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Restoration of ligase activity in E. coli K12 lig ts7 strain by bacteriophage Mu and cloning of a DNA fragment harbouring the Mu 'lig' gene

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ABSTRACT

Restoration of ligase activity has been observed in E. coli K12 ligts7 strain lysogenic for Mu , in presence as well in absence of lysogenic immunity. This restoration consist in phenotypic reversal of temperature sensitivity of E. coli ligts7 which also regain the ability to sustain the complete growth cycle of T4 lig⁻ phages.

It is possible to put under the control of the gal operon the expression of the viral gene responsible for the restoration effect.

This new gene of Mu has been named 'lig'.

A 5 kb fragment responsible for the reported effects and localized between genes gam and lys of Mu genome has been cloned in pBR322. This recombinant plasmid used for transforming ligts7 strain restores in it normal behaviour for ligation of Okazaki pieces.

INTRODUCTION

The thermosensitive strain ligts7 of E. coli K12 is a conditional lethal mutant which ligase activity is progressively reduced above 25° and barely observable at 42° (1, 2).

We have already shown that the bacteriophage Mu 'complements' the ligts7 mutation in E. coli in that it restores in the host both the resistance to lethal action of U.V. and the bacterial replication at a non-permissive temperature, survival after U.V. treatment being a function of multiplicity of infection (3).

Due to these results, we tried to determine the best conditions for the expression of the new phage gene.

In this paper we show that the same effect was observed upon induction of a strain ligts7 lysogenic for a prophage Mu cts62. In addition we found an unexpected result: the restoration by Mu of the bacterial activity is operating

when the prophage is not derepressed, i.e. in a strain lysogenic for Mu c^+, resulting in strains phenotipycally heat resistant and permissive for T4 lig^- lytic cycle.

Because of these results, we have postulated a new gene of Mu and named it 'lig'.

The expression of the Mu 'lig' gene may be put under the control of the galactose operon; this situation restores in ligts7 strains an active growth upon induction of the gal operon at a non-permissive temperature.

Furthermore, we have prepared a recombinant plasmid, pZP2, from the fragments obtained cutting the plasmid pBR322 with EcoRI and BamH1, and phage Mu with EcoRI and BglII. This new plasmid contains a Mu fragment of 5 kb which permits an active bacterial growth at high temperature when introduced in a ligts7 strain. In such a strain a temperature resistant ligase activity has also been found.

These reported data allow us to conclude that Mu codes for the synthesis of a product at least able to replace E. coli K12 and T4 ligases. The role of this product is discussed.

MATERIALS AND METHODS

Bacterial and phage strains. All the strains used are listed in table 1.

Media. LB - L broth (4), for standard cultures. LA - L agar supplemented with either 150 µg/ml streptomycin or ampicilline or 10 µg/ml tetracycline, for standard plates. SM - (5) for dilutions and standard suspension medium. M9 - Minimal medium supplemented with 0.4 % glucose or lactose as a carbon source (6); fucose is used at a concentration of 2×10^{-3}M. McConkey agar plates (7) supplemented with the needed sugars 1% w/v, for the selection of special lysogens.

Lysogenization procedure and mutants selection. Described in a previous paper (3). All the lysogenic strains reported are derived from independent lysogenization events.

Construction of strain R247 ligts7 galE :: Mu. This strain has been obtained crossing R245 with KL 16-5 Hfr according to Miller (7). Thy^+ lac^+ recombinants have been selected and checked for the ts phenotype and for

TABLE 1 A
Bacterial strains

strain	origin	relevant genotype	reference	notes
C600		pm^+ TLB1	Appleyard (28)	lysis indicator
EB93		F^- thy / RP4	Bade collection	episome donor
KL16-5		Hfr galE rec A	Buttin collection	cross
1159	KS269	F^- pm^- lig^+ lacYa482 str^r	Modrich et al. (1)	
1160	KS268	F^- pm^- ligts7 thy lacYa482 str^r	" " (1)	
R198	RS54	lig^+ (Mu cts62 kil) / RP4 (Mu c^+)	Paolozzi collection	episome donor
R235	1160	ligts7 mal :: Mu cts62 λ^r	this paper	
R236	1160	ligts7 man :: Mu cts62	" "	
R237	1160 x EB93	ligts7 lac^+ Tc^r / RP4	" "	
R238	R235 x R198	ligts7 lac^+ Tc^r / RP4 (Mu c^+)	" "	
R239	1160	ligts7 (Mu c^+)	" "	) three independent
R240	1160	" "	" "	) lysogenic
R241	1160	" "	" "	) clones
R242	1160	ligts7 (Mu cts62)	" "	
R243	1159	lig^+ (Mu cts62)	" "	
R244	1160	ligts7 / pZP2	" "	recombinant plasmid
R245	1160	ligts7 lac^+	" "	spontaneous lac^+ revertant
R246	R245 x KL16-5	ligts7 thy^+ lac^+ galE recA	" "	
R247	R246	ligts7 thy^+ lac^+ recA galE :: Mu c^+	" "	Mu prophage under gal operon control
R248	1159	lig^+ (Mu cts62)	" "	
R249	C600	lig^+ pm^+ thy^+ str^s (Mu c^+)	" "	

All "R" strains are F^- pm^- thy lacYa482 str^r amp^s Tc^s, unless otherwise stated.

TABLE 1 B
Phage stocks

strain	reference	notes
Mu c^+	Bade collection	
Mu cts62	Howe (30)	a heat inducible mutant of Mu
T4 lig^-	Ph. Vigier collection	
T4 lig^+	Ph. Vigier collection	

epimerase deficiency. One of these ligts7 galE strains, R246, has been infected with Mu c^+ in LB at 30° and then plated on M9 lactose at 42°. All the clones surviving at 42° and lysogenic for Mu were gal^-. One of them has been purified and used as R247 for the experiments.

Crosses with RP4. Strains R237 and R238 have been obtained by crossing, respectively, strains 1160 and EB93 and strains R235 and R198, to transfer the RP4 episome, according to Datta et al. (8). Lac^+ Tc^R colonies have been selected on McConkey agar plates supplemented with 10 µg/ml tetracycline.

U.V. treatment. Described in a previous paper (3). Lysogenic cultures with a Mu cts prophage are induced for 20 min. at 42° prior to irradiation.

T4 lig^- infection and lytic cycle in ligts7 (Mu) strains. a) Spot test - each strain to be tested is layered in LA on LA plates. Drops of 25µl from 10^7/ml dilutions of both T4 and T4 lig^- are put on top. When dry, plates are incubated at 32° for 18 hours. b) Burst size - Strains 1159 and R248 are infected with either T4 or T4 lig^- at m.o.i. 0.1 and let 10 min. at 42° to allow prophage induction. Anti-T4 serum (K = 3) is then added to inactivate non-adsorbed phage. After 2 min. the mixture is diluted 10^{-3} in LB and divided in two aliquots: one is plated on R249 at 37° to count lytic centers (A); the other is incubated at 37° for 40 min., then $CHCl_3$ is added and the produced phage is plated on R249 at 37° (B). The ratio B/A gives the burst size.

Cloning of Mu 'lig'. a) DNA and enzymes - Mu cts62 lysates have been obtained by induction (9), concentrated with PEG 6000 (10) and purified through a CsCl gradient (11); phage DNA has been extracted with phenol (12). The DNA of pBR322 and the restriction enzymes used have been kindly provided by Dr. F. Rougeon. b) Construction of the recombinant plasmid - DNA fragments are prepared by serial digestions (13); restriction endonucleases EcoRI and BglII are used for Mu, EcoRI and BamHI for pBR322. Five min. at 65° block the reactions. Mu and pBR322 DNA fragments are deproteinized with $CHCl_3$, precipitated with ethanol, mixed in equimolecular proportions and bound with phage T4-induced DNA ligase according to Weiss et al. (14). The serial steps of the procedure are controlled on 0.8% agarose gel electrophoresis according to Helling et al. (15). The fragments are extracted from agarose gel according to Finkelstein and Rownd (16). c) Clone transformation and identification -

The method is described by Rougeon et al. (17). The transformant bacteria are plated in duplicate on LA supplemented with 150 µg/ml ampicillin and incubated both at 32° and 42° for 18 hours. d) Alkaline sucrose density gradient - The procedure used for isolation of DNA pulse labeled with [^{3}H] thymidine, modified from Okazaki et al. (18), is described by Konrad et al. (2).

RESULTS

U.V. resistance of ligts7 (Mu) strains.

Survival after U.V. treatment of independent Mu lysogens of strain ligts7 is higher than for the non-lysogenic parental strain 1160, even when not induced; lysogens for Mu cts62, if heat induced prior to U.V. treatment, show the same resistance rate as the lig$^+$ strain (see fig. 1).

We have found that the rate of U.V. survival varies with the site of prophage insertion onto the bacterial chromosome: maximal nearest to the origin of replication decreases by a factor of 2 when distal (see fig. 2). U.V. survival kinetics are reported in fig. 2 for two sample lysogens, one

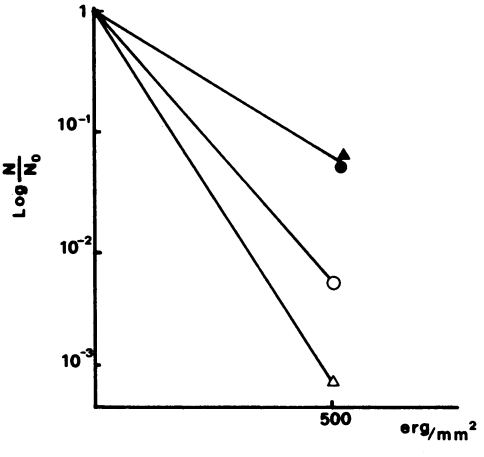

FIG. 1 - U.V. resistance induced by Mu in various strains. - A single total dose of 500 ergs/mm^2 was given.
▲ lig$^+$; ● ligts7 (Mu cts62), induced;
O ligts7 (Mu cts62), not induced;
Δ ligts7.
In both cases bacterial cultures were

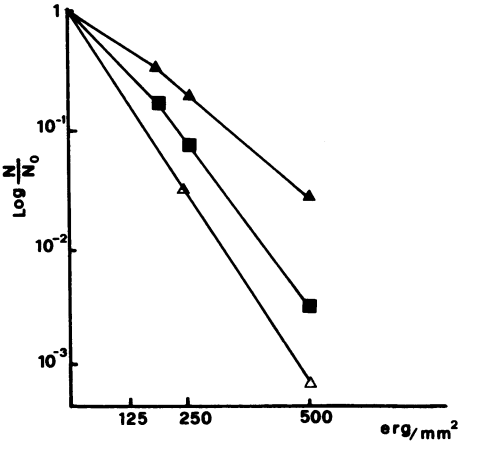

FIG. 2 - U.V. resistance in lysogenic strains related to the site of insertion of the prophage. - Irradiation was in the form of a cumulative dose.
▲ ligts7 mal ::Mu; ■ ligts7 man::Mu;
Δ ligts7.
prepared as previously described (3).

inserted in gene maltose A, proximal to the origin, the other in mannose, a distal gene: they follow the genetic dosage existing between these two genes, according to the equation of Sueoka and Yoshikawa (32).

Heat resistance of ligts7 (Mu) strains.

Strains ligts7 (Mu) at 42° show a higher ratio of colonies/plated cells than the non lysogenic parental strain. The e.o.p. increment varies with the lysogens, being 10 to 100 times higher than the reversion value for the ligts7 mutation (see table 2). The surviving clones have been checked: they are still lig⁻. In addition, we have observed that, besides restoring an active growth at non-permissive temperature, Mu causes a protection effect against the lethal action of temperature. This effect becomes evident if we transfer and incubate at 32° plates with colonies previously fully grown at 42°: new colonies appear up to a total number 10 times higher than before. The non-lysogenic control does not show a similar phenomenon (data not shown).

Furthermore, we have studied the behaviour of the polylysogenic strain

TABLE 2 - Survival to heat treatment for various ligts7 (Mu) lysogens.

strain nr.	relevant genotype		e. o. p. at 32°	e. o. p. at 42°	42°/32°
1160	ligts7	a)	1.6×10^8	1.0×10^3	6.2×10^{-6}
		b)	1.0×10^8	5.7×10^2	5.7×10^{-6}
		c)	1.8×10^8	1.1×10^3	6.1×10^{-6}
R239	ligts7 (Mu c^+)		1.0×10^8	7.5×10^3	7.5×10^{-5}
R240	" "		9.6×10^7	6.9×10^4	7.0×10^{-4}
R241	" "		1.2×10^8	1.0×10^4	8.0×10^{-4}
R237	ligts7 / RP4		1.0×10^8	3.0×10^2	3.0×10^{-6}
R238	ligts7 (Mu cts62) / RP4 (Mu c^+)		1.1×10^8	5.1×10^7	4.6×10^{-1}
R244	ligts7 / pZP2		1.0×10^8	1.0×10^5	1.0×10^{-3}
R246	ligts7 galE		2.0×10^8	1.1×10^3	5.8×10^{-6}
R247	ligts7 galE :: Mu c^+		2.2×10^8	1.1×10^4	5.0×10^{-5}

Strain cultures were grown to 10^8 at 32° in LB with aeration. Serial dilutions were made from each culture and each dilution plated on LA in duplicate. One set was incubated at 32°, the other at 42°. Three independent measurements are reported for the parental strain 1160 to show the minimal variation in behaviour.

R238. This strain is a derivative of R235 ligts7 mal :: Mu cts62 which carries an RP4 (Mu c^+) plasmid derived from R198, i.e. harbors more than one phage genome. Its e.o.p. at 42° is 45% of that at 32°, but full growth of the colonies is achieved only after about 60 hours. However, if the strain is grown at 42° in broth and plated at 32°, a high rate of mortality becomes evident: the kinetics of growth give only 10% of survival after 4 hours (see fig. 3). The two results may represent the same situation due to plasmid abundance and segregation.

Control of the expression of Mu gene 'lig'.

Mutations in gene epimerase of the gal operon are lethal in the presence of galactose, possibly because of the accumulation of UDPgal. Since the first gene of the galactose operon codes for galactose epimerase and since this enzyme catalyses the last step of the galactose metabolism (19), the insertion of Mu in any gene of the gal operon causes a polar mutation which prevents such an accumulation.

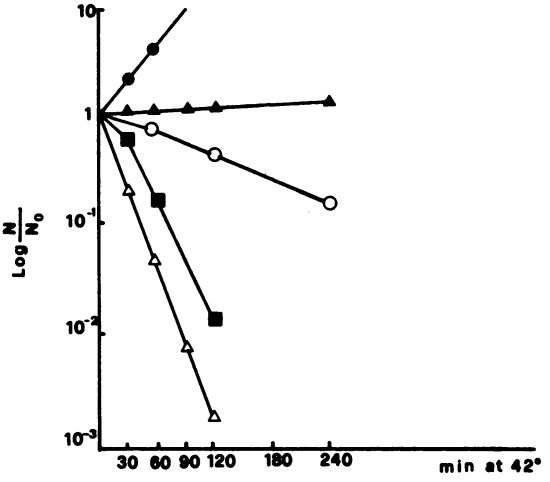

FIG. 3 - Growth kinetics at 42° of ligts7 (Mu) lysogenic strains.

Bacterial cultures were grown in LB at 32° in aeration to 10^8 cells/ml, diluted 1 : 100 in fresh broth, and then transferred at 42° (= T 0'). Standard aliquots of each culture were withdrawn at regular intervals and plated on LA at 32°. - ● lig^+; ▲ ligts7 / pZP2; ■ ligts7 (Mu c^+); o ligts7 mal :: Mu cts62 / RP4 (Mu c^+); Δ ligts7.

A culture of R246 has been infected with Mu $\underline{c}^+$ at 30° and surviving clones have been selected at 42° on M9 supplemented with lactose. The results obtained with one of these clones, R247, are shown in table 3. It can be seen that the e.o.p. of the lysogenic strain is increased by near a factor of 10 on the inductor containing medium (column IV); such a difference is not observed with the non lysogenic strain. It has been verified that the e.o.p. at 30° is identical on any medium (data not shown).

Columns II and III show an increased survival at 42° of R247 strain when grown in M9 as compared to LB. We have no explanation for this observation; however it can be remembered that ligts7 mutants behave differently according to the culture medium: for example, survival at 42° depends strongly on the culture medium (2, 20).

Survival in LB at 42° (column III) of the lysogenic strain tested is compatible with the data obtained with other strains lysogenic for Mu $\underline{c}^+$.

$\underline{Gal}^+$ ligts7 recombinants have been obtained by crossing R247 with a $\underline{gal}^+$ $\underline{lig}^+$ non lysogenic Hfr. They all are sensitive to bacteriophage Mu. Four of these recombinants have been tested for temperature sensitivity: they all behave like R246 in table 2.

All these results agree with the idea of Mu c^+ inserted inside the gal operon and certain of its functions expressed under the control of the promoter of the gal operon.

TABLE 3 - Expression of gene Mu 'lig'.

strain nr.	relevant genotype	rate of number of colonies on M9GF at 42° / M9 at 30°	M9 at 42° / M9 at 30°	L at 42° / L at 30°	I / II
		I	II	III	IV
R246	ligts7	1×10^{-4}	1×10^{-4}	1×10^{-6}	1
R247	ligts7 galE :: Mu c^+	1×10^{-1}	1×10^{-2}	5×10^{-5}	10

Each strain was grown to 10^8 and plated in the same medium. While in the exponential phase, it was spread on agar plates and incubated at the chosen temperature. - M9 = M9 glucose; M9GF = M9 glucose complemented with fucose 2×10^{-3} M as unmetabolized inducer of gal operon.

T4 lig⁻ growth in strains ligts7 (Mu).

A spot test showed that lig⁻ mutants of phage T4 are able to lyse ligts7 (Mu) strains used as indicators; lysis is stronger on the polylysogenic strain R238. On the contrary, it is proved that amber mutants of the T4 gene for ligase give a reduced burst in bacterial strains of the type lig⁺ pm⁻ (21).

We have compared the burst of a T4 lig⁻ lysate on R248 lig⁺ pm⁻ (Mu cts62), when the prophage is induced, and on 1159 the parental non lysogenic strain. As shown in table 4, the presence of Mu prophage increases about 10 times the burst of phage T4 lig⁻.

Cloning of gene Mu 'lig'.

Restriction endonucleases HindIII, EcoRI and BglII cut the genome of phage Mu producing various fragments as shown in fig. 4A. Cuts produced by HindIII, EcoRI and BamHI enzymes in pBR322 genome are shown in fig. 4B.

Hybridization between phage and plasmid fragments is possible between sites HindIII and sites EcoRI, and between the sites BglII and BamHI.

Recombinant plasmids have been obtained, purified and tested for their transforming ability on E. coli ligts7 for both ampicillin and heat resistance. The results reported in table 5.I clearly show that gene Mu 'lig' is included

TABLE 4 - T4 infection and lysis.

strain number	relevant genotype	T4 genotype lig⁻	T4 genotype lig⁺
a)	spot test		
1160	ligts7	-	+++++
R239	ligts7 (Mu c⁺)	+	+++++
R238	ligts7 (Mu cts62) / RP4 (Mu c⁺)	++	+++++
1159	lig⁺ pm⁻	+++	+++++
C600	lig⁺ pm⁺	+++++	+++++
b)	burst size		
1159	lig⁺ pm⁻	1	100
R248	lig⁺ pm⁻ (Mu cts62)	10	100

a) - = no lysis; +++++ = full lysis; +, ++, +++ = intermediate strength of lysis on the spot.
b) for procedure of the experiment see materials and methods.

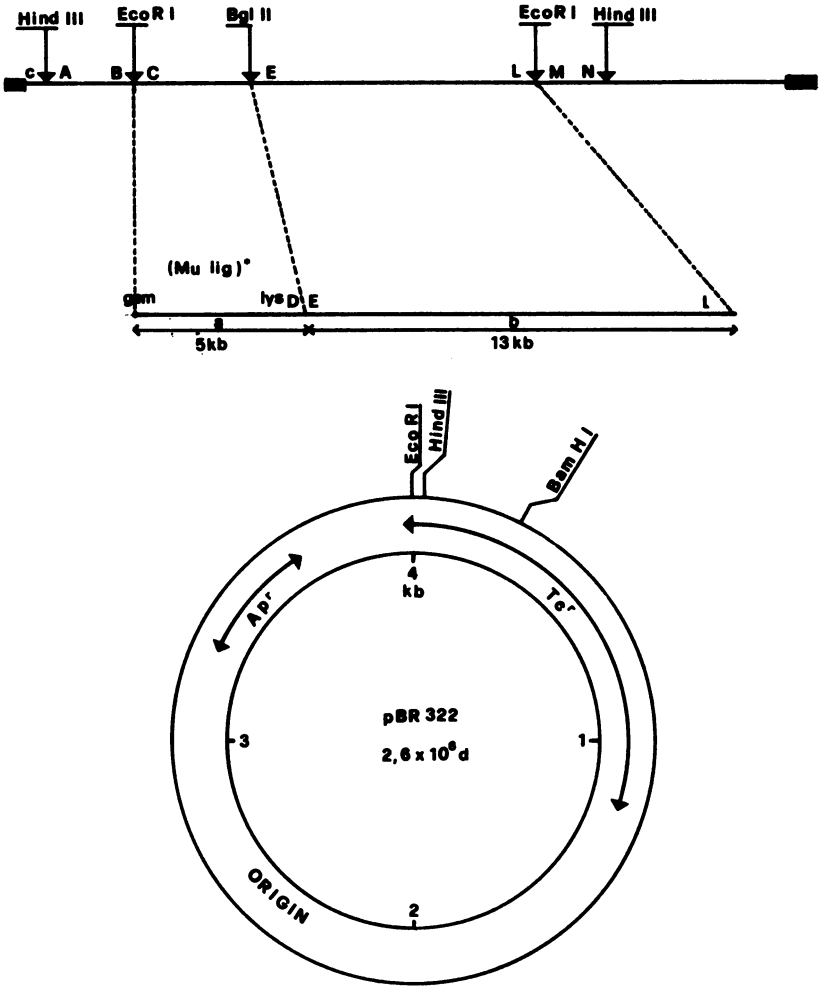

FIG. 4 - Restriction enzymes maps. A - Mu bacteriophage, redrawn after (26, 27). B - pBR322, redrawn after (28).

in the fragment of Mu genome defined by EcoRI sites.

EcoRI and BglII produce two fragments out of Mu genome: one of 5 kb between genes gam and lys and one of 13 kb between genes E and L (see fig. 4A.II); while in pBR322 they cut out a fragment of about 0.3 kb in the gene of tetracycline resistance (see fig. 4B). The corresponding recombinant

TABLE 5 - Transforming ability of Mu fragments produced by different restriction enzymes.

selective conditions for transforming ability	fragments produced by I			II	
	HB	HE	EB	EB a	EB b
amp^R at 32°	+	+	+	+	+
amp^R at 42°	+	-	+	+	-

I - Mu-pBR322 recombinant plasmids obtained cutting the genomes with either HindIII and BglII (HB) or with HindIII and EcoRI (HE) or with EcoRI and BglII (EB) have been tested for their ability to transform 1160 for ampicillin resistance at 32° and 42°. II - The two recombinant plasmids obtained cutting with EcoRI and BglII have been separated on agarose gel and tested for their transforming ability.

plasmids have been separated on agarose gel and tested for their transforming ability. The results are shown in table 5.II. We can conclude that gene 'lig' is included in the smaller of the two fragments, carried by the pZP2 recombinant plasmid, because it is the only one which by transformation confers both heat and ampicillin resistance.

Alkaline sucrose density gradients.

Figure 5 shows ligation kinetics of Okazaki fragments at 30° and 42° in strain 1160 and in R244 which harbors the pZP2 plasmid. 1160 gives results similar to those obtained by Gottesman et al. (21) with the same strain: there is a strong delay in the ligation of Okazaki fragments even at permissive temperature. Strain R244 has a composite behaviour: the major part of the Okazaki fragments are immediately ligated at any temperature, but at permissive temperature a small amount of the radioactivity remains at the top of the gradient, while at non permissive temperature the same radioactivity seems to migrate slowly.

The parental lig^+ 1159 strain shows an immediate ligation of Okazaki

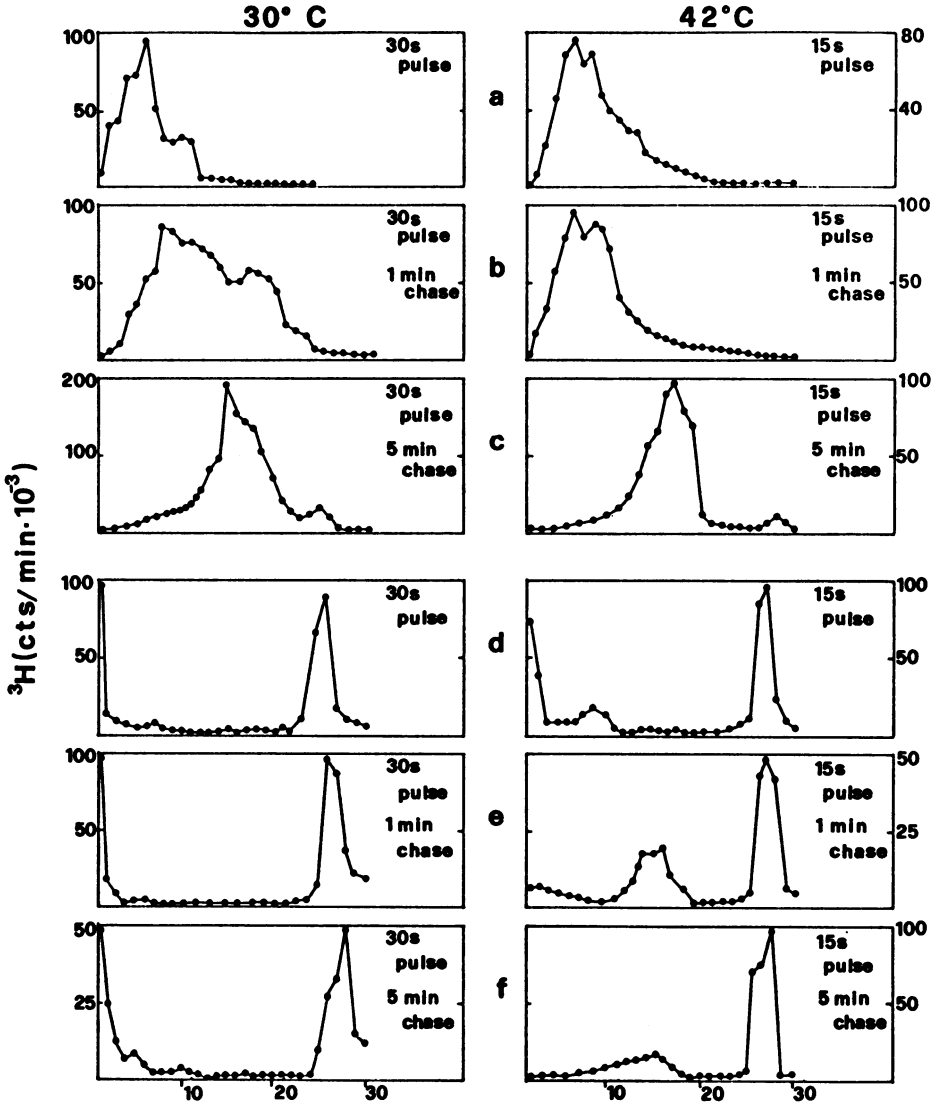

FIG. 5 - Alkaline sucrose density gradients. - DNA pulse labelled at 30° and 42° was extracted from strains 1160 and R244. The experimental procedure was exactly as described by Konrad et al. (2). Gradients were collected from the top. - (a), (b) and (c) = 1160 ; (d), (e) and (f) = R244.

fragments at any temperature.

DISCUSSION

Rescue of the mutation ligts7 in E. coli K12 due to phage Mu infection was reported in a previous paper (3).

Two new results suggested that, even as a non-induced prophage, Mu has the possibility to express the same function in ligts7 strain derivatives: Mu cts62 lysogens are resistant to U.V., while Mu lysogens with a non-thermosensitive repressor show a higher survival to heat treatment and permit T4 lig⁻ superinfecting phages to complete the lytic cycle.

By analogy of function we have named 'lig' the gene of Mu which product is able to substitute the ligase of both E. coli and T4; however the activity of the gene should not be restricted only to this function.

Finding the 'complementation' effect reproduced also by a non-derepressed prophage opens the problem of determining optimal conditions for the maximal expression of the gene involved, even as a prerequisite for the molecular purification and identification of its product.

The highest levels of expression we have found occur in the polylysogenic strain R238, in strain R247 after induction of the galactose operon and in strain R244 which harbors the recombinant plasmid pZP2.

Strain R238 has an efficiency of plating at 42° as high as 45% of that at 32°, but has a quite low rate of survival when grown in broth at 42°. The apparent disagreement between the two observations is due to the high rate of segregation of the plasmid in strain R238 at 42°. In absence of the plasmid protection effect, both the Mu cts prophage induction and the bacterial ligts7 mutation can kill the cell. In fact, in broth at 42° we register the segregation, i.e. killing of segregating cells; on the contrary, inside each colony growing on plate reinfection by segregant plasmids is easily achieved so that we register the survival of the colony as a whole.

Hence, since R238 is a polylysogenic strain and the survival rate seems connected with the segregation rate, there is the possibility that the e.o.p. depends on the number of the prophage genomes, for example episome copies.

In fact, in table 2 it is clearly shown that parental non lysogenic strains (1160, R237) have an e.o.p. ratio of 5×10^{-6}; in single lysogens such as R239, R240, R241 the ratio may go up to 7×10^{-4}, while in strains harboring more than one copy of Mu genome as in R238 and R244 or as in strain R247 in which the prophage expression is under the control of the gal operon, the rate may reach the value of 5×10^{-1} (see tables 2 and 3).

Strain R244, as a carrier of the plasmid pZP2, should have shown the highest rate of grawth, according to the idea of survival to heat exposure in strains ligts7 (Mu) as a function of the number of prophage genome copies. The value of 10^{-3} in table 2 is connected with the apparent slowness in multiplication of this strain; in fact, both are due to the high rate of segregation of the plasmid in a ligts7 strain.

Strain R247 is very interesting because it opens the problem of the control of gene Mu 'lig' expression. This strain is a ligts7 lysogen with the Mu prophage inserted in the gal operon. When the operon is induced, active bacterial growth due to the phage product is restored at 42°.

Actually, in glucose inducing catabolite repression, the induction of the gal operon produced by fucose is less effective than that obtained when using, for example, glycerol. However we could not use glycerol minimal medium in our experiments because of the very poor growth of our strains in this medium even at permissive temperature.

We can postulate that a terminator of phage transcription is located before the promoter of the phage gene 'lig'; upon prophage induction the terminator action may be cancelled. For example, a phage product expressed after the gal operon induction could function as an antiterminator.

If this hypothesis is correct, R247 could be very useful for the selection of regulator mutants in gene 'lig' of Mu.

The recombined plasmid pZP2 has been engineered to map the phage gene 'lig' and to isolate its product. The Mu fragment carried by pZP2 contains a functional phage 'lig' gene, because, when transferred, it confers to strain 1160 the heat resistance phenotype.

The results shown in table 5.II clearly indicate that the gene 'lig' is associated in the recombined plasmid with the Mu fragment of 5 kb delimited by

genes gam and lys. As a support to this conclusion there are several mutants of gene 'lig', recently isolated, which map between genes B and lys (31).

The data presented in this paper complete those previously published (3) and allow us to conclude with the idea that the product of gene 'lig' in Mu has, besides other possibilities, specific ligase functions.

In fact, the data show that Mu, while it is able to produce a substitute for the ligase of both E. coli K12 and T4, also offers a protection against the lethal action of high temperature (secondary growth at 32° on plates previously grown at 42° for ligts7 (Mu) strains). This effect could be due to a protection against DNA degradation, as was already suggested in our previous paper (3).

At permissive temperature, a ligts7 strain does not degrade its DNA detectably (22); delay in ligation at permissive temperature (fig. 5) cannot be attributed to an abnormally high nuclease activity but preferentially to a defect in ligase activity (21). The normal ligation of the major part of the Okazaki fragments observed in R244 strain must be attributed to an increased ligase activity which should persist even at non permissive temperature giving a normal ligation of Okazaki fragments (see fig. 5).

Experiments are performed at present to determine the function of R244 responsible for its composite behaviour in the ligation of the Okazaki fragments.

Until now, the product of Mu gene 'lig' seems to be the only phage product involved in replication for which a definition in enzymatic terms can be given; in fact, genes A and B products are described in that they are involved with phage DNA replication but not in biochemical terms (23, 24, 25).

A more precise definition of the specific role of lig function in Mu can be given upon studying the behaviour in both replication and integration of the corresponding mutants.

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