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On the dual effect of glucose during production of pBAD/AraC-based minicircles

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Abstract

Minicircles are promising vectors for DNA vaccination, gene or cell therapies due to their increased transfection efficacy and transgene expression. The *in-vivo* production of these novel vectors involves recombination of a parental molecule into a minicircircle and a miniplasmid bacterial backbone. Tight control of recombination is crucial to maximize minicircle yields and purity. In this work, a minicircle production system was constructed that relies on the enzymatic activity of ParA resolvase, a recombinase that is expressed under the transcription control of the arabinose inducible expression system pBAD/AraC, and on *E. coli* BWAA, a strain improved for arabinose uptake. Undesired recombination already after 4 hours of incubation in Luria-Bertani broth at 37°C was observed due to the leaky expression from pBAD/AraC. While addition of glucose to the growth media repressed this leaky expression, it triggered a pH drop to 4.5 during exponential phase in shake flasks, which suppressed growth and plasmid production. A quantitative PCR analysis confirmed that only a few copies of the high-copy number parental plasmid remained inside the *E. coli* cells. To ensure the stability of the minicircle-producing system, seed cultures should be grown at 30°C with glucose overnight whereas cells for minicircle production should be grown in shake flasks at 37°C without glucose up to early stationary phase when the recombination is induced by addition of arabinose.

Introduction

Minicircles are small supercoiled DNA molecules devoid of bacterial backbone that exhibit increased transfection efficacy and transgene expression when compared to their parental plasmid molecules. These properties make minicircles particularly attractive as gene delivery vectors for DNA vaccination and gene therapy. The typical minicircle production system encompasses an *in vivo* induced recombination between two direct repeats present in a parental plasmid DNA (pDNA). This event originates two supercoiled pDNA molecules: i) a

26 minicircle (MC) that carries the eukaryotic expression cassette and ii) an undesired
27 miniplasmid (MP) that contains the bacterial elements. The recombination of the parental
28 plasmid is commonly controlled by the arabinose inducible pBAD/AraC expression system
29 [1–3]. Key features of this system are: i) the repression of transcription initiation by AraC in
30 the absence of arabinose and ii) the induction of transcription of recombinase in the presence
31 of arabinose. In addition, the presence of glucose in growth medium further repress the leaky
32 expression of the BAD promoter via reduced levels of cAMP [4].
33 The minicircle model system used in this work relies on ParA resolvase, a recombinase that is
34 under transcription control of the arabinose inducible expression system pBAD/AraC.
35 BWAA, an *E. coli* strain genetically modified to increase uptake of arabinose via the
36 constitutive expression of low-affinity arabinose transporter (AraE) is used as producer host
37 [5]. The undesired recombination driven from the leaky expression of pBAD/AraC is
38 described together with the recombination efficiency in different stages of growth induced by
39 L-(+) arabinose. The secondary effect of glucose addition to minicircle-producing medium in
40 shake flasks on cell metabolism and final plasmid yield is clarified.
41

42 **Material and methods**

43 All primers used in this study were synthetized by Stabvida (Lisbon, Portugal). The PCR
44 reactions were performed using a KOD Hot Start Master Mix (Novagen) according to the
45 manufacturer instructions.

46 *Strains and plasmids*

47 The strain *E. coli* BW27783 improved for arabinose uptake was purchased from The Coli
48 Genetic Stock Center at Yale. In order to obtain a suitable strain for plasmid production, the
49 *endA and recA genes* of the BW27783 strain were knocked out by P1 transduction [6],
50 yielding the strain *E. coli* BWAA. The following plasmid templates were used in the

51 construction of pVAXmini: i) pVAX1GFP (3697 bp) contains the GFP gene and was derived
52 from pVAX1LacZ (Invitrogen, Carlsbad, CA) as described previously [7], ii) pUC57cas
53 contains a cassette with the ParA resolvase gene under pBAD promoter, the AraC repressor
54 in opposite direction and a multimer resolution site (MRS) upstream of the pBAD promoter
55 (obtained from NZYTech Lda, Lisbon, Portugal).

56 *Construction of plasmid pVAXmini*

57 Firstly, the cleavage sites for restriction enzymes NsiI, SalI were introduced into pVAX1GFP
58 [7] by site- directed mutagenesis using 0.3 μM of primer MutNSiISalIfwd
59 (CTATGGCTTCTAATGCATGGTTTTGTCGACAGCAAGCGAACCGG), 0.3 μM of
60 primer MutNsiISalIrev
61 (CCGGTTCGCTTGCTGTCGACAAAACCATGCATTAGAAGCCATAG) and 10 ng of
62 template pVAX1GFP. The MRS for the *parA* resolvase was generated using 0.6 μM of
63 MRS2fwd (CATGCATTCGCGATTGGTCAAATTGGG), a primer containing the NruI
64 restriction site (underlined), 0.6 μM of MRS2rev
65 (TGCAAGCAACGCGTTAGCACATATGTG), a primer containing the MluI restriction site
66 (underlined) and the template plasmid DNA pUC57cas. The PCR fragment was digested by
67 MluI and NruI and was cloned into the naturally occurring restriction sites NruI and MluI on
68 pVAX1GFP by using the Clonables™ 2x ligation premix (Novagen), yielding plasmid
69 pVAXMRS. The pBAD/AraC- MRS cassette was amplified using the primers casParAfwd
70 (CCCTCATGCATTCCCCCTTGG) containing NsiI restriction site and casParArev (5´-
71 AGCCGTCGACTGCCCGGCTTA-3`) containing SalI restriction site and pUC57cas as a
72 template. The digested PCR fragment was subjected to ligation (Clonables™ 2x ligation
73 premix, Novagen) with digested pVAXMRS, yielding pVAXmini (5944 bp).

74 *Minicircle propagation*

75 Five mL of LB (Luria-Bertani) medium supplemented with kanamycin (30 µg/ ml) and 0.5%
76 (w/v) glucose were inoculated with a loop of frozen *E. coli* BWAA previously transformed
77 with pVAXmini and incubated overnight at 30ºC, 250 rpm. Next, an appropriate volume of
78 this seed culture was used to inoculate 50 mL of LB media supplemented with kanamycin (30
79 µg/ ml) up to an OD of 0.1. Cultures were then incubated at 37ºC, 250 rpm until reaching
80 stationary phase. In a typical experiment, the transcription of *parA* resolvase was induced by
81 adding 0.01% L-(+) arabinose directly to the medium and recombination was allowed to
82 proceed for one hour. Culture samples were withdrawn at different time points and
83 centrifuged to obtain cell pellets from the same amount of cells that were stored at -20ºC for
84 further analysis.

85 *Analysis of plasmid replication efficiency by densitometry*

86 Plasmids were isolated with the Qiagen miniprep purification kit according to the
87 manufacturer instructions. Two µl of purified pVAXmini (5.9 kb) was digested with MluI, a
88 restriction enzyme (Biolabs) with one recognition site on the miniplasmid segment (4 kb) and
89 one recognition site on the minicircle segment (1.9 kb), originating two fragments with
90 similar size (~3 kb). Restriction mixtures were loaded onto 1% agarose gels and
91 electrophoresis was carried out at 100 V, for 50 min, with TAE buffer (40 mM Tris base, 20
92 mM acetic acid and 1 mM EDTA, pH 8.0). Gels were photographed with AlphaInnotech
93 digital imager. The recombination efficiency was calculated on the basis of band intensities
94 obtained with the ImageJ software and normalized for molar amounts using the equation Er=
95 (1- PP/(MP+PP))*100%, where Er is the efficiency of recombination, PP is the molar amount
96 of parental plasmid and MP is the molar amount of miniplasmid.

97 *Plasmid copy number determination by quantitative PCR*

98 Parental plasmid copy number was quantified directly from cells by performing quantitative
99 PCR on a 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA) as described
100 previously [8]. The following pair of primers were used to amplify a 182 bp long fragment:
101 ppfwd (TTGCTCACATGTTCTTGCTGC) and pprev (ATCAATGTCAACGCGTTAGCA).
102 Each 25 µl of qPCR reaction mixture contained 1X Brilliant II SYBR Green High ROX
103 Master Mix reagent (Agilent Technologies), 0.2 µM each of the forward and reverse primers,
104 and 50,000 cells carrying pVAXmini in 2 µl of nuclease-free water. The thermal profile
105 consisted of a 10-min hold at 95ºC followed by 40 cycles at 95ºC for 30 s, 55ºC for 1 min and
106 72ºC for 30s. Plasmid copy number was calculated using absolute quantification directly
107 from standard curves that were plotted as Ct values vs. calculated plasmid copy number.

108 **Results**

109 *Optimization of minicircle production in E. coli* BWAA

110 The minicircle production system described here relies on the enzymatic activity of ParA
111 resolvase, a recombinase that is expressed under the transcription control of the arabinose
112 inducible expression system pBAD/AraC. The *in vivo* recombination of the parental plasmid
113 pVAXmini (5944 bp) is induced by the addition of 0.01 % arabinose to cultures of the *E. coli*
114 BWAA host, originating a 1881 bp minicircle that carries the eukaryotic expression cassette
115 and a 4063 bp miniplasmid (Figure 1A). When using this system, special attention has to be
116 taken to environmental factors to avoid uncontrolled recombination when preparing seed
117 cultures for minicircle production and cell banking. The introduction of modifications in the
118 backbone of the parental plasmid can also interfere with the control of recombination. If
119 recombination events occur before arabinose induction, the resulting miniplasmids will
120 replicate faster than parental plasmids since they also contain the bacterial origin of
121 replication and have the advantage of smaller size [1,2]. In glucose-absent medium at 37°C,

122 recombination of the parental plasmid was observed just after 4 hours of growth as can be
123 seen by the presence of the miniplasmid and minicircle bands in an electrophoresis gel
124 (**Error! Reference source not found.**D). However, at 30°C the parental plasmid remains
125 intact for at least 7 hours by maintaining all the other conditions (data not shown). This might
126 be explained by the lower plasmid copy number of parental plasmid observed during growth
127 at 30ºC and hence by a decreased production of ParA resolvase from leaky pBAD/AraC. The
128 RK2 broad host range plasmid originally carrying the *parABCDE* partioning system
129 exhibited higher activity at 30ºC than at 33.5ºC, however the decreased activity was most
130 probably due to temperature-sensitive plasmid loss [9,10]. In order to prevent leaky
131 expression from pBAD, addition of 0.5% (w/v) glucose to LB media was required (**Error!**
132 **Reference source not found.**C). Glucose present in media acts by lowering cAMP levels. As
133 a consequence, less activator protein complex cAMP-CAP binds to DNA and thus AraC
134 remains strongly bound to DNA blocking the transcription initiation [11]. In the presence of
135 0.5% of glucose no undesired recombination was observed. However, yields of plasmids
136 isolated by miniprep or alkaline lysis were abnormally low (Figure 1C). In conclusion, to
137 maintain a tight control of the minicircle producing system it is essential to grow seed
138 cultures in media containing at least 0.5% glucose at lower temperatures (30°C) and then use
139 medium without glucose to generate high yields of minicircles at shake flask scale.
140 The recombination efficiency was studied by performing arabinose induction at different
141 stages of the growth (**Error! Reference source not found.**B). Complete recombination was
142 obtained only in early exponential growth phase. However, under these conditions the total
143 plasmid yield was very low and miniplasmid species kept replicating during the
144 recombination phase thereby increasing the amount of miniplasmid impurity. An 88%
145 efficiency of recombination of the parental plasmid was observed when arabinose was added
146 at early stationary phase, at a time when higher cell density and plasmid yield had already
147 been achieved. The best compromise between cell density and recombination efficiency

148 (98%) was obtained by inducing recombination at late exponential phase. Cells entering the
149 stationary phase are nutrient depleted and consequently the anabolic activity is decreased
150 [12]. To overcome this limitation Kay and co-workers choose the strategy of mixing the cells
151 in stationary phase with a fresh induction media and further incubating for additional 5 hours
152 to allow complete recombination [3,13]. Our results are consistent with previous observation
153 that superior recombination efficiency is obtained when the recombinase is carried on each
154 parental plasmid molecule as compared to systems where recombinase is carried by a helper
155 plasmid molecule [14,15] or integrated in the chromosome [3].

156 *The influence of medium pH on plasmid copy number*

157 Glucose contributes to repress the pBAD/AraC promoter and thus prevent undesired
158 recombination before arabinose induction. Nevertheless, very low yields of total pDNA were
159 obtained following isolation by alkaline lysis when minicircles were produced in LB media
160 supplemented with 0.5% (w/v) glucose. Furthermore, the extracellular pH by the end of
161 growth dropped to 4.5 whereas the pH of a control experiment performed with LB
162 supplemented with glycerol reached 8.5. When the pH in the shake flasks was controlled by
163 pH indicator bromothymol blue and kept at neutral pH by addition of 1 M NaOH neither
164 growth nor plasmid production repression was noticed, whereas the cells in the shake flask
165 without pH control showed lower final OD and low plasmid yield (**Error! Reference source**
166 **not found.**A, B). Basically, the cells kept only few plasmid copies to survive on kanamycin
167 selection pressure as confirmed by qPCR analysis of PCN directly from the cells (**Error!**
168 **Reference source not found.**C).

169 In bacterial aerobic growth with a glucose surplus the pH drop is associated with the
170 production of organic acids. The work of Gonçalves conducted in our group showed high
171 acetic acid production (5 g/L) in *E. coli* DH5α when growing on glucose as a carbon source
172 and only traces amount of acetic acid (0.05 g/L) in the presence of glycerol [16]. A similar
173 behavior was observed in the present work with the *E. coli* BWAA and DH5α strain (data not

174 shown). To counter acid stresses bacteria have developed active mechanisms including pH
175 increase by decarboxylation activity or proton extrusion across the cell membrane to maintain
176 pH homeostasis (for review [17–19]). Apparently, these systems shift metabolism and
177 consume free energy for bacterial survival at the expenses of metabolite production. Even a
178 small variation in pH resulted in a decrease in plasmid production. More than two-fold less
179 volumetric plasmid yield was observed in production media of pH 6 compared to pH 7.5
180 [20].

181 **Conclusions**

182 The tight control of pBAD/AraC expression system of ParA resolvase during minicircle
183 production by glucose addition to growth medium had a deleterious effect on overall plasmid
184 yield as a result of a pH drop. We showed that the presence of glucose in LB medium causes
185 a drop in pH values and consequently cell growth and plasmid production are repressed. On
186 the other hand when no glucose is added, the leaky expression of pBAD/AraC causes
187 undesired recombination of parental plasmid and subsequently the smaller miniplasmid
188 species carrying the origin of replication dominates the plasmid population. To ensure the
189 stability of the minicircle-producing system, parental plasmid modification and/or seed
190 banking and seed culture preparation should be always carried out with glucose addition and
191 at lower temperatures (30°C) whereas cells for minicircle production should be grown in
192 shake flasks at 37°C without glucose for maximum minicircle yield. Based on our
193 observations, we recommend performing metabolite production experiments with minimal
194 pH control (e.g. addition of a pH indicator such as bromothymol blue to the growth media)
195 for reliable shake-flask data interpretation.

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202 The authors have declared no conflict of interest.
203

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262

263 Figure 1: (A) Parental plasmid recombination. The minicircle is generated *in vivo* in host
264 strain *Escherichia coli* BWAA from parental plasmid containing the gene for ParA resolvase
265 under tight transcription control of the pBAD/AraC arabinose promoter. After induction by
266 L-(+)arabinose the ParA resolvase catalyses intramolecular recombination between the MRS
267 sites resulting in two circular DNA molecules, one containing the eukaryotic expression
268 cassette (minicircle) and one containing the bacterial backbone (miniplasmid), (MRS:
269 multimer resolution sites of the *parA* resolvase system; CMV: cytomegalovirus immediate
270 early promoter; GFP: green fluorescence protein gene; BGH: bovine growth hormone
271 polyadenylation signal; AraC: gene encoding for repressor of arabinose operon; parA:
272 resolvase gene; kan: kanamycin resistance gene; ORI: origin of replication). (B)
273 Recombination efficiency across the growth phase. The same amount of cells were harvested
274 before (B) and after (A) induction at OD 1, 2, 3 and 4. Two μl of pDNA isolated by miniprep
275 kit (Qiagen) was digested by MluI restriction enzyme and the band intensity was evaluated by
276 ImageJ analysis. The recombination efficiency is shown under the bar of each growth phase.
277 The results represent average of 12 measurements (three independent growths in duplicates,
278 for each growth two independent digestions with standard deviation of the mean) with
279 confidence level $p<0.05$. (C) Effect of glucose concentrations on pBAD/AraC repression. The
280 same amount of cells grown with different glucose amounts were harvested at the beginning
281 of stationary phase. Following miniprep, 2 μl of plasmid DNA was loaded on the gel. (D)
282 Recombination driven by parA resolvase expressed from leaking expression of pBAD/AraC.
283 The same amount of cells was harvested at different time instants. Following miniprep, 2 μl
284 of plasmid DNA was loaded on the gel.

285

286 Figure 2: (A) The growth curve of *E. coli* BWAA carrying pVAXmini in LB media
287 supplemented by 0.5% (w/v) glucose (empty triangles); LB with 0.5% (w/v) glucose and
288 NaOH (full triangles); and LB medium supplemented with 0.5% (w/v) of glycerol. The
289 dashed line follows the final concentration of added NaOH across the growth. (B) Agarose
290 gel of plasmid DNA when cells were grown on LB+ Glu (1), LB+Glu+NaOH (2) and
291 LB+Gly (3). (C) The quantitative real-time PCR analysis of plasmid copy number. The
292 results represent average of 6 measurements (three independent growths in duplicates) with
293 confidence level $p<0.05$.

294