Chapter 14
Plasmid Regulation and Systems-Level Effects on *Escherichia coli* Metabolism

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Abstract ColE1-type plasmids are multicopy extra-chromosomal vectors with wide-spread applications in many areas of genetic engineering and biotechnology. While the regulation of ColE1 replication is primarily effected by plasmid-encoded factors, the continual discovery of new host-encoded factors modulating ColE1 replication such as RNases and exoribonucleases reveals that the *Escherichia coli* host could exert a considerable effect on plasmid replication as well. On the other hand, the presence of plasmids also imposes a metabolic burden impeding host growth and metabolism. The basis of this metabolic burden is multifaceted

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and appears to involve both the plasmid-related drain of cellular resources from the host cell and the perturbation of cellular regulatory state mediated by global transcriptional regulators. Through the systems-level analysis by "omics" tools and *in-silico* modeling, we are gaining better understanding of plasmid-host interactions. This chapter will discuss the interaction of host-encoded factors with the regulation of ColE1-type plasmid replication and the systems-level effects of these multicopy plasmids on metabolism of the *E. coli* host.

14.1 Plasmids and Its Biotechnological Applications

Plasmids are self-replicating extra-chromosomal DNA elements found in many bacteria and yeasts. Initially revealed as the F factor for conjugative gene transfer in *Escherichia coli* (Hayes 1953, Lederberg 1998, Lederberg et al. 1952), later studies on plasmids led to remarkable contributions to the field of molecular biology and biotechnology (Cohen 1993). Figure 14.1 summarizes the major scientific impacts arising from the studies of plasmids. The first replication origins were isolated and characterized from plasmids (Lovett and Helinski 1976, Timmis et al. 1975), providing the foundation for the construction of artificial chromosomes and our present understanding of DNA replication and topology. Subsequent analysis of plasmid-derived genetic elements such as operon and replicon builds further fundamental knowledge on DNA conjugation and fertility, gene expression, genetic recombination, gene transfer and transposable elements (Cohen 1993).

Early investigation of ColE1 type plasmid replication first led to the discovery of antisense RNA and its control on gene expression (Tomizawa et al. 1981). Subsequent studies on antisense RNA of ColE1 plasmid helped to clarify the mechanism of RNA decay (Lin-Chao and Cohen 1991, Xu et al. 1993). In addition, these plasmids also play a critical role in the development of recombinant DNA technology, gene cloning and genome evolution (Cohen 1993). The discovery of

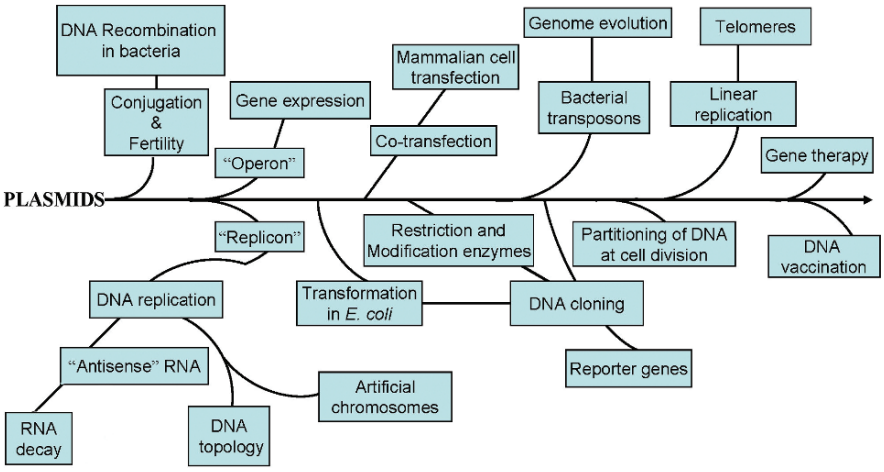

Fig. 14.1 Major scientific impacts rising from the studies of plasmid. (Cohen 1993)

Table 14.1 Commonly-utilized ColE1-type plasmids in biotechnology

Family	Examples	Application
pBR322	pBR322, pBR325, pBR328	Cloning
pBluescript	pBluescript SK, pBluescript KS, pBluescript II SK, pBluescript II KS	Cloning
pUC	pUC18, pUC118, pUC19, pUC119	Cloning/Expression
pET	pET3, pET5, pET7, pET9, pET11, pET12, pET39	Expression
Others	pVAC, pDNAVACCultra, pcDNA series, pCMV series	DNA vaccination

restriction endonucleases and stable transformation of *E. coli* using plasmid DNA led to the invention of recombinant DNA technology and gave rise to the present field of genetic engineering (Cohen 1973, Cohen et al. 1972, Watanabe et al. 1966).

Plasmids used in genetic engineering and biotechnology are commonly known as vectors. Plasmid-based vectors are important tools in biotechnology, where they allow the efficient cloning of genes and expression of desired recombinant proteins in *E. coli* and other microorganisms. Furthermore, there is also emerging interest to apply plasmid DNA as non-viral vectors for delivery of therapeutic or antigenic genes during gene therapy and DNA vaccination (Anderson and Schneider 2007, Ledley 1995, Liu and Huang 2002, Weide et al. 2008). The majority of plasmid vectors used in current recombinant DNA work were high-copy derivatives of ColE1-type plasmids (or its close relative pMB1) (Bolivar et al. 1977, Kahn et al. 1979). Examples of these are high-copy cloning vectors like the well-known pBR322, pBluescript and pUC series (Balbas et al. 1986). Table 14.1 lists some common ColE1-related plasmids currently in use. These high-copy plasmids are usually smaller than low-copy plasmids and, when transformed into the *E. coli* host, are routinely used for gene cloning, recombinant protein production and plasmid DNA production. Despite their widespread utility, we are only beginning to comprehend the complexity of plasmid-host interactions. A better understanding of plasmid-host interactions would allow the design of enhanced plasmid vectors and host strains for biotechnological processes. In view of this, the current review will discuss the interaction of host-encoded molecules with the regulation of ColE1-type plasmid replication, followed by the systems-level effects of these multicopy plasmids on *E. coli* metabolism.

14.2 Host Factor-Mediated Regulation of ColE1 Plasmid Replication

14.2.1 Replication of ColE1 Type Plasmids

ColE1 type plasmids are small circular plasmids naturally occurring in members of the family Enterobacteriaceae and they include plSA, pMB1, RSF1010 (NTP1), CloDF13 (Selzer et al. 1983), NTP16 (Lambert et al. 1987), and other coligenic plasmids (Zverev et al. 1984). The original ColEl is a 6.6 kb *E. coli* plasmid with

a copy number of nearly 20 (Chan et al. 1985). It encodes for a 57 kDa colicin E1 toxin which can kill other *E. coli* cells by depolarizing the bacterial membrane and another protein (*Imm*), offering self-immunity to its colicin. The native plasmid contains the following genes: *cea, imm, kil, inc, RNAII, RNAI, rom, mob, cer* and *exc*; it also carries an origin of replication (*oriV*) and a region (*bom*) from which it can be mobilized for transfer to other bacteria.

The fundamental regulation of ColE1 plasmid replication by plasmid-encoded molecules has been extensively studied (Cabello et al. 1976, Davison 1984, Panayotatos 1984, Schmidt and Inselburg 1982). A region of about 600 bp in the ColE1 plasmid and several *E. coli* enzymes are involved in replication of ColE1. The initiation of replication of ColE1 plasmid proceeds from 555 bp upstream of the *oriV* and leads to the transcription of a pre-primer RNAII by RNA polymerase (Itoh and Tomizawa 1979). The nascent RNAII transcript hybridizes with the DNA template of ColEl and forms a DNA-RNA hybrid with a specific secondary structure which can be recognized and cleaved by RNase H, resulting in a free 3′-OH end which serves as a primer for DNA synthesis by DNA Polymerase I (Tomizawa and Som 1984). Plasmid replication proceeds by covalent extension of RNA primer (pRNA) from the *oriV* region (Fig. 14.2).

The repression of Co1E1 plasmid replication depends on the inhibition of the primer precursor, RNAII, by its plasmid-encoded antisense molecule, RNAI. RNAI is a 108-nucleotide molecule which is transcribed from 445 bp upstream of the *oriV*, in the opposite direction to RNAII, from a promoter located between the RNAII promoter and the origin of replication of ColE1 type plasmids. As the sequence of RNAI is complementary to the 5′-end of RNAII, RNAI can bind to RNAII and form a stable RNA-RNA hybrid (Cesareni et al. 1991, Kues and Stahl 1989). The binding of RNAII to RNAI leads to a conformational change in RNAII, preventing the formation of the DNA-RNA hybrid (Masukata and Tomizawa 1986, Polisky et al. 1990). Consequently, RNAII may not be able to function as a replication primer. Thus, RNAI plays a key role in the control of ColE1 plasmid copy number as the inhibitor of plasmid replication.

In addition to RNAI and RNAII, a third plasmid-encoded factor, Rom or Rop, can also negatively control the replication of ColE1. Rom is a small protein which has been proposed to accelerate the binding of RNAI to RNAII (Tomizawa and Som 1984) and inhibit RNAII primer formation (Cesareni et al. 1982). Thus, the expression of Rom protein reduces plasmid copy number and the *rom* gene is absent in many high-copy plasmids like pUC or pET. In line with that, a point mutation in RNAII that suppresses Rom was shown to increase plasmid copy number (Lin-Chao et al. 1992). Figure 14.2 illustrates the mechanism of ColE1 replication by antisense RNAI regulation.

14.2.2 Host-Mediated Regulation of ColE1 Plasmid Replication

Although copy number control of Co1E1-type plasmid in *E. coli* by plasmid-encoded molecules has been studied at length, there is recent evidence that other

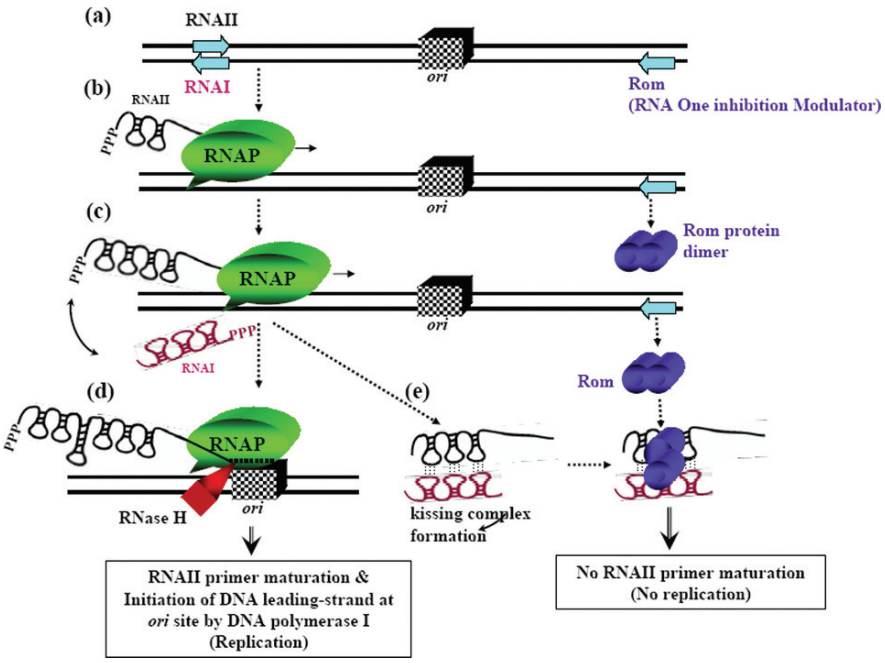

Fig. 14.2 The mechanism of ColE1 plasmid replication by anti-sense RNA regulation. (**a**) A genetic map of ColE1 plasmid replication. The blue arrows ( ) indicate the transcription direction for RNAII (primer RNA,), RNAI (anti-sense RNA,) and Rom (RNA One Modulator). The replication origin (*ori*,) is indicated. (**b–d**) The pre-primer RNAII and anti-sense RNAI are synthesized by the host RNA polymerase (RNAP,). RNase H ( ) cleavage at the DNA-RNA hybrid between RNAII and the DNA template at the origin region generates the 3′-OH end of the RNAII primer (i.e. RNAII primer maturation) for initiation of leading strand synthesis by DNA polymerase I. (**e**) When RNAI interacts with RNAII, the kissing complex is formed and stabilized by the Rom ( ) proteins. This anti-sense and primer RNA interaction inhibits the formation of DNA-RNAII hybrid, and prevents maturation of pre-primer RNAII. As a result, no RNAII primer is available for plasmid replication

host-encoded factors also modulate overall plasmid copy number. In principle, any host-encoded factors affecting the stability or secondary structure of RNAI and RNAII will also interfere with the plasmid copy number. It has been shown that some host-encoded enzymes regulate the degradation of RNAI by endo- or exonucleolytic cleavage: (i) RNase E has been shown to have an endonucleolytic activity in RNAI decay (Lin-Chao and Cohen 1991); (ii) RNase III plays a role in turnover of RNAI (Binnie et al. 1999); (iii) polynucleotide phosphorylase is implicated in degradation of RNAI (Xu and Cohen 1995); and (iv) poly(A) polymerase I has been shown to play a role in the regulation of ColE1 plasmid copy number and RNAI decay (Xu et al. 2002). Figure 14.3 shows how host-encoded proteins interfering with the RNA-RNA interaction or regulating RNAI degradation can affect ColE1 plasmid replication (e.g. r-protein L_4; Singh et al. 2008).

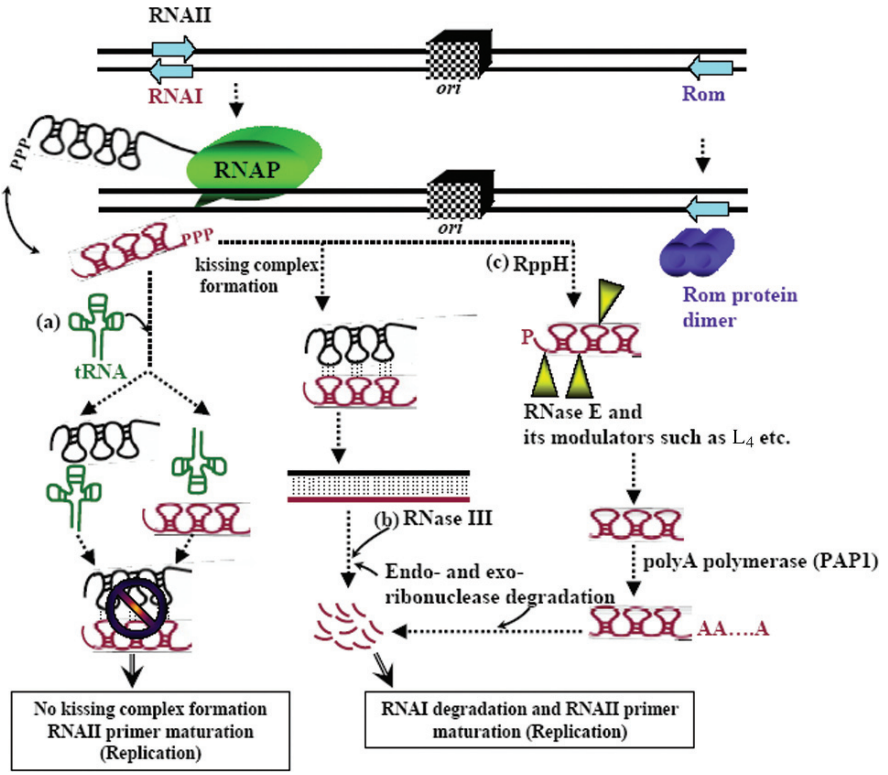

Fig. 14.3 Host-encoded proteins and tRNAs interfering with RNA-RNA interaction or regulating RNAI degradation can affect ColE1 plasmid replication. (**a**) Owing to sequence homologies with RNAII and RNAI, uncharged tRNA ( ) can interact with RNAII or RNAI and block kissing complex formation. Thus, matured primer-RNA is produced which promotes initiation of plasmid DNA replication. (**b**) When RNAII and RNAI form a kissing complex, RNase III can cleave the double-stranded RNAs into single-stranded RNA. These substrates are degraded by endo- and exo-ribonucleases (**c**) When RNAI is transcribed by RNAP, the tri-phosphate RNAI (ppp-RNAI) phosphate groups are removed by RppH (pyrophosphohydrolase) proteins to form monophosphate RNAI (p-RNAI). The p-RNAI substrates are cleaved by RNase E to form $pRNAI_{-5}$. Some RNase E modulators such as inhibitor L_4 are also involved in RNAI metabolism. Poly(A) tails are added to the $pRNAI_{-5}$ substrates by PAPI (polyA polymerase 1) proteins. These intermediates of RNAI are then degraded rapidly by endo- and exo-ribonucleases. When all RNAI intermediates have been degraded, no kissing complex can form and replication starts

14.2.2.1 Role of RNases and Polyadenylation in the Regulation of ColE1 Plasmid Replication

Several RNases encoded by *E. coli* have been shown to be implicated in ColE1 plasmid replication. The endoribonuclease RNase E, which is involved in the maturation of 5S rRNA (Apirion 1978, Mackie 1998), has also been shown to have an endonucleolytic activity in RNAI decay (Lin-Chao and Cohen 1991). It mediates the cleavage of RNAI from the full length triphosphorylated pppRNA I108 to pRNA I105 (Lin-Chao and Cohen 1991). Later, Kaberdin and co-workers also

identified multiple cleavage sites in the stem loops of RNAI by RNase E (Kaberdin et al. 1996). RNase H recognizes and cleaves the RNAII–DNA hybrids at the origin with a sequence of AAAAA of RNAII and then generates mature primer RNAII (Naito and Uchida 1986). RNase III, an endonuclease which recognizes and cleaves double-strand RNA, is a third implicated RNase. It is involved in both the processing of rRNA and the degradation or processing of a variety of mRNA (Babitzke et al. 1993). It has been shown that RNase III is involved in the processing or degradation of RNAI and RNAII during the formation of RNAI and RNAII complex (Binnie et al. 1999).

More recently, polyadenylation has also been shown to be involved in regulating the copy number of ColE1 plasmids (Xu et al. 1993). Polynucleotide phosphorylase (PNPase) is an exoribonuclease which is implicated in mRNA degradation by removing nucleotides from the 3′-end of the RNA(Donovan and Kushner 1986) and it can also degrade RNAI (Xu and Cohen 1995). The degradation of RNAI by PNPase is further promoted by its polyadenylation. When a poly(A) tail is added to the 3′-end of RNAI by poly(A) polymerase (encoded by *pcnB*), this facilitates further exonucleolytic cleavage by PNPase after cleavage by RNase E (Xu et al. 2002). Therefore, the addition of a poly(A) tail hastens the decay of RNAI. In contrast, mutation in the *pcnB* gene stabilizes the RNA intermediate and reduces the copy number of ColE1 plasmid (Masters et al. 1990, Sarkar et al. 2002).

14.2.2.2 Role of tRNA on the Regulation of ColE1 Plasmid Replication

Previous studies have reported that the sequence of the loop II of RNAI and the dihydrouridylic loop of tRNA have close homologies (Yavachev and Ivanov 1988). This therefore implies a possible role for tRNA in the regulation of ColE1 plasmid replication. It has been shown that uncharged tRNA can interact with RNAI to regulate the replication of ColE1 plasmid (Wang et al. 2002, Wang et al. 2004, Wrobel and Wegrzyn 1998). It was speculated that tRNA–RNAI interactions may interfere with hybridization between RNAI and RNA II, thus allowing more maturation of the pre-primer RNAII and initiation of plasmid DNA replication (Wegrzyn 1999). Another effect of tRNA in the control of replication of ColE1-type plasmids, based on the interaction of the 3′-CCA sequence of uncharged tRNA with RNAI, has also been proposed (Wang et al. 2004). Understanding the role of uncharged tRNA in regulating the replication of ColE1-type plasmid is important, because amino acid starvation (which leads to the accumulation of uncharged tRNA) has been considered to be a more effective method than temperature shift for up-regulating the replication of ColE1-type plasmids (Wegrzyn 1999). Figure 14.3 illustrates how tRNAs interference with RNA-RNA interaction can affect ColE1 plasmid replication.

14.2.2.3 Other Host Factor-Mediated Regulation of ColE1 Type Plasmid Replication

Other host factors are also involved in regulation of ColE1 plasmid replication. It has been shown that RraA, a regulator of ribonuclease A activity, interacts with RNase E and inhibits RNase E endonucleolytic cleavage (Lee et al. 2003). Inhibition of RNase E by RraA prolongs the half-life of substrates such as RNAI, and thus in-

terferes with the replication of ColE1 plasmids. Moreover, the degradation of most transcripts of *E. coli* proceeds through a 5′-end-dependent pathway and begins with endonucleolytic cleavage. The endonuclease responsible is RNase E, whose cleavage is the initial, rate-limiting step of mRNA degradation in *E. coli*. Previous study reported that the mechanism of the 5′-end dependent pathway for RNA decay is triggered by 5′-pyrophosphate removal (Celesnik et al. 2007). RppH, an RNA pyrophosphohydrolase which belongs to the Nudix protein family, has been reported to initiate the degradation of mRNA by this 5′-end dependent pathway as it removes the phosphates from the 5′-end of a triphosphorylated primary transcript (Deana et al. 2008). Therefore, RppH triggers RNase E cleavage and controls the rate of RNA decay (such as the degradation of RNAI) and also affects the ColE1 plasmid copy number.

14.3 Systems-Level Effects of Plasmid on *E. coli* Host Metabolism

14.3.1 Cellular Metabolic Burden from the Presence of Multicopy Plasmids

Although multicopy derivatives of ColE1 plasmids are widely-used, the introduction of these plasmids to *E. coli* often imposes a metabolic burden causing systems-level perturbation to cellular metabolism (Glick 1995). Phenotypically, the metabolic burden can be directly observed as reduction in cellular growth rate and final biomass (Fig. 14.4). Growth rate of plasmid-bearing (P+) *E. coli* decline with increasing plasmid copy number or size (Bentley et al. 1990, Cheah et al. 1987, Seo and Bailey 1985). Conversely, with increasing growth rate, the ratio of the RNAI inhibitor of plasmid replication over the replication pre-primer RNAII has been shown to increase correspondingly (Lin-Chao and Bremer 1986), suggesting the existence

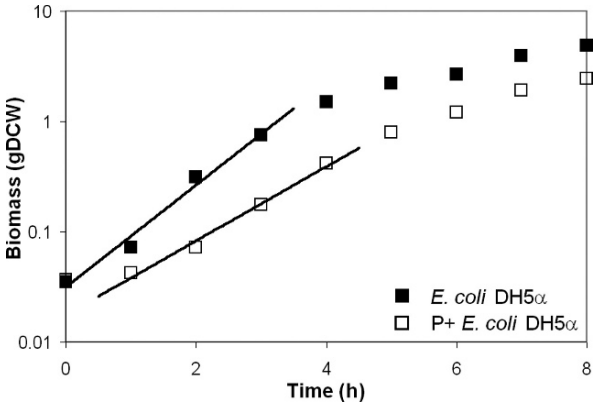

Fig. 14.4 Growth of plasmid-free (■) and plasmid-bearing (□) *E. coli DH5α* cells during 2-L batch cultures. Due to the metabolic burden of maintaining multicopy plasmids, the plasmid-bearing (P+) cells showed a reduced growth rate and final biomass relative to the plasmid-free host cells

of an intricate relationship between plasmid copy regulation and growth rate. The majority of growth-related phenotypic changes from plasmid presence have been extensively covered in two prior reviews (Glick 1995, Ricci and Hernandez 2000). Other than growth retardation, plasmid presence could also incur additional physiological changes, including elevation of oxygen uptake rates (Khosravi et al. 1990), increased glucose uptake and ATP synthesis (Diaz-Ricci et al. 1992), co-localization and presumed interaction with the host replication machinery at the cell membrane (Pogliano 2002, Yao et al. 2007), and loss of viability and cell lysis during fed-batch cultures (Andersson et al. 1996).

Conventionally, the basis of plasmid metabolic burden has been attributed to the metabolic drain of biosynthetic precursors, energy and other cellular resources for the maintenance of multicopy plasmids (Seo and Bailey 1985). As illustrated in Fig. 14.5, plasmid DNA replication and plasmid-encoded mRNA and protein synthesis share the same precursors, energy and enzymatic machinery as the analogous host metabolic processes (Peretti and Bailey 1987). Accordingly, the maintenance of plasmids would inevitably compete with the cellular growth for a limited pool of cellular resources, including biosynthetic precursors like deoxyribonucleotides, ribonucleotides and amino acids and high-energy molecules like ATP, GTP, NADH and NADPH. All these precursors and high-energy molecules required for plasmid maintenance are derived from the distribution of carbon fluxes through the central metabolic pathways (CMP) into assorted branches of biosynthetic and catabolic pathways (Holms 1996).

First proposed by Diaz Ricci and Hernandez (2000), an alternate proposition for the metabolic burden is that the presence of plasmids distresses host metabolism by perturbing the global transcriptional network. In other words, the *E. coli* host could perceive plasmids or plasmid-encoded products as an intracellular stress stimulus,

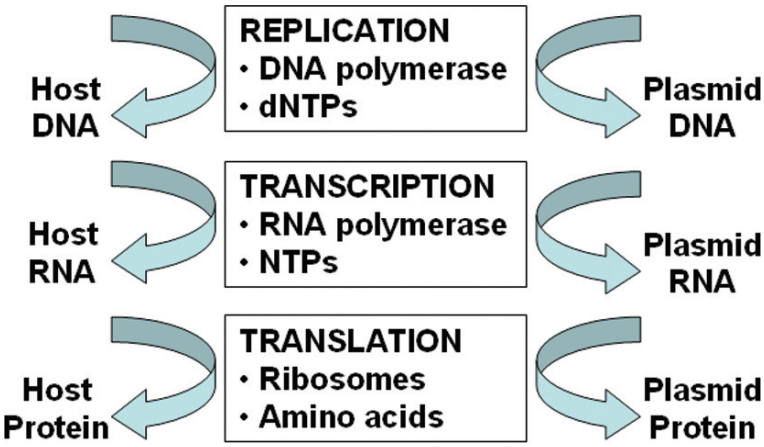

Fig. 14.5 Schematic illustration of competition between plasmid DNA and the bacterial host for cellular resources (Peretti and Bailey 1987). Copyright (1987, John Wiley & Sons, Inc.), reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc

triggering a cascade of stress signals affecting the *E. coli* transcriptional network. That in turn could lead to substantial changes in global gene expression and cellular phenotype.

14.3.2 *Plasmid Perturbation of the Global Transcriptional Regulatory Network in* E. coli

In bacteria, transcription regulation is generally considered the main mode of gene regulation. Figure 14.6 illustrates the multi-layer hierarchical structure of the *E. coli* global transcriptional network (Ma et al. 2004). Of the 4280 transcripts identified in *E. coli*, 267 are known or putative transcriptional regulators (Babu et al. 2004). The overall regulation of transcription in response to environmental and physiological changes is coordinated by a set of specific and global transcriptional regulators. While specific transcriptional regulators mainly regulate single transcriptional units consisting of genes with related functions known as operons, global transcriptional regulators are pleiotropic proteins with the ability to regulate operons belonging to several metabolic pathways or functional classes (Gottesman 1984).

There are now evidences suggesting that global transcriptional regulators play a key role in mediating the plasmid metabolic burden response. The first global

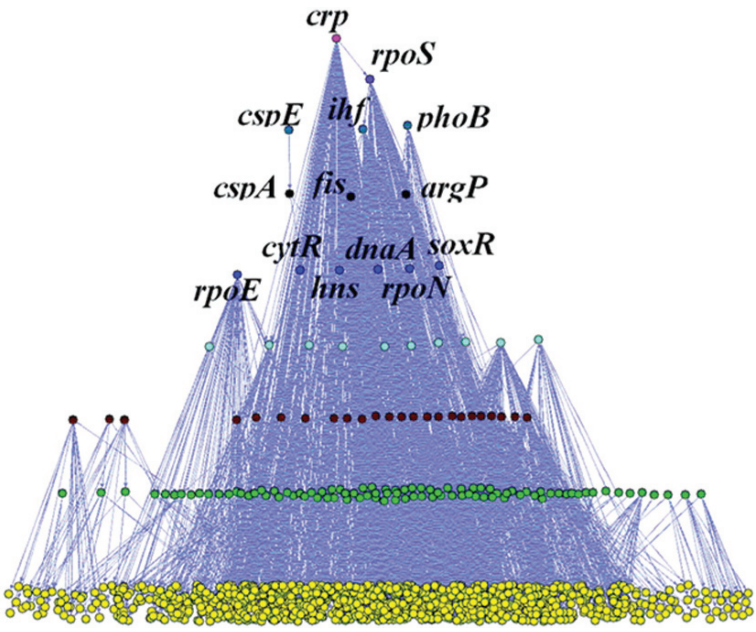

Fig. 14.6 Multi-layer hierarchical structure of the *E. coli* global transcriptional network. In the extended transcriptional regulatory network model containing 1278 genes and 2724 regulatory interactions by Ma and co-workers, the top layer regulators tend to be global transcriptional regulators, while the regulated metabolic enzymes are at the bottom layer (Ma et al. 2004)

transcriptional regulator implicated in plasmid metabolic burden is the cyclic AMP-response protein (CRP). The presence of plasmids is reported to lead to an increase in intracellular concentration of cyclic AMP (cAMP) in three *E. coli* strains (Diaz-Ricci et al. 1995). cAMP is a signal molecule responsible for activating the CRP, a global transcriptional regulator that directly regulates the expression of 197 *E. coli* genes including 22 other transcriptional regulators (Martinez-Antonio and Collado-Vides 2003). The cAMP-CRP complex activates the expression of catabolic operons in response to the availability of glucose and is also involved in cell division, motility, starvation function and anaerobiosis (Botsford and Harman 1992). During the growth of *E. coli* HB101, DH1 and JM109 carrying 3 different plasmids (including the ColE1 derived pUC19), an increase in intracellular cAMP concentration was accompanied by the higher activity of the cAMP-CRP activated β-galactosidase and an elevated rate of glucose uptake relative to the corresponding plasmid-free cells (Diaz-Ricci et al. 1995). Thereafter, the authors proposed, "*plasmids affect host metabolism through the perturbation of the cAMP-CRP complex, which in turn causes the alteration of the regulatory status of host regulations*." (Diaz Ricci and Hernandez 2000). Despite the finding that plasmid presence is related to higher cellular cAMP levels, the exact mechanisms involved remain unclear.

A second global regulator-like molecule that could be involved in the transcription response to plasmid metabolic burden is guanosine tetraphosphate or ppGpp (Magnusson et al. 2005). ppGpp is the effector of the stringent response to amino acid starvation, widely-observed as the overall down-regulation of rRNA biosynthesis and ribosome production (Stent and Brenner 1961). The intracellular level of ppGpp rises in response to amino acid, carbon or energy depletion(Cashel et al. 1996) and is shown to correlate inversely with cellular growth rate (Joseleau-Petit et al. 1994). Recently, ppGpp is proposed to be the master regulator coordinating the binding of various sigma factors with RNA polymerase core enzyme (Nystrom 2004); in doing so, ppGpp in turn regulates the transcription of various stress-responsive genes mediated by alternative sigma factors, including σ^s (regulator of stationary phase response), σ^{32} (regulator of heat shock response) and σ^{54} (regulator of nutrient limitation and alternative carbon utilization). In recombinant plasmid-bearing cells under significant metabolic stress, ppGpp levels could be elevated. An intracellular ppGpp level of 0.45 μMol/gDCW was reported in uninduced pET11ahSOD plasmid-bearing *E. coli* HMS174(DE3) cells not producing the recombinant protein product (Cserjan-Puschmann et al. 1999). The subtle metabolic burden displayed by these plasmid-bearing cells was attributed to the replication and expression of the plasmid and its marker protein.

At present, the sole global transcriptional regulator shown to directly affect the metabolic burden-related retardation of cellular growth is FruR (fructose repressor, also known as Cra or catabolite activator repressor). FruR is a global transcription regulator of major catabolic enzymes using a cAMP-CRP independent mechanism (Saier 1996). Primarily, FruR represses the transcription of catabolic enzymes involved in the glycolytic pathway (*pfkA, pykF, gapA, pgk, eno*), Entner-Doudoroff pathway (*edd, eda*) and alternative sugar catabolism (*fruBAK, mtlADR*). At the same time, it positively activates genes in glyconeogenesis (*fbp, ppsA*), TCA cycle (*acnA,*

icdA), glyoxalate shunt (*aceBA*) and electron transport chain (*cydAB*) (Ramseier 1996). Corresponding to FruR regulation of central metabolic pathways, knockout of the *fruR* gene in *E. coli* was shown to enhance carbon flow though glycolytic pathway and inhibit carbon flow though glyconeogenesis (Ramseier et al. 1995).

The inactivation of FruR by gene knockout was found to significantly improve the growth rates of plasmid-bearing cells relative to the respective wildtype cells (Ow et al. 2007). For *E. coli DH5α* carrying a ColE1-derived pcDNA3.1d/NS3 plasmid, the cellular growth rate during 2-L batch cultures improved from $0.75\,h^{-1}$ to $0.91\,h^{-1}$ after *fruR* knockout. This considerable growth rate recovery from plasmid metabolic burden was accompanied by a corresponding up-regulation of glycolytic enzymes and down-regulation of TCA cycle and stress proteins as revealed from proteomic and transcriptional analyses (Fig. 14.7).

As revealed from these studies, there is mounting evidence that, mediated by the action of global transcriptional regulators, the presence of plasmid leads to alterations in the global transcriptional network. Two implicated global transcriptional regulators, CRP and FruR, are both recognized to be major regulators of central metabolic gene expression. This appears to point towards the prevailing role of central metabolic gene expression in effecting the metabolic burden phenomenon. In all, these findings do not disprove the former proposition of plasmid metabolic drain. It is more likely that both the drain of cellular resources and the perturbation of the cellular regulatory network act synergistically together to contribute to the metabolic burden.

14.3.3 Central Metabolic Gene Expression and Plasmid Metabolic Burden

As metabolic fluxes within pathways have to synchronize with biosynthetic demands for precursors and energy during cell growth, they have evolved to be under tight regulatory control (Nielsen 2003). Regulation of metabolic fluxes can occur at the level of transcription (mRNA synthesis and degradation), translation (protein synthesis and proteolysis) and enzyme activity (allosteric regulation; Table 14.2). Due to the existence of various feedback loops and signaling cascades, these regulatory processes are interlinked to form a complex regulatory network (Vemuri and Aristidou 2005). Despite our extensive knowledge on *E. coli*, the exact nature of the regulatory network and its impact on central metabolism has not been clearly elucidated.

Although many CMP enzymes are constitutively expressed (Fraenkel 1996) and a few key enzymes are regulated by allosteric binding of effector molecules, the transcription of CMP enzymes has been observed to vary in response to different physiological condition (Sabnis et al. 1995). For instance, considerable transcriptional changes were observed within the glycolytic, gluconeogenic and TCA cycle pathways in *E. coli* during growth in acetate versus glucose media (Oh and Liao 2000). These transcriptional changes were found to qualitatively correlate to the actual CMP metabolic fluxes, which indicate the existence of significant regula-

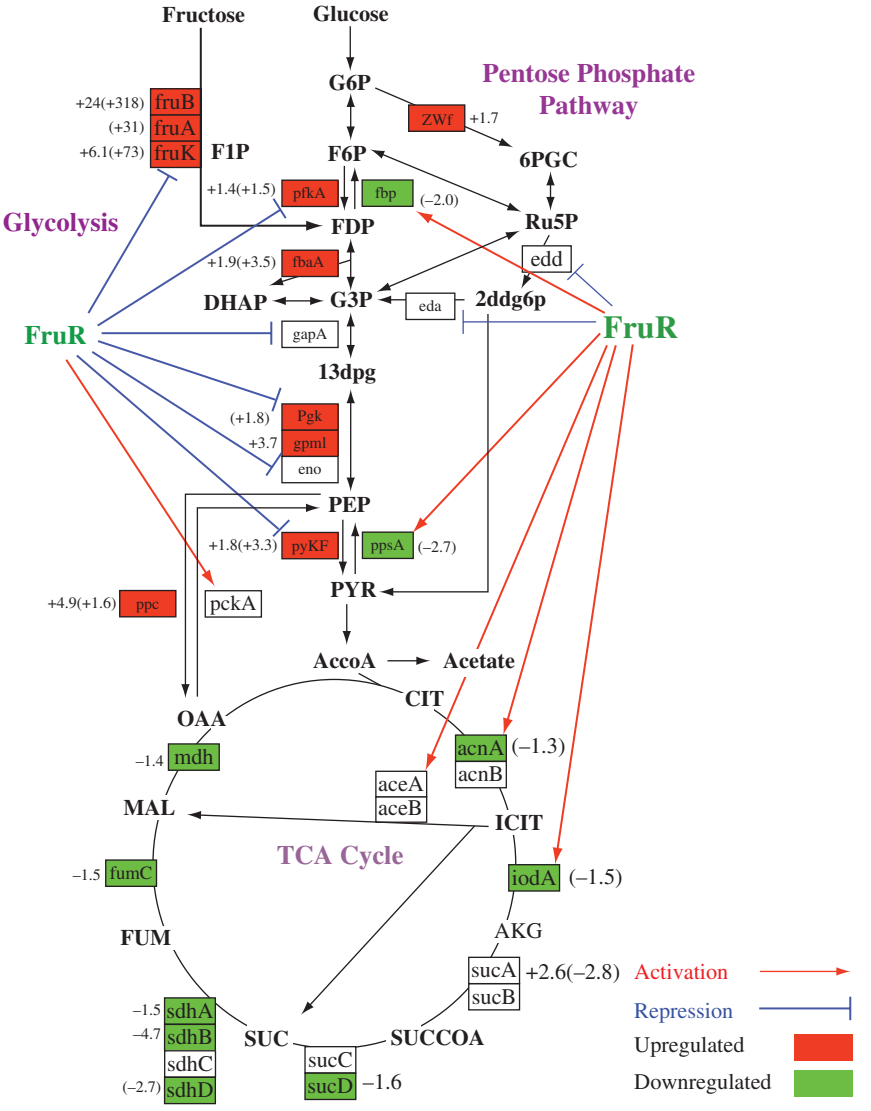

Fig. 14.7 Central metabolic gene expression changes in plasmid-bearing *DH5α* cells after *fruR* gene knockout. Values without brackets are protein expression fold changes, while values in brackets are the transcriptional fold changes. A trend of down-regulated (green boxes) glycolytic genes and up-regulated (red boxes) TCA cycle genes was observed. FruR mediated activation and repression are indicated by → or ⊣ respectively. (Figure from Ow et al. 2007)

tion in these pathways. During high cell density culturing of *E. coli* (Yoon et al. 2003), global transcriptional and proteomic studies showed a pattern of CMP gene expression changes that relates to the various physiological growth phases. These

Table 14.2 Allosteric regulation of enzyme activity in glycolysis and TCA cycle (compiled from EcoCyc database, Keseler et al. 2005)

Enzyme	Activator	Inhibitor
Phosphofructokinase 1	ADP	PEP
Fructose-1,6-biphosphate		AMP
Pyruvate kinase 1	FBP	
Pyruvate kinase 2	AMP	
Citrate synthase		NADH, OAA
Phosphoenolpyruvate carboxylase	FBP, acetyl-CoA	Aspartate, MAL
Phosphoenolpyruvate carboxykinase		NADH

observations indicate that transcriptional regulation of CMP is also of physiological importance (Sabnis et al. 1995).

The apparent role that the central metabolic gene expression plays in affecting plasmid metabolic burden is further supported by another recent study (Flores et al. 2004), whereby the overexpression of the *zwf* gene (encoding for the first enzyme of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase) increased the growth rate of plasmid-bearing *E. coli* JM101 from 0.46 h^{-1} to 0.64 h^{-1} (Fig. 14.8). The growth rate recovery was ascribed to the potential increase in carbon flux to the oxidative branch of the pentose phosphate pathway. Since the PP pathway provides: (1) NADPH, a source of reducing power for many biosynthetic reactions and (2) precursors (ribose-5-phosphate and erythrose-4-phosphate) for nucleotide, histidine, and aromatic amino acids biosynthesis, they hypothesized that the availability

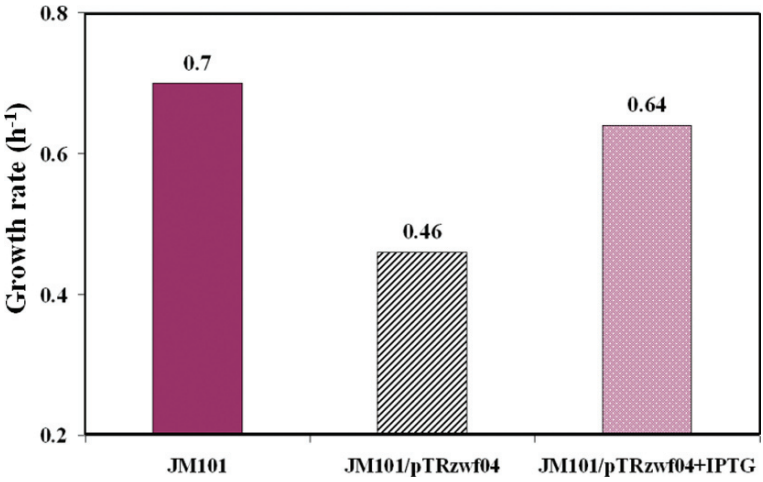

Fig. 14.8 Specific growth rates of *E. coli* JM101 strains over-expressing the *zwf* gene (Flores et al. 2004). The overexpression of *zwf* (encoding for the first enzyme of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase) with IPTG led to an increase in growth rate from 0.46 h^{-1} to 0.64 h^{-1} in cells carrying plasmid pTRzwf04

of some of these metabolites could be limiting for the biosynthesis of plasmids or foreign proteins.

14.3.4 Global Transcriptional and Proteomic Studies of Plasmid Metabolic Burden

The first semi-global proteomic study to investigate protein expression changes due to plasmid presence in exponentially-growing *E. coli* was conducted by Birnbaum and Bailey (Birnbaum and Bailey 1991). In the study, two ColE1-derived plasmids that differ only by a mutated RNAI sequence were used to generate two plasmid copy number mutants with: (1) a mid plasmid copy number of 56 (designated strain P60), and (2) a high plasmid copy number of 240 (designated strain P120). Protein expression trends of the P60 and P120 strains were compared with the plasmid-free HB101 parental strain grown in minimal media supplemented with 20 amino acids. From 93 polypeptides identified, 34 were examined.

It was found that the levels of TCA cycle enzymes increase as the plasmid copy number increases initially from 0 to 56 (Birnbaum and Bailey 1991). Subsequently, at the higher copy number of 240, an increase in the anaplerotic PEP carboxylase expression was accompanied by a corresponding reduction in the expression of pyruvate kinase I, pyruvate dehydrogenase complex and TCA cycle enzymes. This indicates that, when grown on amino acids as the sole carbon source, cells carrying more copies of plasmids replenish TCA intermediates for precursor generation at the expense of TCA cycle flux. In additional, reduced expression of proteins of the protein synthesis machinery (2 elongation factors, 9 ribosomal subunits, asparyl-tRNA synthetase) and increased expression of 4 heat shock proteins were also seen. Together with a decrease in total cellular RNA and ribosome content as revealed from sucrose gradient profiles, the results denote a reduced translational capacity and elevated metabolic stress for cells carrying more plasmid.

Subsequently, a global transcriptome-proteome study was conducted to examine gene expression changes from plasmid presence in *E. coli* $DH5\alpha$ grown on glucose-containing complex media (Ow et al. 2006). The ColE1-type pcDNA3.1d/NS3 plasmid used was a DNA vaccine carrying a non-expressing antigenic gene against Dengue virus and has a copy number of approximately 100–150 during exponential phase (Lee et al. 2006). In the study, pcDNA3.1d/NS3 plasmid-bearing cells showed a 25% drop in growth rate over the plasmid-free host cells. Comparison of the exponentially growing plasmid-bearing cells over the plasmid-free host cells identified 364 genes and 18 proteins with more than 1.2 fold changes in gene expression.

A general downregulation of biosynthetic and key aerobic respiratory genes was observed (Ow et al. 2006). The downregulation of NADH dehydrogenase II (*ndh*) and several aerobic terminal oxidases (*cydA*, *cydB*, *cyoA*, *cyoB*) indicated an overall repression of major respiratory energy pathways in the plasmid-bearing cells. Among the upregulated genes were 6 stress-response heat shock proteins (*lon,*

mopA, clpB, hslV, ibpB, ibpA). In particular, the upregulation of the heat shock *clpB* chaperone and *hslV* protease have not been previously associated with plasmid presence. Consistent with reports of higher cAMP-CRP activity in cells carrying plasmids, most upregulated carbon transporters are activated by the cAMP-CRP global regulator. Moreover, the downregulation of two key glycolytic genes, *pfkA* and *pykF* was seen. Interestingly, the only known transcriptional repressor of *pfkA* and *pykF* is FruR, another global regulator implicated with plasmid metabolic burden.

In the previous two studies, the comparison of gene expression was made on plasmid-bearing and the host cells showing evident variations in growth rates during exponential phase. As any variations in growth rates or physiological conditions could affect the interpretation of gene expression studies, Wang and colleagues used glucose-limited chemostat cultures to equilibrate the growth rates of two BL21 strains carrying a mid or a high copy ColE1 plasmid (copy numbers of 80 and 420) with the plasmid-free host (Wang et al. 2006). At the identical steady-state growth rate of $0.20\,h^{-1}$, glucose consumption rates for the plasmid-bearing cells were higher by approximately 2.5–3 fold relative to the host. Correspondingly, the acetate excretion rates for the plasmid-bearing cells were higher by 5–11 fold.

Microarray transcriptional analysis on these plasmid-bearing cells over the host showed a clear gene expression trend of an increase in glycolysis and TCA cycle and decrease in pentose phosphate pathway (Wang et al. 2006). These central metabolic gene expression trends were found to be consistent with the corresponding data from enzyme activity assays and metabolic flux analysis. In line with the experimental observations of higher glucose consumption and acetate excretion rates, the upregulation of *ptsG* for glucose uptake and acetate metabolic genes (*ackA*, *pta*) was also reported. In contrast with previous studies, only subtle changes in expression of genes related to cellular structure, DNA replication, and transcription/translation processes were observed. The authors reported that only the CMP related genes showed the largest expression changes. Hence, it appeared that, when growing at the same physiological growth rate as the host, CMP gene expression changes in the plasmid-bearing cells are dominant over other functional changes.

14.3.5 In-silico Simulation of Plasmid Metabolic Burden

It is now widely accepted that mathematical modeling and simulation of complex biological systems play a pivotal role in further improving our understanding of systems-level characteristics and functions. To date, various quantitative models have been presented for describing host-plasmid interactions in *E. coli*. Once predictive models are developed, various simulations under different conditions can be conducted by changing relevant parameters, thus allowing us to explore effects of plasmid presence on metabolic burden. As discussed previously, the presence of plasmids in the cell results in metabolic burden effects leading to retarded growth, changes in gene regulation, enzyme activities and metabolic flux. Major mechanistic processes involved in host-vector systems compose of plasmid replication, mRNA transcription, and plasmid-encoded mRNA translation of the foreign protein. Thus,

detailed kinetics and control structures describing those processes along with key factors affecting host physiology can be formulated within plasmid-host interaction models. Experimentally observed metabolic burden is then characterized through *in silico* simulation of the models.

Plasmid replication is the first step in any plasmid-host interactions related to plasmid metabolic burden. In order to understand the underlying strategy of plasmid replication control, many researchers have presented mathematical models for describing the mechanism of ColE1 plasmid replication by anti-sense RNA regulation (Brendel and Perelson 1993). Paulsson and Ehrenberg determined optimal ColE1 copy number to achieve increased segregational stability, thereby reducing metabolic burden to the host cell (Paulsson and Ehrenberg 1998). Peretti and Bailey presented a mechanistically detailed single-cell model for *E. coli*, considering various competitive interactions found in plasmid-host systems (Peretti and Bailey 1987). They simulated recombinant cell growth by changing the relevant factors to metabolic activity, including plasmid copy number, promoter strength and ribosome binding strength. From these simulation experiments, strategies for enhancing cloned-gene productivity or reducing metabolic burden could be evaluated.

The second principal factor affecting metabolic burden is the expression of plasmid-encoded protein, which is commonly the antibiotic resistance marker protein. A simple mathematical model was developed for guiding stable target protein production and excretion (Togna et al. 1993). In the latter study, the empirical expression for the specific rate of plasmid production and less structured model of the *lac* operon induction was included in the dynamic model formulation. Bentley and co-workers presented a metabolically-structured kinetic model where expression of foreign proteins such as chloramphenicol-acetyl-transferease (CAT) and resistance marker protein (b-lactamase) was described based on plasmid content in addition to replication and mRNA transcription (Bentley et al. 1990). They observed the close corelationship between growth rate and foreign protein expression while the effect of plasmid replication on the growth rate was negligible. This implies that the metabolic drain of precursors and energy associated with the expression of proteins prevail over that for the replication of plasmid DNA.

Most plasmid-host interaction models are kinetics-based dynamic models which require extensive kinetic and regulatory information for modeling. Most often than not, experimental measurements are not easily obtained for determining a large number of kinetic parameters (e.g. intracellular reaction rates). The stationary modeling approach is, therefore, a good alternative to the kinetic model for the simulation of plasmid-bearing host metabolism. Assuming the pseudo-steady state, the kinetic model can be simplified into static representation, taking into account the network's connectivity and capacity as time-invariant properties of the metabolic system. To investigate the effect of plasmid-directed synthesis on metabolic stoichiometry, da Silva and Bailey calculated additional energetic and material requirements caused by plasmids (da Silva and Bailey 1986), hence deriving an early stoichiometric model for plasmid synthesis. Subsequently, Ozkan and colleagues developed a metabolic model for cell growth and recombinant protein overproduction in *E. coli* that included precursor balances and energetic requirement for plasmid replication,

and protein expression within the metabolic balance model (Ozkan et al. 2005). Using some of these stoichiometric models for plasmid synthesis, the physiological effect of plasmid metabolic burden on *E. coli* metabolism can be further explored by constraints-based flux modeling (Ow et al. 2009). exploited a genome-scale *E. coli* model using various linear-programming cellular objective functions to identify the most plausible describer of the physiological state within the plasmid-bearing cells. The study demonstrated that flux simulations by maximizing maintenance energy expenditure showed good consistency with experimental data, suggesting that the plasmid-bearing cells are less energetically-efficient and could require more maintenance energy.

Current models are still limited by insufficient knowledge on global regulation and kinetic information. As there are now evidences of global regulatory changes in plasmid-bearing cells, future modeling approaches should systematically combine dynamic and stationary models with regulatory information and high-throughput "omics" data analysis to characterize the metabolic burden, thereby identifying engineering strategies for overcoming plasmid metabolic burden in *E. coli*.

14.4 Conclusions and Future Prospects

ColE1-type plasmids have been extensively characterized and widely applied in biotechnology. Early studies of plasmids and the successive development of ColE1-type plasmid vectors have contributed extensively to the present progress in molecular biology and recombinant DNA technology. Studies on the basic regulation of ColE1 replication have been initialized more than three decades ago. Although it is now well known that the control of ColE1 replication is primarily regulated by plasmid-encoded factors, the continual discovery of new host-encoded factors modulating ColE1 replication reveals that the *E. coli* host could exert a considerable effect on the replication of ColE1 plasmids as well.

While *E. coli* host produces several factors modulating ColE1 replication, plasmids also impose a metabolic burden impeding host growth and metabolism. The basis of this metabolic burden is complex and appears to involve both the plasmid-related drain of cellular resources from central metabolism and the perturbation of cellular regulatory state mediated by global transcriptional regulators. Through the application of systems level "omics" tools and *in-silico* modeling, we are beginning to gain better understanding of plasmid-host interactions. From the initial discovery of plasmids, to successive vector construction and emerging applications like genetic therapy and vaccination, it is anticipated that the current trend towards systems-level studies of plasmid-host interactions will give rise to even more knowledge and further biotechnological applications.

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