

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

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22Depositories: The GenBank accession number for the updated complete sequence of pANS is

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24

25Abbreviations: EPA, eicosapentaenoic acid; MCS, multiple cloning site

26SUMMARY

27To facilitate development of synthetic biology tools for genetic engineering of cyanobacterial
28strains, we constructed pANS-derived self-replicating shuttle vectors that are based on the
29minimal replication element of the *Synechococcus elongatus* strain PCC 7942 plasmid pANS.
30To remove the possibility of homologous recombination events between the shuttle plasmids
31and the native pANS plasmid, the endogenous pANS was cured through plasmid
32incompatibility-mediated spontaneous loss. A heterologous toxin-antitoxin cassette was
33incorporated into the shuttle vectors for stable plasmid maintenance in the absence of antibiotic
34selection. The pANS-based shuttle vectors were shown to be able to carry a large 20-kb DNA
35fragment containing a gene cluster for biosynthesis of the omega-3 fatty acid eicosapentaenoic
36acid (EPA). Based on qPCR analysis, there are about 10 copies of pANS and 3 copies of the
37large native plasmid pANL per chromosome in *S. elongatus*. Fluorescence levels of GFP
38reporter genes in a pANS-based vector were about 2.5-fold higher than when in pANL or
39integrated into the chromosome. In addition to its native host, pANS-based shuttle vectors were
40also found to replicate stably in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120.
41There were about 27 copies of a pANS-based shuttle vector, 9 copies of a pDU1-based shuttle
42vector, and 3 copies of an RSF1010-based shuttle vector per genome when these three
43plasmids co-existed in *Anabaena* cells. The endogenous pANS from our *S. elongatus* laboratory
44strain was cloned in *E. coli*, re-sequenced, and re-annotated to update previously published
45sequencing data.

47INTRODUCTION

48In recent years cyanobacteria, the only group of prokaryotes that perform oxygenic
49photosynthesis, have attracted interest as promising microbial factories for production of
50renewable chemicals and fuels directly from sunlight and CO₂ (Hays & Ducat, 2015; Sarsekeyeva *et al.*, 2015).
51Because many cyanobacterial strains are amenable to genetic modification, extensive genetic
52tools have been developed in model and newly isolated strains to facilitate metabolic
53engineering and strain development (Ruffing, 2011; Taton *et al.*, 2014). Depending on the strain,
54DNA constructs can be introduced into cyanobacteria through natural transformation,
55electroporation, or conjugation from *Escherichia coli*. Homology-based integration vectors are
56commonly used in genetic manipulation of cyanobacteria, but they are highly strain specific. For
57example, genetic engineering of *Synechococcus elongatus* strain PCC 7942 is typically
58performed by integration of recombinant DNA at one of three neutral sites in the chromosome
59by homologous recombination (Clerico *et al.*, 2007; Niederholtmeyer *et al.*, 2010). In
60*Synechococcus* sp. strain PCC 7002, strain engineering is accomplished by homologous
61recombination of linear recombinant DNA fragments that carry selectable markers into neutral
62sites on endogenous plasmids and on the chromosome (Xu *et al.*, 2011).
63Broad-host-range autonomously replicating shuttle vectors based on RSF1010, first identified as
64a self-mobilizable plasmid from *E. coli* (Scholz *et al.*, 1989), have been shown to function in a
65variety of cyanobacterial genera, such as unicellular *Synechococcus* (Brahamsha, 1996) and
66*Synechocystis* (Marraccini *et al.*, 1993) strains, and filamentous strains including
67*Pseudanabaena* (Sode *et al.*, 1992), *Anabaena* (Thiel, 1994; Wolk *et al.*, 2007), and
68*Leptolyngbya* (Taton *et al.*, 2014). In addition to RSF1010, endogenous cyanobacterial plasmids
69have been used to construct chimeric shuttle vectors, but the cyanobacterial origins of
70replication tend to have a narrow host range and replicate in only a limited number of species.
71Derivatives of *Nostoc* sp. PCC 7524 plasmid pDU1 have been widely used in the model
72filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena* PCC 7120) as

73well as other related filamentous strains (Schmetterer & Wolk, 1988). Endogenous plasmid-
74based shuttle vectors were also developed for marine *Synechococcus* species (Matsunaga *et*
75*al.*, 1990).

76The unicellular model cyanobacterium *S. elongatus* (strain PCC 7942) carries two endogenous
77plasmids, the 7.8 kb pANS (Van der Plas *et al.*, 1992) and the 46.2 kb pANL (Chen *et al.*, 2008).
78Because pANS is not essential (Lau & Doolittle, 1979) and has a relatively small size, it was
79used for constructing self-replicating shuttle vectors (Golden & Sherman, 1983; Kuhlemeier &
80van Arkel, 1987). However, structural integrity issues of pANS-derived shuttle vectors that result
81from recombination events between these vectors and the native pANS population have
82significantly limited their use in genetic modification of *S. elongatus* (Gendel, 1987; Kuhlemeier
83*et al.*, 1981). For unknown reasons, RSF1010-based shuttle vectors do not replicate well in *S.*
84*elongatus*. Hence almost all genetic and molecular studies in *S. elongatus* rely on homologous
85recombination-mediated integration of neutral-site vectors into the chromosome (Clerico *et al.*,
862007). The potential of self-replicating shuttle vectors for genetic engineering of *S. elongatus*
87has yet to be fully explored.

88Self-replicating plasmids offer certain advantages over integration plasmids for genetic
89engineering. They can have higher copy number and therefore higher gene expression levels.
90Most, if not all, cyanobacterial species have multiple copies of identical chromosomes (Griese
91*et al.*, 2011); thus chromosome segregation after integration-based gene transfer can be a
92lengthy and troublesome process, especially in filamentous strains. Engineered strains
93containing self-replicating plasmids can be selected without segregation concerns. Furthermore,
94self-replicating shuttle vectors that carry replication origin(s) for both *E. coli* and cyanobacteria
95can be easily recovered from cyanobacteria and introduced back into *E. coli*, which is
96convenient for studying plasmid stability and integrity, and for the isolation of recombinant library
97clones. Development of stable self-replicating shuttle plasmid vectors would enlarge the genetic

98toolbox for genetic engineering and synthetic biology approaches in *S. elongatus* and other
99cyanobacteria.

100Desired properties of robust plasmid shuttle vectors are that they are stably maintained in the
101absence of selection, can carry large DNA inserts, can be transferred through conjugation, have
102relatively high copy numbers, can replicate in different strains, and are compatible with other
103plasmids for experiments that require multiple genetic manipulations. Here, we report
104construction of such shuttle vectors that are based on the minimal 3.76 kb replication region of
105pANS (Van der Plas *et al.*, 1992). The pANS-based shuttle vectors were shown to replicate not
106only in their native *S. elongatus* host, but also in the heterologous strain *Anabaena* PCC 7120,
107and to have the capacity to carry a 20-kb eicosapentaenoic acid (EPA) biosynthesis gene
108cluster.-

109

110METHODS

111Strains and growth conditions.

112*E. coli* and cyanobacterial strains used in this study are listed in Table 1. Growth conditions for

113*E. coli* and cyanobacterial strains *S. elongatus* strain PCC 7942, *Anabaena* sp. strain PCC

1147120, *Leptolyngbya* sp. strain BL0902, *Synechocystis* sp. strain PCC 6803, and *Synechocystis*

115sp. strain WHSYN were as previously published (Taton *et al.*, 2014) unless stated otherwise.

116Cyanobacterial liquid and plate cultures were grown at 30 °C under continuous illumination of 60

117 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Antibiotics were used for the selection of transformed cyanobacterial

118strains at the following concentrations: chloramphenicol (Cm; 7.5 $\mu\text{g ml}^{-1}$), gentamicin (Gm; 2 μg

119 ml^{-1}), kanamycin (Km; 5 $\mu\text{g ml}^{-1}$), neomycin (Nm; 50 $\mu\text{g ml}^{-1}$), nourseothricin (Nt; 50 $\mu\text{g ml}^{-1}$),

120spectinomycin (Sp; 2 $\mu\text{g ml}^{-1}$), and streptomycin (Sm; 2 $\mu\text{g ml}^{-1}$).

121Transformation of cyanobacteria.

122Natural transformation of *S. elongatus* and bi-parental conjugation of plasmids from *E. coli* to

123*Anabaena* PCC 7120, *Leptolyngbya* BL0902, *Synechocystis* PCC 6803, and *Synechocystis*

124WHSYN followed published protocols (Clerico *et al.*, 2007; Elhai & Wolk, 1988; Elhai *et al.*,

1251997; Golden & Sherman, 1984; Taton *et al.*, 2012). The genotypes of transformants or

126exconjugants were confirmed by colony PCR, 25 cycles, using Taq DNA polymerase (NEB).

127Plasmids and primers.

128Plasmids used in this study are described in Table 1 and DNA primers are listed in Table S1.

129Plasmids were assembled using a GeneArt Seamless Cloning and Assembly Kit (Life

130Technologies) following the manufacturer's instructions with slight modifications described

131earlier (Taton *et al.*, 2014). Unless otherwise stated, plasmids were constructed in *E. coli* strain

132DH5 α .

133Plasmid pAM4787 (7032 bp) was designed with the CYANO-VECTOR assembly portal

134(<http://golden.ucsd.edu/CyanoVECTOR/>) (Taton *et al.*, 2014) using the following modules: the

135*aadA* resistance marker for Sp and Sm (1276 bp *EcoRV*-digested fragment from pCVD002), the

136PconII-yemGFP module (945 bp *EcoRV*-digested fragment from pCVD031), the pBR322 oriV-

137oriT fragment (1080 bp *EcoRV*-digested fragment from pCVD026), and the pANS minimal
138replication origin region (3813 bp *ZraI*-digested fragment from pCVD048). Plasmid pAM4789
139(4891 bp) was made with the same modules as pAM4787 but the pANS replication region was
140replaced with the pANL replication origin region (1674 bp *ZraI*-digested fragment from
141pCVD066). Plasmid pAM4788 (7774 bp) was made with a 789 bp pSYSA-TA1 cassette, which
142was amplified with the primer pair yc087/yc088 from *Synechocystis* sp. PCC 6803 gDNA, that
143was cloned into the *SacI* site of pAM4787. To make plasmids pAM5173 (26,643 bp) and
144pAM5174 (27,385 bp), the backbones of pAM4787 (4910 bp) and pAM4788 (5652 bp),
145respectively, were amplified with the primers yc163 and yc164. These PCR fragments were then
146each separately assembled with the aadA-EPA insert (21,779 bp), which was produced by
147digestion of pAM4790 (24,640 bp) with *AscI* and *SmaI*. To make pAM5187 (6194 bp) and
148pAM5188 (6936 bp) plasmids, pAM4787 and pAM4788, respectively, were digested with *AgeI*
149and *XbaI* to remove the PconII-yemGFP fragment, and the largest digestion fragment from each
150plasmid (6081 bp for pAM4787; 6823 bp for pAM4788) was then assembled with a pBluescript
151SK(-) multiple-cloning-site PCR fragment (148 bp) that was amplified with primers yc336 and
152yc337 from pBluescript SK(-) (Stratagene). To make pAM5189 (7962 bp), pAM4787 was
153digested with *SacI* and *AgeI* to release the oriV-oriT-aadA fragment (2310 bp), which was then
154assembled with pUL19L (2659 bp; Thermo Fisher Scientific) and the following 3 PCR fragments:
155pANS-*Bam*HI-up arm (primers yc292 and yc293; 1069 bp of pANS), pANS-*Bam*HI-dn arm
156(primers yc294 and yc295; 1012 bp of pANS), and PconII-LTRBS-GFPmut2 fragment (primers
157yc296 and yc297; 990 bp of pCV0003). Plasmid pAM5190 resulted from in vivo double-
158recombination events in *S. elongatus* PCC 7942 between pAM5189 and the native pANS
159plasmid, such that the oriV-oriT-aadA and PconII-LTRBS-GFPmut2 fragments were inserted into
160the *Bam*HI site of pANS.

161Restriction digestion analysis of shuttle vectors recovered in *E. coli*.

162Shuttle vectors were extracted from cyanobacterial strains with QIAprep Spin Miniprep Kit (Qiagen) using
163the standard protocol. A 1-2 μ l sample of the extracted DNA was introduced into *E. coli* DH10B electro-

164competent cells (50 μ l) using Gene Pulser II (Bio-Rad). Electroporated cells were plated for single
165colonies on LB agar plates containing appropriate antibiotics. Different shuttle vectors carried by the
166same cyanobacterial strain were separately recovered in *E. coli* clones based on their different antibiotic
167resistance markers. The recovered plasmids were then digested with appropriate restriction enzymes
168and analyzed by agarose gel electrophoresis.

169**Genomic DNA extraction.**

170The total DNA of cyanobacterial strains was extracted with the CTAB method (Golden *et al.*,
1711987). Typically, a 10-ml growing culture of cyanobacteria with an optical density (OD₇₅₀) of 0.5
172was collected for each sample.

173**Chromosome copy number determination.**

174A Petroff-Hausser Counting Chamber was used to count *S. elongatus* cells. The cell-count data
175(cell ml⁻¹) were calculated from the averaged cell numbers of 10 blocks in the center grid of the
176chamber. The chromosome copy number per cell was then determined based on the molecular
177weight of the chromosome and the total amount of DNA extracted from a determined number of
178cells.

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

180Total DNA from cyanobacterial strains was used as the template for qPCR analysis. Primers
181were designed using the PrimerQuest online tool (IDT). For each experiment, standard real-time
182PCR reactions, in triplicate, were set up with the Power SYBR Green PCR Master Mix (Thermo
183Fisher Scientific) and run on a StepOne Real-Time PCR System (Thermo Fisher Scientific)
184following the manufacturers' instructions. The qPCR results were calculated from triplicate
185experiments. The copy numbers of endogenous plasmids per chromosome were determined
186based on qPCR analysis using 3 pairs of plasmid-specific primers for each plasmid and 3
187reference primer pairs specific for the chromosome.

188**Reporter fluorescence measurement.**

189For each strain, the original culture was subcultured in 24-well plates once every 7 days at 30
190°C under continuous illumination of 60 μ mol photons m⁻² s⁻¹ in BG-11 medium with or without
191antibiotics (Sp and Sm). The emission intensities of GFP fluorescence from liquid culture
192samples were measured with triplicate readings in Greiner 96-well flat bottom black polystyrene

193plates with a Tecan Infinite M200 plate reader. The excitation and emission wavelengths for
194GFPmut2 and yemGFP were set to EX488/9 and EM518/20. GFP measurements were
195normalized by subtracting the blank measurement and then dividing the GFP fluorescence by
196the corresponding OD₇₅₀.

197**Plasmid maintenance assay.**

198The plasmid maintenance assay protocol was modified for *S. elongatus* from a published
199protocol for *Anabaena* PCC 7120 (Lee *et al.*, 2003). *S. elongatus* strains were subcultured in BG-
20011 liquid medium without antibiotics every 7 days for 5 weeks. At the end of each growth period
201a sample was diluted 10,000 fold with BG-11 medium and 100 μ l was then spread with glass
202beads onto a BG-11 agar plate without antibiotics and then incubated for 7 days. After this
203incubation 24 colonies from each plate were streaked in duplicate onto BG-11 plates without
204and with antibiotics. Plates were incubated for 5-7 days until sufficient growth had occurred for
205scoring. Patches that grew without antibiotics but not with Sp and Sm were scored as having
206lost the plasmid.

207

208RESULTS

209Construction of a pANS-based shuttle vector and transformation of *S. elongatus*

210Earlier work on functional deletion analysis of pANS identified a 3.65-kb *XhoI-BglII* restriction
211fragment as the minimal replication element of pANS that was sufficient for autonomous
212replication (Van der Plas *et al.*, 1992). This region contains the gene (originally annotated as
213genes *repA* and *repB*) necessary for plasmid replication and a 200 bp intergenic region that was
214predicted to contain the replication origin (Van der Plas *et al.*, 1992). Two other genes, *pmaA*
215and *pmaB*, that function in plasmid maintenance of pANS are not included in the defined
216minimal replication element, but the shuttle vectors based on the minimal region nonetheless
217can be maintained in strains by selection with appropriate antibiotics added to the growth
218medium (Van der Plas *et al.*, 1992). We designed improved versatile shuttle vectors based on
219this minimal replication element of pANS.

220A pANS-derived shuttle vector, pAM4787, was constructed using a GC-adaptor assembly (Taton
221*et al.*, 2014) with the pANS minimal replication element, a pBR322 fragment that contained the
222pMB1 replication origin (*oriV*) and the origin of transfer (*oriT*), an *aadA* gene cassette as the
223selectable marker conferring resistance to Sp and Sm, and a copy of the *yemGFP* gene driven
224by the *conII* promoter as a reporter for gene expression levels (Table 1 and Fig. 1).

225The plasmid pAM4787 was introduced into wild-type *S. elongatus* via both natural
226transformation and bacterial conjugation. Colony PCR analysis showed that all Sp+Sm resistant
227*S. elongatus* transformants carried the introduced pAM4787 shuttle vector (Fig. 2, bottom panel
228B), but 2 out of the 6 transformants still retained the endogenous pANS (Fig. 2, top panel A),
229which indicates that selection for pAM4787 does not rapidly exclude the endogenous pANS
230plasmid. In addition, we observed evidence of recombination between pAM4787 and the
231endogenous pANS plasmid, which would be detrimental for genetic engineering experiments.
232Therefore, we produced a new *S. elongatus* genetic-engineering platform strain lacking the
233pANS plasmid.

234 **Plasmid incompatibility-mediated spontaneous loss of pANS**

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

240 To obtain a pANS-cured host cloning strain for pANS-based shuttle vectors, we first transformed
241 the wild-type strain with shuttle vector pAM4787, then identified strains in which the native
242 pANS plasmid had been lost as a result of plasmid incompatibility-mediated spontaneous loss,
243 and then screened for strains that lost the pAM4787 shuttle vector after antibiotic selection was
244 removed. We selected two *S. elongatus* transformants, GLC001 and GLC002 (Table 1), that
245 contained pAM4787 and had completely lost pANS (Fig. 2, lanes 3 and 6). The strains were
246 continuously subcultured in the absence of antibiotic selection in liquid media every 7 days.
247 After subculturing 3 times (21 days of growth and at least 12 generations as estimated from
248 OD₇₅₀ measurements), the subcultured strains were then replica plated on agar plates. Only
249 colonies that grew up on plates without antibiotics, but not on plates with antibiotics, were
250 patched and inoculated into liquid medium for growth and analysis. PCR analyses using primer
251 pairs specific for pAM4787 (Fig. 3, panels A, B), pANS (Fig. 3, panel C), and pANL (Fig. 3, panel
252 D) were performed for four colony-derived cultures. Spontaneous loss of pAM4787 from the
253 pANS-cured strains was observed in all four tested clones (Fig. 3 & Table S1). All four clones
254 still contained the large pANL plasmid.

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

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

265 **A heterologous toxin-antitoxin cassette enhanced plasmid maintenance**

266 Plasmid maintenance without antibiotic selection is highly desirable in biotechnology
267 applications, especially in large bacterial cultures. The native plasmid maintenance genes of
268 pANS (*pmaA* and *pmaB*), however, may not have optimal efficacy because pANS can be cured
269 by chemical treatment (Kuhlemeier *et al.*, 1983) and even spontaneous loss (Lau & Doolittle,
270 1979; Lau *et al.*, 1980). Toxin-antitoxin systems, widely distributed among prokaryotic plasmids
271 and chromosomes, function in plasmid maintenance through a mechanism of post-
272 segregational killing (Pecota *et al.*, 1997). To explore the possibility of using heterologous
273 cyanobacterial toxin-antitoxin cassettes for antibiotic-free plasmid vector maintenance, the
274 pANS-derived shuttle vector pAM4788 (Table 1 & Fig. 1, right panel, TA1 segment) was
275 constructed to carry a toxin-antitoxin cassette from the endogenous megaplasmid pSYSA of
276 *Synechocystis* sp. PCC 6803 (Kopfmann & Hess, 2013).

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

285colonies that grew in the presence of antibiotics by the total number of colonies that grew in the
286absence of antibiotics. The second method relied on GFP reporter fluorescence measurements
287of liquid cultures in 24-well plates. Liquid cultures were subcultured once a week in the
288presence or absence of selective antibiotics (Sp and Sm). The GFP fluorescence data of each
289strain were normalized to the corresponding OD₇₅₀ culture density. Percent plasmid retention
290was calculated after each round of weekly subculturing by dividing the normalized GFP
291fluorescence values of each strain grown in the presence of antibiotics by that in the absence of
292antibiotics.

293As shown in Fig. 4(a), after 5 weekly passages of strains over 35 days of growth (at least 20
294generations), the plasmid retention percentage of *S. elongatus* carrying pAM4787 gradually
295dropped to about 39%. For *S. elongatus* carrying pAM4788, however, the plasmid retention
296remained at about 100%. Similarly, the GFP reporter fluorescence of *S. elongatus* carrying
297pAM4787 dropped from 85% at day 7 to 27% at day 35, while *S. elongatus* carrying pAM4788
298only showed a slight decrease from 96% to 90% (Fig. 4(b)). The results indicate that the
299*Synechocystis* PCC 6803 pSYSA toxin-antitoxin cassette functions to maintain pANS-based
300shuttle vectors in *S. elongatus*.

301pANS-based shuttle vectors can carry large DNA fragments in *S. elongatus*

302The native pANS is a relatively small plasmid of only 7.8 kb. We wanted to determine if pANS-
303based shuttle vectors can carry large DNA inserts. The essential backbone of shuttle vector
304pAM4787 is approximately 6.1 kb, which consists of the 3.81-kb minimal replication element, the
3051.04-kb fragment containing the *E. coli* replication origin and origin of transfer, and the 1.28-kb
306*aadA* selectable marker gene. pAM4787 is slightly larger than the essential backbone because
307it carries a 0.94-kb GFP reporter cassette. The ability of pANS-based shuttle vectors to carry
308large foreign DNA inserts was evaluated by cloning the 20-kb gene cluster for EPA
309(eicosapentaenoic acid) biosynthesis into them (Shulse & Allen, 2011). Restriction digestion
310analysis of the resulting plasmids, pAM5173 (pAM4787-EPA, 26,643 bp) and pAM5174

311(pAM4788-EPA, 27,385 bp), showed the expected digestion patterns (Table 1 & Fig. 5(a)). The
312presence of the intact EPA biosynthetic gene cluster in both constructs was confirmed by
313sequencing the whole gene cluster insert. The pAM5173 and pAM5174 EPA gene cluster clones
314were then successfully introduced into *S. elongatus*(-pANS) as shown by PCR analysis of
315exconjugant clones containing each of the plasmids (Fig. 5(b)). *S. elongatus* strains containing
316pAM5173 and pAM5174 both showed substantial production of EPA indicating that the 20-kb
317biosynthetic gene cluster was fully intact and functional. We did not detect any instability of the
318EPA-producing strains in these experiments. Production of EPA by *S. elongatus* and another
319cyanobacterial strain will be reported elsewhere.

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

321Previous studies showed that there are 3-5 (Griese *et al.*, 2011), 2-10 (Watanabe *et al.*, 2015),
322and 1-10 (Chen *et al.*, 2012) copies of the chromosome in *S. elongatus* cells, but the copy
323number of its native plasmids is not known. In this study, the chromosome copy number of our
324laboratory *S. elongatus* strain grown under our standard conditions was determined from the
325total DNA quantity per cell. We determined that there are about 8 copies of the chromosome per
326cell in wild-type *S. elongatus*, *S. elongatus*(-pANS), and the closely related *S. elongatus* PCC
3276301. The copy number of endogenous plasmids pANS and pANL in wild-type *S. elongatus*, the
328cured *S. elongatus*(-pANS) strain, and *Synechococcus* PCC 6301 was determined by qPCR
329analysis (Fig. 6(a)). The large pANL plasmid was calculated to be present in 2.6 to 3.0 copies
330per chromosome in all three strains. The small pANS plasmid was absent from the cured strain
331and calculated to be present at 9.9 and 2.8 copies per chromosome in *S. elongatus* and PCC
3326301, respectively.

333**Expression levels of a reporter gene in a pANS-based vector are higher than from the reporter gene in 334pANL or in the chromosome in *S. elongatus***

335The relative gene expression level of a GFP reporter (*yemGFP*) was compared between *S.*
336*elongatus* strains carrying the same reporter cassette in a pANS-based vector, in the large
337native plasmid pANL, or recombined into the chromosome at the neutral site 1 (NS1) locus. The

338normalized GFP fluorescence level of the reporter in pANL was similar to that of the reporter in
339the chromosome, while the GFP fluorescence from the reporter in a pANS-based vector was
340almost 3 times higher (Fig. 6(b)).

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

342To test the host range of pANS-based shuttle vectors, conjugation experiments using pAM4787
343were performed with 5 cyanobacterial strains: *S. elongatus*, *Anabaena* PCC 7120, *Leptolyngbya*
344BL0902, *Synechocystis* PCC 6803, and *Synechocystis* WHSYN. In addition to *S. elongatus*,
345exconjugants carrying pAM4787 were obtained for *Anabaena* PCC 7120, but not for the 3 other
346strains. These data extend the utility of a shuttle plasmid based on the endogenous pANS
347plasmid from the unicellular *S. elongatus* to the filamentous *Anabaena* PCC7120 strain.

348Self-replicating plasmids derived from the *Nostoc* plasmid pDU1 (Schmetterer & Wolk, 1988)
349and the *E. coli* broad-host-range plasmid RSF1010 (Thiel, 1994) have been widely used in
350*Anabaena* PCC 7120 for genetic experiments. pDU1- and RSF1010-based plasmids are known
351to be compatible with each other in *Anabaena* PCC 7120 (Wolk *et al.*, 2007). Because plasmid
352compatibility can be beneficial for genetic engineering, we determined if pANS-based shuttle
353plasmids would be compatible with pDU1- and RSF1010-based plasmids in *Anabaena* PCC
3547120. Colony PCR analysis data showed that pAM4788 (pANS origin) was successfully
355introduced into *Anabaena* PCC 7120 cells carrying both pAM4688 (pDU1 origin) and pAM4896
356(RSF1010 origin) shuttle plasmids (Fig. 7(a), Table 1). Restriction digestion analysis of
357recovered plasmids (Fig. 7(b)) demonstrate that pANS-based shuttle vectors can be stably
358maintained in *Anabaena* PCC 7120 in the presence of pDU1- and RSF1010-based plasmids.
359*Anabaena* PCC 7120 has been shown to have about 8 copies of the chromosome per cell (Hu
360*et al.*, 2007). qPCR analysis of *Anabaena* PCC 7120 strains carrying all three plasmids showed
361that there were about 27 copies of pANS-based, 9 copies of pDU1-based, and 3 copies of
362RSF1010-based plasmids per chromosome (Fig. 7(c)).

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

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

371 Resequencing and annotation of pANS

372 The complete sequence of pANS (GenBank # S89470) was previously determined to be 7,835
373 bp in length and to include 8 open reading frames (ORFs; *orfA* to *orfH*) (Van der Plas *et al.*,
374 1992). During verification of the sequence of pANS-based shuttle vectors, several nucleotide
375 polymorphisms were found compared to the published sequence, and some of these altered the
376 identification of ORFs. Therefore, we resequenced and annotated pANS from our laboratory
377 strain of *S. elongatus* PCC 7942.

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

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

389plasmid (GenBank accession # CP006473) of *Synechococcus elongatus* UTEX 2973, a strain
390that is closely related to *S. elongatus* PCC 7942 and *S. elongatus* PCC 6301 (Yu *et al.*, 2015).
391Based on the updated sequence, pANS is 7842 bp in length, with 59.5% GC content. It was re-
392annotated to carry 7 ORFs because the previously annotated *orfE* (*repB*) and *orfF* (*repA*) are
393now joined in a single ORF, pANS_07 (*repA*) (Fig. 1, left panel and Table 2). Additional
394information for the new annotation is listed in Table 2. The protein encoded by the longest ORF,
395pANS_07 (3060 bp), carries conserved domains related to DNA primase, and was previously
396suggested to be involved in autonomous plasmid replication (Van der Plas *et al.*, 1992). The
397small ORF pANS_06 (309 bp) sits immediately downstream of pANS_07 and overlaps it by 4 bp
398(Table 2). No conserved domains or significant BLAST hits were found for pANS_06. The 3.76
399kb minimal replication region cloned in pAM4787 and pAM4788 contains the complete
400pANS_07 (*repA*) ORF, partial sequences for pANS_01 and pANS_06, and the predicted
401replication origin sequence in the intergenic region between pANS_01 and pANS_07 (Van der
402Plas *et al.*, 1992) (Fig. 1 and Table 2).

403

404DISCUSSION

405Ever since the discovery of endogenous plasmids in cyanobacteria (Van den Hondel *et al.*,
4061979) there have been efforts to construct cyanobacterial plasmid-based self-replicating shuttle
407vectors for genetic manipulation in native or heterologous strains (Koksharova & Wolk, 2002;
408Thiel, 1994). Some of the earliest cyanobacterial genetic work was done with pANS (formerly
409pUH24) in *S. elongatus* (formerly *Anacystis nidulans*) strains (Golden & Sherman, 1983;
410Kuhlemeier *et al.*, 1981; Van Den Hondel *et al.*, 1980), and a host-vector system was developed
411to eliminate the interference of native pANS with the stability of introduced shuttle vectors
412(Kuhlemeier *et al.*, 1983). However, the pANS-cured *S. elongatus* strain, which was obtained
413through chemical (SDS) treatment (Kuhlemeier *et al.*, 1983), is currently not available. To
414facilitate the use of cyanobacteria as synthetic biology platforms we have made new stable
415pANS-based shuttle vectors based on the minimal replication element of pANS (Van der Plas *et*
416*al.*, 1992), and produced a transformable *S. elongatus* strain cured of the native pANS plasmid
417as a cloning host strain. The pANS-cured *S. elongatus* strain together with the pANS shuttle
418vectors form a robust vector-host cloning system for *S. elongatus*.

419Somewhat surprisingly, pANS shuttle vectors replicate in the phylogenetically distant
420filamentous *Anabaena* sp. strain PCC 7120, but not in two unicellular *Synechocystis* strains or a
421filamentous *Leptolyngbya* strain. Other cyanobacterial plasmids have been shown to replicate in
422heterologous hosts, including pDU1 of *Nostoc* sp. PCC 7524 (Schmetterer & Wolk, 1988),
423pSY11 of marine *Synechococcus* sp. NKBG 042902 (Matsunaga *et al.*, 1990), and pMA4 of a
424thermophilic *Synechococcus* strain (Miyake *et al.*, 1999), but in most of these cases, the
425heterologous hosts are closely related to the original plasmid hosts. Some synthetic biology
426experiments could benefit from being able to transfer a single plasmid construction into both
427*Synechococcus* and *Anabaena* strains. pANS-based shuttle vectors may also replicate in other
428*Synechococcus* species and other species related to *Anabaena* PCC 7120, such as *Anabaena*
429*variabilis* and *Nostoc punctiforme*. A derivative of pAM4787, in which the *E. coli* replication origin

430was removed, was unable to transform *E. coli* DH5 α , indicating that pANS cannot replicate in *E.*
431*coli*.

432Although the structural stability of pANS-derived shuttle vectors missing the plasmid
433maintenance genes *pmaA* and *pmaB* was reported to be severely reduced (Van der Plas *et al.*,
4341992), we found that shuttle vectors constructed with the minimal replication element and
435lacking these genes can be stably maintained as long as appropriate antibiotic selection is
436applied. Recent work that identified essential genes in *S. elongatus* by RB-TnSeq showed that
437transposon insertions in pANS_07 (*repA*) resulted in plasmid loss, and therefore *repA* is
438essential for maintenance of pANS under standard growth conditions (Rubin *et al.*, 2015). In
439contrast, *pmaA* and *pmaB* were not essential for plasmid maintenance (Rubin *et al.*, 2015).

440Spontaneous loss of cyanobacterial plasmids has been observed before, including the loss of
441pANS (Castets *et al.*, 1986; Lau & Doolittle, 1979; Lau *et al.*, 1980). On the contrary, the large
442native plasmid pANL has been very difficult to eliminate (Chen *et al.*, 2008; Encinas *et al.*,
4432014), probably due to the presence of two toxin-antitoxin cassettes (Chen *et al.*, 2008). We
444were able to exploit the ability of a toxin-antitoxin cassette to confer antibiotic-free plasmid
445selection (Unterholzner *et al.*, 2013) by inclusion of the pSYSA-TA1 toxin-antitoxin cassette in
446pAM4788 (Fig. 4(a), (b)).

447Cyanobacteria are known to contain multiple copies of identical chromosomes per cell (Griese
448*et al.*, 2011). Many cyanobacterial strains also contain one or more endogenous plasmids (Chen
449*et al.*, 2008). The copy number of native cyanobacterial plasmids has been studied in several
450strains and has been reported as: 0.4-7 copies per chromosome in *Synechocystis* PCC 6803
451depending on the plasmid (Berla & Pakrasi, 2012); 1 to 8 copies per chromosome in
452*Synechococcus* PCC 7002 (which was reported to contain 6 copies of the chromosome per
453cell), depending on the plasmid and growth conditions, particularly the salt concentration (Xu *et*
454*al.*, 2011; Yano *et al.*, 1995); 50 copies per chromosome in a marine *Synechococcus* strain,

455which increased at higher salt concentrations (Takeyama *et al.*, 1991); and 350 copies per cell
456in a thermophilic *Synechococcus* strain (Miyake *et al.*, 1999). The broad host range plasmid
457RSF1010 replicates in several unicellular cyanobacterial strains with a copy number of about 10
458per chromosome (Marraccini *et al.*, 1993). Reports on the copy number of shuttle vectors based
459on the *Nostoc* PCC 7524 plasmid pDU1 vary from 1 to 17 copies per chromosome (Wolk *et al.*,
4602007), however a recent study showed that the copy number of the pDU1-based plasmid
461pRL25T to range from 0.53 to 1812 per chromosome in *Anabaena* PCC 7120, depending upon
462the recombinant insert and growth conditions (Yang *et al.*, 2013).

463In the work reported here, the pANL copy number was consistent at about 2 to 3 copies per
464chromosome in the two *S. elongatus* strains PCC 7942 and PCC 6301. The copy number per
465chromosome of pANS, however, was significantly lower in *Synechococcus* PCC 6301 compared
466to *S. elongatus* (Fig. 6(a)). The cause of this copy number difference is not known and the DNA
467sequence of pANS in *Synechococcus* PCC 6301 has not been determined. The copy number of
468a RSF1010 vector in *Anabaena* PCC 7120 was about 3 per genome, which is lower than the 10
469per chromosome reported for several unicellular cyanobacterial strains (Marraccini *et al.*, 1993).
470The co-existing pANS-based vector, on the other hand, was about 27 copies per chromosome
471in *Anabaena* PCC 7120, perhaps due to altered replication and copy number control in the
472heterologous host. Overall, our results show that pANS-based vectors will be useful for genetic
473experiments and synthetic biology applications in *Synechococcus* and *Anabaena* strains.

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481

482REFERENCES

483

484Berla, B. M. & Pakrasi, H. B. (2012). Upregulation of plasmid genes during stationary phase in
485*Synechocystis* sp. strain PCC 6803, a cyanobacterium. *Appl Environ Microbiol* **78**, 5448-5451.

486

487Brahamsha, B. (1996). A genetic manipulation system for oceanic cyanobacteria of the genus
488*Synechococcus*. *Appl Environ Microbiol* **62**, 1747-1751.

489

490Castets, A. M., Houmard, J. & Tandeau de Marsac, N. (1986). Is cell motility a plasmid-
491encoded function in the cyanobacterium *Synechocystis* 6803? *FEMS Microbiol Lett* **37**, 277-
492281.

493

494Chen, A. H., Afonso, B., Silver, P. A. & Savage, D. F. (2012). Spatial and temporal
495organization of chromosome duplication and segregation in the cyanobacterium
496*Synechococcus elongatus* PCC 7942. *PLoS One* **7**, e47837.

497

498Chen, Y., Holtman, C. K., Magnuson, R. D., Youderian, P. A. & Golden, S. S. (2008). The
499complete sequence and functional analysis of pANL, the large plasmid of the unicellular
500freshwater cyanobacterium *Synechococcus elongatus* PCC 7942. *Plasmid* **59**, 176-192.

501

502Clerico, E. M., Ditty, J. L. & Golden, S. S. (2007). Specialized techniques for site-directed
503mutagenesis in cyanobacteria. *Methods Mol Biol* **362**, 155-171.

504

505Elhai, J. & Wolk, C. P. (1988). Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol*
506**167**, 747-754.

507

508Elhai, J., Vepritskiy, A., Muro-Pastor, A. M., Flores, E. & Wolk, C. P. (1997). Reduction of
509conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. *J*
510*Bacteriol* **179**, 1998-2005.

511

512Encinas, D., Garcillan-Barcia, M. P., Santos-Merino, M., Delaye, L., Moya, A. & de la Cruz,
513F. (2014). Plasmid conjugation from proteobacteria as evidence for the origin of xenologous
514genes in cyanobacteria. *J Bacteriol* **196**, 1551-1559.

515

516**Gendel, S. M. (1987)**. Instability of Tn5 inserts in cyanobacterial cloning vectors. *J Bacteriol*
517**169**, 4426-4430.

518

519**Golden, S. S. & Sherman, L. A. (1983)**. A hybrid plasmid is a stable cloning vector for the
520cyanobacterium *Anacystis nidulans* R2. *J Bacteriol* **155**, 966-972.

521

522**Golden, S. S. & Sherman, L. A. (1984)**. Optimal conditions for genetic transformation of the
523cyanobacterium *Anacystis nidulans* R2. *J Bacteriol* **158**, 36-42.

524

525**Golden, S. S., Brusslan, J. & Haselkorn, R. (1987)**. Genetic engineering of the cyanobacterial
526chromosome. *Methods Enzymol* **153**, 215-231.

527

528**Griese, M., Lange, C. & Soppa, J. (2011)**. Ploidy in cyanobacteria. *FEMS Microbiol Lett* **323**,
529124-131.

530

531**Hays, S. G. & Ducat, D. C. (2015)**. Engineering cyanobacteria as photosynthetic feedstock
532factories. *Photosynth Res* **123**, 285-295.

533

534**Hu, B., Yang, G., Zhao, W., Zhang, Y. & Zhao, J. (2007)**. MreB is important for cell shape but
535not for chromosome segregation of the filamentous cyanobacterium *Anabaena* sp. PCC 7120.
536*Mol Microbiol* **63**, 1640-1652.

537

538**Koksharova, O. A. & Wolk, C. P. (2002)**. Genetic tools for cyanobacteria. *Appl Microbiol*
539*Biotechnol* **58**, 123-137.

540

541**Kopfmann, S. & Hess, W. R. (2013)**. Toxin-antitoxin systems on the large defense plasmid
542pSYSA of *Synechocystis* sp. PCC 6803. *J Biol Chem* **288**, 7399-7409.

543

544**Kuhlemeier, C. J., Borrias, W. E., van den Hondel, C. A. & van Arkel, G. A. (1981)**. Vectors
545for cloning in cyanobacteria: construction and characterization of two recombinant plasmids
546capable of transformation of *Escherichia coli* K12 and *Anacystis nidulans* R2. *Mol Gen Genet*
547**184**, 249-254.

548

549Kuhlemeier, C. J., Thomas, A. A., van der Ende, A., van Leen, R. W., Borrias, W. E., van
550den Hondel, C. A. & van Arkel, G. A. (1983). A host-vector system for gene cloning in the
551cyanobacterium *Anacystis nidulans* R2. *Plasmid* **10**, 156-163.

552

553Kuhlemeier, C. J. & van Arkel, G. A. (1987). Host-vector systems for gene cloning in
554cyanobacteria. *Methods Enzymol* **153**, 199-215.

555

556Lau, R. H. & Doolittle, W. F. (1979). Covalently closed circular DNAs in closely related
557unicellular cyanobacteria. *J Bacteriol* **137**, 648-652.

558

559Lau, R. H., Sapienza, C. & Doolittle, W. F. (1980). Cyanobacterial plasmids: their widespread
560occurrence, and the existence of regions of homology between plasmids in the same and
561different species. *Mol Gen Genet* **178**, 203-211.

562

563Lee, M. H., Scherer, M., Rigali, S. & Golden, J. W. (2003). PlmA, a new member of the GntR
564family, has plasmid maintenance functions in *Anabaena* sp. strain PCC 7120. *J Bacteriol* **185**,
5654315-4325.

566

567Liu, X., Wang, D., Wang, H., Feng, E., Zhu, L. & Wang, H. (2012). Curing of plasmid pXO1
568from *Bacillus anthracis* using plasmid incompatibility. *PLoS One* **7**, e29875.

569

570Marraccini, P., Bulteau, S., Cassier-Chauvat, C., Mermat-Bouvier, P. & Chauvat, F. (1993). A
571conjugative plasmid vector for promoter analysis in several cyanobacteria of the genera
572*Synechococcus* and *Synechocystis*. *Plant Mol Biol* **23**, 905-909.

573

574Matsunaga, T., Takeyama, H. & Nakamura, N. (1990). Characterization of cryptic plasmids
575from marine cyanobacteria and construction of a hybrid plasmid potentially capable of
576transformation of marine cyanobacterium, *Synechococcus* sp., and its transformation. *Appl*
577*Biochem Biotechnol* **24-25**, 151-160.

578

579Miyake, M., Nagai, H., Shirai, M., Kurane, R. & Asada, Y. (1999). A high-copy-number plasmid
580capable of replication in thermophilic cyanobacteria. *Appl Biochem Biotechnol* **77-79**, 267-275.

581

582Niederholtmeyer, H., Wolfstadter, B. T., Savage, D. F., Silver, P. A. & Way, J. C. (2010).
583Engineering cyanobacteria to synthesize and export hydrophilic products. *Appl Environ*
584*Microbiol* **76**, 3462-3466.

585

586Pecota, D. C., Kim, C. S., Wu, K., Gerdes, K. & Wood, T. K. (1997). Combining the *hok/sok*,
587*parDE*, and *pnd* postsegregational killer loci to enhance plasmid stability. *Appl Environ Microbiol*
588**63**, 1917-1924.

589

590Rubin, B. E., Wetmore, K. M., Price, M. N. & other authors (2015). The essential gene set of
591a photosynthetic organism. *Proc Natl Acad Sci U S A* **112**, E6634-6643.

592

593Ruffing, A. M. (2011). Engineered cyanobacteria: teaching an old bug new tricks. *Bioeng Bugs*
594**2**, 136-149.

595

596Sarsekeyeva, F., Zayadan, B. K., Usserbaeva, A., Bedbenov, V. S., Sinetova, M. A. & Los,
597D. A. (2015). Cyanofuels: biofuels from cyanobacteria. Reality and perspectives. *Photosynth*
598*Res* **125**, 329-340.

599

600Schmetterer, G. & Wolk, C. P. (1988). Identification of the region of cyanobacterial plasmid
601pDU1 necessary for replication in *Anabaena* sp. strain M-131. *Gene* **62**, 101-109.

602

603Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M. & Scherzinger,
604E. (1989). Complete nucleotide sequence and gene organization of the broad-host-range
605plasmid RSF1010. *Gene* **75**, 271-288.

606

607Shulse, C. N. & Allen, E. E. (2011). Widespread occurrence of secondary lipid biosynthesis
608potential in microbial lineages. *PLoS One* **6**, e20146.

609

610Sode, K., Tatara, M., Takeyama, H., Burgess, J. G. & Matsunaga, T. (1992). Conjugative
611gene transfer in marine cyanobacteria: *Synechococcus* sp., *Synechocystis* sp. and
612*Pseudanabaena* sp. *Appl Microbiol Biotechnol* **37**, 369-373.

613

614Takeyama, H., Burgess, J. G., Sudo, H., Sode, K. & Matsunaga, T. (1991). Salinity-
615dependent copy number increase of a marine cyanobacterial endogenous plasmid. *FEMS*
616*Microbiol Lett* **90**, 95-98.

617

618Taton, A., Lis, E., Adin, D. M., Dong, G., Cookson, S., Kay, S. A., Golden, S. S. & Golden, J.
619W. (2012). Gene transfer in *Leptolyngbya* sp. strain BL0902, a cyanobacterium suitable for
620production of biomass and bioproducts. *PLoS One* **7**, e30901.

621

622Taton, A., Unglaub, F., Wright, N. E. & other authors (2014). Broad-host-range vector system
623for synthetic biology and biotechnology in cyanobacteria. *Nucleic Acids Res* **42**, e136.

624

625Thiel, T. (1994). Genetic Analysis of Cyanobacteria. In *The Molecular Biology of Cyanobacteria*,
626pp. 581-611. Edited by D. A. Bryant. Dordrecht: Springer Netherlands.

627

628Trevors, J. T. (1986). Plasmid Curing in Bacteria. *FEMS Microbiol Lett* **32**, 149-157.

629

630Unterholzner, S. J., Poppenberger, B. & Rozhon, W. (2013). Toxin-antitoxin systems: Biology,
631identification, and application. *Mob Genet Elements* **3**, e26219.

632

633Van den Hondel, C. A., Keegstra, W., Borrias, W. E. & van Arkel, G. A. (1979). Homology of
634plasmids in strains of unicellular cyanobacteria. *Plasmid* **2**, 323-333.

635

636Van Den Hondel, C. A., Verbeek, S., Van Der Ende, A., Weisbeek, P. J., Borrias, W. E. & Van
637Arkel, G. A. (1980). Introduction of transposon Tn901 into a plasmid of *Anacystis nidulans*:
638preparation for cloning in cyanobacteria. *Proc Natl Acad Sci U S A* **77**, 1570-1574.

639

640Van der Plas, J., Oosterhoff-Teertstra, R., Borrias, M. & Weisbeek, P. (1992). Identification of
641replication and stability functions in the complete nucleotide sequence of plasmid pUH24 from
642the cyanobacterium *Synechococcus* sp. PCC 7942. *Mol Microbiol* **6**, 653-664.

643

644Watanabe, S., Ohbayashi, R., Kanesaki, Y., Saito, N., Chibazakura, T., Soga, T. &
645Yoshikawa, H. (2015). Intensive DNA replication and metabolism during the lag phase in
646cyanobacteria. *PLoS One* **10**, e0136800.

647

648Wolk, C. P., Fan, Q., Zhou, R., Huang, G., Lechno-Yossef, S., Kuritz, T. & Wojciuch, E.
649(2007). Paired cloning vectors for complementation of mutations in the cyanobacterium
650*Anabaena* sp. strain PCC 7120. *Arch Microbiol* **188**, 551-563.

651

652Xu, Y., Alvey, R. M., Byrne, P. O., Graham, J. E., Shen, G. & Bryant, D. A. (2011). Expression
653of genes in cyanobacteria: adaptation of endogenous plasmids as platforms for high-level gene
654expression in *Synechococcus* sp. PCC 7002. *Methods Mol Biol* **684**, 273-293.

655

656Yang, Y., Huang, X. Z., Wang, L., Risoul, V., Zhang, C. C. & Chen, W. L. (2013). Phenotypic
657variation caused by variation in the relative copy number of pDU1-based plasmids expressing
658the GAF domain of Pkn41 or Pkn42 in *Anabaena* sp. PCC 7120. *Res Microbiol* **164**, 127-135.

659

660Yano, S., Kawata, Y. & Kojima, H. (1995). Salinity-dependent copy number change of
661endogenous plasmids in *Synechococcus* sp strain PCC-7002. *Curr Microbiol* **31**, 357-360.

662

663Yu, J., Liberton, M., Cliften, P. F., Head, R. D., Jacobs, J. M., Smith, R. D., Koppenaal, D.
664W., Brand, J. J. & Pakrasi, H. B. (2015). *Synechococcus elongatus* UTEX 2973, a fast growing
665cyanobacterial chassis for biosynthesis using light and CO₂. *Sci Rep* **5**, 8132.

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668

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

Cyanobacterial Strains	Relevant characteristics	Source
<i>Anabaena</i> sp. PCC 7120	Wild type	Lab collection
<i>Leptolyngbya</i> sp. BL0902	Wild type	Lab collection
<i>Synechococcus elongatus</i> PCC 7942	Wild type	Lab collection
<i>Synechococcus elongatus</i> PCC 6301	Wild type	Lab collection
<i>Synechocystis</i> sp. PCC 6803	Wild type	Lab collection
<i>Synechocystis</i> sp. WHSYN	Wild type	Lab collection
GLC001	<i>S. elongatus</i> carrying pAM4787 isolate #S2-T1 (no native pANS); for competing out the native pANS; Sp ^r Sm ^r	This work
GLC002	<i>S. elongatus</i> carrying pAM4787 isolate #S1-1-C1 (no native pANS); for competing out the native pANS; Sp ^r Sm ^r	This work
GLC003	<i>Anabaena</i> PCC 7120 carrying pAM4688, pAM4896, and pAM4788 isolate #5-1; for testing compatibility of plasmids in <i>Anabaena</i> PCC 7120; Nm ^r Nt ^r Sp ^r Sm ^r	This work
GLC004	<i>Anabaena</i> PCC 7120 carrying pAM4688, pAM4896, and pAM4788 isolate #5-2; for testing compatibility of plasmids in <i>Anabaena</i> PCC 7120; Nm ^r Nt ^r Sp ^r Sm ^r	This work
GLC005	<i>S. elongatus</i> carrying pAM5190; for following GFP fluorescence in <i>S. elongatus</i> ; Sp ^r Sm ^r	This work
GLC006	<i>Anabaena</i> PCC 7120 carrying pAM5190; for following GFP fluorescence in <i>Anabaena</i> PCC 7120; Sp ^r Sm ^r	This work
AMC2302	<i>S. elongatus</i> (-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 <i>S. elongatus</i> 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 <i>S. elongatus</i> chromosome; Cm ^r	Lab collection
pAM4688	pDU1-ori-(PconII-GFPmut2); for testing plasmid compatibility in <i>Anabaena</i> 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 <i>pfaA-E</i> gene cluster from <i>Shewanella pealeana</i>	Chen <i>et al.</i> , in prep.
pAM4896	RSF1010-(PconII-GFPmut2); for testing plasmid compatibility in <i>Anabaena</i> PCC 7120; Nt ^r	Lab collection (Taton <i>et al.</i> , 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 <i>E. coli</i> 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 <i>et al.</i> , 2014)
pCV0003, pCV0035	CYANO-VECTOR shuttle plasmids	(Taton <i>et al.</i> , 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	<i>orfG, orfH</i>	M744_14255	61.81%	22 - 1626 (c)	1605/534	hypothetical
pANS_02	<i>orfA</i>	M744_14260	61.05%	1664 - 2197 (c)	534/177	hypothetical
pANS_03	<i>orfB (pmaA)</i>	-	56.31%	2545 - 2988 (d)	444/147	plasmid maintenance
pANS_04	<i>orfC (pmaB)</i>	M744_14235	57.69%	2998 - 3543 (d)	546/181	plasmid maintenance
pANS_05	<i>orfD</i>	M744_14240	63.56%	3704 - 4219 (c)	516/171	hypothetical
pANS_06	-	M744_14245	62.14%	4324 - 4632 (c)	309/102	hypothetical
pANS_07	<i>orfE (repB)</i> <i>orfF (repA)</i>	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.

676 Figure Legends

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

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

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

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

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

702 **Fig. 5.** pANS can carry large inserts. **(a)** Restriction digestion analysis of pANS-based shuttle
703 plasmids containing the 20-kb EPA biosynthetic gene cluster. Expected digestion pattern:
704 pAM5173 digested with *Bst*BI: 7927, 6400, 5839, 4183, 2294 bp; pAM5174 digested with *Bst*BI:
705 8669, 6400, 5839, 4183, 2294 bp. Samples: 1-2, independent pAM5173 isolates; 3-4,
706 independent pAM5174 isolates; M, 1-kb DNA ladder. **(b)** PCR analysis of exconjugants carrying
707 large pANS-EPA constructs pAM5173 (26,643 bp) or pAM5174 (27,385 bp). Primer pairs:
708 yc124/yc057 (*pfaD* specific; expected 645 bp). Samples: M, 100-bp DNA ladder (NEB); P,
709 pAM5173 plasmid DNA; 1-4, isolates of *S. elongatus*(-pANS) carrying pAM5173; 5-8, isolates of
710 *S. elongatus*(-pANS) carrying pAM5174.

711 **Fig. 6.** Plasmid copy number and reporter-gene expression levels from pANS-based shuttle
712 vectors. **(a)** Copy number of pANS and pANL per chromosome in *S. elongatus* PCC 7942
713 (S7942(WT)), pANS-cured *S. elongatus* PCC 7942 (S7942(-pANS)), and *Synechococcus* PCC
714 6301 (S6301(WT)). Standard deviation was calculated from biological triplicates. See Methods
715 for experimental details of qPCR analysis and Table S1 for specific primer pairs; **(b)** GFP
716 fluorescence measurement of *S. elongatus* PCC 7942 strains harboring a GFP reporter gene on
717 the chromosome at NS1 (pCV0035), on pANL (pAM4789), or on pANS (pAM4787). Standard
718 deviation was calculated from biological triplicates. Relative fluorescence units (RFU) were
719 normalized to OD₇₅₀.

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

725products from genomic DNA of independent *Anabaena* PCC 7120 exconjugant isolates; lanes P
726are PCR products from control plasmid DNA for each plasmid; and M, marker 1-kb DNA ladder
727(NEB). **(b)** Restriction digestion with *XmnI* of shuttle vectors recovered from *Anabaena* PCC
7287120. Plasmids: pAM4788 (pANS-based, total 7774 bp; expected fragment sizes 390 and 7384
729bp); pAM4896 (RSF1010-based, total 7729 bp; expected fragment sizes 2014 and 5715 bp);
730pAM4688 (pDU1-based, total 7192 bp; expected fragment sizes 1254, 1584, 1931, and 2423
731bp). Samples: P, marker plasmid controls isolated from *E. coli*; 1-4, individual recovered
732plasmids from 4 *Anabaena* PCC 7120 isolates that carry all three plasmids; M, marker 1-kb
733DNA ladder (NEB). The different plasmids were separately recovered in *E. coli* clones based on
734their differences in antibiotic resistance. **(c)** Copy number of pAM4788 (pANS), pAM4688
735(pDU1), and pAM4896 (RSF1010) shuttle vectors in *Anabaena* PCC 7120. Standard deviation
736was calculated from biological triplicates. See Methods for experimental details of qPCR
737analysis and Table S1 for specific primer pairs.

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