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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
minicircle (MC) that carries the eukaryotic expression cassette and ii) an undesired miniplasmid (MP) that contains the bacterial elements. The recombination of the parental plasmid is commonly controlled by the arabinose inducible pBAD/AraC expression system [1–3]. Key features of this system are: i) the repression of transcription initiation by AraC in the absence of arabinose and ii) the induction of transcription of recombinase in the presence of arabinose. In addition, the presence of glucose in growth medium further repress the leaky expression of the BAD promoter via reduced levels of cAMP [4].

The minicircle model system used in this work relies on ParA resolvase, a recombinase that is under transcription control of the arabinose inducible expression system pBAD/AraC. BWAA, an *E. coli* strain genetically modified to increase uptake of arabinose via the constitutive expression of low-affinity arabinose transporter (AraE) is used as producer host [5]. The undesired recombination driven from the leaky expression of pBAD/AraC is described together with the recombination efficiency in different stages of growth induced by L-(+)-arabinose. The secondary effect of glucose addition to minicircle-producing medium in shake flasks on cell metabolism and final plasmid yield is clarified.

**Material and methods**

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

**Strains and plasmids**

The strain *E. coli* BW27783 improved for arabinose uptake was purchased from The Coli Genetic Stock Center at Yale. In order to obtain a suitable strain for plasmid production, the *endA* and *recA* genes of the BW27783 strain were knocked out by P1 transduction [6], yielding the strain *E. coli* BWAA. The following plasmid templates were used in the
construction of pVAXmini: i) pVAX1GFP (3697 bp) contains the GFP gene and was derived from pVAX1LacZ (Invitrogen, Carlsbad, CA) as described previously [7], ii) pUC57cas contains a cassette with the ParA resolvase gene under pBAD promoter, the AraC repressor in opposite direction and a multimer resolution site (MRS) upstream of the pBAD promoter (obtained from ZYTech Lda, Lisbon, Portugal).

Construction of plasmid pVAXmini

Firstly, the cleavage sites for restriction enzymes NsiI, SalI were introduced into pVAX1GFP [7] by site-directed mutagenesis using 0.3 µM of primer MutNSiISalIfwd (CTATGGCTTTCAATGCATGGTTTTGTCGACAGCAAGCGAACCGG), 0.3 µM of primer MutNSiISalIrev (CCGGTTCGCTTGCTGTCGACAAAACCATGCATTAGAAGCCATAG) and 10 ng of template pVAX1GFP. The MRS for the parA resolvase was generated using 0.6 µM of MRS2fwd (CATGCATTCGCGATTTGGTCAAATTGGG), a primer containing the NruI restriction site (underlined), 0.6 µM of MRS2rev (TGCAAGCAACGGCTTTAGCACATATGTG), a primer containing the MluI restriction site (underlined) and the template plasmid DNA pUC57cas. The PCR fragment was digested by MluI and NruI and was cloned into the naturally occurring restriction sites NruI and MluI on pVAX1GFP by using the Clonables™ 2x ligation premix (Novagen), yielding plasmid pVAXMRS. The pBAD/AraC- MRS cassette was amplified using the primers casParAfwd (CCCTCATGCGATTCCCCCTTGG) containing NsiI restriction site and casParArev (5’-AGCCGTGACTGCCCCGCTTA-3’) containing SalI restriction site and pUC57cas as a template. The digested PCR fragment was subjected to ligation (Clonables™ 2x ligation premix, Novagen) with digested pVAXMRS, yielding pVAXmini (5944 bp).
Minicircle propagation

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

Analysis of plasmid replication efficiency by densitometry

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

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

Results

Optimization of minicircle production in E. coli BWAA

The minicircle production system described here 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. The in vivo recombination of the parental plasmid pVAXmini (5944 bp) is induced by the addition of 0.01 % arabinose to cultures of the E. coli BWAA host, originating a 1881 bp minicircle that carries the eukaryotic expression cassette and a 4063 bp miniplasmid (Figure 1A). When using this system, special attention has to be taken to environmental factors to avoid uncontrolled recombination when preparing seed cultures for minicircle production and cell banking. The introduction of modifications in the backbone of the parental plasmid can also interfere with the control of recombination. If recombination events occur before arabinose induction, the resulting miniplasmids will replicate faster than parental plasmids since they also contain the bacterial origin of replication and have the advantage of smaller size [1,2]. In glucose-absent medium at 37ºC,
recombination of the parental plasmid was observed just after 4 hours of growth as can be seen by the presence of the miniplasmid and minicircle bands in an electrophoresis gel (Error! Reference source not found.D). However, at 30°C the parental plasmid remains intact for at least 7 hours by maintaining all the other conditions (data not shown). This might be explained by the lower plasmid copy number of parental plasmid observed during growth at 30°C and hence by a decreased production of ParA resolvase from leaky pBAD/AraC. The RK2 broad host range plasmid originally carrying the parABCDE partitioning system exhibited higher activity at 30°C than at 33.5°C, however the decreased activity was most probably due to temperature-sensitive plasmid loss [9,10]. In order to prevent leaky expression from pBAD, addition of 0.5% (w/v) glucose to LB media was required (Error! Reference source not found.C). Glucose present in media acts by lowering cAMP levels. As a consequence, less activator protein complex cAMP-CAP binds to DNA and thus AraC remains strongly bound to DNA blocking the transcription initiation [11]. In the presence of 0.5% of glucose no undesired recombination was observed. However, yields of plasmids isolated by miniprep or alkaline lysis were abnormally low (Figure 1C). In conclusion, to maintain a tight control of the minicircle producing system it is essential to grow seed cultures in media containing at least 0.5% glucose at lower temperatures (30°C) and then use medium without glucose to generate high yields of minicircles at shake flask scale.

The recombination efficiency was studied by performing arabinose induction at different stages of the growth (Error! Reference source not found.B). Complete recombination was obtained only in early exponential growth phase. However, under these conditions the total plasmid yield was very low and miniplasmid species kept replicating during the recombination phase thereby increasing the amount of miniplasmid impurity. An 88% efficiency of recombination of the parental plasmid was observed when arabinose was added at early stationary phase, at a time when higher cell density and plasmid yield had already been achieved. The best compromise between cell density and recombination efficiency
(98%) was obtained by inducing recombination at late exponential phase. Cells entering the stationary phase are nutrient depleted and consequently the anabolic activity is decreased [12]. To overcome this limitation Kay and co-workers choose the strategy of mixing the cells in stationary phase with a fresh induction media and further incubating for additional 5 hours to allow complete recombination [3,13]. Our results are consistent with previous observation that superior recombination efficiency is obtained when the recombinase is carried on each parental plasmid molecule as compared to systems where recombinase is carried by a helper plasmid molecule [14,15] or integrated in the chromosome [3].

The influence of medium pH on plasmid copy number

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

In bacterial aerobic growth with a glucose surplus the pH drop is associated with the production of organic acids. The work of Gonçalves conducted in our group showed high acetic acid production (5 g/L) in *E. coli* DH5α when growing on glucose as a carbon source and only traces amount of acetic acid (0.05 g/L) in the presence of glycerol [16]. A similar behavior was observed in the present work with the *E. coli* BWAA and DH5α strain (data not
shown). To counter acid stresses bacteria have developed active mechanisms including pH increase by decarboxylation activity or proton extrusion across the cell membrane to maintain pH homeostasis (for review [17–19]). Apparently, these systems shift metabolism and consume free energy for bacterial survival at the expenses of metabolite production. Even a small variation in pH resulted in a decrease in plasmid production. More than two-fold less volumetric plasmid yield was observed in production media of pH 6 compared to pH 7.5 [20].

**Conclusions**

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

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Figure 1: (A) Parental plasmid recombination. The minicircle is generated in vivo in host strain *Escherichia coli* BWAA from parental plasmid containing the gene for ParA resolvase under tight transcription control of the pBAD/AraC arabinose promoter. After induction by L- (+)arabinose the ParA resolvase catalyses intramolecular recombination between the MRS sites resulting in two circular DNA molecules, one containing the eukaryotic expression cassette (minicircle) and one containing the bacterial backbone (miniplasmid). (B) Recombination efficiency across the growth phase. The same amount of cells were harvested before (B) and after (A) induction at OD 1, 2, 3 and 4. Two µl of pDNA isolated by miniprep kit (Qiagen) was digested by MluI restriction enzyme and the band intensity was evaluated by ImageJ analysis. The recombination efficiency is shown under the bar of each growth phase. The results represent average of 12 measurements (three independent growths in duplicates, for each growth two independent digestions with standard deviation of the mean) with confidence level p<0.05. (C) Effect of glucose concentrations on pBAD/AraC repression. The same amount of cells grown with different glucose amounts were harvested at the beginning of stationary phase. Following miniprep, 2 µl of plasmid DNA was loaded on the gel. (D) Recombination driven by parA resolvase expressed from leaking expression of pBAD/AraC. The same amount of cells was harvested at different time instants. Following miniprep, 2 µl of plasmid DNA was loaded on the gel.
Figure 2: (A) The growth curve of *E. coli* BWAA carrying pVAXmini in LB media supplemented by 0.5% (w/v) glucose (empty triangles); LB with 0.5% (w/v) glucose and NaOH (full triangles); and LB medium supplemented with 0.5% (w/v) of glycerol. The dashed line follows the final concentration of added NaOH across the growth. (B) Agarose gel of plasmid DNA when cells were grown on LB+ Glu (1), LB+Glu+NaOH (2) and LB+Gly (3). (C) The quantitative real-time PCR analysis of plasmid copy number. The results represent average of 6 measurements (three independent growths in duplicates) with confidence level p<0.05.