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Method for Determining Whether a Gene of *Escherichia coli* Is Essential: Application to the *polA* Gene

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We have developed a general method for determining whether a gene of *Escherichia coli* is essential for viability. The method requires cloned DNA spanning the gene in question and a reasonably detailed genetic and physical map of the cloned segment. Using this information, one constructs a deletion of the target gene in vitro. For convenience, the deletion can be marked by an antibiotic resistance gene. A DNA segment containing the deletion is then cloned onto an *attΔ* phage λ vector. Integration of this phage, by homologous recombination at the target locus, and subsequent excision provide an efficient route for crossing the marked deletion onto the bacterial chromosome. Failure to delete the target gene indicates either that the resulting deletion was not viable or that the desired recombinational event did not take place. The use of prophage excision to generate the deletion allows one to estimate the fraction of deletion-producing events by analysis of the other product of the excision, the phage produced on induction of the prophage. In this way one can determine whether failure to recover a particular chromosomal deletion was due to its never having been formed, or, once formed, to its failure to survive. Applying this method to the *polA* gene, we found that *polA* is required for growth on rich medium but not on minimal medium. We repeated the experiment in the presence of plamids carrying functional fragments of the *polA* gene, corresponding to the 5′-3′ exonuclease and the polymerase–3′-5′ exonuclease portions of DNA polymerase I. Surprisingly, either of these fragments, in the absence of the other, was sufficient to allow growth on rich medium.

The most convincing proof that a gene product is nonessential is provided by showing the viability of a strain from which the relevant gene has been deleted. However, the inability to obtain a deletion of a particular gene does not necessarily prove that the gene is essential. It could result simply from a low frequency of the events leading to the desired deletion. Moreover, the presence of a nearby essential gene could influence the outcome of the experiment. We have developed a general experimental strategy that allows us to address these two possibilities and therefore to determine whether the failure to recover a particular deletion is due to lethality. In this paper, we describe the use of this method in our studies of the *polA* gene of *Escherichia coli*.

The *polA* gene is located at 86.6 min on the *E. coli* genetic map (1). Its gene product, DNA polymerase I (PolI), is a complex enzyme which functions both in excision repair and in the processing of Okazaki fragments into high-molecular-weight DNA (24). Partial proteolysis experiments suggest that PolI has two relatively independent functional units, a small N-terminal domain containing the 5′-3′ exonuclease activity and a larger C-terminal domain having the polymerase and associated 3′-5′ exonuclease activity (subsequently called Klenow fragment) (3, 22). A number of *polA* mutants have been described that are deficient in one or more of the enzymatic activities of PolI. However, analysis of these mutants has failed to provide conclusive evidence as to whether PolI is essential for cell viability. An obvious difficulty in interpreting the data on these mutants is caused by the multifunctional nature of PolI; many of the mutant proteins retain some enzymatic functions to almost wild-type levels. For example, the two amber mutations (*polA1* and *resA1*) produce isolable amber fragments having substantial 5′-3′ exonuclease activity (19, 25). The viability of these mutants, even in strongly nonsuppressing backgrounds (19), therefore argues against an essential role for the whole polymerase but does not rule out an essential function for the 5′-3′ exonuclease. The importance of the 5′-3′ exonuclease has also been inferred from the existence of conditional lethal *polA*(Ts) mutations in which this activity is either absent or is thermolabile (26). In this work, we have investigated two questions. First, can a strain having a deletion of *polA* be viable, and second, what contribution does each of the functional domains make to viability?

MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from New England Biolabs and Boehringer-Mannheim Biochemicals. T4 DNA ligase was from Collaborative Research, Inc., and Boehringer-Mannheim Biochemicals. Oligonucleotide linkers were from Collaborative Research, Inc., and New England Biolabs.

Media. The media used are described by Davis et al. (8). Kanamycin was used at 40 μg/ml, chloramphenicol was used at 20 μg/ml, methyl methanesulfonate (MMS) was used at 0.06%, and isopropyl-β-D-thiogalactopyranoside (IPTG) was used at 1 mM.

Plasmids. The vectors used in our plasmid constructions were pNG16 (10), a deletion derivative of pBR322, and pCJ56, in which the *Bam*HI site of pNG16 is replaced by a *Sac*I linker. pCJ1 contains a 5-kilobase-pair (kb) *Hin*dIII fragment carrying the *polA1*(Am) allele cloned in pNG16 (17). pGH54 contains the kanamycin resistance (Km^r) gene of Tn*903* (nucleotides 1,082 to 2,038 [28]), cloned with *Bam*HI linkers into the *Bam*HI site of pNG16 (31).

Since the $polA^+$ allele is unstable on a multicopy plasmid (18), we propagated the wild-type gene as two separate *Hin*dIII-*Sac*I clones in the vector pCJ56; pDF2 contains the 2.8-kb (upstream) fragment, and pDF1 contains the 2.1-kb (downstream) fragment. pGH78 was derived from pDF1 by

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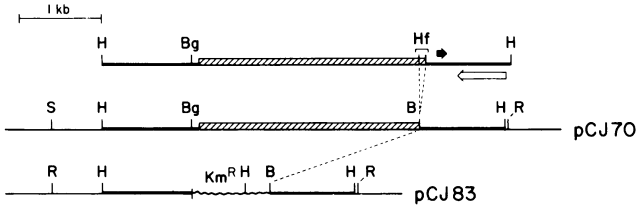

FIG. 1. Construction of a marked *polA* deletion. The top line represents the 5-kb *Hin*dIII fragment that carries *polA*. The hatched region indicates the *polA* coding sequence, reading from left to right. Two other genes encoded by this fragment are shown, *spf* (black arrow) and the gene for a 22-kilodalton protein (open arrow) (15). The lower lines represent plasmids described in the text. In each case, the vector pNG16 is indicated by a thin line and is not shown in its entirety. The *polA* flanking sequences are shown as thick lines, and the Km^r fragment is shown as a wavy line. Restriction sites relevant to this work are marked as follows: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Hf, *Hin*fI (only two sites are shown); R, *Eco*RI; S, *Sal*I.

insertion of a *Bam*HI linker in the *Alu*I site 46 base pairs (bp) beyond the end of the *polA* coding sequence. Plasmids pDF1 and pDF2 were kindly provided by David Fujii, and pGH54 and pGH78 were provided by Graham Hatfull.

In vitro construction of a genetically marked *polA* deletion. Figure 1 shows the important features of the plasmids used in this construction. pCJ70 was derived from pCJ1 by destroying the vector *Bam*HI site and inserting a *Bam*HI linker in place of the small *Hin*fI fragment at the end of the *polA* coding region (nucleotides 2,701 to 2,788 [17]). The large *Bam*HI-*Bgl*II fragment of pCJ70 (containing the vector and *polA* flanking sequences) was purified by agarose gel electrophoresis and ligated to the 950-bp *Bam*HI fragment from pGH54 carrying the Km^r gene. After transforming strain S165 (*his* Δ*galS165 rpsL*) to kanamycin and ampicillin resistance, we obtained a plasmid in which the *polA* structural gene had been replaced by the Km^r gene, transcribed in the same direction. The *Sal*I site within the vector portion of this plasmid was replaced by an *Eco*RI linker, and the resulting 3.7-kb *Eco*RI fragment carrying the *polA* deletion was recovered by cloning into the *Eco*RI site of pNG16 (to give pCJ83) and then introduced into the *Eco*RI vector λgt1-λB (4). The structure of the resulting phage (λCJ83) was verified by Southern blot analysis.

Isolation and analysis of lysogens. Lysogens of λCJ83 in strain CM4722 [F$^+$ Δ(*gal-bio*) *thi-1 relA1 spoT1*] were selected at 30°C on LB plates seeded with λ *ch*80del9 (27) and subsequently checked for λ immunity and kanamycin resistance. Chromosomal DNA from candidate lysogens was analyzed by Southern blots to determine the structure at the *polA* locus.

Curing of lysogens. Lysogens of λCJ83 were grown overnight at 30°C in LB. Appropriate dilutions were plated on LB at 30°C to determine the total number of viable cells and at 42°C to obtain cured cells. Since the 42°C incubation results in considerable release of phage, we usually included 1 mM EDTA in the 42°C plates to minimize phage attack on the cured survivors. (We checked that the inclusion of EDTA merely aided in the recovery of cured cells and did not in any way affect the outcome of the experiment). The proportion of survivors that retained kanamycin resistance was determined by replica plating. The alternative approach of plating the lysogen culture directly on kanamycin at 42°C (with or without EDTA) often gave as much as a 10-fold-lower frequency of Km^r cells, presumably owing to poor plating efficiency on kanamycin under these conditions. Chromosomal DNA isolated from cured cells was analyzed by Southern blots.

Analysis of phage. A sample of the same lysogen culture used in the curing experiment above was diluted 10-fold in broth and grown at 30°C to mid-log phase. The phage was induced by shifting the culture to 42°C for 30 min followed by growth at 37°C until the cells lysed (1 to 2 h). The lysate was treated with chloroform and then titrated on strain LE392 (*lacY galK galT metB trpR supE supF hsdR*) at 30°C. Lysogens were picked from the center of about 100 plaques and propagated on a plate seeded with λ *ch*80del9 at 30°C. The proportion of phage that transduced Km^r was determined by replica plating the lysogen grid.

In vitro construction of F′ *polA* derivatives. In all of these constructions, we inserted the desired *polA*-containing fragments into the unique *Hin*dIII site of pOX38 (13). A chloramphenicol resistance (Cm^r) gene inserted downstream from the *polA* sequences provided a selectable marker on the resulting F derivative. The Cm^r gene was isolated from pACYC184 (5) as an *Hae*II fragment (ca. 1.5 kb) and cloned with *Bam*HI linkers into the *Bam*HI site of pGH78 to give pCJ89 (see Fig. 2). F′ *polA*$^+$ contains the upstream *Hin*dIII-*Sac*I fragment (2.8 kb) of the wild-type gene (from pDF2) and the downstream *Sac*I-*Hin*dIII fragment (3.6 kb) from pCJ89 (Fig. 2A).

To construct F′ 5′ Exo, which can only produce the 5′-3′ exonuclease activity of PolI, we made use of a plasmid carrying a *polA* deletion, ΔC1546. This plasmid carries the *polA1* amber allele, which produces an active 5′-3′ exonuclease fragment; in addition, formation of any readthrough product is eliminated by deletion of all *polA* sequences beyond nucleotide 1,546. The deletion endpoint is marked by a *Bam*HI site, which we used for insertion of restriction fragments derived from pCJ89, carrying Cm^r and the flank-

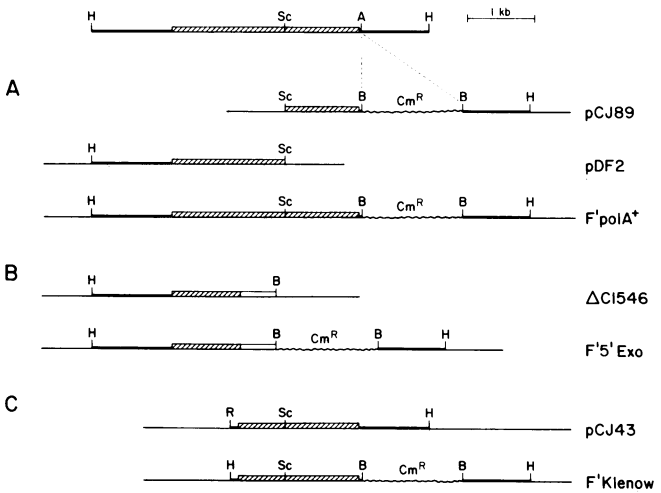

FIG. 2. Construction of F′*polA* derivatives. The top line represents the 5-kb *Hin*dIII fragment that carries *polA*. The rest of the diagram shows plasmid structures involved in the construction of (A) F′ *polA*$^+$, (B) F′ 5′ Exo, and (C) F′ Klenow. Throughout, the thin lines indicate vector DNA, and the thick lines represent the *polA* flanking sequences. The Cm^r fragment is shown as wavy lines. The *polA* coding sequence is boxed; the hatched regions show functional translated sequences, and the open regions indicate untranslated sequences beyond the *polA1* amber mutation. Restriction sites relevant to this work are marked as follows: A, *Alu*I (only one site is shown); B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; Sc, *Sac*I.

ing DNA downstream of *polA*. The resulting 5.3-kb *Hin*dIII fragment was then inserted into pOX38 (Fig. 2B).

To construct F′ Klenow, which contains only the polymerase and 3′-5′ exonuclease regions of the *polA* gene, we started from plasmid pCJ43 which carries a functional gene for the Klenow fragment portion of PolI, expressed from the *lacUV5* promoter (16). We replaced the *Eco*RI site upstream of the *lac* promoter with a *Hin*dIII linker and then attached the upstream portion of the Klenow fragment gene to the downstream *Sac*I-*Hin*dIII fragment of pCJ89. The resulting 4.4-kb *Hin*dIII fragment was then cloned into pOX38 (Fig. 2C).

In each of these experiments, the resulting Cm^r transformants (in strain LE392) were screened for sensitivity to phage R17 and for the ability to transfer the Cm^r marker. The extent of the *polA* sequences carried on each F′ was verified by Southern blot analysis after digestion with *Hin*fI, *Hae*III, and *Sau*96I, each of which gives fragments clearly diagnostic of the upstream or downstream portions of the *polA* gene. The essentially complementary nature of the F′ 5′ Exo and F′ Klenow constructs was also shown by their ability or inability to rescue certain *polA* mutations whose position within the gene is known (17, 19, and unpublished data).

The F derivatives obtained in this way were transferred into the λCJ83 lysogens used in the curing experiment, selecting for Km^r Cm^r exconjugants. (Since the lysogens were derived from strain CM4722, which is F^+, we used saturated cultures of the recipients to provide F^- phenocopies.)

DNA preparation and analysis. DNA, of both multicopy plasmids and F derivatives, was prepared by the alkaline lysis method (2). Phage and bacterial DNAs were prepared by the rapid procedure described by Davis et al. (8), except that we omitted the diethyl pyrocarbonate treatment and phenol extracted the DNAs before use.

Phage, bacterial, and F′ DNAs were analyzed by Southern blotting, with nick-translated pCJ1 as probe to identify *polA*-containing bands.

RESULTS

Our strategy for determining whether cells having a deletion of a particular gene remain viable is illustrated in Fig. 3, with *polA* used as an example. First, the gene of interest must be available on a cloned fragment that also contains DNA from both the upstream and downstream flanking regions. A marked deletion of the gene is then constructed by incorporating a kanamycin resistance (Km^r) marker between the flanking DNA sequences. This construction is cloned into a phage λ vector that lacks the normal attachment site and is thermoinducible. At the permissive temperature the resulting phage can form lysogens by homologous recombination at the target locus, giving a structure in which the integrated prophage is flanked on one side by a wild-type gene and on the other by a deletion which is marked by Km^r. Spontaneous curing of such a lysogen can take place by a subsequent homologous recombination. Depending on the position of the recombinational crossover, this will either regenerate the wild-type configuration or else leave behind the Km^r-marked deletion. Failure to recover cured cells that retain kanamycin resistance will indicate that cells carrying the deletion are nonviable, provided that it can be established that the recombinational event producing such cells actually took place. Although it is not possible to monitor this event directly, the relative frequency of recombination within the two flanking regions can be inferred by analysis of the progeny phage produced on induction of the same lysogen. The production of phage which do not transduce Km^r is diagnostic for the recombinational event that leaves a deletion of the target gene in the bacterial chromosome during the curing experiment (Fig. 3).

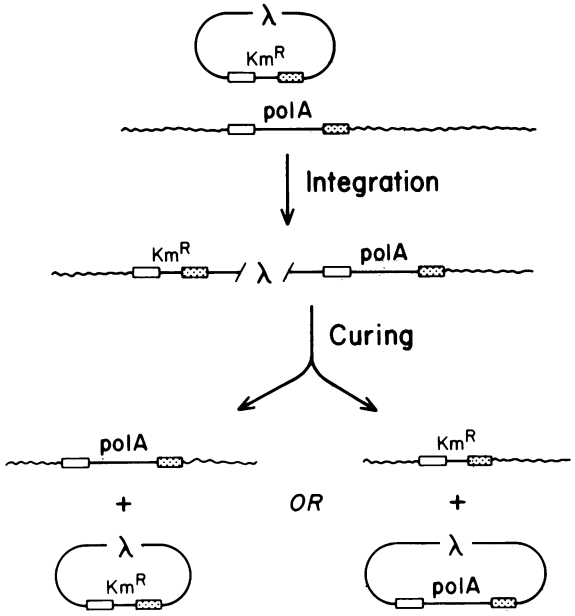

FIG. 3. Method for determining whether the *polA* gene can be deleted. Phage integration and excision are used to cross a *polA* deletion onto the bacterial chromosome. For details see the text. The regions of homology between the phage and the chromosome are boxed; the open boxes indicate the upstream flanking DNA, and the stippled boxes indicate the downstream flanking DNA. Chromosomal DNA external to these regions of homology is shown as wavy lines. (Note that only one of the two possible integrated structures is shown.)

Phage and lysogen structure. The in vitro construction of λCJ83 carrying a marked *polA* deletion is described in detail above and in Fig. 1. For this construction, we chose deletion endpoints that coincided as nearly as possible with the functional limits of the *polA* gene, to avoid leaving behind potentially functional polymerase fragments and yet to leave intact any genes within the flanking regions.

Lysogens of λCJ83 in strain CM4722 were selected, and the DNA from six of them was analyzed by Southern blots to determine the structure at the *polA* locus. Digestion with *Eco*RI and *Sal*I can distinguish between lysogens formed by homologous recombination in the upstream flanking region (structure A) and those formed by recombination within the downstream flanking region (structure B) (Fig. 4). We chose one lysogen of each structure (designated 83A and 83B) for the curing experiment. The main purpose of the Southern blot analysis (Fig. 5) was to eliminate lysogens of the type shown in lane 3, which have the wild-type configuration at *polA* (compare lane 1) and the 3.7-kb band that corresponds to the cloned *Eco*RI fragment on λCJ83 (lane 2). These lysogens presumably arise by integration of the phage elsewhere on the bacterial chromosome and cannot therefore be used in the curing experiment. Another lysogen (lane 7) shows bands indicative of integration at *polA* (structure A) but has an additional, more intense band at 3.7 kb. We interpret this structure as a polylysogen, probably having

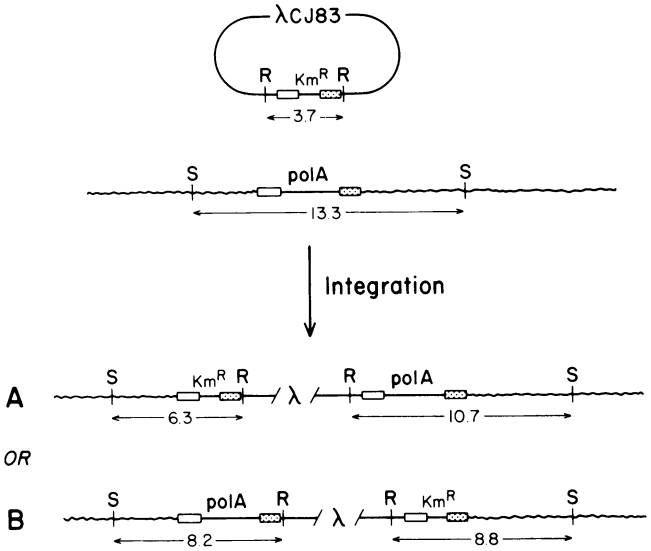

FIG. 4. Integration of λCJ83. Two alternative structures, designated A and B, can result from integration of λCJ83 via homologous recombination at the *polA* locus. The upstream flanking DNA carried on λCJ83 is represented by the open boxes, and the downstream flanking DNA is represented by the stippled boxes. Chromosomal DNA external to the region of homology with λCJ83 is shown as wavy lines. The positions of relevant *Eco*RI (R) and *Sal*I (S) sites are indicated, along with the sizes (in kb) of fragments that would hybridize to a pCJ1 probe.

tandemly integrated phage genomes at the *polA* locus. Although the curing experiment can be carried out on such polylysogens, we prefer to exclude them since the input phage (λCJ83) will be overrepresented in the induced phage lysate.

Curing of lysogens. Spontaneous curing of the prophage from the recombination-proficient lysogens 83A and 83B took place at a frequency of about 10^{-4} during nonselective growth at 30°C. Cured cells were selected by plating at 42°C and then tested by replica plating on kanamycin. If the recombination that results in excision of the prophage could occur with equal frequency in either of the two flanking regions, then 50% of the cured cells would be Km^r. In fact, no Km^r-cured cells were obtained (Table 1), indicating either that the associated *polA* deletion is lethal or that all the recombinational events took place within just one of the flanking regions. (The latter explanation is unlikely since it would mean that recombination took place exclusively within the upstream flanking region for lysogen 83A and within the downstream region for lysogen 83B; [Fig. 3 and 4].) We determined the relative recombination frequencies in the two flanking regions by examining the other product of the excision event, the phage produced on thermal induction of the same lysogen culture. Only a fraction of the resulting lysate was able to transduce Km^r (Table 1). (Moreover, the phage that did not carry Km^r transduced *polA*$^+$, as predicted from Fig. 3.) The recovery of both types of phage indicated that excision was taking place via both of the routes shown in Fig. 3 and allowed us to conclude that the absence of Km^r-cured cells resulted from the inability of cells with a *polA* deletion to survive under the conditions of the experiment.

An alternative strategy for establishing the relative amounts of recombination in the two flanking regions is to determine the numbers of *polA*$^+$ Km^s and Δ*polA* Km^r cells that are obtained when the curing is carried out with an additional copy of the wild-type gene in *trans* so that there is no disadvantage associated with the chromosomal *polA* deletion. To do this, we introduced into lysogens 83A and 83B an in vitro-constructed F′ that carries both *polA*$^+$ and a chloramphenicol resistance (Cm^r) gene as a selectable marker (F′ *polA*$^+$). When we repeated the curing in the presence of this F′, at least half of the cured cells were Km^r (Table 1). Figure 6 shows a Southern blot of a *Hin*dIII digest of cured cells derived from lysogen 83A with and without F′ *polA*$^+$. The result is exactly as predicted in Fig. 7; the wild-type chromosomal *polA* fragment was present in Km^s cells and missing from Km^r cells.

Table 1 indicates that the numbers of Km^r and Km^s cells obtained by curing in the presence of F′ *polA*$^+$ do not agree with the relative recombination frequencies inferred from the analysis of the corresponding induced-phage lysates. All of the phage lysates analyzed showed a deficit of Km^r-transducing phage. This is curious since, although one would not necessarily expect equal numbers of the two progeny phage (Fig. 3), one might expect the alternative lysogen

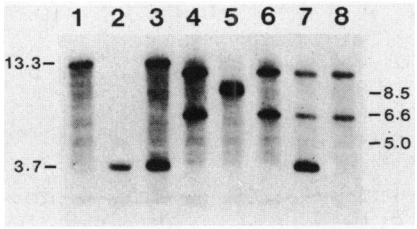

FIG. 5. Southern blot analysis of six λCJ83 lysogens (lanes 3 to 8). Chromosomal DNA from these lysogens was digested with *Eco*RI and *Sal*I, fractionated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pCJ1. Lane 1 contained DNA from the host strain, CM4722, and shows the 13.3-kb *Sal*I fragment diagnostic of an unrearranged *polA* locus. Lane 2 contained DNA from λCJ83 and shows the 3.7-kb cloned *Eco*RI fragment. The positions of other size markers (in kb) are indicated. Lanes 4 and 5 correspond to the lysogens 83A and 83B used in subsequent experiments.

TABLE 1. Curing of λCJ83 lysogens

Lysogen[a]	Medium	Cured cells		Phage[b]	
		Km^r	Km^s	Km^r	Km^s
83A	LB	0[c]	52	11	79
83B	LB	0[c]	59	7	89
83A F′ *polA*$^+$	LB	30	34	16	75
83B F′ *polA*$^+$	LB	53	18	6	92
83A F′ 5′ Exo	LB	50	40	14	62
83B F′ 5′ Exo	LB	57	33	11	86
83A F′ Klenow	LB	33	42	ND	ND
83A F′ Klenow	LB + 1 mM IPTG	46	37	ND	ND
83B F′ Klenow	LB	34	43	ND	ND
83B F′ Klenow	LB + 1 mM IPTG	57	23	ND	ND
83A	M9 glucose	28	49	ND	ND
83B	M9 glucose	36	48	ND	ND

[a] The structures of lysogens 83A and 83B are shown in Fig. 4.
[b] ND, Not determined.
[c] By replica plating larger numbers of survivors, we estimated the frequency of Km^r cells to be less than 1 in 10^3. The few Km^r survivors that were obtained were probably the result of reversion of the *c*I857 mutation since they remained λ immune.

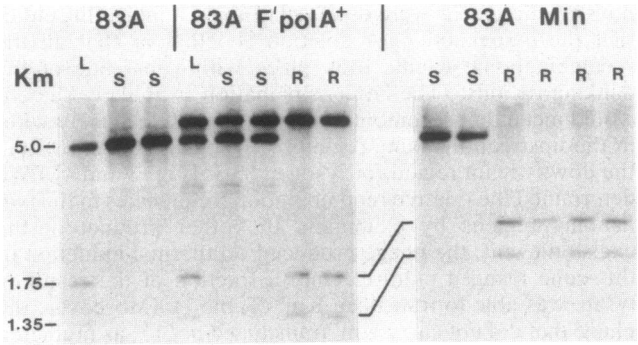

FIG. 6. Southern blot analysis of some of the survivors obtained in various curing experiments. In each case, chromosomal DNA was digested with *Hin*dIII, fractionated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pCJ1. The first three lanes show lysogen 83A (L) and two Km^s survivors isolated on broth plates at 42°C. The next five lanes correspond to curing of lysogen 83A in the presence of F′ *polA*$^+$. The starting lysogen (L) is shown, along with two Km^s and two Km^r survivors. (The largest hybridizing band in these samples corresponds to the 6.4-kb *polA*-containing fragment of F′ *polA*$^+$.) The final six lanes show two Km^s and four Km^r survivors obtained when curing of lysogen 83A was carried out in minimal medium.

structures 83A and 83B to give opposite biases in the composition of the phage lysate. DNA extracted from several of the phage lysates was analyzed by *Eco*RI digestion and Southern blotting. In every case, there was an excess of the *polA*$^+$ fragment relative to the Δ*polA* Km^r fragment, suggesting that at least some of the bias was due to a larger burst size of λ *polA*$^+$ compared with λ Km^r during the induction. (These phage are *red* and therefore dependent on host *polA* function; we speculate that the increased gene dosage of *polA* during growth of λ *polA*$^+$ may facilitate growth of this phage.) An additional bias in favour of λ *polA*$^+$ is probably introduced during the analysis of the lysate, a procedure that involves forming lysogens and testing their ability to grow on kanamycin (see above). The Km^r-transducing phage often gave very sparse patches on the lysogen master plate (presumably because the smaller extent of homology with the bacterial chromosome, compared with λ *polA*$^+$, reduced the efficiency of lysogenization). Consequently, any lysogens that failed to grow on the master plate (and were therefore omitted from the analysis) were more likely to have been Km^r than Km^s. Consistent with this idea, there appeared to be a slight increase in the proportion of Km^r-transducing phage if we picked plaques from our initial plate onto a lawn of sensitive cells at 30°C to allow the growth of larger plaques before attempting to pick and analyze lysogens. In another study, where we used the same approach to obtain a deletion of the *spf* gene, the phage analysis agreed well with the composition of the population of cured cells (G. Hatfull and C. M. Joyce, unpublished data). This observation also supports the preceding reasoning since the small size of the *spf* gene would be unlikely to cause a detectable difference in lysogenization efficiency between *spf*$^+$- and Δ*spf*-transducing phage. We therefore believe that the discrepancy in the phage analysis in the *polA* experiment is due to the particular structure and genetic constitution of the phage involved and does not indicate an inherent weakness in the general experimental design.

Curing in the presence of functional polymerase fragments. The experimental strategy described above, in which the curing was carried out in the presence of F′ *polA*$^+$, can also be used to determine whether either of the functional domains of PolI (5′-3′ exonuclease or Klenow fragment) can compensate for the absence of whole PolI from the cell. To do this, we supplied, on an F′, a manipulated copy of the *polA* gene that encoded one of these functions in the absence of the other and determined whether the presence of the corresponding PolI fragment allowed the recovery of Km^r cells in the curing experiment.

The cloning of the necessary fragments of the *polA* gene onto the F-derived vector, pOX38 (13), is described above and in Fig. 2. To construct a gene specifying solely the 5′-3′ exonuclease we made use of a cloned fragment that carries the *polA1* mutation (which produces an amber fragment having 5′-3′ exonuclease activity [25]) and has a substantial deletion of the coding sequence beyond the mutation site to eliminate the readthrough product. To provide polymerase and 3′-5′ exonuclease activities in the absence of 5′-3′ exonuclease, we used a previously constructed plasmid in which Klenow fragment is expressed from the *lacUV5* promoter (16).

The in vitro constructs (designated F′ 5′ Exo and F′ Klenow) were introduced into the lysogens 83A and 83B, and cured cells were selected and analyzed, giving the results shown in Table 1. In the presence of F′ 5′ Exo, comparable numbers of Km^r and Km^s colonies were obtained, with no apparent difference in colony size or morphology. Although the result was superficially the same as that with F′ *polA*$^+$, the majority of the Km^r-cured cells lacked a wild-type *polA* gene, as shown by sensitivity to MMS, whereas all the Km^s cells were resistant to MMS. (A few [less than 10%] of the Km^r cells were also resistant to MMS; at least some of these were the result of reversion of the *c*I857 mutation since they remained λ immune.) Southern blot analysis of representative cured cells (not shown) was exactly analogous to the data shown in Fig. 6 and confirmed that the Km^r cells had indeed lost the wild-type chromosomal *polA* fragment but retained the *polA* fragment derived from F′ 5′ Exo.

Since the Klenow fragment gene on F′ Klenow is expressed from the *lac* promoter, we carried out the curing experiment both in the absence and the presence of IPTG.

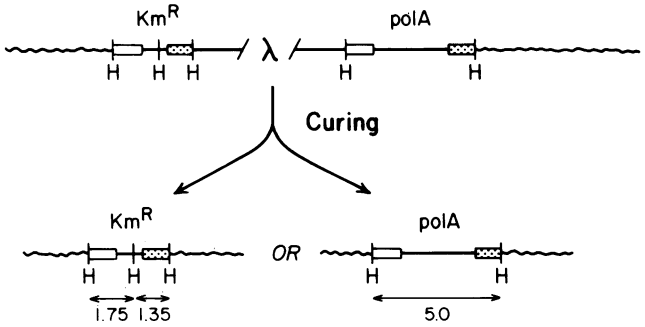

FIG. 7. Curing of λCJ83 lysogens. Two alternative structures can be generated at the *polA* locus on excision of the prophage from lysogen 83A. The open boxes indicate the upstream flanking homology between λCJ83 and the chromosome, and the stippled boxes represent the downstream homology. Chromosomal DNA external to the region of homology is shown as wavy lines. The positions of relevant *Hin*dIII sites are indicated, along with the sizes (in kb) of fragments that would hybridize to a pCJ1 probe.

This allowed us to compare the effect of a basal level and a fully induced level of expression. In each case, the curing procedure yielded both Km^r and Km^s cells (Table 1). In the presence of IPTG, the proportion of Km^r cells was similar to that obtained from the same lysogens containing F′ *polA*$^+$. Moreover, there was no apparent difference in the growth of Km^r and Km^s cells on the original selection plate. In the absence of IPTG, the proportion of Km^r cells was slightly lower, suggesting that the Δ*polA* Km^r cells (containing only a low level of Klenow fragment) either had been growing slightly more slowly than their *polA*$^+$ counterparts before the selection of cured cells or had lower viability when plated. This growth difference was also apparent on the selection plates; the Km^r colonies had about half the diameter of the Km^s colonies. As with the previous experiment, Southern blotting (not shown) confirmed the absence of a wild-type *polA* gene from the Km^r cells and its presence in the Km^s cells. We found that Δ*polA* cells containing F′ Klenow are sensitive to MMS even in the presence of IPTG, indicating that the Klenow fragment activities alone are not sufficient for excision repair. (As discussed by Wahl et al. [30], this is probably due to formation of structures that cannot be sealed by DNA ligase.)

These experiments therefore demonstrated that either of the functional fragments of PolI, in the absence of the other, is sufficient to allow survival under the conditions of the curing experiment. We have eliminated the trivial explanation that some other gene on the pOX38 derivatives is able to substitute for *polA* by introducing a control plasmid (pOX38 containing just the cloned Cm^r marker) into lysogens 83A and 83B. When the curing experiment was repeated, no Δ*polA* Km^r survivors were obtained. We have also ruled out the unlikely possibility that our supposed chromosomal deletions contain subtle rearrangements that allow persistence of wild-type gene function, by a more detailed Southern blot analysis (Fig. 8). A *Hin*fI digest, which displays characteristic fragments from the 5′-3′ exonuclease and Klenow fragment regions, showed that our chromosomal *polA* deletion strains contain only the expected portions of the *polA* gene.

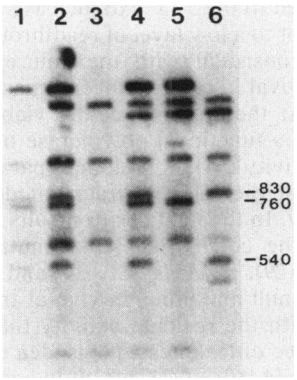

FIG. 8. *Hin*fI analysis of Δ*polA* strains. Chromosomal DNA was digested with *Hin*fI and *Hin*dIII, fractionated on a 1.2% agarose gel, transferred to Gene Screen (New England Nuclear Corp.), and hybridized with nick-translated pCJ1. The 760-bp fragment is derived from the 5′-3′ exonuclease portion of the *polA* gene; the 830- and 540-bp fragments are from the Klenow fragment portion. Lane 1, strain CM4722 (*polA*$^+$); lane 2, lysogen 83A; lane 3, Δ*polA*, obtained on minimal medium; lane 4, Δ*polA* F′ *polA*$^+$; lane 5, Δ*polA* F′ 5′ Exo; lane 6, Δ*polA* F′ Klenow.

Cells with a *polA* deletion are viable on minimal medium. It is known that some conditional lethal mutations in *polA* show increased survival at the nonpermissive temperature when plated on minimal media (7, 23). We therefore repeated the curing of lysogens 83A and 83B, using a minimal glucose medium both for the nonselective growth at 30°C and for the selection of cured cells at 42°C. Under these conditions, comparable numbers of Km^r and Km^s cells were obtained (Table 1). The proportion of Km^r cells was slightly lower than in the presence of F′ *polA*$^+$, perhaps indicating slower growth or lower viability of the Δ*polA* cells. Southern blot analysis confirmed that the Km^r cells had completely lost the *polA* gene (Fig. 6 and 8). As might be expected, cells which completely lack the *polA* gene were more sensitive to MMS than those containing the 5′-3′ exonuclease or Klenow fragment activities (data not shown).

We have found that Δ*polA* cells grown in M9 glucose plate with equal efficiency on both M9 glucose and Trypticase agar but with <1% efficiency on LB agar. Since the background strain (CM4722) grows at the same rate in M9 glucose as in Trypticase, it seems likely that the requirement for the *polA* gene is related to the growth rate. When Δ*polA* cells are shifted from M9 glucose to LB, the majority form long filaments. By plating a Δ*polA* strain on LB, we have obtained survivors that replate with high efficiency on LB. Such suppressors of Δ*polA* may allow us to determine the cause of the Δ*polA* lethality in rich media.

DISCUSSION

We have developed a new experimental strategy for determining whether a particular gene of *E. coli* is essential for viability. The method can be applied to any gene, regardless of function, provided that cloned DNA spanning the gene in question is available and that the location of the gene sequence within the cloned segment is known. This approach offers substantial advantages over existing alternatives by eliminating several sources of ambiguity in the interpretation of the results. The in vitro manipulation of the target gene allows one to design a deletion that does not leave behind a functional fragment of the gene. Deletion or insertion mutations obtained in vivo should only be taken as proof that the affected gene product is inessential if it can be shown that no gene functions remain. Our demonstration that either functional half of the *polA* gene can substitute for the intact gene shows that it is not safe to assume that an insertion mutation or a partial deletion will satisfy this condition, particularly for a multifunctional gene product. Equally important, the in vitro deletion can be designed to avoid interfering with neighboring genes, thus excluding the possibility that attempts to obtain deletions of a particular gene may be compromised by the presence of a nearby essential gene.

The major advantage of our method is that it provides an independent estimate of the frequency at which deletion of the target gene should occur and thus allows one to interpret a negative outcome. If no deletions are recovered in a situation where a substantial fraction of deletion-producing events have occurred, then one can conclude unambiguously that cells carrying the deletion have failed to survive. Moreover, if deletions are obtained, but at a substantially lower frequency than expected, this suggests that a secondary chromosomal mutation may be necessary for the deletion mutant to be viable (as is the case for deletions of *topA* [9]).

The use of an antibiotic resistance gene to mark the

deletion is an added convenience. Cells carrying the deletion can easily be identified, even if there is no phenotype associated with deletion of the target gene. Southern blot analysis need only then be used as a final check on the chromosomal structure of the deletion mutant rather than as a routine screening method.

An analogous strategy has been used to introduce sequences altered in vitro onto the bacterial chromosome by means of plasmid integration and subsequent excision (12). Clearly, this method could also be used to introduce deletions of particular genes and thus to assess whether the corresponding gene products are essential for viability. However, it would be more difficult in this system to interpret a negative result (failure to obtain the desired chromosomal deletion). Direct analysis of the frequency of deletion-forming events is precluded by the inability to recover the excised product and the greater complexity of the integrated structures (12).

In vivo role of PolI. Our results show that some PolA function is required for growth on rich medium. Surprisingly, however, either the 5′-3′ exonuclease or the Klenow fragment portion of the molecule is sufficient. On minimal medium this requirement no longer exists. Deletions of the chromosomal *polA* gene, obtained either on minimal medium or on rich medium in the presence of either of the complementing fragment activities, occurred at about the same frequency as in the presence of F′*polA*$^+$, which eliminates selective pressure on the *polA* gene. This observation argues against survival of the deletion being contingent upon a secondary mutation.

How can we interpret our results in terms of the role of PolI in vivo? PolI is known to function both in excision repair and in the processing of Okazaki fragments during discontinuous replication (24). The viability of a complete *polA* deletion on minimal medium but not on LB is compatible with the possibility that either of these functions is of primary importance. If the primary function of PolI is to repair small amounts of DNA damage (such as might occur even in the absence of exogenous DNA damaging agents), then growth on rich medium might be more demanding for either of the following reasons. In the first place, chemicals present in LB could cause additional DNA damage. Alternatively, the longer generation time on minimal medium would allow more time for DNA repair between cycles of chromosome replication and could therefore allow the use of a less efficient repair pathway in the absence of PolI. An argument that DNA repair is not the primary function of PolI is provided by the substantial repair deficiency of strains carrying just the 5′-3′ exonuclease or the Klenow fragment activity (as judged by MMS sensitivity). These strains, nevertheless, grow normally on rich medium.

We believe that our data are more compatible with the generally accepted hypothesis that, in the absence of DNA damage, the important function of PolI is the processing of Okazaki fragments into high-molecular-weight DNA (24, 26). Under normal conditions, the nick-translation activity of PolI is thought to accomplish simultaneous extension from the 3′ end of one Okazaki fragment while removing the RNA primer from the 5′ end of the downstream fragment. This process is clearly essential for the continued integrity of the bacterial chromosome and thus for survival. If we assume that this is the primary function of PolI, the ability of cells that completely lack PolI activity to survive on minimal medium suggests that, under conditions of slow growth, some other enzyme(s) can fulfill the same function. The two other DNA polymerases, II and III, are obvious candidates for this alternative pathway. PolIII, although capable of the same enzymatic reactions as PolI, would be less efficient because the small numbers of PolIII molecules are likely to be sequestered at replication forks, particularly during rapid growth. PolII would be able to extend the 3′ end of an Okazaki fragment, but it lacks both a 5′-3′ exonuclease activity and the ability to carry out strand displacement synthesis (24). PolII would therefore be unable to convert Okazaki fragments into high-molecular-weight DNA in the absence of an additional function (such as RNase H) to remove the RNA primers.

The ability of either the 5′-3′ exonuclease or Klenow fragment portions to compensate for intact PolI in allowing growth on rich medium suggests that even partial participation by PolI functions can improve the efficiency of Okazaki fragment processing. On the one hand, the 5′-3′ exonuclease activity could remove RNA primers, leaving a DNA duplex with short gaps, the substrate of choice for PolII (24). On the other hand, the Klenow fragment activity could extend the 3′ end of an Okazaki fragment while displacing the obstructing RNA primer, making the latter more accessible to digestion by other cellular nucleases.

Our results suggest that a relatively small amount of either fragment (5′-3′ exonuclease or Klenow fragment) alone is adequate to speed up the processing of Okazaki fragments until it no longer limits the growth rate. The only growth impairment that we observed was for the Δ*polA* F′ Klenow strain in the absence of IPTG, where the basal expression from the *lacUV5* promoter probably provides less than 10 molecules of the polymerase fragment per cell. The F′ 5′ Exo plasmid probably supplies the 5′-3′ exonuclease fragment at a level equal to the normal cellular level of PolI (about 400 molecules per cell [24]); however, the rate of exonuclease action is reduced about 10-fold in the absence of coupled polymerization (20). The likelihood that a relatively low level of activity of either fragment can substitute for whole PolI bears out the notion that most of the normal cellular complement of PolI is used in DNA repair, and very little is required for replication.

The result that either functional fragment of PolI allows survival on rich medium provides a framework within which to reexamine the behavior of known *polA* mutants. The viability of the two amber mutants (*polA1* and *resA1*) can now be attributed to the 5′-3′ exonuclease activity that each produces and not to a low level of readthrough product. This conclusion is consistent with the failure to observe any decrease in survival in more stringent nonsuppressing backgrounds (19). At the same time, the viability of *polA107*, which produces a functional polymerase but lacks the 5′-3′ exonuclease activity (14), is no longer paradoxical since we have shown that the Klenow fragment domain alone can provide viability. In the light of our results, we are forced to conclude that the conditional lethal mutations *polA480ex* (23), *polA4113* (29), *polA214* (11, 21) and *polA34* (6) must approximate to null mutations in vivo at the nonpermissive temperature, with the residual activity that remains determining the degree of leakiness. (This idea contradicts the in vitro data for *polA480ex* (23), which indicates that only the 5′-3′ exonuclease activity is temperature sensitive. However, it is possible that the mutant enzyme becomes susceptible to degradation in vivo at the nonpermissive temperature.)

Finally, the availability of an isogenic set of strains in which PolI activity is either completely absent or is strictly limited to one of the functional domains should greatly help in establishing the role of the various enzymatic activities of PolI in replication and repair.

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