Journal of Biotechnology 146 (2010) 130–137

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Marker-free plasmids for gene therapeutic applications—Lack of antibiotic resistance gene substantially improves the manufacturing process

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ARTICLE INFO

Article history:
Received 16 November 2009
Received in revised form 19 January 2010
Accepted 29 January 2010

Keywords:
Plasmid DNA
Gene therapy
DNA vaccines
Plasmid addiction
Antibiotic resistance gene
Lon protease

ABSTRACT

Plasmid DNA is being considered as a promising alternative to traditional protein vaccines or viral delivery methods for gene therapeutic applications. DNA-based products are highly flexible, stable, are easily stored and can be manufactured on a large scale. Although, much safer than viral approaches, issues have been raised with regard to safety due to possible integration of plasmid DNA into cellular DNA or spread of antibiotic resistance genes to intestinal bacteria by horizontal gene transfer. Accordingly, there is interest in methods for the production of plasmid DNA that lacks the antibiotic resistance gene to further improve their safety profile. Here, we report for the first time the gram-scale manufacturing of a minimized plasmid that is devoid of any additional sequence elements on the plasmid backbone, and merely consists of the target expression cassette and the bacterial origin of replication. Three different host/vector combinations were cultivated in a fed-batch fermentation process, comparing the progenitor strain JM108 to modified strains JM108*murselect*, hosting a plasmid either containing the aminoglycoside phosphotransferase which provides kanamycin resistance, or a marker-free variant of the same plasmid. The metabolic load exerted by expression of the aminoglycoside phosphotransferase was monitored by measuring ppGpp- and cAMP-levels. Moreover, we revealed that JM108 is deficient of the Lon protease and thereby refined the genotype of JM108. The main consequences of Lon-deficiency with regard to plasmid DNA production are discussed herein. Additionally, we found that the expression of the aminoglycoside phosphotransferase, conferring resistance to kanamycin, was very high in plasmid DNA producing processes that actually inclusion bodies were formed. Thereby, a severe metabolic load on the host cell was imposed, detrimental for overall plasmid yield. Hence, deleting the antibiotic resistance gene from the vector backbone is not only beneficial with regards to safety and potency of the end-product but also regarding the overall process performance.

1. Introduction

The demand for plasmid DNA (pDNA) for vaccine and gene therapy applications has increased during the past few years in response to several technical improvements and the recent licensing of four DNA products for veterinary applications (Redding and Weiner, 2009). A detailed review on the current progress in this field can be found elsewhere (Kutzler and Weiner, 2008; Redding and Weiner, 2009). pDNA, used for gene delivery, has attracted a lot of interest since it offers several advantages over viral gene delivery, e.g. weak immunogenicity, an improved safety profile and the ease of manufacturing. Due to the fact that the efficiency and duration of expression of current pDNA-based products is very limited, considerable amounts of pharmaceutical grade pDNA per patient are required (milligram-scale). A detailed description on the ambitious efforts made in the upstream processing of pDNA processes can be found elsewhere (Bower and Prather, 2009; Tejeda-Mansir and Montesinos, 2008; Williams et al., 2009). Manufacturing and preclinical development concerns, associated with the use of pDNA-based therapeutics, are similar to those for other biological products. To allow for selective retention in *Escherichia coli* (*E. coli*), plasmid vectors, used for therapeutic applications, typically encode for the aminoglycoside phosphotransferase (*nptII*), mediating resistance to kanamycin; this marker gene is the only one tolerated by regulatory authorities (FDA, 1996). As previously described (Mairhofer and Grabherr, 2008), selectable markers and non-essential bacterial sequences in general are responsible for many drawbacks of pDNA performance. They cause possible safety problems, may provoke inflammatory reactions in vivo (Yew et al., 2000), hamper transfection efficiency by increased pDNA size

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doi:10.1016/j.jbiotec.2010.01.025

(Maucksch et al., 2009; Yin et al., 2005) and disturbing sequences, and cause an additional metabolic load for the host cell during manufacturing (Rozkov et al., 2004). Bioprocesses, designed for the manufacturing of pDNA, tend to overburden the host machinery by enormous over-replication of pDNA, induced by so-called runaway replication (Yau et al., 2008) or thermal shift (Carnes et al., 2006). This metabolic burden, caused by pDNA replication and consumption of precursor metabolites is manifested by inhibition of biomass growth (Diaz Ricci and Hernández, 2000), structural instabilities of cultivated pDNA (Cooke et al., 2004) and segregational instability (Paulsson and Ehrenberg, 1998), impairing the overall process performance. The major cause for metabolic burden exerted on the host cell, is the constitutive expression of the antibiotic resistance gene. Usually, the marker protein represents up to 20% of total cellular protein, thereby exceeding the levels that are needed for proper selection and maintenance (Panayotatos, 1988; Rozkov et al., 2004). Due to the fact that these sequences represent a safety problem, are non-essential for the later product characteristics, and reduce the potency of the final pDNA-based product, it is desirable to exclude these sequences from the vector backbone. We have recently shown that antibiotic resistance-free maintenance of plasmids is possible by using the origin of replication (ori)-encoded RNAI instead of the antibiotic resistance marker (Mairhofer et al., 2008). Here, pDNA selection and maintenance is achieved by functionally linking the RNAI, encoded on the ori of common ColE1-based plasmids, to a repressor protein, encoded on the host genome that further controls the expression of an essential gene (for details see Fig. 1). The recent years have shown numerous approaches for antibiotic-resistance-free pDNA production (Cranenburgh et al., 2001; Goh and Good, 2008; Luke et al., 2009; Mayrhofer et al., 2008; Soubrier et al., 2005). However, most of these approaches are dependent on additional sequences that need to be introduced in the plasmid backbone or have failed to be applicable in large-scale processes. Here, we report for the first time the gram-scale production of pDNA, devoid of antibiotic resistance genes or other additional sequences, in synthetic, chemically defined media.

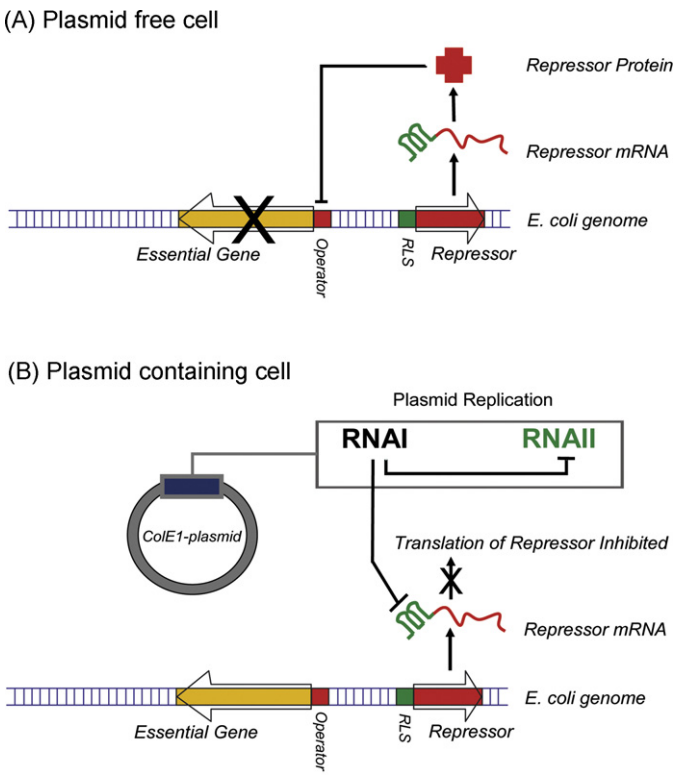

Fig. 1. Schematic overview on the RNA-based plasmid maintenance system. The E. coli genome was modified by chromosomal engineering: an operator was introduced in front of an essential gene; the repressor gene corresponding to this operator is fused to an RNAII-like sequence (RLS). Inhibition is marked by ⊥, Activation is marked by ↑. (A) Plasmid-free cell: the repressor gene is transcribed and mRNA thereof is translated to the repressor protein which inhibits transcription of the essential gene by blocking its operator. Thereby, cell growth is inhibited in plasmid-free cells. (B) Plasmid containing cell: two RNA-molecules, RNAI (plasmid replication inhibitor) and RNAII (primer for plasmid replication), are naturally encoded on the origin of replication of standard ColE1-plasmids. Plasmid replication is inhibited by base-pairing of the complementary RNAI to RNAII. If plasmid is present in the cell then RNAI is deduced from the plasmid replication control and translation of the repressor is inhibited with high efficiency by anti-sense hybridization of the RNAI to the RNAII-like sequence (RLS) fused to the mRNA of the repressor. Expression of the essential gene is provided, leading to cell survival.

2. Methods

2.1. Strains

Plasmids were propagated in *E. coli* K-12 strain JM108 (ATCC No. 47107; DSMZ No. 5585). The genotype of JM108 is annotated as: *F*⁻ *thi* Δ(*lac-proAB*) *end A*1 *gyrA*96 *relA*1 *phx hsdR*17 *supE*44 *recA* (Yanisch-Perron et al., 1985).

2.2. Plasmids

The pMCP-1 (4892 bp) and pMINI/MCP-1 (3477 bp) vectors (Fig. 2) encode for the monocyte chemoattractant protein-1 (MCP-1) that has been shown to improve arteriogenesis (Muhs et al., 2004). The major parts of pMCP-1 were derived from the plasmid pcDNA3 (Invitrogen, Karlsbad), the kanamycin resistance gene was derived from pZErO-2 (Invitrogen, Karlsbad). pMINI/MCP-1 is a derivative of pMCP-1 and was created by two PCR reactions, subsequently ligated. The following primers have been used: MCP1-PvuI-pUCori-back (5′-ATG ATG CGA TCG GGC GCT CTT CCG CTT CCT C-3′) and MCP1-BamHI-pUCori-for (5′-ATG ATG GGA TCC TCA TGA CCA AAA TCC CTT AAC-3′) for the amplification of the ori and pMINI-BamHI-CMV-back (5′-ATG ATG GGA TCC GTT GAC ATT GAT TAT TGA CTA G-3′) and pMINI-PvuI-polyA-for (5′-ATG ATG CGA TCG CCT CCC CCT TGC TGT CC-3′) for the amplification of the CMV promoter, the MCP-1 gene and the bovine growth hormone polyA signal. The resulting plasmid comprises 3477 bp, which represents a size reduction of 29% compared to pMCP-1. pMCP-1 was propagated in JM108 (resulting in JM108pMCP-1) and JM108*murselect* (resulting in JM108*murselect*pMCP-1, while pMINI-MCP-1 was propagated in JM108*murselect* only (resulting in JM108*murselect*pMINI/MCP-1), using a fed-batch process (for a overview on the different strains used see Table 1). *E. coli* JM108

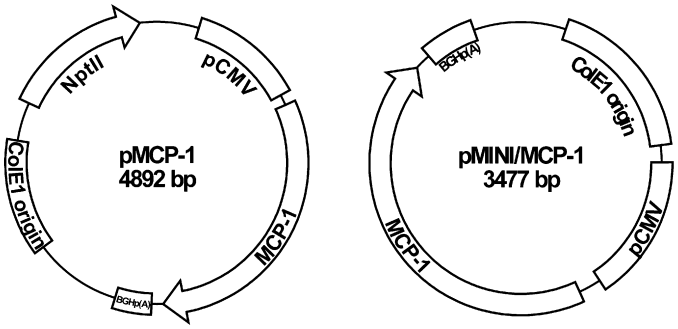

Fig. 2. Plasmid map of the progenitor vector pMCP-1 and its antibiotic resistance-free derivative pMINI/MCP-1, indicating the origin of replication (ColE1 origin), the kanamycin resistance gene (NptII), the cytomegalovirus early promoter (pCMV), the therapeutic gene monocyte and chemo attractant protein (MCP-1) and the bovine growth hormone polyadenylation site (BGHp(A)).