Metabolic Engineering **2**, 328–338 (2000)
doi:10.1006/mben.2000.0161, available online at http://www.idealibrary.com on IDEAL®

Low-Copy Plasmids can Perform as Well as or Better Than High-Copy Plasmids for Metabolic Engineering of Bacteria

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Received April 10, 2000; accepted July 24, 2000; published online October 30, 2000

Multicopy plasmids are often chosen for the expression of recombinant genes in *Escherichia coli*. The high copy number is generally desired for maximum gene expression; however, the metabolic burden effects that usually result from multiple plasmid copies could prove to be detrimental for maximum productivity in certain metabolic engineering applications. In this study, low-copy mini-F plasmids were compared to high-copy pMB1-based plasmids for production of two metabolites in *E. coli*: polyphosphate (polyP) and lycopene derived from isopentenyl diphosphate (IPP). The stationary-phase accumulation of polyP on a per cell basis was enhanced approximately 80% when either high- or low-copy plasmids were used, from 120 μmol/g DCW without augmented polyP kinase (PPK) activity to ~220 μmol/g DCW. The cell density of the high-copy plasmid-containing culture at stationary phase was approximately 24% lower than the low-copy culture and 30% lower than the control culture. This difference in cell density is likely a metabolic burden effect and resulted in a lower overall product concentration for the high-copy culture (~130 μmol/L culture) relative to the low-copy culture (~160 μmol/L culture). When the gene for DXP (1-deoxy-D-xylulose 5-phosphate) synthase, the first enzyme in the IPP mevalonate-independent biosynthetic pathway, was expressed from the *tac* promoter on multicopy and low-copy plasmids, lycopene production was enhanced two- to threefold over that found in cells expressing the chromosomal copy only. Cell growth and lycopene production decreased substantially when isopropyl β-D-thiogalactosidase (IPTG) was added to the high-copy plasmid-containing culture, suggesting that overexpression of DXP synthase was a significant metabolic burden. In the low-copy plasmid-containing culture, no differences in cell growth or lycopene production were observed with any IPTG concentrations. When *dxs* was placed under the control of the arabinose-inducible promoter (P_{BAD}) on the low-copy plasmid, the amount of lycopene produced was proportional to the arabinose concentration and no significant changes in cell growth resulted. These results suggest that low-copy plasmids may be useful in metabolic engineering applications, particularly when one or more of the substrates used in the recombinant pathway are required for normal cellular metabolism. © 2000 Academic Press

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INTRODUCTION

Metabolic engineering generally involves the introduction of new metabolic activities into the host organism or the improvement of existing processes (Stephanopoulos, 1999). This type of genetic engineering presents a set of challenges different from those typically encountered when one seeks to overexpress a protein as the final product of interest. Consequently, the choice of an appropriate expression vector for the recombinant genes must be made with these challenges in mind.

Multicopy plasmids have long been the vector of choice for recombinant gene expression, particularly the overexpression of genes, and many have been developed for this purpose. The advantages of these vectors are well known. (1) They are typically small, on the order of 5 kbp, and easily manipulated for controlled gene expression. (2) Large quantities of the vector can be isolated in high concentration for the cloning reactions because of the high copy number. (3) The high copy number generally results in good expression of the recombinant genes in the host organism. The use of these plasmids, however, also carries some disadvantages. The recombinant vector may be structurally unstable (O'Connor *et al.*, 1989; Balbas and Bolivar, 1990) or segregationally unstable (Noack *et al.*, 1981; Jones and Melling, 1984). The presence of a high-copy plasmid may also cause a significant shift in the normal metabolism of the host cell, which can increase the risk of plasmid instability. This metabolic burden has been attributed to the increased demand for nucleotides for replication of plasmid DNA (Birnbaum and Bailey, 1991) and to the translation of plasmid-encoded genes (Bentley *et al.*, 1990; Vind *et al.*, 1993).

The nature of these disadvantages suggests that multicopy plasmids may not be the best option for some metabolic engineering applications, even when the objective is to increase the intracellular concentration of an enzyme in order to improve flux through an existing pathway. In this case, the specific activity of the gene product is not important in and of itself, but rather only as it contributes to increased production of some metabolite of interest. These

1096-7176/00 $35.00

Metabolic Engineering **2**, 328–338 (2000)
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reaction pathways often include substrates that are important, even critical, for general cell metabolism and host survival. Overproducing a protein whose turnover is ultimately limited by the availability of such precursors may needlessly burden the cell with the task of creating that protein. Further, the overconsumption of metabolites involved in general metabolism may result in decreased production of other vital cell components and thus reduced production of the compound of interest. A balance must therefore be achieved between optimizing production of the desired final compound and ensuring the viability of the host cell.

Low-copy-number plasmids could prove useful for these purposes. Mini-F plasmids, based on the *Escherichia coli* F plasmid, are maintained at one to two copies per chromosome. These low-copy plasmids have been shown to be at least 2.5 times more stable than pMB1-derived high-copy plasmids in nonselective medium, to be more tightly regulated as demonstrated with a 10-fold lower basal level of expression, and to impose minimal metabolic burden on the host cell when expression of a plasmid-encoded *lac*Z gene was induced (Jones and Keasling, 1998). These plasmids enable recombinant expression at gene dosage levels similar to that of the chromosome without interfering with the genome. Chromosomal insertion could result in the interruption of native genes that are not required under the conditions of selection but that may be desired under a different set of growth conditions. Expression of genes in the chromosome is also affected by their position relative to the origin of replication (Sousa *et al*., 1997).

We compared low-copy, mini-F vectors to high-copy vectors for expression of genes encoding rate-limiting enzymes in two metabolic pathways. The first metabolic pathway investigated was the production of long-chain inorganic polyphosphate (polyP) by polyP kinase (PPK). PolyP is synthesized when the terminal phosphate from ATP is transferred (reversibly) to a growing polyP chain:

$$\mathrm{ATP} + \mathrm{polyP}_n \xleftrightarrow{\mathrm{PPK}} \mathrm{polyP}_{n+1} + \mathrm{ADP}. \qquad (1)$$

The gene is naturally transcribed as part of an operon that includes the enzyme polyphosphatase (PPX). PPX processively degrades long-chain polyP to inorganic phosphate (P_i):

$$\mathrm{polyP}_n \xrightarrow{\mathrm{PPX}} \mathrm{polyP}_{n-1} + P_i. \qquad (2)$$

PolyP is quite ubiquitous in nature and is implicated in many roles: storage of high-energy phosphoanhydride bonds and P_i, chelation of cations and metals, and as a buffer against changes in environmental conditions (Kornberg, 1995). The role of polyP as a storage polymer of phosphate and energy is important for biological phosphorus removal for the treatment of municipal wastewater (Stensel, 1991). In this process, a waste stream containing high amounts of phosphate (P_i) is fed to activated sludge that is cycled through anaerobic and aerobic zones. During these shifts, intracellular polyP is alternately degraded and synthesized for energy and to cause a net uptake of P_i in the system. The balance of PPK and PPX activities in the EBPR process provides an effective means of storing energy and phosphate when both are in excess for later release when they are limiting. The cycling between high and low energy zones is important for establishing this behavior of alternately forming and degrading polyP. Similarly, *E. coli* accumulates polyP in response to a shift from low P_i to high P_i medium. PPK activity is low during P_i starvation (low P_i) and increases after a shift to excess P_i, while PPX activity decreases following the shift (Sharfstein and Keasling, 1994).

Several factors make the polyP biosynthetic reaction a good model to study metabolic engineering for enhanced productivity by using low-copy plasmids. First, the reaction requires a substrate, ATP, that is necessary for maintaining cell viability. Diverting too much ATP from normal cellular processes toward polyP formation, perhaps as a result of overexpression of *ppk* from a high-copy plasmid, could significantly alter the growth dynamics of the cell. Second, in the native system product accumulation and high enzyme activity are dependent upon a shift in the composition of the growth medium. Presumably this shift results in induction of gene expression or enzyme activation. This induction pattern can be mimicked using a recombinant system by adding a chemical inducer at the time of shift to P_i starvation. Although activity remains high prior to P_i shifts in cultures that utilize a recombinant *ppk* gene under the control of a strong promoter, the activity still rises by a factor of about two after the addition of excess P_i (Sharfstein and Keasling, 1994). Finally, the one-step pathway is easy to manipulate, allowing one to effectively alter the productivity by focusing on a single enzyme.

The second pathway investigated was the production of isopentenyl diphosphate (IPP) through the mevalonate-independent pathway. IPP is the common precursor for all isoprenoids, a ubiquitous class of naturally occurring molecules that includes more than 30,000 compounds (Eisenreich *et al*., 1998). The first step in the mevalonate-independent pathway is the production of 1-deoxyxylulose 5-phosphate (DXP) from pyruvate and glyceraldehyde 3-phosphate (Harker and Bramley, 1999). The gene responsible for the DXP synthase (DXS) activity, *dxs*, has been identified and cloned (Lois *et al*., 1998; Sprenger *et al*., 1997). Overexpression of this gene on a multicopy plasmid results in increased production of lycopene, a representative carotenoid, when the *Erwinia herbicola crtEBI* genes are

expressed in the same cell (Kim and Keasling, in press). This system is similar to the polyP production pathway because it consumes key metabolites, pyruvate and glyceraldehyde 3-phosphate, involved in normal cellular metabolism, and flux through the pathway can be increased by overexpression of a single gene, *dxs*. These simplified model systems provide insights into the potential benefits as well as limitations of using low-copy plasmids for metabolic engineering applications.

1. MATERIALS AND METHODS

Bacterial strains and plasmids (Table 1). *E. coli* DH10B [F^- *mcr*A *Δ*(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lacZΔ*M15 *ΔlacX*74 *deo*R *rec*A1 *end*A1 *ara*D139 *Δ*(*ara*, *leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G] and DH5α[F^- ϕ80d*lacZΔ*M15 *Δ*(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1] were purchased from Life Technologies (Grand Island, NY). *E. coli* DPA1 [F^- *ppk*::*kan ΔaraBAD* *leu*::Tn10] was constructed by P1 transduction using standard techniques (Miller, 1992). To produce DPA1, the mutation in the arabinose catabolic genes (*araBAD*) along with the proximal Tn10(tet^R)-disrupted leucine marker was transduced from LMG194 (Guzman *et al*., 1995) into the *ppk*::*kan* mutant CF5802 (Ault-Riché *et al*., 1998). Transductants were selected on LB plates with 10 μg/ml tetracycline, and the absence of arabinose catabolism was verified using EMB plates (Miller, 1992).

The low-copy mini-F plasmid pKLJ12 contains the arabinose-inducible P_{BAD} promoter and associated *araC* gene and has been described previously (Jones and Keasling, 1998). Multicopy plasmid pKLJ01 was constructed by insertion of a 1.5-kbp *Ssp*I fragment containing the *lac*I^q gene from pMMB206 (Morales *et al*., 1991) into the *Bam*HI site (Klenow-filled) of pMEX7 (Bio 101, Vista, CA), upstream of the *tac* promoter. The origin of replication was removed from this plasmid by digestion with *Alw*NI (Klenow-filled) and *Nae*I, and the resulting fragment (4.7 kbp) was ligated to a previously described mini-F fragment from pML31 to construct the low-copy plasmid pKLJ03 (Jones and Keasling, 1998).

The multicopy plasmid pSVD8 contains the *E. coli ppk* gene, which encodes PPK, under the control of the arabinose-inducible *ara*BAD promoter, P_{BAD} (S. Van Dien and J. D. Keasling, unpublished). This plasmid was constructed by inserting the 2-kbp *Pst*I–*Hin*dIII DNA fragment containing *ppk* from pSK14 (Van Dien *et al*., 1997) into the vector pBAD18 (Guzman *et al*., 1995). The *ppk* gene from pSVD8 was isolated first by digestion with restriction enzyme *Hin*dIII, followed by filling the recessed ends using Klenow enzyme. Digestion of the linearized plasmid with *Nhe*I produced a 2-kbp fragment containing the *ppk* gene. This fragment was ligated to vector pKLJ12 that had been digested with *Nhe*I and *Sal*I (Klenow-filled) to produce plasmid pF12-ppk.

Plasmids pK1-dxs and pF3-dxs contain the *E. coli dxs* gene, which produces DXP (1-deoxy-D-xylulose 5-phosphate) synthase. These plasmids were constructed by inserting the 1.9-kbp *Eco*RI–*Sph*I *dxs* gene fragment from pTdxs (Kim and Keasling, in press) into the vectors pKLJ01 and pKLJ03, respectively, under control of the IPTG-inducible *tac* promoter. The low-copy plasmid pF12-dxs, containing *dxs* under the control of the arabinose-inducible P_{BAD} promoter, was constructed by inserting the 1.9-kbp *Nhe*I–*Sph*I fragment from pDdxs (Kim and Keasling, submitted) into the mini-F plasmid pKLJ12. Plasmid pAC-LYC04, which expresses the *E. herbicola crtEBI* genes necessary for lycopene biosynthesis in *E. coli*, was a gift from F. X. Cunningham of University of Maryland. The plasmid also contains the *Haematococcus pluvialis ipi* gene that encodes IPP isomerase. The restriction digests and ligations were performed according to standard protocols (Sambrook *et al*., 1989), and the

TABLE 1

Plasmids Used in This Study

Plasmid	Parental plasmid	Promoter	Gene	Copy number
pKLJ12	—	P_{BAD}	—	Low
pBAD18	—	P_{BAD}	—	High
pKLJ03	—	P_{tac}	—	Low
pKLJ01	—	P_{tac}	—	High
pF12-ppk	pKLJ12	P_{BAD}	*ppk*	Low
pSVD8	pBAD18	P_{BAD}	*ppk*	High
pF12-dxs	pKLJ12	P_{BAD}	*dxs*	Low
pF3-dxs	pKLJ03	P_{tac}	*dxs*	Low
pK1-dxs	pKLJ01	P_{tac}	*dxs*	High

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enzymes were purchased from New England Biolabs (Beverly, MA).

Growth media and conditions. For the polyP accumulation experiments, cultures were grown at 37°C in MOPS-buffered minimal medium (Neidhardt *et al.*, 1974) supplemented with 0.4% glycerol, 105 μg/ml leucine, and ampicillin at 100 μg/ml (multicopy plasmid) or 50 μg/ml (low-copy plasmids). To accumulate high levels of intracellular polyP, cultures were grown initially in the presence of 1.32 mM potassium phosphate (1 × concentration for MOPS medium). At an optical density at a wavelength of 600 nm (OD_{600}) of 0.4–0.5, the cells were harvested and resuspended in P_i-free MOPS medium prewarmed to 37°C, containing 1.33 mM arabinose. After 2 h, the cultures were shifted to excess P_i at a final concentration of 13.2 mM and sampled periodically. Aliquots of 1 ml were removed for measurement of intracellular polyP levels and polyP kinase activity. For OD_{600} less than 1.0, aliquots of 2–4 ml were taken for PPK activity assays. The samples were centrifuged and the cell pellets were stored at −20°C pending further analysis.

For the lycopene production experiments, a seed culture was made by inoculating cells into 2 × YT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol and growing the cells overnight at 37°C. An aliquot of the seed culture was inoculated into 5 ml of 2 × YT medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol to an OD_{600} of 0.1 and incubated at 29°C for 24 h. IPTG and arabinose induction studies were initiated at OD_{600} of 0.8 and 0.1, respectively.

Measurement of intracellular polyP. Intracellular polyP was assayed by the method of Ault-Riché *et al.* (1998). Frozen cell pellets were thawed at room temperature. Briefly, the method involves lysis of the cells in 4 M guanidine thiocyanate at high temperatures and binding of long-chain inorganic polyP to silica beads ("Glassmilk" purchased from Bio 101). The polyP was eluted in 50 mM Tris buffer, pH 8.0, at high temperature and converted enzymatically to ATP *in vitro* using the reverse PPK reaction with purified enzyme from *E. coli*. The ATP concentration was measured using a luminometer (Turner Designs, Sunnyvale, CA) and the Sigma Chemical ATP bioluminescent assay kit (St. Louis, MO). PolyP levels are reported as micromoles of P_i incorporated into polyP per gram of dry cell weight.

Measurement of PPK activity. Cell pellets were thawed on ice and resuspended in 50 mM Tris–Cl, pH 7.5, 10% sucrose. An equal volume of lysis buffer consisting of 50 mM Tris–Cl, pH 7.5, 10% sucrose, 300 mM NaCl, 90 mM EDTA, and 3 mg/ml lysozyme was added, followed by incubation on ice for 1 h. The suspensions were subjected to five freeze–thaw cycles, alternating between liquid nitrogen and a 30–40°C water bath. After chilling on ice, the samples were sonicated at 4°C twice for 5 s each, with a minimum 5-s incubation on ice between rounds. The lysed cell solution was then centrifuged for 30 min at 4°C, and the supernatant was transferred to a fresh microcentrifuge tube. Total protein was quantified using the Bio-Rad protein assay solution (Hercules, CA). The PPK activity in the soluble protein extract was assayed using a previously described non-radioactive method (Tinsley *et al.*, 1993). One unit is defined as picomoles of P_i incorporated into polyP per minute at room temperature; PPK activity is reported as units per microgram of total protein.

Lycopene assays. The lycopene content of the cells was determined by harvesting aliquots of cells by centrifugation at 13,000*g* for 3 min, washing once in water, and recentrifuging. The cells were resuspended in acetone (200 μl) and incubated at 55°C for 15 min in the dark. The samples were centrifuged again at 13,000*g* for 10 min, and the acetone supernatant containing the lycopene was placed in a clean tube. The lycopene content of the extracts was quantified by measuring the absorbance at 470 nm and comparing to a lycopene standard (purchased from Sigma Chemical) using a Beckman DU Series 640 spectrophotometer.

2. RESULTS

Enhanced production of polyP utilizing low-copy plasmids. *E. coli* DH10B contains the *ppk* gene under control of the native promoter. Cells harboring the plasmid pF12-ppk [DH10B(pF12-ppk)] were initially grown at 37°C in MOPS-buffered medium containing 1.32 mM potassium phosphate and were harvested during midexponential phase (OD_{600} = 0.4–0.5) and resuspended in P_i-free medium. To mimic the observed natural expression pattern of *ppk* in response to shifts in inorganic P_i concentration, induction of *ppk* gene expression from the low-copy plasmid was initiated at this point by the addition of arabinose to a final concentration of 1.33 mM. Inducing during the shift to low P_i allows for the initiation of transcription prior to the shift to excess P_i, immediately after which native PPK activity is known to be high. The arabinose concentration was chosen to ensure maximum induction from the low-copy plasmid, without regard to growth rate effects since induction occurred late in the growth cycle. Cultures were not washed prior to resuspension, but were incubated for 2 h at 37°C to enable complete consumption of residual P_i and induction of the native phosphate starvation response. Separate

experiments showed that growth stopped, presumably due to P_i limitations, 2 h after the shift to P_i-free medium, as indicated by optical density measurements (data not shown). At this time, P_i was added to a final concentration of 13.2 mM, and samples were collected to assay for polyP content. DH10B(pKLJ12) served as a control.

The intracellular polyP content of each culture was essentially zero immediately prior to the first shift to P_i-free medium (-2 h) and before the second shift to excess P_i (0 h) (Figs. 1a and 1b). At 3 h, the polyP content was approximately 20-fold higher in the augmented culture containing pF12-ppk relative to the control culture containing pKLJ12 (2.8 μmol polyP/g DCW versus 0.1 μmol polyP/g DCW). The 20-fold enhancement was maintained until at least 9 h after the second shift, at which time the augmented culture contained 45 μmol polyP/g DCW versus 2.3 μmol polyP/g DCW for the control. In the 1.5-h interval between 9 and 10.5 h, the polyP level in the control culture rose sharply, from 2.3 to 41 μmol/g DCW, with only a 70% increase in the augmented culture during the same time interval. However, the polyP levels remained higher in the augmented culture throughout the course of the experiment. The stationary-phase polyP content, measured at 24 h, was approximately 2-fold higher in the augmented culture, at 219 μmol/g DCW versus 120 μmol/g DCW in the control culture.

Growth rates were not measured for these cultures. Any burden effects on growth rate as a result of the presence of the *ppk*-containing plasmid should only be significant after the addition of the inducer (Jones and Keasling, 1998). Because arabinose addition was concomitant with a shift in culture medium, it is probably not possible to separate any effects of metabolic burden caused by plasmid presence from those caused by a response to changes in medium composition, as measured by growth rates. Further, the addition of inducer late in the growth cycle allows only a small time window for true exponential growth after adjusting to the medium shift and before approaching stationary phase. However, a comparison of optical densities (OD_{600}) taken at each time point should give some indication of any metabolic burden effects that may impact the absolute cell density of the cultures, irrespective of the growth rate. As shown in Fig. 1c, the presence of an additional copy of the *ppk* gene in DH10B(pF12-ppk) did not appear to significantly affect the maximum achievable cell densities of the polyP-producing cultures.

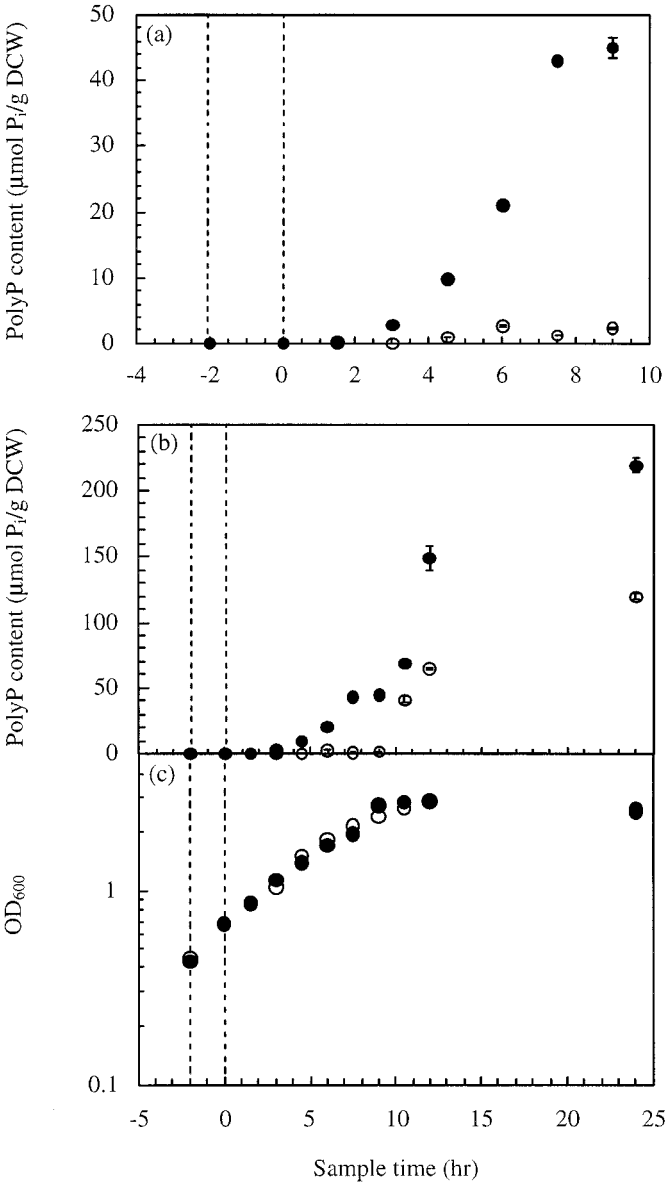

FIG. 1. Intracellular polyP content of DH10B cultures (a, b) and optical densities (c) as a function of sample time. The complete polyP profile (b) is shown in detail in (a) to indicate the differences at early time points. (c) Optical density, measured at 600 nm. At -2 h, the culture was shifted from $1 \times P_i$ to P_i-free medium. At 0 h, excess P_i was added to a final concentration of 13.2 mM. Open circles, pKLJ12 (control); filled circles, pF12-ppk (augmented). Data shown are averages of two measurements from a single experiment and are representative of several experiments conducted similarly. Error bars are standard deviations of the measurements.

Comparison of polyP formation from low- and multicopy plasmids. Plasmid pSVD8 contains the *ppk* gene in the same configuration behind the *ara*BAD promoter (P_{BAD}) as pF12-ppk. This plasmid was used to determine the enhancement of polyP production that could be achieved using a multicopy plasmid. Cultures were grown in medium with varying P_i concentrations as described previously; however, a full-time course profile was not examined. Instead, polyP levels were measured prior to the first and second shifts, at 3 and 24 h. The first two measurements should give insights into any effects of leaky expression on product formation

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from the multicopy plasmid, while the 24-h data point will provide a measure of the levels of polyP present in the stationary phase. (The importance of the stationary phase levels stems from an interest in polyP as a storage polymer.)

Prior to the shift to P_i-free medium, the polyP levels in DH10B(pSVD8) were higher than in the pF12-ppk and pKLJ12 (control) cultures, but the content was still very low (Fig. 2). The small amount of polyP that was present (1.1 μmol/g DCW) was reduced to the same level as the control culture during the 2-h phosphate starvation phase. The stationary phase level of polyP obtained with the high-copy plasmid was approximately equal to the content in the culture augmented with pF12-ppk, at 223 μmol/g DCW. The polyP content for DH10B(pSVD8) at 3 h was significantly higher than the stationary phase level. This fourfold increase, to 909 μmol/g DCW, represents a two orders of magnitude increase over the same time point utilizing the low-copy plasmid; however, this enhancement was not maintained.

The native *ppk* gene in *E. coli* is part of an operon that also includes the polyphosphatase gene *ppx*. It is possible that the presence of PPX activity in the cell prevents the multicopy culture from maintaining high levels of polyP. To investigate this scenario, pSVD8 was used to transform the mutant strain DPA1, which has a kanamycin resistance gene insertion in the open reading frame of *ppk*. This insertion prevents the expression of both *ppk* and *ppx*. DPA1

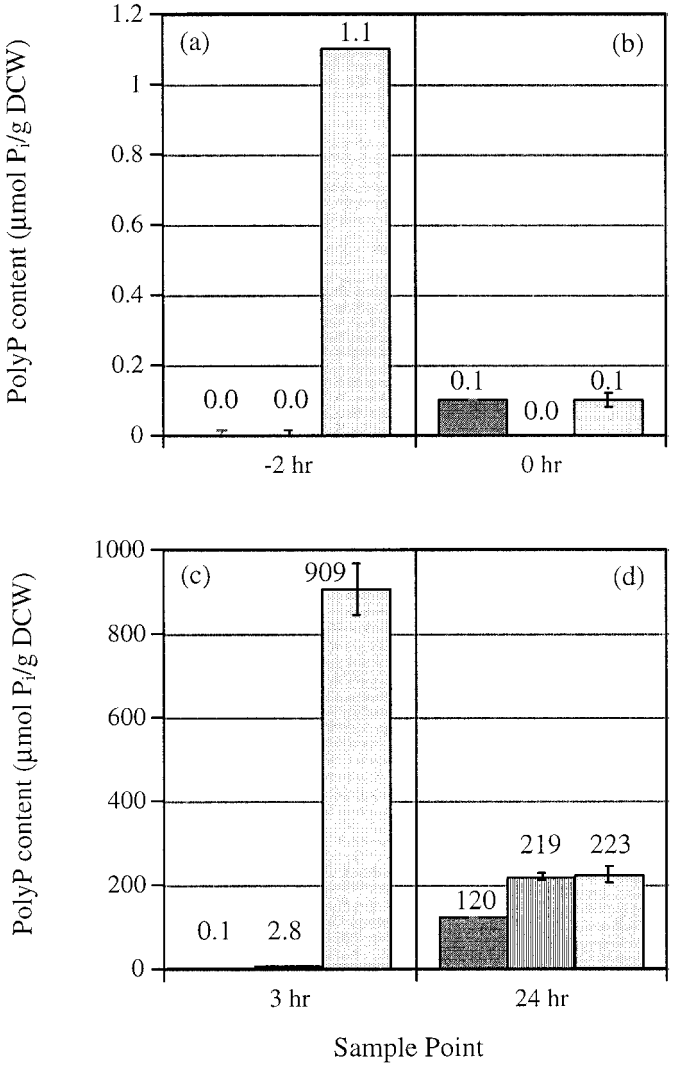

FIG. 2. Comparison of polyP content in DH10B control and augmented cultures. Cultures were grown in varying P_i concentrations as described previously. Sample points at -2 h (a), 0 h (b), 3 h (c), and 24 h (d). Solid gray bars (left), pKLJ12 (control); striped bars (center), pF12-ppk (low-copy augmented); stippled bars (right), pSVD8 (multicopy augmented). Data for pKLJ12 and pF12-ppk are the same as in Fig. 1 at the indicated times. Values shown are averages of at least two measurements from a single experiment. Error bars are standard deviations between measurements.

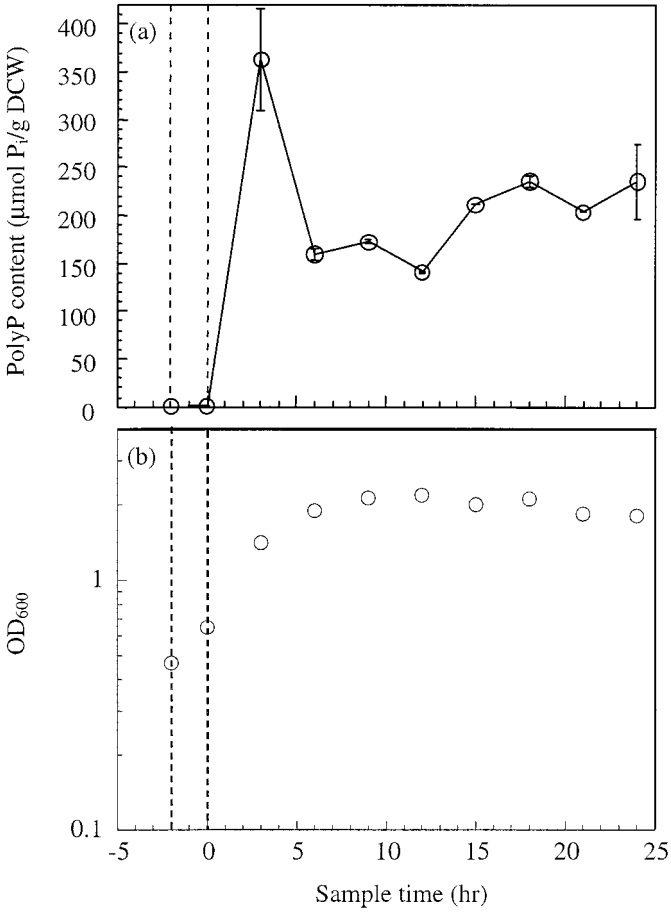

FIG. 3. (a) Intracellular polyP content as a function of sample time for the *ppx* mutant DPA1(pSVD8). Cultures were grown in varying P_i concentrations as described previously. Data shown are averages of at least two measurements from a single representative experiment. Error bars represent standard deviations between measurements. Lines are drawn to indicate trends. (b) Optical density, measured at 600 nm.

cultures containing pSVD8 were grown as described previously, except that samples were taken to assay for polyP content every 3 h after the shift to excess P_i, up to 24 h. The polyP content showed a maximum at 3 h, similar to that observed with DH10B(pSVD8), and dropped to the lower levels seen previously by 6 h (Fig. 3a). Over the same 3-h interval, the optical density of the culture only increased from 1.4 to 1.9 (Fig. 3b). The polyP content rose slightly over the next 9 h until reaching a stationary phase level of approximately 235 μmol/g DCW (measured at 24 h). This profile indicates that the failure of the DH10B cells to maintain polyP at higher levels is not due to the presence of the *ppx* gene, but rather results from some other limitation or point of regulation in the system.

The overall product concentration for polyP accumulation in the culture can be estimated based on the polyP content per cell and the cell density of the culture. The average cell density at 24 h (stationary phase), expressed as OD measured at 600 nm, for each culture is given in Table 2. These values are the averages of two separate experiments. The stationary-phase cell density of the culture augmented with a multicopy plasmid was consistently lower than that of either the control or the low-copy augmented culture, by averages of 30 and 24%, respectively. The cell density of the low-copy augmented culture was slightly lower than that of the control, by about 7%. The significantly lower cell density of the multicopy culture may represent a metabolic burden imposed upon the cell. The OD_{600} can be converted to a gram per liter basis by assuming an average dry weight of 0.3×10^{-12} g/cell and that an OD_{600} of 1 represents 10^{12} cells/L. The product concentration, expressed as micromoles of P_i in polyP per liter culture medium, is then calculated as

$$P = 0.3 \times OD_{600}\,(\text{g DCW/L}) \times \text{polyP content}\,(\mu\text{mol/g DCW}).$$

As expected, the lower cell density observed with the multicopy augmented culture resulted in a lower overall product concentration relative to the low-copy plasmid (Table 2). Utilizing a low-copy plasmid results in a 69% increase in product concentration relative to the control, whereas the increase when utilizing a multicopy plasmid is 31%.

Finally, PPK activity was measured in DH10B. In these cultures, the activity should be considered the net reaction rate toward production of polyP since *ppx* was still expressed from the chromosome. In general, the PPK activity mirrored the polyP content, i.e., both the polyP content and the PPK activity increased with time and were higher in the augmented cultures relative to the control. At 24 h, the activity of the low-copy culture was slightly higher than the multicopy culture, and both were more than threefold higher than the control (Table 2).

Comparison of lycopene production using low- and multicopy plasmids. The multicopy plasmid pK1-dxs and the low-copy plasmid pF3-dxs carry the *dxs* gene under the control of the IPTG-inducible P_{tac} promoter. This promoter has been shown to be stronger than the P_{BAD} promoter used in the previous set of experiments; however, it is also less tightly regulated (Guzman *et al.*, 1995). These plasmids were introduced into *E. coli* DH5α transformed with pAC-LYC04 (*crtEBI* and *ipi* genes), which enables *E. coli* to produce lycopene. The cells were cultured for 24 h at 29°C in 2 × YT medium. DH5α(pAC-LYC04) served as a control. When cell growth reached an OD_{600} of 0.8, IPTG was added to a final concentration of 0.0 to 0.9 mM. With no IPTG added, the cultures harboring pK1-dxs or pF3-dxs accumulated 2.4- or 2.9-fold, respectively, more lycopene than the control; the growth of the three cultures was similar (Fig. 4). However, when IPTG was added to the cultures, the cells harboring pK1-dxs produced less lycopene and grew to a lower OD_{600} than either the control or the culture harboring pF3-dxs. The culture harboring pF3-dxs produced high levels of lycopene, independent of the IPTG concentration; cell growth was unaffected by addition of IPTG.

TABLE 2

Overall Product Concentration and PPK Activity of DH10B Cultures at 24 h

Culture	Average OD_{600}	Product conc (μmol/L)	PPK activity (U/μg protein)
pKLJ12 (control)	2.7	97	145 ± 4
pF12-ppk (low-copy)	2.5	164	527 ± 11
pSVD8 (multicopy)	1.9	127	457 ± 20

Note. Product concentration was calculated assuming $OD_{600} \sim 1$ equals 0.3 g DCW/L culture. The average OD_{600} was calculated from two separate experiments. For PPK activity, one unit is defined as pmol Pi incorporated into polyP per minute at room temperature. Data are reported as averages of two measurements ± the standard deviation.

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doi:10.1006/mben.2000.0161

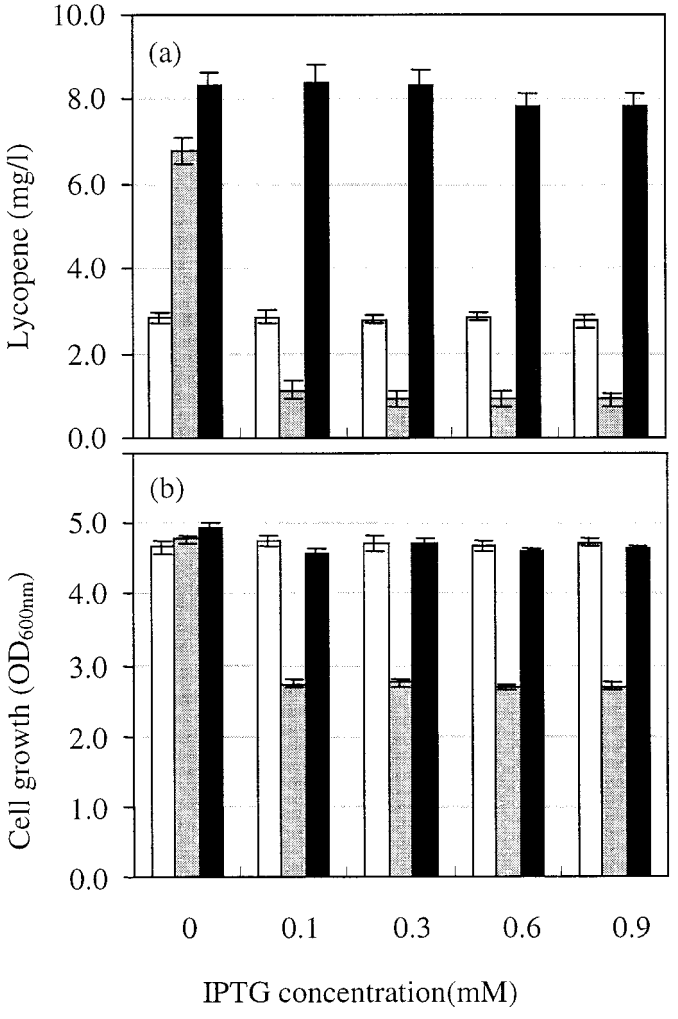

FIG. 4. Comparison of lycopene production (a) and cell growth (b) in DH5α expressing *dxs* from the *tac* promoter on high- and low-copy plasmids. Cultures were grown in the presence of 0.0 to 0.9 mM IPTG. White bars (left), pAC-LYC04 alone (control); gray bars (center), pAC-LYC04 and pK1-dxs; black bars (right), pAC-LYC04 and pF3-dxs. Error bars indicate standard deviations in triplicate cultures.

To reduce metabolic burden and to control expression of DXP synthase, *dxs* was introduced into pKLJ12, the low-copy F-based plasmid that carries the tightly regulated *araBAD* promoter P_{BAD}. Arabinose was added to a final concentration of 0 to 13.3 mM to induce expression of *dxs* at an OD_{600} of 0.1. These cultures were induced at a lower OD_{600} than the P_{tac} cultures to allow for longer expression from the weaker P_{BAD} promoter. Lycopene production increased with the arabinose concentration up to 1.33 mM (Fig. 5). At this and higher concentrations, lycopene production was 2.8-fold higher than without arabinose. Cell growth was not affected by arabinose induction. Previous experiments with pDdxs, the high-copy plasmid similar to pF12-dxs, also showed that lycopene production increased

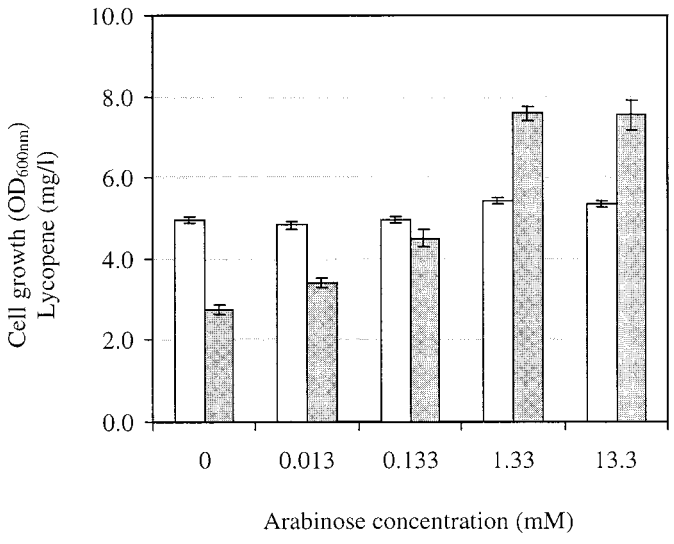

FIG. 5. Lycopene production and cell growth in DH5α transformed with pAC-LYC04 and pF12-dxs. Cultures were grown in the presence of 0 to 13.3 mM arabinose. Cell growth, white bars; lycopene production, gray bars. Error bars indicate standard deviations in triplicate cultures.

with arabinose concentration, up to 13.3 mM (Kim and Keasling, submitted). At this concentration, lycopene production from pDdxs was 30% higher than that reported here for pF12-dxs, and there was no difference in cell growth between the cultures. Given that the copy number of pF12-dxs is 1–2 per cell and the copy number of pDdxs is approximately 130 per cell, the plasmid-specific expression from the low-copy plasmid was significantly higher than from the high-copy counterpart.

3. DISCUSSION

We examined two metabolic pathways to determine the performance of low-copy plasmids versus high-copy plasmids for the enhanced production of two metabolites. In the first example, augmenting PPK activity with a low-copy plasmid caused a 20-fold increase in polyP content at early times following the shift to excess P_i, although the content per cell was low (less than 50 μmol P_i/g DCW). At these early times, there appears to have been a delay in the onset of gene expression under native control that was overcome using chemical induction. In stationary phase, the enhancement was $\sim$80% with respect to polyP content. Product concentration with respect to culture volume was calculated based on the cell density in stationary phase and was found to increase by $\sim$70% relative to the control. The smaller increase in product concentration per volume versus product content per cell is the result of a slightly lower

average cell density of the strain containing the additional *ppk*.

The profile obtained using a multicopy plasmid to augment PPK activity depicted a different behavior of product content per cell versus culture time. PolyP increased dramatically within the first 3 h following the shift to excess P_i; however, the high product levels were not maintained in stationary phase. At 24 h, the polyP levels were the same as those obtained using a low-copy plasmid, dropping to one-fourth of that observed at 3 h. The loss of polyP could be due to degradation by polyphosphatase, the gene which is expressed natively from an operon that includes *ppk*. However, a profile of polyP content versus time using a strain lacking native PPK and PPX activities also showed an increase to maximum product content at 3 h followed by a decrease to less than half that amount within the next 3 h. If the polyP was not degraded, then the decrease in product content could indicate dilution by growth while no new polyP was synthesized. For the *ppx* mutant culture, the cell density increased by approximately 35% between 3 and 6 h, from 0.42 to 0.57 g DCW/L. Assuming no additional synthesis, a polyP content of 360 μmol/g DCW at 3 h should be reduced by about 25% to 265 μmol/g DCW at 6 h. The actual reduction was more than 50%, to 160 μmol/g DCW. Thus, although dilution by growth may contribute to the observed decrease in polyP content, it does not appear to be the only relevant factor. Recall that the activity of the PPK enzyme is reversible such that polyP can be processively degraded to form ATP from ADP. The sudden increase in polyP content caused by the presence of the *ppk* gene on a multicopy plasmid could push the product level above the equilibrium concentration, causing the reaction to reverse toward polyP degradation. Degradation may also be assisted by the activity of other polyphosphatases present in the cell (Keasling *et al.*, 1993). Cultures augmented with multicopy plasmids also achieved lower cell densities in stationary phase such that the increase in product concentration with respect to culture volume was only $\sim$30%, although the enhancement of polyP accumulation per cell was similar to the low-copy system at $\sim$80%. The lower cell density may reflect a metabolic burden that results because of the consumption of ATP for the production of polyP, particularly at early times when the product level is substantially higher than in stationary phase. The subsequent reduction in polyP content does not appear to eliminate these burden effects such that the average cell density at 24 h was approximately 30% lower than the control cultures.

In the second system examined, the *dxs* gene was augmented using multi- or low-copy plasmids to increase the production of lycopene. The expression of *dxs* from pK1-dxs, a multicopy plasmid containing the *tac* promoter, became toxic to the cell upon addition of the inducer IPTG. Lycopene production and cell growth decreased drastically when IPTG was added, even in the presence of 0.1 mM IPTG. When *dxs* was expressed from pF3-dxs, the mini-F plasmid with the *tac* promoter, there were no differences in lycopene production and cell growth at all IPTG concentrations. One possible explanation for this observation is that the *tac* promoter on pF3-dxs becomes saturated with inducers (galactosides, lactose, and their analogues) present in the rich medium, 2 × YT, which causes full induction of the gene even without IPTG. (The repression–activation of the promoter in the parental plasmid pKLJ01 was verified using the nontoxic *lac*Z gene, indicating that the recombinant *lac*I^q used to create the vector is indeed functional; K. L. Jones and J. D. Keasling, unpublished.) With no IPTG added, the lycopene production from pF3-dxs was approximately 20% higher than from pK1-dxs, suggesting that even in the absence of inducer, there is some metabolic burden that impedes maximum production from the high-copy plasmid. This effect is amplified upon the addition of the inducer, resulting in a significant loss of cell growth. In contrast, expression of *dxs* on the low-copy pF12-dxs could be controlled by the arabinose concentration while still yielding high levels of lycopene relative to the control cultures. The lycopene production obtained using this plasmid was comparable to that of the similar multicopy plasmid at high arabinose concentrations, though the latter exists at two orders-of-magnitude higher copy number within the cell.

The two examples given here of metabolic engineering for the purposes of augmenting natural activities in the cell highlight the challenges that one may face when the pathway of interest uses a metabolite that plays an important role in normal cellular metabolism. In the first example, ATP is converted to ADP in the process of extending a growing polyP chain. ATP is the primary "energy currency" of the cell and is involved in countless biochemical reactions, including many for synthesis of compounds, transport of substances across the cell membrane, and cell motility. It then stands to reason that recombinant metabolic pathways that utilize ATP as a substrate may somehow become limited by the availability of that substrate and that decreasing the amount of free ATP that can be used for other biochemical reactions may cause a metabolic burden. In the second example, the substrates for the production of DXP by DXS are glyceraldehyde 3-phosphate (G3P) and pyruvate. G3P is an intermediate in glycolysis and the starting compound for the energy-generating portion of that pathway. Diverting substantial amounts of G3P toward alternative biochemical reactions could alter the energy state of the cell. Similarly, pyruvate, the final product of glycolysis, is converted to acetyl-CoA, which can be shunted to the energy-generating TCA cycle.

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doi:10.1006/mben.2000.0161

Both of these examples indicate that gratuitous overproduction of recombinant enzymes could alter the energy state of the cell such that overall productivity may be compromised; however, low-copy plasmids may play a role in overcoming this obstacle. Multicopy plasmids enhanced product formation by roughly two- to threefold. These enhancements were of the same order of magnitude as those achieved using low-copy plasmids. In contrast, experiments with β-galactosidase suggest that at maximum induction, the amount of protein that can be produced should be more than an order-of-magnitude higher with multicopy pMB1-based plasmids relative to the low-copy, mini-F plasmids (Carrier *et al*., 1998). However, the extra resources diverted toward recombinant protein synthesis from a multicopy plasmid can severely burden the host cell, as indicated by the slow growth of the multicopy plasmid-bearing cell. The difference between those results and the results reported here is most likely due to the nature of the protein produced. Besides the burden incurred on the cell in polymerizing amino acids to form PPK and DXS, there is an additional burden from the metabolites consumed by the overproduced enzyme.

Low-copy plasmids may provide a means to optimize productivity by reducing the burden effects associated with high plasmid copy number while allowing the use of strong promoters to maximize gene expression. Low-copy mini-F plasmids have also been shown to be extremely stable and to be capable of maintaining very large DNA fragments (Jones and Keasling, 1998). These characteristics suggest that these vectors may prove especially beneficial for long-term expression of entire multistep metabolic pathways. The insights gained here may also be useful in metabolic engineering applications where the heterologous pathways produce novel products, but do so while using substrates involved in the normal metabolism of the host organism.

ACKNOWLEDGMENTS

This research was supported in part by the ERC Program of the National Science Foundation under Award No. EEC-9731725 and by National Science Foundation Grants BES-9409603 and BES-9502495. K.L.J. was funded by the Department of Defense–National Defense Science and Engineering Graduate Fellowship, The College Fund/UNCF–Merck Company Foundation Graduate Fellowship, and the Ford Foundation Dissertation Fellowship.

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