



## Plasmid amplification in *Escherichia coli* after temperature upshift is impaired by induction of recombinant protein synthesis

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Received 22 August 2001; Accepted 18 September 2001

**Key words:** *Escherichia coli*, inclusion bodies, plasmid amplification, recombinant protein

### Abstract

Production of recombinant proteins often interferes with the physiology of the host organism by causing stress responses. In recombinant *Escherichia coli*, the cellular content of ColE1-derived plasmids and, consequently, the synthesis of the constitutively synthesized plasmid-encoded proteins generally increases after a temperature upshift. Simultaneous induction of inducible recombinant proteins that are synthesized at high levels and tend to form inclusion bodies, however, attenuates the plasmid amplification. This phenomenon was observed using temperature- as well as IPTG-inducible expression systems. Thus, high-level recombinant gene expression in connection with inclusion body formation does not only interfere with host cell but also with plasmid-related functions.

### Introduction

A major parameter to optimize recombinant protein production is the temperature. A temperature upshift enhances the synthesis of plasmid-encoded proteins by two ways: relative to cellular proteins by an increase in the plasmid copy number at higher temperatures (Kaprálék *et al.* 1998) and absolutely by accelerating protein synthesis in general (Farewell & Neidhardt 1998). For example, the synthesis rate of a recombinant protein can equal 160% of the total protein synthesis of uninduced cells with a temperature-inducible expression system (Hoffmann & Rinas 2000). Also, a temperature upshift from 30 °C to 42 °C simultaneous with the induction of recombinant protein synthesis using an IPTG-inducible expression system increases the total protein synthesis (including host proteins) three times (Pilon *et al.* 1996). Temperature-inducible expression systems are popular for their ease and low cost of induction. On the other hand, higher temper-

atures encourage inclusion body formation, thereby reducing the yield of active product (Schein 1989).

Moreover, plasmid presence and recombinant protein production entail negative consequences on the host physiology, as summarized by Bailey (1993) and Glick (1995). Recombinant protein production can trigger the major stress responses, such as the heat-shock response (Goff & Goldberg 1985) or the stringent response (Harcum & Bentley 1999). In some cases, recombinant protein production can even lead to destruction of ribosomes (Dong *et al.* 1995).

In this study, we examine how the induction of recombinant protein synthesis simultaneous to a temperature upshift affects the plasmid content of the cells and the synthesis of constitutive plasmid-encoded proteins.

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## Materials and methods

### Strains and plasmids

*Escherichia coli* strains TG1 *supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZΔM15]* (Carter *et al.* 1985) and BL21 *hsdS gal<sup>-</sup> Lon<sup>-</sup> OmpT<sup>-</sup>* (Studier & Moffat 1986) were used. The plasmid pλFGFB, derived from the expression vector pCYTEXP1, carries the gene encoding human basic fibroblast growth factor (hFGF-2) under the control of the bacteriophage λP<sub>R</sub>P<sub>L</sub> promoter tandem (Seeger *et al.* 1995). Both plasmids, pCYTEXP1 and pλFGFB, carry the gene encoding the temperature sensitive λcI857 repressor and the *amp* gene (Seeger *et al.* 1995). The plasmid pJHLbFGF carries the hFGF-2 gene under the control of the *tac* promoter, the gene encoding the LacI repressor and the *amp* gene (Estapé *et al.* 1998). The plasmid pPLac8 carries a gene encoding an aprotinin β-galactosidase (Ap::β-Gal) fusion protein under the control of the lacUV5 promoter, the gene encoding the LacI repressor and the *amp* gene (Hellmuth *et al.* 1994). Plasmid amplification is controlled by the ColE1 site in all plasmids.

### Culture conditions

For shake flask cultures, Luria–Bertani medium (Sambrook *et al.* 1989) was supplemented with 50 μg ampicillin ml<sup>-1</sup> and inoculated with 1% (v/v) of an overnight preculture. After incubation at 30 °C until the culture reached OD<sub>600</sub> ≈ 0.6, cells were induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at concentrations specified in the results section and shifted to the indicated temperatures. High-cell density cultivations of TG1:pλFGFB and TG1:pCYTEXP1 were performed as described previously (Hoffmann & Rinas 2000).

### Protein gel electrophoresis

Cell pellets were resuspended in 50 mM phosphate buffer (pH 7) to an OD<sub>600</sub> of 4.5 and disrupted by sonication. Soluble and insoluble cell fractions were separated by centrifugation (45 min at 38 000 × g), washed and recentrifuged. One-dimensional SDS-PAGE was carried out as described previously (Hoffmann & Rinas 2000). The position of LacI was identified by N-terminal sequencing of blotted protein (sequence MKPVTLYDVAEYAGV). Protein synthesis rates were quantified by two-dimensional non-

equilibrium pH-gel electrophoresis of <sup>35</sup>S-methionine labelled proteins as described before (Hoffmann & Rinas 2000).

### Plasmid content

Plasmids were extracted from cell pellets corresponding to a biomass of 2 mg dry cell wt using the alkaline lysis protocol and purified with the Jetstar 2.0 set (Genomed, Germany) according to the supplier's instructions. Plasmids were linearized with *Bam*H1 (or *Xho*I in case of pPLac8). After separation on a 1% (w/v) agarose gel containing 1.1 mg ethidium bromide l<sup>-1</sup>, the plasmid content was quantified by densitometry. Equal volumes of a culture carrying an appropriate reference plasmid were added to the samples before plasmid extraction to allow correction for different extraction yields.

## Results

### Temperature-induced production of a human protein in fed-batch cultures interferes with plasmid amplification

The temperature-inducible cI857/λP<sub>R</sub>P<sub>L</sub> expression system was employed to produce the human basic fibroblast growth factor (hFGF-2) in high-cell density cultures of recombinant *E. coli* TG1:pλFGFB. When the optical density reached OD<sub>600</sub> = 100 during the fed-batch process at 30 °C, the temperature was shifted to 42 °C to induce hFGF-2 production. Within 4 h, 50 mg hFGF-2 accumulated per gram cell dry wt, whereof 60% were in the insoluble cell fraction (Hoffmann *et al.* 2001). Analysis of protein synthesis rates by <sup>35</sup>S-methionine labelling revealed a threefold increase of the total protein synthesis after the temperature upshift (Hoffmann & Rinas 2001). 2-D gel analysis of the cellular proteome demonstrated that synthesis of hFGF-2 amounted to 60% of the total protein synthesis (Hoffmann & Rinas 2001), whereas the constitutive plasmid-encoded proteins (β-lactamase and the cI857 repressor) were produced at low rates (Figure 1A) and did not contribute in significant amounts to the total protein synthesis. However, in the control culture of *E. coli* TG1:pCYTEXP1 not carrying a structural gene under the control of the temperature-inducible promoter and grown under identical conditions, the synthesis rates of constitutive plasmid-encoded proteins started to increase rapidly after the temperature upshift (Figure 1A) and amounted to over

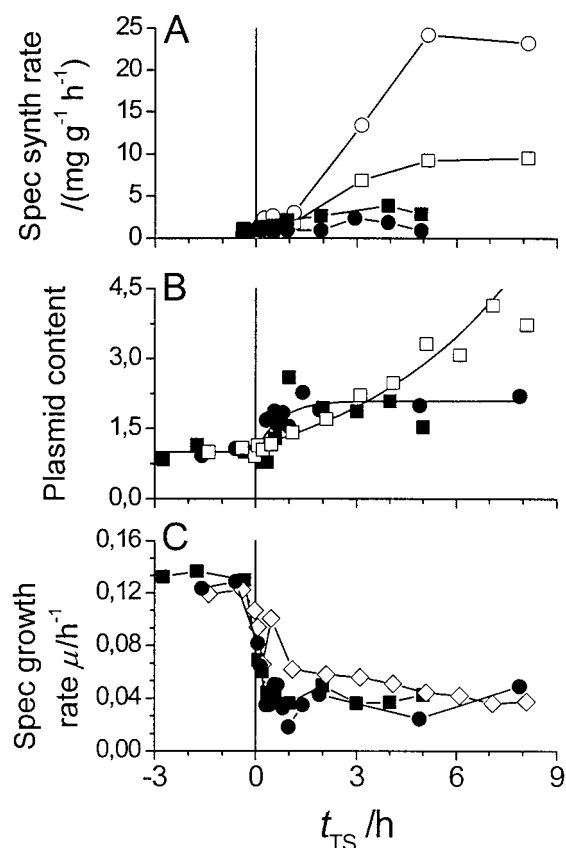


Fig. 1. Plasmid amplification after temperature upshift in high-cell density cultivations of recombinant *E. coli* TG1:p $\lambda$ FGFB producing hFGF-2 (closed symbols) and control strain TG1:pCYTEXP1 (open symbols). (A) Synthesis of constitutive plasmid-encoded proteins. Specific synthesis rates of (■, □)  $\beta$ -lactamase (including pre- $\beta$ -lactamase) and (●, ○) repressor cI857 (including cI')\* with (■, ●) TG1:p $\lambda$ FGFB and (□, ○) TG1:pCYTEXP1. (B) Mass of plasmid DNA per biomass, relative to the values obtained at 30 °C. (●, ■) Two independent high-cell density cultivations of TG1:p $\lambda$ FGFB, (□) control high-cell density cultivation of TG1:pCYTEXP1. (C) Specific growth rate vs. time. Time is given relative to the temperature upshift from 30 °C to 42 °C.

\*In addition to the full form of cI857, a slightly more acidic, shorter form with intact *N*-terminus (termed cI') was detected by two-dimensional gel electrophoresis (Hoffmann & Rinas 2001).

50% of the total protein synthesis 5 h later (Hoffmann & Rinas 2001).

Plasmid analysis revealed a rapid doubling of the specific plasmid content after the temperature upshift in the culture producing hFGF-2 (Figure 1B). Thereafter, the plasmid content did not increase any further. In contrast, with TG1:pCYTEXP1 carrying the 'empty' parental expression vector and grown under the same culture conditions, the increase of the specific plasmid content continued for at least 7 h (Fig-

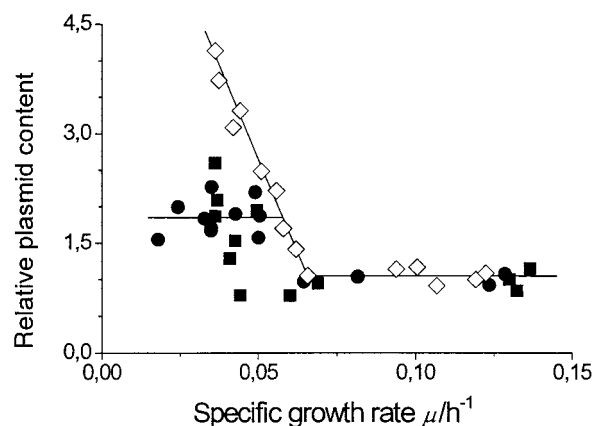


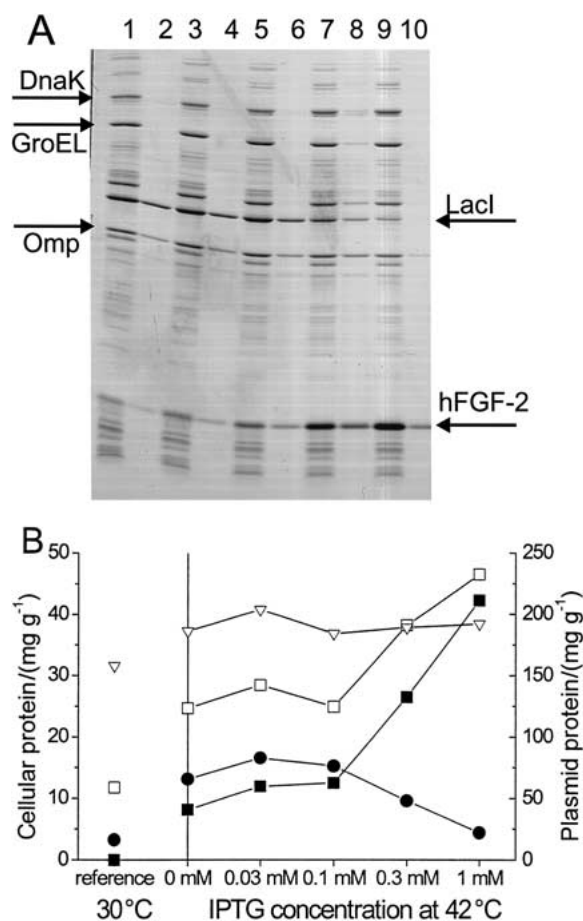
Fig. 2. Plasmid content vs. specific growth rate, which decreased after the temperature upshift. The specific growth rate  $\mu$  was calculated from the slope of the logarithm of cell density times culture volume versus time. (●, ■) Two independent high-cell density cultivations of TG1:p $\lambda$ FGFB, (□) control high-cell density cultivation of TG1:pCYTEXP1.

ure 1B). The synthesis rates of constitutive plasmid-encoded proteins increased nearly linearly with the plasmid content in TG1:pCYTEXP1 (not shown). In both cultures, the temperature upshift resulted in reduced biomass yields (Hoffmann & Rinas 2001) and, thus, lower specific growth rates (Figure 1C).

When the growth rate decreased after the temperature upshift, TG1:pCYTEXP1 showed an inverse correlation of plasmid content and specific growth rate,  $\mu$ , below a threshold value of about  $\mu = 0.07\ h^{-1}$ , while the plasmid content of TG1:p $\lambda$ FGFB did not show this correlation (Figure 2).

#### *Chemical induction of hFGF-2 by IPTG also interferes with plasmid amplification after a temperature upshift*

To rule out that interference with plasmid amplification upon a temperature upshift occurs only with temperature-inducible expression vectors, an IPTG-inducible expression system was investigated. Cultures of *E. coli* BL21, transformed with the expression vector pJHLbFGF, carrying the hFGF-2 gene under control of the LacI repressed *tac* promoter, were grown at 30 °C in complex medium to an  $OD_{600}$  of 0.3–0.6 and subsequently shifted to 42 °C. Even without IPTG addition, more than 30 mg of hFGF-2 per g cell dry wt accumulated 2 h after the temperature upshift; likewise, the concentration of the constitutively synthesized plasmid-encoded repressor LacI increased more than fivefold compared to 30 °C (Figure 3). With



**Fig. 3.** Change in synthesis of plasmid-encoded proteins by inducer. BL21:pJHLhFGF was grown at 30 °C and hFGF-2 synthesis was induced by the addition of indicated concentrations of IPTG simultaneous to a temperature shift to 42 °C. (A) Coomassie-stained SDS-PAGE of samples taken 2 h after the shift. Cell pellets resuspended to the same OD are loaded. Lanes 1, 2: without IPTG; 3, 4: 0.03 mM; 5, 6: 0.1 mM; 7, 8: 0.3 mM; 9, 10: 1 mM. Lanes 1, 3, 5, 7, 9: total cell protein; 2, 4, 6, 8, 10: insoluble cell fraction obtained by centrifugation of disrupted cells and washing the obtained pellet fraction with 50 mM sodium phosphate buffer (pH 7). Positions of the inducible protein hFGF-2 and the constitutive protein LacI are indicated. (B) Specific concentrations of plasmid encoded proteins (the inducible protein (■) hFGF-2 and the constitutive protein (●) LacI) and cellular proteins (heat-shock protein (□) DnaK and (▽) 38 kDa outer membrane protein) were obtained by densitometric analysis of Coomassie stained SDS-PAGE gels; peak areas are normalized to the sum of the peak areas of the cellular proteins.

full induction by IPTG, hFGF-2 accumulated up to 30% of the cellular protein (Figure 3). While typical cellular proteins like outer membrane proteins (Omps) were not affected significantly by the production of hFGF-2, the concentration of the plasmid-encoded LacI repressor was progressively lower at higher inducer concentrations (Figure 3).

Temperature-induced production of hFGF-2 results in inclusion body formation and prolongates the heat-shock response after the temperature upshift, leading to elevated levels of the major heat-shock chaperone DnaK (Hoffmann & Rinas 2000). With IPTG-induced production of hFGF-2 concomitant to a temperature upshift, also about 30% of the product were found in the insoluble cell fraction (Figure 3A). As therefore expected, the concentration of the heat-shock chaperone DnaK, which was threefold higher at 42 °C than at 30 °C, increased further at higher IPTG concentrations paralleling the increase of hFGF-2 (Figure 3).

The plasmid content of the cells showed similar trends as the LacI concentration: it increased after the temperature upshift, but simultaneous induction of hFGF-2 attenuated the plasmid amplification (Table 1). At 42 °C, already low IPTG concentrations reduced the increase of the plasmid content markedly. These effects were also observed with a moderate temperature upshift to 37 °C, although to a lesser extent, while induction of hFGF-2 without a temperature upshift at 30 °C had no significant effect on the plasmid content (Table 1).

#### *Effect of temperature upshift and induction of another recombinant protein on plasmid amplification*

As an unrelated system, *E. coli* TG1 transformed with the expression vector pLac8 encoding a large tetrameric aprotinin  $\beta$ -galactosidase (Ap:: $\beta$ -Gal) fusion protein of 1050 amino acids per subunit under the control of the lacUV5 promoter was tested. Cultures were shifted to 42 °C with or without induction of Ap:: $\beta$ -Gal by IPTG. Growth was not affected by induction, as judged from similar increase of OD<sub>600</sub> of the induced and non-induced cultures. More than 50 mg of the Ap:: $\beta$ -Gal fusion protein per g cell dry mass accumulated. The protein was mainly found in the insoluble cell fraction. As with the other systems, plasmid amplification was observed after the temperature upshift in the absence of induced recombinant protein synthesis. However, simultaneous induction of the Ap:: $\beta$ -Gal gene interfered with the temperature-dependent plasmid amplification (Table 2). Thus, the impairment of plasmid amplification is not specific to high level expression of a particular recombinant gene, but occurs during production of completely unrelated proteins.

Table 1. Effect of temperature upshift and simultaneous induction of hFGF-2 synthesis by addition of IPTG on plasmid content and specific concentrations of plasmid-encoded proteins in BL21:pJHLbFGF.

Temperature (°C)	IPTG (mM)	2 h after temperature shift		
		Plasmid content <sup>a</sup>	hFGF-2 <sup>b</sup>	LacI <sup>b</sup>
30	0	1.3	0	14
	1	1.1	45	12
37	0	3.3	7	20
	1	2.2	120	18
42	0	5.8	37	77
	0.03	5	48	77
	0.1	3	62	75
	0.3	nd	103	49
	0.7	0.9	nd	nd
	1	1	175	27

<sup>a</sup>Mass of plasmid DNA per biomass, values relative to reference (at time zero and 30 °C).

<sup>b</sup>Specific protein concentration (mg g<sup>-1</sup> biomass); mean of four experiments; 95% confidence interval: <5% (concentration 20–40 mg g<sup>-1</sup>) – 15% (high concentrations) of mean value.

nd: not determined.

Table 2. Effect of recombinant protein induction by addition of IPTG (at 1 mM) on the increase of plasmid content 2 h after a temperature shift to 42 °C.

Plasmid	Plasmid content <sup>a</sup>		Induced protein		OD/OD <sub>TS</sub>	
	– IPTG	+ IPTG	Sp. conc. (mg g <sup>-1</sup> )	% insoluble	– IPTG	+ IPTG
pJHLbFGF	5.8	1	160–190	30	6.1	4.1
pPLac8	3.6	2.1	50–65	85	4.6	4.3

<sup>a</sup>Mass of plasmid DNA per biomass, values relative to reference (at time zero and 30 °C).

## Discussion

The synthesis rates of the constitutively synthesized proteins  $\beta$ -lactamase and repressor cI857 encoded on the ‘empty’ expression vector pCYTEXP1 increased considerably after a temperature upshift in high-cell density cultures of TG1:pCYTEXP1, finally accounting for 50% of the total protein synthesis. In contrast, these rates remained low when the temperature shift induced the strong synthesis of hFGF-2 from the pCYTEXP1-derived plasmid p $\lambda$ FGFB under the same culture conditions. Likewise, the concentration of the constitutively synthesized repressor LacI encoded on the plasmid pJHLbFGF increased after a temperature-upshift in *E. coli* BL21:pJHLbFGF, whereas simultaneous induction of hFGF-2 by IPTG attenuated the increase of the LacI concentration in a IPTG-dependent manner. In all cases, the changes in the synthesis of

constitutive plasmid-encoded proteins are attributed to corresponding changes in the plasmid contents.

Several factors determine the plasmid content of *E. coli*. Plasmid content and specific growth rate are inversely correlated, as is widely observed in steady state cultures (Bentley *et al.* 1990, Seo & Bailey 1986). Accordingly, the plasmid content increased when the specific growth rate decreased after the temperature upshift in high-cell density-cultivation of TG1:pCYTEXP1. Thus, the inverse relationship of plasmid content and growth rate also holds under the dynamic conditions with changes in the specific growth rate with time.

Although the growth rate increases at elevated temperatures, the plasmid content is higher at 40 °C than at 28 °C in *E. coli* HB101 (Kaprálék *et al.* 1998). Likewise, we found an increase in the content of the various plasmids after a temperature upshift in *E. coli*

TG1 and BL21 despite faster growth. This increase of the plasmid content is not a function of the growth rate.

Induction of a recombinant protein can influence the plasmid content. With proteins whose production exerts a strong negative effect on growth and reduces translation as well as DNA replication, an increase of the plasmid content was found after induction (Teich *et al.* 1998). A similar observation was made with BL21(DE3):pET29c(+), which after addition of IPTG strongly produces a peptide of 75 amino acids, leading to an arrest of growth and a sixfold increase of the plasmid content within 2 h (not shown).

In contrast, the synthesis of the small protein hFGF-2 (16 kDa monomeric protein) strongly interfered with plasmid amplification after the temperature upshift, both with glucose-limited TG1:p $\lambda$ FGFB in high-cell density-cultivation and with unlimited growing BL21:pJHLbFGF in complex medium with chemically induced hFGF-2 synthesis. A similar effect was found with the large aprotinin  $\beta$ -galactosidase fusion protein (four subunits of 120 kDa each) under the control of the lacUV5 promoter. Both proteins tend to form inclusion bodies. Low level induction of proteins that neither affect growth nor accumulate into inclusion bodies did not interfere with temperature-dependent plasmid amplification (data not shown).

While chromosomal DNA replication depends on continuous protein synthesis, plasmid replication is more stable, leading to plasmid amplification upon inhibition of translation by chloramphenicol (Clewell 1972). It seems possible that the stability of proteins involved in plasmid replication depends on chaperones that become limiting upon accumulation of inclusion bodies, while chromosomal replication is affected to a lesser extent as long as *de-novo* protein synthesis is unaffected. Also, it is known that another stress response interfering with protein stability, the stringent response, inhibit the replication of ColE1 type plasmids (Hecker *et al.* 1983).

## Acknowledgements

Technical assistance of M. Schmidt and S. Marten during high-cell density cultivation is gratefully acknowledged. Also, we gratefully acknowledge protein sequencing for protein identification by M. Kieß and R. Getzlaff.

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