectors based on pANS, a small endogenous plasmid of the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942

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Abbreviations: EPA, eicosapentaenoic acid; MCS, multiple cloning site

SUMMARY

To facilitate development of synthetic biology tools for genetic engineering of cyanobacterial strains, we constructed pANS-derived self-replicating shuttle vectors that are based on the minimal replication element of the *Synechococcus elongatus* strain PCC 7942 plasmid pANS. To remove the possibility of homologous recombination events between the shuttle plasmids and the native pANS plasmid, the endogenous pANS was cured through plasmid incompatibility-mediated spontaneous loss. A heterologous toxin-antitoxin cassette was incorporated into the shuttle vectors for stable plasmid maintenance in the absence of antibiotic selection. The pANS-based shuttle vectors were shown to be able to carry a large 20-kb DNA fragment containing a gene cluster for biosynthesis of the omega-3 fatty acid eicosapentaenoic acid (EPA). Based on qPCR analysis, there are about 10 copies of pANS and 3 copies of the large native plasmid pANL per chromosome in *S. elongatus*. Fluorescence levels of GFP reporter genes in a pANS-based vector were about 2.5-fold higher than when in pANL or integrated into the chromosome. In addition to its native host, pANS-based shuttle vectors were also found to replicate stably in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. There were about 27 copies of a pANS-based shuttle vector, 9 copies of a pDU1-based shuttle vector, and 3 copies of an RSF1010-based shuttle vector per genome when these three plasmids co-existed in *Anabaena* cells. The endogenous pANS from our *S*. *elongatus* laboratory strain was cloned in *E. coli*, re-sequenced, and re-annotated to update previously published sequencing data.

INTRODUCTION

In recent years cyanobacteria, the only group of prokaryotes that perform oxygenic photosynthesis, have attracted interest as promising microbial factories for production of renewable chemicals and fuels directly from sunlight and CO_2 (Hays & Ducat, 2015; Sarsekeyeva *et al.*, 2015). Because many cyanobacterial strains are amenable to genetic modification, extensive genetic tools have been developed in model and newly isolated strains to facilitate metabolic engineering and strain development (Ruffing, 2011; Taton *et al.*, 2014). Depending on the strain, DNA constructs can be introduced into cyanobacteria through natural transformation, electroporation, or conjugation from *Escherichia coli*. Homology-based integration vectors are commonly used in genetic manipulation of cyanobacteria, but they are highly strain specific. For example, genetic engineering of *Synechococcus elongatus* strain PCC 7942 is typically performed by integration of recombinant DNA at one of three neutral sites in the chromosome by homologous recombination (Clerico *et al.*, 2007; Niederholtmeyer *et al.*, 2010). In *Synechococcus* sp. strain PCC 7002, strain engineering is accomplished by homologous recombination of linear recombinant DNA fragments that carry selectable markers into neutral sites on endogenous plasmids and on the chromosome (Xu *et al.*, 2011).

Broad-host-range autonomously replicating shuttle vectors based on RSF1010, first identified as a self-mobilizable plasmid from *E. coli (Scholz et al., 1989)*, have been shown to function in a variety of cyanobacterial genera, such as unicellular *Synechococcus* (Brahamsha, 1996) and *Synechocystis (Marraccini et al., 1993)* strains, and filamentous strains including *Pseudanabaena* (Sode *et al.*, 1992), *Anabaena* (Thiel, 1994; Wolk *et al.*, 2007), and *Leptolyngbya* (Taton *et al.*, 2014). In addition to RSF1010, endogenous cyanobacterial plasmids have been used to construct chimeric shuttle vectors, but the cyanobacterial origins of replication tend to have a narrow host range and replicate in only a limited number of species. Derivatives of *Nostoc* sp. PCC 7524 plasmid pDU1 have been widely used in the model filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena* PCC 7120) as

well as other related filamentous strains (Schmetterer & Wolk, 1988). Endogenous plasmid-based shuttle vectors were also developed for marine *Synechococcus* species (Matsunaga *et al.*, 1990).

The unicellular model cyanobacterium *S. elongatus* (strain PCC 7942) carries two endogenous plasmids, the 7.8 kb pANS (Van der Plas *et al.*, 1992) and the 46.2 kb pANL (Chen *et al.*, 2008). Because pANS is not essential (Lau & Doolittle, 1979) and has a relatively small size, it was used for constructing self-replicating shuttle vectors (Golden & Sherman, 1983; Kuhlemeier & van Arkel, 1987). However, structural integrity issues of pANS-derived shuttle vectors that result from recombination events between these vectors and the native pANS population have significantly limited their use in genetic modification of *S. elongatus* (Gendel, 1987; Kuhlemeier *et al.*, 1981). For unknown reasons, RSF1010-based shuttle vectors do not replicate well in *S. elongatus*. Hence almost all genetic and molecular studies in *S. elongatus* rely on homologous recombination-mediated integration of neutral-site vectors into the chromosome (Clerico *et al.*, 2007). The potential of self-replicating shuttle vectors for genetic engineering of *S. elongatus* has yet to be fully explored.

Self-replicating plasmids offer certain advantages over integration plasmids for genetic engineering. They can have higher copy number and therefore higher gene expression levels. Most, if not all, cyanobacterial species have multiple copies of identical chromosomes (Griese *et al.*, 2011); thus chromosome segregation after integration-based gene transfer can be a lengthy and troublesome process, especially in filamentous strains. Engineered strains containing self-replicating plasmids can be selected without segregation concerns. Furthermore, self-replicating shuttle vectors that carry replication origin(s) for both *E. coli* and cyanobacteria can be easily recovered from cyanobacteria and introduced back into *E. coli*, which is convenient for studying plasmid stability and integrity, and for the isolation of recombinant library clones. Development of stable self-replicating shuttle plasmid vectors would enlarge the genetic

toolbox for genetic engineering and synthetic biology approaches in *S. elongatus* and other cyanobacteria.

Desired properties of robust plasmid shuttle vectors are that they are stably maintained in the absence of selection, can carry large DNA inserts, can be transferred through conjugation, have relatively high copy numbers, can replicate in different strains, and are compatible with other plasmids for experiments that require multiple genetic manipulations. Here, we report construction of such shuttle vectors that are based on the minimal 3.76 kb replication region of pANS (Van der Plas *et al.*, 1992). The pANS-based shuttle vectors were shown to replicate not only in their native *S. elongatus* host, but also in the heterologous strain *Anabaena* PCC 7120, and to have the capacity to carry a 20-kb eicosapentaenoic acid (EPA) biosynthesis gene cluster.

METHODS

Strains and growth conditions.

E. coli and cyanobacterial strains used in this study are listed in Table 1. Growth conditions for *E. coli* and cyanobacterial strains *S. elongatus* strain PCC 7942, *Anabaena* sp. strain PCC 7120, *Leptolyngbya* sp. strain BL0902, *Synechocystis* sp. strain PCC 6803, and *Synechocystis* sp. strain WHSYN were as previously published (Taton *et al.*, 2014) unless stated otherwise. Cyanobacterial liquid and plate cultures were grown at 30 °C under continuous illumination of 60 µmol photons m^{-2} s^{-1}. Antibiotics were used for the selection of transformed cyanobacterial strains at the following concentrations: chloramphenicol (Cm; 7.5 µg ml^{-1}), gentamicin (Gm; 2 µg ml^{-1}), kanamycin (Km; 5 µg ml^{-1}), neomycin (Nm; 50 µg ml^{-1}), nourseothricin (Nt; 50 µg ml^{-1}), spectinomycin (Sp; 2 µg ml^{-1}), and streptomycin (Sm; 2 µg ml^{-1}).

Transformation of cyanobacteria.

Natural transformation of *S. elongatus* and bi-parental conjugation of plasmids from *E. coli* to *Anabaena* PCC 7120, *Leptolyngbya* BL0902, *Synechocystis* PCC 6803, and *Synechocystis* WHSYN followed published protocols (Clerico *et al.*, 2007; Elhai & Wolk, 1988; Elhai *et al.*, 1997; Golden & Sherman, 1984; Taton *et al.*, 2012). The genotypes of transformants or exconjugants were confirmed by colony PCR, 25 cycles, using Taq DNA polymerase (NEB).

Plasmids and primers.

Plasmids used in this study are described in Table 1 and DNA primers are listed in Table S1. Plasmids were assembled using a GeneArt Seamless Cloning and Assembly Kit (Life Technologies) following the manufacturer's instructions with slight modifications described earlier (Taton *et al.*, 2014). Unless otherwise stated, plasmids were constructed in *E. coli* strain DH5α.

Plasmid pAM4787 (7032 bp) was designed with the CYANO-VECTOR assembly portal (http://golden.ucsd.edu/CyanoVECTOR/) (Taton *et al.*, 2014) using the following modules: the *aadA* resistance marker for Sp and Sm (1276 bp *Eco*RV-digested fragment from pCVD002), the PconII-yemGFP module (945 bp *Eco*RV-digested fragment from pCVD031), the pBR322 oriV-

oriT fragment (1080 bp *Eco*RV-digested fragment from pCVD026), and the pANS minimal replication origin region (3813 bp *Zra*I-digested fragment from pCVD048). Plasmid pAM4789 (4891 bp) was made with the same modules as pAM4787 but the pANS replication region was replaced with the pANL replication origin region (1674 bp *Zra*I-digested fragment from pCVD066). Plasmid pAM4788 (7774 bp) was made with a 789 bp pSYSA-TA1 cassette, which was amplified with the primer pair yc087/yc088 from *Synechocystis* sp. PCC 6803 gDNA, that was cloned into the *Sac*I site of pAM4787. To make plasmids pAM5173 (26,643 bp) and pAM5174 (27,385 bp), the backbones of pAM4787 (4910 bp) and pAM4788 (5652 bp), respectively, were amplified with the primers yc163 and yc164. These PCR fragments were then each separately assembled with the aadA-EPA insert (21,779 bp), which was produced by digestion of pAM4790 (24,640 bp) with *Asc*I and *Sma*I. To make pAM5187 (6194 bp) and pAM5188 (6936 bp) plasmids, pAM4787 and pAM4788, respectively, were digested with *Age*I and *Xba*I to remove the PconII-yemGFP fragment, and the largest digestion fragment from each plasmid (6081 bp for pAM4787; 6823 bp for pAM4788) was then assembled with a pBluescript SK(-) multiple-cloning-site PCR fragment (148 bp) that was amplified with primers yc336 and yc337 from pBluescript SK(-) (Stratagene). To make pAM5189 (7962 bp), pAM4787 was digested with *Sac*I and *Age*I to release the oriV-oriT-aadA fragment (2310 bp), which was then assembled with pUL19L (2659 bp; Thermo Fisher Scientific) and the following 3 PCR fragments: pANS-*Bam*HI-up arm (primers yc292 and yc293; 1069 bp of pANS), pANS-*Bam*HI-dn arm (primers yc294 and yc295; 1012 bp of pANS), and PconII-LTRBS-GFPmut2 fragment (primers yc296 and yc297; 990 bp of pCV0003). Plasmid pAM5190 resulted from in vivo double-recombination events in *S. elongatus* PCC 7942 between pAM5189 and the native pANS plasmid, such that the oriV-oriT-aadA and PconII-LTRBS-GFPmut2 fragments were inserted into the *Bam*HI site of pANS.

Restriction digestion analysis of shuttle vectors recovered in *E. coli*.

Shuttle vectors were extracted from cyanobacterial strains with QIAprep Spin Miniprep Kit (Qiagen) using the standard protocol. A 1-2 □l sample of the extracted DNA was introduced into *E. coli* DH10B electro-

competent cells (50 μl) using Gene Pulser II (Bio-Rad). Electroporated cells were plated for single colonies on LB agar plates containing appropriate antibiotics. Different shuttle vectors carried by the same cyanobacterial strain were separately recovered in *E. coli* clones based on their different antibiotic resistance markers. The recovered plasmids were then digested with appropriate restriction enzymes and analyzed by agarose gel electrophoresis.

Genomic DNA extraction.

The total DNA of cyanobacterial strains was extracted with the CTAB method (Golden *et al.*, 1987). Typically, a 10-ml growing culture of cyanobacteria with an optical density (OD_{750}) of 0.5 was collected for each sample.

Chromosome copy number determination.

A Petroff-Hausser Counting Chamber was used to count *S. elongatus* cells. The cell-count data (cell ml^{-1}) were calculated from the averaged cell numbers of 10 blocks in the center grid of the chamber. The chromosome copy number per cell was then determined based on the molecular weight of the chromosome and the total amount of DNA extracted from a determined number of cells.

Plasmid copy number determination with quantitative PCR (qPCR) analysis.

Total DNA from cyanobacterial strains was used as the template for qPCR analysis. Primers were designed using the PrimerQuest online tool (IDT). For each experiment, standard real-time PCR reactions, in triplicate, were set up with the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and run on a StepOne Real-Time PCR System (Thermo Fisher Scientific) following the manufacturers' instructions. The qPCR results were calculated from triplicate experiments. The copy numbers of endogenous plasmids per chromosome were determined based on qPCR analysis using 3 pairs of plasmid-specific primers for each plasmid and 3 reference primer pairs specific for the chromosome.

Reporter fluorescence measurement.

For each strain, the original culture was subcultured in 24-well plates once every 7 days at 30 °C under continuous illumination of 60 μmol photons m^{-2} s^{-1} in BG-11 medium with or without antibiotics (Sp and Sm). The emission intensities of GFP fluorescence from liquid culture samples were measured with triplicate readings in Greiner 96-well flat bottom black polystyrene

plates with a Tecan Infinite M200 plate reader. The excitation and emission wavelengths for GFPmut2 and yemGFP were set to EX488/9 and EM518/20. GFP measurements were normalized by subtracting the blank measurement and then dividing the GFP fluorescence by the corresponding OD_{750}.

Plasmid maintenance assay.

The plasmid maintenance assay protocol was modified for *S. elongatus* from a published protocol for *Anabaena* PCC 7120 (Lee *et al.*, 2003). *S. elongatus* strains were subcultured in BG-11 liquid medium without antibiotics every 7 days for 5 weeks. At the end of each growth period a sample was diluted 10,000 fold with BG-11 medium and 100 □l was then spread with glass beads onto a BG-11 agar plate without antibiotics and then incubated for 7 days. After this incubation 24 colonies from each plate were streaked in duplicate onto BG-11 plates without and with antibiotics. Plates were incubated for 5-7 days until sufficient growth had occurred for scoring. Patches that grew without antibiotics but not with Sp and Sm were scored as having lost the plasmid.

RESULTS

Construction of a pANS-based shuttle vector and transformation of *S. elongatus*

Earlier work on functional deletion analysis of pANS identified a 3.65-kb *Xho*I-*Bgl*II restriction fragment as the minimal replication element of pANS that was sufficient for autonomous replication (Van der Plas *et al.*, 1992). This region contains the gene (originally annotated as genes *repA* and *repB*) necessary for plasmid replication and a 200 bp intergenic region that was predicted to contain the replication origin (Van der Plas *et al.*, 1992). Two other genes, *pmaA* and *pmaB,* that function in plasmid maintenance of pANS are not included in the defined minimal replication element, but the shuttle vectors based on the minimal region nonetheless can be maintained in strains by selection with appropriate antibiotics added to the growth medium (Van der Plas *et al.*, 1992). We designed improved versatile shuttle vectors based on this minimal replication element of pANS.

A pANS-derived shuttle vector, pAM4787, was constructed using a GC-adaptor assembly (Taton *et al.*, 2014) with the pANS minimal replication element, a pBR322 fragment that contained the pMB1 replication origin (*oriV*) and the origin of transfer (*oriT*), an *aadA* gene cassette as the selectable marker conferring resistance to Sp and Sm, and a copy of the *yemGFP* gene driven by the *conII* promoter as a reporter for gene expression levels (Table 1 and Fig. 1).

The plasmid pAM4787 was introduced into wild-type *S. elongatus* via both natural transformation and bacterial conjugation. Colony PCR analysis showed that all Sp+Sm resistant *S. elongatus* transformants carried the introduced pAM4787 shuttle vector (Fig. 2, bottom panel B), but 2 out of the 6 transformants still retained the endogenous pANS (Fig. 2, top panel A), which indicates that selection for pAM4787 does not rapidly exclude the endogenous pANS plasmid. In addition, we observed evidence of recombination between pAM4787 and the endogenous pANS plasmid, which would be detrimental for genetic engineering experiments. Therefore, we produced a new *S. elongatus* genetic-engineering platform strain lacking the pANS plasmid.

Plasmid incompatibility-mediated spontaneous loss of pANS

The pANS plasmid was cured via plasmid incompatibility with an introduced pANS-based shuttle plasmid that was maintained in the cells by antibiotic selection. Compared to plasmid elimination with the use of intercalating dyes that interfere with replication, plasmid incompatibility-mediated spontaneous plasmid loss minimizes the possibility of mutations being introduced in the cell's chromosomes during the curing process (Liu *et al.*, 2012; Trevors, 1986).

To obtain a pANS-cured host cloning strain for pANS-based shuttle vectors, we first transformed the wild-type strain with shuttle vector pAM4787, then identified strains in which the native pANS plasmid had been lost as a result of plasmid incompatibility-mediated spontaneous loss, and then screened for strains that lost the pAM4787 shuttle vector after antibiotic selection was removed. We selected two *S. elongatus* transformants, GLC001 and GLC002 (Table 1), that contained pAM4787 and had completely lost pANS (Fig. 2, lanes 3 and 6). The strains were continuously subcultured in the absence of antibiotic selection in liquid media every 7 days. After subculturing 3 times (21 days of growth and at least 12 generations as estimated from OD_{750} measurements), the subcultured strains were then replica plated on agar plates. Only colonies that grew up on plates without antibiotics, but not on plates with antibiotics, were patched and inoculated into liquid medium for growth and analysis. PCR analyses using primer pairs specific for pAM4787 (Fig. 3, panels A, B), pANS (Fig. 3, panel C), and pANL (Fig. 3, panel D) were performed for four colony-derived cultures. Spontaneous loss of pAM4787 from the pANS-cured strains was observed in all four tested clones (Fig. 3 & Table S1). All four clones still contained the large pANL plasmid.

Because the capability for natural transformation can be lost in *S. elongatus* cloned lineages (Golden & Sherman, 1984), 4 independent pANS-cured strains were tested for their transformation efficiency with both integration and shuttle vectors (pAM1303 (NS1), pAM1573 (NS2), and pAM4787). No significant difference in transformation efficiency was found between

S. elongatus wild type and pANS-cured strains (designated as *S. elongatus*(-pANS)) (Table S2). The cured *S. elongatus*(-pANS) strains had normal morphology and growth on agar plates and in liquid media under standard laboratory growth conditions. These results are consistent with a previous report in which a strain of *S. elongatus* (previously named *Anacystis nidulans*) that spontaneously lost pANS showed no difference in growth characteristics compared to a strain of *S. elongatus* that carried pANS (Lau & Doolittle, 1979).

A heterologous toxin-antitoxin cassette enhanced plasmid maintenance

Plasmid maintenance without antibiotic selection is highly desirable in biotechnology applications, especially in large bacterial cultures. The native plasmid maintenance genes of pANS (*pmaA* and *pmaB*), however, may not have optimal efficacy because pANS can be cured by chemical treatment (Kuhlemeier *et al.*, 1983) and even spontaneous loss (Lau & Doolittle, 1979; Lau *et al.*, 1980). Toxin-antitoxin systems, widely distributed among prokaryotic plasmids and chromosomes, function in plasmid maintenance through a mechanism of post-segregational killing (Pecota *et al.*, 1997). To explore the possibility of using heterologous cyanobacterial toxin-antitoxin cassettes for antibiotic-free plasmid vector maintenance, the pANS-derived shuttle vector pAM4788 (Table 1 & Fig. 1, right panel, TA1 segment) was constructed to carry a toxin-antitoxin cassette from the endogenous megaplasmid pSYSA of *Synechocystis* sp. PCC 6803 (Kopfmann & Hess, 2013).

Two different methods were used to evaluate the maintenance of the pANS shuttle vectors in the absence of antibiotic selection. A plasmid maintenance assay was based on the loss of antibiotic resistance. Liquid cultures of *S. elongatus* strains cured of the native pANS (strain AMC2302; Table 1) and carrying either the pAM4787 or pAM4788 shuttle vector were subcultured once a week in the absence of selective antibiotics. These cultures were plated once a week onto agar plates without antibiotics and the resulting colonies were then tested for plasmid retention by patching them onto agar plates with and without Sp and Sm antibiotics. The percent plasmid retention for each strain was calculated by dividing the number of patched

colonies that grew in the presence of antibiotics by the total number of colonies that grew in the absence of antibiotics. The second method relied on GFP reporter fluorescence measurements of liquid cultures in 24-well plates. Liquid cultures were subcultured once a week in the presence or absence of selective antibiotics (Sp and Sm). The GFP fluorescence data of each strain were normalized to the corresponding OD_{750} culture density. Percent plasmid retention was calculated after each round of weekly subculturing by dividing the normalized GFP fluorescence values of each strain grown in the presence of antibiotics by that in the absence of antibiotics.

As shown in Fig. 4(a), after 5 weekly passages of strains over 35 days of growth (at least 20 generations), the plasmid retention percentage of *S. elongatus* carrying pAM4787 gradually dropped to about 39%. For *S. elongatus* carrying pAM4788, however, the plasmid retention remained at about 100%. Similarly, the GFP reporter fluorescence of *S. elongatus* carrying pAM4787 dropped from 85% at day 7 to 27% at day 35, while *S. elongatus* carrying pAM4788 only showed a slight decrease from 96% to 90% (Fig. 4(b)). The results indicate that the *Synechocystis* PCC 6803 pSYSA toxin-antitoxin cassette functions to maintain pANS-based shuttle vectors in *S. elongatus*.

pANS-based shuttle vectors can carry large DNA fragments in *S. elongatus*

The native pANS is a relatively small plasmid of only 7.8 kb. We wanted to determine if pANS-based shuttle vectors can carry large DNA inserts. The essential backbone of shuttle vector pAM4787 is approximately 6.1 kb, which consists of the 3.81-kb minimal replication element, the 1.04-kb fragment containing the *E. coli* replication origin and origin of transfer, and the 1.28-kb *aadA* selectable marker gene. pAM4787 is slightly larger than the essential backbone because it carries a 0.94-kb GFP reporter cassette. The ability of pANS-based shuttle vectors to carry large foreign DNA inserts was evaluated by cloning the 20-kb gene cluster for EPA (eicosapentaenoic acid) biosynthesis into them (Shulse & Allen, 2011). Restriction digestion analysis of the resulting plasmids, pAM5173 (pAM4787-EPA, 26,643 bp) and pAM5174

(pAM4788-EPA, 27,385 bp), showed the expected digestion patterns (Table 1 & Fig. 5(a)). The presence of the intact EPA biosynthetic gene cluster in both constructs was confirmed by sequencing the whole gene cluster insert. The pAM5173 and pAM5174 EPA gene cluster clones were then successfully introduced into *S. elongatus*(-pANS) as shown by PCR analysis of exconjugant clones containing each of the plasmids (Fig. 5(b)). *S. elongatus* strains containing pAM5173 and pAM5174 both showed substantial production of EPA indicating that the 20-kb biosynthetic gene cluster was fully intact and functional. We did not detect any instability of the EPA-producing strains in these experiments. Production of EPA by *S. elongatus* and another cyanobacterial strain will be reported elsewhere.

The copy number of endogenous plasmids in *S. elongatus*

Previous studies showed that there are 3-5 (Griese *et al.*, 2011), 2-10 (Watanabe *et al.*, 2015), and 1-10 (Chen *et al.*, 2012) copies of the chromosome in *S. elongatus* cells, but the copy number of its native plasmids is not known. In this study, the chromosome copy number of our laboratory *S. elongatus* strain grown under our standard conditions was determined from the total DNA quantity per cell. We determined that there are about 8 copies of the chromosome per cell in wild-type *S. elongatus*, *S. elongatus*(-pANS), and the closely related *S. elongatus* PCC 7942 and *Synechococcus* PCC 6301. The copy number of endogenous plasmids pANS and pANL in wild-type *S. elongatus*, the cured *S. elongatus*(-pANS) strain, and *Synechococcus* PCC 6301 was determined by qPCR analysis (Fig. 6(a)). The large pANL plasmid was calculated to be present in 2.6 to 3.0 copies per chromosome in all three strains. The small pANS plasmid was absent from the cured strain and calculated to be present at 9.9 and 2.8 copies per chromosome in *S. elongatus* and PCC 6301, respectively.

Expression levels of a reporter gene in a pANS-based vector are higher than from the reporter gene in pANL or in the chromosome in *S. elongatus*

The relative gene expression level of a GFP reporter (yemGFP) was compared between *S. elongatus* strains carrying the same reporter cassette in a pANS-based vector, in the large native plasmid pANL, or recombined into the chromosome at the neutral site 1 (NS1) locus. The

normalized GFP fluorescence level of the reporter in pANL was similar to that of the reporter in the chromosome, while the GFP fluorescence from the reporter in a pANS-based vector was almost 3 times higher (Fig. 6(b)).

pANS-based shuttle vectors replicate in *Anabaena* PCC 7120

To test the host range of pANS-based shuttle vectors, conjugation experiments using pAM4787 were performed with 5 cyanobacterial strains: *S. elongatus*, *Anabaena* PCC 7120, *Leptolyngbya* BL0902, *Synechocystis* PCC 6803, and *Synechocystis* WHSYN. In addition to *S. elongatus*, exconjugants carrying pAM4787 were obtained for *Anabaena* PCC 7120, but not for the 3 other strains. These data extend the utility of a shuttle plasmid based on the endogenous pANS plasmid from the unicellular *S. elongatus* to the filamentous *Anabaena* PCC7120 strain.

Self-replicating plasmids derived from the *Nostoc* plasmid pDU1 (Schmetterer & Wolk, 1988) and the *E. coli* broad-host-range plasmid RSF1010 (Thiel, 1994) have been widely used in *Anabaena* PCC 7120 for genetic experiments. pDU1- and RSF1010-based plasmids are known to be compatible with each other in *Anabaena* PCC 7120 (Wolk *et al.*, 2007). Because plasmid compatibility can be beneficial for genetic engineering, we determined if pANS-based shuttle plasmids would be compatible with pDU1- and RSF1010-based plasmids in *Anabaena* PCC 7120. Colony PCR analysis data showed that pAM4788 (pANS origin) was successfully introduced into *Anabaena* PCC 7120 cells carrying both pAM4688 (pDU1 origin) and pAM4896 (RSF1010 origin) shuttle plasmids (Fig. 7(a), Table 1). Restriction digestion analysis of recovered plasmids (Fig. 7(b)) demonstrate that pANS-based shuttle vectors can be stably maintained in *Anabaena* PCC 7120 in the presence of pDU1- and RSF1010-based plasmids.

Anabaena PCC 7120 has been shown to have about 8 copies of the chromosome per cell (Hu *et al.*, 2007). qPCR analysis of *Anabaena* PCC 7120 strains carrying all three plasmids showed that there were about 27 copies of pANS-based, 9 copies of pDU1-based, and 3 copies of RSF1010-based plasmids per chromosome (Fig. 7(c)).

pANS shuttle vectors carrying a standard multiple cloning site (MCS)

To facilitate pANS-based expression of heterologous genes, the PconII-yemGFP module of the pAM4787 and pAM4788 shuttle plasmids was replaced by a standard pBluescript SK(-) MCS. The resulting vectors, pAM5187 and pAM5188 (Table 1) were introduced into *S. elongatus*(-pANS) and *Anabaena* PCC 7120. Maintenance and stability of the plasmids in these hosts was analyzed by restriction digestion of plasmid DNA recovered from cyanobacterial cells. The digestion patterns indicated that these new vectors replicate well and are stable in the both *S. elongatus*(-pANS) and *Anabaena* PCC 7120 (Fig. S1).

Resequencing and annotation of pANS

The complete sequence of pANS (GenBank # S89470) was previously determined to be 7,835 bp in length and to include 8 open reading frames (ORFs; *orfA* to *orfH*) (Van der Plas *et al.*, 1992). During verification of the sequence of pANS-based shuttle vectors, several nucleotide polymorphisms were found compared to the published sequence, and some of these altered the identification of ORFs. Therefore, we resequenced and annotated pANS from our laboratory strain of *S. elongatus* PCC 7942.

To simplify the production of pANS DNA for sequencing, we cloned an *E. coli* replication origin into pANS. First, a shuttle vector (pAM5189) was constructed to introduce a pBR322 *oriV-oriT* fragment and the *aadA* gene into the unique *Bam*HI site in pANS via double homologous recombination (Table 1). Second, the recombinant pANS plasmid, named pAM5190, which contained the complete pANS sequence disrupted at the *Bam*HI site, was recovered from *S. elongatus* and transferred into *E. coli* (Table 1).

The pANS plasmid was sequenced with primers that were originally used for qPCR analysis and additional primers that were required to obtain the complete sequence (Table S1). There are 11 sequence differences (most of them single nucleotide changes) compared to the previous GenBank entry (accession # S89470). The new sequence was submitted to GenBank as accession # KT751091. Our new pANS sequence is identical to the sequence of the small

plasmid (GenBank accession # CP006473) of *Synechococcus elongatus* UTEX 2973, a strain that is closely related to *S. elongatus* PCC 7942 and *S. elongatus* PCC 6301 (Yu *et al.*, 2015). Based on the updated sequence, pANS is 7842 bp in length, with 59.5% GC content. It was re-annotated to carry 7 ORFs because the previously annotated *orfE* (*repB*) and *orfF* (*repA*) are now joined in a single ORF, pANS_07 (*repA*) (Fig. 1, left panel and Table 2). Additional information for the new annotation is listed in Table 2. The protein encoded by the longest ORF, pANS_07 (3060 bp), carries conserved domains related to DNA primase, and was previously suggested to be involved in autonomous plasmid replication (Van der Plas *et al.*, 1992). The small ORF pANS_06 (309 bp) sits immediately downstream of pANS_07 and overlaps it by 4 bp (Table 2). No conserved domains or significant BLAST hits were found for pANS_06. The 3.76 kb minimal replication region cloned in pAM4787 and pAM4788 contains the complete pANS_07 (*repA*) ORF, partial sequences for pANS_01 and pANS_06, and the predicted replication origin sequence in the intergenic region between pANS_01 and pANS_07 (Van der Plas *et al.*, 1992) (Fig. 1 and Table 2).

DISCUSSION

Ever since the discovery of endogenous plasmids in cyanobacteria (Van den Hondel *et al.*, 1979) there have been efforts to construct cyanobacterial plasmid-based self-replicating shuttle vectors for genetic manipulation in native or heterologous strains (Koksharova & Wolk, 2002; Thiel, 1994). Some of the earliest cyanobacterial genetic work was done with pANS (formerly pUH24) in *S. elongatus* (formerly *Anacystis nidulans*) strains (Golden & Sherman, 1983; Kuhlemeier *et al.*, 1981; Van Den Hondel *et al.*, 1980), and a host-vector system was developed to eliminate the interference of native pANS with the stability of introduced shuttle vectors (Kuhlemeier *et al.*, 1983). However, the pANS-cured *S. elongatus* strain, which was obtained through chemical (SDS) treatment (Kuhlemeier *et al.*, 1983), is currently not available. To facilitate the use of cyanobacteria as synthetic biology platforms we have made new stable pANS-based shuttle vectors based on the minimal replication element of pANS (Van der Plas *et al.*, 1992), and produced a transformable *S. elongatus* strain cured of the native pANS plasmid as a cloning host strain. The pANS-cured *S. elongatus* strain together with the pANS shuttle vectors form a robust vector-host cloning system for *S. elongatus*.

Somewhat surprisingly, pANS shuttle vectors replicate in the phylogenetically distant filamentous *Anabaena* sp. strain PCC 7120, but not in two unicellular *Synechocystis* strains or a filamentous *Leptolyngbya* strain. Other cyanobacterial plasmids have been shown to replicate in heterologous hosts, including pDU1 of *Nostoc* sp. PCC 7524 (Schmetterer & Wolk, 1988), pSY11 of marine *Synechococcus* sp. NKBG 042902 (Matsunaga *et al.*, 1990), and pMA4 of a thermophilic *Synechococcus* strain (Miyake *et al.*, 1999), but in most of these cases, the heterologous hosts are closely related to the original plasmid hosts. Some synthetic biology experiments could benefit from being able to transfer a single plasmid construction into both *Synechococcus* and *Anabaena* strains. pANS-based shuttle vectors may also replicate in other *Synechococcus* species and other species related to *Anabaena* PCC 7120, such as *Anabaena variabilis* and *Nostoc punctiforme*. A derivative of pAM4787, in which the *E. coli* replication origin

was removed, was unable to transform *E. coli* DH5, indicating that pANS cannot replicate in *E. coli*.

Although the structural stability of pANS-derived shuttle vectors missing the plasmid maintenance genes *pmaA* and *pmaB* was reported to be severely reduced (Van der Plas *et al.*, 1992), we found that shuttle vectors constructed with the minimal replication element and lacking these genes can be stably maintained as long as appropriate antibiotic selection is applied. Recent work that identified essential genes in *S. elongatus* by RB-TnSeq showed that transposon insertions in pANS_07 (*repA*) resulted in plasmid loss, and therefore *repA* is essential for maintenance of pANS under standard growth conditions (Rubin *et al.*, 2015). In contrast, *pmaA* and *pmaB* were not essential for plasmid maintenance (Rubin *et al.*, 2015).

Spontaneous loss of cyanobacterial plasmids has been observed before, including the loss of pANS (Castets *et al.*, 1986; Lau & Doolittle, 1979; Lau *et al.*, 1980). On the contrary, the large native plasmid pANL has been very difficult to eliminate (Chen *et al.*, 2008; Encinas *et al.*, 2014), probably due to the presence of two toxin-antitoxin cassettes (Chen *et al.*, 2008). We were able to exploit the ability of a toxin-antitoxin cassette to confer antibiotic-free plasmid selection (Unterholzner *et al.*, 2013) by inclusion of the pSYSA-TA1 toxin-antitoxin cassette in pAM4788 (Fig. 4(a), (b)).

Cyanobacteria are known to contain multiple copies of identical chromosomes per cell (Griese *et al.*, 2011). Many cyanobacterial strains also contain one or more endogenous plasmids (Chen *et al.*, 2008). The copy number of native cyanobacterial plasmids has been studied in several strains and has been reported as: 0.4-7 copies per chromosome in *Synechocystis* PCC 6803 depending on the plasmid (Berla & Pakrasi, 2012); 1 to 8 copies per chromosome in *Synechococcus* PCC 7002 (which was reported to contain 6 copies of the chromosome per cell), depending on the plasmid and growth conditions, particularly the salt concentration (Xu *et al.*, 2011; Yano *et al.*, 1995); 50 copies per chromosome in a marine *Synechococcus* strain,

which increased at higher salt concentrations (Takeyama *et al.*, 1991); and 350 copies per cell in a thermophilic *Synechococcus* strain (Miyake *et al.*, 1999). The broad host range plasmid RSF1010 replicates in several unicellular cyanobacterial strains with a copy number of about 10 per chromosome (Marraccini *et al.*, 1993). Reports on the copy number of shuttle vectors based on the *Nostoc* PCC 7524 plasmid pDU1 vary from 1 to 17 copies per chromosome (Wolk *et al.*, 2007), however a recent study showed that the copy number of the pDU1-based plasmid pRL25T to range from 0.53 to 1812 per chromosome in *Anabaena* PCC 7120, depending upon the recombinant insert and growth conditions (Yang *et al.*, 2013).

In the work reported here, the pANL copy number was consistent at about 2 to 3 copies per chromosome in the two *S. elongatus* strains PCC 7942 and PCC 6301. The copy number per chromosome of pANS, however, was significantly lower in *Synechococcus* PCC 6301 compared to *S. elongatus* (Fig. 6(a)). The cause of this copy number difference is not known and the DNA sequence of pANS in *Synechococcus* PCC 6301 has not been determined. The copy number of a RSF1010 vector in *Anabaena* PCC 7120 was about 3 per genome, which is lower than the 10 per chromosome reported for several unicellular cyanobacterial strains (Marraccini *et al.*, 1993). The co-existing pANS-based vector, on the other hand, was about 27 copies per chromosome in *Anabaena* PCC 7120, perhaps due to altered replication and copy number control in the heterologous host. Overall, our results show that pANS-based vectors will be useful for genetic experiments and synthetic biology applications in *Synechococcus* and *Anabaena* strains.

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669**Table 1.** Cyanobacterial strains and plasmids used in this study

Cyanobacterial Strains	Relevant characteristics	Source
Anabaena sp. PCC 7120	Wild type	Lab collection
Leptolyngbya sp. BL0902	Wild type	Lab collection
Synechococcus elongatus PCC 7942	Wild type	Lab collection
Synechococcus elongatus PCC 6301	Wild type	Lab collection
Synechocystis sp. PCC 6803	Wild type	Lab collection
Synechocystis sp. WHSYN	Wild type	Lab collection
GLC001	*S. elongatus* carrying pAM4787 isolate #S2-T1 (no native pANS); for competing out the native pANS; Sp^r Sm^r	This work
GLC002	*S. elongatus* carrying pAM4787 isolate #S1-1-C1 (no native pANS); for competing out the native pANS; Sp^r Sm^r	This work
GLC003	*Anabaena* PCC 7120 carrying pAM4688, pAM4896, and pAM4788 isolate #5-1; for testing compatibility of plasmids in *Anabaena* PCC 7120; Nm^r Nt^r Sp^r Sm^r	This work
GLC004	*Anabaena* PCC 7120 carrying pAM4688, pAM4896, and pAM4788 isolate #5-2; for testing compatibility of plasmids in *Anabaena* PCC 7120; Nm^r Nt^r Sp^r Sm^r	This work
GLC005	*S. elongatus* carrying pAM5190; for following GFP fluorescence in *S. elongatus*; Sp^r Sm^r	This work
GLC006	*Anabaena* PCC 7120 carrying pAM5190; for following GFP fluorescence in *Anabaena* PCC 7120; Sp^r Sm^r	This work
AMC2302	*S. elongatus*(-pANS) cured of the native pANS plasmid	This work

Plasmid name	Relevant characteristics*	Source
pAM1303	pBR322-based NS1 integrative shuttle vector; for inserting heterologous DNA sequences into the neutral site 1 (NS1) of *S. elongatus* chromosome; Sp^r Sm^r	Lab collection
pAM1573	pBR322-based NS2 integrative shuttle vector; for inserting heterologous DNA sequences into the neutral site 2 (NS2) of *S. elongatus* chromosome; Cm^r	Lab collection
pAM4688	pDU1-ori-(PconII-GFPmut2); for testing plasmid compatibility in *Anabaena* PCC 7120; Nm^r	Lab collection
pAM4787	pANS-ori-(pBR322-oriV-oriT)-aadA-(PconII-yemGFP); for testing the function of the minimal replication element of pANS; Sp^r Sm^r	This work
pAM4788	pAM4787-(pSYSA-TA1); for testing the function of the pSYSA-TA1 toxin-antitoxin cassette; Sp^r Sm^r	This work

pAM4789	pANL-(pBR322-oriV-oriT)-aadA-(PconII-yemGFP); for testing the function of the minimal replication element of pANL; Sp^r Sm^r	This work
pAM4790	pBR322-based NS1 integrative shuttle vector harboring *pfaA-E* gene cluster from *Shewanella pealeana*	Chen *et al*., in prep.
pAM4896	RSF1010-(PconII-GFPmut2); for testing plasmid compatibility in *Anabaena* PCC 7120; Nt^r	Lab collection (Taton *et al.*, 2014)
pAM5173	pAM4787-EPA; addition of EPA genes for testing cloning capacity of pANS shuttle vectors; Sp^r Sm^r	This work
pAM5174	pAM4788-EPA; addition of EPA genes for testing cloning capacity of pANS shuttle vectors; Sp^r Sm^r	This work
pAM5187	pAM4787-(pBS MCS); addition of a standard MCS in pAM4787; Sp^r Sm^r	This work
pAM5188	pAM4788-(pBS MCS); addition of a standard MCS in pAM4788; Sp^r Sm^r	This work
pAM5189	pUC19L-(pANS-BamHI-up)-(pBR322-oriV-oriT)-aadA-(PconII-GFPmut2)-(pANS-BamHI-down); for integration of an *E. coli* origin into pANS; Sp^r Sm^r	This work
pAM5190	pANS-(pBR322-oriV-oriT)-aadA-(PconII-GFPmut2); which contains the complete sequence of pANS; Sp^r Sm^r	This work
pCVD002, pCVD026, pCVD031, pCVD048, pCVD066	CYANO-VECTOR plasmids carrying modular devices (including a pBR322-oriV-oriT, pANS and pANL minimal replicons, a Sp Sm resistance gene and a GFP reporter gene) for the construction of shuttle plasmids	(Taton *et al.*, 2014)
pCV0003, pCV0035	CYANO-VECTOR shuttle plasmids	(Taton *et al.*, 2014)

670* Abbreviations for antibiotics: chloramphenicol (Cm), gentamicin (Gm), kanamycin (Km), neomycin (Nm),
671nourseothricin (Nt), spectinomycin (Sp), and streptomycin (Sm).

672**Table 2.** Annotation of pANS ORFs

ORF ID	pUH24 (S89470)*	UTEX (CP006473)†	GC content	Coordinates (Orientation)‡	ORF size (bp/aa)	Predicted function
pANS_01	*orfG*, *orfH*	M744_14255	61.81%	22 - 1626 (c)	1605/534	hypothetical
pANS_02	*orfA*	M744_14260	61.05%	1664 - 2197 (c)	534/177	hypothetical
pANS_03	*orfB* (*pmaA*)	-	56.31%	2545 - 2988 (d)	444/147	plasmid maintenance
pANS_04	*orfC* (*pmaB*)	M744_14235	57.69%	2998 - 3543 (d)	546/181	plasmid maintenance
pANS_05	*orfD*	M744_14240	63.56%	3704 - 4219 (c)	516/171	hypothetical
pANS_06	-	M744_14245	62.14%	4324 - 4632 (c)	309/102	hypothetical
pANS_07	*orfE* (*repB*) *orfF* (*repA*)	M744_14250	60.16%	4629 - 7688 (c)	3060/1019	plasmid replication

673* Corresponding ORFs in the published version of pANS in *S. elongatus*. † Corresponding ORFs in
674published small plasmid of *Synechococcus elongatus* UTEX 2973. ‡ c, complementary; d, direct.
675hypothetical, hypothetical protein.

Figure Legends

Fig. 1. Illustration of native pANS from *S. elongatus* and pANS-based shuttle vector plasmids. Open arrows, open reading frames; grey box between pANS_01 and pANS_07, predicted replication origin sequence (Van der Plas *et al.*, 1992). pANS contains a unique *Bam*HI site. For the pAM4787 vector map, lines with arrowheads indicate DNA fragments used for assembly: pBR322 *oriV/oriT*, *aadA*, *yemGFP*, and the pANS minimal replication element (mre); the *Sac*I site used for insertion of the TA1 addiction cassette (grey arrow boxes below) to make pAM4788 is indicated.

Fig. 2. Colony PCR analysis of *S. elongatus* transformants carrying pAM4787. Primer pairs: Panel A, yc059/yc057 (pANS specific; expected 448 bp); Panel B, yc054/yc057 (pAM4787 specific; expected 378 bp). M, 100-bp DNA ladder (NEB). Sample templates: S, wild-type *S. elongatus* total DNA; P, pAM4787 plasmid DNA isolated from *E. coli*; 1-6, *S. elongatus* total DNA from transformant colonies. All transformants contain pAM4787. Colonies in lanes 1 and 5 also still contain the native pANS plasmid. Horizontal arrows: expected PCR products. Extraneous higher MW bands in the lane 2 pAM4787 control are non-specific products.

Fig. 3. PCR analysis of pANS-cured strains of *S. elongatus* that have lost pAM4787 after growth for several generations without antibiotic selection. Primer pairs: A, yc056/yc055 (pAM4787 specific; expected 481 bp); B, yc054/yc057 (pAM4787 specific; expected 379 bp); C, yc056/yc058 (pANS specific; expected 603 bp – indicated with arrow); D, yc060/yc052 (pANL specific, expected 801 bp). Samples: S, *S. elongatus* total DNA; P, pAM4787 plasmid DNA; 1-4, *S. elongatus* clones cured of pANS and pAM4787; M, 100-bp DNA ladder (NEB).

Fig. 4. Stability of pANS-based shuttle vectors. **(a)** Maintenance of plasmids pAM4787 and pAM4788 in *S. elongatus* over time based on the ratio of antibiotic resistant clones compared to the total number of clones obtained from 3 replicate liquid cultures for each plasmid; **(b)** Plasmid

maintenance over time based on GFP fluorescence levels measured from 3 replicate liquid cultures for each plasmid.

Fig. 5. pANS can carry large inserts. **(a)** Restriction digestion analysis of pANS-based shuttle plasmids containing the 20-kb EPA biosynthetic gene cluster. Expected digestion pattern: pAM5173 digested with *Bst*BI: 7927, 6400, 5839, 4183, 2294 bp; pAM5174 digested with *Bst*BI: 8669, 6400, 5839, 4183, 2294 bp. Samples: 1-2, independent pAM5173 isolates; 3-4, independent pAM5174 isolates; M, 1-kb DNA ladder. **(b)** PCR analysis of exconjugants carrying large pANS-EPA constructs pAM5173 (26,643 bp) or pAM5174 (27,385 bp). Primer pairs: yc124/yc057 (*pfaD* specific; expected 645 bp). Samples: M, 100-bp DNA ladder (NEB); P, pAM5173 plasmid DNA; 1-4, isolates of *S. elongatus*(-pANS) carrying pAM5173; 5-8, isolates of *S. elongatus*(-pANS) carrying pAM5174.

Fig. 6. Plasmid copy number and reporter-gene expression levels from pANS-based shuttle vectors. **(a)** Copy number of pANS and pANL per chromosome in *S. elongatus* PCC 7942 (S7942(WT)), pANS-cured *S. elongatus* PCC 7942 (S7942(-pANS)), and *Synechococcus* PCC 6301 (S6301(WT)). Standard deviation was calculated from biological triplicates. See Methods for experimental details of qPCR analysis and Table S1 for specific primer pairs; **(b)** GFP fluorescence measurement of *S. elongatus* PCC 7942 strains harboring a GFP reporter gene on the chromosome at NS1 (pCV0035), on pANL (pAM4789), or on pANS (pAM4787). Standard deviation was calculated from biological triplicates. Relative fluorescence units (RFU) were normalized to OD_{750}.

Fig. 7. pANS replicates in *Anabaena* PCC 7120 and is compatible with pDU1- and RSF1010-based shuttle vectors. **(a)** Colony PCR analysis of 3 *Anabaena* PCC 7120 exconjugants, each carrying the 3 shuttle vectors pAM4688 (pDU1), pAM4896 (RSF1010), and pAM4788 (pANS). Primer pairs: yc159/yc160 (pDU1 specific, expected 552 bp); yc161/yc162 (RSF1010 specific, expected 414 bp); and yc054/yc057 (pANS specific, expected 379 bp). Lanes 1-3 are PCR

products from genomic DNA of independent *Anabaena* PCC 7120 exconjugant isolates; lanes P are PCR products from control plasmid DNA for each plasmid; and M, marker 1-kb DNA ladder (NEB). **(b)** Restriction digestion with *Xmn*I of shuttle vectors recovered from *Anabaena* PCC 7120. Plasmids: pAM4788 (pANS-based, total 7774 bp; expected fragment sizes 390 and 7384 bp); pAM4896 (RSF1010-based, total 7729 bp; expected fragment sizes 2014 and 5715 bp); pAM4688 (pDU1-based, total 7192 bp; expected fragment sizes 1254, 1584, 1931, and 2423 bp). Samples: P, marker plasmid controls isolated from *E. coli*; 1-4, individual recovered plasmids from 4 *Anabaena* PCC 7120 isolates that carry all three plasmids; M, marker 1-kb DNA ladder (NEB). The different plasmids were separately recovered in *E. coli* clones based on their differences in antibiotic resistance. **(c)** Copy number of pAM4788 (pANS), pAM4688 (pDU1), and pAM4896 (RSF1010) shuttle vectors in *Anabaena* PCC 7120. Standard deviation was calculated from biological triplicates. See Methods for experimental details of qPCR analysis and Table S1 for specific primer pairs.