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Gene Expression Enhancement due to Plasmid Maintenance

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Analysis of the chromosomic β-galactosidase activity in strains of *Escherichia coli* with and without plasmids indicated that plasmid maintenance enhances gene expression. Cyclic AMP (cAMP) determinations confirmed that the gene enhancement observed in strains carrying plasmids was due to a small increase in the intracellular concentration of cAMP. Also, cells carrying plasmids displayed higher specific glucose uptake rates than did cells without plasmids. The increases in the expression of β-galactosidase and the glucose uptake rate suggest a cAMP-mediated release of the glucose effect due to plasmid maintenance. Our results suggested that this effect is independent of the host and type and number of plasmids.

Since plasmids became obligatory genetic tools for cloning and expressing recombinant DNA, a great deal of literature showing effects of plasmids on hosts has been accumulated (1, 4, 9, 11, 16, 19, 20). It has been suggested that cells carrying plasmids suffer some kind of "metabolic burden" (2, 12–14) and that for that reason the growth rates of plasmid-bearing cells are affected negatively, plasmid-free cells therefore overgrowing plasmid-bearing cells. Although real reasons for that behavior are still unclear, certain hypothetical mechanisms based on realistic assumptions have been accepted (i.e., consumption of limiting intracellular metabolite and extra ATP consumption for plasmid maintenance, etc.).

In this communication, we present some new and totally unexpected evidence that suggests that the metabolic burden induced by plasmids can be explained by an increase in the intracellular concentration of cyclic AMP (cAMP) which renders the alteration of the regulation and control of the glucose effect. Also, we show that the phenomenon of plasmid-host interaction has to be considered on a case-by-case basis because the genetic background of the host exerts a strong influence.

Selection of microorganisms and plasmids. Strains with different genetic backgrounds and plasmids of different incompatibility groups were selected to test the influence of hosts and plasmids on β-galactosidase gene expression (Table 1). We show results obtained with hosts carrying only one plasmid (pBR322, pACYC184, or pRK248) and the same strains bearing two and three plasmids. Hosts with two plasmids carried pEcoR4 and pRK248, and hosts with three plasmids carried pEcoR4, pRK248, and pMTC48 (Table 1). The *Escherichia coli* strain CA8224.1, which bears the mutation *lacZL37UV5* (*lac* promoter completely independent of cAMP), was used to test plasmid effect on the induction of the *lacZ* gene through a cAMP-independent promoter. The control strain CA8224.1 was transformed with plasmids pACYC184 and pBR322 and double-transformed with pACYC184 plus pBR322. Transformation and DNA manipulation were carried out according to the methods of Sambrook et al. (26).

Medium and growth conditions. LB (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) was used as the regular medium and was supplemented with glucose whereindicated (Fig. 1 and Tables 3 and 4). Because we wanted to test only the effect of plasmid maintenance on the host, all the experiments were carried out at 30°C (agitation rate, 150 rpm) to avoid the expression of the gene cloned into pMTC48 (18), which is controlled by a temperature-sensitive repressor (e.g., cIts857) from the plasmid pRK248 (3). Determination of the activity of the fusion protein spa::ecor1 cloned in the plasmid pMTC48 (18) confirmed that the level of expression of the gene was negligible at 30°C. Precultures were prepared with LB and grown to mid-log phase (optical density at 600 nm [OD_{600}] = 0.5). A 30-ml volume of sterile LB was inoculated with 0.1 ml of the corresponding preculture. Samples were withdrawn from the culture every hour for OD, dry weight, glucose, and acid determinations. Antibiotics were always added in the media when strains carrying plasmids were used (ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; tetracycline, 10 μg/ml) in order to maintain the selective pressure to avoid plasmid loss. Controls for strains and plasmids were carried out systematically, and experiments were repeated many times to rule out possible artifacts.

Analytical procedures. Growth was monitored by measuring OD_{600}. Glucose and fermentation by-products (i.e., ethanol and succinic, acetic, and lactic acids) were determined by high-performance liquid chromatography (HPLC) (IONpak Shodex S-801 column at 40°C; eluent, 0.005 M H_2SO_4, 0.5 ml/min) with a differential refractometer detector. Protein, β-galactosidase, and cAMP were determined from freeze-dried extracts. Cells were grown in LB and harvested at mid-log phase. Suspensions from each strain were washed three times with 0.1 M cold phosphate buffer (pH 7.0), centrifuged, and resuspended in the same buffer up to an OD_{600} of 1.0. Aliquots of cell suspensions were withdrawn for dry weight determination, sonicated for 10 s three times (on ice), centrifuged (14,000 × *g*), and freeze-dried for β-galactosidase and cAMP analysis. Dry weight was determined by drying washed cells (24 h at 105°C). β-Galactosidase activity was determined according to the method of Miller (21), total protein was determined according to the method of Bradford (8), and cAMP was determined according to the method of Kaever and Resch (17). Activity of the fusion protein spa::ecor1 was determined according to the method of Maschke et al. (18).

Analysis of the basal level of chromosomic β-galactosidase. HB101, DH1, and CA8224.1 have an intact *lacZ* gene in the chromosome (Table 1); therefore, the activity of β-galactosidase should not be affected by any plasmidic effect (e.g., copy number, plasmidic limiting factors, and cooperative plasmid

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TABLE 1. Strains and plasmids used in this study[a]

E. coli strain or plasmid	Genotype or markers[b] and replicon	Reference
Strains		
DH1	*recA1 endA1 supE44 hsdR17 gyrA96 thi-1 relA1*	15
JM109	*recA1 endA1 supE44 hsdR17 gyrA96 thiA relA1* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	28
HB101	*recA23 hsdS20 supE44* (r_B^- m_B^-) *ara-14 lacY1 galK2 xyl-5 rpsL20 mtl-1 proA2 leu*	7
CA8224.1	*lacZL37UV5 relA1 spoT1 thi* λ$^-$	27
Plasmids		
pACYC184	Tc Cm; P15A	10
pBR322	Tc Ap; ColE1	5
pEcoR4	*ecor1-methylase*, Cm; ColE1	6
pMTC48	*spa*::*ecor1*, Ap; ColE1	18
pRK248	cIts857, Tc; RK2	3

[a] Plasmids and strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany, except strain CA8224.1, which was obtained from the *E. coli* Genetic Stock Center (Yale University), and plasmids pEcoR4, pMTC48, and pRK248.

[b] Tc, tetracycline resistance; Cm, chloramphenicol resistance; Ap, ampicillin resistance.

promoters, etc.). JM109 has a deletion in the chromosomic *lacZ* gene; accordingly, it was used as a negative control. In Table 2 we show that plasmid maintenance induced a higher level of β-galactosidase expression in all strains tested, and it was even higher in strains carrying two and three plasmids. Table 2 also shows that when IPTG (isopropyl-β-D-thiogalactopyranoside) was added (0.1 mM), the level of β-galactosidase induction was much higher than in cultures without it; nevertheless, the plasmid effect was still clearly observable. The β-galactosidase activities determined for the control strain CA8224.1 with one or two plasmids and without a plasmid revealed that the expression of β-galactosidase was not affected by the presence of plasmids at all (Table 2).

In order to rule out any antibiotic effect, β-galactosidase activities of all strains were determined in control experiments in which different antibiotics were used. Strains carrying pBR322 were grown with ampicillin or tetracycline only, and strains carrying pACYC184 were grown with chloramphenicol or tetracycline only. Control experiments with and without streptomycin (100 μg/ml) were also carried out with strain HB101, which bears the mutation *rpsL20* conferring chromosomic resistance to streptomycin (Table 1), and its transformants (results not shown). Our results confirmed that antibiotics have no significant influence on the induction of the β-galactosidase gene; hence, we ruled out any antibiotic-mediated gene expression enhancement and confirmed that this phenomenon was due to plasmid maintenance. Furthermore, strains grown in antibiotic-free media up to mid-log phase showed results almost identical to those presented in Table 2 (results not shown).

TABLE 2. β-Galactosidase activities of different strains of *E. coli* carrying different plasmids[a]

Plasmid(s)[b]	β-Galactosidase activity (U/mg of protein) of strain[c]:					
	HB101		DH1		CA8224.1	
	−IPTG	+IPTG	−IPTG	+IPTG	−IPTG	+IPTG
None	0.23	840	0.41	950	0.53	805
pACYC184	0.89	1,370	1.21	1,550	0.48	810
pBR322	0.98	1,290	1.15	1,480	0.55	780
pRK248	0.67	1,220	0.84	1,380	ND	ND
Two	1.39	1,858	1.64	1,860	0.53	790
Three	1.85	1,970	1.98	2,100	ND	ND

[a] Cells were grown in LB supplemented with 10 μg of tetracycline per ml with (+) or without (−) IPTG at 30°C and harvested at mid-log phase. IPTG was used at 0.1 mM.

[b] Strains HB101 and DH1 with two plasmids carried pEcoR4 and pMTC48; strain CA8224.1 with two plasmids carried pACYC184 and pBR322; strains with three plasmids (HB101 and DH1) carried pEcoR4, pMTC48, and pRK248. Transformants of JM109 carrying no, one, two, or three plasmids were used as negative controls.

[c] One unit of β-galactosidase hydrolyzes 1 μmol of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) per min at pH 7.0 and 37°C. Data are averages from three independent determinations. The mean error was 10%. ND, not determined.

TABLE 3. Intracellular concentrations of cAMP of different strains of *E. coli* carrying different plasmids[a]

Plasmid(s)[b]	cAMP concn (pmol/mg of protein) of strain[c]:					
	HB101		DH1		JM109	
	−G	+G	−G	+G	−G	+G
None	17	10	24	18	22	17
pACYC184	41	22	51	27	50	30
pBR322	54	24	49	26	54	29
pRK248	35	18	40	23	42	ND
Two	63	28	74	31	55	35
Three	76	32	89	36	58	40

[a] Cells were grown in LB supplemented with 10 μg of tetracycline per ml without glucose (−G) or with glucose (10 g/liter) (+G) at 30°C and harvested at mid-log phase.

[b] Strains with two plasmids carried pEcoR4 and pMTC48, and strains with three plasmids (HB101 and DH1) carried pEcoR4, pMTC48, and pRK248.

[c] Values are averages from three independent determinations. The mean error was ±5. ND, not determined.

Analysis of intracellular concentration of cAMP. In order to test a plausible hypothesis to explain this behavior, we measured the intracellular concentration of cAMP. Table 3 shows that all strains bearing plasmids also showed higher levels of cAMP than did hosts without plasmids. It is interesting that although we could not see in strain JM109 the plasmid-mediated gene expression enhancement effect observed with strains HB101 and DH1 (Table 2), measurements of cAMP suggested that plasmids affected JM109 as well (Table 3). In Table 3 we also show that the cAMP contents of hosts (e.g., JM109, HB101, and DH1) carrying two or three plasmids were even higher than those of strains bearing one or no plasmid were. Control experiments carried out with cells grown in LB supplemented with glucose (10 g/liter) showed that the intracellular concentrations of cAMP were lower than they were in strains cultured without glucose, as expected (because of catabolic repression); nevertheless, the pattern of plasmid effect was similar (Table 3). The plasmid effects were further confirmed when we analyzed the basal levels of β-galactosidase activity of all strains from the same experiment. β-Galactosidase activity was repressed to about 70% in cells grown with glucose, but the pattern of plasmid effect was similar to that presented in Table 2 (i.e., without IPTG or supplemented with IPTG). By contrast, β-galactosidase activity of strain CA8224.1 displayed no variation when cells were grown in glucose-supplemented medium (results not shown).

Although the changes in the intracellular concentration of cAMP observed are relatively small, they support our findings. We speculate that real values for the intracellular concentration of cAMP may be even higher because (i) we did not use any phosphodiesterase inhibitor to prevent the hydrolysis of cAMP during sample preparation and (ii) cAMP efflux in *E.*

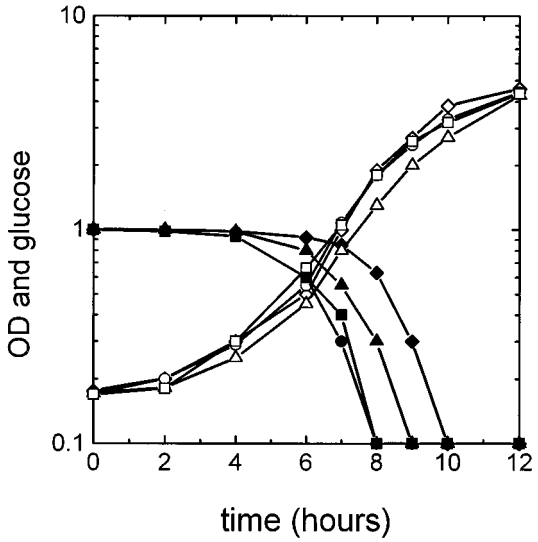

FIG. 1. Effect of plasmid maintenance on growth (OD_{600}, unshaded symbols) and glucose consumption (grams per liter, solid symbols) of *E. coli* HB101 carrying only one but different plasmids, growing in a glucose-supplemented (1 g/liter) complex medium (LB). Symbols: ◆, HB101 carrying no plasmid; ■, ●, and ▲, HB101 carrying plasmids pBR322, pACYC184, and pRK248, respectively.

coli, as a mechanism of control of the physiological activity, is reported to proceed quickly (25).

Glucose uptake enhancement effect. In Fig. 1 we show the patterns of growth and glucose consumption of HB101 carrying only one plasmid (pBR322, pACYC184, or pRK248). Figure 1 shows that although HB101 grew more slowly when bearing plasmids (as expected), glucose was consumed faster than it was in HB101 without plasmids. Rates of by-product formation during glucose catabolism confirmed these results. Higher levels of glucose consumption correlated with higher rates of formation of acetic, lactic, and succinic acids, and of ethanol as well (results not shown). Measurements of dry weight confirmed that the OD/dry weight ratio did not change during growth. Experiments shown in Fig. 1 were repeated three times, and we always observed identical behavior. Since growth patterns did not correlate with patterns of glucose consumption and it was difficult to evaluate the specific glucose uptake rate (s_g), under growing conditions, the latter was evaluated in separate experiments under nongrowing conditions. For s_g determination, cells were grown in 50 ml of LB supplemented with glucose (10 g/liter) and antibiotic up to mid-log phase (OD_{600} = 0.5), harvested (14,000 × *g* at 4°C), and washed three times with 0.1 M cold phosphate buffer (pH 7.0). Pellets were resuspended in 10 ml of the same buffer, and glucose was added to a final concentration of 2 g/liter. Suspensions were allowed to homogenize and stabilize for 10 min. Samples were withdrawn every 15 min, rapidly centrifuged (4°C), and filtered through Millipore membrane (pore size, 0.22 μm). Glucose and acids in the supernatant were analyzed by HPLC. In Table 4 we present maximum specific growth rates (μ^{max}) and glucose uptake rates (s_g) of all the strains and plasmids tested. Values for μ^{max} indicated that plasmid maintenance decreased host growth rates (as was expected), but these values did not correlate with s_g values. Whereas HB101 and DH1 showed clear increases in s_g, JM109 displayed a much smaller increase. We observed that in strains carrying more than one plasmid, μ^{max} decreased dramatically as the number of plasmids increased but s_g did not decrease accordingly, being 0.45 g/g/h for HB101 and DH1 (average) and 0.4 g/g/h for JM109 when the strains each carried three plasmids. For strain JM109, results are more difficult to analyze because this strain carries a big plasmid (F′ [Table 1]). Most probably this plasmid stresses the host metabolism already, so that the presence of any other plasmid has dramatic effects on the host metabolism. However, it is interesting that although the s_g of strain JM109 carrying plasmids was not significantly higher than that for JM109 without a plasmid, their μ^{max} values were almost unaffected (Table 4). The latter suggests that the presence of plasmids also affects the overall efficiency of carbon utilization, which agrees again with the hypothesis of the release of the glucose effect (e.g., by increase of cAMP levels [Table 3]). Furthermore, the unusual behavior of JM109 when growing in minimal medium has already been reported (24). Values for μ^{max} shown in Table 4 are somewhat lower than expected, but confirmatory experiments showed that this phenomenon was due to the suboptimal temperature used in experiments.

The correlation among levels of cAMP, β-galactosidase activity, and s_g strongly suggests that a plasmid-cAMP-mediated derepression effect was taking place. We propose the hypothesis that plasmids can alter cellular metabolism by increasing the levels of cAMP and that this plasmid-mediated effect is independent of the host and the type of plasmid. As a result of this, every biological and genetic mechanism of regulation in which cAMP participates would be altered. This hypothesis was further confirmed, because no plasmid effect was observed with strain CA8224.1, which carries the mutation *lacZL37UV5*. This mutation eliminates the influence of cAMP on the regulation of the *lac* operon (27). As consequence of this, the plasmid-cAMP-mediated derepression effect was not observed (Table 2).

The activation of adenylate cyclase by phosphoenolpyruvate (22) and the transcription enhancement of the *pts* operon by cAMP (23) have already been reported. This would explain why plasmid maintenance could partially release the repression exerted by glucose on the *lac* operon and increase the s_g (25). For that reason, when cells carrying plasmids are forced to metabolize more glucose under conditions of partial derepression, higher acid production levels should be expected. Higher acid production levels could easily explain the observed physiological advantage of plasmid-free cells over plasmid-bearing cells and the well-known effect of plasmids on cell growth, as we show in Fig. 1. If the effect of catabolite repression is regarded as a beneficial mechanism that prevents metabolic burden or physiological stress (14), then plasmids would affect hosts by relaxing the mechanisms involved in catabolite

TABLE 4. μ^{max} and s_g values for different strains of *E. coli* bearing different plasmids[a]

E. coli strain	Result with plasmid:							
	None		pACYC184		pBR322		pRK248	
	μ^{max}[b]	s_g[c]	μ^{max}	s_g	μ^{max}	s_g	μ^{max}	s_g
HB101	0.43	0.36	0.41	0.61	0.41	0.59	0.40	0.50
DH1	0.42	0.32	0.41	0.43	0.41	0.43	0.41	0.42
JM109	0.46	0.27	0.46	0.30	0.46	0.32	0.44	0.34

[a] For μ^{max} determination, cells were grown in LB supplemented with 2 g of glucose and tetracycline per ml (10 μg/ml); for s_g determination, cells were grown in the same medium, harvested, and suspended in phosphate buffer at an OD_{600} of 1.0 (see Materials and Methods).

[b] Values per hour are shown. The mean error was ±0.02. Data are averages from three independent determinations.

[c] Grams of glucose per gram of dry cells per hour. The mean error was ±0.03. Data are averages from three independent determinations.

repression. We would like to stress that although the real mechanism underlying this phenomenon remains elusive, the nature of the interaction between plasmids and hosts is totally different from what was hypothesized earlier. The hypothesis of a metabolic burden due to an intracellular metabolite limitation would not agree with the results presented in this communication. Furthermore, our results agree with previous reports for HB101 carrying pUC19 and other plasmids (13, 14). Experiments to test this hypothesis under conditions of gene expression are now being conducted in our laboratory.

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