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WIDE HOST RANGE PLASMID PROBE FOR THE HETEROLOGOUS TRANSMISSION OF *Escherichia coli* *lac* GENES.

*(Sonda plasmidial de amplio rango de hospedero para la transmisión heteróloga de los genes lac de **Escherichia coli**)*

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SUMMARY

*The **lac** operon of **Escherichia coli** is a genetic system proven useful in the elucidation of basic principles of genetic variation and expression and in microbial strain construction for applied purposes. In this context, and using the in vivo cloning vehicle pULB113 (IncPa) we derived the corresponding wide host range plasmid pUCV3, containing the **E. coli** lac genes, which can now be delivered with ease to all microorganisms capable of incorporating replicons of the IncPa incompatibility group. In our experiments, pUCV3 was transmitted to representative marine bacteria and to the gliding, soil bacterium **Cytophaga johnsonae**. These microorganisms showed regulated expression of the heterologous **lac** system. Thus, pUCV3 could be used as a probe to evaluate behavior of the **lac** operon in varied, microbial genetic backgrounds.*

RESUMEN

*El operon **lac** de **Escherichia coli**, es un sistema genético que ha sido útil para elucidar principios básicos de variación y expresión genética y para la construcción de cepas microbianas de proyección industrial. En este contexto, hemos derivado por clonación in vivo, un plasmidio de amplio rango de hospedero (pUCV3), que contiene los genes **lac** de **E. coli**, los cuales pueden ser ahora transmitidos con facilidad a todos los microorganismos capaces de incorporar replicones IncPa, grupo de compatibilidad al que pertenece pUCV3. Como ejemplo este plasmidio fue transmitido a bacterias marinas y a **Cytophaga johnsonae**, microorganismos en los cuales se apreció la expresión regulada del sistema **lac**. En consecuencia, pUCV3 puede ser usado como sonda para evaluar el comportamiento del operón **lac** en variados transfondos genéticos microbianos.*

INTRODUCTION

The genes for lactose utilization from ***Escherichia coli***, have gathered a long standing interest, being therefore, the subject of a number of studies pertaining to both basic and applied research. Undoubtly, of prime relevance are the investigations of Jacob & Monod (1961), based on the use of various mutations in the ***E. coli*** *lac* region in conjunction with F'*lac* factors. These inquiries, marked by the analyses of haploid and merodiploid strains for the ***E. coli*** *lac* genes, finally led to the definition of negative control of gene expression in a coordinately regulated set of genes: the lactose operon.

The *lac* system has also proven to be useful in addressing crucial issues contingent to the process of mutation. It has been a valuable tool to elucidate principles of both spontaneous and mutagen-induced mutation in bacteria (Eisenstadt, 1987) and in eukaryotic cells, into which tha *lac* genes are incorporated by means of appropriate shuttle vectors (Calos et al.,1983).

In a similar vein, the formation of papillae due to

reversion of *lacZ* mutations, has been a fruitful analytical approach in the identification of mutator alleles (Nghiem et al., 1988). In addition, the *lac* system has been widely used to investigate non-sense suppression (Miller & Albertini,1983; Kleina et al., 1990) and to gain insight into the mobility of transposable genetic elements, through the analyses of *lac* segregation patterns (Bender & Kleckner, 1986).

On the other hand, the concept of adaptive mutation in bacteria originated in studies on the pattern of reversion of a *lacZam* mutation under non-lethal selective conditions (Cairns et al., 1988). These observations have triggered a substantial amount of controversy (for a review see Sniegowsky & Lenski, 1995) and continue to motivate experiments using the *lac* system (Galitski & Roth, 1996), aimed at testing one of the basic tenets of evolutionary theory, namely that genetic variation precedes adaptation.

Certainly, one of the most important applications of the ***E.coli*** *lac* genes has been the use of genetic fusions between these genes and other target genetic units, with the purpose of monitoring the expression of genes lacking an easily assayable phenotype by means of the ß-galactosidase activity coded by the fused *lacZ* gene, that acts as a reporter gene informing on the expression of the target gene (for a review see Slauch & Silhavy, 1991).

Further practical applications of the *lac* system have used ß-galactosidase activity to monitor individual, viable non-culturable cells of ***E.coli*** and ***Salmonella enteritidis*** by means of fluorescence microscopy (Nwoguh et al., 1995) and non-culturable coliforms in marine waters (Davies et al., 1995).

Finally, the ***E. coli*** *lac* genes have been used as a substrate for the construction of lactose-utilizing, genetically engineered strains of ***Xanthomonas campestris***, a bacterium that is naturally unable to use lactose as sole source of carbon. Nevertheless, ***X. campestris*** is useful industrially, as producer of xanthan gum. Consequently, the Lac^+ engineered strains, could be used for polysaccharide production from whey, the lactose- rich by-product of milk processing (Fu & Tseng, 1990; Papoutsopoulou et al.,1994; Drahovskà & Turna, 1995).

Considering the relevance of the ***E.coli*** *lac* genes, outlined above, we thought it would be useful to derive a plasmid probe that would allow evaluation of the behavior of the ***E.coli*** *lac* module in heterologous microbial systems and in a context of genetic variation, gene expression and assessment of potential new applications. Consequently, our aim was to clone the ***E.coli*** *lac* genes in a wide host range, conjugative plasmid vehicle to generate a plasmid probe that would allow transmission of the *lac* genes to a wide variety of microorganisms. To achieve this objective we used pULB113 (Van Gijsegem & Toussaint, 1982), a plasmid vector for in vivo cloning that has been useful in generating molecular clones in several microbial systems, owing to its capacity for horizontal transmission, conferred in turn, by its wide host range, typical of replicons belonging to the IncP*a* incompatibility group (Van Gijsegem & Toussaint, 1982; Chatterjee et al., 1985).

MATERIALS AND METHODS

1.- Bacterial strains, plasmids and phages

Table 1. shows a list of bacteria, plasmids and phages used in this work. Additional bacterial strains are described in the Results section.

2.- Culture media, microbial growth and characterization tests

All bacterial strains were grown routinely in LB broth or agar (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl and 1.5% Agar for solid medium). MacConkey Agar Base was used to determine fermentation of sugars added at 1% final concentration. Medium M9 (Miller, 1972) was used as minimal growth medium. Culture media and components were from Difco (Detroit, Michigan) and salts for media and buffers from Merck (Darmstadt, Germany). For marine bacteria, 3% NaCl was used in culture media.

For phenotypic characterization tests and the preparation of selective plates used in genetic crosses, antibiotics (Sigma, St. Louis. Mo., unless otherwise indicated) were used in the following concentrations, in µg/ml: ampicillin (Ap, 25), tetracycline (Tc, 25), kanamycin (Km, 50), chloramphenicol (Cm,20), streptomycin (Sm, 200), nalidixic acid (Nal, 100) and rifampicin (Rif, 100; P-L Biochemicals, Milwaukee, Wis.).

Arabinose (Ara, Pfanstiehl, Waukegan, Ill.) and lactose (Lac, NBC, Cleveland,Ohio) were used in fermentation tests and the latter also as carbon source in M9 medium.

3.- Assay of ß-galactosidase activity

The qualitative and quantitative assays of ß-galactosidase were performed according to the the standard methodology described by Miller (1972), using suspensions of toluenized cells and ortho-nitrophenyl-thio-galactoside (ONPG, Sigma) as enzyme susbstrate. For the induction of *lac* genes, iso-propyl-thio-galactoside (IPTG, Sigma) was used, as recommended by the same author.

4.- Analyses of plasmid DNA

Preparation of plasmid DNA was according to

Kado & Liu (1981), except that bacterial cells were initially suspended in sterile, deionized water. Cell lysis was for one hour at 65°C. Plasmid DNA was resolved by horizontal gel electrophoresis in 0.4% agarose prepared in Tris-Borate-EDTA buffer, pH 8.

The gel was stained in ethidium bromide , viewed under UV light and photographed according to Sambrook et al. (1989).

5.- Genetic crosses and phage assays

For the construction of pUCV3 and bacterial strains with different plasmids (see Results), conjugative genetic crosses were performed by the spin mating technique. We used exponentially growing cultures, in LB broth,at a density of approximately $1x10^8$ cells/ml. Equal volumes (0,5 ml) of donor and recipient populations were mixed together in sterile 1.5 ml Eppendorf tubes and spun for 1 minute in an Eppendorf Model 5414 microcentrifuge. Tubes were then incubated for 24 h at 28°C without discarding the supernatant. Cells were then resuspended by vortexing and 0.1 ml samples of the mating mixture seeded on selective plates for the recovery of transconjugants. Separate cultures of donors and recipients, treated similarly, served as controls.

Phage susceptibility or resistance of bacterial strains was determined by cross-streak, using exponentially growing cultures of bacteria and freshly prepared cell-free lysates of GU5 and Mu *cts* 62.

RESULTS AND DISCUSSION

Our main objective was to obtain a plasmid probe containing the ***E.coli*** *lac* genes, such that it could be transmitted to a wide variety of microorganisms and thereby allow evaluation of basic issues concerning *lac* gene expression in heterologous genetic backgrounds. Likewise, potential new applications of the *lac* system could be visualized and explored.

To achieve our goal, we used the in vivo cloning vector pULB113. This is a plasmid of the IncP*α* incompatibility group, a set of wide host range replicons, characterized by their ability to flow among different genera of Proteobacteria and to transfer ,as well, to other phylogenetic groups (for reviews, see Thomas, 1989). pULB113, in particular, is a derivative of RP4 containing a Mu3A insertion, itself a transposable genetic module derived from phage Mu (Van Gijsegem & Toussaint, 1982). In fact, Mu3A is a defective phage capable only of transposition, since it does not code for any viral proteins. Therefore, pULB113 codes for the pattern of antibiotic resistance conferred by RP4 (Ap, Tc, Km) and acts as a vector for the delivery of Mu3A. Thus, upon entrance of pULB113 into bacterial cells, Mu3A can transpose to many different sites in the chromosome of the recipient , adjacent to corresponding chromosomal loci such as the *lac* operon, thereby generating regions of homology with pULB113, such that integrative recombination is possible. In this way, the following genetic configuration is likely to arise: *lac*-Mu3A-RP4-Mu3A. A further translocation of Mu3A to the left of *lac* is feasible, creating a new region of homology that allows recombination with the distal copy of Mu3A so that a new plasmid is reconstituted in the form of an R-prime factor, carrying the *lac* genes that have been cloned in vivo (see Van Gijsegem & Toussaint, 1982, for further details).

In our experiments to derive pUCV3, we crossed ***E. coli*** MxR with ***E. coli*** VAL53 selecting transconjugants in MacConkey Agar plates containing Cm, Ap, Km and Tc. The resulting strain, ***E. coli*** VAL55, is Lac^+ and resistant to all the specified antibiotics. VAL 55 was then grown to saturation to allow the in vivo generation of recombinant plasmids that were outcrossed to ***E. coli*** 2908. Transconjugants containing pULB113 derivatives with cloned lac genes were selected on M9-lactose plates containing Sm at a frequency of about 2×10^{-6} transconjugants/donor cell. One of these transconjugants, designated VAL 102 and containing the recombinant plasmid pUCV3 was used in further experiments.

Figure 1 shows that , in fact, pUCV3 contains cloned genetic material from VAL 55, since its molecular mass is incremented in relation to the parental vector molecule pULB113. In addition, and according to phenotypic analyses shown in Table 1, pUCV3 contains the *lac* system genes, as it confers a Lac^+ phenotype on strain VAL 102, complementing the *lac* deletion of the parental ***E. coli*** strain 2908. It also confers the same pattern of resistance to antibiotics as pULB113 and a pattern of GU5 and Mu cts62 susceptibility that is the inverse of that shown by strain 2908. This was expected because VAL 102 should express P type pili and immunity to phage Mu owing, respectively, to the *tra* functions coded in pUCV3 and the copies of Mu3A that form part of its genetic structure. A similar situation is observed for VAL 55 with respect to VAL 53. We should also point out that pUCV3 complements the *lacI*$^-$ mutation, affecting the lactose permease in the ***E.coli*** strain C600 (Nal-r). In fact, ***E.coli*** C600 transconjugants containing pUCV3 which were the product of a cross with Val 102, all exhibited a Lac^+ phenotype on MacConkey agar plates. All these analyses, taken together with the fact that the cloned *lac* genes are inducible by IPTG (Table 3), indicate that pUCV3 contains the complete version of the ***E.coli*** *lac* operon. These results confirm the relative ease of manipulation of the pULB113 cloning system in ***E. coli*** as in other Gram negative bacteria (Van Gijsegem &

TABLE 1. Bacterial strains, plasmids and phages.

Biologicals	Relevant features	Source
Bacteria		
Escherichia coli K-12 M x R	*lac* genes deleted. Lac⁻ Contains pULB 113	F. V. Gijsegem
VAL 53	Prototrophic. Contains Tn9 in chromosome. Cm-r	This work
VAL 55	Same as VAL 53. Contains pULB 113	This work
2908	Arabinose sensitive. Rec⁻. *lac* genes deleted.Sm-r.	R. Curtiss
VAL 102	Same as 2908. Contains pUCV3	This work
Plasmids		
pULB 113	RP4::Mu3A. Confers resistance to Ap, Tc and Km	F.V. Gijsegem
pUCV 3	In vivo cloning product of *lac* genes from ***E. coli.*** Confers resistance to Ap, Tc, Km and Lac+ phenotype	This work
Phages		
GU5	Phage specific for P-type pili	F.V.Gijsegem
Mu*cts62*	Derivative of phage Mu with thermosensitive repressor	R. Hull

Toussaint, 1982; Chaterjee et al., 1985).

We should also mention that in our experiments we recovered other genetic markers by cloning in pULB113: for example, genes for arabinose utilization, that conferred an Ara-r phenotype on ***E.coli*** 2908 (data not shown).

We were also interested in probing transmission of pUCV3 to other microbial groups to evaluate expression of the ***E. coli*** *lac* genes in other genetic backgrounds, through the assay of *ß*-galactosidase activity coded by the *lacZ* gene. For this purpose, VAL 102 was crossed with Rif-r derivatives of the marine bacteria ***Vibrio harveyi*** and BM-E and the gliding bacterium ***Cytophaga johnsonae.***

TABLE 2: Phenotypes conferred by pULB 113 and pUCV 3 on strains of *E. coli*.

	Tests								
Strains	**Lac**	**Ara**	**Ap(25)**	**Tc(25)**	**Km(50)**	**Rec**	**GU5**	**Mu**	**ONPG**
2908	-	S	S	S	S	-	R	S	-
VAL 102	+	S	R	R	R	-	S	R	+
VAL 53	+	R	S	S	S	+	R	S	+
VAL 55	+	R	R	R	R	+	S	R	+

Positive test (+) and negative (-) R = resistant and S = sensitive.

TABLE 3: Expression of ß-galactosidase coded by pUCV3 in various bacterial hosts.

Bacterial strain (a)	**Plasmid content**	**Relative activity of ß-galactosidase (b)**		**Growth in M9 lactose (c)**
		+ IPTG	**- IPTG**	
E. coli K-12				
M x R	pULB 113	< 1	< 1	NO
2908	None	< 1	< 1	NO
	pULB 113	< 1	< 1	NO
	pUCV 3	100	1.8	YES
Vibrio harveyi BB7-1	None	< 1	< 1	NO
	pULB 113	< 1	< 1	NO
	pUCV 3	113.3	10	YES
BM-E	pULB 113	< 1	< 1	NO
	pUCV 3	115.1	< 1	YES
Cytophaga johnsonae	None	ND	ND	NO
UW101	pULB 113	ND	ND	NO
	pUCV 3	ND	ND	YES

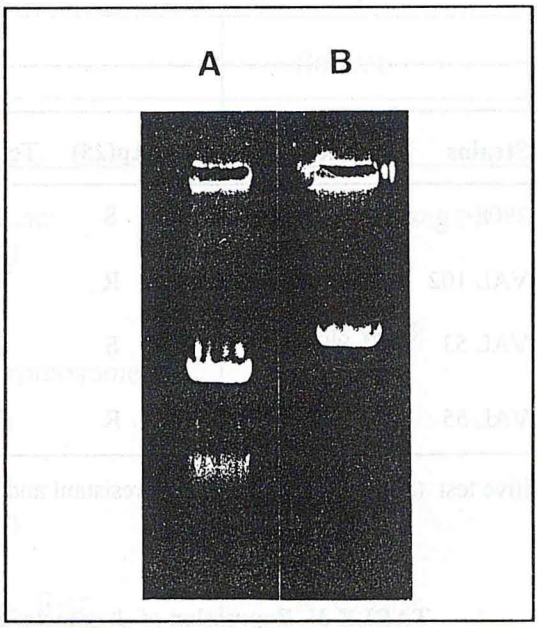
(a) ***V. harveyi*** BB7-1 (obtained from M. Silverman) and BM-E (isolated in our laboratory) are both marine bacteria. ***C. johnsonae*** UW 101, is a gliding, soil bacterium obtained from J. Pate.
(b) Activity of ß-galactosidase in ***E.coli*** Val 102 was given the value of 100%, in relation to which the activities in other strains were expressed.
(c) Media for the growth of marine bacteria contained 3% NaCl.

Analyses of β-galactosidase activities in pUCV3 and pULB113 transconjugants of these bacterial strains together with enzyme activity in the corresponding parental bacteria are shown in Table 3. We note the fact that the ***E.coli*** *lac* genes are fully functional in the marine bacteria tested, which show activities over the ***E. coli*** control and maintenance of the system's repression and inducibility by IPTG. This is confirmed by the fact that these bacteria are capable of growth on lactose as sole source of carbon and energy. This genetic compatibility could be understood in terms that ***E.coli*** and marine bacteria such as ***V. harveyi*** and BM-E belong to the same phylogenetic group of Proteobacteria. However, we also observed that the lac system allows growth of ***C. johnsonae*** in M9-lactose , in spite of the fact that this bacterium belongs to another phylogenetic group, distant from the Proteobacteria. In this case, we did not determine β-galactosidase activity, because ***C. johnsonae*** cells produce a yellow pigment that inter-feres with readings of the product of ONPG hydrolysis, itself of yellow color. Nevertheless, the pUCV3 resistance markers were present in the corresponding ***C. johnsonae*** derivative , although we could not detect plasmid DNA in this strain. It is possible that it had integrated in the ***C. johnsonae*** chromosome, in a manner analogous to what has been described for ***Myxococcus xanthus***, a gliding bacterium in which chromosomal integration of the plasmid RP4 has been shown, following conjugation with ***E.coli*** (Breton et al., 1985). An interesing alternative to consider would be that pULB113 had integrated into the ***C. johnsonae*** chromosome by homologous recombination with a transposed copy of Mu3A. To prove this point would be worthwhile since there are no data on the behavior of Mu or Mu-like phage in gliding bacteria of the ***Cytophaga*** group.

The data in Table 3 suggest that pUCV3, or derivatives thereof containing specific mutations in the ***E coli*** *lac* operon could be used to explore aspects of variation and expression of that genetic module in other bacteria, in a vein similar to that reported for ***Agrobacterium*** by Bezdek & Soska (1984) and for ***Proteus*** by Roberts & Baumberg (1984). However, the pULB113 system offers the advantage of avoiding the more cumbersome derivation of in vitro-generated constructs.

Another further use of pUCV3 could be concieved within the context of conjugative transmission of genes from bacteria to the yeast ***Saccharomyces cerevisiae*** (Heinemann & Sprague, 1989). In fact, replicons such as pUCV3 could be used for the transmission of bacterial genes into yeast cells to study their behavior in an eukaryotic background. There is already a report concerning these studies, in which wide host range bacterial plasmids of other incompatibility groups were used to test gene transfer from ***E. coli*** to the yeast

Figure 1



Photograph under UV light of an agarose gel (0.4% in TBE buffer,pH 8.0), stained with ethidium bromide (0.5 mg/ml), in which plasmid bands are seen,corresponding to pULB113 (lane A) and pUCV3 (lane B). The latter shows an increase in molecular mass in relation to the parental plasmid. Diffuse bands under plasmid DNA correspond to chromosomal DNA fragments. DNA in A was prepared from ***E. coli*** MxR and that in B from ***E. coli*** Val 102.

Saccharomyces kluyveri (Inomata et al., 1994). In addition, given the recognized suitability of yeast for industrial fermentation processes, it is of interest to project the specific design of novel yeast strains for applied purposes, using both eukaryotic and prokaryotic genes as raw materials. Evidence for this line of activity is available in the work of Compagno et al. (1995), who tailored yeast strains for the utilization of whey and starch, and that of Adam et al. (1995) who designed a strain of ***S. cerevisiae*** for the production of a bacterial β-glucosidase.

In summary, we have constructed a plasmid vehicle for the lateral transmission of ***E.coli*** *lac* genes, that allows evaluation of the behavior of this genetic system in other microbial backgrounds.

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