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Effects of Human Interferon-gamma Gene Expression and Structure of ColE1-like Plasmids on Plasmid Segregation in *Escherichia coli*

PhD THESIS

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Declaration

This PhD thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

12. 03. 2012 Mladen Popov

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Abbreviations

ATP – adenosine triphosphate

Ap – ampicillin

bp – base pairs

FISH – fluorescence *in situ* hybridization

hIFNγ – human interferon gamma

IPTG – isopropyl thiogalactosidase

LB – Luria-Bertani medium

nt – nucleotides

SD – Shine & Dalgarno consensus sequence

tet – tetracycline transacetylase gene

Nomenclature

D – dilution rate	[h^{-1}]
G_{in} – intracellular plasmid concentration	[g plasmid / L biomass]
G_{inmax} – maximal intracellular plasmid concentration	[g plasmid / L biomass]
k_d – decay constant of cloned gene messenger RNA	[min^{-1}]
k_e – decay constant of recombinant protein	[min^{-1}]
k_p^0 – overall transcription rate constant	[min^{-1}]
k_q^0 – overall translation rate constant	[min^{-1}]
K_s – Monod constant	[g/L]
m – exponent of plasmid vector inhibition	
m_{in} – intracellular concentration of cloned gene messenger RNA	[moles RNA / L biomass]
n – exponent of product inhibition term	
N_p – average number of plasmids per cell	
p – concentration of recombinant protein related to the culture volume	[g protein / L culture broth]
p_{in} – intracellular concentration of recombinant protein	[g protein / L dry biomass]
p_{inmax} – maximal intracellular concentration of recombinant protein	[g protein / L dry biomass]
p_0 – initial concentration of recombinant protein related to the culture volume	[g protein / L culture broth]
s – concentration of limiting nutrient (glucose)	[g/L]
s_F – concentration of substrate (glucose) in the fresh nutrient medium	[g/L]
s_0 – initial concentration of substrate (glucose)	[g/L]

t – time	[h]
x – total biomass concentration (= x^++x^-)	[g/L]
x^+ – concentration of plasmid-harbouring cells	[g/L]
x^- – concentration of plasmid-free cells	[g/L]
x_0^+ – initial concentration of plasmid-harbouring cells	[g/L]
x_0^- – initial concentration of plasmid-free cells	[g/L]
$Y_{x/s}$ – biomass / substrate yield factor	[g biomass / g substrate]
z – plasmid-harbouring cell fraction (= x^+/x)	

Greek symbols

Δ – difference in the specific growth rate between plasmid-free and plasmid-harbouring cells	[h^{-1}]
γ – gene expression parameter	
η – transcription efficiency	[moles RNA / g plasmid]
θ – relative plasmid loss rate	
Θ – specific plasmid loss rate	[h^{-1}]
μ^+ – specific growth rate of plasmid-harbouring cells	[h^{-1}]
μ^- – specific growth rate of plasmid-free cells	[h^{-1}]
μ^* – specific growth rate, defined by Eq. 9	[h^{-1}]
μ_{max} – maximal specific growth rate of wild-type cells	[h^{-1}]
ξ – translation efficiency	[g protein / moles RNA]
ρ_B – cell density	[g dry biomass/L dry biomass]

BACKGROUND AND LITERATURE REVIEW

1. Plasmid segregational instability

Plasmids are nonessential extrachromosomal circular double stranded DNA molecules that replicate independently of bacterial chromosome. They are indispensable in genetic engineering for cloning and expression of foreign genes as well as in studying fundamental molecular processes in bacteria.

Plasmid segregational instability 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 (Bentley and Kompala 1990; Seo and Bailey 1985; Siegel and Ryu 1985). Plasmid segregation is related with their irregular distribution between the daughter cells during cell division. Under non-selective growth conditions, this results in generation of heterogeneous cell populations, where the non-productive plasmid-free cells overgrow the plasmid-harbouring cells (because of their metabolic burden with plasmid replication and foreign gene expression), (Summers and Sherratt 1984; Imanaka and Aiba 1981; Boe et al. 1987).

Theoretically there are three ways in which plasmids are inherited at cell division: (i) plasmids are actively portioned, (ii) distributed randomly, (iii) or their inheritance incorporates elements of active and random partitioning (Summers and Sherratt 1985).

In case of random plasmid distribution, plasmid-free cells arise in a frequency that is dependent upon the plasmid copy-number. The probability of generation of a plasmid free-cell after maternal cell division (θ) is $\theta=2^{(1-q)}$, where q is the plasmid copy-number in the cell immediately before division (Nordström and Austin 1989).

Most of the natural bacterial plasmids (usually low copy-number) are partitioned actively by a specific mechanism and therefore exhibit extremely high segregational stability ($\theta=10^{-7}$), (Nordström and Aagaard Hansen 1984).

The most commonly used in genetic engineering cloning and expression vectors are multicopy plasmids, in which the native partitioning function is inactivated. These plasmids are usually unstable and are lost from cultures at a frequency of about 10^{-2}-10^{-5} per cell per generation under non-selective conditions (Summers and Sherratt 1984). According to the above equation, the high copy-number plasmids (more than 20 copies

per cell) distributing randomly between the daughter cells should be stably maintained in the population under non-selective growth conditions. In reality, however, they are lost often at frequencies much higher than predicted. This indicates that their partitioning is not fully random, which is not due to their inactivated partitioning mechanism but rather than to other factors affecting their random distribution between daughter cells (Summers and Sherratt 1985; Dunn et al. 1993).

2. Factors affecting plasmid segregational instability

Plasmid segregational instability is a very complex phenomenon, influenced by a large number of factors that could be classified into two main groups - *biological* (cellular) and *environmental* (cell-independent) factors (Fig.1). The first group includes factors related to the host cell genotype and physiology, whereas the second group comprises the factors associated with the cultivation conditions.

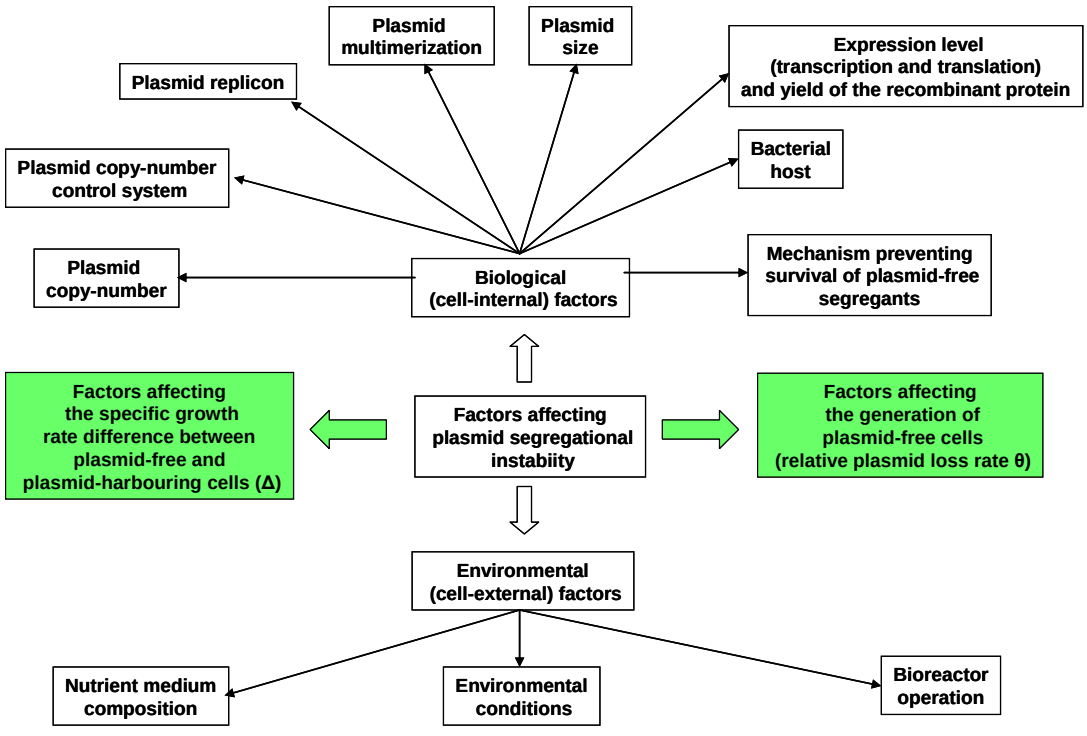


Fig. 1 Factors affecting plasmid segregational instability.

According to their effect on plasmid segregational instability the above factors can be divided into the following two groups: *factors affecting specific growth rate* of the

plasmid-harbouring cells (μ^+), (i.e. influencing the difference in the specific growth rate between plasmid-free and plasmid-harbouring cells (Δ)) and *factors affecting the generation rate* of plasmid-free cells in the bacterial population (relative plasmid loss rate θ). Both factors drive the population dynamics between plasmid-free and plasmid-harbouring cells under non-selective conditions (see "Mathematical models describing plasmid segregational instability"). It has to be mentioned that some factors could influence both Δ and θ.

2.1. Biological factors

2.1.1. Plasmid copy-number

If plasmid molecules are distributed randomly by cell division, segregational instability is influenced by the plasmid copy-number in the cells. At population level both the mean (average plasmid copy-number per cell, N_p) and the variance of plasmid copy-number are important determinants of segregational instability (Summers 1998). These parameters are highly dependent on the plasmid–encoded copy-number control system (regulating the number of the plasmid copies in the cell through the plasmid replication rate). The variance is determined by the efficiency of the control system to detect deviations and to correct plasmid copy number (Summers 1998). Nordström and Austin (1989) have shown that the broadened copy-number distribution results in increasing of plasmid segregation rate. The N_p should follow a narrow Gaussian distribution when the control system functions under optimal conditions and it should not be valid for a replicon lacking specific control functions (Solar and Espinosa 2000). Plasmid multimerization (see below) is also a factor, responsible for the asymmetrical plasmid copy-number distribution in the cell population, which results in higher frequency of generation of plasmid free cells (Summers and Sherratt 1984).

The control system is responsible for the inverse correlation between plasmid copy-number and plasmid replication rate. Summers (1998) discussed two extreme cases of plasmid copy-number control kinetics – *step function kinetics* (the replication rate drops down to zero at a critical plasmid copy number and *hyperbolic kinetics* (reflecting a simple inverse proportionality between plasmid copy-number and plasmid replication rate). The step function kinetics results in rapid correction and minimum variance of

plasmid copy-number, whereas the hyperbolic kinetics corrects relatively slow copy-number deviations thus ensuring significantly greater variance compared to the step function kinetics. A borderline case between the kinetics mentioned above is the *exponential kinetics* whose variance lies between the variances of the hyperbolic and the step function kinetics (Summers 1998).

Plasmid copy-number is strongly affected by the specific growth rate of the plasmid-harbouring cells. Generally, plasmid copy-number decreases with increasing the specific growth rate. Such results are observed for R1 plasmids (Engberg and Nordström 1975), pBR322 (Stueber and Bujard 1982), ColE1 (Siegel and Ryu 1985), etc. In batch cultures no significant variation in plasmid copy-number in the exponential growth phase is expected (cells grow with a maximal specific growth rate), whereas in the transient and stationary growth phase a decrease in specific growth rate and increase in plasmid-copy number is observed (Stueber and Bujard 1982; Ryan and Parulekar 1990).

It is shown that in a two-stage continuous culture system the plasmid content of the cells in the growth stage (where recombinant gene is repressed and specific growth rate is high) is lower than the plasmid content in the production stage (where the recombinant gene is expressed and specific growth rate is low) (Park et al. 1990).

Plasmid copy-number (Fig. 1) belongs to the factors that influences plasmid segregational instability not only through the relative plasmid loss rate θ (see above), but also via the difference in the specific growth rate between plasmid-free and plasmid-harbouring cells Δ. Plasmids require for their replication and plasmid-encoded gene expression various cell components (DNA and RNA polymerases, ribosomes, metabolites etc.), as well as energy supply. Due to this the plasmid-harbouring cells have a reduced specific growth rate in comparison with the plasmid-free cells (Ensley 1986; Bentley et al. 1990; Bhattacharya and Dubey 1995; Glick 1995). The specific growth rate of plasmid-harbouring cells is also reduced by other plasmid-related factors such as plasmid size (see below) and expression of plasmid-encoded proteins. For example, in plasmid pBR322 the *tet* gene expression (Chopra 1986) reduces considerably the specific growth rate of plasmid-harbouring cells (Lee and Edlin 1985). The reduction of specific growth rate related with heterologous gene expression is discussed below.

2.1.2. Type of the plasmid copy-number control system

Plasmid segregational instability is related with the plasmid-copy number through a copy-number control system, which ensures that each plasmid, on the average, replicates once per cell cycle. Solar and Espinosa (2000) distinguish three types of plasmid copy-number control systems, depending on the type of negative control elements employed: (i) directly repeated sequences (iterons) that complex with cognate replication (Rep) initiator proteins; (ii) antisense RNAs that hybridize to a complementary region of an essential RNA (termed countertranscribed or ctRNAs) (Wagner and Brantl 1998; Athanasopoulus et al. 1999); and (iii) ctRNA and a protein. The latter is subdivided into two categories: (iii-a) ctRNA is the major regulatory element and the protein plays an auxiliary role; and (iii-b) ctRNA and protein act on different targets and correct fluctuations in the plasmid copy-number at the steady state (del Solar et al. 1995; 1998). In the current work we have focused mainly on case (iii-a), to which belongs the most widely studied plasmids R1 and ColE1.

2.1.3. Type of plasmid replicon

The smallest section of plasmid DNA required for plasmid replication is defined as *basic replicon*, consisting of sequences responsible for start and regulation of the replication events. The basic replicon includes origin of replication (*ori*) (i); gene (*rep*) coding for replication initiation protein (Rep) (ii); and one or more genes controlling the rate of replication initiation (*cop*) (iii), (Dunn et al. 1993). The type of the plasmid replicon specifies the mechanism of plasmid replication that strongly affects segregational plasmid instability.

There are two major mechanisms of plasmid replication: *circle to circle* (theta), (i) and *rolling-circle* (sigma), (ii), (del Solar et al. 1998; Khan 2005). Generally, theta and sigma replication predominate in plasmids from Gram-negative and Gram-positive eubacteria (and archea), respectively (Wegrzyn 2005). Plasmids with similar replicons belong to the same incompatibility group. Such replicons can not coexist in the same cell (Novick 1987; Austin and Nordström 1990).

Plasmid replicon specifies the type of plasmid copy-number control system, although to one and the same control system different replicons can be related. It is known that

average plasmid copy-number, as well as variance of plasmid copy-number are highly dependent on the type of plasmid replicon.

In addition to the basic replicon, many natural plasmids (most of them low copy-number) bear different genetic systems (partition cassettes, *par* cassettes, *par* loci) ensuring even distribution of the plasmid copies between the daughter cells prior to cell division. This type of plasmids is characterized by an extremely stable maintenance and high segregational stability. Plasmids devoid of partition cassettes are lost from the population at a frequency that is dependent on plasmid copy-number (Nordström and Gerdes 2003). Generally, *par* loci function independently of the basic replicon on which they reside (Ebersbach and Gerdes 2005).

The *par* loci encode two *trans*-acting proteins expressed from one operon and one or more *cis*-acting centromere-like sites, at which the two proteins act (Ebersbach and Gerdes 2005). The first gene of the *par* operon encodes two alternative types of ATPases and accordingly to this characteristic, *par* loci are divided into: (i) *par* loci encoding actin-like ATPases (Type II *par* loci) and (ii) *par* loci encoding Walker box ATPases (Type I *par* loci). Type I *par* loci are harboured by the majority of plasmids with partition cassettes. R1 is the best investigated plasmid carrying Type II *par* locus (Nordström 2006).

The ColE1 replicon

The ColE1 replicon, native of ColE1 and pMB1 plasmids, has been studied for many years (Davidson 1984; Thomas 1988; Kues and Stahl 1989; Wegrzyn 1999; Grabherr and Bayer 2002). It consists of an origin of replication (*ori*), two *cop* genes (coding for RNAI and Rop protein) and a *rep* gene (encoding RNAII), (Dunn et at. 1993).

The ColE1 replication mechanism is summarized in Fig. 2. The transcription initiated from the P^{r2} promoter (located 555 bp upstream of *ori*) results in a 555 nt long pre-primer RNAII with a very specific (clover like) structure at the 5' terminus (Masukata and Tomizawa 1984; 1986; Wong and Polisky 1985). This RNA forms a hybrid with the template DNA that is recognized and cleaved by a specific RNase H up to the site of *ori*. Thus an RNA primer is formed to allow DNA polymerase I to begin unidirectional DNA synthesis (plasmid replication) at the promoter P^{d} (Itoh and Tomizawa 1980; Selzer and

Tomizawa 1982). The RNAII primer is further removed from the template by RNase H. It is worth mentioning that additionally to the described replication mechanism, two alternative pathways of replication without RNase H or Pol I have also been described (Dasgupta et al. 1987; Ohmori et al. 1987; Masukata et al. 1987).

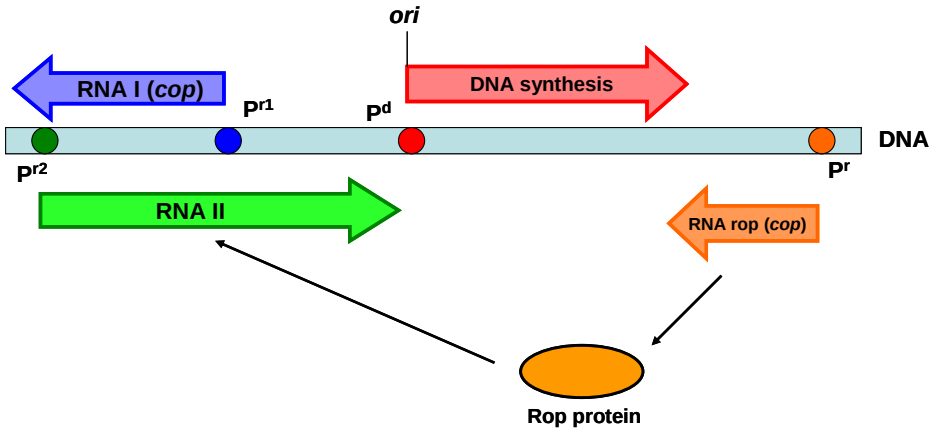


Fig. 2 Structure and function of the ColE1 replicon. Thick arrows denote the direction of transcription from the relevant promoters. All symbols are given in the text. (The figure is derived from Dunn et al. 1993).

Initiation of ColE1 plasmids replication is assisted also by other two molecules - RNAI and Rop protein. The transcription of the 108 nt long RNAI starts from the promoter P^{rI} (located 445 bp upstream of *ori*) and proceeds in the opposite (to RNAII) direction using the other DNA strand. Therefore the RNAI is fully complementary to the 5'-terminus of RNAII and can acts as an antisense RNA. The RNAI-RNAII duplex thus formed is strong enough to prevent hybridization of RNAII with the template DNA and therefore to suppress formation of an RNAII primer necessary for initiation of plasmid replication.

The mechanism of RNAI-RNAII duplex formation is very well studied (Tomizawa 1986). Both RNAI and RNAII have similar (clover like) secondary structures characterized by three loops (I, II and III). The three loops in RNAII are located at its 5'-terminus. RNAI and RNAII interact initially through a reversible contact (called "kissing") between the loops II, followed by a complete pairing of their complementary regions by a zipping mechanism (Tomizawa 1984; 1985). The RNAI-RNAII pairing is assisted by a small (63 amino acids) protein Rop/Rom, encoded by a gene staying downstream from *ori* (Lacatena et al. 1984; Tomizawa 1990). The Rop protein (in the

form of homodimer) stabilizes the “kissing” step (Dooley and Polisky 1987) thus enhancing RNAI-RNAII binding, which suppresses the formation of RNAII primer. As a result, the combined action of RNAI and Rop results in a decrease in the rate of plasmid replication and reduction of plasmid copy-number. That is way any deletions or mutations in the Rop gene have increasing effect on the plasmid copy-number (Lin-Chao et al. 1992). Such effect is also observed with mutant RNAI and RNAII (Cesareni et al. 1991) particularly those bearing mutations in the loop regions (Grabherr and Bayer 2002; Grabherr et al. 2002).

How the two antisense RNAs RNAI and RNAII maintain the plasmid copy-number level? The RNAI is transcribed constitutively and its half life is only 0.55 min (for comparison, the half-life of the Rop protein is 69 min) so that its concentration in the cell closely follows the plasmid copy-number. Immediately after transformation the content of plasmids in bacterial cell is low and consequently the concentration of RNAI in bacterial cytoplasm is low too. The latter allow RNAII to form easily functional primers and to initiate plasmid replication. The efficient plasmid replication results in increasing of plasmid copy-number, which leads to an increase in the concentration of RNAI in the cytoplasm. Higher concentration of RNAI however, is a predisposition for RNAI-RNAII duplex formation and therefore for a decrease in the plasmid replication rate. Finally, a steady state condition is established (a constant concentration of RNAI and RNAII) characterized with constant plasmid content. Its level (i.e. the plasmid copy-number value) is determined by the genetic structure of the ColE1 replicon.

The secondary (clover like) structure of RNAI and that of the 5’ end of RNAII resemble that of the tRNAs. It has been observed that some tRNA’s interfere with the regulation of ColE1-like plasmid replication. The latter could be explained by close homology between loop II of RNAI and the dihydrouridilic loop of tRNAs (i) (Yavachev and Ivanov 1988), by similarities of anticodon loops of tRNAs and RNAI or RNAII (ii) (Wrobel and Wegrzyn 1998), or by the 3’ CCA sequences of tRNAs and loops I, II and III of RNAI or RNAII (iii) (Wang et al. 2002).

The kinetics of ColE1 replication control is also studied and the obtained results and conclusions are disputable (Ehrenberg 1996). Whereas Brendel and Perelson (1993)

proposed a hyperbolic kinetics, Brenner and Tomizawa (1991) as well as Paulsson and Ehrenberg (1998) predicted an exponential rather than hyperbolic kinetics.

2.1.4. Plasmid multimerization

One of the most significant factors affecting the random distribution of plasmids between daughter cells is their multimerization (Summers and Sherratt 1984). The plasmid multimers can be either concatamers (several plasmids covalently linked in a single ring), or concatenanes (several plasmid molecules joined together in a chain), (Dunn et al. 1993).

Concatamers arise in the cells as a result of homologous recombination between plasmids. A single cross-over between two plasmid monomers results in a formation of a plasmid dimer, where the two plasmid monomers are covalently linked in a head-to-tail arrangement. The plasmid dimer may be resolved into two monomeric plasmids as the result of *intramolecular* recombination between the two plasmid units, or it may serve as substrate for generating a trimeric or tetrameric plasmid by *intermolecular* recombination with monomeric or a dimeric plasmid (Boe and Tolker-Nielsen 1997).

The plasmid multimers (concatamers and concatenanes) segregate as individual units irrespective of the number of plasmids associated together. The plasmid replicon can not improve this because it "counts" the number of origins of replication and not the number of plasmids. This is predicted by the so called "origin counting" hypothesis of copy-number control proposed by Pritchard (1978). The origin counting model is explained by the *inhibitor dilution theory*, (Pritchard 1984), according to which the cellular concentration of the *cop* gene product (inhibiting the *rep* gene product) is proportional to the number of the plasmids in the cytoplasm. Consequently, the plasmid multimerization reduces the number of free (randomly) segregating units in the cell thus increasing plasmid segregational instability.

Summers and Sherrat (1984) have shown that even a small number of plasmid dimers in the cell population greatly affect plasmid segregation. Obviously, the effect of plasmid multimerization on plasmid segregation exceeds the expected effect of the reduced number of randomly distributed segregation units. An explanation of this phenomenon is given by the *dimer catastrophe hypothesis*, proposed by Summers et al.

(1993). They show that plasmid monomers and dimers are heterogeneously distributed in the plasmid-carrying cell population. The majority of cells harbour mainly plasmid monomers ("monomer-only cells"), whereas a small portion of cell population bears exclusively plasmid dimers ("dimer-only cells"). The obviously non random distribution of monomers and dimers in the microbial population is due to the replication advantage of plasmid multimers over plasmid monomers. This is explained by the random choice of a plasmid (respectively *ori*) for initiation of plasmid replication (Gustafsson and Nordström 1975) so that dimers have twice the probability of replication than monomers because dimers have two origins of replication. Due to their replication advantage dimers are accumulated rapidly after their appearance in the cell population – a phenomenon known as a "dimer catastrophe". As a result of the dimer catastrophe approximately half the plasmid dimers in the population are distributed in dimer-only cells at steady state conditions (Summers et al. 1993).

According to the dimer catastrophe hypothesis plasmid dimers should be displaced by trimers, they by tetramers etc. On the other hand, dimer (multimer)-containing cells grow more slowly than monomer-containing cells (Summers et al. 1993; Summers 1998) thus a progressive runaway multimerization is limited in the microbial population. The slower growth of cells containing plasmid multimers can be considered also as a strategy for prevention of the dimer catastrophe. For plasmids lacking a multimer resolution system (see below) it is the only way to avoid dimer catastrophe (Summers 1998).

As a strategy against multimerization and dimer catastrophe, many naturally occurring multicopy plasmids evolved highly specific recombination sites, where certain proteins act to resolve multimers to monomers (Summers 1998; Summers and Sherratt 1988). The best studied plasmid resolution system is *cer*-Xer of the plasmid ColE1 (Summers and Sherratt 1984). This plasmid contains a 240 bp recombination site (*cer*) that when present in more than one copy per molecule (as in plasmid multimers), is involved in site-specific recombination, resulting in resolution of ColE1 multimers to monomers. At least four host-encoded proteins participate in the recombination process at *cer*: XerC and XerD form a heterodimeric recombinase which catalyzes strand exchange (Colloms et al. 1990; Blakely et al. 1993). In addition, two accessory proteins, ArgR and PepA, are required as mediator in the recombination process (Stirling et al. 1988; Stirling

et al. 1989; Colloms et al. 1996). It has been mentioned that common recombination cell enzyme reveals an extremely low specificity/activity for recombination at *cer* (compared to the specific recombinase enzyme Xer) and its efficiency to convert multimers to monomers is negligible.

Molecular models for structure and function of the nucleoprotein complexes accomplishing recombination at *cer* have been proposed by Hodgman et al. (1998) and Strater et al. (1999). Additionally, Balding et al. (2006) proposed a checkpoint mechanism performed by the *cer*-Xer resolving system that delays the cell division until multimer resolution is complete. In multimer-containing cells the promoter P_{cer} located within *cer* directs a transcription of a 70 nt RNA, called Rcd, that delays the division of multimer-containing cells. The crucial role of Rcd against plasmid multimerization places this RNA among the factors preventing plasmid segregation.

The plasmid ColE1 is characterized by a very low level of multimerization due to the *cer*-Xer multimer resolution system. The common cloning and expression vectors are devoid of *cer* sequence. They are prone to multimerization and show higher segregational instability in recombinant proficient hosts (Summers and Sherratt 1984).

Multimer resolution systems similar to *cer*-Xer were identified also for other naturally occurring plasmids. Most of them employ the four chromosome-encoded proteins mentioned above. The plasmid pSC101 (harbouring the recombination site *psi*), (Cornet et al. 1994) is independent of ArgR, which is replaced by the DNA binding protein ArcA (Colloms et al. 1998). A notable exception is the plasmid P1 (containing the recombination site *lox*), that encodes the recombinase Cre (Austin et al. 1981).

Some plasmids contain “hot spots” of recombination (James and Kolodner 1983) that favours plasmid multimerization and plasmid segregational instability (Summers and Sherratt 1984). Kolot et al. (1989) have shown that a small DNA fragment localized in the area of *Hin*d III in the plasmid pBR322 (within the region of the Tet^R gene promoter) decreases substantially plasmid segregational stability (both in *cis* and in *trans*). They suggest that this region (responsible also for the abnormally high supercoiling of plasmids in topoisomerase I mutants of *E. coli*, (Pruss and Drlica 1986)) could be a “hot spot” of plasmid recombination.

2.1.5. Plasmid size

Large plasmids are known to decrease the specific growth rate of bacteria (Summers and Sherratt 1984; Ryan et al. 1989). Cheah et al. (1987) showed that such plasmids do not affect microbial exponential growth, but decrease maximal cell density and accelerate cell death after approaching the stationary phase. It was shown also that large plasmids are responsible for a longer lag growth phase (Smith and Bidochka 1998). Corchero and Villaverde (1998) showed, however, that the size of the insert rather plasmid size decreases the specific growth rate of plasmid-harbouring cells. Additionally, a very large insert can accelerate plasmid loss (Smith and Bidochka 1998).

In some cases an inverse correlation between plasmid size and plasmid copy-number is observed (Gelfand et al. 1978; Bron and Luxen 1985; Smith and Bidochka 1998).

2.1.6. Expression level (transcription and translation) and yield of the recombinant protein

The level of gene expression (transcription and translation) and the resulting yield of recombinant protein are factors strongly affecting plasmid segregational instability. They can influence both the difference in specific growth rates between plasmid-free and plasmid-harbouring cells Δ and the relative plasmid loss rate θ.

Recombinant gene expression and plasmid replication (see Plasmid copy-number) cause significant reduction in the specific growth rate of plasmid-harbouring cells, μ^+ (respectively an increase of Δ), (Ensley 1986; Bentley et al. 1990; Bhattacharya and Dubey 1995; Glick 1995; Kyslik et al. 1993). Generally, recombinant gene expression is a much higher metabolic burden than the plasmid replication itself (Bentley et al. 1990). The reduction of μ^+ can be referred to the following burdens in recombinant bacteria: metabolic and energetic cell resources, necessary for recombinant gene expression and plasmid maintenance (i), competition with components engaged in chromosomal replication and host gene expression (ii), and recombinant protein toxicity (iii). According to Corchero and Villaverde (1998), the recombinant protein toxicity is a major factor affecting negatively the physiology of the recombinant bacterial cell. Some properties of the recombinant protein such as hydrophylicity/hydrophobicity can also influence the cell growth. It was shown that highly hydrophobic recombinant proteins

strongly inhibit cell growth (Zabeau and Stanley 1982; Padan et al. 1983; Remaut et al. 1983).

In some plasmid constructs the recombinant gene expression can affect plasmid segregational instability through plasmid replication. Stueber and Bujard (1982) and Bujard et al. (1985) have shown that transcription from a strong promoter can interact with the ColE1 replicon of pBR322 derivative plasmids and reduces their copy-number and hence plasmid segregational stability. Stueber and Bujard (1982) have shown that transcriptional readthrough into the ColE1 replicon towards *ori* reduces plasmid-copy number through the following effects: (i) leads to overproduction of Rop protein which reduces plasmid copy-number and (ii) interferes negatively with plasmid replication initiation. It is not clear whether the latter is due to an enhanced synthesis of RNAI, to a decreased synthesis of RNAII, or both (Bujard et al. 1985). The insertion of an efficient transcriptional terminator that prevents the transcriptional readthrough into the replication region, however, increases plasmid copy-number again.

2.1.7. Bacterial host

Wang et al. (2004) listed a large number of genes that are associated with the ColE1 replication and therefore affecting plasmid copy-number and plasmid segregational instability. Homologous recombination between plasmids depends on the bacterial strain and leads to plasmid multimer formation that strongly reduces plasmid segregational instability (Summers and Sherratt 1984). In some host strains up to 50% of the plasmids are found in oligomeric form (James et al. 1982).

2.1.8. Mechanisms preventing survival of plasmid-free cells

Some natural plasmids possess mechanisms allowing to kill (or strongly inhibit the growth of) the plasmid-free cells thus resulting in a cell population consisting mainly of plasmid-harbouring cells irrespective of the plasmid segregation process (i.e. at population level $\theta \approx 0$). For example the plasmid R1 “kills” plasmid-free cells through the so called *hok-sok* system (Gerdes et al. 1986). In plasmid-harbouring cells the *hok* gene is transcribed in a stable $mRNA_{hok}$ whose translation is blocked by the unstable antisense $mRNA_{sok}$ (a product of the *sok* gene). In cells that lose their plasmids, the concentration

of $mRNA_{sok}$ rapidly drops off thus enabling the translation of $mRNA_{hok}$ to a toxic protein hok.

Similar mechanisms preventing survival of plasmid-free cells are observed by other plasmids such as Col plasmids (Luria and Suit 1987) and F (Miki et al. 1988).

2.2. Environmental conditions

Environmental factors play a crucial role in plasmid segregational instability. Generally, they can be classified as related with the: (i) nutrient medium composition; (ii) cell cultivation conditions (temperature, pH, pO_2, etc) and (iii) bioreactor construction and operation.

2.2.1. Nutrient medium composition

Generally, glucose limitation increases plasmid segregational stability (Noack et al. 1981; Jones and Melling 1984). Phosphate and magnesium limitations decrease plasmid segregational stability (Jones et al. 1980). Nitrogen limitation does not influence plasmid stability (Noack et al. 1981). Some ingredients as casamino acids can improve plasmid segregational stability (Brownlie et al. 1990). Low copy-number plasmids show higher segregational stability under continuous cultivation with phosphate, nitrogen and potassium limitation but lower stability with sulphate limitation (Caulcott et al. 1987).

2.2.2. Cultivation conditions

Plasmid segregational stability decreases with increasing the cultivation temperature (Wouters et al. 1980). Plasmid-free and plasmid-harbouring cells can expose different pH preferences and therefore their growth competition can be influenced by pH. Ryan and Parulekar (1990) have shown that the optimal pH for growth of *E. coli* JM103 cells harbouring the plasmid pUC8 is 6.8 whereas for plasmid-free cells it is pH 7.4. Both short-term and long-term oxygen limitations decrease significantly plasmid segregational stability (Chen et al. 1991; Nielsen et al. 1991).

2.2.3. Bioreactor operation

Plasmid segregational instability is influenced also by the bioreactor configuration and operation. The dilution rate in continuous fermentation is one of the most important factors affecting plasmid segregational stability (Chew et al. 1988; Karbasi and Keshavarz 1997).

To increase productivity and decrease plasmid instability in recombinant fermentation processes a two stage continuous culture system consisting of a separated growth and production stage has been proposed (Siegel and Ryu 1985; Lee et al. 1988; Park and Ryu 1990). In the growth stage the expression of the recombinant protein is repressed and bacteria grow with a high specific growth rate resulting in a rapid accumulation of biomass. Conversely, in the production stage (where optimal biomass is reached) the expression of the recombinant gene is induced (usually after derepression of an inducible promoter). The two stage continuous culture system is often combined with a runaway replication plasmid system (e.g. plasmids that are temperature-sensitive for replication) that enables an increase of plasmid copy-number, i.e. an increased gene dosage effect in the production stage. To improve plasmid stability other specific bioreactor configurations have also been proposed (see 4.2.6).

Cell immobilization is another factor increasing plasmid segregational instability (Berry et al. 1990; de Taxis du Poet et al. 1987; Nasri et al. 1987; Joshi and Yamazaki 1987, etc.). Generally, this is due to the mechanical properties of the gel beads that are responsible for limited number of cell divisions so that the competition between plasmid-free and plasmid-harbouring cells is significantly reduced (Barbotin 1994; Barbotin et al. 1990). In some cases the cell immobilization leads also to an increase in plasmid copy-number that additionally contributes to enhanced segregational stability (Sayadi et al. 1989).

3. Plasmid clustering

The random loss of multicopy plasmids suggests that the plasmid segregation unit is a single plasmid copy (Nordström et al. 1980). It was found, however, that the plasmids (both multicopy and low copy-number) are grouped in clusters rather than being randomly distributed in bacterial cytoplasm (Eliasson et al. 1992; Reich et al. 1994;

Gordon et al. 1997; Niki and Hiraga 1997; Bignell et al. 1999; Jensen and Gerdes 1999; Weitao et al. 2000a; Pogliano et al. 2001; Ho et al. 2002; Li and Austin 2002; Lawley and Taylor 2003; Gordon et al. 2004). The latter raises the question of how plasmid molecules can segregate randomly if they have cluster organization.

Clusters are observed as fluorescent foci using FISH (fluorescence *in situ* hybridization) and fluorescence microscopy. Unfortunately, it has not been possible to isolate real clusters until now (e.g. by sucrose gradients) that incredibly troubles their investigation. Due to this, very little is known about their structure and the mechanisms of their formation. Not known are also the factors essential for plasmid clustering and how the plasmid molecules are bound together. It was found, however, that the cluster formation is dependent on protein and/or RNA synthesis (Yao et al. 2006) but not on DNA synthesis (Johnson et al. 2005). Observation of clusters with both Par^- and Par^+ plasmids suggests that the *par* locus is dispensable for cluster formation. The presence of a *par* locus, however, affects the cluster localization in bacterial cytoplasm (Nordström and Gerdes 2003; Yao et al. 2006). Generally, Par^+ plasmids occupy defined domains in the cell (mid-cell area in new-born cells and ¼ to ¾ positions to the cell poles in older cells (Weitao et al. 2000b). Moreover, Ho et al. (2002) and Ebersbach et al. (2005) have shown that clusters of Par^+ plasmids with different replicons are located preferably in the cytoplasm (at the mid-cell or at the quarter of the cell). In contrast to the Par^+ plasmids, the clusters of Par^- plasmids are distributed more randomly inside the cell (Weitao et al. 2000b).

Nordström and Gerdes (2003) proposed a hypothetical model for plasmid partitioning and segregation that defines the single plasmid but not the plasmid cluster as a unit of random segregation. According to this model plasmid replication occurs in replication factories located stationary in the cell centre (experimentally supported by Onogi et al. (2002) and Møller-Jensen et al. (2002)) and not in the clusters, that are localized far away from the replication machinery (experimentally supported by Ho et al. (2002)). The model postulates that a single plasmid is released from the cluster and moved to the replication factory located in the cell centre where it doubles (replicates). After replication Par^+ and Par^- plasmids follow different pathways of distribution in the maternal cell. The two Par^+ plasmid copies are actively moved in opposite directions to

the two cell halves so that each daughter cell will receive one of the copies after cell division. Related to all plasmids in the mother cell, each daughter cell will receive the half of the copies (in reality approximately the half of the copies, if some plasmids can not be replicated in the cell generation time). At the end of replication the plasmid copies do not remain randomly distributed in the cytoplasm but they move back to the already existing cluster or participate in the formation of a new cluster (in cells with a single cluster a new one have to be created so that every daughter cell can inherit a cluster). In contrast to the Par^+ plasmids, the Par^- plasmids are not actively moved after replication by a partition mechanism but turn randomly to the clusters (to the cell halves). As a result, the daughter cells have two opportunities: i) both cells inherit plasmid copies and ii) one of the cells inherits all plasmid copies and the other one remains plasmid-free cell. Related to all plasmids of the maternal cell (q), they will segregate randomly as the probability of generation of plasmid-free cells is $\theta=2^{(1-q)}$ (see above).

The model proposed by Nordström and Gerdes (2003) introduces a new concept about plasmid segregational instability, according to which the random plasmid segregation is a continuous process closely related to plasmid replication but not to the cell division process.

4. Methods for overcoming plasmid segregational instability

Segregational instability of multicopy plasmids with a relaxed control of replication is a major factor reducing the yield of recombinant proteins. Several approaches for improving plasmid segregational stability have been developed (for review see Friehs and Reardon (1993)). Generally they can be classified as selective (plasmid-free cells are eliminated) and non-selective (plasmid-free cells have a growth advantage over the plasmid-harbouring cells).

4.1. Selective methods

4.1.1. Antibiotic selection

The most common selective method for retaining the expression plasmid in recombinant bacteria is the insertion of an antibiotic-resistance gene (genes) in the plasmid and cultivation in the presence of the same antibiotic. This method is simple but

it has several disadvantages at an industrial scale, including antibiotic cost, separation of the antibiotic from the final product, detoxication of the antibiotic in the wastes, etc. The presence of antibiotics in growth media decreases also the specific growth rate of the plasmid-harbouring cells (Porter et al. 1990; Friehs and Reardon 1993, Balbas 2001). Moreover, sometimes arising of plasmid-free cells cannot be completely prevented, because the effective antibiotic concentration in the growth medium can decrease upon a long-term cultivation because of dilution and/or enzymatic degradation (Balbas 2001; Porter et al. 1990).

4.1.2. Mutant host/plasmid selection systems

This selection method consists in insertion in the plasmid of an indispensable for the cell metabolism gene, which is mutated (or deleted) in the host chromosome.

To this end a variety of hosts have been created containing mutant genes such as: *serB* gene (coding for serine production)/*serB*$^{-}$ host, *valS* gene (coding for valyl tRNA-synthetase)/*valS*^{ts} host, *ssb* gene (coding for SSB protein)/*ssb*$^{-}$ host (Friehs and Reardon 1993), *infA* (coding for translation initiation factor 1, IF1)/*infA*$^{-}$ host (Hägg et al. 2004). Disadvantages of the method are the low specific growth rate of the plasmid-harbouring cells as well as spontaneous reverse mutations.

4.1.3. Systems for post-segregational killing of plasmid-free cells

In this approach a natural cell killer system preventing survival of plasmid free cells is incorporated into the plasmid vector. For example the *hok*/*sok* killer locus of the naturally occurring plasmid R1 (see 2.1.8) was used for preventing plasmid segregation (Wu and Wood 1994).

A similar technique for lysis of plasmid-free cells is the incorporation of phage λ repressor gene in the plasmid and infection of the host cells with the phage λ (Rosteck and Hershberger 1983).

4.2. Non-selective methods

A significant disadvantage of the selective methods is that they are not applicable for maintaining high copy-number plasmids (usually one plasmid copy per cell is sufficient

to make the cell viable). Unlike selective methods, the non-selective (genetic) methods allow stable maintenance of high copy-number of plasmids.

4.2.1. Incorporation of a strong transcription terminator in the plasmid

The insertion of a strong transcription terminator aims to overcome the interference between transcription of the foreign gene with that of the genes involved in plasmid replication (Bujard et al. 1985; Chen and Morrison 1987).

4.2.2. Incorporation of a stabilization partition locus in the plasmid

Many naturally occurring low copy-number plasmids contain partition cassettes, (*par* cassettes, *par* loci) ensuring a precise distribution of the plasmids between the daughter cells and therefore an extremely high plasmid segregational stability (see 2.1.3.). Incorporation of a *par* locus into the plasmid vector (e.g. the *par* locus of the plasmid pSC101, (Nashimura et al. 1989; Meacock and Cohen 1980)) increases its stability. This effect is better expressed with low copy-number rather than with high copy-number plasmids (Meacock and Cohen 1980).

4.2.3. Incorporation of the *cer* locus in plasmid vectors

The naturally occurring plasmid ColE1 evolves a highly specific recombination site *cer* for resolving multimers to monomers (see 2.1.4). Incorporation of the *cer* locus in plasmid vectors ensures their stable maintenance in *xer*$^{+}$ (genes for site-specific recombination) hosts (Summers and Sherratt 1984).

4.2.4. Addition of complex nutrients

Plasmid segregational instability can be improved by addition of complex nutrients like casamino acids (Brownlie et al. 1990).

4.2.5. Runaway replication systems

The use of adjustable copy-number (runaway replication) plasmids ensures both high yield of recombinant protein and high segregational stability (Nordström and Uhlin 1992). This approach is suitable for continuous cultivation in a two-stage chemostat (see

2.2.3.), where low copy-number and high copy-number of plasmids is maintained in the growth and production stage, respectively (Siegel and Ryu 1985).

4.2.6. Bioreactor configuration methods and cell immobilization

Alternatively to the traditional batch and chemostat operation mode several bioreactor configurations have been applied to increase plasmid segregational stability including two-stage chemostat (Siegel and Ryu 1985) and configurations with cyclic changes of dilution rates (Impoolsup et al. 1989), substrate concentrations (Stephens and Lyberatos 1988) and temperatures (Di Pasquantonio et al. 1987). Cell immobilization has also been used to increase plasmid stability (see 2.2.3).

5. Mathematical models describing plasmid segregational instability

5.1. Plasmid segregational instability and cell population dynamics

The plasmid segregational instability (due to irregular distribution of plasmids between the daughter cells) leads to a rise of plasmid-free cells and plasmid-harbouring cells with different content of plasmids per cell. The generation rate of plasmid-free cells in the population (specific $\boldsymbol{\Theta}$, or relative $\boldsymbol{\theta}$, see Mathematical background) and the difference in the specific growth rate between plasmid-free and plasmid-harbouring cells $\boldsymbol{\Delta}$ are prerequisites for a complex population dynamics depending on various biological and environmental factors (see Fig. 3). The rate of generation of plasmid free-cells is a parameter reflecting the tendency of establishing plasmid-free cell population (Fig. 3, the black, the yellow and the blue lines compared with the mangenta, green and red lines, respectively). The difference between the specific growth rates $\boldsymbol{\Delta}$, however, can tend to the establishment of either plasmid-free or plasmid-harbouring cell populations depending on the absence/existence of selection pressure during cultivation. In the absence of selection pressure, the plasmid free cells (exposing higher specific growth rate comparing with the plasmid-harbouring cells) will overgrow the plasmid bearing cells (Fig. 3, red and blue lines). Conversely, in the presence of selection pressure the plasmid-harbouring cells will be at growth advantage (despite of their metabolic and energetic burden) and therefore they will overgrow the plasmid-free cells (Fig 3, mangenta and black lines). However, the investigation of the plasmid segregational instability is

performed in the absence of selection pressure, i.e. the case when $\Delta<0$ is not under considerations in the current work.

The time course of the plasmid-harbouring cell fraction (segregation curve) is defined by both the generation rate of plasmid-free cells and the growth rate difference between plasmid-free and plasmid-harbouring cells (Fig. 4). Therefore, in order to analyze the factors affecting population dynamics it is necessary to distinguish their influence on these two parameters, which requires implementation of mathematical modelling.

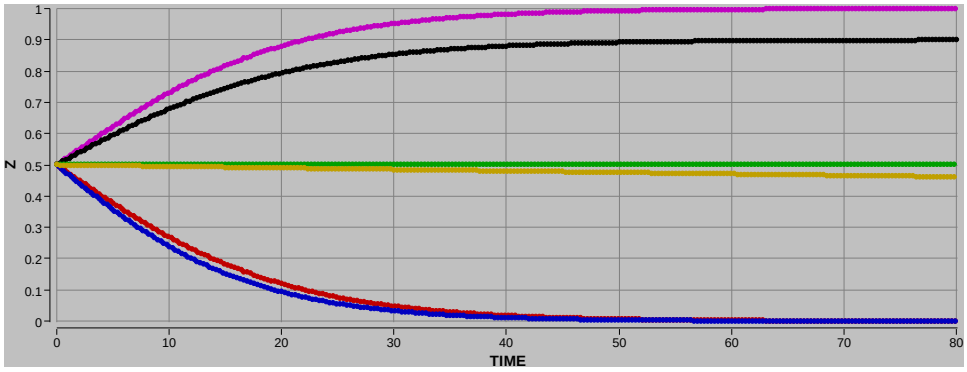


Fig. 3 Simulated plasmid-harbouring cell fractions with the time under selective and non-selective growth conditions obtained by the model of Stewart and Levin (1977). The simulations were performed employing Eq. 23 with the following parameter values: Δ=0.1 h^{-1} , Θ=0 (red line); Δ=0.1 h^{-1} , Θ=0.001 h^{-1} (blue line); Δ=-0.1 h^{-1}, Θ=0; (mangenta line); Δ=-0.1 h^{-1} , Θ=0.001 h^{-1} (black line); Δ=0, Θ=0 (green line) and Δ=0, Θ=0.001 h^{-1} (yellow line). (According to Eq. 26 $\Delta<0$ if $\mu^+ > \mu^-$). Initial condition for all simulations: z=0.5 at t=0.

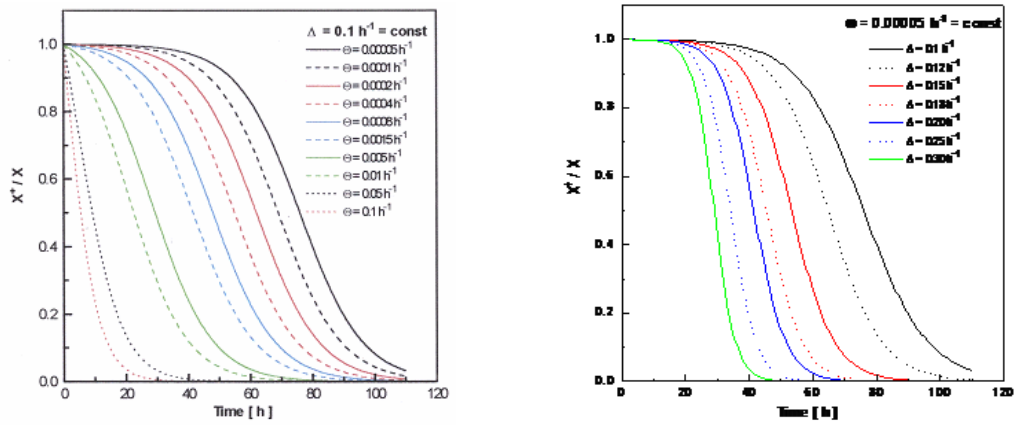


Fig. 4 Simulated plasmid-harbouring cell fractions with the time under non-selective conditions obtained by the model of Stewart and Levin (1977). Panel A: Simulations performed employing Eq. 23 with Δ=0.1 h^{-1}=const and different values of Θ. **Panel B:** Simulations performed employing Eq. 23 with Θ=0.1 h^{-1} = const and different values of Δ. Initial condition for all simulations: z=0 at t=0.

Usually plasmid segregational instability is investigated in a chemostat, which allows prolonged study under constant cultivation conditions. The transition from batch to continuous operation results in establishment of a quasi-stationary state (for relative low values of **Λ** and **Θ** (**θ**)), where the culture is dominated by plasmid-bearing cells despite of the slow appearance of plasmid-free cells. In the quasi stationary state many process variables such as total cell mass concentration, dissolved oxygen level, plasmid copy-number etc. remain fairly constant. The quasi stationary state is followed by a transition period where the plasmid-free cells rapidly overgrow plasmid-harbouring cells. During this phase the process variables change significantly as the plasmid-harbouring cells, recombinant protein concentration and plasmid-copy number decrease and finally vanish (Ryan and Parulekar 1990).

5.2. Modelling of fermentation processes and plasmid segregational instability

Five levels of complexity have been introduced in bioprocess modelling (Fig 5), (Bailey et al. 1983; Moser 1985). According to them the following type of models are distinguished: molecular level model (1); single cell model (2), population model (3), bioreactor model (4), and bioplant model (5).

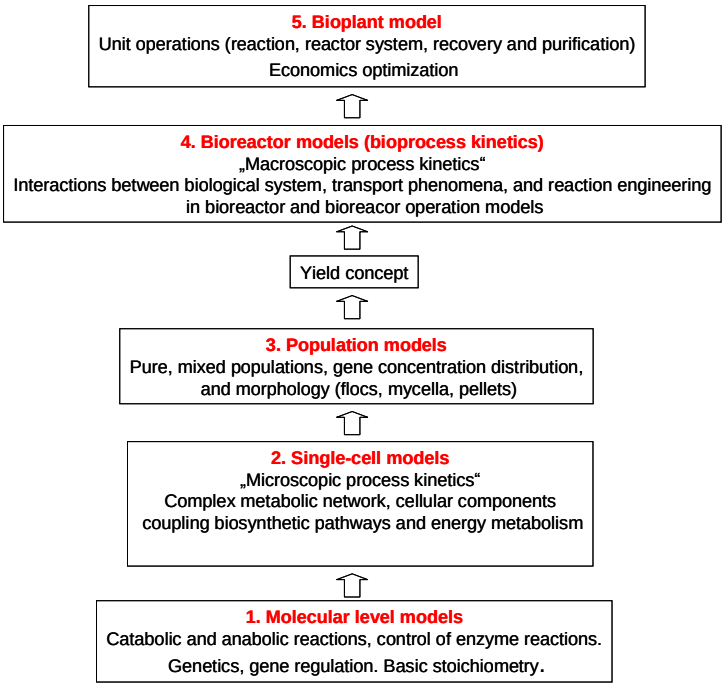


Fig. 5 The five levels of complexity in bioprocess modelling (Bailey et al. 1983; Moser 1985). This figure is borrowed from Ryu et al. (2008).

The model proposed by Stewart and Levin (1977) describes growth of plasmid-harbouring and plasmid-free cells at population level. The population dynamics is dependent on the specific growth rates of plasmid-free and plasmid-harbouring cells as well as on the specific generation rate of plasmid-free cells (specific plasmid loss rate). The specific plasmid loss rate, however, is a product of the specific growth rate of the plasmid-harbouring cells and the relative plasmid loss rate (probability of generation of plasmid-free cell per cell division per plasmid-harbouring cell). The latter is introduced in the population model of Imanaka and Aiba (1981). The model of Ollis and Chang (1982) employs equations for substrate consumption and product formation together with the equations describing growth of plasmid-harbouring and plasmid-free cells. Thus this model covers both population and bioreactor levels. The specific growth rate of plasmid-free and plasmid-harbouring cells is described by the Monod equation where the maximal specific growth rates of both cell types are presumed to be constants. The product formation is given by the Leudeking-Piret equation. The model proposed by Lee et al. (1985) comprises single cell, population and bioreactor level. The product formation kinetics is based on quasi-steady-state transcription-translation model. The specific growth rate of the plasmid-harbouring cells is given by an extended Monod equation containing empirical terms for plasmid content and recombinant protein inhibition on cell growth. These inhibition effects are dependent on a large number of factors such as plasmid design, properties of the recombinant protein, genetic background and physiology of the host cells, environmental conditions, etc. Due to this variations of the inhibition terms are also proposed. For example, Patnaik (2000) has reviewed model concepts describing inhibition of plasmid content on specific bacterial growth rate. Beside the model of Lee et al. (1985), the models of Satyagal and Agraval (1989), Keasling and Palsson (1989) and Bentley and Quiroga (1993) are also discussed. Usually single cell models explore the specific growth rate as a characteristic of the overall metabolic activities of the cell population.

Molecular level models based on genetic control and regulatory mechanisms have been proposed for the *lac* promoter-operator system (Lee and Bailey 1984 a, b) and for the λdv plasmid replication (Lee and Bailey 1984 c, d). If molecular level models describing plasmid segregational instability would be developed, such models should

relate the relative plasmid loss rate with plasmid design and mechanisms of plasmid multimerization, plasmid replication, plasmid copy-number control and partitioning. Such models would describe entirely plasmid segregational instability but inevitably would come up against practical restrictions because of a large number of parameters.

6. Human interferon gamma

Interferon-gamma is a cytokine endowed with multiple biological activities and broad pharmaceutical applications (Tsanev and Ivanov 2001). 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 antiparasitic cell activity, affecting cell proliferation, apoptosis, etc.

Human interferon-gamma (hIFNγ) is a 17 kDa single polypeptide protein consisting of 143 amino acids (aa), of which 28 are basic (lysine and arginine). It is organized in six α-helices comprising 62% of the molecule, which are linked by short unstructured regions. The hIFNγ is devoid of β-sheets. Under physiologic conditions it is organized in a robust non-covalent homodimer, in which the two monomers are associated in antiparallel orientation (Ealick et al. 1991). Besides the six α-helices and connecting regions, hIFNγ contains also a long unstructured C-terminal region consisting of 21 aa, of which 8 aa are Lys and Arg. In a study on the electrostatic interactions in both hIFNγ free homodimer and homodimer bound to the hIFNγ receptor, it is shown that most of the basic amino acids in the globular part of the hIFNγ homodimer are buried inside the molecule. They are not accessible to the water and therefore are not ionized. This means that the effective (for intermolecular electrostatic interactions) positive charges in hIFNγ are located in the unstructured C-terminal domain. Therefore it is logical to expect that deletions in the C-terminal region, particularly affecting Arg/Lys, would change the physic-chemical and/or biological properties of the resulting protein. Aiming to shed light on the role of the C-terminal part of hIFNγ for its biological activities, in a previous study Nacheva et al. (2003) performed gradual (by three codons) deletions at hIFNγ gene 3'-terminus until the whole unstructured C-terminal region of hIFNγ was removed. Investigating antiviral and antiproliferative activities of the derivative proteins, they

observed an increase in biological activity (up to ten times) upon deletion of 9 amino acids followed by a sharp decrease (to zero) on further (up to 24 aa) deletions. Nacheva et al. (2003) also found that C-terminal deletions resulted in a significant variation in the yield of both recombinant protein and mRNA.

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.

MATHEMATICAL BACKGROUND

1. Mathematical model of Lee et al. (1985)

Bacterial growth and product formation kinetics in unstable recombinant fermentations were described by the mathematical model of Lee et al. (1985). This model describes kinetic relations for plasmid-harbouring and plasmid-free biomass, limiting substrate and recombinant product, used in the relevant material balances.

Kinetic relations

The following kinetic relations for biomass and limiting substrate correspond to the case of pure segregational plasmid instability, i.e. the bacterial population is assumed to consist only of two cell types – plasmid-harbouring and plasmid-free cells:

Plasmid-harbouring biomass:
$$r_{x^+} = \frac{dx^+}{dt} = \mu^+ \cdot x^+ - \theta \cdot \mu^+ \cdot x^+ \qquad (1)$$

where $\mathbf{x^+}$ is the concentration of plasmid-harbouring biomass, $\boldsymbol{\mu^+}$ is the specific growth rate of the plasmid-harbouring cells and $\boldsymbol{\theta}$ denotes the relative plasmid loss rate (i.e. a ratio of cloning vector loss rate to the specific growth rate of the plasmid-harbouring cells). The relative plasmid loss rate $\boldsymbol{\theta}$ describes plasmid segregation and is assumed constant irrespective to the specific growth rate of plasmid-harbouring cells and cultivation conditions (Lee et al. 1985).

Plasmid-free biomass:
$$r_{x^-} = \frac{dx^-}{dt} = \mu^- \cdot x^- + \theta \cdot \mu^+ \cdot x^+ \qquad (2)$$

$\mathbf{x^-}$ is the concentration of plasmid-free biomass and $\boldsymbol{\mu^-}$ is the specific growth rate of the plasmid-free cells.

Limiting substrate:
$$r_s = \frac{ds}{dt} = -\frac{1}{Y_{x/s}} \cdot \left(r_{x^+} + r_{x^-} \right) \qquad (3)$$

s is the limiting substrate concentration in the broth. It is assumed that substrate consumption for synthesis of the recombinant product is negligible and that the yield factor $\mathbf{Y_{x/s}}$ is identical for both plasmid-harbouring and plasmid-free cells.

Specific growth rate of plasmid-free and plasmid-harbouring cells:

The specific growth rate of the plasmid-free cells is described by the classical Monod equation:

$$\mu^{-} = \mu_{\max} \cdot \left(\frac{s}{K_s + s} \right) \qquad (4)$$

where $\boldsymbol{\mu_{max}}$ is the maximal specific growth rate of the host cells and $\mathbf{K_s}$ is the Monod constant.

Lee et al. (1985) have proposed an "extended" Monod equation, where the specific growth rate of the plasmid-harbouring cells is also a function of the intracellular concentration of recombinant protein $\mathbf{p_{in}}$ and the intracellular plasmid concentration $\mathbf{G_{in}}$:

$$\mu^{+} = \mu_{\max} \cdot \left(1 - \frac{G_{in}}{G_{in\max}} \right)^{m} \cdot \left(1 - \frac{p_{in}}{p_{in\max}} \right)^{n} \cdot \left(\frac{s}{K_s + s} \right) \qquad (5)$$

The terms $\left(1 - \frac{G_{in}}{G_{in\max}} \right)^{m}$ and $\left(1 - \frac{p_{in}}{p_{in\max}} \right)^{n}$ represent the inhibitory effect on cell growth of the plasmid content and recombinant protein, respectively. The parameters $\mathbf{G_{inmax}}$ and $\mathbf{p_{inmax}}$ denote maximal intracellular concentrations of plasmid DNA and recombinant protein at which the cell growth is not more possible, i.e. $\mu^{+} = 0$. **m** and **n** are exponents for plasmid and recombinant product inhibition. It is assumed that both plasmid-harbouring and plasmid-free cells have equal Monod constant $\mathbf{K_s}$. The plasmid concentration $\mathbf{G_{in}}$ [g/L] for representative plasmid size and cell volume of *E. coli* can be calculated using the following relationship (Lee et al. 1985):

$$G_{in} = 0.0001186 \cdot N_p \qquad (6)$$

where $\mathbf{N_p}$ is the average plasmid copy-number per cell.

Recombinant product:

Assuming a first order kinetics for the decay of mRNA and the recombinant protein, Lee et al. have proposed the following material balances for the intracellular concentrations of both cloned gene mRNA ($\mathbf{m_{in}}$) and recombinant protein ($\mathbf{p_{in}}$):

$$\frac{dm_{in}}{dt} = k_p^0 \cdot \eta \cdot G_{in} - k_d \cdot m_{in} - \mu^* \cdot m_{in} \quad (7)$$

$$\frac{dp_{in}}{dt} = k_q^0 \cdot \xi \cdot m_{in} - k_e \cdot p_{in} - \mu^* \cdot p_{in} \quad (8)$$

μ* denotes overall specific growth rate of plasmid-harbouring biomass that describes growth of plasmid-harbouring cells by simultaneous appearance of plasmid-free cells, i.e.:

$$\mu^* = \mu^+ \cdot (1-\theta) \quad (9)$$

η and **ξ** denote transcription efficiency and translation efficiency, respectively. $\mathbf{k_p^0}$, $\mathbf{k_q^0}$, $\mathbf{k_d}$ and $\mathbf{k_e}$ are overall transcription rate constant, overall translation rate constant, decay constant of cloned-gene mRNA, and decay constant of recombinant protein, respectively. A quasi-steady-state approximation applied to $\mathbf{m_{in}}$ (i.e. $dm_{in}/dt = 0$) and a substitution of Eq. 7 in Eq. 8 leads to the following kinetic relation for intracellular recombinant product formation:

$$r_{p_{in}} = \frac{dp_{in}}{dt} = f\left(\mu^*\right) \cdot \gamma \cdot G_{in} - k_e \cdot p_{in} - \mu^* \cdot p_{in} \quad (10)$$

where the function $\mathbf{f(\mu^*)}$ is defined by

$$f(\mu^*) = \frac{k_p^0 \cdot k_q^0}{k_d + \mu^*} \quad (11)$$

and the gene expression parameter **γ** by

$$\gamma = \eta \cdot \xi \quad (12)$$

The concentration of the recombinant product in the culture volume **p** can be calculated using the intracellular recombinant product concentration $\mathbf{p_{in}}$ by the following equation:

$$p = \frac{p_{in} \cdot x^+}{\rho_B} \quad (13)$$

where $\boldsymbol{\rho_B}$ is the cell density [cell mass/unit volume cell].

After differentiation of Eq. 13 by ρ_B=const and substitution of Eq. 10, the following kinetic relation for recombinant product concentration in the culture volume is obtained:

$$r_p = \frac{dp}{dt} = \frac{1}{\rho_B} \cdot \left[f\left(\mu^*\right) \cdot \gamma \cdot G_{in} \right] \cdot x^+ - k_e \cdot p \qquad (14)$$

Using characteristic average values of the model parameters $\mathbf{k_p^0}$, $\mathbf{k_q^0}$ and $\mathbf{k_d}$ for recombinant gene expression in *E. coli* (Lee and Bailey 1984e), Lee et al. (1985) have proposed the following evaluation for the function $\mathbf{f(\mu^*)}$, Eq. 11 by $\mu^* < \ln 2$:

$$f\left(\mu^*\right) = \frac{4.5 \cdot 10^{10} \cdot \mu^{*4}}{\left(78 \cdot \mu^{*2} + 233\right) \cdot \left(145 \cdot \mu^* + 82.5\right) \cdot \left(27.6 + \mu^*\right)}, \ [\mathrm{h}^{-1}] \qquad (15)$$

Material balances

The material balances for biomass, limiting substrate and product for a chemostat culture, consisting of plasmid-harbouring and plasmid-free cell types (and by sterile feed) are:

Plasmid-harbouring biomass:
$$\frac{dx^+}{dt} = r_{x^+} - D \cdot x^+ \qquad (16)$$

Plasmid-free biomass:
$$\frac{dx^-}{dt} = r_{x^-} - D \cdot x^- \qquad (17)$$

Limiting substrate:
$$\frac{ds}{dt} = D \cdot \left(s_F - s\right) + r_s \qquad (18)$$

Recombinant product:
$$\frac{dp}{dt} = r_p - D \cdot p \qquad (19)$$

where **D** and $\mathbf{s_F}$ denote the dilution rate of the chemostat and the concentration of limiting substrate in the fresh nutrient medium, respectively.

2. Mathematical model of Stewart and Levin (1977)

For description of population dynamics of plasmid-harbouring and plasmid-free cells a model proposed by Stewart and Levin (1977) was used. The equations are obtained by substitution of the kinetic relations for plasmid-harbouring and plasmid-free cells (Eq. 1 and Eq. 2) into the relevant material balances of the chemostat (Eq. 16 and Eq. 17, respectively):

$$\frac{dx^+}{dt} = \mu^+ \cdot x^+ - \Theta \cdot x^+ - D \cdot x^+ \qquad (20)$$

$$\frac{dx^-}{dt} = \mu^- \cdot x^- + \Theta \cdot x^+ - D \cdot x^- \qquad (21)$$

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 $\boldsymbol{\mu}^+$:

$$\Theta = \mu^+ \cdot \theta \qquad (22)$$

Davidson et al. (1990) propose 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. If the total cell concentration is assumed to be constant, then Eq. 20 and Eq. 21 can be combined into a single equation describing the population dynamics by segregational plasmid instability in both batch and continuous culture (Cooper et al. 1987):

$$\frac{dz}{dt} = \Delta \cdot z^2 - (\Delta + \Theta) \cdot z \qquad (23)$$

z (fraction of plasmid-harbouring cells in the bacterial population) is the ratio between concentration of plasmid-harbouring cells $\mathbf{x}^+$ and total biomass concentration **x**:

$$z = \frac{x^+}{x} \qquad (24)$$

The total biomass concentration is defined as

$$x = x^+ + x^- \qquad (25)$$

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

$$\Delta = \mu^- - \mu^+ \qquad (26)$$

In general, **Δ** and **Θ** are functions of genetic characteristics, cell physiology and cultivation conditions. However, under the apparent steady-state conditions (i.e. by negligible variations in the total cell concentration and in limiting substrate concentration) the values of **Δ** and **Θ** may be assumed to be nearly constant.

Assuming that **Λ** and **Θ** are constant and z = 1 at t = 0, Eq. 23 can be solved for a biological relevant situation to:

$$z = \frac{\Delta + \Theta}{\Theta \cdot e^{(\Delta+\Theta)\cdot t} + \Delta} \tag{27}$$

AIMS AND OBJECTIVES

I To investigate the effect of hIFNγ-gene expression on plasmid segregation

Vectors for recombinant gene expression are usually multicopy plasmids with a relaxed control of replication, carrying a strong promoter and a strong ribosome binding site. The high levels of gene expression thus achieved usually decrease the specific growth rate of plasmid-harbouring cells. A little is known, however, about the effect of recombinant gene expression on the generation of plasmid-free cells and plasmid segregation. To investigate the effect of this factor on plasmid segregational instability and particularly on the relative plasmid loss rate (θ), the following objectives were set:

1. Construction of a series of pBR322-based plasmid constructs in which the recombinant hIFNγ gene is placed under the control of different regulatory elements (promoter and ribosome-binding sites) allowing variations in the efficiency of both transcription and translation.

2. Investigation of plasmid segregational instability and growth/product formation kinetics of *E. coli* cells producing human IFNγ with modulated expression in a chemostat culture.

3. Modelling and analysis of bacterial growth, product formation and plasmid segregational instability.

II To investigate the effect of hIFNγ-gene 3'-terminal deletions on plasmid segregation

The structure of the recombinant gene is supposed to be a potential factor affecting plasmid segregational instability. To prove this hypothesis, the following objectives were set:

1. Investigation of segregational instability of a series of plasmids constitutively expressing 3'-end truncated hIFNγ genes.

2. Modelling of the population dynamics of plasmid-free and plasmid-harbouring cells and analysis of the plasmid segregational instability.

MATERIALS AND METHODS

1. Bacterial strain

E. coli LE392 (*supE*44 *supF*58 *hsdR*514 *galK*2 *galT*22 *metB*1 *trpR*55 lac*Y*1), (Sambrook et al. 1989) was used as a host throughout this work. We choose to use this strain for investigation of plasmid segregational stability because of the following criteria:

(i) *E. coli* LE392 is a good producer of recombinant hIFNγ thus all data about recombinant hIFNγ synthesis and plasmid segregational instability derived in this work could be beneficial for the large scale production of recombinant hIFNγ.

(ii) This strain ensures a moderate plasmid segregational stability (deviations are easy to register).

2. Plasmids

2.1. Plasmids designed to investigate effects of hIFNγ gene expression on plasmid segregation

The plasmids used are listed in Table 1.

Table 1 Plasmid constructs.

Plasmid	Properties			
	Promoter P_1	**Ribosome-binding site**	**Transcription of hIFNγ gene**	**Translation of hIFNγ-mRNA**
pP_1-(SD)-hIFNγ	(+)	(+) (1 × SD)	(+)	(+)
$p\Delta P_1$-(ΔSD)-hIFNγ	(-)	(-)	(-)	(-)
pP_1-(ΔSD)-hIFNγ	(+)	(-)	(+)	(-)
pP_1-(4SD)-hIFNγ	(+)	(+) (4 × SD)	(+)	(+)

The plasmid pP_1-(SD)-hIFNγ (Fig. 6) is a derivative of pBR322 where the fragment between *Eco*RI and *Bam*HI restriction sites (the latter located in the *tet* (Tc^R) gene) is replaced by a cassette containing a strong constitutive promoter P_1 (analogue of the T5 bacteriophage early promoter), a ribosome-binding site (the consensus Shine & Dalgarno

sequence AAGGAGGT) and a hIFNγ gene (Nacheva et al. 2003). The mRNA transcribed from the P_1 promoter is dicistronic and consists of the complete hIFNγ sequence plus a part of the *tet* gene (downstream of the *Bam*HI site). A translation stop codon TAA is introduced after the last codon of the hIFNγ gene.

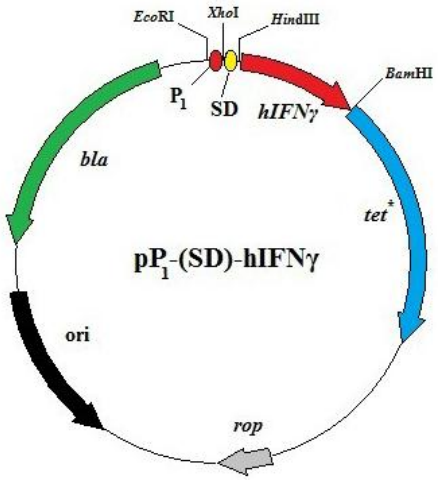


Fig. 6 Functional map of the plasmid pP_1-(SD)-hIFNγ. P_1 - constitutive (T5P25) promoter; SD - Shine & Dalgarno consensus sequence; *hIFNγ* - human interferon gamma gene coding for 143 amino acids; *bla* (Ap^R) - β-lactamase gene; *tet** - truncated (residual) tetracycline resistance gene; *rop* - gene coding for the Rop protein; ori - origin of replication.

Plasmids $p\Delta P_1$-(ΔSD)-hIFNγ, pP_1-(ΔSD)-hIFNγ and pP_1-(4SD)-hIFNγ are derivatives of the plasmid pP_1-(SD)-hIFNγ. The construct $p\Delta P_1$-(ΔSD)-hIFNγ was derived from pP_1-(SD)-hIFNγ by removing the *Eco*RI/*Hin*dIII fragment (bearing both the P_1 promoter and the SD sequence), blunting and ligation of the rest of the plasmid. The plasmid pP_1-(ΔSD)-hIFNγ was constructed by removing the SD sequence from the plasmid pP_1-(SD)-hIFNγ (by *Xho*I and *Hin*dIII), blunting and ligation. The construct pP_1-(4SD)-hIFNγ is derivative of the plasmid pP_1-(SD)-hIFNγ in which a cluster of four tandemly repeated SD sequences (AAGGAGGTTTAACGTAAGGAGGTTTATCGAG-AAGGAGGTTTAACGTAAGGAGGT, where the consensus SD sequence is underlined) was substituted for the single SD sequence. As seen from the map (Fig. 6), all plasmids have the phenotype Ap^R,Tc^S.

2.2. Plasmids used to investigate effects of the 3'-terminal truncation of hIFNγ gene on plasmid segregation

A series of 3'-end gradually (by 9 bp) truncated hIFNγ genes (designated hIFNγΔ1 to hIFNγΔ7), (Fig. 7) have been constructed by PCR using specific primers and cloned in *Hin*dIII/*Bam*HI restriction sites of pP_1-(SD)-hIFNγ (Nacheva et al. 2003) (Fig. 6). The derivative plasmids (designated pP_1-(SD)-hIFNγΔ1 to pP_1-(SD)-hIFNγΔ7, respectively) express hIFNγ proteins with systematically deleted (by three amino acids) C terminus (Nacheva et al. 2003). We use the same series of truncated hIFNγ genes to investigate the effect of the 3'-end truncation of the hIFNγ gene (i.e. the hIFNγ C-terminal deletions) on the segregation of the corresponding expression plasmids.

IFNγC
CAG…CCC GCG GCT AAA ACA GGG AAG CGT AAA CGT AGT CAG ATG CTG TTT CGT GGT CGT CGT GCA TCC CAG
Gln[1] ... Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met Leu Phe Arg Gly Arg Arg[140] Ala Ser Gln

IFNγΔ1
CAG…CCC GCG GCT AAA ACA GGG AAG CGT AAA CGT AGT CAG ATG CTG TTT CGT GGT CGT CGT
Gln[1] ... Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met Leu Phe Arg Gly Arg Arg[140]

IFNγΔ2
CAG…CCC GCG GCT AAA ACA GGG AAG CGT AAA CGT AGT CAG ATG CTG TTT CGT
Gln[1] ... Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met Leu Phe Arg

IFNγΔ3
CAG…CCC GCG GCT AAA ACA GGG AAG CGT AAA CGT AGT CAG ATG
Gln[1] ... Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met

IFNγΔ4
CAG…CCC GCG GCT AAA ACA GGG AAG CGT AAA CGT
Gln[1] ... Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg

IFNγΔ5
CAG…CCC GCG GCT AAA ACA GGG AAG
Gln[1] ... Pro Ala Ala Lys Thr Gly Lys

IFNγΔ6
CAG…CCC GCG GCT AAA
Gln[1] ... Pro Ala Ala Lys

IFNγΔ7
CAG…CCC
Gln[1] ... Pro

Fig. 7 3'-Terminal nucleotide sequence of hIFNγ gene and 3'-end truncated hIFNγ genes. The C-terminal amino acid sequences of the corresponding recombinant proteins are also given.

The plasmids pP_1-(SD)-hIFNγ-λ(+) and pP_1-(SD)-hIFNγ-λ(-) were constructed from pP_1-(SD)-hIFNγ by digestion with *Pvu* II (located in the *rop* gene) and insertion of a strong transcription terminator (λ phage t_0 terminator), (McKinney et al. 1981) in both functional and non-functional orientation.

2.3. Plasmid used as a calibrator in the quantitative real-time PCR (QPCR)

The plasmid pGEM-BD is described elsewhere (Lee et al. 2006).

3. Media

M9 minimal medium (Sambrook et al. 1989) supplemented with trace element stock solution (1:1000), glucose (final concentration 1.96 g/L), L-Methionine and L-Tryptophan (both at 40 μg/ml) was used for preparation of seed cultures and for both batch and continuous fermentations in studying the effects of recombinant hIFNγ gene expression on plasmid segregation. The trace element stock solution consisted of 1.38 g $ZnSO_4 \cdot 7H_2O$, 5.4 g $FeCl_3 \cdot 6H_2O$, 1.80 g $MnSO_4 \cdot H_2O$, 0.17 g $CuCl_2$, 0.56 g $CoSO_4 \cdot 7H_2O$, 0.06 g H_3BO_3 and 10 ml 37% HCl per liter.

LB (Luria-Bertani) medium (Sambrook et al. 1989) was used in seed cultures and for batch fermentations in studying the effects of the 3'-terminal truncation of hIFNγ gene on plasmid segregation. LB medium was used also for preparation of competent cells and in the plasmid stability assay.

Both LB and M9 media were supplemented (when required) with ampicillin to a final concentration given in the text.

4. Preparation and transformation of competent *E. coli* cells

Competent *E. coli* LE392 cells were prepared and transformed by the calcium chloride procedure (Sambrook et al. 1989) and finally selected on LB agar plates containing 100 μg/ml ampicillin.

5. Cell cultivation

Cell cultivation to study growth kinetics of E. coli LE392 cells under batch cultivation conditions

Batch growth kinetics of *E. coli* LE392 was studied in both LB and minimal M9 medium with glucose. Cultivations were performed in both flask and bioreactor.

Inocolum: A single cell colony was transferred to 100 ml flask with 10 ml sterile LB (or M9) medium. The flask was incubated over night at 37 ˚C and 200 rpm.

Fermentation conditions: An inoculum (100 µl or 6 ml for flask and bioreactor, respectively) was added to a 500 ml shake flask, containing 100 ml medium or 1L-Biostat® Bplus bioreactor, containing 600 ml medium, respectively. The flask was incubated at 37 °C and 200 rpm. The cultivation in the bioreactor was performed under the conditions described below.

Cell cultivation to study effects of recombinant hIFNγ gene expression on plasmid segregation

E. coli LE392 cells transformed with the plasmids pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ, $p\Delta P_1$-(ΔSD)-hIFNγ and pP_1-(ΔSD)-hIFNγ were used for batch and chemostat cultivations.

Inoculum: Single colonies of transformed *E. coli* LE392 cells were transferred to 100 ml flasks with 10 ml sterile M9 medium (pH 7.0) containing 100 µg/ml ampicillin. The flasks were incubated at 37˚C and 200 rpm until an optical density of A_{600} = 1.5-1.8.

Fermentation conditions: Batch and chemostat cultivations were performed in M9 medium containing 1.96 g/L glucose (without antibiotic) in a Biostat® Bplus Bioreactor (Sartorius BBI Systems) with a working volume of 600 ml. The pH value was maintained during cultivation at 7.0 ± 0.1 by 2M NaOH, temperature and stirrer speed were kept constant at 37˚C and 600 rpm, respectively. Dissolved oxygen was monitored using a pO_2-Elektrode Oxyferm FDA160 (Hamilton), and pO_2 was controlled at 80-90% of air saturation by the airflow. Feeding of chemostat cultivations was initiated after the initial batch phase (cell density of A_{600} = 1.5-1.6) at a constant dilution rate of 0.3 h^{-1}. The inoculum in all experiments was 1% v/v of the final culture volume. Samples were aseptically collected at different time intervals and used for determination of cell concentration, plasmid copy-number, glucose and hIFNγ quantification, as well as for plasmid stability assay.

Cell cultivation to study effects of the 3'-terminal truncation of hIFNγ gene on plasmid segregation

E. coli LE392 cells transformed with the series of plasmids expressing 3'-truncated hIFNγ genes and the plasmid pP_1-(SD)-hIFNγ were used.

Inoculum: A single colony of transformed cells was picked from a selective plate and transferred to a 100 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 until an optical density of about 0.6.

Cultivation procedure: 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 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. In every subculture the maximum cell density was kept under A_{595} = 0.8 in order to maintain cultures in exponential growth phase.

6. Analytical methods

6.1. Determination of cell concentration

Cell growth was monitored by measuring the optical density at 600 nm (in triplicates) using a Ultrospec 500 *pro* Visible Spectrophotometer (GE Healthcare Life Sciences). Optical density was converted to dry cell mass concentration using a standard curve determined before. To determine dry cell weight bacterial cells were collected from 5 ml cell suspension by centrifugation at 5000 × g for 5 min at 4˚C, washed twice with distilled water and dried at 100˚C to constant weight.

6.2. 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. 250 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).

6.3. Glucose quantification

Samples of 1-5 ml bacterial culture were centrifuged at 5000 × g for 5 min at 4˚C. The glucose concentration in the supernatant (in triplicates) was determined using a BioProfile 100 Plus Analyzer (Nova Biomedical).

6.4. hIFNγ quantification

hIFNγ quantification in studying the effects of recombinant hIFNγ gene expression on plasmid segregation

Samples of 1-5 ml bacterial culture were centrifuged at 5000 × g for 5 min at 4˚C. The harvested bacteria were lysed by boiling (5 min) in 1 ml 7M guanidine hydrochloride (GnHCl) and after appropriate dilution of the samples (so that they remained in the linear range of reading) the content of hIFNγ was determined by ELISA (in 6 repetitions for each probe) using the Ready-Set-Go! kit for human interferon gamma (NatuTec), following the manufacturer's instructions.

hIFNγ quantification in studying the effects of the 3'-terminal truncation of hIFNγ gene on plasmid segregation

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 (Nacheva et al. 2003).

The yield of recombinant hIFNγ was measured by ELISA (Nacheva et al. 2003) using a sequence specific anti-hIFNγ monoclonal antibody (Nacheva et al. 2002).

6.5. hIFNγ-mRNA determination

In studying the effects of recombinant hIFNγ gene expression on plasmid segregation *E. coli* LE392 cells transformed with the plasmids pΔP_1-(ΔSD)-hIFNγ and pP_1-(ΔSD)-hIFNγ were grown in M9 medium containing 100 μg/ml ampicillin in 100-ml Erlenmeyer flasks (working volume of 10 ml) at 37˚C and 200 rpm to A_{600} = 0.7.

In studying the effects of the 3'-terminal truncation of hIFNγ gene on plasmid segregation 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 (Nacheva et al. 2003).

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 already described (Nacheva et al. 2003).

6.6. Determination of plasmid copy-number

In studying the effects of recombinant hIFNγ gene expression on plasmid segregation samples for plasmid copy-number determination were derived from the chemostat cultures of the transformed *E. coli* cells 20 h after switch to continuous cultivation.

In studying the effects of the 3'-terminal truncation of hIFNγ gene on plasmid segregation 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.

Plasmid copy-number (N_p) was determined by real-time quantitative PCR (QPCR) as described by Lee et al. (2006). Total DNA was isolated from the samples 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 primers sets, calibrator (plasmid pGEM-BD, carrying both *bla* and *dxs* gene) and thermal cycling protocol as proposed by Lee et al. (2006), 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

I Effect of the recombinant human interferon-gamma gene expression on plasmid segregation

1. Growth, product formation and plasmid segregational instability of *E. coli* cells expressing hIFNγ gene with varying efficiency in chemostat culture

To study the influence of transcription and translation on plasmid segregation four expression plasmids based on the cloning vector pBR322 were used. Plasmids were designed to express a synthetic hIFNγ under the control of different regulatory elements (see Materials and Methods) in order to vary the efficiency of transcription and translation. The plasmid pP_1-(SD)-hIFNγ, (Fig 6) bears a strong constitutive promoter (P_1) and a strong synthetic ribosome binding site (SD) thus insuring a high level of constitutive gene expression. In the construct pP_1-(4SD)-hIFNγ a cluster of four identical SD sequences is substituted for the single SD in the previous plasmid. As shown earlier (Alexiev et al. 1989), the repetition of the SD sequence can have a strong suppressive effect on translation and therefore on the yield of recombinant protein. The constructs $p\Delta P_1$-(ΔSD)-hIFNγ and pP_1-(ΔSD)-hIFNγ are derivatives of the plasmid pP_1-(SD)-hIFNγ in which both promoter and SD sequence (in $p\Delta P_1$-(ΔSD)-hIFNγ) or the SD sequence (in pP_1-(ΔSD)-hIFNγ) were deleted. Therefore, the hIFNγ gene in the first plasmid can not be transcribed and in the second plasmid can not be translated. To prove this experimentally *E. coli* LE392 cells transformed with the plasmids $p\Delta P_1$-(ΔSD)-hIFNγ and pP_1-(ΔSD)-hIFNγ were cultivated in flasks with M9 medium to A_{600} = 0.7 and the content of hIFNγ and hIFNγ-mRNA was measured as already described (Nacheva et al. 2003). As expected, no hIFNγ-mRNA and no protein (hIFNγ) were detected in cells harbouring $p\Delta P_1$-(ΔSD)-hIFNγ and pP_1-(ΔSD)-hIFNγ, respectively.

To study segregational plasmid instability *E. coli* LE392 cells transformed with the above mentioned constructs were cultivated in a chemostat in M9 medium supplemented with glucose. Following the initial batch phase, feeding was initiated at a cell density of A_{600} = 1.5-1.6 at a constant dilution rate of 0.3 h^{-1}. Time dependence of plasmid-

harbouring, plasmid-free and total biomass concentrations (x^+, x^- and x, respectively) , concentration of hIFNγ in the culture volume (p), glucose concentration (s), as well as fraction of plasmid-harbouring cells in the total cell population (z) during the continuous phase of cultivation for the plasmids pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ are shown in Fig. 8A – 8C.

The fraction of plasmid-harbouring cells (**z**) determined for the construct $p\Delta P_1$-(ΔSD)-hIFNγ and for the other investigated plasmids are presented comparatively in Fig. 9.

Results demonstrated that the alterations in gene control elements lead to well distinguished differences in the population dynamics of plasmid-harbouring and plasmid-free cells. As seen in Fig. 9, the plasmid $p\Delta P_1$-(ΔSD)-hIFNγ (devoid of a promoter) demonstrated an extremely high segregational stability. In this case, the fraction of plasmid-free cells did not exceed 5% after 190 h of cultivation. The plasmid-free cell population outgrow the plasmid-harbouring once after about 180, 160 and 80 hours of cultivation for the constructs pP_1-(ΔSD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(SD)-hIFNγ, respectively. A juxtaposition of the segregation curves with the hIFNγ expression levels shows that the higher the expression of the recombinant protein, the more rapidly the plasmid-free cells overgrow the plasmid-harbouring cells. The population dynamics depends on the growth rate difference between plasmid-free and plasmid-harbouring cells, as well as on the plasmid segregation process, i.e. on the generation of plasmid-free cells in the population. The observed relationship between population dynamics and recombinant gene expression level are analyzed further by the mathematical models of Lee et al. (1985) and Stewart and Levin (1977).

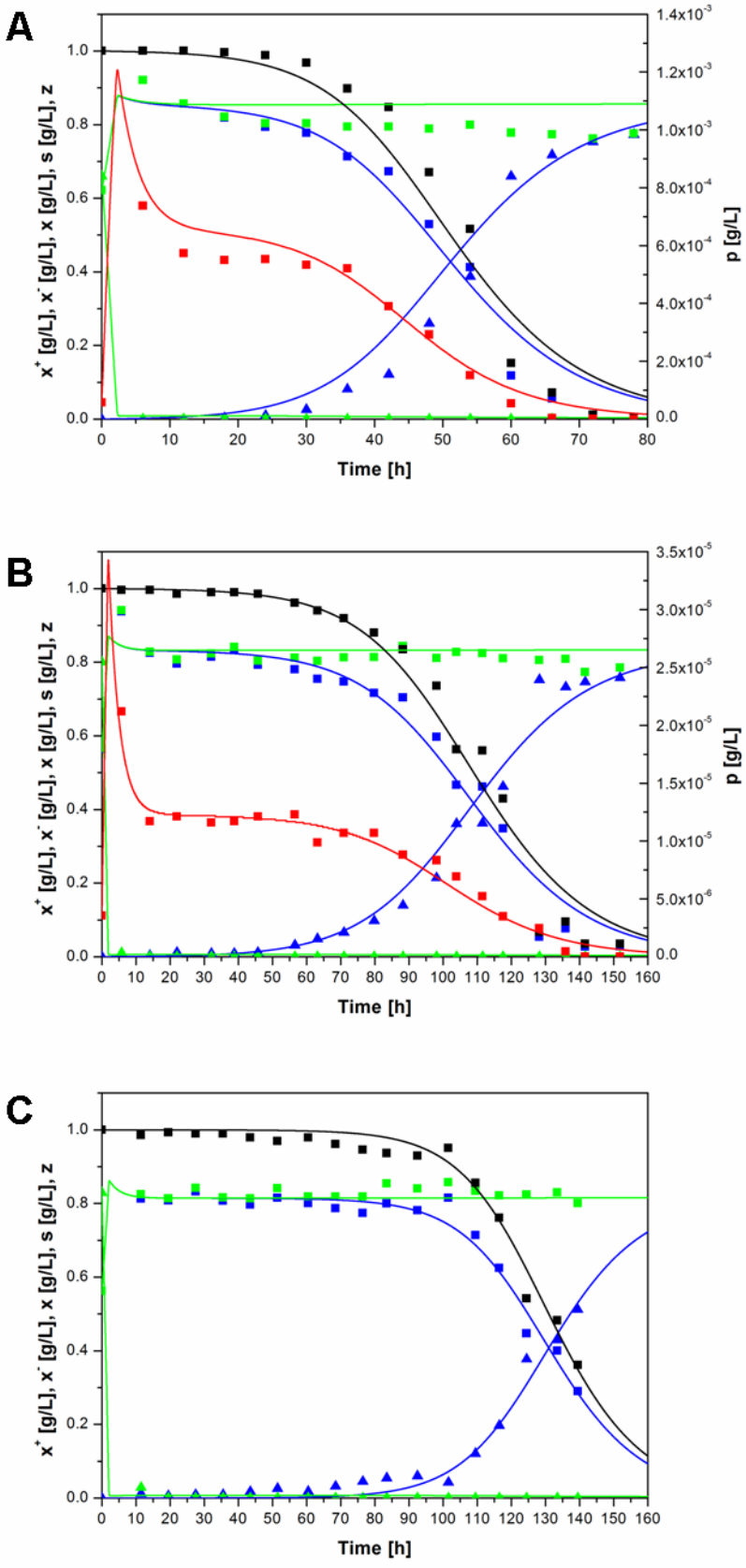


Fig. 8 Growth and plasmid loss kinetics of transformed *E. coli* LE392 cells in a chemostat culture. Experimental data (data points) and simulation curves obtained by the model of Lee et al. (lines) for plasmid-harbouring biomass, $\mathbf{x}^+$ (blue squares), plasmid-free biomass, $\mathbf{x}^-$ (blue triangles) and total biomass, **x** (green squares), glucose concentration, **s** (green triangles), hIFNγ concentration, **p** (red squares) and plasmid-harbouring cell fraction, **z** (black squares) for cells carrying the plasmid pP1-(SD)-hIFNγ (Fig. **8A**), pP1-(4SD)-hIFNγ (Fig. **8B**) and pP1-(ΔSD)-hIFNγ (Fig. **8C**).

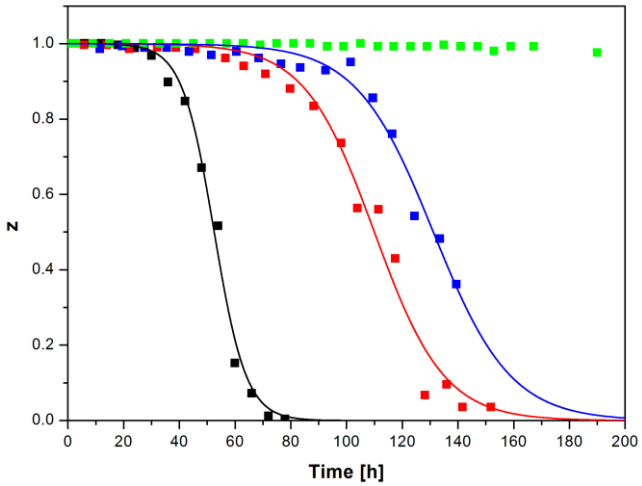


Fig. 9 Plasmid-harbouring cell fraction z as a function of cultivation time. Experimental results were processed and simulations were carried out employing Eq. 23 and using the software product Berkeley Madonna, Version 8.3.9. Black squares - pP_1-(SD)-hIFNγ; red squares - pP_1-(4SD)-hIFNγ; blue squares - pP_1-(ΔSD)-hIFNγ; green squares - $p\Delta P_1$-(ΔSD)-hIFNγ. Data points and lines represent experimental results and trajectories predicted by the model.

2. Kinetic description of cell growth and product formation by the model of Lee et al. (1985)

The genetically structured model of Lee et al. (1985) (see Mathematical background) was employed to describe bacterial growth in a chemostat, hIFNγ production and plasmid loss kinetics of *E. coli* LE392 cells transformed with the plasmids pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ. Since recombinant hIFNγ is a non-secretion protein, the concept for intracellular product formation/inhibition, as given by Eq. 5, was used.

The model of Lee et al. involves numerous parameters whose values were: i) obtained by experiments; ii) taken after consideration; iii) calculated by fitting model equations to experimental data.

Parameter values determined by experiments

The maximum specific growth rate μ_{max} in Eq. 4 and Eq. 5 refers to the host cells (plasmid-free). In the current study a parameter value of 0.66 h^{-1} was estimated from the growth kinetics of *E. coli* LE392 cells cultivated in minimal (M9) medium supplemented with glucose in a batch reactor (see Appendix I).

The intracellular plasmid concentration $\mathbf{G_{in}}$ (Eq. 5, Eq. 14) is a linear function of the average plasmid copy-number $\mathbf{N_p}$. For a representative plasmid size and *E. coli* cell volume $\mathbf{G_{in}}$ can be calculated by Eq. 6 (Lee et al. 1985).

The model of Lee et al. (1985) is based on the assumption that the plasmid copy-number per cell remains constant during cultivation. Employing QPCR (Lee et. al. 2006), the copy-number of the investigated plasmids was measured during chemostat cultivations in M9 medium 20 h after initiation of feeding, i.e. when a quasi steady-state for the plasmid copy-number was established. The plasmid copy-number values determined under this condition (where the bacterial population consists of plasmid-harbouring cells only) is considered as corresponding to the $\mathbf{N_p}$ in the model of Lee et al. (1985). The plasmid copy-number values corresponding to the constructs pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ, $p\Delta P_1$-(ΔSD)-hIFNγ and pP_1-(ΔSD)-hIFNγ were 41±3.2, 24±1.8, 22±1.6 and 30±2.1, respectively.

The dilution rate **D** (Eq. 16-19), as well as the inlet substrate concentration $\mathbf{S_F}$ (Eq. 18) were determined experimentally and remained constant for all chemostat cultivations.

Parameter values taken after consideration

The exponent **m** and the maximum intracellular plasmid concentration $\mathbf{G_{inmax}}$ in Eq. 5 describe mathematically the inhibitory effect of the plasmid copy-number on cell growth. Both parameters might be highly dependent on some factors related to plasmid structure and host cells genotype, as well as on the cultivation conditions (Lee et al. 1985). For *E. coli* Lee et al. considered 1 and 0.036 g/L as reasonable for **m** and $\mathbf{G_{inmax}}$, respectively. This $\mathbf{G_{inmax}}$ value corresponds to a plasmid copy-number of about 300 (Eq. 6). Palaiomylitou et al. (2002) and Altinash et al. (2001) have used similar values for $\mathbf{G_{inmax}}$, but different values for **m**, applying the model of Lee et al. to describe the cell growth and production of recombinant proteins in *E. coli* and *S. cerevisiae*. Palaiomylitou et al. (2002) set in their calculations m = 1, whereas Altinash et al. (2001) obtained better results with m = 0.01. The latter indicates that the **m** value is of great importance for the adequate cell growth modelling. In the following a $\mathbf{G_{inmax}}$ of 0.036 g/L (as given by Lee et al. (1985)) was assumed. Using nonlinear fitting of the model equations to the experimental data obtained with the plasmid pP_1-(ΔSD)-hIFNγ (see below), a value of

2.399 for **m** was determined. Since the plasmids used in this study have similar molecular characteristics (molecular mass and genetic structure), the same **m** value was also accepted for the plasmids pP_1-(SD)-hIFNγ and pP_1-(4SD)-hIFNγ.

Analogously, the exponent **n** and the maximal intracellular concentration of the recombinant product $\mathbf{p_{inmax}}$ define the inhibitory effect of the recombinant product on the growth of plasmid-harbouring bacteria (Eq. 5). These two parameters can also be affected by other factors such as properties of the recombinant protein or the genotype of host bacteria (Lee et al. 1985). Lee et al. (1985) proposed two values for the product inhibition exponent **n**: n = 0 (no product inhibition) and n = 1 (product inhibition). Based on the maximal recombinant product levels reported in literature, they have proposed 150 g/L for the parameter $\mathbf{p_{inmax}}$.

Yield of hIFNγ expressed in *E. coli* LE392 can approach 30% of the total cell protein (own data, not published). Considering the gross chemical composition of *E. coli* cells (70% water, 15% proteins and 15% other compounds (Watson 1972), and assuming a cell density of 1000 g dry biomass/L dry biomass (Lee et al. 1985), the recombinant product concentration was estimated to be of about 150 g hIFNγ/L dry biomass, i.e. the same $\mathbf{p_{inmax}}$ value as used by Lee et al. (1985). This parameter $\mathbf{p_{inmax}}$ represents the maximal intracellular concentration of recombinant product at which the cell growth is impossible, i.e. $\mu^+ = 0$ (Eq. 5). Assuming that the maximal intracellular product concentration experimentally determined corresponds to the theoretical value of $\mathbf{p_{inmax}}$, 150 g/L and 1 were assumed for $\mathbf{p_{inmax}}$ and **n**, respectively.

The Monod constant $\mathbf{K_s}$ in Eq. 4 and Eq. 5 is assumed to be equal for both plasmid-harbouring and plasmid-free cells, as proposed by Lee et al. (1985). In the following, $\mathbf{K_s}$ was set as 0.005 g/L, as proposed by Atkinson and Mavituna (1983) for *E. coli* grown in minimal medium containing glucose.

For the overall transcription and translation rate parameters $\mathbf{k_p^0}$ and $\mathbf{k_q^0}$ (both growth rate-dependent), as well as for the mRNA decay constant $\mathbf{k_d}$ (Eq. 11), the values obtained for the wild-type λdv plasmid replicon (Lee and Bailey 1984e; Bailey and Ollis 1986) were used. Furthermore, it was assumed that the cell density parameter $\mathbf{\rho_B}$ (Eq. 13) had

the same value as in Lee et al. (1985), and that the recombinant protein degradation rate constant $\mathbf{k_e}$ (Eq. 10) is negligible. All parameter values are summarized in Table 2.

Table 2 Summary of parameter values used for model simulations.*

Parameter	Value	Units
D	0.3	h^{-1}
G_{inmax}	0.036	g plasmid DNA / L biomass
K_s	0.005	g/L
k_d	0.46	min^{-1}
k_e	0	min^{-1}
k_p^0	$2400/(233\mu^{-2}+78)$	min^{-1}**
k_q^0	$3600\alpha/(82.5\mu^{-1}+145)$ $\alpha = 1$ for $\mu > \ln 2$ $\alpha = \mu/\ln 2$ for $\mu < \ln 2$***	min^{-1}**
m	2.399****	-
N_p	41 (pP_1-(SD)-hIFNγ) 24 (pP_1-(4SD)-hIFNγ) 30 (pP_1-(ΔSD)-hIFNγ)	-
n	1	-
p_{inmax}	150	g protein / L dry biomass
s_F	1.96	g/L
μ_{max}	0.66	h^{-1}
ρ_B	1000	g dry biomass /L dry biomass

*In the absence of translation (as in pP_1-(ΔSD)-hIFNγ) the gene expression parameters are ignored.

**Calculating $\mathbf{k_p^0}$ and $\mathbf{k_q^0}$ the specific growth rate $\boldsymbol{\mu}$ is expressed in h^{-1}.

***By $\alpha = \mu/\ln 2$ (for $\mu < \ln 2$) and Eq. 9, the substitution of $\mathbf{k_p^0}$, $\mathbf{k_q^0}$ and $\mathbf{k_d}$ in Eq. 11 results in Eq. 15.

****The **m** value was determined by nonlinear fitting for pP_1-(ΔSD)-hIFNγ and set in the calculations for both pP_1-(SD)-hIFNγ and pP_1-(4SD)-hIFNγ (see below).

Parameters determined by fitting model equations to experimental data

To determine the exponent **m** describing the plasmid vector inhibition experimental data for pP_1-(ΔSD)-hIFNγ were used. Since the hIFNγ gene in this construct is transcribed but the hIFNγ-mRNA is not translated, the Monod equation describing the specific growth rate of plasmid-harbouring cells (Eq. 5) is simplified and the kinetic relation for recombinant product formation (Eq. 14) drops off.

The yield factor $\mathbf{Y_{xs}}$ (Eq. 3) is assumed to be the same for both plasmid-harbouring and plasmid-free cells (Lee et al. 1985). Since substrate consumption necessary for formation of the recombinant product is low it can be neglected. For the sake of simplicity substrate consumption for the endogenous cell metabolism was also neglected. By batch cultivations of both non-transformed and plasmid bearing *E.coli* LE392 cells a yield factor Y_{xs} = 0.5 g biomass/g glucose was obtained. Different $\mathbf{Y_{xs}}$ values were expected however, for chemostat cultivations depending on the dilution rate. Seo and Bailey (1986) have shown experimentally that this parameter decreases with increasing dilution rate, which contradicts the theoretical expectations.

The parameter **γ** in Eq. 12 represents the efficiency of transcription and translation of the cloned gene and therefore it should have different values for the plasmids pP_1-(SD)-hIFNγ and pP_1-(4SD)-hIFNγ. However, for $p\Delta P_1$-(ΔSD)-hIFNγ (devoid of promoter) and pP_1-(ΔSD)-hIFNγ (lacking SD sequence) γ = 0 because of the lack of transcription and translation, respectively.

The relative plasmid segregation rate **θ,** which is a key parameter in analyzing plasmid segregation, is assumed to be constant in the model of Lee et al. (1985).

The values of **θ**, $\mathbf{Y_{xs}}$ and **γ** for the constructs pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ, and the exponent **m** for the plasmid pP_1-(ΔSD)-hIFNγ were determined numerically using experimental data obtained from continuous cultivations. To this aim the model equation system was fitted to the data for the plasmid-harbouring biomass $\mathbf{x^+}$, overall recombinant product concentration **p** and plasmid-harbouring cell fraction **z** (= x^+/x) using the software product Berkeley Madonna, Version 8.3.9 (see Appendix II). The initial conditions (experimental data determined immediately before starting continuous cultivations) are listed in Table 3.

Table 3 Initial values used for parameter estimation.

Variable [Units]	Plasmid construct		
	pP_1-(SD)-hIFNγ	**pP_1-(4SD)-hIFNγ**	**pP_1-(ΔSD)-hIFNγ**
$x^+(0)$ [g/L]	0.62	0.56	0.56
$x^-(0)$ [g/L]	0	0	0
s(0) [g/L]	0.66	0.8	0.83
p(0) [g/L]	5.78×10^{-5}	3.54×10^{-6}	-

Experimental data and model simulations for plasmid-harbouring, plasmid-free and total biomass, limiting substrate, recombinant product and plasmid-harbouring cell fraction for the plasmids pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ are shown in Fig. 8 A-C, respectively.

The calculated curves obtained by the model of Lee et al. (1985) fit well the experimental data for all investigated plasmids. The root mean square deviation (RMSV) was 0.137, 0.116 and 0.057 for the plasmids pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ, respectively.

The estimated values of the parameters **θ**, $\mathbf{Y_{xs}}$ and **γ** are listed in Table 4. A value of 2.399 for **m** was determined for the plasmid pP_1-(ΔSD)-hIFNγ.

Table 4 Parameter values estimated numerically by the model of Lee et al. (1985).

Parameter	Plasmid construct		
	pP_1-(SD)-hIFNγ	**pP_1-(4SD)-hIFNγ**	**pP_1-(ΔSD)-hIFNγ**
θ	7.435×10^{-4}	1.829×10^{-4}	4.488×10^{-6}
Y_{xs}	0.438	0.426	0.417
γ	0.110	0.0036	-

The gene expression parameter **γ** (reflecting transcription and translation efficiency, see Eq. 12) depends on the strength of both promoter and ribosome binding site (Lee et al. 1985). In particular the replacement of the single SD sequence in pP_1-(SD)-hIFNγ with a tetrameric SD sequence (as in the construct pP_1-(4SD)-hIFNγ) led to a sharp decrease in the yield of hIFNγ (Fig. 8A and 8B). As seen from Table 4, the predicted γ values for the plasmids pP_1-(SD)-hIFNγ and pP_1-(4SD)-hIFNγ correlate well with the experimentally observed reduction in the yield of hIFNγ.

The model predicts almost identical values of the yield factor $\mathbf{Y_{xs}}$ at continuous cultivation conditions for all investigated plasmids (about 0.43 g biomass/g glucose, Table 4).

The plasmid loss probability **θ** predicted by the model of Lee et al. (1985), (Table 4) clearly demonstrates that the alterations in the ribosome binding site (affecting the efficiency of hIFNγ-mRNA translation) interfere with the plasmid segregational stability. A reverse correlation between the yield of recombinant protein and the segregational plasmid stability was observed.

3. Results of simulation studies using the model of Lee et al. (1985)

The model of Lee et al. (1985) was employed for simulations (see Appendix III) to study different trends that can be expected for chemostat cultivation of *E. coli* LE392 cells transformed with the plasmid pP_1-(SD)-hIFNγ at different dilution rates.

4. Description of plasmid segregation by the model of Stewart and Levin (1977)

To describe the population dynamics of plasmid-harbouring and plasmid-free cells the nonlinear fitting method of Davidson et al. (1990), which is based on the equations proposed by Stewart and Levin (1977), was also applied (see Mathematical background). These equations describe the growth kinetics of plasmid-harbouring and plasmid-free cells in chemostat culture (see Eq. 20 and Eq. 21), which can be combined into a single equation representing the fraction of plasmid-harbouring cells in the population **z** as a time dependent function (Eq. 23). Eq. 23 includes the parameters **Δ** (difference in the specific growth rate between plasmid-free and plasmid-harbouring cells as in Eq. 26) and **Θ** (specific rate of generation of plasmid-free cells, or specific plasmid loss rate as in Eq.

22). Employing Eq. 23 the values of **Δ** and **Θ** were estimated for the plasmids pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ (Table 5).

Table 5 Estimated values of Δ and Θ for the constructs pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ.

Parameter [Units]	**Plasmid**		
	pP_1-(SD)-hIFNγ	**pP_1-(4SD)-hIFNγ**	**pP_1-(ΔSD)-hIFNγ**
Δ [h^{-1}]	0.1741	0.0799	0.0723
Θ [h^{-1}]	1.8632×10^{-5}	1.2152×10^{-5}	5.4836×10^{-6}

As the fraction of plasmid-harbouring cells **z** did not change significantly during cultivation both parameters were not determined for the plasmid $p\Delta P_1$-(ΔSD)-hIFNγ. The calculated fraction of plasmid-harbouring cells in the total population **z** versus cultivation time is shown in Fig. 9 for all investigated plasmids.

As seen from Table 5, the highest Δ value was observed for the plasmid pP_1-(SD)-hIFNγ and this is correlated with the highest cellular content of recombinant product (hIFNγ) and the highest plasmid copy-number compared to the other two plasmids. Simulations performed using the model of Lee et al. (1985) also confirmed the highest specific growth rate difference between cells harbouring pP_1-(SD)-hIFNγ and plasmid-free cells (see Appendix IV). These results agree with the fact that **Δ** increases with increasing the yield of recombinant protein and plasmid content in the cell, which is related to the increased metabolic burden of the plasmid-harbouring cells. Gene expression efficiency affects also the generation rate of plasmid free-cells **Θ** (Table 5). It decreases in the order pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ, pP_1-(ΔSD)-hIFNγ, i.e. with decreasing the expression efficiency of the recombinant protein. Simulations performed with the model of Lee et al. (1985) confirm this tendency (see Appendix IV, Fig. 20).

II Effect of the 3'-terminal truncation of the human interferon-gamma gene on plasmid segregation

1. Population dynamics of plasmid-harbouring cells expressing intact and 3'-end truncated hIFNγ genes

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 (pP_1-(SD)-hIFNγ) containing a strong constitutive promoter P_1 and a strong ribosome binding site (see Materials and Methods). These plasmids, designated as pP_1-(SD)-hIFNγΔ1 to pP_1-(SD)-hIFNγΔ7 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 Eq. 24) versus the cultivation time is shown in Fig. 10.

To describe the population dynamics of plasmid-harbouring and plasmid-free cells the nonlinear fitting method of Davidson et al. (1990), based on the equations proposed by Stewart and Levin (1977), was applied. The values of the parameters **Δ** and **Θ** for all investigated plasmids were evaluated by nonlinear fit of Eq. 27 to the experimental data points using OriginPro 8 software (Table 6). The fractions of plasmid-harbouring cells, **z** predicted by Eq. 27 are presented in Fig. 10.

The specific plasmid loss rate **Θ** depends on the specific growth rate of the plasmid-harbouring cells $\boldsymbol{\mu}^+$ as well as on the relative plasmid loss rate **θ,** (Eq. 22). The relative plasmid loss rate **θ** is assumed to be constant irrespective of the host cells' specific growth rate and cultivation conditions (Lee et al. 1985) and is influenced mainly by the plasmid segregation process. The value of **θ** for each investigated plasmid was calculated using Eq. 22 and Eq. 26 where the corresponding values of **Θ** and **Δ** were estimated by the method of Davidson et al. (1990), (Table 6).

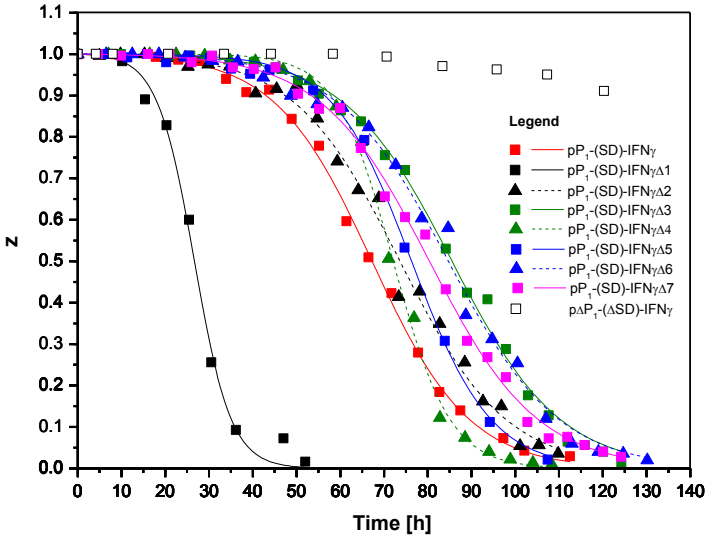


Fig. 10 Plasmid-harbouring cell fraction z versus cultivation time. Experimental data (points) and fittings (lines) obtained by the model of Stewart and Levin (1977).

Table 6 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 rate Θ [h^{-1}]	Relative plasmid loss rate θ	Plasmid copy-number per cell
pP_1-(SD)-hIFNγ (0)	0.0899±0.0024	$(2.10±0.29)\times10^{-4}$	$(1.89±0.26)\times10^{-4}$	14.1±1.4
pP_1-(SD)-hIFNγΔ1 (9)	0.2365±0.0200	$(4.30±0.77)\times10^{-4}$	$(4.46±0.81)\times10^{-4}$	21.1±1.5
pP_1-(SD)-hIFNγΔ2 (18)	0.0828±0.0037	$(1.85±0.43)\times10^{-4}$	$(1.66±0.39)\times10^{-4}$	19.8±2.9
pP_1-(SD)-hIFNγΔ3 (27)	0.0811±0.0025	$(7.84±1.47)\times10^{-5}$	$(7.01±1.32)\times10^{-5}$	17.4±2.1
pP_1-(SD)-hIFNγΔ4 (36)	0.1547±0.0068	$(2.21±0.10)\times10^{-6}$	$(2.11±0.95)\times10^{-6}$	16.1±2.4
pP_1-(SD)-hIFNγΔ5 (45)	0.1105±0.0042	$(2.41±0.68)\times10^{-5}$	$(2.21±0.62)\times10^{-5}$	22.6±1.9
pP_1-(SD)-hIFNγΔ6 (54)	0.0794±0.0036	$(9.48±2.48)\times10^{-5}$	$(8.46±2.22)\times10^{-5}$	18.4±2.3
pP_1-(SD)-hIFNγΔ7 (63)	0.0808±0.0022	$(1.22±0.19)\times10^{-4}$	$(1.09±0.17)\times10^{-4}$	37.6±4.0

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} (see Appendix I). The estimated values of **θ** for all investigated plasmids are also presented in Table 6.

To explore the role of hIFNγ gene transcription on plasmid segregation we investigated the population dynamics of *E. coli* LE392 cells harbouring the plasmid pΔP1-(ΔSD)-IFNγ, a derivative of pP_1-(SD)-IFNγ devoid of P_1 promoter. Our results demonstrated that the switching off of 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. 10).

2. Comparison between plasmid copy-number, hIFNγ (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 as described in "Materials and Methods" and their copy-number was determined using QPCR (Table 6). Except for the plasmid pP_1-(SD)-IFNγΔ7, the determined values for the plasmids carrying truncated genes varied within the range of 16-23 plasmid copies per cell. These values are slightly higher in comparison with the control plasmid (pP_1-(SD)-IFNγ) expressing the full size hIFNγ gene (about 14 plasmid copies per cell). Presently, we have no reasonable explanation of the observed drastic increase in plasmid copy-number (up to 38 copies per cell) following the deletion of 63 3'-terminal base pairs in the pP_1-(SD)-IFNγΔ7 construct. The copy-number of the plasmid $p\Delta P_1$-(ΔSD)-IFNγ was 13±1.9 only.

The yields of recombinant protein (hIFNγ) and hIFNγ-mRNA have been examined earlier by Nacheva et al. (2003). As potential factors interfering with plasmid segregation they were now compared with the plasmid copy-number and with the model parameters Δ and θ (Fig. 11).

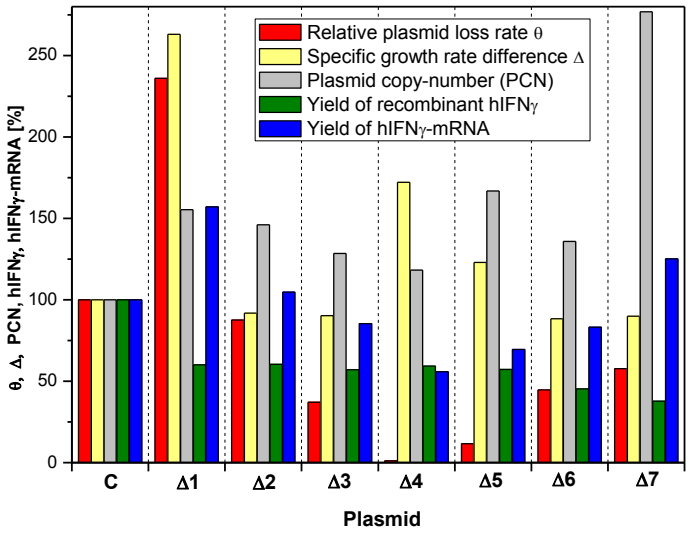


Fig. 11 Comparison of the experimental data (PCN values, recombinant hIFNγ and hIFNγ-mRNA yields) with the calculated Δ and θ values for the expression plasmids carrying 3'-truncated hIFNγ genes. Data (in %) are related to the corresponding values obtained for the pP_1-(SD)-IFNγ construct (designated here as C) carrying the full size hIFNγ gene (100%). The plasmids expressing 3'-truncated hIFNγ genes are designated as Δ1, Δ2 etc. hIFNγ and hIFNγ-mRNA yields are taken from Nacheva et al. (1993).

As seen from the figure, the systematic 3'-end deletions of the hIFNγ gene resulted in significant variations in the yields of both recombinant protein and 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. 10 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 6). These findings raise the question of how the 3'-end truncation of the hIFNγ-gene affects the model parameters.

As seen from Table 6 and Fig. 11, the **Δ** values for most of the plasmids were similar except for the constructs pP_1-(SD)-IFNγΔ1, pP_1-(SD)-IFNγΔ4 and pP_1-(SD)-IFNγΔ5 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 cut off correlation between **Δ** and the plasmid copy-number, yield of recombinant protein, and hIFNγ-mRNA was not observed.

The results presented in Table 6 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 6 and Fig. 11) do 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. (2003) 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.

The analysis of the effect of recombinant gene mRNA variations upon 3'-end gene deletions on the relative plasmid loss rate (Fig. 11) 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. 12).

The deviation of pP_1-(SD)-IFNγΔ4 from the linearity shown in Fig. 12 might mean that the latter is not valid for hIFNγ-mRNA yields lower than 30% of that of the referent pP_1-(SD)-IFNγ construct. We assume that a minimal threshold value of **θ** might exist that is independent on the content of hIFNγ-mRNA.

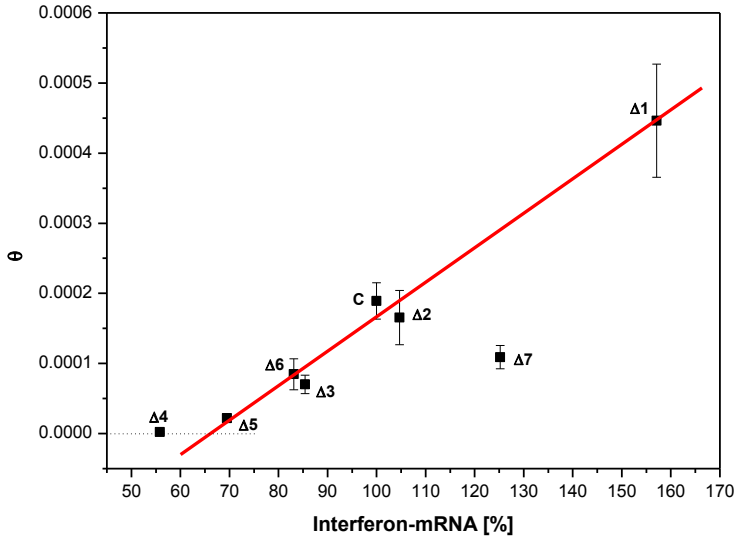


Fig. 12 Correlation between yield of hIFNγ-mRNA and relative plasmid loss rate θ. In the figure the construct pP_1-(SD)-IFNγ is designated as C and the plasmids expressing 3'-truncated hIFNγ genes are designated as Δ1, Δ2 etc.

A lower than expected θ value (according to the linear correlation) was observed for the pP_1-(SD)-IFNγΔ7 construct. Although we are unable to give a reasonable explanation of its high segregational stability, it is worth mentioning that this construct is characterized by a much higher plasmid copy-number value in comparison with the 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. 13) indicates a negative effect of transcription on plasmid stability.

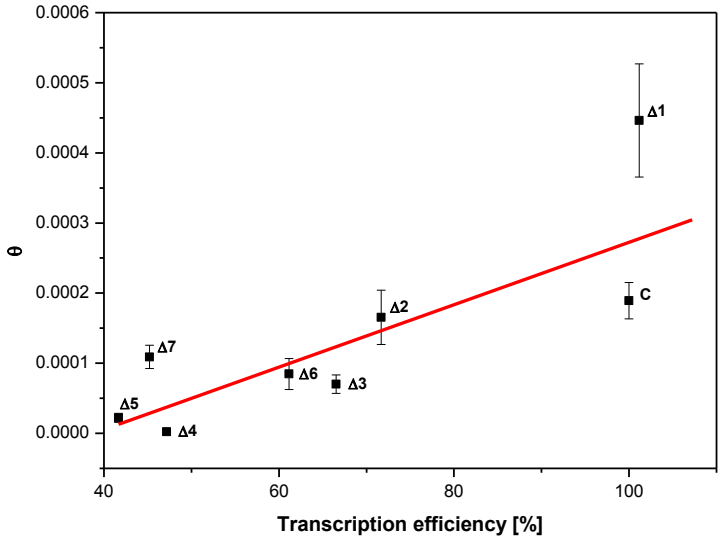


Fig. 13 Relationship between the relative plasmid loss rate θ and the transcription efficiency of the truncated hIFNγ genes. Transcription efficiency data are presented in % and are related to the construct pP_1-(SD)-IFNγ (taken as 100%). In the figure the construct pP_1-(SD)-IFNγ is designated as C and the plasmids expressing 3'-truncated hIFNγ genes are designated as Δ1, Δ2 etc.

3. Construction and copy-number of the plasmids pP_1-(SD)-IFNγ-λ(+) and pP_1-(SD)-IFNγ-λ(-)

Stueber and Bujard (1982) 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 (Remaut et al. 1981, 1983; Sambrook et al. 1989). 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 (Stueber and Bujard 1982). 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 (1982). 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 pP_1-(SD)-IFNγ-λ(+) and pP_1-(SD)-IFNγ-λ(-). They were obtained from the plasmid pP_1-(SD)-IFNγ in which a strong transcription terminator (the λ phage t_0 terminator (McKinney et al. 1981)) 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 pP_1-(SD)-IFNγ-λ(+) and pP_1-(SD)-IFNγ-λ(-), 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.

DISCUSSION

I Effect of the recombinant human interferon-gamma gene expression on plasmid segregation

The plasmids pΔP_1-(ΔSD)-hIFNγ, pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ carrying different gene expression control elements were studied to evaluate the role of heterologous gene expression on plasmid segregation. These plasmids were transformed in *E. coli* LE392 cells and their segregation was investigated under continuous fermentation conditions in non-selective M9 medium containing glucose. For description of population dynamics the mathematical models proposed by Lee et al. (1985) and Stewart and Levin (1977) were applied. The obtained results clearly demonstrate that the alterations in the structure of gene expression control elements result in variations in both the yield of hIFNγ and plasmid copy-number (Fig. 14A) and on the other hand determine (directly or indirectly) plasmid segregational instability (the relative plasmid loss rate **θ**). The question rises of how these two factors could affect the relative plasmid loss rate **θ**.

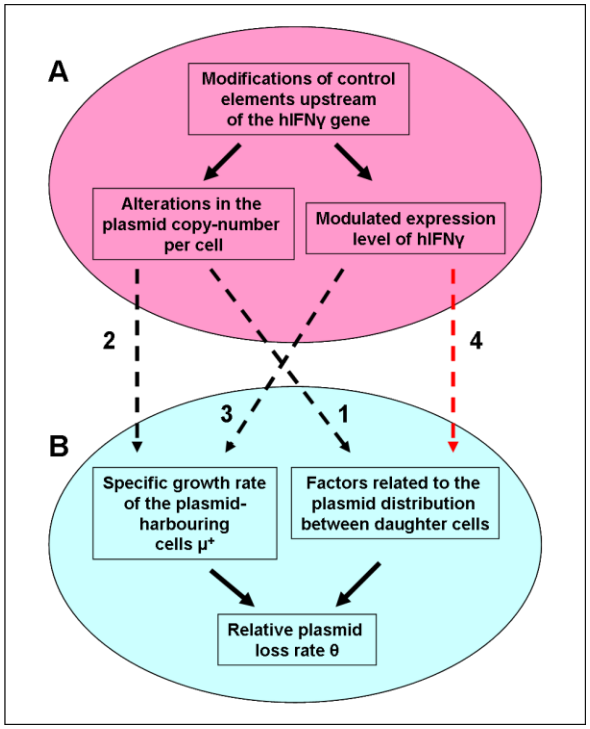


Fig. 14 Relationship between recombinant gene control elements and relative plasmid loss rate. Interconnections between the effects caused by the modifications of the recombinant gene control elements (**A**) and factors influencing the relative plasmid loss rate θ (**B**).

In general, **θ** is affected by various factors related with plasmid distribution between the daughter cells during cell division or to the specific growth rate of the plasmid-harbouring cells (Fig. 14B).

Amongst the factors affecting plasmid partitioning (Fig. 14B) are the plasmid copy-number $\mathbf{N_p}$ (Summers 1991), plasmid multimerization (Summers and Sherratt 1984), concatameric replication (Viret et al. 1991), presence of partitioning elements in the plasmid (Summers and Sherratt 1984), host cell genotype (James et al. 1982), etc. In the focus of this study was the average (related to the whole cell population) plasmid copy-number (Fig. 14A), whose reduction often leads to segregational plasmid instability, i.e. to an increased plasmid loss (**θ**) (see Fig. 14, *Arrow 1*), (Summers et al. 1993). Based on our results, however, it seems unlikely that the variations in the segregational plasmid instability are related to the differences in their copy-number. It seems to be no correlation between plasmid content and the relative plasmid loss rate **θ**. Moreover, the highest plasmid copy-number value was observed with the plasmid pP_1-(SD)-hIFNγ (Table 4), showing the highest **θ** value (Table 6).

It is clear that the level of gene expression and plasmid copy-number interfere with the specific growth rate of plasmid-harbouring cells. Both high plasmid copy-number and high recombinant protein yield lead to a decrease in specific growth rate of the plasmid-harbouring cells $\boldsymbol{\mu}^+$ (Fig. 14, *Arrows 2 and 3*) as described in the model of Lee et al. (1985) by Eq. 5. As a result, the decreased specific growth rate $\boldsymbol{\mu}^+$ leads to a decrease in the relative plasmid loss rate **θ**, respectively to increased plasmid stability (Mosrati et al. 1993). However, the observed variation in stability/instability of the investigated plasmids can not be well explained by the difference in the specific growth rate of plasmid-harbouring cells in relation with the level of gene expression and plasmid copy-number. The highest **Δ** value determined by the model of Stewart and Levin (1977), (respectively the lowest value of $\boldsymbol{\mu}^+$, according to Eq. 26) for the plasmid pP_1-(SD)-hIFNγ should result in a minimal **θ** value. Conversely, a high plasmid loss rate should be expected for the two plasmids pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ (both characterized by high $\boldsymbol{\mu}^+$ values). In contrast, however, no correlation between $\boldsymbol{\mu}^+$ and plasmid loss probability **θ** (as proposed by Mosrati et al. (1993)) was observed.

The observed relative plasmid loss rate (**θ**) of the expression plasmids used in this study is difficult to be explained by either the alterations in the plasmid copy-number (Fig. 14, *Arrows 1* and *2*) or gene expression efficiency (affecting $\boldsymbol{\mu}^{+}$; see Fig. 14, *Arrow 3*). This is an indication for the existence of other factors that might interfere with plasmid segregation. One can assume that hIFNγ gene expression interfere with plasmid segregation (i.e. the relative plasmid loss rate **θ**) at the level of plasmid partitioning during cell division (Fig. 14, *Arrow 4*). The latter might be related with the specificity of prokaryotic gene expression itself. Unlike in eukaryotes, the three processes replication, transcription and translation in prokaryotes are conjugated and all occur in one compartment (bacterial cytoplasm). This enables interactions between molecules that are principally engaged in different cell processes (replication, transcription or translation). It is shown for instance that the initiation of chromosomal DNA replication in *E. coli* is dependent on transcriptional activation (Baker and Kornberg 1988) where a direct interaction between DnaA (a bacterial replication initiator protein) and RNA polymerase is found (Atlung 1984; Flatten et al. 2009). Moreover, Szambowska et al. (2011) provide evidence that during the initiation of λ phage DNA replication the λO protein (a replication initiator of phage λ) interacts directly with the β subunit of the bacterial RNA polymerase. Therefore, it might be assumed that especially in the presence of a strong constitutive promoter and a strong SD sequence (i.e. in case of extensive gene expression) the plasmid is involved in formation of very complex aggregates consisting of the replicating plasmid, growing mRNA(s), translating ribosomes/polysomes and growing polypeptide chains. Apparently, the risk of a non-random distribution of such huge complexes between the daughter cells is much greater compared to the naked (silent/cloning) plasmids. Among the factors affecting this process are the structure and aminoacid composition of the recombinant protein, its solubility in bacterial cytoplasm, affinity to the cell membrane, etc. For instance, in case that the growing polypeptide chain is hydrophobic or bears an N-terminal secretion signal, it might “stick” to the plasma membrane. Membrane association on the other hand is a predisposition for a non-random partitioning and changes in the probability for appearance of plasmid-free cells (therefore the relative plasmid loss rate θ will be changed too). A potential factor promoting non-random plasmid partitioning could also be the formation of inclusion

bodies (huge intracellular aggregates of unfolded recombinant protein), typical for the expression of many eukaryotic genes in bacteria. Usually, they have polar localization and could entangle growing polypeptide chains.

The plasmid pΔP_1-(ΔSD)-hIFNγ is devoid of promoter and therefore the inserted hIFNγ gene is not active. As seen in Fig. 9, this plasmid demonstrates an extremely high segregational stability (the fraction of plasmid-free cells in the population after 190 h of cultivation does not exceed 5%). The same figure shows that the next stable plasmid is pP_1-(ΔSD)-hIFNγ (devoid of a SD sequence), where the synthesis of recombinant protein is also turned off. Comparing the results obtained with these constructs, one can estimate the impact of transcription on plasmid segregation. Fig. 9 shows that the inactivation of translation (deletion of the SD sequence) shifts the segregation curve to the right. However, comparing with the results obtained with the active plasmids pP_1-(SD)-hIFNγ and pP_1-(4SD)-hIFNγ, we conclude that the inactivation of translation does not prevent plasmid segregation. The latter happens only after shutting down the transcription. This phenomenon might be explained by a mechanism proposed by Stueber and Bujard (1982). They showed that the extensive transcription interferes with plasmid replication and leads to a reduction in plasmid copy-number and segregational plasmid instability (Remaut et al. 1981, 1983; Sambrook et al. 1989). In this study, however, we demonstrated that the plasmid copy-number of the transcriptionally inactive construct (pΔP_1-(ΔSD)-hIFNγ) was even lower (22 copies per cell) compared to that of the other three plasmids, i.e. the complete inactivation of the hIFNγ gene did not increase the plasmid copy-number but had a strong stabilizing effect against plasmid segregation. Apparently this result cannot be explained by the above mentioned mechanism and agrees well with the hypothesis raised in this study about the possible role of extensive constitutive gene expression on plasmid segregation. When interpreting these results, however, it should be taken into account that the data obtained by Stueber and Bujard (1982) refer to plasmids bearing chloramphenicolacetyltransferase (CAT) gene. In both cases (CAT and hIFNγ expression plasmids) a high level of gene expression can be achieved (up to 30-40% of the total protein) but the two proteins differ in their behaviour/solubility in bacterial cytoplasm (CAT is soluble and hIFNγ is insoluble and forms inclusion bodies). In future studies (already undertaken) we are going to shed more

light on the relationship between plasmid stability/instability and the intracellular state of the expressed protein.

In conclusion, the results presented above demonstrate that the efficiency of recombinant gene expression strongly affects plasmid segregation. The relative plasmid loss rate (**θ**) increases (i.e. plasmid stability decreases) with improving the constitutive gene expression. This correlation, however, can not be explained by changes neither in the plasmid copy-number nor in the specific growth rate of the plasmid-harbouring cells. Switching-off of transcription has a stabilizing effect against segregation, which is not due to an increase in the plasmid copy-number.

II Effect of the 3'-terminal truncation of the human interferon-gamma gene on plasmid segregation

A series of plasmids expressing the recombinant hIFNγ gene and its 3'-truncated derivatives 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 of plasmid-harbouring and plasmid-free cells, the mathematical model proposed by Stewart and Levin (1977) was applied. The obtained results demonstrate that small changes in the 3'-terminus of the hIFNγ gene strongly affect plasmid segregation. To explain this influence, the model parameter **θ** (relative plasmid loss rate) was compared with experimental data characterizing hIFNγ gene expression such as yield of recombinant protein, hIFNγ-mRNA and plasmid copy-number. A clear cut correlation was found between **θ** and hIFNγ-mRNA content (Fig. 12), which could be explained by two 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. 15).

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 (Nacheva et al. 2003).

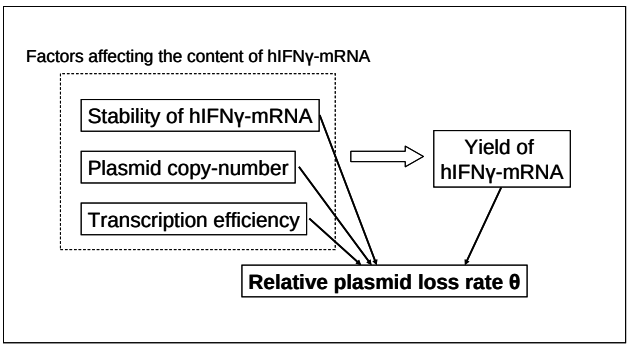


Fig. 15 Hypothetical factors affecting plasmid segregation.

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 favours the irregular distribution of plasmids during cell division thus increasing the relative plasmid loss rate **θ** (Summers et al. 1993). 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 6 and Fig. 11) do not show any correlation between **θ (**plasmid segregation**)** and the plasmid copy-number.

The observed variations in copy-number values of the plasmids carrying hIFNγ genes with different extent of truncation could be explained in the light of the hypothesis of Yavachev and Ivanov (1988), predicting a possible interference of other cellular RNAs with plasmid replication through RNAI/RNAII interactions. Therefore, we searched for a homology and hybrid formation between truncated hIFNγ-mRNAs and RNAI/RNAII. No such homology was found.

Transcription efficiency of the recombinant gene is another factor that could interfere with plasmid segregation. The observed negative effect of transcription efficiency on plasmid stability in our experimental system (Fig. 13) could be explained by the observation of Stueber and Bujard (1982) showing that the extensive constitutive transcription results in reduction of copy-number of plasmids containing ColE1 replicon

and leads to an increase in plasmid segregational instability (Remaut et al. 1981, 1983; Sambrook et al. 1989). To check this hypothesis we constructed the plasmids pP_1-(SD)-IFNγ-λ(+) and pP_1-(SD)-IFNγλ(-), 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 plasmid copy-number. In the right orientation (pP_1-(SD)-IFNγ-λ(+)), the t_0 terminator should suppress the transcriptional readthrough in the replication region and therefore a further increase in plasmid copy-number is expected. In the case of reverse orientation (pP_1-(SD)-IFNγ-λ(-)), the transcription will not be interrupted and lower copy-number value (compared to the plasmid pP_1-(SD)-IFNγ-λ(+)) has to be expected. The obtained copy-number values demonstrate, however, that the insertion of the functional transcription terminator into the *rop* gene (pP_1-(SD)-IFNγ-λ(+)) does not lead to an increase in plasmid copy-number as compared to the pP_1-(SD)-IFNγ-λ(-) construct. This means that the mechanism proposed by Stueber and Bujard (1982) 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 of plasmid copy-number. This conclusion was supported also by the estimated low copy-number value of the plasmid $p\Delta P_1$-(ΔSD)-IFNγ (see below).

In addition to the factors affecting the yield of hIFNγ-mRNA considered above (Fig. 15), Tartaglia et al. (2007, 2009) 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 for avoiding protein aggregation at maximum levels of gene expression. Our data, however, cannot be explained in the light of this hypothesis. The hypothesis that hIFNγ-mRNA itself interferes with plasmid segregation (Fig. 15) however, 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 their random distribution between the daughter cells during cell division.

To estimate the impact of hIFNγ gene transcription on plasmid segregation we used the plasmid pΔP_1-(ΔSD)-IFNγ, 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. 10). This is a clear evidence that inactivation of transcription has a strong stabilizing effect on plasmids against segregation. This finding is supported also by our experiments, where the role of transcription on plasmid segregation was investigated in a chemostat culture (see above).

In conclusion, the results presented in this thesis 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, can not be explained by variations in the plasmid copy-number. Switching-off transcription has a strong stabilizing effect against segregation, which also is unrelated to the increase of plasmid copy-number.

CONCLUSIONS

I Effect of the recombinant human interferon-gamma gene expression on plasmid segregation

1. The efficiency of constitutive gene expression is a major factor affecting ColE1-like plasmid segregation. Any increase in the efficiency of gene expression increases the relative plasmid loss rate, i.e. enhances the plasmid segregational instability. This, however, is not associated with a decrease in plasmid copy-number.
2. Depriving the hIFNγ gene of transcription has a stabilizing effect on ColE1-like plasmids, which is also not associated with an increase in plasmid copy-number.

II Effect of 3'-terminal truncation of the human interferon-gamma gene on plasmid segregation

1. hIFNγ gene 3'-terminal truncations strongly affects plasmid segregational stability.
2. hIFNγ gene transcription interferes with the relative plasmid loss rate and therefore with the plasmid segregational instability, which (again) is not associated with the plasmid copy-number.

SCIENTIFIC CONTRIBUTIONS

Plasmid segregational instability, growth and product formation kinetics of recombinant *E. coli* LE392 cells expressing constitutively hIFNγ in chemostat culture are described for the first time by the kinetic model of Lee et al. (1985). To our knowledge, this is the first application of the model for studying plasmid segregation in chemostat cultures.

PUBLICATIONS AND PRESENTATIONS

Publications

1. Popov M, Petrov S, Kirilov K, Nacheva G, Ivanov I (**2009**) Segregational instability in *E. coli* of expression plasmids carrying human interferon gamma gene and its 3'-end truncated variants. *Biotechnology and Biotechnological Equipment* **23**(2), Special edition, 840-843. (**IF 0.503**)

2. Popov M, Petrov S, Nacheva G, Ivanov I, Reichl U (**2011**) Effects of a recombinant gene expression on ColE1-like plasmid segregation in *Escherichia coli*. *BMC Biotechnology* **11**:18. (**IF 2.97**)

3. Popov M, Nacheva G, Reichl U, Ivanov I (**2012**) Effect of the 3'-terminal truncation of the human interferon-gamma gene on plasmid segregation in *Escherichia coli*. *Biotechnology and Biotechnological Equipment*, in press. (**IF 0.503**)

Presentations

1. Poster presentation: Popov M, Petrov S, Kirilov K, Nacheva G, Ivanov I. Segregational instability in *E. coli* of expression plasmids carrying human interferon gamma gene and its 3'-end truncated variants. XI Anniversary Scientific Conference with International Attendance "Biology - Traditions and Challenges", May 27-29, 2009, Sofia, Bulgaria.

2. Poster presentation: Popov M, Petrov S, Receb M, Ivanov I, Nacheva G, Reichl U. Segregational instability of expression plasmids carrying the human interferon gamma gene in *E. coli.* 34th FEBS Congress "Life's Molecular Interactions", July 4-9, 2009, Prague, Czech Republic.

3. Poster presentation: Popov M, Petrov S, Nacheva G, Ivanov I, Reichl U. Segregation of bacterial plasmids expressing 3'-end truncated human interferon-gamma genes in *E. coli* cells. BIOMATH 2011, International Conference on Mathematical Methods and Models in Biosciences, June 15-18, 2011, Sofia, Bulgaria.

4. Poster presentation: Popov M, Petrov S, Nacheva G, Ivanov I, Reichl U. Effects of human interferon-gamma gene expression on ColE1-like plasmid segregation in *Escherichia coli*, BioProcessing, Biologics & Biotherapeutics Congress (BBBC), July, 20-21, 2011, Edinburgh, UK.

5. Poster presentation: Popov M, Petrov S, Nacheva G, Ivanov I, Reichl U. Fermentation kinetics of recombinant *E. coli* cells producing human interferon-gamma in a chemostat culture, 6th PhD Students and Young Scientists Conference "Young Scientists Towards the Challenges of Modern Technology", September, 19-22, 2011, Warsaw, Poland.

Appendix I

Growth kinetics of *E. coli* LE392 under batch fermentation conditions

The batch growth kinetics of *E. coli* LE392 cells was studied under different cultivation conditions: LB medium and flask (Fig. 16A); LB medium and bioreactor (Fig. 16B); M9 medium and flask (Fig. 16C); M9 medium and bioreactor (Fig. 16D). The maximal specific growth rate of the cells (μ_{max}) was found as a slope of the straight line lnX =f(t) with the data points derived from the exponential growth phase. The value of μ_{max} calculated for LB and M9 medium is approximately 1.20 h^{-1} (generation time 35 min) and 0.66 h^{-1} (generation time 63 min), respectively.

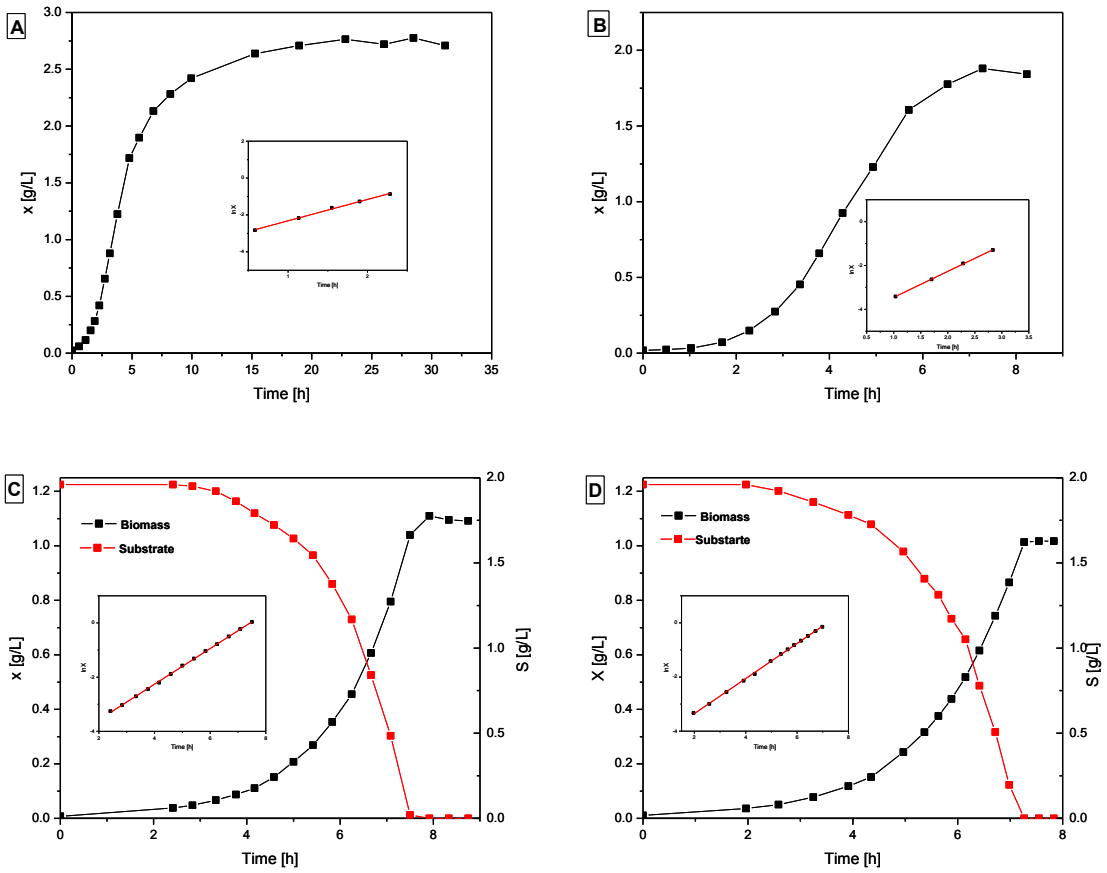


Fig. 16 Cell growth and substrate consumption of *E. coli* LE392 cells under batch fermentation conditions: LB medium, flask (Panel A), LB medium, bioreactor (Panel B), M9 medium with glucose, flask (Panel C), M9 medium with glucose, bioreactor (Panel D). The inserted figures represent the linearity region of lnX=f(t) with data points derived from the exponential growth phase.

Appendix II

Parameter calculation (curve fitting) and simulation applying the model of Lee et al. (1985) in Berkeley Madonna software

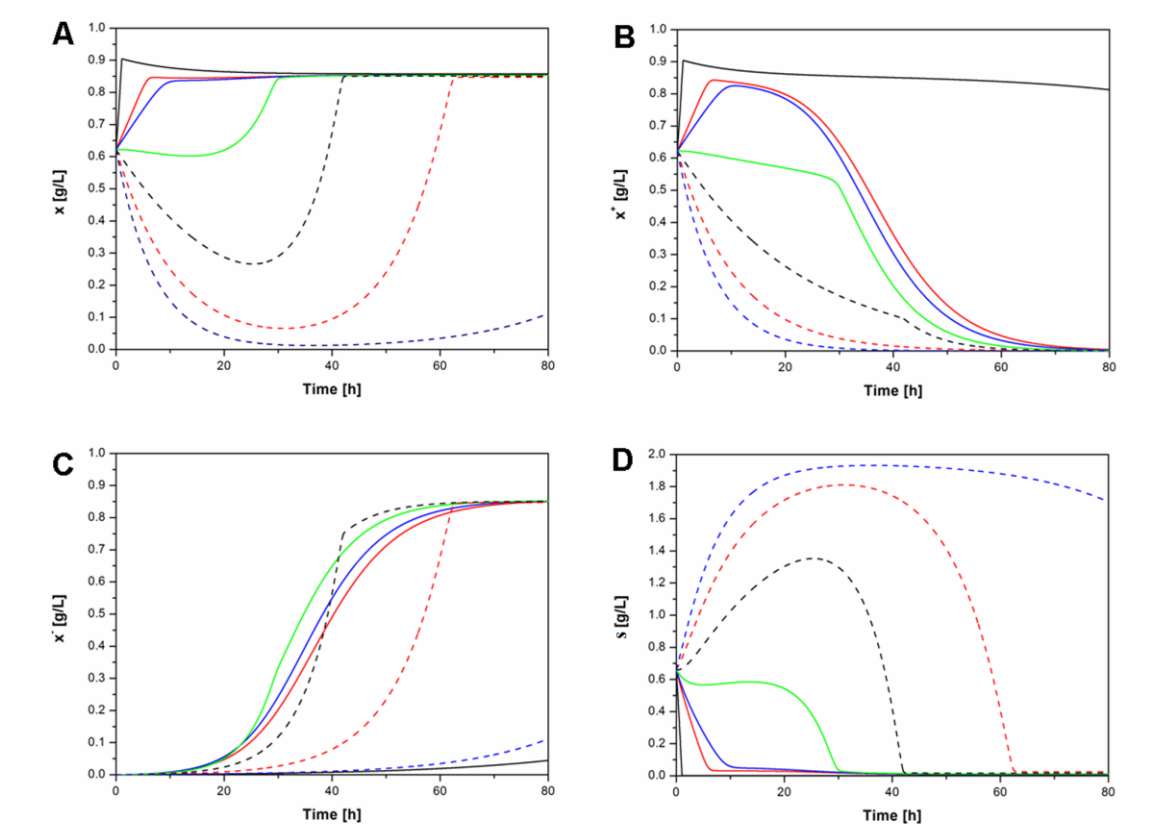
```
METHOD RK4

STARTTIME = 0
STOPTIME=80
DT = 0.002

{SEGREGATIONAL PLASMID INSTABILITY}

{MODEL PROPOSED BY LEE; SERESSIOTIS & BAILEY FOR THE INTRACELLULAR PRODUCT INHIBITION}


{CONSTANTS}

D1 = 0.3                ; 1/h                    Dilution rate
SF = 1.96               ; g/l                    Feed concentration
mumax = 0.66            ; 1/h                    Maximum specific growth rate of plasmid-free cells
Ks = 0.005              ; g/L                    Monod constant
Ginmax = 0.036          ; g plasmid DNA /L biomass  Maximum intracellular cloning vector concentration
Pinmax = 150            ; g/l                    Maximum intracellular product concentration
Np = 41                 ; -                      Number of plasmids per cell
m = 2.399               ; -                      Exponent of vector inhibition term
n = 1.0                 ; -                      Exponent of product inhibition term
Y = 0.438               ; g cells / g substrate  g cells / g substrate yield factor
Ro = 1000               ; g/l                    Cell density
Kp = 0                  ; 1/h                    Decay constant of protein
GAMA = 0.110            ; -                      Gene expression parameter
TITA = 7.435E-4         ; -                      Relative plasmid loss rate

X10 = 0.6216            ; g/l                    Initial concentration of plasmid-bearing cells
X20 = 0                 ; g/l                    Initial concentration of plasmid-free cells
S0 = 0.6599             ; g/l                    Initial substrate concentration in the medium
P0 = 0.00005782         ; g/l                    Initial product concentration in the medium


{INITIAL CONDITIONS}

Init X1 = X10
Init X2 = X20
Init S = S0
Init P = P0


{MASS BALANCES}

X1' = -D1*X1+RX1        ; Balance equation for the plasmid-harbouring cells
X2' = -D1*X2+RX2        ; Balance equation for the plasmid-free cells
S' = D1*(SF-S)+RS       ; Substrate balance equation
P' = -D1*P+RP           ; Product balance equation


{KINETICS}

Gin = 0.0001186*Np      ; Intracellular cloning vector concentration

Pin = P*Ro/X1           ; Intracellular product concentration

mu2 = mumax*(S/(Ks+S))  ; Specific growth rate of plasmid-free cells
```

```
mu1 = mumax*H*Q*(S/(Ks+S))          ;Specific growth rate of plasmid-harbouring cells
H = (1-Gin/Ginmax)^m                ; Term for plasmid inhibition
Q = (1-Pin/Pinmax)^n                ; Term for intracellular product inhibition
R = mu1*(1-TITA)                    ; Help relation
W1 = (4.5*10^10)*(R^4)              ; Help relation
W2 = (78*R^2)+233                   ; Help relation
W3 = 145*R+82.5                     ; Help relation
W4 = 27.6+R                         ; Help relation
W5 = (31.104*10^9)*(R^3)
F1 = W5/(W2*W3*W4)
F2 = W1/(W2*W3*W4)                  ; The function F = f (R)

RX1 = mu1*X1*(1-TITA)               ; Rate equation for the plasmid-harboring cells
RX2 = mu2*X2+mu1*X1*TITA            ; Rate equation for the plasmid-free cells
RS = (-1/Y)*(mu1*X1+mu2*X2)         ; Substrate rate equation
RP = (1/Ro)*(F*GAMA*Gin*X1)-Kp*P    ; Product rate equation

{OTHER RELATIONS}
Z = X1/(X1+X2)          ;  -        Productive (plasmid-harboring) cell fraction
X = X1+X2               ;  g/l      Total biomass concentration
DELTA = mu2-mu1         ; 1/h       Difference in the specific growth rates between plasmid-free
                                    and plasmid-harbouring cells
TETA = TITA*mu1         ; 1/h       Specific plasmid loss rate

{CONDITIONAL}
F = if R > 0.6931 then F1 else F2
{LIMITS}
Limit S>=0.0
```

Appendix III

Results of simulation studies using the model of Lee et al. (1985)

A number of simulations employing the model of Lee et al. (1985) were performed to study growth and hIFNγ formation kinetics of *E. coli* cells cultivated in a chemostat at the following dilution rates: D = 0.1, 0.4, 0.42, 0.46, 0.5, 0.55 and 0.6 h^{-1} (Fig. 17). All parameter values necessary for the calculations were obtained by the model of Lee et al. (1985) for *E. coli* LE392 transformed with pP_1-(SD)-hIFNγ and grown under continuous cultivation conditions (Table 4 and Table 6). For all simulations the same initial conditions were used (Table 5).



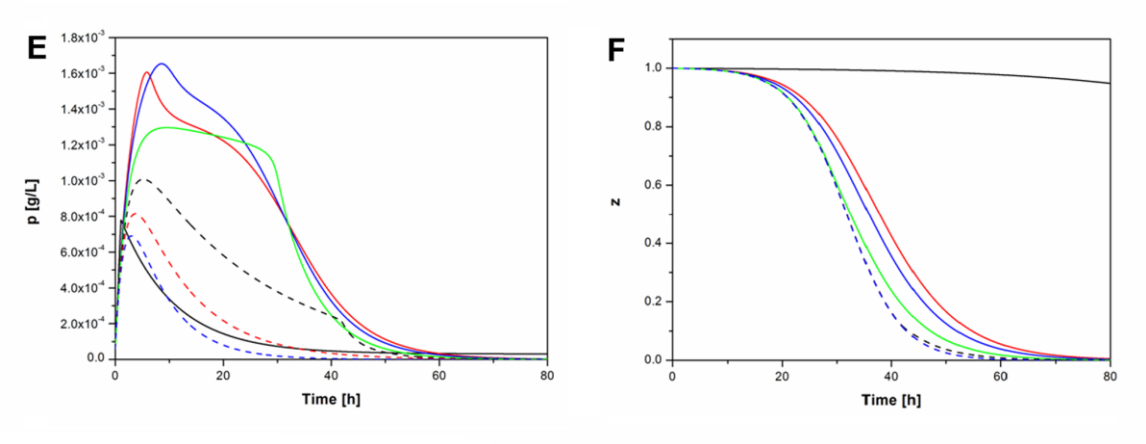


Fig. 17 Simulated chemostat cultivations of *E. coli* LE392 cells carrying pP_1-(SD)-hIFNγ at different dilution rates. Time trajectories for total cell concentration (A), plasmid-harbouring cell concentration (B), plasmid-free cell concentration (C), limiting substrate (D), recombinant product (E) and productive cell fraction (F) at different dilution rates, simulated by the model of Lee et al. Dilution rates: D = 0.1 h^{-1} (black solid line), 0.4 h^{-1} (red solid line), 0.42 h^{-1} (blue solid line), 0.46 h^{-1} (green solid line), 0.5 h^{-1} (black dashed line), 0.55 h^{-1} (red dashed line) and 0.6 h^{-1} (blue dashed line).

Fig. 17A presents time trajectories of the total cell concentration **x**, calculated for different dilution rates. As predicted by Lee et al. (1985), at certain dilution rates (in this study higher than 0.46 h^{-1}) the total cell concentration drops and then increases, which is not observed for θ = 0, i.e. for a stable plasmid copy-number (data not shown). This simulation indicates that (related to the total biomass) the concentration of the limiting substrate increases for certain dilution rates followed by a decrease (Fig. 17D). Fig. 17B, presenting the simulated time trajectories of plasmid-harbouring cells for different dilution rates, indicates that at dilution rates lower than 0.46 h^{-1} the curves are characterized by maxima, where the initial plasmid-harbouring cell concentration is exceeded. For higher dilution rates, however, the maxima of the simulation curves correspond to the initial plasmid-harbouring cell concentration. The dependence of the maximal plasmid-harbouring cell concentration on the dilution rate is presented in Fig. 18.

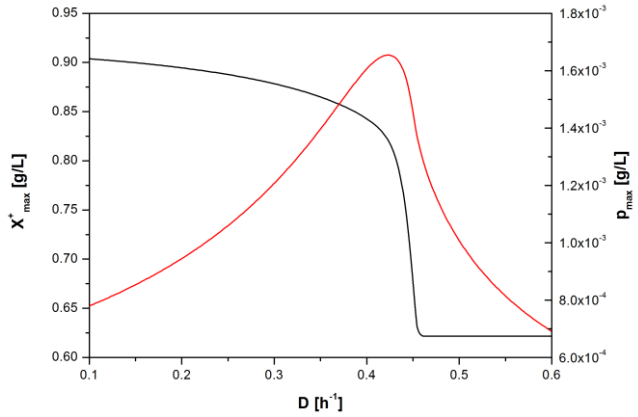


Fig. 18 Simulated maximal plasmid-harbouring cell concentration and maximal concentration of recombinant protein at different dilution rates. The maximal plasmid-harbouring cell concentration $\mathbf{x^+_{max}}$ (black line) and the maximal concentration of recombinant protein $\mathbf{p_{max}}$ (red line) are predicted by the model of Lee et al. (1985) for a chemostat cultivation of *E. coli* LE392 cells carrying pP_1-(SD)-hIFNγ.

Fig. 17C shows that at dilution rates lower than 0.46 h^{-1} the cultivation time required for the plasmid-free cells to reach a certain concentration (e.g. 0.3 g/L) decreases with increasing dilution rate. However, at dilution rates higher than 0.46 h^{-1}, the opposite tendency is observed. Time course of recombinant protein concentration shows sharp maxima at very low and very high dilution rates (Fig. 17E). Both maximum concentration of recombinant protein reached in chemostat cultivations (Fig. 18) and population dynamics of plasmid-harbouring and plasmid-free cells (Fig. 17F) are dependent on the dilution rate. The latter is in accordance with the experimental data obtained by Chew et al. (1988).

Appendix IV

Difference in the specific growth rate between plasmid-free and plasmid-harbouring cells (Δ) and specific plasmid loss rate (Θ) in the models describing plasmid segregation

Mathematically, the cell population dynamics is influenced mainly by the following two factors: a) difference in the specific growth rate between plasmid-free and plasmid-harbouring cells **Δ** and b) probability of generation of plasmid-free cells (specific plasmid loss rate) **Θ** due to the plasmid loss. The latter itself is a function of the specific growth rate of the plasmid-harbouring cells $\boldsymbol{\mu}^{+}$ and the relative plasmid loss rate **θ** (Eq. 22). In some models (Stewart and Levin (1977); Cooper et al. (1987); Noak et al. (1984)) **Δ** and **Θ** are expressed as *constants* assuming apparent steady-state conditions. In general, however, these parameters are complex functions depending on cell genetics and cell physiology as well as on the corresponding cultivation conditions (Park et al. 1991). In the model of Stewart and Levin (1977) **Δ** and **Θ** are considered to be *constants*, whereas in the model of Lee et al. (1985) they are considered to be *functions*. In the model of Lee et al. (1985) the dimensionless relative plasmid loss rate **θ** describing plasmid segregation is assumed to be a *constant* irrespective of the specific growth rate of host cells and cultivation conditions.

The Δ and Θ values of the plasmids pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ calculated by the model of Stewart and Levin (1977) are presented in Table 6. **Δ** and **Θ** of the same plasmids presented as time dependent functions by the model of Lee et al. (1985) are presented in Fig. 19 and Fig. 20, respectively.

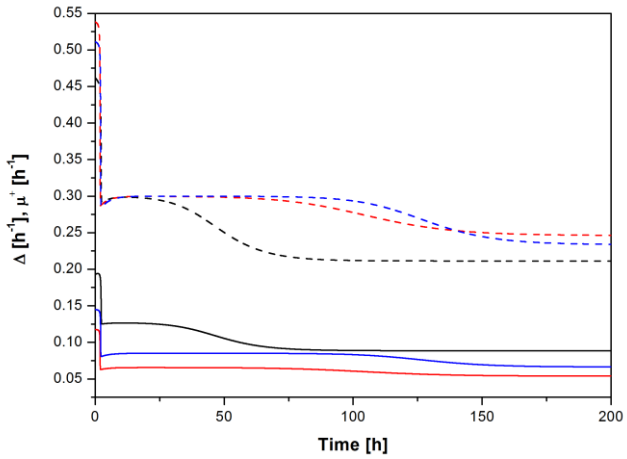


Fig. 19 Simulated specific growth rate difference and specific growth rate of plasmid-harbouring cells by the model of Lee et al (1985). Specific growth rate difference **Δ** (solid lines) and specific growth rate of plasmid-harbouring cells $\boldsymbol{\mu}^+$ (dash lines) as a function of cultivation time predicted by the model of Lee et al. (1985) for the plasmids pP_1-(SD)-hIFNγ (black lines), pP_1-(4SD)-hIFNγ (red lines) and pP_1-(ΔSD)-hIFNγ (blue lines).

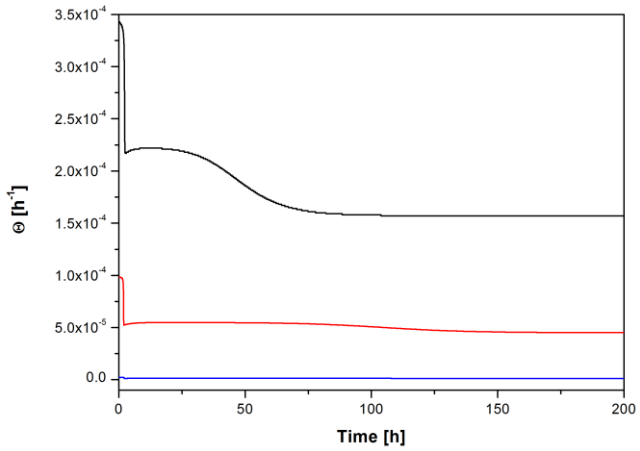


Fig. 20 Specific rate of generation of plasmid-free cells Θ simulated by the model of Lee et al. (1985). The simulated graphs of Θ for pP_1-(SD)-hIFNγ, pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ are presented with black, red and blue line, respectively.

The time course of **Θ** according to the model of Lee et al. (1985), (Fig. 19) confirms the tendency already reported for **Θ** values obtained by the model of Stewart and Levin, (1977), (Table 5).

The specific growth rate of plasmid-free cells ($\boldsymbol{\mu}^-$) is described by the Monod-equation (Eq. 4), whereas the corresponding rate of plasmid-harbouring cells ($\boldsymbol{\mu}^+$) depends on the cellular content of both recombinant protein and expression plasmid (Eq. 5). $\boldsymbol{\mu}^+$ can be presented as time-dependent function applying the model of Lee et al.

(1985), (Fig. 4). The time courses of $\boldsymbol{\mu}^{+}$ clearly show that the specific growth rates of the cells transformed with the plasmids pP_1-(4SD)-hIFNγ and pP_1-(ΔSD)-hIFNγ are almost equal and higher than those of the cells transformed with the construct pP_1-(SD)-hIFNγ.

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