J Ind Microbiol Biotechnol (2009) 36:521–530
DOI 10.1007/s10295-008-0522-7

ORIGINAL PAPER

DNA plasmid production in different host strains of *Escherichia coli*

Adam Singer · Mark A. Eiteman · Elliot Altman

Received: 21 August 2008 / Accepted: 19 December 2008 / Published online: 10 January 2009

Abstract We compared plasmid DNA production in 13 strains of *Escherichia coli* in shake flasks using media containing glucose or glycerol. DNA yield from either carbon source showed small correlation with maximum growth rate. Three strains, SCS1-L, BL21 and MC4100, were selected for a controlled exponential fed-batch process at a growth rate of 0.14 h^{-1} to an optical density of about 70, followed by a four-hour heat treatment. Prior to heat treatment, SCS1-L generated 15.4 mg DNA/g, BL21 generated 11.0 mg DNA/g and MC4100 generated 7.9 mg DNA/g, while after heat treatment the strains attained DNA yields, respectively, of 18.0, 15.0 and 6.8 mg/g. The strains also varied in their percentage of supercoiled DNA after heat treatment, with SCS1-L averaging 66% supercoiled, BL21 17% and MC4100 40%. We further investigated the two strains that yielded the highest percentage of supercoiled DNA (SCS1-L and MC4100) at a higher growth rate of 0.28 h^{-1}. At this condition, a slightly lower DNA yield was generated faster, and the percentage of supercoiled DNA increased. Heat treatment improved DNA yield, and surprisingly did so to a greater extent at the higher growth rate. As a consequence of these factors, higher growth rates might be advantageous for DNA production.

Keywords Fed-batch process · Supercoiled DNA · DNA yield · Heat treatment

A. Singer
Merial Ltd, 115 Transtech Drive, Athens, GA 30605, USA

M. A. Eiteman (✉) · E. Altman
Center for Molecular BioEngineering,
Driftmier Engineering, University of Georgia,
Athens, GA 30602, USA
e-mail: eiteman@engr.uga.edu

Introduction

Currently over 100 human DNA vaccines are in clinical trials [26], and plasmid DNA vaccines target major human diseases, including HIV, cancer, enteric pathogens, malaria and influenza [6, 14, 19, 23, 27, 41, 46]. The market for gene therapy-based medicines is expected to reach US $45 billion by 2010 [15]. Unfortunately, DNA vaccines require milligram dosages to elicit proper immune responses [3, 8, 26, 30]. Therefore, the volumetric and specific yields of DNA must be maximized during production. In order to achieve high DNA yields, high-density cultures of *Escherichia coli* have been widely utilized for DNA production [5, 42]. Studies have generally focused on only a very few strains such as DH5 [26, 30] or DH5α [37]. Recently, a comprehensive study of 17 different *E. coli* strains demonstrated significant differences in DNA yield and supercoiling, although for this study complex medium was used in shake flasks [52]. Currently, supercoiled DNA is thought to be the most effective form at transferring gene expression [33], and the US FDA recommends a minimum specification for supercoiled plasmid content (of greater than 80%) be established [47]. Industrially relevant production of plasmid DNA depends on many factors including choice of plasmid, media components, fermentation type/strategy and downstream processing [4, 9].

Although some studies indicate an optimal specific growth rate for maximal DNA yield [35, 39], low specific growth rates are generally associated with favoring plasmid DNA synthesis on a per cell basis [2, 12, 22, 25, 43]. Since a nutrient-limited fed-batch process routinely achieves cell densities of 40 g/L, volumetric plasmid yield is maximized by a process operated at a low feeding (i.e., growth) rate. For example, [7] observed a fivefold greater specific yield in a DO-stat, nutrient-limited fed-batch process ($\mu = 0.13\ h^{-1}$)

compared to non-limiting process ($\mu = 0.5–1.0\ h^{-1}$). Similarly, 70% greater specific yield was observed during the late growth phase ($\mu = 0.1\ h^{-1}$) compared to the early growth phase ($\mu = 0.48\ h^{-1}$) during a linear-feeding process [36].

Additional strategies such as temperature shock, chemical treatment, and amino acid starvation can increase yields for certain plasmids [40]. In particular, a temperature shift from 37°C to 42°C–45°C using pUC-based plasmids containing a G → A point mutation that affects initiation of DNA replication from the CoIE1 origin results in an elevated plasmid copy number [24, 28]. Because of this benefit, the current industrial practice for plasmid DNA production involves fed-batch growth of strains transformed with temperature-responsive plasmids [4]. Previous studies have not compared temperature shift in different strains, nor considered the relationship between specific growth rate and the effect of a temperature shift. Moreover, a strain screening procedure that correlates small-scale DNA production with an ultimate larger-scale process would be desirable for industrial production.

The goal of this study was to compare several *E. coli* strains for plasmid DNA production in a chemically defined medium suitable for commercial vaccine production. The strains each were transformed with a replication temperature sensitive plasmid derived from pUC and then compared for DNA production in shake-flasks using defined medium with either glycerol or glucose as the sole carbon source. Subsequently, plasmid DNA rate of production, yield and supercoiling by selected strains were compared at a larger scale using carbon-limited fed-batch conditions with a temperature shift. Within a limited group of strains, we sought first to determine whether shake flask (i.e., batch) results correlated with fed-batch results and second, to determine whether heat treatment and growth rate impacted DNA yield and supercoiling.

Materials and methods

Strains and plasmids

Strains used in this study are shown in Table 1. Transformed strains were maintained in a chemically defined medium (CDM) supplemented with 25% glycerol (v/w) at −70°C. The 4.655 kb plasmid pLL14 (Merial Ltd., Lyon France) used in this study is a derivative of pVR1012 [17]; Vical Inc, San Diego, CA) which contains a single promoter for the kanamycin resistance gene. A chemical transformation kit (Qbiogene, Irvine, CA) was used to transform pVR1012 into the different strains. Transformed strains were plated on CDM agar plates with 5 g/L glycerol and 100 mg/L kanamycin and incubated at 37°C for 24 h.

Clonal selection

After transformation on LB plates containing antibiotic, five single colonies of each strain were adapted to CDM plates using two passes. Each clone was grown in 3.0 mL for 10–12 h at 37°C and 200 rpm. Miniprep DNA was prepared and quantified, and for each strain one clone with high DNA production and growth was chosen. Working seed batches were produced and then frozen at −70°C with 15% (v/v) glycerol.

Table 1 Strains used in this study

Strain	Genotype	Source
BL21	B F- *dcm ompT hsdS* (r_B^-, m_B^-) *gal*	Stratagene
DH1	F- *λ- supE44 hsdR17 recA1 gyrA96 relA1 endA1 thi*-1	ATCC 33849
DH5α	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*argF*)U169 *recA1 endA1 hsdR17*(r_k^-, m_k^+) *phoA supE44 λ*$^-$ *thi-1 gyrA96 relA1*	Invitrogen
JM105	F- Δ(*lac proAB*) *lacI*^{q} *thi repsL endAl slcB15 hadR4 traD36 proAB* Δ(ZM15)	Pharmacia
JM109	e14$^-$(McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}ZΔ*M15*]	Promega
Mach 1	F- Φ80(*lacZ*) ΔM15 Δ*lac*X74 *hsd*R(r_K^- m_K^+) Δ*recA*1398 *endA1 tonA*	Invitrogen
MG1655	F- *λ- ilvG rfb*-50 *rph*-1	ATCC 700926
MC1061	F- Δ*lacX74 rpsL araD139* Δ*(ara leu)7697 galU galK hsdR mcrB thi*	Laboratory collection
MC4100	F- *araD*139 Δ(*argF-lac*)U169 *rpsL150 relA1 deoC1 rbsR fthD*5301 *fruA*25 *λ*	Laboratory collection
NM554	MC1061 *recA13*	Laboratory collection
SCS1-L	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1*	Merial Ltd., Lyon France, previously from Stratagene
SCS1-S	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1*	Stratagene
SE5000	MC4100 *recA1*	Laboratory collection

Media

Chemically defined medium (CDM) contained (per L) 6.0 g citric acid, 20.0 mg $MnSO_4 \cdot H_2O$, 8.0 mg $CoCl_2 \cdot 6H_2O$, 4.0 mg $ZnSO_4 \cdot 7H_2O$, 3.6 mg $AlCl_3 \cdot 6H_2O$, 3.97 mg $Na_2MoO_4 \cdot 4H_2O$, 3.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg H_3BO_3, 800 mg $MgSO_4 \cdot 7H_2O$, 52.8 mg $CaCl_2 \cdot 2H_2O$, 74.0 mg $FeSO_4 \cdot 7H_2O$, 8.0 g KH_2PO_4, 2.4 g $Na_2HPO_4 \cdot 2H_2O$, 750.0 mg $(NH_4)_2SO_4$, 8.0 g $(NH_4)_2HPO_4$, 130.0 mg NH_4Cl, and 10 mg thiamine HCl. For MC1061 and NM554, the medium also contained 20.0 mg/L leucine. Luria–Bertani (LB) medium contained (per L): 10.0 g bacto-tryptone, 5.0 g yeast extract, and 10.0 g NaCl. The pH of CDM was adjusted to 6.8 using 28% NH_4OH. Kanamycin (Km) was added after sterilization to all media at 100 mg/L.

Growth conditions

Flask cultures were grown at 37°C and 200 rpm in 250 mL baffled flasks containing 35 mL CDM with 5 g/L glycerol or glucose. Cultures were grown to an OD of 1. Fed-batch fermentations were carried out in a 7 L bioreactor (Applikon Biotechnology, Foster City, USA) using 3.5 L CDM with 5 g/L glucose and 2 mL of antifoam (Antifoam 1500, Dow Corning, Midland, USA). Vessels were inoculated with 35 mL of a flask culture containing CDM with 5 g/L glycerol grown to an OD of 1. Control was provided by BioXPert software, ADI 1030 Bio-controller and an ADI 1035 Bio-console (Applikon). Fermentations were carried out at 37°C, a pH of 6.8 (controlled with 15% NH_4OH) and an agitation of 1,000 rpm. Dissolved O_2 (DO) was maintained above 20% with aeration at 2.45 L/min (O_2 and air mixed as necessary). Aeration commenced when the DO initially reached 30%. Fed-batch mode was initiated when the initial glucose was exhausted, as indicated by the increase in DO. The feed solution contained 60% glucose (w/v), 8.5 g/L $MgSO_4 \cdot 7H_2O$ and 1 mL/L antifoam. Feed rates were controlled at exponentially increasing flow rates using a mini-pulse pump (Gilson Inc, Middleton, USA) and control algorithms [20] to maintain a constant specific growth rate (at either 0.14 or 0.28 h^{-1} as indicated). When the OD reached 60–70, the temperature was raised to 42°C over the course of about 10 min, while the feeding rate was reduced by 10% and held constant for 4-h heat treatment.

Sampling and analysis

Cell growth was measured as optical density (OD) at 562 nm (Pharmacia LKB Ultraspec III, St Albans, UK). This measurement was correlated to dry cell weight (DCW). For DCW measurement, 20 mL of cell culture was centrifuged (10 min at 3,000×g), the supernatant decanted, the pellet washed with DI water and the tube centrifuged twice again. The pellet was resuspended in DI water, poured into a tared weighing boat and dried at 60°C for 24 h**.** DNA samples were obtained using a column miniprep kit (Qiagen, CA) using the equivalent of 3 mL of an OD = 1 sample to ensure the binding capacity of the column was not exceeded. The absorbance was measured at 260, 280 and 320 nm, and the DNA concentration calculated via the spectrophotometer software for samples that fell in the linear range ($0.1 \leq A_{260} \leq 1.0$). All samples were deemed of high DNA purity as indicated by A_{260}:A_{280} of 1.8–2.0. Absence of contaminating chromosomal DNA was verified by gel analysis. Volumetric DNA yield is expressed as mass plasmid DNA per culture volume whereas specific DNA yield is expressed per dry cell weight. Percentage of plasmid supercoiled DNA was determined after the heat treatment using the protocol of [32]. Acetate was measured by liquid chromatography using a refractive index detector as previously described [10].

Results

DNA yield comparisons in shake flask cultures

DNA yields from plasmid pLL14 were compared in 13 strains of *E. coli* using a defined medium in shake flasks. Plasmid pLL14 contains a single G → A mutation that increases copy number in response to a temperature shift to 42°C [24]. These strains were compared for plasmid DNA yield and growth rates in an initial "screen" using glycerol and glucose (Table 2). Since these shake flasks were conducted in a batch mode only to an OD of 1 and without a temperature shift, the strains each grew at their maximum growth rate. Thus, the calculated DNA "volumetric" yields represent the plasmid DNA generated during each strain's maximum specific growth rate, but not one consistent growth rate. Volumetric yields using glycerol were 1–3 mg/L, while volumetric yields using glucose were 0.5–3 mg/L. SCS1-L, SCS1-S, BL21, SE5000 and NM554, JM109 consistently generated high DNA yields using either carbon source. Studies using the plasmid pUC9 (data not shown) also substantiated that the two strains consistently generating the least quantity of plasmid DNA were MC4100 and MG1655. The highest volumetric yield (SCS1-S) corresponds to a specific DNA yield of 8.7 mg/g cell.

Figure 1 shows the distribution of maximum growth rates and DNA yields for all the strains. We observed a slight negative relationship between DNA yield and maximum specific growth rate ($R^2 = 0.254$). Although exceptions did occur, generally the use of glucose as a carbon source resulted in higher maximum specific growth rate and lower DNA yield than the use of glycerol.

Table 2 DNA yield, maximum specific growth rate and acetate concentration for strains containing plasmid pLL14

Strain	DNA yield (mg/L)		μ_{MAX} (h^{-1})		Acetate (mg/L)	
	Glycerol	Glucose	Glycerol	Glucose	Glycerol	Glucose
BL21	2.61 (0.02)	2.34 (0.08)	0.45	0.77	0	0
DH1	1.76 (0.02)	1.09 (0.05)	0.61	0.90	0	60
DH5α	2.06 (0.16)	1.42 (0.03)	0.58	0.99	0	180
JM105	1.42 (0.09)	0.70 (0.08)	0.44	0.64	0	190
JM109	1.97 (0.18)	1.94 (0.06)	0.51	0.70	0	190
Mach 1	2.37 (0.16)	1.40 (0.03)	0.63	0.84	0	60
MC1061	1.78 (0.07)	1.62 (0.11)	0.32	0.35	0	80
MC4100	1.27 (0.05)	0.85 (0.06)	0.79	0.92	80	360
MG1655	1.02 (0.07)	0.50 (0.04)	0.49	0.80	0	80
NM554	2.62 (0.10)	1.81 (0.04)	0.39	0.56	0	210
SCS1-L	3.00 (0.12)	2.06 (0.13)	0.45	0.90	0	180
SCS1-S	3.04 (0.09)	3.03 (0.18)	0.42	0.38	0	80
SE5000	2.61 (0.20)	1.56 (0.08)	0.57	1.00	0	200

Strains were grown in CDM in shake flasks to an OD of 1. Standard deviation of yield measurements is shown in parentheses

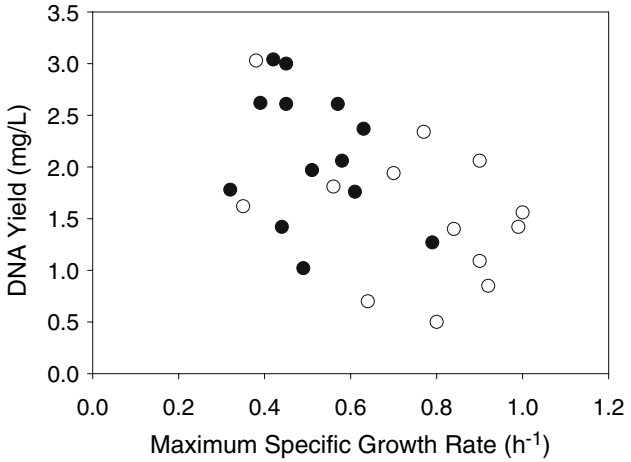

Fig. 1 Growth rates and volumetric DNA yields for various *E. coli* strains (all those listed in Table 1) containing the pLL14 plasmid and grown in defined medium containing either glycerol (*filled circle*) or glucose (*open circle*). Strains were grown in CDM in shake flask to an OD of 1

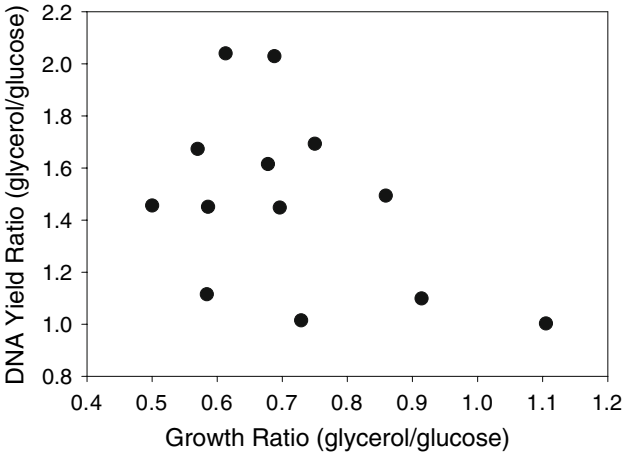

Fig. 2 Comparison of DNA yield ratio and specific growth ratio for *E. coli* strains (all those listed in Table 1) containing the pLL14 plasmid (*filled circle*). Strains were grown in CDM in shake flask to an OD of 1

As another means to compare the two carbon sources, for each strain we calculated the growth ratio: the maximum specific growth rate (μ_{MAX}) observed with glycerol divided by the μ_{MAX} observed with glucose. Similarly, the yield ratio for a particular strain was calculated as the DNA yield observed when glycerol was the carbon source divided by the DNA yield observed with glucose. Because strains generally had a higher μ_{MAX} with glucose than with glycerol, the growth ratio was generally less than 1 whereas the yield ratio was greater than 1. Figure 2 shows all yield ratios for all strains as functions of the respective growth ratios. Although a few strains with a low growth ratio also had a low yield ratio, generally the highest yield ratios were found with strains having growth ratios between 0.5 and 0.75. Thus strains with the greatest differences in maximum growth rate for each substrate generally had a greater difference in yield for each substrate. Of the strains studied, only SCS1-S attained a higher specific growth rate using glycerol than using glucose, and this strain also showed a very low DNA yield ratio, with the DNA yield observed when the strain grew on glucose being approximately equal to the DNA yield using glycerol.

Acetate is generated by "overflow" metabolism, and its formation from glucose has long been associated with an imbalance between substrate uptake and the utilization of building block compounds needed for protein synthesis [11, 16]. Because plasmid production similarly increases metabolic demands on a cell, we wished to determine whether any relationship existed between acetate formation and DNA yield in these strains. Acetate formation from

glucose correlated poorly with growth rate ($R^2 = 0.077$). Although no strain generated both a high acetate concentration and a high DNA yield, there was also poor correlation ($R^2 = 0.087$) between DNA yield and acetate formation when glucose was the carbon source (Fig. 3). Interestingly, the strain with the highest growth rate on glycerol (MC4100), also was the only strain generating acetate from that carbon source (Table 2), and was one of the worst plasmid DNA producers. High acetate generation by MC4100 can be attributed to its higher growth rate [11] and the elevated expression of RpoS, which is known to be related to acetate-stress response [1, 44].

A *recA* mutation is thought to reduce plasmid degradation [4, 5], and therefore two sets of isogenic *recA*$^+$/*recA*$^-$ pairs were examined in this study. These pairs were MC4100-SE5000 and MC1061-NM554. Both SE5000 and NM554 are *recA* mutants of their respective parent strain. In each case (two carbon sources and two isogenic pairs), the *recA* strain yielded more DNA than the control strain containing this gene. The difference between the pairs was greatest for shake flasks in which glycerol was the carbon source (for example, a 106% improvement for the SE5000 versus MC4100). Also, except for MC4100/SE5000 on glycerol, the *recA* strain showed a higher maximum growth rate than the control strain in the defined medium.

Fed-batch processes

Three strains of *E. coli* containing the pLL14 plasmid were selected for the comparison of plasmid DNA production using a fed-batch process: SCS1-L, BL21 and MC4100. These strains reflected a range of DNA yields in the shake flask studies. Each strain was grown under glucose-limited conditions (using defined medium) at a controlled specific growth rate of 0.14 h^{-1}. Glucose was selected because this carbon source is typically used for industrial fed-batch fermentations. After reaching an OD of about 70, each process was subject to a 4-h heat treatment at 42°C as described in "Materials and methods".

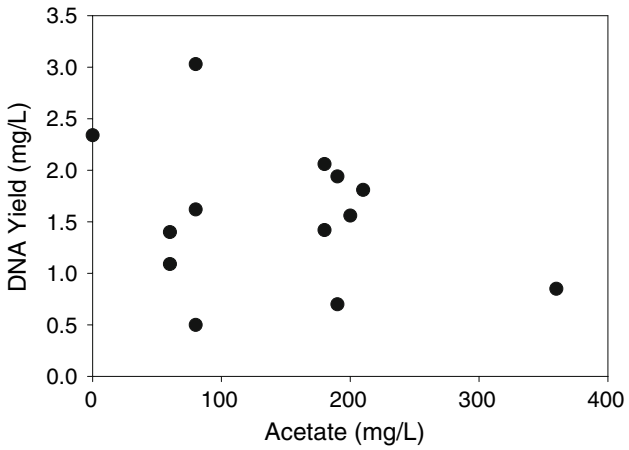

Fig. 3 Acetate concentrations and volumetric DNA yields for various *E. coli* strains (all those listed in Table 1) containing the pLL14 plasmid (*filled circle*). Strains were grown in CDM in shake flask to an OD of 1

Figure 4 shows a representative time course for temperature, OD, dissolved oxygen, and total volume for the fed-batch process using SCS1-L. Similar to other experiments at this growth rate, the glucose feed began at about 13 h (at an OD of 8), oxygen-enriched air commenced at about 27 h (OD of 40), heat treatment commenced at 33 h (OD of 70), and the final cell harvest was at 37 h. We also measured volumetric and specific DNA yields, and acetate at selected times during each duplicated fermentation (Table 3). Of the three forms of DNA (supercoiled, open circular and linear) linear and open circular are undesirable for DNA vaccine production because of their greater risk in integrating into the host genome, and because these forms give lower levels of expression once transfected, and hence have lower efficacy [29, 49]. Therefore, in our experiments the percentage of supercoiled DNA was also determined at the end of the heat treatment.

These three strains have differing capacities to generate plasmid DNA. Prior to heat treatment, SCS1-L generated the most DNA (average of 15.4 mg/g), followed by BL21 (11.0 mg/g) and MC4100 (7.9 mg/g). The order of DNA yields for these strains was identical to the results obtained for those shake flasks containing glycerol (Table 2), despite the shake flask experiments achieving lower cell density and being conducted at their maximum growth rate. In the shake flask studies using glucose, BL21 produced a greater DNA yield than SCS1-L, but SCS1-L grew at a much higher maximum growth rate than BL21. In the fed-batch experiment, we also observed significantly different values for the percentage of supercoiled DNA among the three strains. SCS1-L averaged 66% supercoiled DNA, BL21 17% and MC4100 40%. Thus, SCS1-L generated 10.2 mg of supercoiled DNA/g, BL21 generated 1.9 mg/g, and MC4100 generated 3.2 mg/g.

We next conducted fed-batch processes operated at a growth rate of 0.28 h^{-1} for the two strains that yielded the highest percentage of supercoiled DNA, SCS1-L and MC4100. These fed-batch processes obviously had shorter durations than the 0.14 h^{-1} processes, with the final harvest reached in 23–25 h. The results are also summarized in Table 3. For this higher growth rate, SCS1-L again generated DNA at a higher yield than MC4100 (10.7 vs. 6.2 mg/g). Surprisingly, for both strains the percentage of supercoiled DNA was greater at a growth rate of 0.28 h^{-1} than at 0.14 h^{-1}. The increased growth rate resulted in an 8% increase in the percentage of supercoiled DNA for SCS1-L, and an 88% increase in the percentage of supercoiled DNA for MC4100. The percentage of supercoiling was very consistent between duplicate experiments. Figure 5a, b show the DNA yields for one set of experiments with

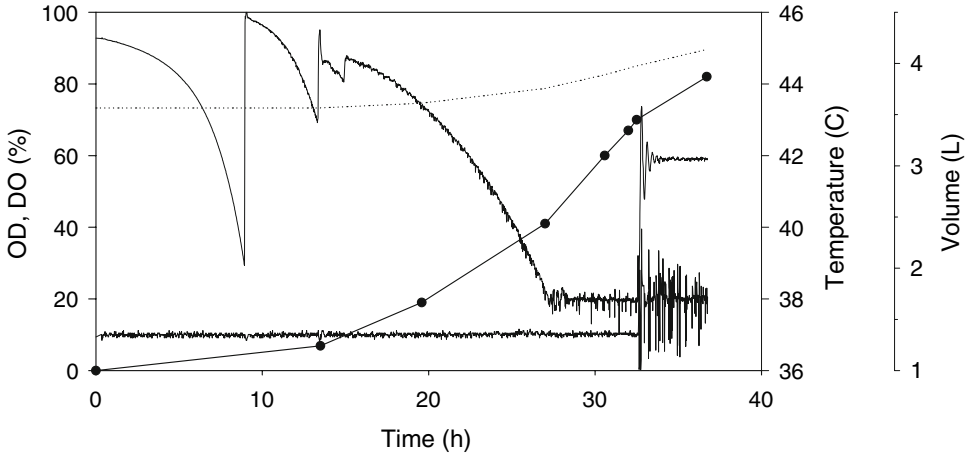

Fig. 4 Time course of fermentation showing dissolved oxygen (DO), OD (*filled circle*), temperature (step change between 37 and 42°C) and volume (*dotted line*). This fermentation is SCS1-L growing at a growth rate of 0.14 h^{-1}

Table 3 All fed-batch experiments conducted using three strains transformed with pLL14 plasmid

Strain	Prior to 4-h heat treatment				After 4-h heat treatment				
	OD	Acetate (mg/L)	Volumetric yield (mg/L)	Specific yield (mg/g)	OD	Acetate (mg/L)	Volumetric yield (mg/L)	Specific yield (mg/g)	Percent supercoil (%)
Growth rate = 0.14 h^{-1}									
BL21	54	20	254 (19)	11.7	71	100	438 (6)	15.4	17.6
BL21	66	80	265 (26)	10.3	88	100	504 (17)	14.4	17.0
MC4100	65	10	200 (8)	8.1	82	150	225 (13)	7.4	38.9
MC4100	65	10	189 (3)	7.6	80	100	193 (6)	6.2	41.2
SCS1-L	70	30	416 (22)	15.2	82	0	595 (7)	18.6	67.1
SCS1-L	71	0	430 (18)	15.5	86	10	595 (5)	17.7	65.6
Growth rate = 0.28 h^{-1}									
MC4100	67	0	179 (9)	7.0	101	20	344 (8)	9.0	78.4
MC4100	53	0	109 (5)	5.4	86	20	291 (18)	8.9	72.3
SCS1-L	61	0	241 (11)	10.1	91	0	599 (14)	16.9	73.2
SCS1-L	61	0	269 (40)	11.3	90	0	584 (21)	16.6	70.5

Glucose-limited fed-batch processes were operated at constant growth rates. DNA yields are the mean of triplicate samples (standard deviation in parentheses)

SCS1-L at the specific growth rates of 0.14 and 0.28 h^{-1}, respectively. Figure 6 shows the volumetric DNA yield for all fed-batch experiments, demonstrating the lower formation of DNA in MC4100 compared to the other strains.

As noted in "Materials and methods", the temperature was raised to 42°C for 4 h, and generally this heat treatment improved the specific DNA yield. For example, for SCS1-L, the heat treatment increased the specific yield by 18%. Since the cell density also continued to increase during the four-hour treatment period, the increase in specific yield corresponds to an even greater percentage increase in volumetric yield (for example, 41% for SCS1-L). More than 30% of the total plasmid DNA was commonly generated during the heat treatment. With SCS1-L at a growth rate of 0.14 h^{-1} as an example, of the 2,530 mg of total DNA generated by the cells ending at just over 4 L volume, more than 800 mg of DNA were generated in the final 4 h during the heat treatment. Similarly for SCS1-L at the 0.28 h^{-1} growth rate, 1,600 mg of the total 2,600 mg plasmid DNA (over 60%) was generated during the heat treatment. The exception to the rule that heat treatment improved yield was MC4100 at a low growth rate. In this case heat treatment reduced the DNA yield by about 20%.

Growth rate also impacted the DNA yield, and there was a relationship between growth rate and heat treatment. Prior to the heat treatment, DNA yield was consistently lower in cells grown at the higher growth rate. For example, SCS1-L grown at 0.28 h^{-1} generated 30% less DNA per cell than SCS1-L grown at 0.14 h^{-1} (10.7 vs. 15.4 mg/g). Interestingly, this difference disappeared or reversed after the heat treatment: SCS1-L grown at 0.28 h^{-1} generated only 8% less DNA per cell than SCS1-L growing at 0.14 h^{-1}, while MC4100 generated 32% more DNA per cell when grown at the higher growth rate. In other words, heat treatment had a

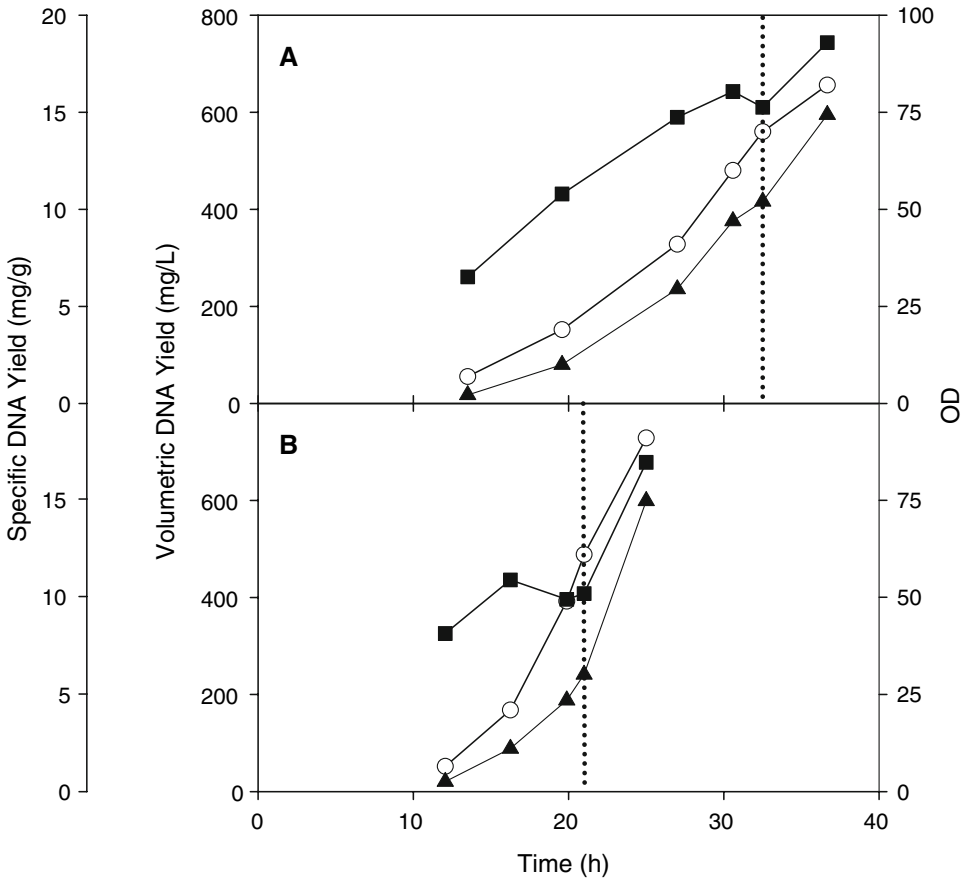

Fig. 5 Production of plasmid DNA using SCS1-L during a glucose-limited fed-batch process with nominal specific growth rate of 0.14 h^{-1} (**a**) and 0.28 h^{-1} (**b**): volumetric DNA yield (*filled triangle*), specific DNA yield (*filled square*), and OD (*open circle*). In each case, the initiation of the 4 h period of heat treatment is shown as a *vertical dotted line*

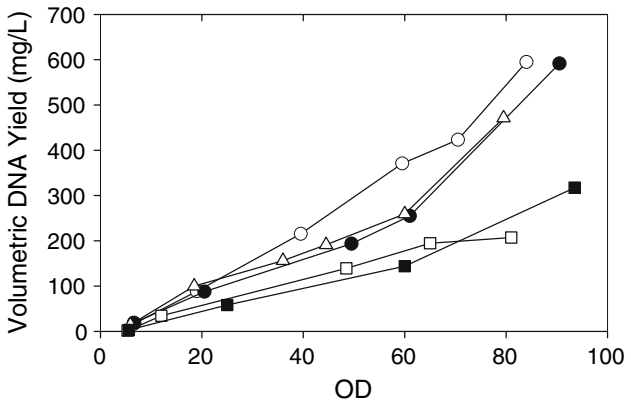

Fig. 6 Comparison of volumetric DNA yields by SCS1-L (*open circle*, *filled circle*), MC4100 (*open square*, *filled square*) and BL21 (*open triangle*) during glucose-limited fed-batch processes having nominal specific growth rates of 0.14 h^{-1} (*hollow symbols*) and 0.28 h^{-1} (*filled symbols*)

much more beneficial effect on DNA production to cells that were growing at the higher growth rate. This observation indicates that DNA production during the 4-h heat treatment for faster growing cells was remarkably high (Table 4). For example, SCS1-L generated DNA at a volumetric rate of 47 mg/L h during the heat treatment only when grown at 0.14 h^{-1}, whereas the cells generated DNA at 95 mg/L h when grown at 0.28 h^{-1}. This comparison also is true for the rate of DNA produced per cell during heat treatment. For SCS1-L during heat treatment the specific rate of plasmid DNA production was 1.56 mg/gh (±0.05 mg/gh) at 0.14 h^{-1} compared with 3.26 mg/gh (±0.17 mg/gh) at 0.28 h^{-1}. Cells were able to generate DNA twice as quickly during heat treatment when grown twice as quickly. Although heat treatment was also accompanied by a constant feed rate, both of which decreased the growth rate, the results demonstrate two effects. First, the results demonstrate that a heat treatment significantly increases specific and volumetric DNA yield. Second, heat treatment is particularly beneficial for cells grown at a high growth rate prior to the heat treatment.

A higher growth rate permitted the cells to reach the same OD in less time, and as noted above heat treatment particularly benefited cells grown at the higher growth rate. Thus, the overall rate of DNA production as reflected by the volumetric productivity was higher at the higher growth rate (Table 4). For SCS1-L, the overall volumetric DNA productivity was 46% greater when the fed-batch process was conducted at 0.28 h^{-1} compared to a growth rate of 0.14 h^{-1}. For MC4100, the volumetric DNA productivity was 130% greater when the fed-batch process was conducted at the higher growth rate. Since both of these two

Table 4 Effect of exponential fed-batch growth rate and 4 hour heat treatment on volumetric DNA productivity

Strain	DNA productivity (mg/L h)		
	At start of heat treatment	During heat treatment	At end of heat treatment
Growth rate = 0.14 h^{-1}			
BL21	8.5	57.5	13.6
MC4100	6.5	6.9	6.1
SCS1-L	13.1	47.0	16.2
Growth rate = 0.28 h^{-1}			
MC4100	7.8	47.6	14.0
SCS1-L	12.2	95.0	23.7

strains also generated a higher percentage of supercoiled DNA at the higher growth rate, the improvement in the rate of supercoiled DNA production was even greater.

Like the shake flask results, no correlation was observed between acetate formation and DNA yield. SCS1-L at both growth rates resulted in very low acetate accumulation. Although BL21 and MC4100 showed similar acetate concentrations after heat treatment, these two strains generated quite different amounts of DNA. The most acetate formed during the heat treatment occurred for MC4100 at low growth rate, which also was the one instance in which heat treatment did not benefit DNA production.

Discussion

Recent work on DNA plasmid production generally has focused on maximizing yield, and most studies have examined only a very few variables, for example, medium components [31, 51] or slight changes in growth rate [7, 36]. The cost in manpower, materials and time associated with commercial-scale processes encourages the development of a small-scale screening protocol for candidate strains and fermentation conditions. One goal of this work was to determine if results at a small-volume flask scale would correlate with production at a larger (3.5–4.5 L) fed-batch process. Shake flask studies indicated that BL21, SCS1-S, and SCS1-L generated the greatest DNA from glucose, while MC4100, JM105, and MG1655 generated the least. In fed-batch studies SCS1-L produced significantly greater DNA yields than MC4100 at two different growth rates. Our results show that small-scale shake flask experiments can aide in strain selection for DNA production, even though they operate exclusively at the maximum growth rate. Another recent study similarly compared 17 strains and three plasmids in shake flasks using complex medium instead of defined medium [52]. In this previous study for a 5.6 kb plasmid, MG1655 and BL21 yielded the most DNA at the small scale, but larger scale studies were not completed.

The fed-batch processes at the low specific growth rate of 0.14 h^{-1} yielded similar DNA (about 15 mg/g prior to heat treatment) to previous studies [7, 36]. Considering the results before the heat treatment, our fed-batch process generated about 2–3 times the specific DNA yield of the shake flask studies (for example, 6.7 mg/g in glucose shake flask versus 11.0 mg/g in 0.14 h^{-1} fed-batch process for BL21, 2.4 vs. 7.8 mg/g for MC4100). A previous study with lower DNA yields reported a greater difference in yield between shake flasks and a lower growth rate fed-batch process [32]. Using a higher growth rate of 0.28 h^{-1}, we reduced the DNA yields achieved prior to the heat treatment by 28% and 20% for SCS1-L and MC4100, respectively, as compared to the low growth rate. It is widely accepted that copy numbers of ColE1-type plasmids are inversely proportional to the specific growth rate [21, 25, 34, 35, 38, 39], and the results of this study are consistent with previous observations. The conclusions are more complicated, however, when the impact of heat treatment is also considered. Although previous studies have suggested that the optimal growth rate for DNA production is less than 0.20 h^{-1} [7, 39], our study shows when heat treatment is included using pUC-based plasmids, higher growth rates (at least prior to the heat treatment) may provide an advantage.

Heat treatment was previously demonstrated to improve volumetric and specific yields [24, 50]. The point mutation on the pLL14 plasmid maps upstream of the RNAI promoter and affects the negative regulation of replication at high temperatures. RNAI inhibition alters the initiation of RNA transcription [13, 48], and two proteins that regulate priming and copy number by interacting with RNAI have also been implicated [45, 48]. Although the fed-batch operation at 0.28 h^{-1} did result in lower DNA yield than the operation at 0.14 h^{-1} in the absence of heat treatment, the difference was not as great as expected. Using heat treatment surprisingly caused the benefit of operating at lower growth rates to disappear. At the growth rate of 0.14 h^{-1}, heat treatment improved the DNA yield in SCS1-L and BL21, but not MC4100. At the growth rate of 0.28 h^{-1}, heat treatment improved the DNA yield in both SCS1-L and MC4100 substantially.

Hecker et al. [18] and Reinikainen et al. [34] found that plasmid yields were highest after the culture had entered the stationary phase. Heat treatment, which was accompanied by a reduction in feed rate, resulted in a 50% decrease in growth rate for strains at either growth rate, and therefore a heat treatment may mimic a stationary phase for DNA production. Since different strains responded differently to heat treatment, the results also suggest that heat treatment does not exclusively affect the plasmid, but that the strain

also plays a role in determining the benefit that heat treatment provides.

Higher growth rate did not adversely affect the "quality" of the DNA as measured by percentage supercoiling. In fact, the higher growth rate improved the percentage of supercoiled DNA, by 88% in the case of strain MC4100. These results are in contrast to a previous report which focused on *E. coli* DH5α and compared the lower growth rates of 0.05 and 0.1 h^{-1} [32]. Since significant differences in supercoiling were observed between the strains, the fraction of supercoiled DNA appears also to be determined by the strain. This conclusion using a fed-batch process at two controlled growth rates is consistent with recent batch results using complex media, in which a wide range of percentage supercoiling was observed among different strains [52].

Finally, the higher growth rate permitted the DNA to be generated more quickly, which resulted in a much greater DNA productivity. In conclusion, although other factors such as oxygenation and heat duty on the fermenter will be factors to consider in designing process, it may be advantageous to operate at greater rates than previously thought "optimal" conditions for DNA production by virtue of the increased rate of production and the benefit of heat treatment.

Acknowledgments The authors acknowledge S. E. Kushner at the University of Georgia for helpful advice, and Amanda Randall at Merial for help with the supercoiling analyses.

References

1. Arnold CN, McElhanon J, Lee A, Leonhart R, Siegele DA (2001) Global analysis of *Escherichia coli* gene expression during the acetate-induced acid tolerance response. J Bacteriol 183(7):2178–2186. doi:10.1128/JB.183.7.2178-2186.2001
2. Bentley WE, Mirjalili N, Andersen DC, Davis RH, Kompala DS (1990) Plasmid-encoded protein: the principal factor in the metabolic burden associated with recombinant bacteria. Biotechnol Bioeng 35:668–681. doi:10.1002/bit.260350704
3. Berzofsky JA, Masaki T, SangKon O, Belyakov IM, Ahlers JD, Janik JE, Morris JC (2004) Progress on new vaccine strategies for the immunotherapy and prevention of cancer. J Clin Invest 113:1515–1525
4. Carnes AE (2005) Fermentation design for the manufacture of therapeutic plasmid DNA. BioProcess Intl 3:36–44
5. Carnes AE, Hodgson CP, Williams JA (2006) Inducible *Escherichia coli* fermentation for increased plasmid DNA production. Biotechnol Appl Biochem 45:155–166. doi:10.1042/BA20050223
6. Catanzaro AT, Roederer M, Koup RA, Bailer RT, Enama ME, Nason MC, Martin JE, Rucker S, Andrews CA, Gomez PL, Mascola JR, Nabel GJ, Graham BS (2007) Phase I clinical evaluation of a six-plasmid multiclade HIV-1 DNA candidate vaccine. Vaccine 25:4085–4092. doi:10.1016/j.vaccine.2007.02.050
7. Chen W, Graham C, Ciccarelli RB (1997) Automated fed-batch fermentation with feed-back controls based on dissolved oxygen (DO) and pH for production of DNA vaccines. J Ind Microbiol Biotechnol 18:43–48. doi:10.1038/sj.jim.2900355
8. Donnelly J, Berry K, Ulmer J (2003) Technical and regulatory hurdles for DNA vaccines. Int J Parasitol 33:457–467. doi:10.1016/S0020-7519(03)00056-0
9. Durland RH, Eastman EM (1998) Manufacturing and quality control of plasmid-based gene expression systems. Adv Drug Deliv Rev 30:33–48. doi:10.1016/S0169-409X(97)00105-1
10. Eiteman MA, Chastain MJ (1997) Optimization of the ion-exchange analysis of organic acids from fermentation. Anal Chim Acta 338:69–75. doi:10.1016/S0003-2670(96)00426-6
11. Eiteman MA, Altman E (2006) Overcoming acetate in *Escherichia coli* recombinant protein fermentations. Trends Biotechnol 24:530–536. doi:10.1016/j.tibtech.2006.09.001
12. Engberg B, Nordström K (1975) Replication of R-factor R1 in *Escherichia coli* K-12 at different growth rates. J Bacteriol 123(1):179–186
13. Fitzwater T, Zhang X, Elblel R, Polisky B (1988) Conditional high copy number ColE 1 mutants: resistance to RNA1 inhibition in vivo and in vitro. EMBO J 7:3289–3297
14. Gilbert SC, Moorthy VS, Andrews L, Pathan AA, McConkey SJ, Vuola JM, Keating SM, Berthoud T, Webster D, McShane H, Hill AV (2006) Synergistic DNA-MVA prime-boost vaccination regimes for malaria and tuberculosis. Vaccine 24:4554–4561. doi:10.1016/j.vaccine.2005.08.048
15. Glaser V (1997) Promise of gene therapy for treating disease remains on trial in the clinic. Genet Eng News 14:33–34
16. Glick BR (1995) Metabolic load and heterologous gene expression. Biotechnol Adv 13:247–261. doi:10.1016/0734-9750(95)00004-A
17. Hartikka J, Sawdey M, Cornefert-Jensen F, Margalith M, Barnhart K, Nolasco M, Vahlsing HL, Meek J, Marquet M, Hobart P, Norman J, Manthorpe M (1996) An improved plasmid DNA expression vector for direct injection into skeletal muscle. Hum Gene Ther 7:1205–1217. doi:10.1089/hum.1996.7.10-1205
18. Hecker M, Schroeter A, Mach F (1985) *Escherichia coli relA* strains as hosts for amplification of pBR322 plasmid DNA. FEMS Microbiol Lett 29:331–334. doi:10.1111/j.1574-6968.1985.tb00885.x
19. Herrmann JE (2006) DNA vaccines against enteric infections. Vaccine 24:3705–3708. doi:10.1016/j.vaccine.2005.07.012
20. Jones RC, Anthony RM (1977) The relationship between nutrient feed rate and specific growth rate in fed batch cultures. Appl Microbiol Biotechnol 4:87–92
21. Jones-Prather K, Edmonds MC, Herod JW (2006) Identification and characterization of IS1 transposition in plasmid amplification mutants of *E. coli* clones producing DNA vaccines. Appl Microbiol Biotechnol 73:815–826. doi:10.1007/s00253-006-0532-1
22. Jones-Prather K, Sager KS, Murphy J, Chartrain M (2003) Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. Enzyme Microb Technol 33:865–883. doi:10.1016/S0141-0229(03)00205-9
23. Laddy DJ, Yan J, Corbitt N, Kobasa D, Kobinger GP, Weiner DB (2007) Immunogenicity of novel consensus-based DNA vaccines against avian influenza. Vaccine 25:2984–2989. doi:10.1016/j.vaccine.2007.01.063
24. Lahijani R, Hulley G, Soriano G, Horn NA, Marquet M (1996) High-yield production of pBR322-derived plasmids intended for human gene therapy by employing a temperature-controllable point mutation. Hum Gene Ther 7:1971–1980. doi:10.1089/hum.1996.7.16-1971
25. Lin-Chao S, Bremer H (1986) Effect of the bacterial growth rate on replication control of plasmid pBR322 in *Escherichia coli*. Mol Gen Genet 203:143–149. doi:10.1007/BF00330395
26. Listner K, Bentley L, Okonkowski J, Kistler C, Wnek R, Caparoni A, Junker B, Robinson D, Salmon P, Chartrain M (2006) Development of a highly productive and scalable plasmid DNA production platform. Biotechnol Prog 22:1335–1345. doi:10.1021/ bp060046h

27. Lowe DB, Shearer MH, Jumper CA, Kennedy RC (2007) Towards progress on DNA vaccines for cancer. Cell Mol Life Sci 64:2391–2403. doi:10.1007/s00018-007-7165-0
28. Miki T, Yasukochi T, Nagatani H, Furuno M, Orita T, Yamada H, Imoto T, Horiuchi T (1987) Construction of a plasmid vector for the regulatable high level expression of eukaryotic genes in *Escherichia coli*: an application to overproduction of chicken lysozyme. Protein Eng 1:327–332. doi:10.1093/protein/1.4.327
29. Nichols WW, Ledwith BJ, Manam SV, Troilo PJ (1995) Potential DNA vaccine integration into host cell genome. Ann N Y Acad Sci 27:30–39. doi:10.1111/j.1749-6632.1995.tb44729.x
30. Okonkowski J, Kizer-Bentley L, Listner K, Robinson D, Chartrain M (2005) Development of a robust, versatile, and scalable inoculum train for the production of a DNA vaccine. Biotechnol Prog 21:1038–1047. doi:10.1021/bp040041p
31. O'Kennedy RD, Baldwin C, Keshavarz-Moore E (2000) Effects of growth medium selection on plasmid DNA production and initial processing steps. J Biotechnol 76:175–183. doi:10.1016/S0168-1656(99)00187-X
32. O'Kennedy RD, Ward JM, Keshavarz-Moore E (2003) Effects of fermentation strategy on the characteristics of plasmid DNA production. Biotechnol Appl Biochem 37:83–90. doi:10.1042/BA20020099
33. Prazeres DMF, Ferreira GNM, Monteiro GA, Cooney CL, Cabral JMS (1999) Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks. Trends Biotechnol 17:169–174. doi:10.1016/S0167-7799(98)01291-8
34. Reinikainen P, Korpela K, Nissinen V, Olkku J, Soderlund H, Markkanen P (1989) *Escherichia coli* plasmid production in a fermenter. Biotechnol Bioeng 33:386–393. doi:10.1002/bit.260330403
35. Reinikainen P, Virkajärvi I (1989) *Escherichia coli* growth and plasmid copy numbers in continuous cultivations. Biotechnol Lett 11(4):225–230. doi:10.1007/BF01031568
36. Rozkov A, Avignone-Rossa CA, Ertl PF, Jones P, O'Kennedy RD, Smith JJ, Dale JW, Bushell ME (2006) Fed batch culture with declining specific growth rate for high-yielding production of a plasmid containing a gene therapy sequence in *Escherichia coli* DH1. Enzyme Microb Technol 39:47–50. doi:10.1016/j.enzmictec.2005.09.005
37. Schmidt T, Friehs K, Flaschel E, Schleef M (2003) Method for the isolation of plasmid DNA. US Patent 6,664,078
38. Seo J, Bailey JE (1985) Effects of recombinant plasmid content on growth properties and cloned gene product formation in *Escherichia coli*. Biotechnol Bioeng 27:1668–1674. doi:10.1002/bit.260271207
39. Seo J, Bailey JE (1986) Continuous cultivation of recombinant *Escherichia coli*: existence of an optimum dilution rate for maximum plasmid and gene product concentration. Biotechnol Bioeng 28:1590–1594. doi:10.1002/bit.260281018
40. Shamlou PA (2003) Scaleable processes for the manufacture of therapeutic quantities of plasmid DNA. Biotechnol Appl Biochem 37:207–218. doi:10.1042/BA20030011
41. Sheets RL, Stein J, Manetz S, Duffy D, Nason M (2006) Biodistribution of DNA plasmid vaccines against HIV-1, ebola severe acute respiratory syndrome, or west Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. Toxicol Sci 91:610–619. doi:10.1093/toxsci/kfj169
42. Shiloach J, Fass R (2005) Growing *E. coli* to high cell density: a historical perspective on method development. Biotechnol Adv 23:345–357. doi:10.1016/j.biotechadv.2005.04.004
43. Siegel R, Ryu DD (1985) Kinetic study of instability of recombinant plasmid pPLc23*trpAI* in *E. coli* using two-stage continuous culture system. Biotechnol Bioeng 27(1):28–33. doi:10.1002/bit.260270105
44. Spira B, Hu X, Ferenci T (2008) Strain variation in ppGpp concentration and RpoS levels in laboratory strains of *Escherichia coli* K-12. Microbiology 154:2887–2895. doi:10.1099/mic.0.2008/018457-0
45. Tamm T, Polisky B (1985) Characterization of the ColE1 primer-RNA1 complex: analysis of a domain of ColE1 RNA1 necessary for its interaction with primer RNA. Proc Natl Acad Sci USA 82:2257–2261. doi:10.1073/pnas.82.8.2257
46. Tuteja R (2002) DNA vaccine against malaria: a long way to go. Crit Rev Biochem Mol Biol 37:29–54. doi:10.1080/10409230290771447
47. US Dept of Health and Human Services, Food and Drug Administration (2007) Guidance for industry: considerations for plasmid DNA vaccines for infectious disease indications, November 2007
48. Wang Z, Yuan Z, Henggeb UR (2004) Processing of plasmid DNA with ColE1-like replication origin. Plasmid 51:149–161. doi:10.1016/j.plasmid.2003.12.002
49. Weintraub H, Cheng PF, Conrad K (1986) Expression of transfected DNA depends on DNA topology. Cell 46:115–122. doi:10.1016/0092-8674(86)90865-2
50. Wong EM, Muesing MA, Polisky B (1982) Temperature-sensitive copy number mutants of ColE1 are located in an untranslated region of the plasmid genome. Proc Natl Acad Sci USA 79:3570–3574. doi:10.1073/pnas.79.11.3570
51. Xu Z, Shen W, Chen H, Cen P (2005) Effects of medium composition on the production of plasmid DNA vector potentially for human gene therapy. J Zhejiang Univ Sci 6B:396–400. doi:10.1631/jzus.2005.B0396
52. Yau SY, Keshavarz-Moore E, Ward J (2008) Host strain influences on supercoiled plasmid DNA production in *Escherichia coli*: implications for efficient design of large-scale processes. Biotechnol Bioeng 101:529–544. doi:10.1002/bit.21915