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Plasmid Effects on *Escherichia coli* Metabolism

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ABSTRACT: The idea that plasmids replicate within hosts at the expense of cell metabolic energy and preformed cellular blocks depicts plasmids as a kind of molecular parasites that, even when they may eventually provide plasmid-carrying strains with growth advantages over plasmid-free strains, doom hosts to bear an unavoidable metabolic burden. Due to the consistency with experimental data, this idea was rapidly adopted and used as a basis of different hypotheses to explain plasmid-host interactions. In this article we critically discuss current ideas about plasmid effects on host metabolism, and present evidence suggesting that the complex interaction between plasmids and hosts is related to the alteration of the cellular regulatory status.

KEY WORDS: *Escherichia coli*, plasmid, metabolism, interaction, metabolic burden.

I. INTRODUCTION

By definition, plasmids are extrachromosomal fragments of DNA that can autoreplicate with different degrees of autonomy from chromosomal DNA and can be found free in the cytoplasm or associated with the cellular membrane and macromolecules (Clowes, 1972; Hardy, 1986; Sasakawa et al., 1980; Firshein et al., 1982; Tomizawa, 1984; Tomizawa, 1985; Perri et al., 1991; Michaels et al., 1994; Mei et al., 1995; Firshein and Kim, 1997; Radnedge and Richards, 1999). Any attempt to give a more precise definition of plasmids will be under the risk of excluding other plasmids that do not fit into this definition, due to the great variety of plasmids reported for microorganisms, most of which were not properly described yet. Plasmid characterization involves not only the determination of some structural features, for example, size, molecular weight, type of plasmid, number of genes carried, restriction and genetic map, sequence, etc., but also the study of other molecular motifs such as origin of replication, mechanism of replication and partition, characterization of plasmid genes involved in replication, as well as other host proteins necessary for plasmid replication and partition (Austin, 1988; Kues and Stahl, 1989; Nordström, 1989; del Solar et al., 1998). From a technological point of view, all this information is important, because the more knowledge we have about a particular plasmid, the easier will be to use and transform it as a useful genetic tool for biotechnological applications (Bolivar and Backman, 1979; Radnedge and Richards, 1999). However, the information we have about most plasmids is regrettably scarce and hence insufficient to predict their behavior or influence on host metabolism. Even for well-characterized plasmids, systematic studies of plasmid-host interactions have not yet been carried out, presumably due to the complexity of the interactions involved. The introduction of plasmids into *Escherichia coli* induces a large

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number of changes, ranging from perturbation in the mechanism of DNA replication, transcription, and translation (Katz et al., 1973; Hasunuma and Sekiguchi, 1977; Weinberger and Helmstetter, 1979; Zund and Lebek, 1980; Lee and Bailey, 1984; Peretti and Bailey, 1987; Wood and Peretti, 1990, 1991; Birnbaum and Bailey, 1991), interaction with cellular membrane (Gustafsson et al., 1983; Firshein and Kim, 1997), alteration of carbon and energy metabolisms (Klemperer et al., 1979; DaSilva and Bailey, 1986; Cheah et al., 1987; Khosravi et al., 1990; Diaz Ricci et al., 1992; George et al., 1992; Andersson et al., 1996), and the alteration of other functions provoked by the modification of the mechanisms already mentioned (Bailey, 1993). Furthermore, because plasmids rarely encode functions that are absolutely necessary for the host growth under laboratory conditions, one may be inclined to think that they will always negatively affect hosts, but that is not necessarily so. In nature, plasmids usually provide hosts with some growth advantage over strains lacking those plasmids, and this therefore constitutes the first line of evidence showing that under certain cultural conditions plasmids can positively affect host performance.

Experimental evidence also shows that plasmid-carrying hosts tend to get rid of their plasmids when growing under condition of nonselective pressure. The question is then, what makes a host keep or lose a plasmid? Experience demonstrates that the phenomenon of plasmid loss is neither a plasmid nor host choice, but rather a statistical event that allows daughter cells to inherit a genetic repertoire deprived of plasmids, or plasmids carrying an impaired replicative capacity (Helinski et al.,1996). The rest is only a matter of time and the opportunity of plasmid-free cells to grow in a nonselective medium (Lenski and Bouma, 1987). However, although faster-growing organisms (usually plasmid-free cells) will always be selected over the plasmid-containing cells, the process by which this selection operates is not clear or easy to understand, because it lays hidden somewhere behind extremely complicated biological mechanisms linked to cellular metabolism, and depends on the nature of the metabolic alterations induced by plasmids. In fact, there is a broad range of host-plasmid interactions that affect plasmid stability and rate of plasmid loss (Basset and Kushner,1984; Ream et al., 1978; Godwin and Slater, 1979; Jones et al., 1980; Nordström et al., 1980; Helling et al., 1981; Noack et al., 1981; Hakkaart et al., 1982; Ray and Skurray, 1984; Tucker et al., 1984; Lee and Edlin, 1985; Biek and Cohen, 1986; Warnes and Stephenson, 1986; Hopkins et al., 1987; Austin,1988; Nasri et al., 1988; Brownlie et al., 1990; Williams and Thomas, 1992; McLoughlin, 1994; Helinski et al., 1996; Corchero et al., 1998).

If a major concern of biotechnology is to preserve genetic information contained in plasmids, it is therefore critical that we manipulate hosts and plasmids so that the information is not lost during cell growth. Based on new findings about plasmid maintenance effects on the metabolism of *Escherichia coli*, we discuss in this communication possible strategies that may greatly contribute to improve plasmid stability.

This review is focussed specifically on the effect of plasmids on the metabolism of *E. coli* and does not discuss plasmid effects on other organisms or the effect of the expression of heterologous genes on host cell metabolism. This posture is necessary, because the physiology of other organisms may greatly differ from *E. coli* and also because when recombinant proteins are overexpressed they can generate a tremendous variety of interactions that would mask any subtle effect induced by plasmid replication on host metabolism (DaSilva and Bailey, 1986; Bentley et al., 1990). For more comprehensive reviews on this subject, we suggest the reviews of Bailey (1993) and Glick (1995).

II. INFLUENCE OF PLASMIDS ON THE HOST GROWTH RATE

Among all possible alterations induced by plasmids in hosts, the growth rate is the most noticeable cellular function affected. Perhaps this is the topic that has accumulated the most

significant amount of evidence showing that plasmids do affect cell growth. Batch and continuous cultures of mixed populations of plasmid-free and plasmid-containing strains have been used in a great variety of experiments (many of which were not carried out specifically to demonstrate this phenomenon) to show the effect of plasmids on cell growth (Dale and Smith, 1979; Godwin and Slater, 1979; Jones et al., 1980; Helling et al., 1981; Noack et al., 1981; Seo and Bailey, 1985; De Bernardez and Dhurjati, 1987; Nasri et al., 1988; Stephanopoulos and Lapidus, 1988; Reinikainen and Virkajärvi, 1989; Mason and Bailey, 1989; Bentley and Kompala, 1990; Brownlie et al., 1990; Diaz Ricci et al., 1992; Mosrati et al., 1993, Andersson, 1996; Tierny et al., 1999). All the evidence confirms that in mixed populations the plasmid-free strain will always overgrow the plasmid-bearing strain, provided that experiments are carried out without selective pressure (i.e., antibiotics), or that the plasmid does not grant the host an essential metabolic function necessary to grow in poor or mineral media.

If we want to analyze the effect of plasmids on cell growth, there are three situations that can take place and should be considered separately. The first situation is when only one type of plasmid replicates within a host; that is the case of cloning vectors expressing only the genes necessary to replicate themselves and to segregate into daughter cells and do not overexpress any foreign gene. The second case is when a plasmid overexpresses homologous or heterologous cloned genes. That is the case of expression vector during induction, which should be considered separately, although it unavoidably includes the first case. Finally, the last situation takes place when hosts harbor more than one type of compatible plasmid (Novick et al., 1976); therefore, they can coexist in the same host and influence simultaneously the same genetic background.

It has been shown that plasmid size and copy number are the variables that impact most negatively on cell growth when the plasmids do not overexpress any cloned gene (Zund and Lebek, 1980; Seo and Bailey, 1985; DaSilva and Bailey, 1986; Cheah et al., 1987; Ryan et al., 1989; Smith and Bidochka, 1998), and both variables are somehow indirectly related. Table 1 shows that small plasmids usually exist in cells in a large number of copies, for example, 30 to 500 copy/genome, whereas larger plasmids are usually present in moderate (i.e., 10 copy/genome)

TABLE 1
Size and Copy Number of Commonly Used Plasmids

Plasmid	Size [kb]	Copy number[a]	Ref.
pUC19	2.7	~500	Norrander et al., 1983
pGEM	2.7	~500	Sambrook et al., 1989
pACYC184	3.9	~18	Chang and Cohen, 1978
pBR322	4.3	~18	Balbas et al., 1986
pSC101	5.6	6–7	Cohen et al., 1977 Hasunama et al., 1977
ColE1	6.6	~24	Chan et al., 1985
pRK248	9.6	8	Bolivar et al., 1979
RK6	40[b]	15–20	Jacob et al., 1979
RK2/RP4/RP1	56	4–7	Figurski et al., 1979 Thomas, 1981
NR1(R100)	95.5	1	Womble and Rownd, 1988
F′	95.5[b]	1–2	Low, 1972

[a] Per chromosome equivalent.
[b] Calculated from MW [660 Da/bp].

to a single copy. In any case, theoretical and experimental work confirmed that the higher the copy number or the size of plasmids, the more severe will be the impact on cell growth (Zund and Lebek, 1980; Seo and Bailey, 1985; DaSilva and Bailey, 1986; Warnes and Stephenson, 1986; Ryan et al., 1989; Bentley and Kompala, 1989). However, no matter how large the effect of copy number or plasmid size on the cell growth rate, the effect of overexpressing any gene from a plasmid on cell growth is strikingly higher. The latter, which corresponds to the second situation mentioned above, was clearly demonstrated by DaSilva and Bailey (1986) when they evaluated the consumption of ATP under different situations by using stoichiometric analysis (Stouthamer, 1973; Stouthamer and Bettenhaussen, 1973). Their results showed that ATP consumption for plasmid replication (i.e., maintenance), regardless of the size or copy number, is small compared with the energy consumed when overexpressing a gene cloned in the plasmid. This means that if we assume that the amount of ATP consumed is directly related to the duplication rate, the impact on the cell growth of plasmids expressing recombinant proteins will be much larger than in cells only maintaining plasmids. The latter was also confirmed by numerous reports (Lee and Bailey, 1984; DaSilva and Bailey, 1986; Bentley and Kompala, 1989; Bentley et al., 1990; George et al., 1992), although the real mechanism of such behavior has not yet been properly characterized.

In the third situation mentioned above, we should consider the case that a single organism may harbor several distinct plasmids. Unfortunately, only scarce information is available about the real contribution of each plasmid to the host growth rate. It is assumed that each species of plasmid would contribute independently to affect the cell, and the overall effect on growth rate would be greater than in the case of single-plasmid-harboring cells (Rhee et al., 1994; Diaz Ricci et al.,1995).

This information leads to the conclusion that a host can sustain the replication of a certain amount of extrachromosomal DNA without extremely severe effects on its growth rate, but the more the biosynthetic machinery is dedicated to replicate the plasmid and/or synthesize recombinant proteins, the slower a microorganism will grow. The intriguing thing is that, despite experimental evidence confirming that plasmids negatively affect host growth rates, the real mechanism underlying this process remains elusive and unclear.

Let us now take a closer look at how the experimental evidence regarding the plasmid effect on cell growth is obtained. Experiments in batch cultures can be carried out with pure or mixed populations of plasmid-harboring and plasmid-free strains, and specific growth rates of strains are evaluated by measuring the change of cell concentration in a given period of time. Cell concentration is determined either by plating, direct counting, optical density, dry weight, fluorescence, or any other more sophisticated method. When plasmid-containing and plasmid-free strains are grown separately, the same medium containing or lacking the compound that exerts the selection pressure is used, respectively. In this case, experience indicates that cultures of plasmid-containing cells will grow more slowly than plasmid-free cells, as depicted in Figure 1.

On the contrary, when experiments are carried out with mixed cultures, we should let the plasmid-free and plasmid-harboring strains grow in the same medium without selection pressure if we want to observe the evolution of both populations. In this case, the analytical method recommended for monitoring both populations must change, because now we need to evaluate the number of individuals that belong to each population, that is, a method that would allow us to discriminate plasmid-containing and plasmid-free cells is required. The latter can be accomplished by two methodologies: using a flow microfluorimeter, which is probably the most accurate analytical method available but implies a relatively cumbersome procedure (Srienc et al., 1983, 1986), or using a plasmid-encoded gene product that would provide the host a cultural advantage over the plasmid-free cell (i.e., an antibiotic-resistance marker). In practice, the latter

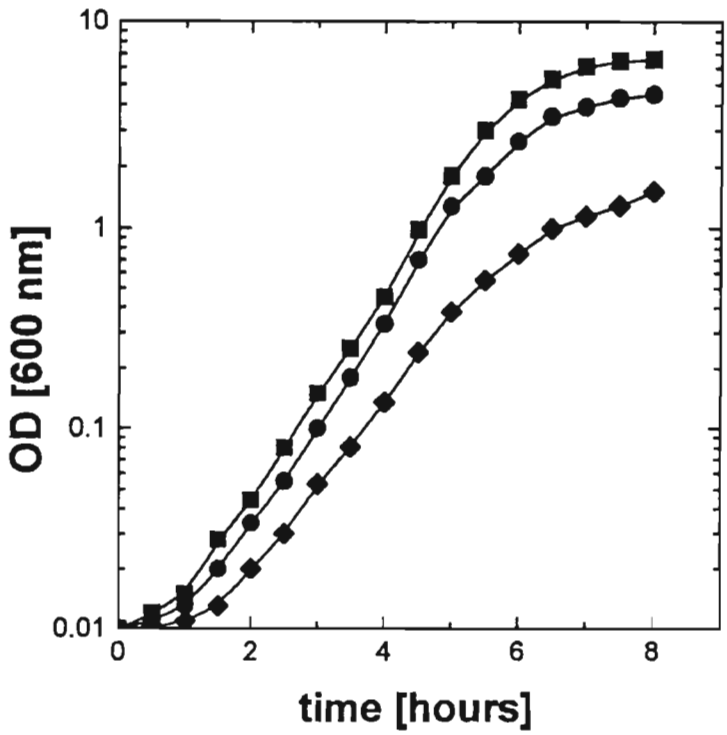

FIGURE 1. Influence of plasmid maintenance on the growth rate of the *E. coli* strain HB101 carrying no plasmid (square), the plasmid pUC19 (circle), and the plasmid pRK248 (diamond). Strains were cultivated in a shaker (200 rpm, at 30°C), separately, using LB medium. Ampicillin (100 μg/ml) and tetracycline (15 μg/ml) were added to the medium where the strain bearing pUC19 or pRK248 were cultivated, respectively. Plasmids pUC19 (2.7 kb) and pRK248 (9.6 kb) have no cloned insert and express genes involved in replication and antibiotic resistance only. pRK248 is a RK2 derivative lacking the *tra* genes (Bolivar et al., 1979).

is carried out simply by plating samples of cell suspension in media with and without the element used to generate the selective pressure (i.e., antibiotic). The results of a typical experiment are presented in Figure 2; there we can see that the population of plasmid-free cells will slowly replace plasmid-containing cells.

Although these two experimental approaches show similar results, they provide qualitatively distinct kinds of information. Whereas in pure cultures each strain grows under conditions that allow it to maintain its original genetic extrachromosomal background due to the selection pressure, in the second case, plasmid-containing cells grow in a medium without selection pressure. This apparently simple change introduced in the medium allow plasmid-free cells to compete with plasmid-containing cells, rendering the slow replacement of the latter population by the former; if we had started with a pure culture of plasmid-containing cells, the question then is where did the plasmid-free cells come from? Clearly, this phenomenon is due to a spontaneous process called plasmid loss, which contributes to an increase in the plasmid-free population at the expense of the plasmid-containing population (Lenski and Bouma, 1987; Helinski et al., 1996)).

In continuous culture, these experiments can be carried out with pure or mixed cultures. When using pure cultures, the plasmid-bearing

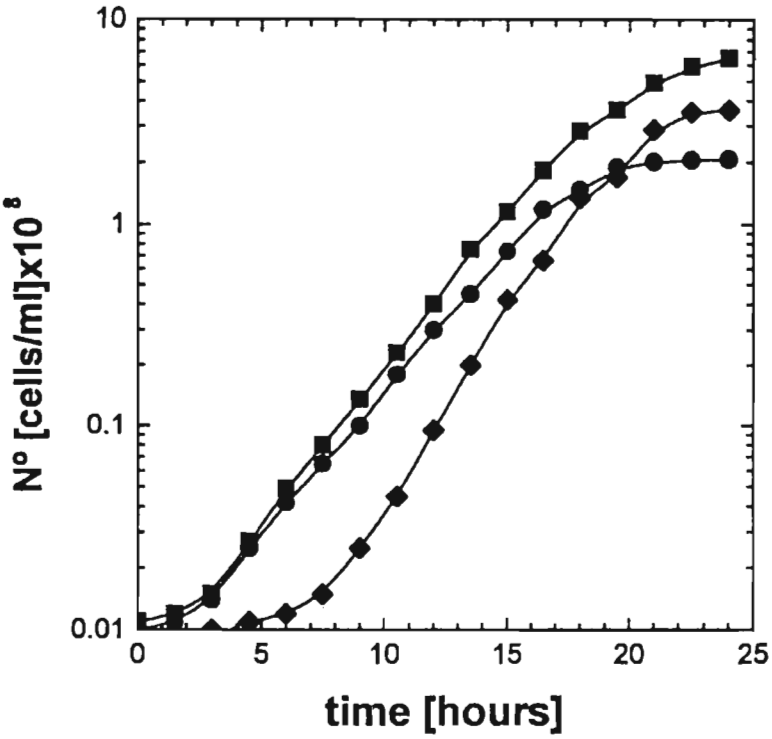

FIGURE 2. Growth kinetics of the strain HB101 of *E. coli* carrying the plasmid pRK248. Cells were grown in a shaker (200 rpm, at 30°C) with antibiotic-free LB medium. The inoculum used (1/100 final volume) was an overnight culture of HB101 carrying the plasmid pRK248 grown in LB medium without antibiotic. The evolution of plasmid-free segregant and plasmid-bearing population were determined by plate counting. Suspension samples were conveniently diluted and spread in LB plates supplemented with tetracycline (15 μg/ml) and plates without tetracycline. Symbols denote: total cell suspension (square), plasmid-containing cells (circle), and plasmid-free cells (diamond).

and the plasmid-free strains are grown separately in the same media with or without selection pressure, respectively. In this case, not only the cell concentration at steady states are affected, but critical dilution rates (Dc) as well. Figure 3 shows that the higher the dilution rate (e.g., growth rate), the more severe the effect of the plasmid on growth. In continuous cultures of mixed populations, however, the phenomenon of population displacement is much easier to observe, because the culture system permits a longer period of selection at fixed dilution rates. This type of experiment allows the evaluation of the rate of population exchange or the so-called "plasmid loss rate" at different dilution rates (Godwin and Slater, 1979; Jones et al., 1980; Helling et al., 1981; Noack et al., 1981). In Figure 4 we present a typical experiment where the influence of the dilution rate can be observed (D) on the plasmid loss rate (r_{pl}). Figure 4 shows that the higher the dilution rate, the higher the plasmid loss rate.

Let us now take a brief look at the phenomenon of plasmid loss and its effect on cell growth. Two major events contribute to plasmid loss: (1) a failure of plasmid replication, and (2) an anomalous partition of plasmids during cell division (Summers and Sherrat, 1984; Williams and Thomas, 1992; Helinski et al., 1996). However, although both are important events determining plasmid stability, they cannot be detected imme-

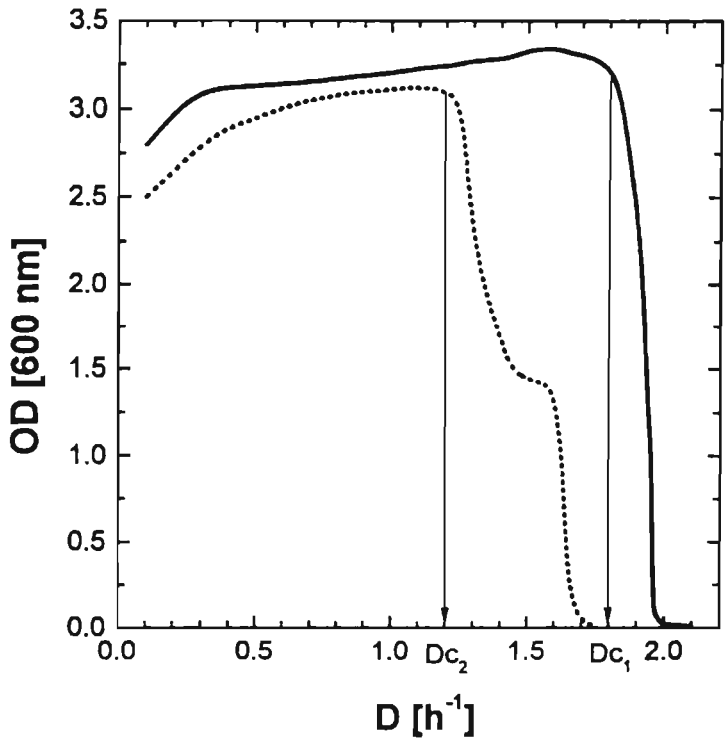

FIGURE 3. Growth pattern of the strain HB101 without plasmid (solid line) and carrying the plasmid pRK248 (dotted line) in a continuous culture. Experiments were carried out under aerobic conditions (500 rpm, 0.5 slpm of air) at pH 7.0 and 30°C. HB101 without plasmid was cultivated in antibiotic-free LB medium and HB101 harboring the plasmid pRK248 was cultivated in LB supplemented with tetracycline (15 g/l) in separate experiments. Critical dilution rates were $Dc_1 = 1.8\ h^{-1}$ for HB101 without plasmid and $Dc_2 = 1.2\ h^{-1}$ for HB101 with pRK248. Plasmid pRK248 has no cloned insert and expresses genes involved in replication and antibiotic resistance only.

diately or directly but through the process of population selection. Moreover, the latter is not a specific process induced by the event of plasmid loss but by the coexistence of two populations with different growth rate. Selection will always operate by letting the faster strain, normally the plasmid-free strain, overgrow the slower one, usually the plasmid-containing strain (Godwin and Slater, 1979; Jones et al., 1980; Lenski and Bouma, 1987; Nasri et al., 1988; Brownlie et al., 1990). If cultures were completely homogeneous and stable, they could be kept as pure cultures and spontaneous plasmid loss would not take place, hence the selection process could not be observed. However, the latter does not happen in the absence of selective pressure. In pure cultures of plasmid-harboring cells growing in the absence of selection pressure, the initially homogeneous plasmid-containing population will always spontaneously segregate plasmid-free cells and then the process of selection will start (Lenski and Bouma, 1987).

In experimental work, plasmid stability or plasmid loss rate is regularly evaluated by plating samples of cell suspension in selective and nonselective media, and by counting colonies of plasmid-free and plasmid-harboring cells (Godwin and Slater, 1979; Jones et al., 1980; Meacock and Cohen, 1980; Nordström et al., 1980; Helling et al., 1981; Noak et al., 1981; Ray and Shurray,

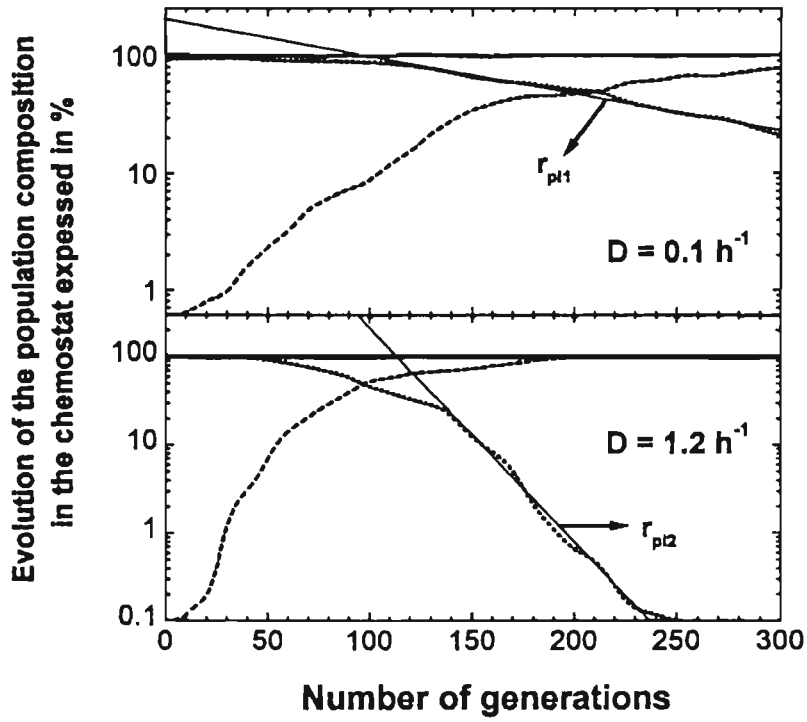

FIGURE 4. Segregation of plasmid-free (dashed line) from plasmid-containing (pRK248) HB101 strain (dotted line) grown in continuous culture at different dilution rates. Solid line denotes total cell population (plasmid-containing + plasmid-free cells). Experimental set up as described in Figure 3 except that the medium (LB) was not supplemented with antibiotic and the evolution of plasmid-free and plasmid-bearing population were determined by plate counting, as described in Figure 2. Plasmid loss rate (r_{pl}) is evaluated from the slope of the linear regression (dashed-dotted line) of the evolution profile of plasmid-containing cells. Plasmid loss rate evaluated from these data shows that the population of plasmid-containing cells decreases in rate of 0.4% (r_{pl1}) and 1% (r_{pl2}) of the original population (100%) per generation time, at D = 0.1 h^{-1} and D = 1.2 h^{-1}, respectively.

1984; Tucker et al., 1984; Lee and Edlin, 1985; Austin, 1988; Biek and Cohen, 1986; Warnes and Stephenson, 1986; Nasri et al., 1988; Bentley et al., 1990; Bentley and Kompala, 1990; Brownlie et al., 1990). However, if we carefully analyze the process that takes place in the fermentor and the information obtained with colony counts, we would realize that the only phenomenon we can observe is a population replacement from plasmid-bearing to plasmid-free cells. Hence, with this procedure what we evaluate is not strictly the "rate of plasmid loss" as it is usually reported but the "rate of population exchange". Consequently, we would conclude that this experimental approach is totally inappropriate to evaluate the real event of plasmid loss. Plasmid loss is related to unpaired segregation, uneven partition during cell division or plasmid replication, and these events are spontaneous and rare (Helinski et al., 1996; Firshein and Kim, 1997). The confusion probably comes from the method used for detecting these processes and from the fact that under nonselective pressure there is no way to avoid an event of plasmid loss. On the other hand, it has been reported that cells carrying plasmids show a "dormancy" effect that could further affect the evaluation of plasmid-containing populations by plate counting (Andersson et al., 1996).

In practice it is extremely difficult to obtain and maintain a pure population of plasmid-harboring cells if a selection pressure is not used. The latter was clearly discussed by Lenski and Bouma (1987). They observed that the real rate of plasmid loss is too low to be measured; therefore, segregation and selection should be evaluated simultaneously, although they are different phenomena. Furthermore, their experiments suggested that the rate of selection observed could not be attributed to a single event of plasmid loss but rather to multiple successive or parallel plasmid-loss events occurring during cell growth (Lenski and Bouma, 1987). However, it is clear that regardless how many plasmid-loss events take place, the mechanism of selection operates by letting the population of cells that show an enhanced metabolism, and ultimately the growth rate, to overgrow the other population; usually that happens to plasmid-free cells. Because different rates of plasmid loss have been reported for different plasmids and different culture conditions (Godwin and Slater, 1979; Jones et al., 1980; Meacock and Cohen, 1980; Noack et al., 1981; Nasri et al., 1988; Brownlie et al., 1990), they may indicate that: (1) the selection process affects some hosts more severely than others, (2) the segregation process operates differently in some hosts, (3) there are different mechanisms of plasmid segregation that induce the uneven plasmid partitioning between mothers and daughters, (4) there are different mechanisms of plasmid loss due to multiple impaired replication mechanisms, and (5) a combination of any of the preceding factors. Answers to all these questions are not easy to find because systematic studies to survey the stability of plasmids on different hosts, and the contribution of segregation and selection of the same plasmid in different hosts or different plasmids in the same host have not been done.

From a technological point of view, it would be highly desirable to eliminate plasmid loss. For years the major effort in biotechnology was devoted to try to understand the mechanism of plasmid loss in order to provide plasmids the maximum stability possible. The effort was concentrated on designing plasmids that would synchronize their replication clocks with the host DNA replicative machinery so that they would guarantee a regular partition during cell division (Meacock and Cohen, 1980; Löbner-Olesen et al., 1987), or proposed different genetic and cultural strategies that would contribute to stabilize plasmids (Basset and Kushner, 1970; Hakkaart et al., 1982; Ream et al., 1978; Godwin and Slater, 1979; Jones et al., 1980; Nordström et al., 1980; Helling et al., 1981; Noak et al., 1981; Ray and Shurray, 1984; Tucker et al., 1984; Lee and Edlin, 1985; Austin, 1988; Biek and Cohen, 1986; Warnes and Stephenson, 1986; Nasri et al., 1988; Brownlie et al., 1990). Unfortunately, all attempts proved to be only partially successful due to the complexity of the mechanisms involved and the stochastic nature of the process of plasmid loss. In any case, as long as the effect of plasmids on cell growth remains unclear, it is impossible to develop rational biotechnological strategies that would guarantee plasmid stability through a positive selection process.

If we think about a plausible mechanism by mean of which plasmids could affect host growth rates, it seems reasonable, at least from stoichiometric standpoint, that plasmids may affect hosts due to the consumption of a hypothetic limiting intracellular metabolite that would lead to a metabolic bottle-neck or would cause a biosynthetic collapse. With the aim of characterizing that hypothetic limiting intracellular metabolite, many theories have been proposed in an effort to explain this "simple" kinetic phenomenon. One of the most solid candidates was ATP, because it is involved not only with the energy available for cell synthesis, but provides structural blocks for the synthesis of DNA and RNA as well. Detailed mass and energy balances of *E. coli*-carrying plasmids suggested that the hypothesis of the existence of a fundamental intracellular metabolite that can become limiting, and for that reason affect the cellular metabolism, was credible indeed, and ATP (and GTP to a lesser extent) fulfilled these requirements (DaSilva and Bailey, 1986; Glick, 1995). Under

this prespective, a plasmid can be visualized as an intracellular molecular parasite that withdraws vital elements from hosts. Furthermore, if ATP is regarded as the most important intracellular metabolite related to cell growth and we assume that growth rate depends on the availability of ATP, then if the maintenance of a plasmid consumes certain amounts of ATP equivalents, then plasmid-free cells would grow faster than plasmid-harboring cells. The same reasoning was applied to plasmid overexpressing recombinant protein (DaSilva and Bailey, 1986). However, as we discuss in the following section, there was an important variable that was not included in these considerations. Theoretical analyses of plasmid effects on host growth rates have concentrated almost exclusively on the growth process, making assumptions that turned out to be not completely correct. In theoretical models and discussion of experimental results, it was systematically assumed that the glucose metabolism and oxygen uptake rate of plasmid-free and plasmid-bearing cells did not change. Therefore, when the amount of material and energy required for plasmid maintenance was evaluated, it was assumed that the amount and rate of synthesis of ATP remained unchanged in plasmid-harboring and plasmid-free cells. Although at that time there were no reports about the influence of plasmids on host cell glucose and oxygen metabolism, we now know that this assumption was incorrect because cells carrying plasmids do modify their metabolism. These new findings not only demand a change in the hypothesis about possible mechanisms of plasmid effects on host growth rates, but also lead to new strategies to increase plasmid stability and diminish the negative impact of plasmid maintenance on host growth rate.

III. INFLUENCE OF PLASMIDS ON THE HOST METABOLISM

As theoretical and experimental results demonstrated that the maintenance of plasmids exerts only a slight burden onto the global metabolism of the host while the overexpression of recombinant proteins contributes greatly to increments that burden (DaSilva and Bailey, 1986; Bentley et al., 1990), no alternative mechanism aiming to explain the phenomenon of overgrowth was proposed. Hypotheses about the influence of plasmid maintenance in hosts were totally based on the phenomenon of selection of plasmid-free cells against plasmid-containing cells, ergo, by the evaluation of cell growth rates exclusively. Although this experimental observation is correct, it is not sufficient to validate a general hypothesis about the effects of plasmids on hosts. Somehow, it seems that it was overlooked that cellular growth is always accompanied by carbon consumption, glucose being the main source of carbon and energy, and other metabolic processes that contribute to sustain that growth. It was relatively easy to extrapolate results obtained from stoichiometric modeling, and to infer that if the "metabolism" of ATP changes as result of plasmid replication, or gene overexpression, then most of the cellular mechanism in which ATP is directly or indirectly involved would change (Bailey, 1993; Glick, 1995). Although polyacrylamide gels showed different protein-fingerprinting patterns between plasmid-harboring and plasmid-free cells due to a perturbation of gene expression, it was attributed to a shift in the distribution of RNA polymerase and other "pleiotropic" effects induced by plasmids on the host's regulatory mechanisms (Birnbaum and Bailey, 1991; Bailey, 1993; Peretti et al., 1989: Wood and Peretti, 1990,1991).

However, a novel plasmid effect reported by Diaz Ricci et al. (1991a) raised new questions about the real target of plasmid maintenance on cellular metabolism. Interestingly, they reported that the plasmid-containing *E. coli* strain HB101 showed an enhanced glucose uptake rate, with respect to the plasmid-free strain, and that apparently "irrelevant" increase of the glucose uptake was accompanied with a faster drop of the extracellular and intracellular pH, higher accumulation of lactic, acetic, formic, and succinic acids (Diaz Ricci et al., 1991a). The evaluation

of the intracellular metabolites concentration (e.g., NTP, NDP, UDPG, and FDP) also showed higher values for HB101 carrying the plasmid pUC19 (Diaz Ricci et al., 1991a). The latter was suggested earlier by Shimosaka et al. (1982); they reported a slight increase of the ATP formation activity in the strain C600 carrying the plasmid pBR322 with respect to the same strain carrying no plasmid. Nonetheless, those authors did not comment about this phenomenon, probably due to the error involved in each measurement. Later, the effect of plasmids on glucose metabolism was confirmed by Diaz Ricci et al. (1992), who showed that although plasmid pUC19 slightly affected the host growth rate, as expected, the glucose metabolism exhibited a dramatic boost. The evaluation of intracellular fluxes also revealed a dramatic increase in the glucose uptake rate accompanied with the increase of ATP and fermentation byproducts synthesis rates (Diaz Ricci et al., 1992).

These data let us conclude that the original hypothesis that ATP was the intracellular limiting-component proposed in earlier papers (Lee and Bailey, 1984; DaSilva and Bailey, 1986; Bentley and Kompala, 1989; Bentley et al., 1990) is not correct. Curiously, with the exception of results obtained for *Bacillus subtilis* by Byrne (1990), the paper published by Diaz Ricci et al. (1992) is the only one that we are aware of that clearly shows the effect of plasmid maintenance on growth and glucose metabolism. Furthermore, results shown in these papers together with those reported by Khosravi et al. (1990) indicated, for the first time, that plasmids might affect hosts through a totally different mechanism than that initially proposed, and this may have important consequences when testing hypotheses of the effect of plasmids on cell metabolism. Later, Diaz Ricci et al. (1995) confirmed that plasmids affect hosts by enhancing the glucose uptake rate, but the intensity of the effect was somewhat plasmid and host dependent. Figure 5 shows that although growth rates are slightly affected by the type of plasmid, glucose consumption rates increased significantly in HB101 carrying any plasmid. In order to test whether this phenomenon could be observed with other hosts, similar experiments were carried out with the *E. coli* strains DH1 and JM109 under identical experimental conditions and the results are presented in Table 2. There we can see that although this phenomenon takes place with all plasmids and hosts tested, the intensity of induced plasmid effects on specific growth and glucose uptake rates depends on the particular host-plasmid pair. In Figure 6 and Table 2, we can also see that for an increasing number of compatible plasmids maintained within a host, the influence on the growth and glucose metabolism becomes more severe. From all of the evidence presented, we can conclude that plasmids dramatically alter the metabolism of glucose regardless of the host or plasmid under study. Diaz Ricci et al. (1995) suggested that the increase of glucose uptake rate may be due to the enhanced expression of proteins participating in the mechanism of glucose uptake, that is, proteins involved in the phosphotransferase system (PTS), which is the main mechanism of glucose uptake in *E. coli* (Postma et al., 1996).

The mechanism of glucose transport through the PTS system involves four different proteins, for example, EI, HPr, and IIA^{glu} and glucose permease, which interact with each other in a cascade reaction, transporting a phosphate from the phosphoenolpyruvate (PEP) to glucose and each protein is encoded by the genes *ptsI*, *pstH*, *crr*, and *ptsG*, respectively (see scheme shown in Figure 7). It has been shown that not only the *pts* operon is subjected to positive regulation mediated by the complex cAMP-CRP (Reuse et al., 1992; Postma et al., 1996; Saier et al., 1996), but also the activity of the enzyme adenylate cyclase can be activated by the phosphorylated form of the protein IIA (P~IIA) (see Figure 7) (Peterkofsky and Gazdar, 1978; Postma et al., 1996; Notley-McRobb et al.,1997). Furthermore, because the complex cAMP-CRP exerts a positive effect on the *ptsG* gene that encodes for the glucose permease, an enhanced glucose uptake rate should be expected. Consequently, the intracellular concentration of PEP would increase through the glycolytic pathway and hence the

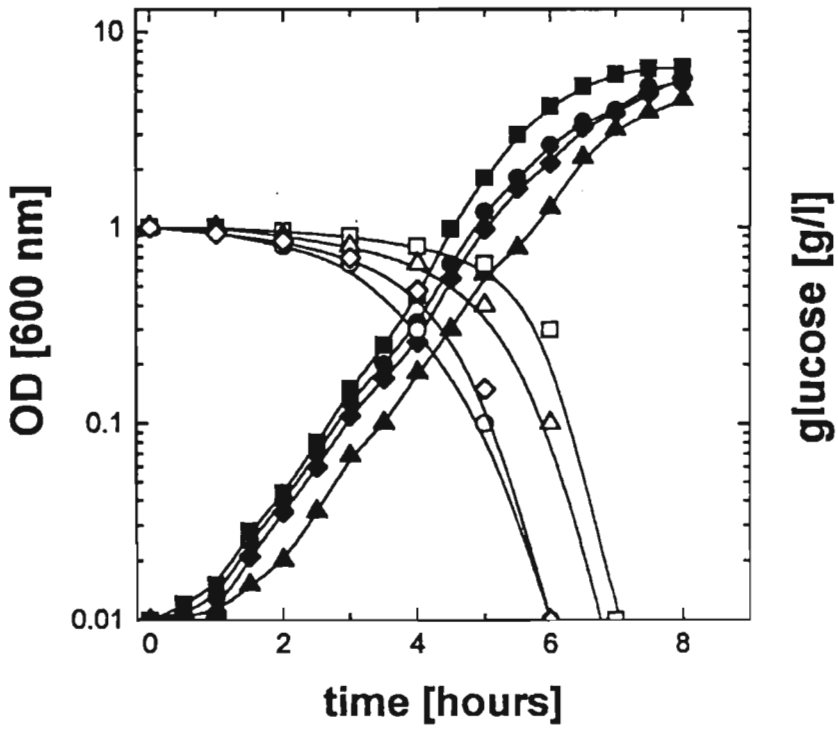

FIGURE 5. Effect of plasmid maintenance on the growth (solid) and glucose consumption (open) of strain HB101 carrying no plasmid (square), and carrying plasmids pME01 (circle), pACYC184 (diamond), and pRK248 (triangle). Experimental conditions as described in Figure 1 and in Diaz Ricci et al. (1995). Cultures were carried out separately and antibiotics were added according the plasmid used: pME01, Ap (100 μg/ml); pACYC184, Cm (30 μg/ml); pRK248, Tc (10 μg/ml). pME01 (2.4 kb) is a pUC19 derivative lacking the β-*gal* gene. Plasmids pUC19, pACYC184, and pRK248 have no cloned insert and express the genes involved in replication and antibiotic resistance only.

phosphate available to fuel the PTS system (see Figure 7). This scenario clearly depicts a self-activating system that may explain the correlation found by Diaz Ricci et al. (1995) between the intracellular concentration of cAMP and the glucose uptake rate. In Table 3 we show the intracellular values of cAMP and chromosomal β-galactosidase activity determined in different strains of *E. coli* carrying different plasmids. From data presented in Tables 2 and 3 we can see that there is a clear correlation between the intracellular cAMP content, the glucose uptake rate, and the activity of chromosomal β-galactosidase, which is also positively regulated by the complex cAMP-CRP (see Figure 7). We could thereby infer that the glucose uptake rate enhancement observed may be due to the activation or the enhanced expression of the PTS operon. However, as we show in Figure 7, the regulatory status of the whole system is hard to assess, because it presents an intricate network of positive and negative regulatory loops, many of which have not been fully characterized. It is noteworthy that plasmids somehow alter the regulatory status of this complex system that seems to be very sensitive to perturbations of glucose metabolism and catabolite repression. Hence, we propose the hypothesis that *plasmids affect host metabolism through the perturbation of the cAMP-CRP complex, which in turn causes the alteration of the regulatory status of host regulons.*

TABLE 2
Effect of Plasmid Maintenance on the Specific Growth and Glucose Uptake Rates of Different *E. coli* Strains

	μ^{max}			Glucose uptake rate		
Plasmid	**HB101**	**DH1**	**JM109**	**HB101**	**DH1**	**JM109**
None	0.43	0.42	0.46	0.36	0.32	0.27
pUC19	0.40	0.40	0.44	0.58	0.48	0.35
pACYC184	0.41	0.41	0.46	0.61	0.43	0.30
pRK248	0.40	0.41	0.44	0.50	0.42	0.34
Two	0.30	0.38	0.40	0.60	0.55	0.45
Three	0.20	0.25	0.35	0.63	0.60	0.48

Note: Cells were grown in shaker (300 rpm, at 30°C) using LB medium supplemented with glucose (2 g/l) and antibiotic according to the plasmid used (see Figure 5). Glucose uptake rate was determined in cells harvested at mid-log phase, washed and suspended in a phosphate buffer (50 m*M*, pH = 7.0) supplemented with glucose (2 g/l). Strains with two plasmids carried pUC19 and pACYC184 and strains with three plasmids carried pUC19, pACYC184, and pRK248. Specific growth rate expressed in h^{-1} and glucose uptake rate expressed in gram of glucose consumed/gram of cell dry weight per hour. Data partially extracted from Diaz Ricci et al. (1995).

Regardless of whether the adenylate cyclase (*cya*), the CRP protein (*crp*), or a PTS protein (*crr, ptsG*) are the primary target of a plasmid's influence on *E. coli* metabolism (see Figure 7), if this mechanism is confirmed by further experimentation, it would contribute to a better understanding of the complex interaction between plasmids and hosts.

The literature indicates that the influence of plasmids on host gene regulation has already been detected but not clearly remarked by authors, probably due to the magnitude of the deviations observed—hardly noticeable changes—or the error involved in each measurement. Shimosaka et al. (1982) reported that in control experiments they observed a slightly higher expression of the chromosomal gene *tpi* (triosephosphate isomerase) in the strain C600 when carrying the plasmid pBR322 with respect to the same strain carrying no plasmid. Also, Diaz Ricci et al. (1992) reported a slight increase of the level of expression of heterologous alcohol dehydrogenase (*adh*) and pyruvate decarboxylase (*pdc*) cloned into pUC18 and regulated by a *plac* promoter, with respect to the level of expression of the plasmid-encoded β-lactamase.

Furthermore, if we analyze some plasmid effects on host metabolism reported in the literature, we would also realize that the hypothesis proposed in this communication can easily explain many of these observations, despite explanations suggested by other hypotheses. For instance, if we assume that plasmids can enhance the glucose uptake rate, that would be enough to induce the synthesis and accumulation of all fermentation byproducts, including some intermediate metabolites. Hence, the phenomenon of growth inhibition displayed by plasmid-harboring cells could be easily explained by the metabolic stress induced by the accumulation of end-products (Chou et al., 1994; Aristidou et al.,1999) or any sugar phosphates, as it was reported by Kadner et al. (1992), instead of the limitation imposed by an hypothetic intracellular metabolite. The former explanation would be much more convincing from a biochemical and physiological

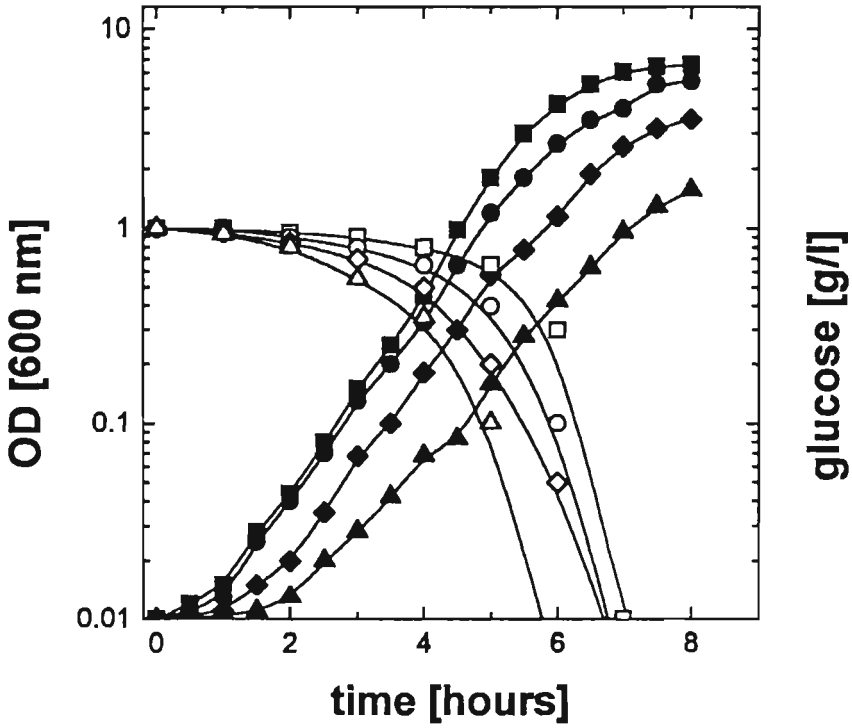

FIGURE 6. Effect of plasmid maintenance on the growth (solid) and glucose consumption (open) of the strain HB101 carrying no plasmid (square), one (pME01, circle), two (pME01 and pACYC184, diamond), and three (pME01, pACYC184, pRK248, triangle) plasmids. Experimental conditions as described in Figure 5.

point of view than the latter. Furthermore, the hypothetical limitation of ATP or GTP cannot be sustained after the results reported by Diaz Ricci et al. (1991a, 1992). They have demonstrated that neither the intracellular concentration of ATP nor the specific rate of synthesis of ATP become limiting or rate controlling. On the contrary, they showed that the steady state concentration of ATP (Diaz Ricci et al., 1991a) and the rate of ADP phosphorylation at substrate level increased during glucose consumption in the presence of plasmid (Diaz Ricci et al., 1992).

Seo and Bailey (1985) reported increasing values of Ki (e.g., glucoside uptake inhibition constant) for α-methylglucoside when HB101 carried plasmids with higher copy number. These results indicated that strains with a higher plasmid content showed lower inhibitory effect of α-methylglucoside, which is a competitive inhibitor of glucose in the glucose uptake process (Postma et al., 1996). In other words, because methylglucoside and glucose compete for the same active site, and experiments were carried out at constant and saturating glucose concentration, the only explanation possible is that the number of active sites would increase, which is the amount of glucose/α-methylglucoside receptor (e.g., permease of the phosphotransferase system) (Saier et al., 1996). From the point of view of our hypothesis, the latter could be explained by the enhanced expression of PTS proteins, particularly the *ptsG* gene (e.g., the permease) that is positively regulated by cAMP-CRP (Figure 7).

Ryan et al. (1989) reported results obtained with a set of recombinant plasmids that expressed only their own β-lactamase. Conceptually, the experimental approach was similar than the one proposed by Seo and Bailey (1985), whereas the latter used a set of plasmids of

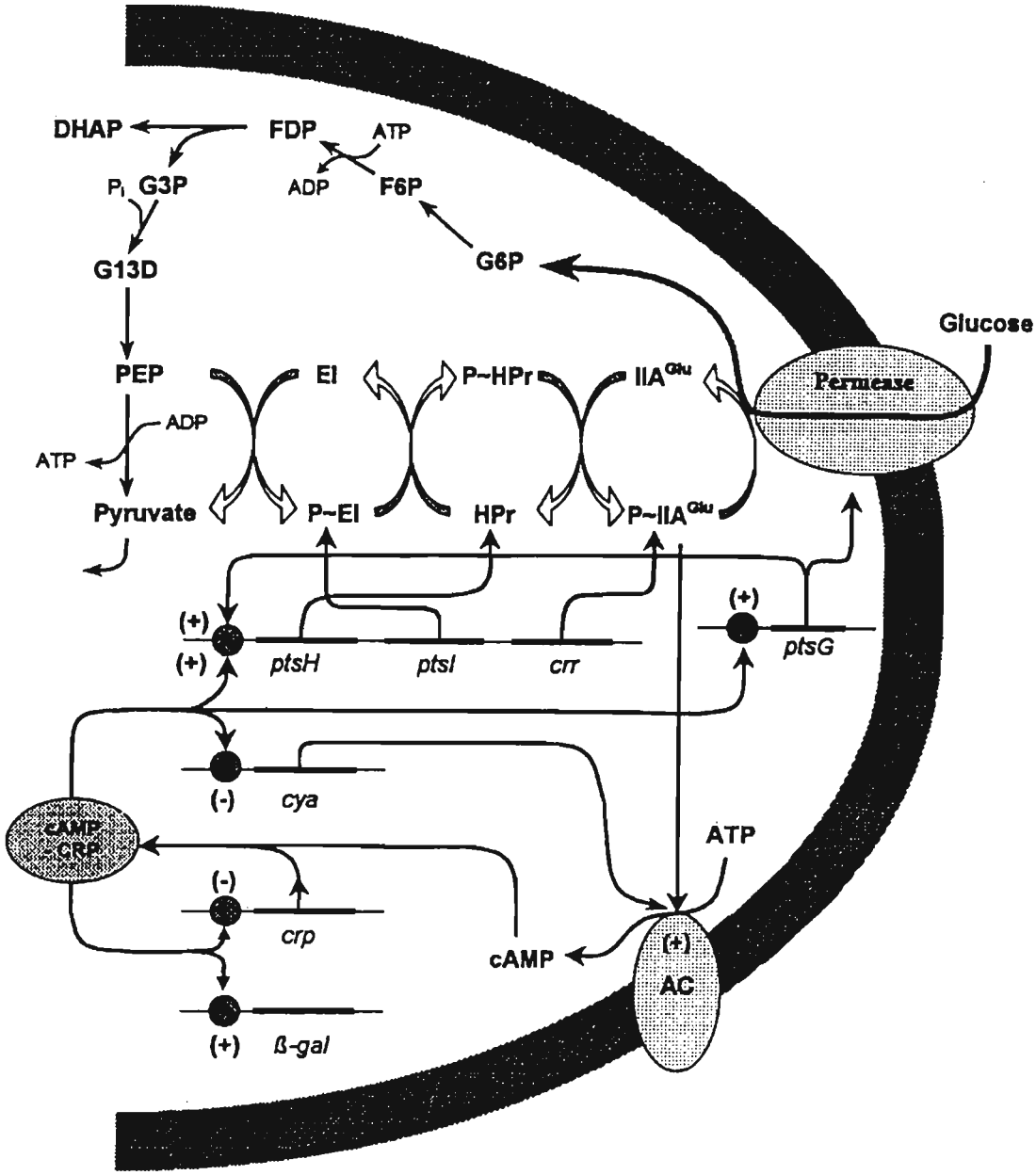

FIGURE 7. Integrated scheme of the gene expression regulation of PTS components and the adenylate cyclase (AC) activity in relation to the glucose metabolism (Saier et al.,1996; Postma et al.,1996). Abbreviations: **G6P,** glucose-6-phosphate; **F6P,** fructose-6-phosphate; **FDP,** fructose-1,6-diphosphate; **DHAP,** dihydroxyacetone-phosphate; **G3P,** glyceraldehyde-3-phosphate; **G13D,** glycerate-1,3-diphosphate; **PEP,** phosphoenolpyruvate; **EI,** PEP phosphotransfer protein; **P~EI,** phosphorylated form of protein EI; **HPr,** histidine-containing protein; **P~HPr,** phosphorylated form of protein P~HPr; **IIA^{glu},** glucose-specific phosphotransfer protein; **P~IIA^{glu},** phosphorylated form of protein IIA^{glu}; ***ptsH,*** gene-encoding protein HPr; ***ptsI,*** gene-encoding protein EI; ***crr,*** gene-encoding protein IIA^{glu}; ***ptsG,*** gene-encoding glucose permease; ***cya,*** gene-encoding adenylate cyclase; **cAMP,** cyclic AMP; **cAMP-CRP,** cAMP-binding protein; ***crp,*** gene-encoding CRP-cAMP; β-***gal,*** gene-encoding β-galactosidase (Saier et al.,1996; Postma et al.,1996).

TABLE 3
Effect of Plasmid Maintenance on the Specific Growth and Glucose Uptake Rates of Different *E. coli* Strains

	cAMP			β-galactosidase		
Plasmid	**HB101**	**DH1**	**JM109**	**HB101**	**DH1**	**JM109**
None	10	18	17	0.84	0.95	—
pUC19	30	30	32	1.40	1.60	—
pACYC184	22	27	30	1.30	1.48	—
pRK248	18	23	22	1.22	1.38	—
Two	30	32	35	1.90	1.90	—
Three	35	36	40	1.90	2.00	—

Note: cAMP and β-galactosidase were determined from extracts of cells grown in LB medium supplemented with glucose (2 g/l) and harvested at mid-log phase. cAMP and β-galactosidase are expressed in pmol/mg protein and U/mg protein, respectively. One unit of β-galactosidase hydrolyzed 1 μmol of ONPG (*O*-nitrophenyl-β-D-galactopyranoside) per min at 37°C and pH 7.0. Cells with two plasmids carried pUC19 and pACYC184 and cells with three plasmids carried pUC19, pACYC184, and pRK248. Other experimental conditions as mentioned in Table 2. Data partially extracted from Diaz Ricci et al. (1995).

[a] The strain JM109 has the chromosomal β-galactosidase gene inactivated (Yanisch-Perron et al., 1985).

approximately the same size but different copy number, Ryan et al. (1989) used a set of plasmids of different sizes. They reported that although the maximum specific growth rate declined for larger plasmid sizes (as expected), the activity of β-lactamase displayed a maximum value when HB101 harbored plasmids with sizes ranging from 2.7-8 kb (Ryan et al., 1989). They also showed that the activity of β-lactamase was higher at intermediate air flow rates, which means that the efficiency of oxygen consumption and protein synthesis is higher when HB101 harbors a plasmid of intermediate size (e.g., 5.3 kb). According to our hypothesis, whereas the effect of smaller plasmids on cellular metabolism is negligible, the effect of larger plasmids is so strong that it becomes deleterious to the host physiology. However, for intermediate plasmid size, the metabolism experiences a moderate enhancement that can be detected through the evaluation of the oxygen consumption efficiency and protein synthesis. These results suggest that the effect of a plasmid on host metabolism depends on the plasmid-DNA content (e.g., by copy number or plasmid size), and it can be observed only at intermediate ranges. The latter was clearly demonstrated by Khosravi et al. (1990) in experiments with strains of *E. coli* carrying no plasmid or two plasmids with different sizes. They showed that the oxygen uptake rate of strain JM103 harboring plasmids of 8.7 and 2.7 kb size was 18.5 and 7.5 times higher than plasmid-free JM103, respectively, although the growth rate of strains decreased with increasing plasmid sizes (Khosravi et al.,1990). Measurements of oxygen consumption rate during glucose and glycerol respiration were carried out in our laboratory and confirmed these observations (unpublished results). Figure 8 shows the oxygen consumption rates determined in three strains of *E. coli* carrying zero, one, two, or three plasmids. From Figure 8 we can see that the influence of plasmids on the host metabolism depends on the genetic background of the host,

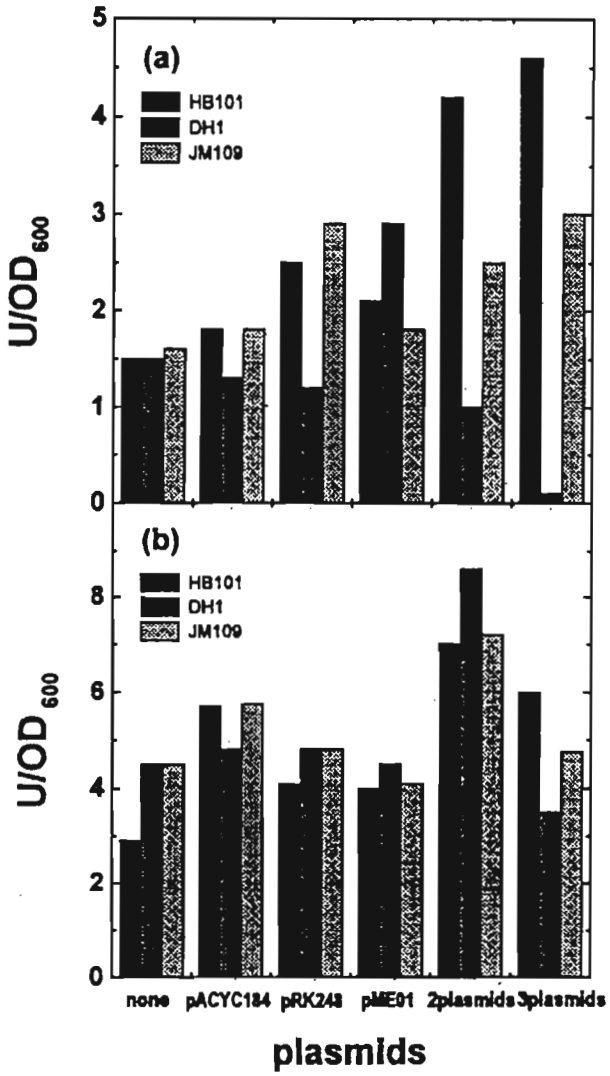

FIGURE 8. Respiration rate of strains HB101, DH1, and JM109 grown in a rich medium (LB) supplemented with glucose (a) or glycerol (b). Cell suspensions were harvested at early mid-log phase ($OD_{600} = 0.5$), washed and resuspended in phosphate buffer (50 m*M*, pH 7.0). Experiments started the addition of a conveniently diluted cell suspension to an air-saturated phosphate buffer supplemented 10 g/l of glucose (a) or 20 g/l of glycerol (b). Dissolved oxygen was measured with a Gilson Oxymeter (2-ml chamber). One unit of oxygen consumption rate corresponds to 1 μmol of oxygen per min at 30°C. Each value comes from five individual experiments and the average standard deviation was 0.3. The rest of experimental conditions as described in Table 2.

strain HB101 being more sensitive to the presence of plasmids than strains DH1 or JM109. Furthermore, the fact that oxygen uptake rate increases in strains carrying plasmids during the catabolism of glucose (Figure 8a) and glycerol (Figure 8b) indirectly supports the hypoth-

esis that the PTS is involved in this phenomenon (Novotny et al., 1985; Saier et al., 1996; Charpentier et al., 1998).

Peretti et al. (1989) and Wood and Peretti (1990) conducted experiments to elucidate the effect of plasmid maintenance and cloned gene expression on host RNA and protein metabolism. They showed that the level of expression of the β-lactamase gene carried by similar plasmids replicating with different copy numbers in HB101 exhibited a maximum value for the plasmid replicating at a ratio of 60 copies per genome (Peretti et al., 1989). The "derepression" phenomenon observed was attributed to three factors: (1) an increase in the synthesis of total RNA polymerase, (2) an increase of the fraction of active RNA polymerase directly involved in transcription, and (3) an increase of the *novo* synthesis of ribosomes that would enhance the mRNA translation efficiency (Peretti et al., 1989). However, further studies showed that the host induced the accumulation of total RNA, rRNA, and proteins by the sole presence of a plasmid (Wood and Peretti, 1991). Byrne (1990) also reported an enhanced biosynthetic capacity of a strains *Bacillus subtilis* (BD170) when carrying plasmid pE194-COP6. He observed in continuous culture experiments, a significant increase of the total RNA when strain BD170 carried a plasmid (McLoughlin, 1994).

Birnbaum and Bailey (1991) also reported that the mere presence of plasmids in *E. coli* HB101 induced an increase in the amount of several enzymes related to the tricarboxylic acids cycle, ribosome structure (e.g., S5, S6a, S6b, L3, L7/12), protein biosynthesis factors (e.g., elongation factor G), heat shock proteins, and total protein in general. They showed that this phenomenon was more severe with intermediate copy number plasmids (e.g., 56 copies per genome), and with lower or higher copy number plasmids the protein pattern changed. They demonstrated that the plasmid burden influenced the expression of protein involved in different cellular mechanisms differentially (Birnbaum and Bailey, 1991). Moreover, they reported that among the proteins induced by the presence of plasmids, the enzymes PEP carboxylase and succinate dehydrogenase showed a significant increase at higher plasmid copy number, and, interestingly, the *pck* and *sdh* genes encoding for those enzymes, respectively, are regulated by the cAMP-CRP system (Botsford and Harman, 1992). In our laboratory we have also confirmed the effect of plasmid maintenance on cellular protein contain. We observed that the values of the β-galactosidase activity in *E. coli* HB101 were strongly dependent on how the activity was expressed. The activity of β-galactosidase was different when evaluated per unit of optical density (600 nm) or milligram of protein (unpublished results). In Figure 9a we show that with the exception of HB101 carrying plasmid pRK248, the rest of the strains displayed higher β-galactosidase activity when expressed per unit of OD_{600} than per mg of protein. This observation suggested that the presence of plasmids induced an augmentation of the total protein content of strains that carried one or more plasmids. In Figure 9b we present results of the ratio milligram of total protein/unit of optical density (at 600 nm) obtained for different strains carrying different plasmids. In this figure we can see that the effect is more severe in strains carrying two and three plasmids and strain HB101 is more sensitive to the plasmid effect than DH1 and JM109.

If plasmids induce the enhanced expression of genes positively regulated by the cAMP-CRP system, and particularly those related to the glucose uptake, we may hypothesize that any anabolic pathway that could take advantage of that extra carbon and energy source would redirect the carbon flux toward synthesis instead of to the formation of inhibitory byproducts. The latter seems to happen when alcohol dehydrogenase (*adh*) and pyruvate decarboxylase (*pdc*) genes from *Zymomonas mobilis* were expressed in *E. coli* (Ingram et al., 1987; Ingram and Conway, 1988; Diaz Ricci et al., 1992). It was reported that the expression of *adh* and *pdc* genes from *Z. mobilis* into the *E. coli* strain HB101 induce a metabolic enhancement effect. The growth was significantly improved and the synthesis of byproducts (i.e., lactic acid, acetic acid, glycerol, etc.) was redirected toward etha-

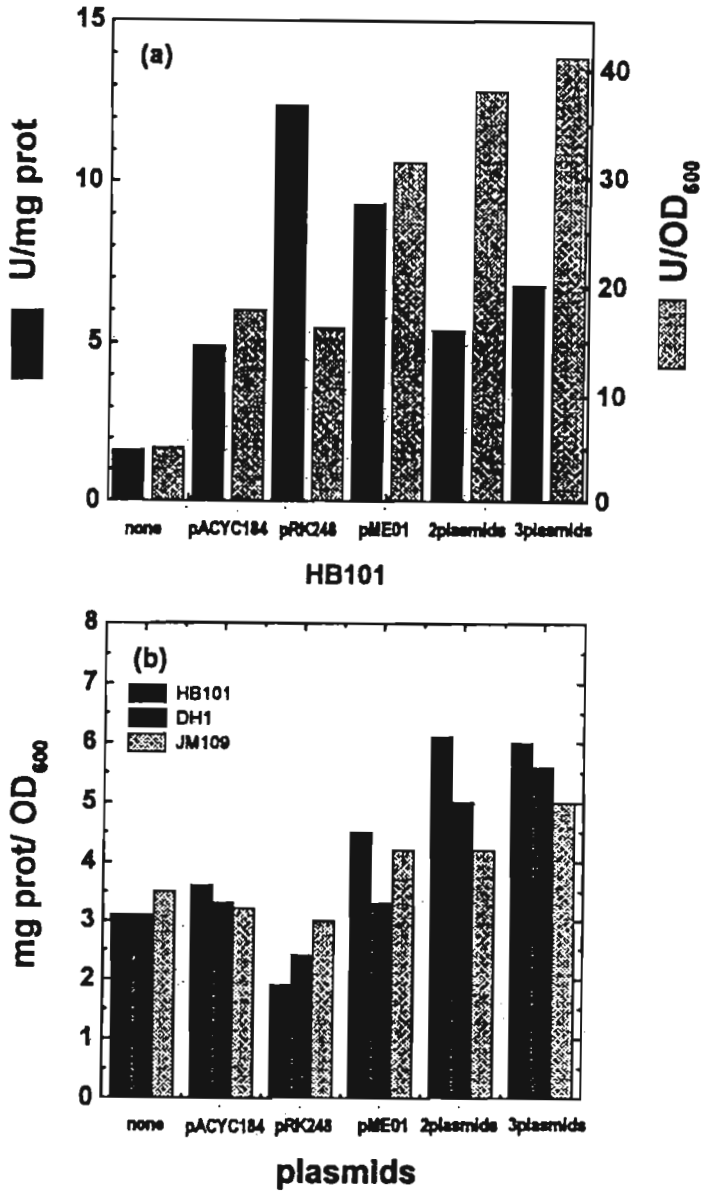

FIGURE 9. (a) β-galactosidades activity of the strain HB101 of *E. coli* carrying no plasmid, one, two (pME01, pACYC184), or three (pME01, pACYC184, pRK248) plasmids, expressed per milligram of protein and per unit of optical density (600 nm). One unit of β-galactosidase hydrolyses 1 μmol of ONPG (*O*-nitrophenyl-β-D-galactopyranoside) per minute of 37°C (b) Ratio between milligrams of protein and unit of optical density (600 nm) of strains HB101, DH1, and JM109 of *E. coli*. Each value comes from five individual experiments, and the average standard deviations were 0.5. Experimental procedures as described in Table 3.

nol production. An explanation of this extraordinary physiological behavior is not easy to find. Initially, it was proposed that the phenomenon could be explain by the shift of fermentative metabolism that allowed cells to accumulate ethanol instead of acids, thus avoiding the drop

of intracellular pH, but experimental evidence ruled out the intracellular pH hypothesis (Diaz Ricci et al., 1991a, 1992). Instead, it was suggested that the effect was due to a metabolic enhancement induced by the expression of the *pet* operon (Diaz Ricci et al., 1992) that yielded the shift of the metabolic flux toward the synthesis of ethanol and CO_2 formation. Nonetheless, mass balances showed that the formation of ethanol and CO_2 were not exclusively at the expense of the other byproducts but from the increase of the glucose input. Surprisingly, that increase of the glucose uptake rate was not only observed in those strains expressing the *pet* operon or the *pdc* gene, but in strains carrying any plasmid, including the control plasmid used, for example, pUC19 (Diaz Ricci et al., 1991a; Diaz Ricci et al., 1992). The latter indicated that two effects were taking place in these experiments. One was the effect induced by the plasmid maintenance (e.g., increase of the glucose uptake rate and metabolic burden by "carbon overflow"), and the other was the effect exerted by the activity of the *adh* and *pdc* products (e.g., redirection of the carbon overflow). They showed that whereas strains carrying the control plasmid pUC19 and the plasmid pLOI284 expressing *adh* alone did not allow the host to get rid of the "carbon overflow", strains carrying plasmids that expressed the *pet* operon (pLOI295, pLOI304) or the *pdc* gene (pLOI276) could do that job successfully and by using a mechanism that avoids the harmful accumulation of inhibitory byproducts (Diaz Ricci et al., 1992). The contribution of the expression of the *pet* operon alone, without the influence of any plasmid, on the growth enhancement effect and the importance of the metabolic pathway used to release the "carbon overflow" was demonstrated by Ohta et al. (1991) when using *E. coli* strains that had the *pet* operon integrated into the host chromosome. They showed that the effect of growth enhancement was still observable with strains expressing the *pet* operon from the chromosome (Ohta et al., 1991). Also, from their results we concluded that the influence of the *pet* operon on the biomass yield is dependent on the genetic background of the strains used (Ohta et al., 1991; Diaz Ricci et al., 1992; Hespell et al., 1996; Lawford and Rousseau, 1996).

All these results together suggest that if we provide *E. coli* with a suitable mechanism to take over the carbon overflow induced by plasmid maintenance, it would be possible to improve growth rates and yields of plasmid-containing cell cultures, ergo we would endow plasmid-carrying cells with the chance to overgrow plasmid-free cells.

Khosla and Bailey (1988) have shown that when the *Vitreoscilla* (*vhb*) hemoglobin gene was expressed in *E. coli* JM101, the bacterium exhibited a significant improvement in cell growth under microaerophilic conditions compared with the same strain carrying the control plasmid pUC9. Following the same reasoning, and although they attributed this phenomenon exclusively to the activity of the *Vitreoscilla* hemoglobin, we could speculate that what the product of the gene *vhb* does is to redirect the carbon overflow induced by the multicopy plasmid pUC9 toward the complete catabolism of glucose to CO_2 via respiration. That would avoid the accumulation of inhibitory byproducts (Khosla and Bailey, 1988; Khosla et al., 1990) as it was suggested by Diaz Ricci et al. (1992) by taking advantage of the respiration efficiency enhancement induced by the expression of the *Vitreoscilla* hemoglobin (Khosla et al., 1990, 1991; Kallio et al, 1996) and also by the pUC9, as it was reported by Ryan et al. (1989) and Khosravi et al. (1990) for other plasmids. Interestingly, they also reported that the recombinant cells expressing the *vhb* gene displayed a protein synthesis enhancement (Kohsla et al., 1990). Although they attributed this biosynthetic enhancement exclusively to the activity of the *Vitreoscilla* hemoglobin, the question that arises is whether the effect they observed also comes from the plasmid effect on the regulatory system cAMP-CRP. Unfortunately, they did not show the cytochrome content of extracts coming from cells harboring the recombinant plasmid, carrying only the expression vector (pUC9) and plasmid-free cells. It has been reported that the expression of endog-

enous haem groups of *E. coli* may be controlled by oxygen and the cAMP-CRP system (Cox and Charles, 1973; Borman and Dobrogosz, 1974; Beale, 1996). If plasmid maintenance affects glucose uptake rate and the regulatory status of hosts, we should therefore expect to also see perturbations of the expression level of their own haem prosthetic groups in cells carrying only plasmid pUC9. The latter was suggested by Ryan et al. (1989) (see above), and clearly demonstrated by Khosravi et al. (1990) when they showed that the amount of cytochromes *b*, *o*, and *d* present in the JM103 strain increased when carrying plasmids of different sizes.

With the examples analyzed above, we feel stimulated to seriously consider the new hypotheses of plasmid effect on host metabolism as a suitable alternative approach to interpret the phenomenon of plasmid-host interaction, because it proved to be sufficiently robust to explain all of the cases discussed in this review.

Nevertheless, we must keep in mind that even when experimental results indicate that this mechanism may actually take place, the real interaction between plasmids and hosts remains unknown. From a biochemical point of view, it is not clear how the mechanism of replication of plasmids such as pBR322, pACYC184, pUC19, or any other can interact with such a complex regulon that involves the phosphotransferase and the adenylate cyclase systems without any apparent connection between them. The only precedents that we found in the literature that could shed some light on this phenomenon comes from Clewell and Helinski (1972) and Katz et al. (1973). They showed that the addition of cAMP to cells growing in glucose medium stimulated the rate of synthesis of the proteins involved in the relaxation complex and the replication rate of the plasmid ColE1 and ColE2 (Katz et al., 1973). Perhaps the answer should include a consideration of the interaction of plasmids with the cellular membrane, because that is the place where the chromosome and some plasmids anchor at the time of segregation (Norris, 1995; Firshein and Kim, 1997), where the active adenylate cyclase (Groelz and Cronan, 1982; Aiba et al., 1984) and the permease of the PTS can be found (Postma et al., 1996; Notley-McRobb et al., 1997), and redox processes modifying the transmembrane potential take place (Reider et al., 1979; Konings and Robillard, 1982; Epstein, 1983; Seo and Bailey, 1985; Axe and Bailey, 1987).

IV. ANOTHER PUZZLING EXAMPLE

Refutationists would say that it is enough for a single example contradicting a hypothesis to refute it, and that is so, because the hypothesis was proven to be false and should be changed. Consequently, any hypothesis about plasmid-host interaction—asserting that plasmids would always negatively affect the growth of the host under laboratory conditions—would be susceptible to refutation if we can find a single example contradicting it. Curiously, it is possible to show that under certain conditions cells harboring a plasmid expressing only their own genes can grow much better than plasmid-free cells. Rhee et al. (1994) reported that *E. coli* JM109 harboring any plasmid can sustain active growth in a minimal media, whereas the same strain without plasmid cannot. They showed that this phenomenon was independent of the plasmid used, but seemed to depend on the bacterial strain. They also showed that this unusual and novel behavior was only observed when cells were cultivated in a poor medium (e.g., supplemented M9) in which the metabolic capacity of the microorganism is tested at its maximum. These authors reported that even under very demanding biosynthetic conditions, strain JM109 was able to grow acceptably well, showing active anabolic and respiratory metabolism only when harboring a plasmid (Rhee et al., 1994). In our laboratory we have confirmed those results using different cultural conditions and other plasmids; results are shown in Figure 10. By looking at that figure we may ask ourselves: What do plasmids provide the host that allow it to enhance metabolic activity? What is the mechanism underlying this plasmid-induced metabolic

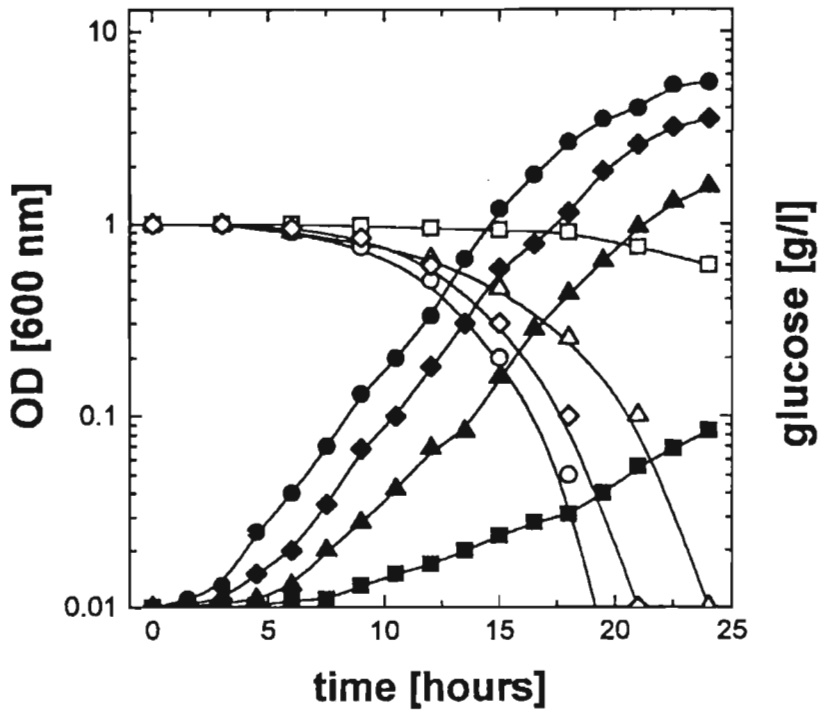

FIGURE 10. Effect of plasmid maintenance on the growth (solid) and glucose consumption (open) of the strain JM109 carrying no plasmid (square), one (pME01, circle), two (pME01 and pACYC184, diamond), or three plasmids (pME01, pACYC184, and pRK248, triangle). Experimental conditions as described in Figure 5 except that the minimal medium M9 supplemented with glucose (10 g/l), thiamine (30 μg/ml), and antibiotics (as required) was used in experiments (Rhee et al., 1994).

enhancement? Until we could come up with any reasonable answer to these questions, experimental evidence suggests that plasmid should no longer be considered an intracellular parasite that would always have negative effects on host growth or metabolism.

Whether the phenomenon of plasmid-induced metabolic enhancement observed with strain JM109 is a consequence of the same mechanism discussed above is still a matter of speculation. However, if the hypothesis that plasmids affect the regulatory status of the host through the cAMP-CRP system proves to be consistent, it would be much easier to explain possible alterations of carbon uptake metabolism as well as the role of other proteins involved in carbon catabolism and protein synthesis, as was suggested by Rhee et al. (1994) and reported by Birnbaum and Bailey (1991) and Wood and Peretti (1991).

V. BIOTECHNOLOGICAL PROSPECTIVE

A major effort in biotechnology is devoted to developing expression systems that allow the maximum expression of recombinant genes and the optimal recovery of the protein of interest, or in other cases the controlled expression of the genes under study. Hence, it is extremely important to keep in mind which is the goal pursued with the expression of recombinant genes because the technological approach can differ significantly. If we need to express the gene of interest at a low level and under strictly controlled conditions, it would most likely require the immobilization of the gene into the host chromosome. Instead, if the idea is to obtain a large amount of a particular protein, it would demand the use of a multicopy expression vec-

tor. Whereas with the former approach the problem of plasmid stability is meaningless, in the latter case that problem becomes extremely serious. If the concern is the overproduction of a protein, it is thus essential to understand and try to control three important biotechnological variables: (1) mechanisms of plasmid loss, (2) consequences of plasmid maintenance on host metabolism, and (3) potential effects of the gene overexpression on host functions. For the first biotechnological variable it would be highly desirable to maximize plasmid stability by minimizing plasmid impaired segregation-partition process during cell division. The latter can only be achieved by selecting the appropriate combination of plasmid-host-cultural conditions in order to provide plasmid-bearing cells a continuous positive selection pressure against plasmid-free cells (Godwin and Slater, 1979; Jones et al., 1980; Meacock and Cohen, 1980; Noak et al., 1981; Tucker et al., 1981; Ray and Shurray, 1984; Austin, 1988; Warnes and Stephenson, 1986; Bentley and Kompala, 1989; Bentley and Kompala, 1990; Brownlie et al., 1990; Mosrati et al., 1993; Rothen et al., 1998; Smith and Bidochka, 1998). That selection pressure could be exerted by the addition of an external compound (i.e., antibiotic, amino acids, vitamins) or by using any particular metabolic feature induced by the plasmid on host metabolism. The latter alternative is directly related to the second technological variable mentioned above. An innovative proposal therefore would consist of designing a plasmid that could take advantage of the unavoidable alterations induced on the host physiology (e.g., a faster glucose metabolism, higher respiratory efficiency, or improvement of protein synthesis), to redirect the carbon flux toward the formation of nontoxic byproducts such as carbon dioxide, ethanol, or active proteins (i.e., hemoglobins) that could contribute to release of the metabolic burden. A plasmid like that could be maintained in a host as a stable genetic element without the necessity of adding any external compound to maintain selection pressure (Khosla et al., 1990; Ohta et al., 1991; Diaz Ricci et al., 1992; Hespell et al., 1996; Lawford and Rouseau, 1996).

Finally, the third variable to take into account concerns the particular gene we would like to express. As mentioned earlier, gene products can be active within the host or not, and in the former case it could interact with the host metabolism or not. In any case, the host biosynthetic capacity, protein processing, folding, and secretion rates should be considered carefully in order to avoid deleterious effects on the host metabolism, the formation of inclusion bodies, or other serious problems during gene expression (Bailey et al., 1986; Bailey, 1993; McLoughlin, 1994; Glick, 1995).

The hypothesis presented in this article may have important biotechnological implications. As the phenomenon is based on the modulation of the regulatory system cAMP-CRP, it can exhibit alterations at different levels of the regulation of cellular metabolism, which potentially could be manipulated, including the enhancement of glucose uptake, respiration, and protein synthesis rates. However, more than one effect may impact on cell metabolism, and they could yield antagonistic effects. For instance, whereas protein synthesis enhancement can be viewed as a positive influence of plasmids on host metabolism, this effect may be totally masked by the high accumulation of acids provoked by the glucose uptake enhancement, also induced by plasmids.

Attempts to redirect the metabolic flux with the aim of helping *E. coli* to overcome the carbon overflow has been done and results proved that it is possible to improve the growth rate of plasmid-bearing cells (Khosla and Bailey, 1988; Diaz Ricci et al., 1991b; Diaz Ricci et al., 1992). An interesting example was proposed by Hespell et al. (1996). They showed that plasmids pLOI295 and pLOI297 expressing the *pet* operon (Ingram et al., 1987) could be stabilized when maintained in a *ldh* (lactate dehydrogenase) and *pfl* (pyruvate formate lyase) mutant of *E. coli*, that is, strain FMJ39, which is unable to regenerate NAD^+ under anaerobic conditions. They have demonstrated that by selecting the appropriate set of plasmid, host, and culture conditions, they could maintain plasmids pLOI295 and pLOI297 for more than 50 generations in

batch and continuous cultures in the absence of selection pressure (e.g., tetracycline) without the apparent loss of plasmid-encoded antibiotic resistance and ethanologenic properties (Hespell et al., 1996). Likewise, Lawford and Rouseau (1996) reported interesting advances in this direction when using the *pet* operon. Although they did not observe a growth enhancement induced by the *pet* operon in *E. coli* strain ATCC11303 when growing in a glucose-supplemented rich medium at pH 7.0, the growth effect was clear in glucose and xylose-supplemented rich medium at pH 6.3.

VI. CONCLUDING REMARKS

Although the real mechanism underlying the phenomenon of glucose uptake enhancement induced in hosts by plasmids is still unknown, experimental evidence shows that it yields the rapid accumulation of inhibitory intermediate metabolites and byproducts. We speculate that if the host or the plasmid provides a suitable alternative to overcome such metabolic stress, then the host would acquire a clear physiological advantage (Khosla et al., 1990; Ohta et al., 1991; Diaz Ricci et al.,1992; Hespell et al., 1996). This mechanism would provide further support for explaining the great stability observed in cryptic plasmids that confer no apparent phenotypic marker to the host (Macrina et al., 1978) and might explain the effect of overgrowth of plasmid-free hosts over the plasmid-containing strains under nonselective pressure. Hence, it is very important to characterize the changes induced by plasmids in the host of choice before analyzing the effect of the expression of recombinant genes. The impact of gene overexpression on the cellular metabolism will always masks subtler physiological changes induced by the vector, and these smaller changes can make the difference between success or failure in a biotechnological process.

Another interesting observation is that as a consequence of a change of the genetic background *E. coli* is able to catabolize glucose faster under identical cultural conditions. This suggests that as a consequence of the plasmid-host interaction the enzymes involved in this metabolic pathway would be activated, derepressed, or would attain different steady state levels. The idea of a multiplicity of physiological states is not a totally new concept in *E. coli*. Even though it has not been confirmed experimentally, multiple steady states were proposed for the regulatory state of bacteriophage λ and the *lac* operon (Chung and Stephanopoulos, 1996). Interesting research fields to explore new modes of regulation and the impact of maintenance of extra DNA on a particular genetic background open in this direction and would help to explain and predict plasmid-host interactions (Neidhardt and Savageau, 1996).

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