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Genetics

Large-scale automated isolation of *Escherichia coli* mutants with thermosensitive DNA replication

(colony morphology/*dna* mutations)

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ABSTRACT **We have screened about 1.4 million colonies of *Escherichia coli* K-12 for their ability to grow on nutrient agar at 30° and 41°. Among the 2266 temperature-sensitive mutants found, 110 were defective in DNA synthesis but not in protein synthesis at 41°. Three of these *dna* mutations mapped at two previously undescribed loci on the *E. coli* genetic map and may represent new genes involved in DNA replication in *E. coli*. The mutant isolation was aided by novel automatic machinery that inoculated agar-filled petri dishes with mutagenized *E. coli* cells laid down in square arrays of evenly spaced rows and columns on the agar. Time-lapse photographs taken before and after a temperature shift were used to find colonies of temperature-sensitive mutants. These mutations were mapped by interrupted conjugation and viral cotransduction methods, and the mutants were divided into three classes according to the kinetics of DNA synthesis at the restricted temperature. Some of the mutants exhibited mutator activity at partially restrictive temperatures. It is argued that some genes involved in DNA synthesis remain to be discovered.**

About 10 years ago, when an *Escherichia coli* DNA polymerase enzyme was purified and its function became known, it was generally believed that replication of the chromosome of *E. coli* consisted of a simple DNA polymerase-mediated conversion of a duplex circle of DNA to two duplex circles (1). Results from genetic and biochemical work in the last few years have shown that DNA replication is not a simple reaction but a complex series of events in which diverse multienzyme systems function.

The effort to elucidate the mysteries of DNA replication has been conducted mainly in two ways: the genetic (isolation of conditionally lethal mutants—*dna* mutants—that are defective in DNA replication in some restrictive environment, usually high temperature), and the biochemical (proteins required for *E. coli* chromosome replication are resolved *in vitro* with the aid of simple viral DNA templates such as M13, G4, and φX174). Several of the known mutant gene products have been purified in this fashion as well as some other proteins whose physiological functions, as regards DNA replication, remain unclear and for which there are no known genetic loci. Examination of the relationship between bacterial function and viral bacteriophage DNA replication has helped identify the specific steps involved in both bacterial DNA replication and dependence of viral DNA replication on host cell functions.

The current results of this effort to solve the DNA replication puzzle can be summarized as follows. (*i*) For the geneticists: at least 10 genetic map loci have been identified that are in some way involved with DNA replication *in vivo;* these genes, discovered and located on the *E. coli* genetic map through isolation and study of temperature-sensitive *dna* mutants (dna_{ts}) are known as *dnaA, dnaB, dnaC, dnaE, dnaG, dnaH, dnaI, dnaP, dnaS,* and *dnaZ* (2–10). (*ii*) For the biochemists: at least 10 purified protein fractions have been shown to be required for the *in vitro* conversion of single-stranded viral φX174 DNA to replicative form II (RFII) in an *E. coli* host; they are *dnaB, dnaC, dnaG, dnaZ* proteins, DNA-binding protein, DNA polymerase III (*dnaE* protein), DNA elongation factors I and II, and DNA replication factors X, Y, and Z (11–13).

The most effective means of assessing the action of a gene (and its product) is the possibility of studying a mutant organism deficient in it; in this paper we describe a novel method for automated screening of large numbers of bacterial colonies in order to identify and isolate temperature-sensitive mutants defective in DNA replication. We present data for 110 *dna* alleles isolated with our method and which are to be included with the approximately 98 *dna* alleles available from previous work by other investigators (e.g., 2–10, 14). All but three of the new mutations map near or within known genes affecting DNA replication. The latter may define two new genes involved with the mechanisms of DNA replication in *E. coli*. The steps of the isolation procedure and its results are discussed below.

Automated inoculation of petri dishes

Bacterial cultures were mutagenized by treatment with nitrosoguanidine according to the method of Adelberg *et al.* (15). After mutagenesis and two division cycles for separation and expression of mutant phenotypes, the cells were inoculated onto prepoured petri dishes.

The inoculation was performed with the help of a computer-controlled prototype machine affectionately nicknamed "Cyclops." The automatic cell inoculator part of Cyclops consisted of an electromechanical system that delivered cell-containing drops of liquid cell suspension onto a moving agar surface beneath it. It was designed and built as an adaptation of a technique originally developed by Fulwyler in 1965 (16). This is a method of forming, charging, and electrostatically deflecting a high-speed jet of small fluid droplets, each with a diameter of about 100 μm and a volume of approximately 10^{-9} liters and containing one bacterium on the average.§ They are

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Abbreviation: dna_{ts} mutant, temperature-sensitive mutant defective in DNA replication.

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§ The distribution of bacteria inside the forming droplets follows Poisson statistics. The cell concentration in the inoculating solution is adjusted to maximize the probability of a single cell per droplet (1/e droplets will probably contain one cell; 1/e will contain no cells; and 1–2/e will carry two or more cells). Empty spots are apparent in Fig. 2.

delivered onto the agar surface of the petri dishes, at adjustable constant spacing (5 mm in our experiment) from each other, in regular rows and columns. In a typical experiment, 100,000 colony-forming units (approximately 80 colonies per petri dish) were inoculated with this method. When the inoculation procedure was completed, all dishes were incubated at the permissive temperature (30°) for 19 hr. At that time they were transferred to a 41° incubation chamber. Incubation at 30° for 19 hr permits the formation of small (1- to 1.5-mm diameter) but clearly visible colonies.

Time-lapse photography of bacterial colonies

All petri dishes were photographed twice, first at 6 hr after the transfer to the restrictive temperature, and then 22 hr later. Photography also was performed with Cyclops. The camera, fixed above the petri dishes and loaded with 35-mm black-and-white film, takes approximately two photographs per second. Each frame contained the entire area of one petri dish. The colonies were illuminated from below by a stable source of parallel white light (high-pressure xenon-filled arc lamp). The camera was looking through the colonies toward the light source, in a bright-field situation. In this optical arrangement the colonies behave as miniature lenses which refract the light but also absorb, scatter, and reflect various colors of light selectively. When the camera was focused accurately on the agar surface, the colony image was generally uninteresting and did not contain much information for subsequent use (scanning) for colony identification purposes. With the camera focused about 10 mm above the agar surface, the image usually was complex and contained features that resulted from refraction and scattering of light by turbid regions of the colonies. Hence, the out-of-focus photographs are preferred for subsequent analysis (17).

Film scanning; isolation of temperature-sensitive mutants

The next step in the automated procedure for mutant isolation consisted of analyzing the data collected on the 35-mm film during time-lapse photography.

The film was developed and loaded into a flying spot scanner (17). The second film was scanned first. The following operations were performed for each film frame. (*i*) *Dish identification:* each dish was identified and numbered by means of a photograph of a data panel containing binary information. (*ii*) *Dish location:* the boundary of the dish was located first and subsequent scanning was done only in the interior of this region. (*iii*) *Object location:* the interior of the dish was scanned in order to locate all objects on the photograph. (*iv*) *Colony identification and measurement:* each located object was subjected to a series of circularity tests to determine whether it was a round, single colony. (This step eliminates scratches on the agar surface, overgrown colonies merged into one another, colonies around the edges of the dish, some contamination, and, in general, anything not *appearing* to be a single bacterial colony.) Objects not passing the circularity test were represented in the computer files as rectangles and colonies were represented as circles whose sizes were directly proportional to the actual colony dimensions. The diameter and the area of each single colony were measured independently.

After completion of the scanning of the second photographs, the first photographs were scanned and the new object/colony dimensions (diameter, area) were recorded after circularity was verified. At the completion of film scanning, data from the earlier and later photographs were combined into a single file. This completed *scan file* was stored in computer memory and

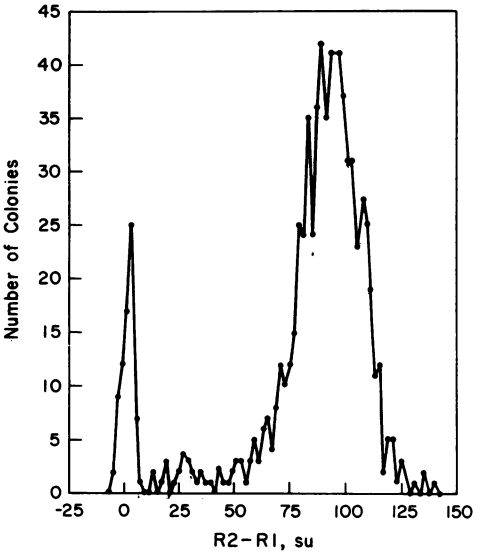

FIG. 1. Illustration of automated system's power to discriminate between two bacterial populations with different growth characteristics. A mutagenized population of wild type *E. coli* cells, enriched with a known temperature-sensitive mutant population, was inoculated and photographed twice as described in the *text*. The histogram of all colonies as a function of the growth exhibited during the elapsed time between photographs reveals two peaks, one for the wild types (right) and the other for the temperature-sensitive mutants (left). Colony growth was measured by comparing radii of colonies as computed by scanning the two photographs; radii given in "scanning units," 1 su $\simeq$ 13.4 μm.

data from this file were retrieved during further computer analysis to identify temperature-sensitive mutants.

Detection of temperature sensitivity from scanning data

In the next step the computer read data for each individual colony from both photographs, compared them, and then set aside, in another file, those colonies whose parameters indicated little or no change during the incubation time between photographs. Fig. 1 illustrates the results of such a scan-file search in which colonies were catalogued as a function of their change in radius (R2 − R1) between time points. The separation between temperature-sensitive and non-temperature-sensitive populations is clearly visible. Another computer program actually displayed, onto a cathode ray tube screen at actual size, the image of every dish that contained a mutant colony, pinpointing the location of the mutants; the operator could match colony and image by simply superimposing the actual petri dish with its display on the television screen. Fig. 2 shows such a dish image plotted on paper with temperature-sensitive clones indicated by numerals on their real position within the dish boundaries (non-temperature-sensitive colonies are indicated by squares). In this fashion, every colony that was identified as temperature-sensitive by the automated procedure was located and made accessible for further experimentation.

Identification and phenotypic classification of dna_{ts} mutants

All colonies with temperature sensitivity as defined by these methods were purified for confirmation of their temperature-sensitive phenotype. All such strains were then screened for incorporation of radioactive protein and DNA precursors at 41°; the incorporation of radioactive leucine and radioactive thymine provided a measure of *in vivo* protein and DNA syntheses, respectively. Fig. 3 summarizes the results of these in-

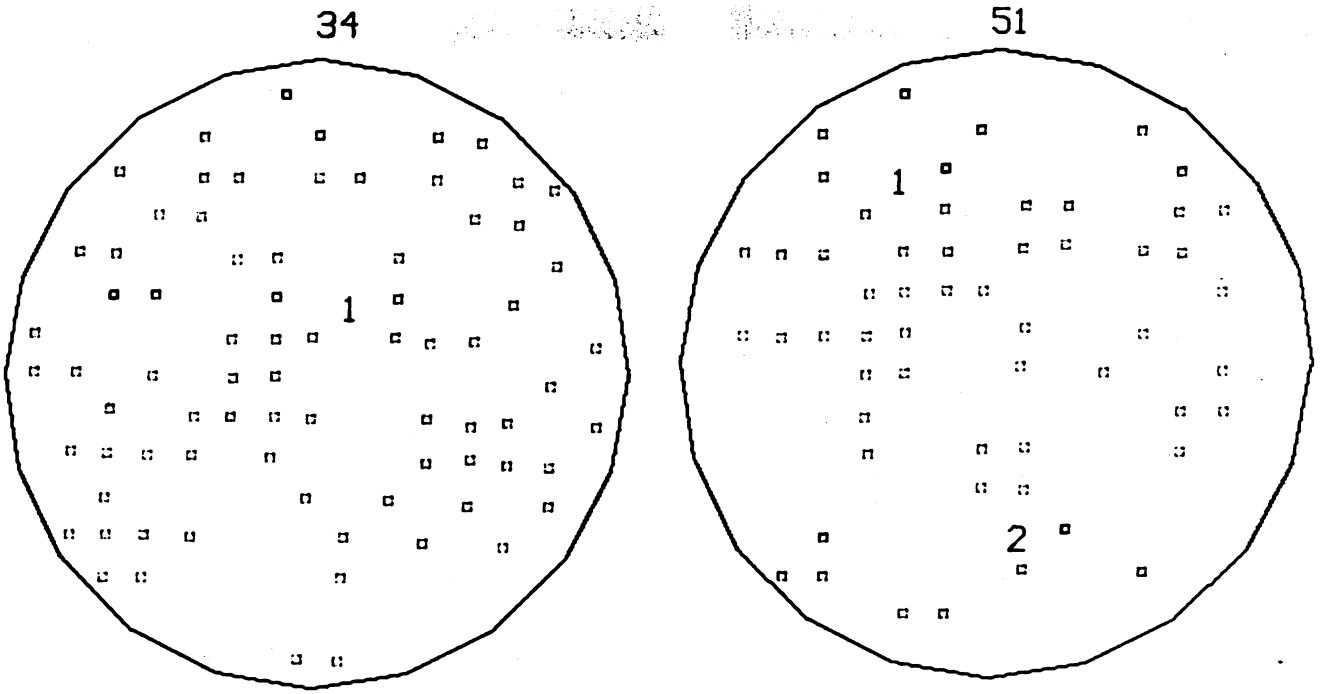

FIG. 2. Excerpt from computer drawing of dishes containing temperature-sensitive mutant colonies. The dish outline is actual size so that the petri dish can be superimposed on its image, with the small squares of the drawing directly below the actual colonies. Mutants were located above numerals inside dish outline; hence, there was a one-to-one correspondence between each colony on the agar surface and its representation on the drawing. On this excerpt, dish 34 contained one mutant (*1*) and dish 51 contained two mutant colonies (*1*, *2*) at the locations shown. Dishes 35–50 are not drawn because no mutant colonies were detected in them.

corporation experiments. Mutants with defective incorporation of radioactive thymine (i.e., defective in DNA synthesis at 41°) exhibited one of the three profiles shown, depending on how DNA synthesis was affected after the shift to the restrictive temperature. Also shown are the incorporation characteristics of the wild-type parental strain (DG17).

It is assumed that dna_{ts} mutants exhibiting the "quick stop" phenotype at high temperature carry mutations in a gene whose product is required during the *elongation* phase of the DNA replication cycle in *E. coli;* at restrictive temperature, the three-dimensional structure of this gene product is affected in such a manner that its function, alone or in cooperation with other proteins of the replication system, ceases. This results in immediate interruption of DNA synthesis. Similarly, mutants that display "slow stop" phenotype at high temperature probably carry mutations in a gene whose product plays a required

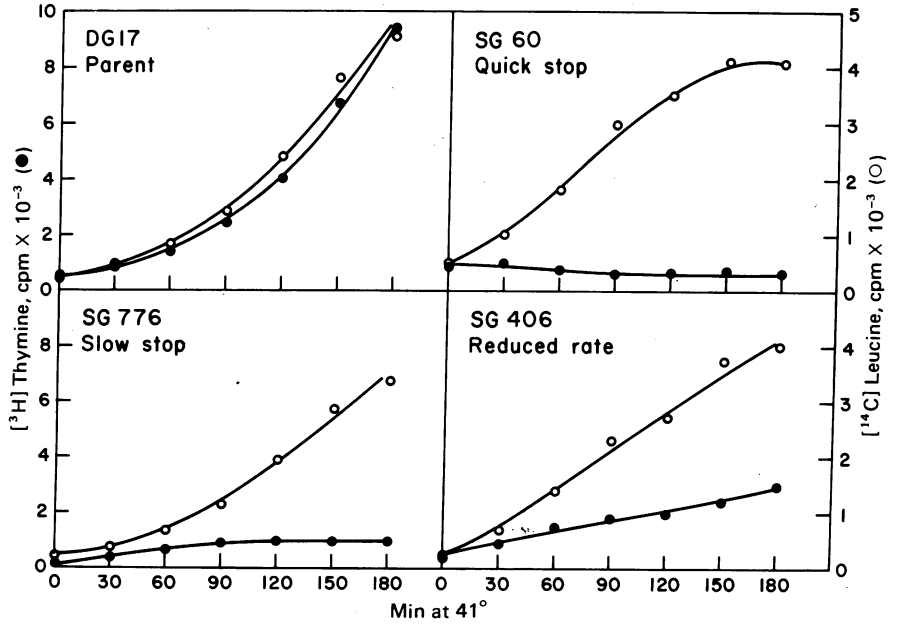

FIG. 3. Incorporation of radioactive leucine (a measure of protein synthesis) and radioactive thymine (a measure of DNA synthesis) at the restrictive temperature (41°) by three representative dna_{ts} mutants (SG strains) and their parent wild-type strain (DG17). The three classes of defective DNA synthesis (quick stop, slow stop, reduced rate) are discussed in the *text*.

Table 1. DNA synthesis by SG dna_{ts} mutants at 41°

Allele no.	SG mutant	Phenotype*	DNA increase after 3 hr, %†	% of parental DNA‡
601	67	SS	99	13.3
602	68	SS	89	12.2
603	69	SS	86	12.5
604	70	SS	71	11.0
605	71	SS	55	10.4
606	79	SS	95	13.1
607	268	RR	442	39.0
608	351	SS	60	10.7
609	861	SS	108	10.0
610	993	RR	212	17.0
611	1097	SS	95	7.9
612	1857	RR	359	23.1
613	2125	QS	−10	14.2
614	60	QS	−27	4.9
615	118	QS	−28	4.8
616	148	QS	0	6.7
617	191	RR	351	30.3
618	347	RR	373	19.2
619	374	RR	436	37.7
620	474	QS	−30	4.5
621	479	RR	541	44.2
622	587	RR	722	30.9
623	666	SS	301	15.1
624	668	SS	257	13.4
625	764	SS	167	12.2
626	767	RR	429	24.0
627	776	SS	525	28.4
628	787	SS	571	30.5
629	795	SS	126	10.3
630	806	QS	−30	3.2
631	857	RR	98	9.6
632	873	SS	85	8.9
633	883	SS	85	8.9
634	936	QS	−30	3.8
635	1018	SS	80	9.8
636	1020	QS	−28	3.9
637	1051	SS	99	8.1
638	1117	QS	−67	1.3
639	1167	RR	201	12.2
640	1250	QS	−69	1.7
641	1284	QS	−32	3.7
642	1536	RR	365	19.1
643	1566	SS	75	12.8
644	1612	SS	218	14.6
645	1625	QS	−13	4.0
646	1664	SS	134	10.8
647	1683	SS	122	10.2
648	1791	SS	66	8.4
649	1953	RR	109	15.3
650	1962	SS	127	16.5
651	1985	QS	30	9.4
652	2007	QS	29	9.4
653	2010	RR	90	30.1
654	2043	RR	49	23.7
655	2058	QS	13	18.0
656	2067	RR	43	22.7
657	2074	QS	15	18.3
658	247	RR	350	32.4
659	300	SS	91	12.8
660	729	SS	136	8.9
661	756	SS	110	7.9
662	1017	SS	41	7.7
663	1144	QS	15	4.7
664	1380	RR	136	11.1
665	1719	QS	26	6.3
666	2022	RR	110	33.3
667	175	QS	30	8.8
668	230	QS	14	7.7
669	345	SS	106	13.8
670	406	RR	462	39.6
671	790	RR	773	39.7
672	902	RR	162	12.7
673	921	SS	65	8.0
674	980	RR	150	13.6
675	1319	SS	190	16.0
676	1424	RR	453	22.7
677	1484	RR	88	7.7
678	1556	RR	313	17.0
679	1758	RR	205	15.4
680	1788	RR	227	16.4
681	1794	RR	254	17.8
682	1887	SS	108	15.2
683	2095	RR	41	22.4
684	251	QS	−4	6.9
685	851	QS	−4	4.6
686	894	QS	13	5.5
687	925	SS	202	16.5
688	143	RR/QS§	−16	9.7
689	1421	RR/QS	80	13.1
690	1775	RR/QS	163	13.2
691	66	SS	62	10.5
692	281	RR	450	35.9
693	282	RR	390	34.5
694	369	QS	−25	5.3
695	426	SS	61	10.4
696	490	SS	66	11.5
697	859	QS	−23	3.7
698	886	QS	19	5.7
699	892	QS	−33	3.2
700	986	RR	350	24.5
701	1041	QS	−18	3.3
702	1147	RR	171	11.0
703	1205	SS	52	7.1
704	1481	SS	199	12.3
705	1691	RR	125	10.4
706	133	SS	59	10.7
707	1019	SS	61	8.8
708	915	QS	31	6.4
709	1137	RR	189	11.8
710	1635	QS	−12	4.0

Incorporation (cpm) of [^{3}H]thymine (e.g., Fig. 3) provided a measure of DNA synthesized at 41°.

* QS, quick stop; SS, slow stop; RR, reduced rate (see *text*).

† Negative values denote DNA degradation.

‡ Amount of mutant DNA, after 3 hr at 41°, expressed as percentage of DNA made by parent strain DG17, which was used as control in all measurements.

§ DNA synthesis at 41° for strains SG143, SG1421, and SG1775 continued at reduced rates (RR) but after 60–90 min was followed by degradation of DNA (QS).

Table 2. Genetic mapping of SG dna_{ts} mutations

Allele no.	Cotransducing marker used (frequency range, %)	*dna* locus assigned
601–613	*ilvD* (4–13)	*dnaA*
614–657	*uvrA* (6–97)	*dnaB*
658–666	*serB* (9–43)	*dnaC*
667–683	*pan* (1–8)	*dnaE*
684–687	*nalA* (6–7)	*nrdA* (*dnaF*)
688–690	*tolC* (11)	*dnaG*
691–705	*thyA* (2–97)	*dnaH*
706	*purE* (11)	*dnaZ*
707	*pyrE* (98)	*dnaS*
708	*pyrF* (96)	*dnaL*
709–710*		*dnaM*

* Interrupted conjugation mapping placed both *dna709* and *dna710* in the *malA-xyl* region of the chromosome at approximately 76 min on the *E. coli* K-12 genetic map (20). Cotransduction was not attempted, and it is not clear whether one or two loci are so defined.

role during *initiation* of DNA replication; therefore, any elongation going on at the time of transfer to high temperature is completed but no reinitiation takes place. For mutants with "reduced rate" phenotype it is assumed that the affected unit of the replication system is partially incapacitated at the restrictive temperature, so that DNA synthesis does continue but at a slower rate.

Of 2266 temperature-sensitive strains isolated with the automated procedure after screening of approximately 1.4 million mutagenized colonies from strain DG17, 110 exhibited defective DNA synthesis at 41° and were selected as dna_{ts} mutants. Of these, 31 exhibited the "quick stop" profile, 40 exhibited the "slow stop" profile, and 39 exhibited the "reduced rate" profile (Table 1).

Genetic sorting of dna_{ts} mutants

The location of the new mutations on the *E. coli* genetic map was determined by interpreting results of bacterial conjugational and bacteriophage transduction crosses. This was done according to the following procedure. Each *dna* mutant was crossed with Hfr (male) strains by a replica-plating technique (18). The results of these crosses helped to define the genetic map interval in which the *dna* mutation belongs. Also, they indicated the most appropriate Hfr strain (the Hfr strain producing the heaviest recombination on the dish) to be used for interrupted conjugational crosses. The selected Hfr strain was used for interrupted conjugational crosses with the *dna* mutants. The transfer of temperature-resistance with other control wild-type alleles, from the Hfr strain to the female *dna* mutant (SG strains), was plotted against time. The knowledge of the map positions of the control wild-type alleles coupled with the results of the interrupted matings defined the position of the unknown mutants.[¶] After the map positions were roughly defined through interrupted matings, most mutations were mapped more accurately through generalized transduction crosses mediated by bacteriophage P1.

This mapping procedure provides sufficient information to assign map locations for the unknown mutations. The genetic loci assignments on the *E. loci* K-12 genetic map (20) for the SG *dna* alleles are given in Table 2. Most of our strains carry mutations mapping near or within known *dna* genes, but the last three alleles (708–710) may define new map loci connected with DNA replication in *E. coli*.

[¶] Experimental methods for interrupted conjugational and transduction crosses were described by Wehr *et al.* (19).

Conclusion

Genetic analysis of 110 dna_{ts} mutants found by examination of 1.4×10^6 colonies of a mutagenized population yielded examples of all previously known *dna* mutations plus some new ones. All these mutants were defective in DNA synthesis at 41° with no immediate deterioration in the rate of protein synthesis.

Three separate mutations, *dna708*, *dna709*, and *dna710*, map at previously undescribed loci, and *dna707* (SG1019) may also define a new locus.

By screening all the *dna* mutations for mutator activity, we found several mutants, with mutations at or near *dnaE*, that exhibited strong mutator activities at partially restrictive and nonrestrictive temperatures (21). Detailed descriptions of these mutator strains will be published elsewhere.

Because some mutations occur rarely or only once in our collection, while others are common, we believe that there are more new loci yet to be discovered that are involved in DNA synthesis. A novel screening method or extension of the present method on a larger scale will probably be required to discover these new genes.

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