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EFFECT OF THE 3'-TERMINAL TRUNCATION OF THE HUMAN INTERFERON-GAMMA GENE ON PLASMID SEGREGATION IN *ESCHERICHIA COLI*

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ABSTRACT

Segregation of plasmids with relaxed control of replication is due to their non-random distribution between the daughter cells during cell division. Plasmid segregation is a biological phenomenon influenced by many factors like type of replicon, presence of partitioning elements, plasmid copy-number, cultivation conditions, host genotype, etc.

The aim of this study was to investigate the effect of systematic changes in the human interferon-gamma (hIFNγ) gene on the segregation of expression plasmids in E. coli cells. To this end a series of 3'-truncated hIFNγ genes were cloned in a pBR322-based expression plasmid containing a strong constitutive promoter (T5P25) and a synthetic ribosome binding site. The series of plasmids thus constructed was transformed in E. coli LE392 cells and their segregation was investigated under batch fermentation conditions in non-selective Luria-Bertani medium. To describe the population dynamics, a mathematical model proposed by Stewart and Levin was applied. Using nonlinear fitting technique, the difference in specific growth rate between plasmid-free and plasmid-harbouring cells and the specific generation rate of plasmid-free cells (as well as the relative plasmid loss rate) were determined.

The obtained results demonstrated that small changes in the 3'-terminus of the hIFNγ gene strongly affect plasmid segregation. To explain this influence the model parameters were compared with experimental data characterizing hIFNγ gene expression such as yield of recombinant protein, hIFNγ-mRNA and plasmid copy-number. A clear correlation was found between the relative plasmid loss rate and hIFNγ-mRNA content, which however was not associated with the plasmid copy-number value.

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Keywords: *Escherichia coli*, human interferon-gamma, hIFNγ, Stewart and Levin model, plasmid segregation, plasmid instability

Symbols:

D: dilution rate [h^{-1}]
t: time [h]
x: cell concentration [g/L]
z: plasmid-harbouring cell fraction [-]
μ: specific growth rate [h^{-1}]
θ: relative plasmid loss rate [-]
Δ: difference in specific growth rate between plasmid-free and plasmid-harbouring cells [h^{-1}]
Θ: specific plasmid loss rate [h^{-1}]

Subscripts and Superscripts:

max: maximum value
(+): phenotype of plasmid-harbouring cells
(-): phenotype of plasmid-free cells

Introduction

Human interferon-gamma (hIFNγ) is a cytokine endowed with multiple biological activities and broad pharmaceutical applications. It is one of the major macrophage stimulating factors and its main biological effects include: enhancing antigen presentation and lysosome activity of macrophages and NK cells, suppressing Th2 cell activity, promoting Th1 cell differentiation, stimulation of antiviral and anti-parasitic cell activity, affecting cell proliferation, apoptosis, etc. (31). For therapeutic use hIFNγ is produced mainly in *E. coli* cells. Because of that in this study hIFNγ expressing *E. coli* cells are chosen as a model for investigation of plasmid segregation – a major factor affecting the yield of recombinant proteins from bacteria.

Segregation of expression plasmids is a well known phenomenon in recombinant DNA biotechnology leading to a reduced viability of bacteria cultivated under selective conditions and lowering the yield of recombinant proteins (1, 22, 23). Plasmid segregation is due to the irregular distribution of plasmids between the daughter cells during cell division. Under nonselective growth conditions, this results in generation of heterogeneous cell populations, where the nonproductive plasmid-free cells overgrow the plasmid-harbouring cells (3, 10, 28). Plasmid segregation is a phenomenon influenced by a large number of factors such as type of plasmid replicon, presence of partitioning elements (28), plasmid copy-number value (26), formation of plasmid concatemers during replication (4, 28, 32), cultivation conditions (2, 5, 8, 9, 17, 20, 33), and host cell genotype (11), etc.

In a recent study (18) we have employed the mathematical models of Stewart and Levin (24) and Lee et al. (13) to study the effect of transcription and translation on the segregation of pBR322-based plasmids expressing hIFNγ gene in *E. coli* LE392 cells under constitutive gene expression conditions. A reverse correlation between the yield of protein and plasmid stability was observed, which did not correlate with the variations in either plasmid copy-number or the specific bacterial growth rate.

The aim of the present study was to investigate another potential factor affecting plasmid segregation – the structure of the recombinant gene. To this end a series of expression plasmids was generated in which the hIFNγ gene was gradually truncated at the 3'-end by 9 base pairs (corresponding to 3 amino acids in the resulting protein) and expressed in *E. coli* LE392 cells under the control of a strong constitutive promoter. The mathematical model proposed by Stewart and Levin (24) was employed to evaluate the impact of the recombinant hIFNγ gene modifications on plasmid segregation. The obtained results demonstrated that the 3'- terminal variations in the structure of the hIFNγ gene strongly affected plasmid segregation.

Materials and Methods

Plasmids, genes and bacterial strain

Expression plasmids were constructed on the basis of the cloning plasmid pBR322 in which a synthetic constitutive promoter (P_1) followed by a synthetic ribosome binding site (SD sequence) were substituted for the *tet* gene promoter (16). The series of 3'-end gradually (by 9 bp) truncated hIFNγ genes (designated hIFNγΔ1 to hIFNγΔ7) were constructed by PCR using specific primers (16). The hIFNγ gene and its derivatives were cloned in *Hin*dIII/*Bam*HI restriction sites as shown in **Fig. 1**.

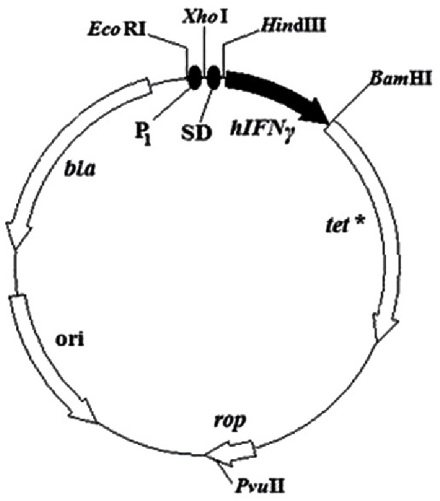

Fig. 1 Functional map of hIFNγ gene expression plasmids. P_1: constitutive (T5P25) promoter; SD: Shine and Dalgarno consensus sequence; *bla*: β-lactamase gene; *tet**: truncated (residual) tetracycline resistance gene; *rop*: gene coding for the Rop protein; ori: origin of replication.

The plasmid pΔP_1IFNγC was derived from the expression plasmid pIFNγC by removing the *Eco*RI/*Hin*dIII fragment (bearing both P_1 promoter and the SD sequence), blunting and ligation (18).

The plasmids pIFNγλ(+) and pIFNγλ(-) were constructed from pIFNγC by digestion with *Pvu*II (located in the *rop* gene) and insertion of a strong transcription terminator (λ phage t_0 terminator) (14) in both functional and non-functional orientation.

The pGEM-BD construction is described elsewhere (12).

Expression plasmids were transformed in *E. coli* LE392 (*sup*E44 *sup*F58 *hsd*R514 *gal*K2 *gal*T22 *met*B1 *trp*R55 lacY1) by the $CaCl_2$ method (21) and selected on LB (Luria-Bertani) agar plates containing 100 μg/ml ampicillin.

Cell cultivation

To prepare the inoculum a single colony of transformed cells was picked from a selective plate and transferred to a 50 ml flask containing 10 ml of sterile LB medium, pH 7.2, supplemented with 50 μg/ml ampicillin. The flask was incubated at 37 °C and 200 rpm to an optical density of about 0.6-0.7.

The following cultivation procedure was applied. The initial batch culture was started by adding 20 μl of inoculum to a 100 ml shake flask containing 20 ml sterile LB medium, pH 7.2. The flask was incubated at 37 °C and 200 rpm until an optical density of A_{595} = 0.6-0.7 was reached. Then another flask was inoculated using 20 μl of this pre-culture and incubated as described above. This procedure was repeated many times until the cell population was cured from the corresponding expression plasmid, i.e. it consisted of plasmid-free cells only. The maximum cell density was kept under A_{595} = 0.8 in order to maintain cultures in exponential growth phase.

Plasmid stability assay

To determine the percentage of plasmid-harbouring cells, culture samples were diluted with 0.9% (w/v) NaCl, spread on LB-agar plates and incubated at 37 °C for 12 h. Two hundred and fifty single colonies were picked with sterile applicator sticks and transferred to LB-agar plates containing 100 μg/ml ampicillin. After 12 h the resulting colonies were counted. The segregational plasmid instability was represented by the ratio of colonies grown on antibiotic to all transferred colonies (250).

Protein yield and hIFNγ-mRNA determination

Samples of 20 ml LB medium supplemented with 50 μg/ml ampicillin were inoculated in a ratio of 1:50 with fresh overnight cultures of transformed *E. coli* LE392 cells and cultivated to a cell density of A_{595} = 0.7 at 37 °C (16). The yield of recombinant hIFNγ was measured by ELISA (16) using a sequence specific anti-hIFNγ monoclonal antibody (15). The relative content of hIFNγ-mRNAs was determined by hybridization with a 19 nt long ^{32}P-labeled oligonucleotide specific for the hIFNγ gene as previously described (16).

Determination of plasmid copy-number

Cells transformed with the series of expression plasmids were cultivated as described above. Plasmid copy-number was

determined by real-time quantitative PCR (qPCR) as described by Lee et al. (12). To this end total DNA was isolated using a QIAamp® DNA Mini Kit (Qiagen), following the method for bacterial cultures. Since all plasmids in this study bear *bla* gene (target gene) and the host *E. coli* LE392 cells harbour chromosomal D-1-deoxyxylulose-5-phosphate synthase gene (*dxs*) (housekeeping gene) the same primer sets, calibrator (plasmid pGEM-BD, carrying both *bla* and *dxs* gene) and thermal cycling protocol as proposed by Lee et al. (12), were used. qPCR amplification was carried out in a Rotor-Gene™ instrument (Corbett Research, Qiagen) using MESA GREEN qPCR MasterMix Plus for SYBR® Assay No ROX kit (Eurogentec) in 9 repetitions for each probe.

Results and Discussion

Modelling of population dynamics of plasmid-harbouring cells at segregational plasmid instability

To investigate the effect of 3'-end truncation of the hIFNγ gene on segregational plasmid stability, a series of gradually (by 9 bp) truncated hIFNγ genes (designated hIFNγΔ1 to hIFNγΔ7) was cloned in a pBR322-based expression plasmid containing a strong constitutive promoter P_1 and a strong ribosome binding site. These plasmids, designated as pIFNγΔ1 to pIFNγΔ7, were transformed in *E. coli* LE392 cells and their segregational stability was studied under batch cultivation conditions. The bacteria were grown in non-selective LB medium in shaking flasks where the cell cultures were maintained at exponential growth phase. The fraction of plasmid-harbouring cells **z** in the total cell population (defined by Equation 4 in the Appendix) versus the cultivation time is shown in **Fig. 2**. To describe the population dynamics of plasmid-harbouring and plasmid-free cells the nonlinear fitting method of Davidson et al. (7), based on the equations proposed by Stewart and Levin (24), was applied (see the Appendix). The values of the parameters **Δ** and **Θ** for all investigated plasmids were evaluated by nonlinear fit of Equation 6 to the experimental data points using OriginPro 8 software (**Table 1**). The fractions of plasmid-harbouring cells, **z**, predicted by Equation 6 are presented in **Fig. 2**. The specific plasmid loss rate, **Θ**, depends on the specific growth rate of the plasmid-harbouring cells, μ^+, as well as on the relative plasmid loss rate, **θ** (Equation 7). The relative plasmid loss rate, **θ**, is assumed to be constant irrespective of the host cells' specific growth rate and cultivation conditions (13) and is influenced mainly by the plasmid segregation process. The value of **θ** for each investigated plasmid was calculated using Equation 7 and Equation 5 where the corresponding values of **Θ** and **Δ** were estimated by the method of Davidson et al. (7) (**Table 1**). Since the cell cultures were maintained only in exponential growth phase, it was assumed that the specific growth rate of the plasmid-free cells, μ^-, is equal to the maximum specific growth rate of wild type (non-transformed) *E. coli* LE392 cells grown under the same experimental conditions. The maximum specific growth rate, μ_{max}, for the *E. coli* LE392 cells cultivated in LB medium was 1.20 h^{-1} (data not shown). The estimated values of **θ** for all investigated plasmids are also presented in **Table 1**.

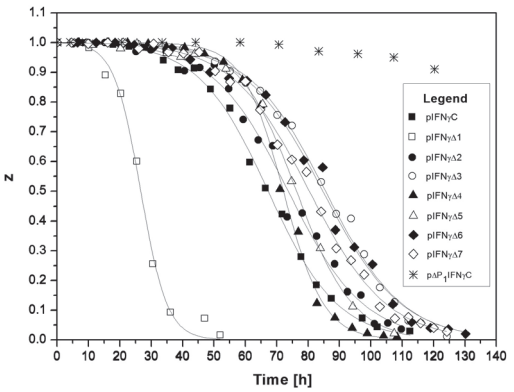

Fig. 2 Plasmid-harbouring cell fraction **z** versus the cultivation time. Experimental results (data points) and fittings of the model proposed by Stewart and Levin (24) (lines).

To explore the role of the hIFNγ gene transcription on plasmid segregation we investigated also the population dynamics of *E. coli* LE392 cells harbouring the plasmid $p\Delta P_1$IFNγ (18), a derivative of pIFNγC, which lacks the P_1 promoter. The switching off of the hIFNγ gene transcription led to extremely high plasmid segregational stability – the fraction of plasmid-free cells in the population after 120 h of cultivation did not exceed 10% (**Fig. 2**).

TABLE 1

Values of the model parameters **Δ**, **Θ** and **θ** and plasmid copy-number per cell (±S.D.)

Plasmid name and number of deleted nucleotides (shown in brackets)	Specific growth rate difference, Δ [h^{-1}]	Specific plasmid loss, Θ [h^{-1}]	Relative plasmid loss rate, θ	Plasmid copy-number per cell
pIFNγC (0)	0.0899 ± 0.0024	(2.10 ± 0.29) × 10^{-4}	(1.89 ± 0.26) × 10^{-4}	14.1 ± 1.4
pIFNγΔ1 (9)	0.2365 ± 0.0200	(4.30 ± 0.77) × 10^{-4}	(4.46 ± 0.81) × 10^{-4}	21.1 ± 1.5
pIFNγΔ2 (18)	0.0828 ± 0.0037	(1.85 ± 0.43) × 10^{-4}	(1.66 ± 0.39) × 10^{-4}	19.8 ± 2.9
pIFNγΔ3 (27)	0.0811 ± 0.0025	(7.84 ± 1.47) × 10^{-5}	(7.01 ± 1.32) × 10^{-5}	17.4 ± 2.1
pIFNγΔ4 (36)	0.1547 ± 0.0068	(2.21 ± 0.10) × 10^{-6}	(2.11 ± 0.95) × 10^{-6}	16.1 ± 2.4
pIFNγΔ5 (45)	0.1105 ± 0.0042	(2.41 ± 0.68) × 10^{-5}	(2.21 ± 0.62) × 10^{-5}	22.6 ± 1.9
pIFNγΔ6 (54)	0.0794 ± 0.0036	(9.48 ± 2.48) × 10^{-5}	(8.46 ± 2.22) × 10^{-5}	18.4 ± 2.3
pIFNγΔ7 (63)	0.0808 ± 0.0022	(1.22 ± 0.19) × 10^{-4}	(1.09 ± 0.17) × 10^{-4}	37.6 ± 4.0

Comparison between plasmid copy-number, recombinant protein and hIFNγ-mRNA yields and model parameters

To study the effect of plasmid copy-number on plasmid segregation *E. coli* cells transformed with the above described plasmids were cultivated and the copy-number was determined using qPCR (**Table 1**). Except for the plasmid pIFNγΔ7, the determined values for the plasmids carrying the truncated genes varied within the range of 16-23 plasmid copies per cell. These values were slightly higher in comparison with the control plasmid (pIFNγC) expressing the full size hIFNγ gene (about 14 plasmid copies per cell). Presently, there is no explanation of the observed drastic increase in the plasmid copy-number to 38 following the 3'-end deletion of 63 base pairs in the pIFNγΔ7 construct. The copy-number of the plasmid $p\Delta P_1IFN\gamma$ was 13±1.9.

The yields of recombinant protein and hIFNγ-mRNA that also can be considered as potential factors interfering with plasmid segregation have been examined earlier by Nacheva et al. (16) and were compared with plasmid copy-number and the values of the model parameters (**Fig. 3**). As it is seen, the systematic 3'-end deletions of the hIFNγ gene resulted in significant variations of the yields of both, the recombinant protein and the hIFNγ-mRNAs. No correlation was observed between the 3'-end deletions of the hIFNγ gene and experimentally obtained data on PCN, recombinant protein and level of hIFNγ-mRNA. On the other hand, **Fig. 2** shows that variations in the hIFNγ-gene 3'-terminus strongly affect the population dynamics of plasmid-harbouring cells. Moreover, the model calculations demonstrate that the variations in the population dynamics are due to the differences in the specific growth rates, **Δ**, as well as the differences in specific (and relative) plasmid loss rates **Θ,** (**θ**), (**Table 1**). These findings raise the question of how the 3'-end truncation of the hIFNγ-gene affects the model parameters.

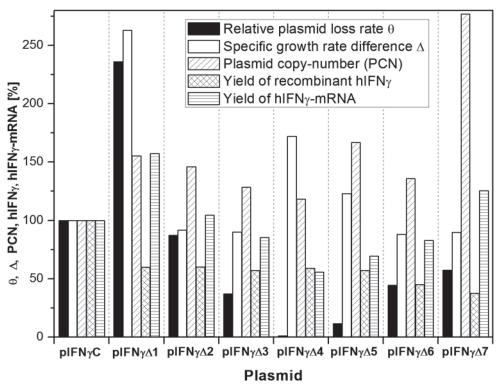

Fig. 3 Comparison of the experimental data (PCN values, recombinant hIFNγ and hIFNγ-mRNA yields) with the calculated **Δ** and **θ** values for the expression plasmids carrying 3'-truncated hIFNγ genes. Data (in %) are related to the corresponding values obtained for the pIFNγC construct (taken as 100%). hIFNγ and hIFNγ-mRNA yields are from Nacheva et al. (16).

As seen from **Table. 1** and **Fig. 3**, the **Δ** values for most of the plasmids were similar except for the constructs pIFNγΔ1, pIFNγΔ4 and pIFNγΔ5, where greater values of **Δ** were observed. These results show that some 3'-terminal deletions of the hIFNγ gene result in a significant reduction of the specific growth rate of the plasmid-harbouring cells. Generally, **Δ** increases with a higher metabolic burden of the plasmid-harbouring cells, which might be due to the presence of plasmids, recombinant protein and mRNA in bacterial cytoplasm as well as to the toxicity of the expressed recombinant protein. It should be mentioned however, that a clear cutoff correlation between **Δ** and the plasmid copy-number, yield of recombinant protein, and hIFNγ-mRNA was not observed.

The results presented in **Table 1** show that the 3'-end truncation of the hIFNγ gene affects the relative plasmid loss rate, **θ**, and can be associated with factors affecting plasmid partitioning. The obtained results (**Table 1** and **Fig. 3**) did not show any correlation between **θ** (plasmid segregation) and the plasmid copy-number. They also show that there is no correlation between the yield of recombinant protein and the relative plasmid loss rate, **θ**. Considering other potential factors that may interfere with plasmid segregation, one could assume that the solubility of the recombinant protein in bacterial cytoplasm and its capability to form inclusion bodies could also influence the random distribution of plasmids during cell division. Usually they have polar localization and therefore might include (drag) growing polypeptide chains, together with the translating ribosomes, mRNAs and expression plasmids. Previously, Nacheva et al. (16) have shown that the systematic truncation of the hIFNγ C-terminal domain from zero to 21 amino acids leads to a gradual solubility increase of the corresponding recombinant protein, i.e. to a gradual decrease of the inclusion bodies formation. However, here we did not observe any correlation between the relative plasmid loss rate **θ** and the tendency of inclusion bodies formation by the recombinant protein.

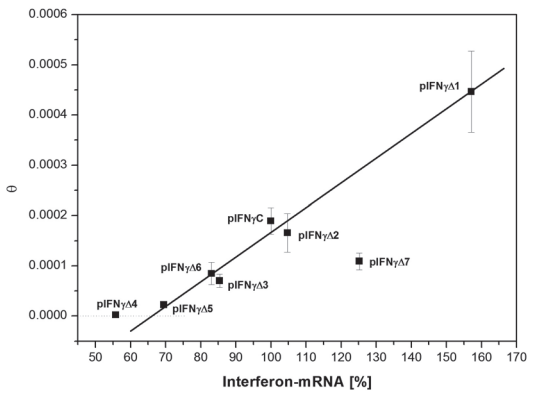

Fig. 4 Correlation between the yield of hIFNγ-mRNA and the relative plasmid loss rate **θ**.

The analysis of the effect of recombinant gene mRNA variations upon 3'-end gene deletions on the relative plasmid loss rate (**Fig. 3**) showed a clear tendency between the yield of hIFNγ-mRNA and **θ**. It was found that **θ** increased linearly with increasing the yield of hIFNγ-mRNA (**Fig. 4**).

The deviation of pIFNγΔ4 from the linearity shown in **Fig. 4** might mean that the latter is not valid for hIFNγ-mRNA yields lower than 30% of that of the referent pIFNγC construct.

We assume that a minimal threshold value of **θ** might exist that is independent of the content of hIFNγ-mRNA.

A lower than expected **θ** value (according to the linear correlation) was observed for the pIFNγΔ7 construct. Although we are unable to give a reasonable explanation of its higher segregational stability, it is worth mentioning that this construct is characterized by a much higher plasmid copy-number value, as compared to other investigated plasmids (see above).

Assuming that all hIFNγ-mRNAs considered in our study have similar half-life, the transcription efficiency of the hIFNγ genes can be expressed as a ratio of the yield of hIFNγ-mRNA to the corresponding plasmid copy-number. The plot of relative plasmid loss rate versus transcription efficiency (**Fig. 5**) indicates a negative effect of transcription on plasmid stability.

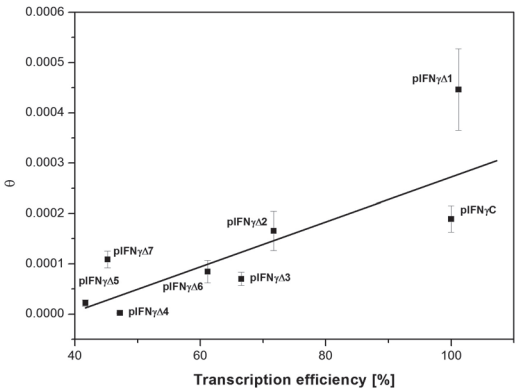

Fig. 5 Relationship between the relative plasmid loss rate **θ** and the transcription efficiency of hIFNγ genes. Transcription efficiency data are presented in % and are related to the construct pIFNγC (taken as 100%).

Construction and copy-number of the plasmids pIFNγλ(+) and pIFNγλ(-)

Stueber and Bujard (25) observed that extensive constitutive transcription from plasmids containing ColE1 replicon resulted in reduction of plasmid copy-number and led to an increase in plasmid segregational instability (19, 21). This is explained by transcriptional readthrough into the replication region (e.g. from the *tet* region of pBR322), which causes overproduction of Rop protein (to reduce the plasmid copy-number) and also interferes negatively with plasmid replication (25). All plasmids used in our study express recombinant proteins under the control of the strong constitutive promoter P_1. The mRNA thus obtained is dicistronic and consists of the entire hIFNγ sequence plus a part of the *tet* gene (downstream of the *Bam*HI site). The transcription of this mRNA terminates downstream of the *tet* gene at an obscure region. If the termination site is near to the origin of replication, it is reasonable to expect that the transcription efficiency might interfere with the plasmid copy-number via the mechanism proposed by Stueber and Bujard (25). To evaluate the effect of transcriptional readthrough from the P_1 promoter on the copy-number of the plasmids expressing hIFNγ derivative genes we constructed the plasmids pIFNγλ(+) and pIFNγλ(-). They were obtained from the plasmid pIFNγC in which a strong transcription terminator (the λ phage t_0 terminator (14) was inserted into the *rop* gene in both right (functional) and reverse (non-functional) orientation, respectively. *E. coli* LE392 cells transformed with these plasmids were cultivated in flasks containing LB medium to A_{595} = 0.7 and the plasmid copy-number was determined by qPCR. The average copy-number values thus obtained were 26 ± 3.6 and 29 ± 3.5 for the plasmids pIFNγλ(+) and pIFNγλ(-), respectively. As expected, the inactivation of the *rop* gene led to a significant increase in the plasmid copy-number of both constructs, however a further increase was not observed when the λ phage t_0 terminator was introduced in a right orientation.

Relationship between hIFNγ gene transcription and plasmid segregation

The observed correlation between the yield of hIFNγ-mRNA and the relative plasmid loss rate, **θ** (**Fig. 4**) could be explained by two possible ways: i) factors that contribute to the hIFNγ-mRNA yield interfere with plasmid segregation and/or ii) hIFNγ-mRNA level itself affects plasmid segregation (**Fig. 6**).

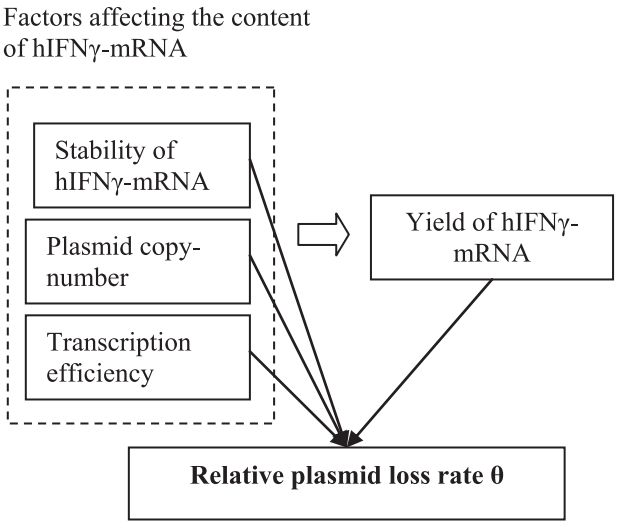

Fig. 6 Hypothetical factors affecting plasmid segregation.

The yield of hIFNγ-mRNA depends on the following factors: i) stability of hIFNγ-mRNA; ii) plasmid copy-number and iii) transcription efficiency of the hIFNγ gene.

Unfortunately, the attempts to register differences in stability (half-life) of hIFNγ-mRNAs obtained from different hIFNγ gene constructs (after blocking the transcription by rifampicin and nalidixic acid) failed because of the extremely short life span (about 60 seconds) of all examined mRNA and the low resolution (±10-15 s) of the employed method (16).

The copy-number of multicopy plasmids with relaxed control of replication is a factor affecting plasmid partitioning between the daughter cells. Usually the low plasmid copy-number favors the irregular distribution of plasmids during cell division thus increasing the relative plasmid loss rate, **θ** (27). Since the plasmids used in this study carry a ColE1-replicon, a reverse correlation between plasmid copy-number and segregational plasmid instability (**θ**) was expected. In contrary to the expectations however, the obtained results

(**Table 1** and **Fig. 3**) do not show any correlation between **θ** (plasmid segregation) and the plasmid copy-number.

The observed variations in the copy-number of the plasmids carrying hIFNγ genes with different extent of truncation could be explained in the light of the hypothesis of Yavachev and Ivanov (34), predicting a possible interference of other RNAs with plasmid replication via RNAI/RNAII interactions. Therefore, we searched for a homology and hybrid formation between the truncated hIFNγ-mRNAs and RNAI/RNAII. However, no such interactions could be identified.

The transcription efficiency of the recombinant gene is another factor which can influence plasmid segregation. The observed negative effect of the transcription efficiency on plasmid stability in our experimental system (**Fig. 5**) could be explained by the observation of Stueber and Bujard (25) that extensive constitutive transcription results in reduction of copy-number of plasmids containing ColE1 replicon and leads to an increase in plasmid segregational instability (19, 21). To check this hypothesis we constructed the plasmids pIFNγλ(+) and pIFNγλ(-), where the λ phage t_0 terminator was inserted into the *rop* gene in both right (functional) and reverse (non-functional) orientations. Thus the t_0 terminator should inactivate the *rop* gene and therefore should result in a significant increase in the plasmid copy-number. In the right orientation (pIFNγλ(+)), the t_0 terminator should suppress the transcriptional readthrough in the replication region and therefore a further increase in plasmid copy-number should be expected. In the case of a reverse orientation (pIFNγλ(-)), the transcription will not be interrupted and lower copy-number value compared to the plasmid pIFNγλ(+) had to be expected. The obtained copy-number values demonstrated, however, that the insertion of a functional transcription terminator into the *rop* gene (pIFNγλ(+)) did not lead to an increase in plasmid copy-number as compared to the pIFNγλ(-) construct, i.e. the mechanism proposed by Stueber and Bujard (25) is invalid for the plasmids used in this study. It seems unlikely that the negative effect of transcription efficiency on plasmid segregation is due to reduction in plasmid copy-number. This conclusion was supported also by the estimated low copy-number value of the plasmid pΔP_1IFNγC (see below).

In addition to the factors affecting the yield of hIFNγ-mRNA considered above (**Fig. 6**), Tartaglia et al. (29, 30) found a relationship between the cellular level of mRNA and protein solubility in *E. coli*. The latter is assumed to be a mechanism for evolutionary pressure to avoid protein aggregation at maximum levels of gene expression. Our data, however, cannot be explained by this hypothesis.

Overall, the hypothesis that hIFNγ-mRNA itself interferes directly with plasmid segregation (**Fig. 6**) cannot be excluded. Taking into consideration that the three biochemical processes – replication, transcription and translation in bacteria occur in a single compartment (bacterial cytoplasm), it can be assumed that the accelerated plasmid loss in association with an increased yield of hIFNγ-mRNA is probably due to the formation of complex aggregates containing replicating plasmids, mRNA, growing polypeptide chains and polysomes. This is supported by our recent finding that hIFNγ inclusion bodies contain both plasmid DNA and ribosomal RNAs (unpublished data). The extremely high molecular mass and low diffusion rate of such aggregates would hamper the random distribution of the expression plasmids between the daughter cells during cell division.

To estimate the impact of hIFNγ gene transcription on plasmid segregation we used the previously constructed plasmid pΔP_1IFNγC (18) in which the P_1 promoter was deleted and therefore the hIFNγ gene was not transcribed. Our results showed that despite the observed low copy-number value (13 ± 1.9), the plasmid was extremely stable (**Fig. 2**). This presents a clear evidence that inactivation of transcription has a strong stabilizing effect on the plasmid against segregation. This finding is supported also by a recent study where the role of transcription on plasmid segregation was investigated in a chemostat culture (18).

In conclusion, the results presented in this paper demonstrate that the 3'-end truncation of the constitutively expressed hIFNγ gene strongly affects plasmid segregation. The relative plasmid loss rate (**θ**) increases with a higher intracellular concentration of hIFNγ-mRNA and/or transcription efficiency of the hIFNγ gene (both influenced by the deletions in the 3'-terminus of the recombinant gene). This correlation, however, cannot be explained by variations in the plasmid copy-number. Switching-off transcription has a stabilizing effect against segregation, which is also unrelated to the increase of the plasmid copy-number.

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Appendix

Mathematical model describing plasmid segregation

For description of population dynamics of plasmid-harbouring and plasmid-free cells a model proposed by Stewart and Levin (24) was applied. It describes the change in the plasmid-harbouring and plasmid-free biomass with time:

$$\frac{dx^+}{dt} = \mu^+ \cdot x^+ - \Theta \cdot x^+ - D \cdot x^+ \quad (1)$$

$$\frac{dx^-}{dt} = \mu^- \cdot x^- + \Theta \cdot x^+ - D \cdot x^- \quad (2)$$

x, **μ**, **Θ** and **D** denote biomass, specific growth rate, specific plasmid loss rate and dilution rate (continuous culture), respectively. The indices (+) and (-) indicate plasmid-harbouring and plasmid-free cell populations, respectively.

Davidson et al. (7) proposed a nonlinear technique to calculate parameters for plasmid segregational instability and differences in specific cellular growth rate for plasmid-harbouring micro-organisms growing in batch or continuous culture. Equation 1 and Equation 2 can be combined into a single equation describing plasmid instability in both batch and continuous cultures (6):

$$\frac{dz}{dt} = \Delta \cdot z^2 - (\Delta + \Theta) \cdot z \tag{3}$$

where **z** is the ratio between the concentration of plasmid-harbouring cells and the total biomass concentration (fraction of plasmid-harbouring cells in the bacterial population):

$$z = x^+ / (x^+ + x^-) \tag{4}$$

and **Δ** is the difference in the specific growth rate between plasmid-free and plasmid-harbouring cells:

$$\Delta = \mu^- - \mu^+ \tag{5}$$

Assuming that **Δ** and **Θ** are constant and z = 1 at t = 0, Equation 3 can be solved for a biological relevant situation to:

$$z = \frac{\Delta + \Theta}{\Theta \cdot e^{(\Delta+\Theta)\cdot t} + \Delta} \tag{6}$$

The specific rate of generation of plasmid-free cells (specific plasmid loss rate), **Θ**, is defined as a product of the relative plasmid loss rate, **θ**, and the specific growth rate of the plasmid-harbouring cells, μ^+:

$$\Theta = \mu^+ \cdot \theta \tag{7}$$

The relative plasmid loss rate, **θ**, describes plasmid segregation and is assumed constant irrespective of the specific growth rate of plasmid-harbouring cells and cultivation conditions (13).

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