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PLASMID-ASSOCIATED ANALOGS OF THE dnab
GENE IN Escherichia coli: GENETIC AND PHYSIOLOGICAL
EVIDENCE FOR OCCURRENCE, DIFFERENCES AND INTERACTIONS

By

Patrick Y. Wang

A thesis submitted to the Faculty of Graduate Studies
in partial fulfilment of the requirement for the degree
of Doctor of Philosophy

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June, 1978.

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Associated Analogs of the dnaB Gene in Escherichia
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ABSTRACT

Several naturally occurring R plasmids have the ability to suppress or enhance the temperature-sensitivity of DnaB(ts) mutants of *Escherichia coli* (E. coli) K-12. This phenomenon appears to be widely distributed among plasmids in various incompatibility groups. Suppression or enhancement can extend to several mutations within the dnaB gene. The mechanism of suppression does not involve the insertion of the plasmid into the genome of E. coli, but rather the production of a DnaB analog (Ban) protein specified by some suppressing R plasmids. This Ban protein can substitute for the product of the dnaB gene in maintaining chromosomal and plasmid DNA replication. In addition, such a Ban protein specified by an R plasmid can interact in vivo with the DnaB protein of E. coli K-12 or the Ban protein specified by mutant P1 bacteriophage (P1Cm bac). As a result of such interactions, changes are observed in the heat or cold-sensitivity of DNA synthesis and colony-forming ability or in the ability of the strains to support the growth of bacteriophage lambda.

ABBREVIATIONS

am	amber
<u>Ap</u>	ampicillin
<u>ara</u>	arabinose
λ	bacteriophage lambda
M13	bacteriophage M13
P1	bacteriophage P1
P7	bacteriophage P7
ϕ X174	bacteriophage ϕ X174
T4	bacteriophage T4
β	beta
CsCl	cesium chloride
Cm	chloramphenicol
ColV	colicinogenic plasmid V
cpm	counts per minute
C	degree centigrade
DNA	deoxyribonucleic acid
<u>drd</u>	derepressed for conjugal transfer
Ban	dnaB <u>an</u> alog (Ban protein)
EM	electron microscopy
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylene diamine tetraacetic acid
F	F plasmid
F'	F' plasmid
fi ⁻	fertility inhibition negative
fi ⁺	fertility inhibition positive

Fig.	figure
<u>gal</u>	galactose
g	gram
Hly	hemolytic plasmid
hr	hour
<u>Kan</u>	kahamycin
<u>lac</u>	lactose
<u>leu</u>	leucine
LA	Luria Agar
LB	Luria Broth
<u>mal</u>	maltose
<u>met</u>	methionine
μ Ci	micro-curie
μ g	micro-gram
μ l	micro-liter
μ	micron
m	milli
mg	milli-gram
ml	milli-liter
MA	minimal agar
MB	minimal broth
mól	mole
$\lambda\pi$	mutant phage of lambda
ω	omega
O.D.	optical density
%	percentage

pfu	plaque forming unit
<u>pro</u>	proline
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
<u>nal</u>	nalidixic acid
NA	nutrient agar
NB	nutrient broth
rpm	revolution per minute
R ⁺	R plasmid-carrying strain
R ⁻	R plasmid negative strain
RNA	ribonucleic acid
sec	second
<u>str</u>	streptomycin
<u>sul</u>	sulphonamide
<u>sup</u>	suppressor
TES	TES buffer
ts	temperature-sensitive
<u>thi</u>	thiamine
<u>thy</u>	thymine
<u>thr</u>	threonine
t-RNA	transfer RNA
<u>trp</u>	tryptophan
vir	virulent
w/v	weight by volume

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GENERAL INTRODUCTION

The term "Plasmid" was first proposed by Lederberg (1952) to describe any extrachromosomal element which can reproduce autonomously; that is, an element which, intracellularly is an independent and stable replicating unit ~~replicon~~. In addition to autonomous replication, some plasmids have the ability to reversibly insert themselves into the bacterial chromosome. Plasmids are widely distributed in many closely and distantly-related genera of bacteria such as *Proteus*, *Salmonella*, *Escherichia*, *Pseudomonas*, *Staphylococcus*, *Rhizobium*, *Bacillus* and *Clostridium*. It seems likely that they will be encountered in nature in virtually all groups of bacteria. It has also been claimed that they occur in the cells of some eukaryotic organisms (Schlessinger (ed.), 1977) although this demonstration has generally been more difficult presumably because of the presence of organelles and organelle DNA in such cells. For this reason, the presence of plasmids in eukaryotes has not been as extensively documented as in the prokaryotes. It is clear, however, that there is a need for studies on the biology of plasmids as a large and distinctive group of genetic elements. Bacterial plasmid groups that have received some attention are those that confer conjugative ability on cells, such as the classical plasmid F; plasmids that specify antibiotic protein, such as the colicin-producing (colicinogenic) plasmids; plasmids that specify toxins such as those producing hemolysins and enterotoxins and plasmids that specify resistance to antibiotics and frequently conjugative ability, such as R plasmids. Certain bacteriophages such as P1 and P7 (formerly ϕ amp) also behave as typical plasmids when they are maintained

in the lysogenic state (Rosner, 1972; Ikeba and Tomizawa, 1969; Smith, 1972; Yun and Vapnek, 1977).

R plasmids are the subject of this study. The term R plasmids has been used for a diverse group of plasmids that carry genetic information for resistance to antibiotics and/or antibacterial drugs. R plasmids are widely distributed in natural isolates of Enterobacteriaceae and most of them are conjugative, that is, they bring about their own transfer by cell to cell contact. Conjugal transfer of R plasmids can also be either interspecies or intergeneric and does not appear to require close genetic homology between cells.

One early approach to R plasmid classification was based upon the ability of R plasmids to inhibit the conjugal or fertility function of the F plasmid. On this basis, R plasmids could be classified into two groups; fi^+ (fertility inhibition⁺) and fi^- (Egawa and Hirota, 1962; Watanabe, 1963; Meynell, Meynell and Datta, 1968). Because of the diversity of R plasmids found in natural isolates of different genera of bacteria, this classification has turned out to be inadequate. One method that has proved to be very useful in R plasmid classification is the incompatibility test (Datta and Hedges, 1971; Datta, 1975; Chabbert *et al.*, 1972). The criterion for incompatibility is that two closely related R plasmids cannot coexist stably in the same host while unrelated plasmids can. When two R plasmids are in the same incompatibility group, one or the other of them is lost from the cell upon cell division. At the present time, it appears that an expedient way to secure a genetically

diverse collection of R plasmids is by choosing R plasmids that belong to different incompatibility groups. It has been found that plasmids of the same incompatibility group show a much greater degree of cross-hybridization of their DNA strands than do plasmids of different incompatibility groups (Guerry and Falkow, 1971; Grindley et al., 1973).

Although the epidemiology of R plasmids with respect to their clinical importance has been studied extensively, our knowledge of the genes and genetic mechanisms determining R plasmid replication and their maintainance in cells is primitive. Thus, for most R plasmids, genes controlling plasmid replication have not been clearly demonstrated and therefore R-specific gene replication products have not been isolated. One approach to the study of the genetics of R plasmid replication is to introduce the test R plasmid into a host strain which is blocked in some functions related to chromosomal DNA replication. Subsequent testing to determine whether the R plasmid or the host and their DNA will replicate may provide some insight into understanding the requirements for R plasmid replication. In such an approach, the class of chromosomal mutants that could be conveniently used is the one that makes chromosomal DNA synthesis temperature-sensitive. The Dna(ts) mutants of E. coli have been shown to be defective in chromosomal DNA replication at a restrictive temperature (usually 37C-42C) but not at a permissive temperature (usually 30C). Two major groups of temperature sensitive mutants have been distinguished with respect to their mode of DNA replication at elevated temperatures. The

first group, called "initiation" mutants, showed a certain amount of residual DNA synthesis at the nonpermissive temperature. An example of this group is the DnaA(ts) mutants. Kohiyama (1968) and Worcel (1970) showed that residual DNA synthesis in the members of this class was the result of the completion of the round of DNA replication already in progress at the time of the temperature shift (from 30C to 41C), but that a new round of DNA replication was not initiated. The second group of temperature-sensitive mutants, first isolated by Bonhoeffer (1966), showed an immediate cessation of DNA synthesis when cells growing at 30C were shifted to 42C. These mutants have also been described as "elongation" mutants (Hirota et al., 1968), implying that they are affected in functions at the DNA replication fork rather than at the chromosome initiation origin.

The "initiation" and "elongation" groups of Dna(ts) mutants are not to be thought of simply as mutants representing two genes. In each of these two groups, there exist several mutants that map at different chromosomal regions implying that "initiation" and "elongation" are individually complex processes that involve the participation of several genes and gene products.

Since this study was to involve a number of R plasmids, we wished to limit it to chromosomal dna(ts) mutations that were known to map in a single gene. The dnaB gene of E. coli K-12 (Wechsler, 1973) was chosen for this purpose because of the following considerations:

- (a) approximately 72% of all dna(ts) mutations that had been isolated and assembled had been found to be dnaB(ts) mutations (Wechsler and Gross, 1971).

- (b) we were aware that biochemical studies on the isolation of the gene product of the dnaB gene were in progress (laboratories of J. Hurwitz, A. Kornberg, H. Schuster and S. Wickner) so that it seemed possible that the function of the gene would soon be defined in molecular terms; these studies are however still in progress.
- (c) studies of a similar nature but on dnaA(ts) mutation (initiation mutant group) had led to the unexpected and interesting set of observations that at the high nonpermissive temperature, the plasmids were being inserted into the chromosome and that this led to the suppression of temperature sensitivity by the strain without the loss of the temperature sensitive dnaA(ts) mutation. With respect to dna(ts) mutations that affect elongation functions, such as the dnaB(ts) mutations, the question of whether a particular R plasmid replicates or does not replicate at a nonpermissive temperature has rarely been examined experimentally. The question of whether an R plasmid can suppress the temperature-sensitivity of such DNA elongation-defective hosts has not been examined before and was undertaken in this study.

Based on information available at the time this study was initiated, a brief summary could be made on plasmid replication with respect specifically to the dnaB gene. Some relatively large, conjugative plasmids (e.g. F, ColV and Hly) that were strictly maintained at a plasmid : chromosomal ratio close to unity were reported to be unable to replicate in DnaB(ts) mutants at restrictive temp-

eratures (Nishimura et al., 1971; Goebel and Schrempf, 1972).

It was reported that under these conditions, both chromosomal DNA and the DNA of these plasmids failed to replicate. On the other hand, small "multicopy" plasmids such as ColE1 were observed to replicate in DnaB(ts) mutants at high temperatures when chromosomal DNA did not replicate. Based on these observations, it has been suggested (Goebel and Schrempf, 1972; Veltkamp and Nijkamp, 1974) that all small "multicopy" plasmids can replicate at a high temperature in DnaB(ts) mutants while all large "single" copy plasmids cannot. As will be seen, this suggestion is not supported by this thesis.

In the first part of this study, a large number of R plasmids originating from geographically dispersed sources and of different incompatibility groups were surveyed for their ability to alter the temperature-sensitivity of DnaB(ts) mutants. Since our early observations showed that DnaB(ts) mutants carrying different dnaB alleles exhibited differences with respect to the degree of temperature-sensitivity, it became necessary to examine not only a large number of plasmids but also each of these plasmids in strains bearing several different dnaB alleles. Such a survey when it was completed allowed us to obtain information on how widely spread is the ability of plasmids to alter the temperature-sensitivity of DnaB(ts) mutants. This survey also enabled the selection of particular plasmids for further study. Subsequent Chapters of this thesis are concerned with experiments relating to the possible mechanisms of suppression or enhancement of the temperature-sensi-

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tivity conferred by dnaB(ts) mutations.

After this general introduction to the subject of my thesis, I have organized each Chapter with (a) a separate introduction which is brief and relevant to the experiments described in that Chapter. (b) Materials and Methods, again pertaining only to experiments in that Chapter. (c) Results. (d) a set of conclusions or discussion and conclusions which often lead into the next Chapter. This mode of presentation was chosen in the belief that it will facilitate a critical reading and comprehension of experiments, and their results and interpretation. A further discussion of all the results taken together and in the context of what is known about DNA replication in E. coli forms the final Chapter, parts of which are speculative.

CHAPTER I

Survey of "Standard" Plasmids and of Thermosensitive Mutants

Introduction.....	8-9
Materials and Methods.....	10-17
Results.....	18-28
Conclusions.....	29-33

INTRODUCTION

The dnaB gene of Escherichia coli (E. coli) K-12 is an essential gene, the product of which is required for chromosomal DNA replication (Kornberg, 1974; Gefter, 1975). The role of this gene in the replication and the maintenance of various bacterial plasmids has been ambiguous. Some plasmids like P1 have been observed to replicate in DnaB(ts) mutants and to suppress temperature-sensitivity (D'Ari et al., 1975; Ogawa, 1975); others like ColV have been reported to neither replicate in nor suppress the temperature-sensitivity of dnaB(ts) mutations (Goebel and Schrempf, 1972) although both of these plasmids are relatively large plasmids (M.W. $> 2 \times 10^7$) that are maintained in cells at low (1-2) copy number.

A purpose of the present study was to identify naturally occurring R plasmids that suppress or enhance the temperature-sensitivity of DnaB(ts) mutants. Naturally occurring R plasmids (wild-type) are usually transferred by conjugation at low frequency (10^{-5} to 10^{-6} per donor cell), but from these wild-type plasmids, conjugally derepressed (drd) mutant plasmids can be isolated that mediate their own transfer at a much higher frequency (Meynell et al., 1968). In the first part of this study, wild-type (with respect to conjugal transfer) R plasmids were introduced into sets of isogenic DnaB(ts) strains which differed only in the dnaB allele, and the plasmids were screened for their ability to suppress or enhance the dnaB(ts) mutations. The use of otherwise isogenic DnaB(ts) strains was important because any observed result could not be due to other mutations carried by the host strains. The R plasmids used

for this study were chosen to belong to different incompatibility groups in order to test whether any observed effect was confined to a particular plasmid group in nature as appears to be the case for the suppression of dnaG(ts) mutations (Wilkins, 1975). Additionally, most of the plasmids used came from bacterial isolates that have widely-dispersed geographic origins (Japan, Germany, France, England, South Africa, USA and Canada).

The observations described in this Chapter indicate that many plasmids suppress temperature-sensitivity but that some enhance temperature-sensitivity. The ability to suppress or enhance dnaB(ts) mutations is widely distributed among wild-type conjugative R plasmids without regard to classification schemes based on incompatibility tests or conjugal transfer system. The efficiency of some conjugally derepressed (drd) R plasmids that conjugate at a higher frequency was also compared to their respective wild-type plasmids. Possible mechanisms of suppression or enhancement of temperature-sensitivity of dnaB(ts) mutations will be considered in Chapters that follow.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Tables I-1 and I-2 list the bacterial strains and conjugative plasmids. Sets of otherwise isogenic strains bearing four different dnaB(ts) mutations were used because of the possibility that the suppressing or enhancing ability of dnaB(ts) mutations by conjugative R plasmids could be an allele-specific property. The mutation dnaB266 is an amber mutation and is suppressible by amber suppressors (D'Ari *et al.*, 1975; Ogawa, 1975). However, in the presence of some amber suppressors (supD and supE), the strain that survives is temperature-sensitive. As far as is known, the other three mutations (dnaB8, dnaB43 and dnaB70) are missense mutations. The R plasmids used were naturally occurring plasmids and they belong to different incompatibility groups. The total DNA from all the plasmid-negative (R⁻) strains was radioactively labeled and examined for supercoiled DNA (plasmid DNA) by cesium chloride-ethidium bromide density centrifugation (Bazara and Helinski, 1968) (for details, see Materials and Methods, Chapter II; p. 34). No supercoiled DNA was detected from such strains (limit of detection: approximately 0.1% of total DNA).

Nomenclature

In this and in subsequent Chapters, the nomenclature for bacterial genotypes and phenotypes follows the widely accepted recommendations of Demerec *et al.* (1966). For example, the genotype for a particular dnaB mutation will be referred to as dnaB43

TABLE I-1
Plasmid-Negative Strains Used

Strain	Genetic markers ^a	Reference
J53	<u>pro</u> <u>met</u>	
Q125	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺	D'Ari <u>et al</u> (1975)
Q1502	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB8</u>	D'Ari <u>et al</u> (1975)
Q1506	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB43</u>	D'Ari <u>et al</u> (1975)
Q1508	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB70</u>	D'Ari <u>et al</u> (1975)
Q1610	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB266</u>	D'Ari <u>et al</u> (1975)
WD00	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>sup</u> ⁺	Ogawa (1975)
WD001	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>supD</u>	Ogawa (1975)
WD002	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>supE</u>	Ogawa (1975)
WD003	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>supF</u>	Ogawa (1975)
WD266I	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>supD</u> <u>dnaB266</u>	Ogawa (1975)
WD266II	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>supE</u> <u>dnaB266</u>	Ogawa (1975)
WD266III	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>supF</u> <u>dnaB266</u>	Ogawa (1975)
CR34	<u>thr</u> <u>leu</u> <u>thy</u> <u>thi</u> <u>str</u> <u>supE</u>	Bonhoeffer (1966)
CR34Ts	<u>thr</u> <u>leu</u> <u>thy</u> <u>thi</u> <u>str</u> <u>supE</u> <u>dnaB43</u>	Bonhoeffer (1966)
CSH56	<u>pro</u> <u>thi</u> <u>ara</u> <u>lac</u> <u>nal</u> <u>supD</u>	Cold Spring Harbor culture kit

^a pro, proline; met, methionine; trp, tryptophane; thi, thiamine; lac, lactose; mal, maltose; ara, arabinose; str, streptomycin; nal, nalidixic acid; sup, suppression; dna, deoxyribonucleic acid; gal, galactose; thy, thymine; leu, leucine; thr, threonine.

TABLE I-2
Wild-Type and Transfer-Derepressed R Plasmids Used

R plasmid	Incompatibility group	Antibiotic and other relevant resistance markers	Reference
RA1	A	<u>sul</u> <u>tet</u>	Hedges & Datta (1971)
R40a	C	<u>amp</u> <u>kan</u> <u>sul</u>	Hedges & Datta (1972)
R386	FI	<u>tet</u>	Dennison (1972)
R100	FII	<u>sul</u> <u>tet</u> <u>str</u>	Egawa & Hirota (1962)
R100-1	FII	<u>sul</u> <u>tet</u> <u>str</u>	Egawa & Hirota (1962)
R1- <u>bla31</u> (R1-am)	FII	<u>bla31</u> (amber)	Kennedy & Crowlesmith (1975) ^b
R124	FIV	<u>tet</u>	Hedges & Datta (1972)
R27	H	<u>tet</u>	Grindley <u>et al</u> (1972)
R621a	I δ	<u>tet</u>	Hedges & Datta (1973)
R64	I Δ	<u>sul</u> <u>tet</u> <u>str</u>	Meynell & Datta (1967)
R64-11	I Δ	<u>sul</u> <u>tet</u> <u>str</u> <u>drd11</u>	Meynell & Datta (1967)
R144	I Δ	<u>kan</u> <u>tet</u>	Meynell & Datta (1967)
R144-3	I Δ	<u>kan</u> <u>tet</u> <u>drd3</u>	Meynell & Datta (1967)
R112	I Δ	<u>kan</u>	Bouanchaud & Chabbert (1969)
R387	K	<u>cam</u> <u>str</u>	Hedges & Datta (1971)
R471a	L	<u>amp</u>	Hedges <u>et al</u> (1974)
R446b	M	<u>tet</u> <u>str</u>	Hedges <u>et al</u> (1973)
R16	O	<u>amp</u> <u>sul</u> <u>tet</u> <u>str</u>	Datta & Olarte (1974)
RP4	P	<u>amp</u> <u>kan</u> <u>tet</u>	Datta <u>et al</u> (1971)
R478	S	<u>cam</u> <u>kan</u> <u>tet</u>	Hedges <u>et al</u> (1974)
S-a	W	<u>cam</u> <u>sul</u> <u>kan</u> <u>str</u>	Watanabe <u>et al</u> (1968)

^a sul, sulfonamides; tet, tetracycline; amp, ampicillin; kan, kanamycin; str, streptomycin; cam, chloramphenicol; amber, amber mutation; drd, derepressed.

^b To conform to current nomenclature practice (Novick et al, 1976), R1-am of Kennedy and Crowlesmith is shown as R1-bla31 to indicate that it carries a point mutation in its β -lactamase locus.

indicating that it carries a particular mutation B43. To pointedly refer to any dnaB mutation that can confer temperature-sensitivity, the symbol dnaB(ts) is used. A strain that is known to carry a dnaB allele and is also temperature-sensitive in DNA synthesis is abbreviated DnaB(ts). The symbol + or sup⁺ should stand for the wild-type strain or wild-type allele (most wild-type strains do not contain suppressor activity) in bacteria. This usage has been distorted in the case of some papers on suppressors in bacterial systems where + conveys the erroneous idea that the suppressor strain has the particular suppressor and su⁻ has been used for the strain lacking suppressor activity. In this thesis and in accordance with the current practice, we use what we believe to be the correct nomenclature; sup⁺ for the wild-type allele and supD, supE and supF for mutant alleles of the sup genes. In the early parts of this thesis, we use sup⁺ (su⁻) to leave no room for ambiguity. The nomenclature for bacterial plasmids and for genotypes and phenotypes follows the recommendations of Demerec et al. (1966), Novick et al. (1976) and the Plasmid Reference Center, Stanford, California. The rationale for the nomenclature system is discussed in detail in the above two references.

Media and Chemicals

For bacterial growth, two media were routinely used:

- (a) Nutrient Broth (NB) and Nutrient Agar (NA).

NB contained per liter of distilled water, 8 g Difco Nutrient Broth (pH 7.2). NA plates contained in addition 1.5% (w/v) Difco Agar.

(b) Davis Minimal Broth (MB) and Davis Minimal Agar (MA).

These media were essentially the same as described by Davis and Mingioli (1950). They contained the following salts per liter of distilled water; K_2HPO_4 , 5.25g; KH_2PO_4 , 2.25g; tri-sodium citrate, 1g and $(NH_4)_2SO_4$, 0.5g. Required supplements added to the medium were; thiamine, 1 μ g/ml; thymine, 10 μ g/ml and 0.5 ml of a 20% $MgSO_4$ (w/v) solution. L-amino acids (40 μ g/ml) were added as required by the particular strain. These supplements were filter-sterilized (sterile cellulose nitrate filters; porosity, 0.45 μ , Millipore Filter Corp.) before use. Glucose (20% w/v) was sterilized separately and added to yield to final concentration of 0.5%. All media were adjusted to pH 7.2 and sterilized by autoclaving for 15 min at 115C.

The sources of antibiotics and the concentrations at which they were used were: ampicillin (Ap), 30 μ g/ml; kanamycin (Kan), 30 μ g/ml; tetracycline (Tc), 20 μ g/ml (all from Bristol Lab., Syracuse, New York); streptomycin (Str), 50 μ g/ml (from Calbiochem., California) and chloramphenicol (Cm), 30 μ g/ml (from Parke Davis and Co., Michigan). (methyl- 3H)thymine (specific activity; 17.86 C/m mol) was purchased from New England Nuclear Corp., Boston, Mass. and ethidium bromide from Sigma Chemical Co., St. Louis, Missouri.

Construction of DnaB(ts) Strains Bearing Different Conjugative

R Plasmids

Plasmids were introduced into various DnaB(ts) mutants by liquid mating. This was achieved by mixing 0.1 ml of a donor culture with 0.9 ml of a recipient culture (both at exponential phase of growth) and incubating in NB for 1 hr at 30C. The mating mixture was appropriately diluted and transconjugants selected on supplemented minimal agar plates containing one antibiotic (usually Tc or Kan), the resistance to which was associated with the conjugative plasmid. Colonies were purified twice by single colony isolation on the same type of selective plate on which they were isolated.

Test of Plasmid-Bearing Strains for Colony-Forming Ability at Different Temperatures

Strains bearing an R plasmid were grown to late exponential phase (approximately 10^9 cells/ml) in NB at 30C and they were appropriately diluted on a series of NA plates that had been prewarmed to the desired incubation temperature. Incubation temperatures in the range of 30C to 42C were used. Care was taken to record and maintain the required temperatures of the forced-draft incubators that were used. Care was also taken to avoid stacking of plates inside the incubators. These precautions were necessary in securing acceptable reproducibility of experimental data.

DNA Synthesis

DNA synthesis was measured by the relative increase in (^3H)thymine incorporation into trichloroacetic acid (TCA) precipitable material. Cultures of plasmid-positive (R^+) and plasmid-negative (R^-) strains growing in supplemented minimal broth were labeled in their DNA with (methyl- ^3H)thymine ($10\text{ }\mu\text{Ci}/2\mu\text{g}/\text{ml}$) for approximately four generations at permissive temperature (30°C). Such cultures in their exponential phase of growth were then diluted 100-fold into similar media containing (^3H)thymine at the same specific activity and prewarmed so that the desired temperature was attained immediately upon dilution. Further DNA synthesis was followed by the accumulation of TCA-precipitable counts. This was achieved by taking samples (0.1 ml) at intervals of 30 min and after absorption onto Whatman 3 MM filter discs (2.3 cm in diameter), they were processed as described by Lark, Repko and Hoffman (1963). This involved TCA-precipitation; washing twice with ice-cold TCA (10%) and finally two times with boiling water. The discs were dried and placed into 10 ml of a toluene-based scintillation fluid (4g of Omnifluor (New England Corp.) per liter toluene) and were counted in a Beckman LS-230 scintillation counter.

Plasmid-Determined Phenotypes

This was routinely determined by spotting a drop of the test culture (in late exponential phase and adjusted to O.D. of 0.7) onto the surface of NA plates supplemented with the appropriate antibiotics. Such spot tests were also used to determine the

degrees of antibiotic resistance. The frequency of antibiotic-resistant survivors was determined quantitatively only when there was need to determine the degree of antibiotic resistance more accurately.

Efficiency of Conjugation

Overnight cultures of donor and recipient strains in NB at 30C were diluted and regrown in the same medium to approximately 2×10^8 cells/ml at the same temperature. Routinely, and at the time of conjugation, the donor strain was checked for its antibiotic resistance markers by streaking on NA plates containing the appropriately antibiotics. Donor and recipient cultures were mixed at a ratio of 1:10 and conjugation was allowed for 45 min at 30C. The ratio of transconjugant colonies (selected on an appropriate selective medium) to viable counts of donor cells was taken as a measure of the efficiency of conjugation.

RESULTS

Stability of the Test R Plasmids

At permissive temperature, all of the plasmids used in this study (Table I-2) were maintained stably in various DnaB(ts) mutants and their corresponding dnaB⁺ strains. This was determined by replica plating a few hundred colonies of the plasmid-bearing strains from NA plates onto antibiotic-containing media selective for the presence of the resistance markers associated with each plasmid. The plasmids in such strains always retain their conjugative ability. R plasmid DNA was always found to be present in DnaB(ts)/R⁺ strains when the total DNA from these strains was isolated and analyzed in ethidium bromide-cesium chloride density gradients (see "Results" in Chapter II, p. 36).

Suppressing Ability of Plasmids of Different Incompatibility Groups

In preliminary experiments to test the temperature-sensitivity of DnaB(ts) mutants, it was found that the degree of temperature-sensitivity of these mutants varied with the particular dnaB allele they carried. It was also apparent that small differences in temperature-sensitivity may be critical for the detection of suppressing ability of R plasmids. These preliminary observations determined the selection of the three "nonpermissive" temperatures used in Table I-3. Strains to be examined were grown to late exponential phase (approximately 10^9 cells/ml) in NB at 30C and were appropriately diluted and spread on NA plates prewarmed to the desired

temperature at which they were incubated. The results are presented in Table I-3 and were compiled from the average of three experiments. As indicated in the footnote to Table I-3, arbitrarily, a plasmid was considered to have a significant effect on the temperature-sensitivity of colony-forming ability when the relative number of temperature-resistant colonies (relative to the number at 30C) of plasmid positive strains differed from those of plasmid-negative (R^-) strains by a factor of 10 or more. This criterion ensured the unambiguous identification of those plasmids which do have an effect. This was preferred at the risk of overlooking the abilities of those plasmids which suppress or enhance the temperature-sensitivity of $DnaB(ts)$ mutants weakly.

It can be seen (Table I-3) that a majority of the wild-type R plasmids belonging to different incompatibility groups isolated from various parts of the world possess the ability to suppress the temperature-sensitivity of colony formation of one or more $dnaB(ts)$ mutations. Therefore, it seems that many wild-type conjugative R plasmids possess the mechanisms for correcting or bypassing the lesion of the $dnaB$ gene. Some of the plasmids (e.g. R27 and R124) enhance rather than suppress the temperature-sensitivity. Even more interestingly, some plasmids will suppress the temperature-sensitivity of a particular $dnaB$ mutation while enhancing that of another (e.g. R16). The introduction of any of these plasmids into the strain carrying the $dnaB^+$ allele (strain Q125) did not increase or decrease the temperature resistance of such strains. When temperature-resistant colonies arising from each of the $DnaB(ts)/R^+$

TABLE I-3
SUPPRESSING OR ENHANCING ABILITY OF PLASMIDS OF DIFFERENT INCOMPATIBILITY GROUPS

Strain	<i>dnaB</i> (ts) allele	R plasmid	Incom- patibility group of plasmid	Colonies at a higher temperature relative to those at 30°C			Inferred suppression pattern of the plasmid ^a
				37°C	39°C	41°C	
Q1502	<i>dnaBR</i>	Nil	Nil	6.5×10^{-1}	3.3×10^{-2}	$<1 \times 10^{-3}$	
Q1506	<i>dnaB43</i>	Nil	Nil	1	1.4×10^{-2}	$<1.3 \times 10^{-3}$	
Q1508	<i>dnaB70</i>	Nil	Nil	7.8×10^{-1}	9.2×10^{-2}	$<5.3 \times 10^{-3}$	
Q1610	<i>dnaB266</i>	Nil	Nil	1.4×10^{-1}	2.1×10^{-2}	$<3 \times 10^{-3}$	
Q1502/R41	<i>dnaBR</i>	R41	A	1	2.7×10^{-2}	2.6×10^{-3}	Suppresses <i>dnaBR</i> , <i>B43</i> , and <i>B70</i> , effect on <i>B266</i> uncertain
Q1506/R41	<i>dnaB43</i>	R41	A	8.5×10^{-1}	6.8×10^{-2}	2×10^{-3}	
Q1508/R41	<i>dnaB70</i>	R41	A	1	7.8×10^{-2}	2×10^{-3}	
Q1610/R41	<i>dnaB266</i>	R41	A	7.0×10^{-2}	7.6×10^{-3}	5×10^{-4}	
Q1502/R40a	<i>dnaBR</i>	R40a	C	1	1	3×10^{-3}	Suppresses all four <i>dnaB</i> mutations
Q1506/R40a	<i>dnaB43</i>	R40a	C	8.3×10^{-1}	1.5×10^{-2}	3×10^{-3}	
Q1508/R40a	<i>dnaB70</i>	R40a	C	9.1×10^{-1}	9.9×10^{-2}	1.6×10^{-3}	
Q1610/R40a	<i>dnaB266</i>	R40a	C	8.9×10^{-1}	6.9×10^{-2}	1.6×10^{-3}	
Q1502/R386	<i>dnaBR</i>	R386	FI	9.6×10^{-1}	4.3×10^{-2}	$<4.3 \times 10^{-3}$	Suppresses <i>dnaB43</i> and <i>B266</i> (at 37°C)
Q1506/R386	<i>dnaB43</i>	R386	FI	1	1	4.2×10^{-3}	
Q1508/R386	<i>dnaB70</i>	R386	FI	9.4×10^{-1}	5.9×10^{-2}	$<5.8 \times 10^{-3}$	
Q1610/R386	<i>dnaB266</i>	R386	FI	7.5×10^{-2}	1.8×10^{-3}	$<1.7 \times 10^{-3}$	
Q1502/R100	<i>dnaBR</i>	R100	FII	8.5×10^{-1}	1.3×10^{-2}	3×10^{-3}	Suppresses <i>dnaB70</i> , enhances <i>B43</i> , effect on <i>dnaB266</i> uncertain
Q1506/R100	<i>dnaB43</i>	R100	FII	9.7×10^{-1}	$<6 \times 10^{-3}$	$<6 \times 10^{-3}$	
Q1508/R100	<i>dnaB70</i>	R100	FII	3×10^{-1}	3×10^{-3}	3×10^{-3}	
Q1610/R100	<i>dnaB266</i>	R100	FII	Not done	Not done	Not done	
Q1502/R124	<i>dnaBR</i>	R124	FIV	6.2×10^{-2}	2.5×10^{-3}	$<2.5 \times 10^{-3}$	Enhances all four <i>dnaB</i> mutations
Q1506/R124	<i>dnaB43</i>	R124	FIV	1.9×10^{-1}	4.5×10^{-3}	$<4.5 \times 10^{-3}$	
Q1508/R124	<i>dnaB70</i>	R124	FIV	9.5×10^{-2}	3×10^{-3}	$<3 \times 10^{-3}$	
Q1610/R124	<i>dnaB266</i>	R124	FIV	2.9×10^{-2}	9×10^{-4}	$<9 \times 10^{-4}$	
Q1502/R27	<i>dnaBR</i>	R27	H	1.9×10^{-1}	1×10^{-3}	$<2.5 \times 10^{-3}$	Enhances all four <i>dnaB</i> mutations
Q1506/R27	<i>dnaB43</i>	R27	H	8.8×10^{-1}	5.5×10^{-2}	$<5.5 \times 10^{-3}$	
Q1508/R27	<i>dnaB70</i>	R27	H	1×10^{-1}	2.5×10^{-3}	$<2.5 \times 10^{-3}$	
Q1610/R27	<i>dnaB266</i>	R27	H	1.4×10^{-2}	3×10^{-3}	$<1.5 \times 10^{-3}$	
Q1502/R621a	<i>dnaBR</i>	R621a	Iy	1	5.8×10^{-4}	$<5 \times 10^{-4}$	Enhances <i>dnaB70</i> , effect on other three mutations uncertain
Q1506/R621a	<i>dnaB43</i>	R621a	Iy	1	6.1×10^{-4}	$<6 \times 10^{-4}$	
Q1508/R621a	<i>dnaB70</i>	R621a	Iy	5.3×10^{-2}	5.3×10^{-4}	$<5 \times 10^{-4}$	
Q1610/R621a	<i>dnaB266</i>	R621a	Iy	2.1×10^{-2}	1.5×10^{-4}	$<1 \times 10^{-4}$	
Q1502/R64	<i>dnaBR</i>	R64	I α	2.6×10^{-1}	1.5×10^{-2}	2.2×10^{-3}	Suppresses all four <i>dnaB</i> mutations
Q1506/R64	<i>dnaB43</i>	R64	I α	5.1×10^{-1}	1×10^{-2}	4.8×10^{-3}	
Q1508/R64	<i>dnaB70</i>	R64	I α	8.2×10^{-1}	9.6×10^{-2}	8.3×10^{-3}	
Q1610/R64	<i>dnaB266</i>	R64	I α	3.4×10^{-2}	2.2×10^{-3}	6.8×10^{-4}	
Q1502/R144	<i>dnaBR</i>	R144	I α	3.8×10^{-1}	4.1×10^{-2}	3.7×10^{-3}	Suppresses all four <i>dnaB</i> mutations
Q1506/R144	<i>dnaB43</i>	R144	I α	9.3×10^{-1}	7.2×10^{-2}	5.3×10^{-3}	
Q1508/R144	<i>dnaB70</i>	R144	I α	8×10^{-1}	1.7×10^{-2}	9.3×10^{-3}	
Q1610/R144	<i>dnaB266</i>	R144	I α	2.8×10^{-2}	1×10^{-3}	3.3×10^{-4}	
Q1502/R112	<i>dnaBR</i>	R112	I α	9×10^{-1}	5.6×10^{-2}	5×10^{-3}	Enhances <i>dnaBR</i> , <i>B43</i> , and <i>B266</i> , effect on <i>B70</i> uncertain
Q1506/R112	<i>dnaB43</i>	R112	I α	1	3.2×10^{-4}	3.5×10^{-4}	
Q1508/R112	<i>dnaB70</i>	R112	I α	7.4×10^{-1}	4.8×10^{-2}	1.9×10^{-3}	
Q1610/R112	<i>dnaB266</i>	R112	I α	3×10^{-2}	$<3 \times 10^{-4}$	$<3 \times 10^{-4}$	
Q1502/R387	<i>dnaBR</i>	R387	K	1	1.8×10^{-3}	2×10^{-4}	Effects uncertain
Q1506/R387	<i>dnaB43</i>	R387	K	5.7×10^{-2}	6.4×10^{-4}	2.5×10^{-4}	
Q1508/R387	<i>dnaB70</i>	R387	K	1	1.2×10^{-4}	5.4×10^{-5}	
Q1610/R387	<i>dnaB266</i>	R387	K	7.7×10^{-2}	4×10^{-5}	4.6×10^{-5}	
Q1502/R471a	<i>dnaBR</i>	R471a	L	7.7×10^{-1}	7.6×10^{-2}	3.2×10^{-3}	Suppresses <i>dnaBR</i> , <i>B43</i> , and <i>B70</i> , enhances <i>B266</i> slightly
Q1506/R471a	<i>dnaB43</i>	R471a	L	6×10^{-1}	5.8×10^{-2}	1.3×10^{-3}	
Q1508/R471a	<i>dnaB70</i>	R471a	L	8.1×10^{-1}	7.9×10^{-2}	2.3×10^{-3}	
Q1610/R471a	<i>dnaB266</i>	R471a	L	1.3×10^{-2}	1.8×10^{-3}	4×10^{-4}	

TABLE 1-3 (CONT'D)

Strain	<i>dnaB</i> (ts) allele	R plasmid	Incompatibility group of plasmid	Colonies at a higher temperature relative to those at 30°C			Inferred suppression pattern of the plasmid ^a
				37°C	39°C	41°C	
Q1502/R446b	<i>dnaBA</i>	R446b	M	2.1×10^{-3}	1.6×10^{-4}	1.7×10^{-4}	Suppresses <i>dnaB43</i> , <i>B70</i> , <i>B266</i> ; effect on <i>BA</i> uncertain
Q1506/R446b	<i>dnaB43</i>	R446b	M	1.9×10^{-3}	3.4×10^{-4}	3.6×10^{-4}	
Q1508/R446b	<i>dnaB70</i>	R446b	M	7.1×10^{-3}	6.7×10^{-4}	6×10^{-4}	
Q1610/R446b	<i>dnaB266</i>	R446b	M	2.2×10^{-3}	2.6×10^{-4}	1.8×10^{-4}	
Q1502/R16	<i>dnaBA</i>	R16	O	6.3×10^{-3}	2.4×10^{-4}	1.7×10^{-4}	Suppresses <i>dnaB43</i> and <i>B70</i> ; enhances <i>dnaB266</i> ; effect on <i>dnaBA</i> uncertain
Q1506/R16	<i>dnaB43</i>	R16	O	6.5×10^{-3}	8.4×10^{-4}	5.4×10^{-4}	
Q1508/R16	<i>dnaB70</i>	R16	O	8.6×10^{-3}	5.8×10^{-4}	1.1×10^{-3}	
Q1610/R16	<i>dnaB266</i>	R16	O	5.4×10^{-4}	3×10^{-5}	$< 3 \times 10^{-5}$	
Q1502/RP4	<i>dnaBA</i>	RP4	P	1.3×10^{-3}	2.2×10^{-4}	$< 7 \times 10^{-5}$	Suppresses <i>dnaB43</i> and <i>dnaB70</i> ; enhances <i>B266</i>
Q1506/RP4	<i>dnaB43</i>	RP4	P	1	1	$< 5 \times 10^{-5}$	
Q1508/RP4	<i>dnaB70</i>	RP4	P	7.4×10^{-3}	1	4×10^{-5}	
Q1610/RP4	<i>dnaB266</i>	RP4	P	2.2×10^{-4}	6.2×10^{-5}	2×10^{-5}	
Q1502/R478	<i>dnaBA</i>	R478	S	8.8×10^{-3}	5.4×10^{-4}	5.3×10^{-4}	Suppresses <i>dnaB8</i> and <i>B43</i> ; enhances <i>B266</i>
Q1506/R478	<i>dnaB43</i>	R478	S	1	2×10^{-3}	2.1×10^{-3}	
Q1508/R478	<i>dnaB70</i>	R478	S	9.7×10^{-3}	5.8×10^{-4}	3.2×10^{-4}	
Q1610/R478	<i>dnaB266</i>	R478	S	4.1×10^{-4}	4.7×10^{-5}	$< 4 \times 10^{-5}$	
Q1502/S-a	<i>dnaB8</i>	S-a	W	9.7×10^{-3}	6.6×10^{-4}	1×10^{-4}	Suppresses <i>dnaBA</i> , <i>B43</i> , and <i>B70</i> ; enhances <i>B266</i>
Q1506/S-a	<i>dnaB43</i>	S-a	W	1	1	4.9×10^{-5}	
Q1508/S-a	<i>dnaB70</i>	S-a	W	7.4×10^{-3}	6.4×10^{-4}	1.5×10^{-4}	
Q1610/S-a	<i>dnaB266</i>	S-a	W	2.9×10^{-4}	2.8×10^{-5}	2×10^{-5}	

^a The results are an average of three experiments. In drawing these inferences, a reproducible suppression or enhancement effect has been considered to be significant only when the relative number of temperature-resistant colonies of plasmid-positive strains differs from those of plasmid-negative strains by a factor of 10 or more.

^b Notations such as $< 1 \times 10^{-4}$ mean that no colony was observed at this temperature when 10^4 colony-forming units (as determined from platings at 30°C) were spread.

^c Note: The derepressed plasmid R100-1 suppresses all four *dnaB* mutations in this genetic background.

strains were regrown at 30°C and retested, the frequency of temperature-resistant colonies from these regrown cultures was similar to that of the parent strains. The appearance of these temperature-resistant colonies does not therefore reflect genetic heterogeneity in the parent strains or the appearance of temperature-resistant mutants.

Suppression and Enhancement of the Temperature-Sensitivity of DNA Synthesis

From the plasmids that were surveyed and found to either suppress or enhance the temperature-sensitivity of colony formation (Table I-3), plasmids R446b and R27 were chosen as examples of a suppressing and an enhancing plasmid respectively. Suppression or enhancement of temperature-sensitivity in DNA synthesis in strains Q1506 (dnaB43), Q1506/R446b and Q1506/R27 was studied by following the incorporation of (³H)thymine into TCA-insoluble counts after a shift from one selected temperature to another. The results are shown Fig. I-1. They confirmed that R446b suppresses the mutation dnaB43 and that R27 enhances it.

The Efficiency of Suppression by Wild-Type Plasmids and by Mutants Derepressed for Conjugal Transfer

Derepressed (drd) mutant plasmids are those that conjugate at a higher efficiency and they are believed to no longer produce an inhibitor component of the transfer system (Meynell et al., 1968; Willetts, 1972). Wilkins (1975) observed that the temperature-

Figure I-1.

DNA synthesis at different temperatures in the plasmid-negative strain Q1506 (dnaB43) and in the strains Q1506/R446b and Q1506/R27 carrying R plasmids that suppress or enhance the temperature sensitivity.

sensitivity of dnaG(ts) mutations could be suppressed by plasmids of the I α incompatibility group, but this phenomenon was only limited to derepressed plasmids. That is, wild-type plasmids were found to be unable to do so at detectable frequencies. A comparison was made on the ability of wild-type plasmids and their corresponding drd mutants to suppress the mutation dnaB266 (strain WD266II). The results are shown in Table I-4. This strain was chosen for this study because the efficiency of suppression is most easily measured in this strain (Table I-5). Both derepressed plasmids, R64-11 and R100-1 belonging to two different incompatibility groups (group I α and FII respectively), suppressed the dnaB266 mutation with a higher efficiency than their corresponding wild-type plasmids R64 and R100. However, such a result was not observed with the drd mutant plasmid R144-3 and the corresponding wild-type plasmid R144 (Table I-4). In this case, the wild-type plasmid (R144) suppressed dnaB266 with a high efficiency and little further increase in the suppression efficiency was observed with the derepressed plasmid R144-3. There was no consistent correlation between the conjugal transfer efficiency of a wild-type plasmid and its ability to suppress dnaB(ts) mutations. For example, plasmid R446b and R40a suppressed dnaB(ts) mutations with high efficiencies (Table I-3), but these plasmids are conjugally transferred at very low frequencies (approximately 10^{-5} per donor cell). Several rare temperature-resistant colonies of the dnaB266-bearing mutants carrying either R64 or R100 were regrown at 30C and tested for their conjugal transfer efficiency. These isolates transferred the plasmid at frequencies characteristic of the wild-type

TABLE 1-4

Effect of the Derepression of Conjugal Transfer^a on the
Efficiency of Suppression by Some Suppressing Plasmids

Donor strain	Colonies at high temperatures relative to the number at 30C			Percentage of donor cells that trans- ferred the plasmid at 30C
	37C	39C	41C	
WD266II	7.5×10^{-6}	2.5×10^{-7}	5×10^{-8}	
WD266II/R64	1.5×10^{-4}	1.5×10^{-4}	1×10^{-7}	1.2×10^{-4}
WD266II/R64-11	1	1×10^{-1}	8×10^{-8}	1.1×10^{-3}
WD266II/R144	8.8×10^{-1}	5×10^{-1}	5.2×10^{-3}	1.7×10^{-4}
WD266II/R144-3	9.8×10^{-1}	7.3×10^{-1}	5.6×10^{-3}	6.6×10^{-1}
WD266II/R100	5.1×10^{-4}	5×10^{-6}	5×10^{-7}	1.5×10^{-3}
WD266II/R100-1	4.4×10^{-2}	4.2×10^{-3}	3×10^{-3}	4.6×10^{-1}

^a The recipient was strain CSH56. Conjugation was for 30min
at a donor to recipient ratio of 1 : 10.

repressed plasmid rather than the mutant drd plasmid, indicating that they were still wild-type with respect to conjugation proficiency. As pointed out earlier, such temperature-resistant colonies are not mutants; when regrown at 30C and tested, they were no more temperature-resistant than the plasmid-positive strains used initially.

Suppression of Some dnaB(ts) Mutations By a Plasmid when these Mutations were Present in Different Strains

The efficiency of suppression by R64-11 of different strains carrying dnaB43 and dnaB266 was compared and the results are shown in Table I-5. Although plasmid R64-11 suppressed both mutations dnaB43 and dnaB266, there were marked differences in the efficiency of suppression when these mutations were present in different strains. Since the amber suppressor, supE is present in most of these strains, it can only be concluded that other unknown differences between the strains contribute to the differences in the efficiency of suppression. From the results presented in Table I-5, it was also evident that the degree of temperature-sensitivity varied when the same dnaB(ts) mutation was present in different host strains. These latter observations only served to emphasize the need for using isogenic strains in such studies.

Loss of Suppression Upon the Displacement of R64-11 by R112

Plasmid R64-11 was found to suppress the mutation dnaB266 and plasmid R112 was found to be unable to do so. Conjugation was performed using recipient strain WD266II/R64-11 and a donor strain carrying R112, a plasmid that belongs to the same incompatibility

TABLE I-5

Differences in the Efficiency of Plasmid-Induced Suppression
of a *dnaB* Allele in Different Genetic Background

Strain	<u><i>dnaB</i></u> allele	Colonies at a high temperature relative to those at 30C (for <u><i>dnaB(ts)</i></u> or 37C (for <u><i>dnaB</i>⁺</u>)	
		39C	41C
CR34	<u><i>dnaB</i>⁺</u>	1	1
CR34/R64-11	<u><i>dnaB</i>⁺</u>	1	1
CR34Ts	<u><i>dnaB43</i></u>	3.9×10^{-6}	$< 1 \times 10^{-6}$
CR34Ts/R64-11	<u><i>dnaB43</i></u>	1×10^{-2}	7.6×10^{-6}
Q125	<u><i>dnaB</i>⁺</u>	9×10^{-1}	5.5×10^{-1}
Q125/R64-11	<u><i>dnaB</i>⁺</u>	9×10^{-1}	1.2×10^{-1}
Q1506	<u><i>dnaB43</i></u>	1.4×10^{-3}	1.3×10^{-6}
Q1506/R64-11	<u><i>dnaB43</i></u>	1.9×10^{-1}	1.1×10^{-5}
Q1610	<u><i>dnaB266</i></u>	2.1×10^{-4}	$< 3 \times 10^{-7}$
Q1610/R64-11	<u><i>dnaB266</i></u>	2.3×10^{-4}	1.3×10^{-6}
WD002	<u><i>dnaB</i>⁺</u>	Not done	2.7×10^{-4}
WD002/R64-11	<u><i>dnaB</i>⁺</u>	Not done	9.3×10^{-4}
WD266II	<u><i>dnaB266</i></u>	2.5×10^{-7}	4.5×10^{-8}
WD266II/R64-11	<u><i>dnaB266</i></u>	1×10^{-1}	8×10^{-8}

group as R64-11, and selection was for the R112-associated kanamycin-resistant marker. All of the 50 kanamycin-resistant trans-conjugants tested had lost the R64-11 associated tetracycline resistance as well as the ability to give rise to colonies at 37C. Thus, upon the displacement of R64-11 by R112, the suppressing ability of the former plasmid was lost. This demonstrated the plasmid-specificity of suppression.

CONCLUSIONS

From the results presented in Table I-3, it can be seen that almost all of the plasmids tested had an observable effect on dnaB(ts) mutations. The majority of the plasmids suppressed temperature-sensitivity (e.g. R64-11) and some plasmids enhanced temperature-sensitivity (e.g. R27). Suppression and enhancement of the temperature-sensitivity of colony-forming ability was accompanied by the suppression and enhancement of the temperature-sensitivity of DNA synthesis (Fig. I-1). That is, a suppressing plasmid will usually suppress a dnaB(ts) mutation in colony formation as well as DNA synthesis. On the other hand, an enhancing plasmid will enhance the temperature-sensitivity of both colony formation and DNA synthesis. Suppressing or enhancing abilities are clearly found to be dispersed widely among wild-type conjugative plasmids of various incompatibility groups. A definite pattern of suppression or enhancement was not observed. For example, within a single incompatibility group (I α), both suppressing and enhancing plasmids were encountered (R64-11 is a suppressing plasmid and R112 is an enhancing plasmid, Table I-3). Among those plasmids which suppressed dnaB(ts) mutations, only some were observed to suppress all four dnaB(ts) mutations (Table I-3). It was also observed that in some cases the detection of suppressing ability is critically dependent on the selected nonpermissive temperature or on the strain in which suppression is studied. (see Table I-3 and I-6). The use of a single dnaB(ts) mutation or a single "nonpermissive" temperature would therefore have yielded an under-

estimate of the prevalence of this property, among wild-type conjugative plasmids. We suspect that in the past, the use of a single mutant and/or a single temperature resulted in this phenomenon being overlooked. In previous studies, a few low copy-number plasmids have been reported to be unable to replicate in *DnaB(ts)* mutants at a nonpermissive temperature (Nishimura *et al.*, 1971; Goebel and Schrempf, 1972). This gave rise to a suggestion (Veltkamp and Nijkamp, 1974) that low copy-number conjugative plasmids, as a group, may be unable to replicate in *DnaB(ts)* mutants at high temperatures. However, the present observations indicate that such a belief would not be warranted because all the R plasmids used in this study are maintained at low copy number. It would appear that at least low copy-number R plasmids have the ability to modify the temperature-sensitivity of *dnaB(ts)* mutations. Such modification usually results in the suppression of temperature-sensitivity, but can sometimes results in an enhancement of temperature-sensitivity. It can thus be concluded that we are concerned here with a general phenomenon involving most low copy-number conjugative R plasmids.

The efficiency of suppression by a plasmid (R64-11) on two different strains carrying the same mutation (*dnaB266*) varied to a great extent (Table I-5). For example, R64-11 suppressed strain WD266II (*dnaB266*) with a much higher efficiency than strain Q1610 (*dnaB266*). Although both of these strains carried the mutation *dnaB266* and *supE*, some other unknown differences between the strains might contribute to the differences in the efficiency of suppression. The presence of the same *dnaB(ts)* mutation in two different strains

may also result in a difference of the degree of temperature-sensitivity.

Suppression of dnaB(ts) mutations is a reversible phenomenon. The loss of a suppressing plasmid, or more properly, the replacement of a suppressing plasmid by a non-suppressing one (replacement of R64-11 by R112) leads to the restoration of temperature-sensitivity. For all suppressing plasmids, it seems that the continued presence of the suppressing plasmid is necessary to secure suppression.

Table I-6 is a summary of the ability of different conjugative plasmids of various incompatibility groups to alter the temperature-sensitivity of four different dnaB(ts) mutations. Since two or more dnaB(ts) mutations are always affected, it may be concluded that dnaB(ts) suppression or enhancement is not frequently allele-specific. A single plasmid may however alter the temperature-sensitivity of different dnaB(ts) mutations in different ways. For example, RP4 suppressed one dnaB(ts) mutation, enhanced another and had no observable effect on the third. Even if a plasmid suppressed all four dnaB(ts) mutations, the efficiency of suppression varied from one dnaB(ts) mutation to another (Table I-6).

In addition to the conclusion that the phenomenon of plasmid-induced suppression and enhancement of dnaB(ts) mutations is a general one, the results in this Chapter have also provided clues to the possible mode of suppression. It appears unlikely that these various plasmids act indirectly and nonspecifically by altering, for example, the cell surface properties or internal environment

TABLE I-6

Suppression or Enhancement of the Temperature-Sensitivity
of DnaB(ts) Mutants by Conjugative Plasmids: Summary^a

Plasmid(s)	<u>dnaB(ts)</u> mutations affected and the nature of the effect	Comment
R40a, R100-1, R64 R64-11, R144, R144-3	all four mutations suppressed	R64-11 suppresses more efficiently than R64
R446b	suppresses <u>B43</u> , <u>B70</u> , <u>B266</u> ; effect on <u>B8</u> uncertain	this plasmid carries an amber suppressor (see Appendix II)
RA1	suppresses <u>B8</u> , <u>B43</u> , <u>B70</u> ; effect on <u>B266</u> uncertain	
R386	suppresses <u>B43</u> , <u>B266</u>	
R27, R124	all four mutation enhanced	see also Fig. I-1
R112	enhances <u>B8</u> , <u>B43</u> <u>B266</u> ; effect on <u>B70</u> uncertain	
R621a	enhances <u>B70</u> ; effect on other three mutat- ions uncertain	
R471a, R100, R16, RP4, R478; S-a	some mutations sup- pressed and other enhanced	for details, see Table I-3, compare the effect of R100 to R100-1; R478 carries an amber suppres- sor (see Appendix II)
R387	effects uncertain	

^a Derived from the results detailed in Tables I-3 and I-5.

of the cell which in turn has an effect on the temperature-sensitivity of the DnaB protein as is apparently the case when temperature-sensitivity is reversed by high salt or sugar concentrations in the growth medium (Ricard and Hirota, 1969; Hirota et al., 1971; Gross, 1971). Instead, the results suggest that the basis of these observations may be interactions between products of particular plasmid genes and particular dnaB(ts) mutations. The level at which such interactions can occur cannot be specified from the results presented here but is examined in further Chapters.

CHAPTER II

Evidence that the Mode of Suppression or Enhancement Does Not Involve the Insertion of the Plasmid into the Chromosome

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INTRODUCTION

In the general introduction (p. 5 para. 4), it was pointed out that a precedent exists for the observation that plasmids (including R plasmids) can suppress the temperature-sensitivity of DnaB(ts) mutations in E. coli. The earlier observations involve the mutations in the dnaA genetic region, a region that is concerned with the initiation of chromosomal DNA replication. In all of the observations on the suppression of the temperature-sensitivity of dnaA(ts) mutations (Nishimura et al., 1971; A. Nishimura et al., 1973; Moody and Runge, 1972; Sotomura and Yoshikawa, 1975; Datta and Barth, 1976), it has been found that in the suppressed state, the plasmid no longer exists in an autonomous state in the cell and that all or most of it has been integrated into the chromosome. Consequently, this phenomenon has been referred to in the literature as "suppressive integration" or "integrative suppression".

To test for the possibility that suppression of dnaB(ts) mutations is by a mechanism similar to that described above, replication and maintenance of the suppressing R plasmids in the suppressed state were studied. In this Chapter, experiments are described which indicate that the mode of suppression of dnaB(ts) mutations is not by the insertion of the suppressing R plasmid into the host chromosome.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains and plasmids used were the same as those described in Tables I-1 and I-2.

Media and Chemicals

The composition of NB and MB are described under "Materials and Methods" in Chapter I. LB Broth contained per liter of distilled water: 10g tryptone, 5g yeast extract and 5g NaCl and the pH was adjusted to 7.4. The concentrations of antibiotic used were similar to that described in "Materials and Methods" in Chapter I.

Measurement of the Proportion of Plasmid DNA synthesized at Different Temperatures

The R^+ and R^- test strains were grown to exponential phase (approximately 5×10^8 cells/ml) in supplemented minimal broth (nonradioactive) and were then diluted 20-fold with prewarmed media of the same composition but containing (methyl- 3H) thymine (10 μ Ci/ml; specific activity, 17.86 C/mM) in place of nonradioactive thymine. Viable counts were also taken at this time. The cultures were further incubated at either permissive (30C) or nonpermissive (41C) temperature. The cultures were then centrifuged and the cells were washed twice with TES buffer (0.05 M NaCl, 0.05 M Tris, 0.005 M EDTA, pH 8) at 0C. The

pellets were resuspended in 1 ml of spheroplast-forming mixture (1 mg lysozyme, 500 μ g RNase and 100 mg of sucrose in TES buffer), and incubated at 37C for 10 min. The mixture was then kept on ice for 5 min. 0.4 ml of Sarkosyl detergent solution (2% in water) at room temperature was then added, followed by 1 ml of TES at room temperature. The mixture was mixed with a Vortex Mixer (Fisher Scientific Co., Minimixer) at the maximum possible speed for 30 sec. The resulting sheared lysate (0.4 ml) was then added to a polyallomer tube (5/8" X 3") containing 2.6 g cesium chloride, 0.8 ml ethidium bromide (700 μ g/ml in 0.1 M sodium phosphate buffer, pH 7) and 1.5 ml water. The contents in the tube were mixed thoroughly and overlaid with sufficient light mineral oil to fill the tube. Samples were centrifuged at 40,000 rpm for 44-48 hr. at 20C in a 50 Ti fixed angle rotor, using a Beckman Spinco model L2-65B ultracentrifuge. 10-drop fractions (19 gauge needle) were collected from the bottom of the tubes directly onto filter paper circles (Whatman 3 MM). The discs were then processed in the same manner as described under "DNA Synthesis" in "Materials and Methods" of Chapter I. and the radioactivity counted.

RESULTS

Total DNA from a set of strains carrying the mutation dnaB43 and any one of several plasmids that suppressed this mutation with a high efficiency (see Table I-3; Chapter I) was isolated from growing cultures either at 30C (nonsuppressed condition) or 41C (suppressed condition). The total DNA was analyzed in ethidium bromide-cesium chloride density gradients and the proportion of supercoiled plasmid DNA to total DNA in each culture determined. Ethidium bromide artificially alters the density of DNA so that the supercoiled form of DNA will band in cesium chloride at a density greater than linear DNA even though both DNA's may have the same average guanine and cytosine composition. Since the vast majority of plasmid DNA molecules in a cell-lysate are in supercoils while chromosomal DNA is not, this provides a satisfactory method for determining the proportion of supercoiled DNA in a cell population (Radloff, Bauer and Vinograd, 1967). A result typical of such an experiment is shown in Fig. II-1 and Table II-1 is a compilation of measurements of the proportion of supercoiled DNA (plasmid DNA) at 30C and 41C for various strains bearing the mutation dnaB43 and one other suppressing plasmid. These results indicate that under the condition of dnaB43-suppression, the plasmid was not stably integrated into the chromosome. This conclusion was further supported by the observation that cells isolated from these strains at 41C were as efficient donors for plasmid-associated markers (but not of chromosomal markers) as were the same strains before exposure to the nonpermissive temperature.

Figure 1I-1.

Sedimentation profile of the total (^3H)-labeled DNA from (a) strain Q1506 and (b), (c) strain Q1506/R446b in ethidium bromide-cesium chloride equilibrium gradients.

Fig. (a) Q1506 (dnaB43, supE, plasmid⁻);

30C (O—O); 41C (●—●)

Fig. (b) Q1506/R446b (dnaB43 supE, plasmid R446b)

30C (O—O)

Fig. (c) Q1506/R446b (dnaB43 supE, plasmid R446b)

41C (●—●)

The dashed vertical line indicates a change of scale.

TABLE II-1

Fraction of the Total Labeled DNA that Sediments as Supercoiled DNA when a DnaB(ts) Strain carrying dnaB43 and Different Suppressing Plasmids is Grown at 30°C or at 41°C^a

Strain and plasmid	Temperature (°C)	Percentage supercoiled DNA
Q1506	30	Nil
	41	Insignificant DNA synthesis (see Fig. II-1)
Q1506/R144	30	4.3
	41	4.1
Q1506/R471a	30	3.6
	41	4.4
Q1506/R446b	30	4.6
	41	4.7
Q1506/RA1	30	2.5
	41	2.4
Q1506/R64	30	3.8
	41	4.2
Q1506/R478	30	3.0
	41	2.7

^a From the results of experiments similar to that shown in Fig. II-1.

In similar experiments with dnaA(ts) mutations, the reported observations are that (a) no plasmid DNA is found in suppressed cultures and (b) plasmid-associated markers are no longer transferred efficiently (Nishimura et al., 1971; Sotomura and Yoshikawa, 1975).

CONCLUSION

The results presented in Table II-1 and Fig. II-1 indicate that under a state in which dnaB(ts) mutations were suppressed by conjugative plasmids, there was no integration of the plasmid into the host chromosome. Furthermore, when those temperature-resistant colonies (from 41C) were regrown at 30C and tested for their ability to transfer the antibiotic-associated markers, they transferred the plasmid with an efficiency similar to those strains before the exposure to the nonpermissive temperature. It is concluded that ~~suppression~~ of dnaB(ts) mutations by R plasmids is not by a mechanism similar to that of the integrative suppression of dnaA(ts) mutations. It is likely that in this case suppression involves the production of some plasmid-specified product that can diffuse cytoplasmically and interact with the temperature sensitive dnaB43 protein or at the replication site on the chromosome where this protein acts. This is in agreement with the observed variability of suppression of different dnaB alleles (Chapter I).

CHAPTER III

Evidence for dnaB Analog (ban) Genes Associated with Some Plasmids

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Specific Discussion and Conclusion..65-68

INTRODUCTION

A partial suppression of dnaB(ts) mutations by P1 prophage was first reported by Beyersmann and Schuster (1971). When a DnaB(ts) mutant carrying a wild-type P1 prophage and growing at 30C was shifted to a nonpermissive temperature of 42C P1 was able to replicate its DNA to a limited extent and in addition, chromosomal DNA replication was partially restored. Subsequently, wild-type P1 prophage was shown to determine a product that could partially substitute (in DNA replication) for the protein specified by the dnaB gene of E. coli. More efficient suppression of dnaB(ts) mutations by a mutant P1 prophage, termed P1Cm bac has been reported recently (D'Ari et al., 1975; Ogawa, 1975). These workers showed that prophage P1 suppression of the dnaB lesion was mediated through a protein (termed Ban for B analog) specified by this phage. The production of this Ban protein was found to be repressed in the wild type P1 prophage, but expressed constitutively in the mutant of P1 (P1Cm bac) (D'Ari et al., 1975; Ogawa, 1975).

The mutation dnaBam266 is an amber mutation. This mutation is the same as dnaB266 used in Chapter I. The prefix am before 266 has been added here and elsewhere in the thesis where it is important to draw to the attention of the reader that this is an amber mutation. A strain carrying such an amber mutation will be non-viable if it does not carry an informational amber suppressor (D'Ari et al., 1975; Ogawa, 1975).

The known informational amber suppressors of amber mutations are not gene-specific. They lead to the production of an altered transfer RNA (t-RNA) species, one that will recognize a peptide chain-terminating nonsense codon and lead to the biosynthesis of a complete peptide rather than an incomplete protein. Thus, these authors have concluded that the dnaB gene is essential for chromosomal DNA replication and for cell viability. It has however been shown that a strain carrying the amber mutation dnaBam266 and no other amber suppressor can be constructed if (in addition) it carries bacteriophage PlCm bac (D'Ari et al., 1975). Bacteriophage PlCm bac specifies a Ban protein which can restore viability to the strain which normally lacks the essential DnaB protein.

As shown in Chapter I, many R plasmids were able to alter the temperature-sensitivity of dnaB(ts) mutations. This situation bore some similarity to that reported for Pl (D'Ari et al., 1975; Ogawa, 1975). A hypothesis that needed to be considered was that many R plasmids that occur in nature each have a dnaB analog gene (ban gene) associated with them. The suppression or enhancement of temperature-sensitivity of DnaB(ts) mutants by these R plasmids could be postulated to arise by an interaction between a DnaB(ts) protein and a particular Ban protein specified by the respective R plasmid. A test for this

hypothesis that R plasmids have ban genes associated with them is to construct strains that are sup⁺ (su⁻ in the older literature) dnaBam266 and carrying the R plasmid. Since the plasmid negative strain of the above genotype is by definition non-viable and dnaB is an essential gene for the cell, restoration of viability to this strain by a plasmid will most likely be due to the plasmid specifying an analog of the missing DnaB protein of the cell. However, such a conclusion would be a valid one only if it can also be shown that the R plasmid itself does not specify an informational amber suppressor. The results presented in this Chapter describe the construction of such strains and thus provides evidence for ban genes associated with R plasmids.

MATERIALS AND METHODS

Bacterial Strains, Bacteriophages and Plasmids

The bacterial strains used are listed in Table III-1 and Table III-2 lists the bacteriophages. The R plasmids used were the same as those described in Table I-2 in Chapter I.

Media and Chemicals

Recipes for Nutrient Broth (NB), Nutrient Agar (NA) and Davis minimal agar are described under "Materials and Methods" in Chapter I. LB Broth contained per liter of distilled water, 10g tryptone, 5g yeast extract and 5g NaCl, and was adjusted to pH 7.4 with NaOH. LB Mg Broth was made up with LB Broth supplemented with 0.01 M $MgSO_4$. LB amp agar contained 1.5% agar and 30 μ g/ml ampicillin in LB broth. LB agar contained per liter of distilled water, 10g Bryptone and 2.5g NaCl and the pH was adjusted to 7.2 before autoclaving.

The concentrations of antibiotics used are similar to that described in "Materials and Methods" in Chapter I.

Construction of $dnaB_{am266} sup^+$ (su^-) Strains carrying various R Plasmids

R plasmids that suppressed $dnaB(tg)$ mutations but that were expected not to carry any known amber suppressors were used in this study. R plasmids specifying chloramphenicol resistance were excluded because of the inability to distinguish this phenotype from chloramphenicol resistance specified by PlCm bac. The plasmids were introduced into a recipient strain carrying $dnaB_{am266} sup^+$ (su^-) and PlCm bac (strain

TABLE III-1. Bacterial Strains

Strain	Chromosomal Markers ^a						Plasmid(s)	Source and Reference (if any)
Q125	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaB</u> ⁺	D'Ari et al, 1975
Q126	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>supE</u>	<u>dnaB</u> ⁺	D'Ari et al, 1975
Q1550	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	D'Ari et al, 1975
Q1550/RA1	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R40a	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R386	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R100-1	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R124	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R27	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R621a	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R64-11	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R144-3	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/R112	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study
Q1550/RP4	<u>trp</u>	<u>thy</u>	<u>thi</u>	<u>lac</u>	<u>mal</u>	<u>str</u> <u>sup</u> ⁺	<u>dnaBam266</u>	This study

^a trp, tryptophan; thy, thymine; thi, thiamine; lac, lactose; mal, maltose; str, streptomycin; sup (suppressor; sup⁺ denotes absence of suppressor and is equivalent to su⁻ used in the older literature); dna, deoxyribonucleic acid synthesis.

TABLE III-2

Bacteriophages

Bacteriophage	Relevant characteristics	Source
P7 <u>cl48ts</u>	ampicillin-resistant and heat inducible	J. Scott
T4am B22	a gene 43 mutant (T4 DNA polymerase)	J. Drake
T4am E509	a gene 23 mutant (major capsid protein)	J. Drake
λ am	λ carrying an amber mutation	C. Fuerst
λ^+	wild-type λ phage	C. Fuerst
Plvir	virulent Pl phage	J. Rosner

Q1550) by filter mating. Filter mating was carried out by passing 1 ml of late exponential recipient culture through a sterile cellulose nitrate filter (Sartorius: porosity, 0.45 μ), followed by 1 ml of an exponentially growing LB culture of the donor strain. Filters were layered on NA plates, incubated at 37C for 1 hr, and resuspended in 1 ml of NB. Transconjugants were selected by plating appropriately diluted conjugation mixtures on supplemented Davis minimal agar plates containing chloramphenicol and one antibiotic to which the test R plasmid conferred resistance. This procedure had been developed for a different purpose in this laboratory.

Test of Spontaneous Loss of PlCm bac (loss of chloramphenicol resistance)

Transconjugants selected from the method described above were replica-plated on supplemented Davis minimal agar plates with chloramphenicol added (50 μ g/ml) and also on NA plates containing one antibiotic resistance to which was specified by the R plasmid (Tc was used wherever possible). To test for the loss of chloramphenicol-resistance (Cm^R), sometimes it was necessary to replica-plate up to 2000 transconjugant colonies (in some strains, PlCm bac was quite stably maintained). The criterion used for the loss of PlCm bac was the loss of Cm^R . As will be shown later, the validity of this criterion was established by DNA hybridization tests.

Test of P7-induced loss of PlCm bac

Phage P7 (formerly known as ϕ amp) belongs to the same incompatibility group as Pl (group Y) (Hedges *et al.*, 1975; Wandersman and Yarmolinsky, 1977) and confers resistance to ampicillin (Smith, 1972; Rosner, 1972). P7 cl48ts is a heat inducible mutant of P7 (Scott, personal communication) that has an ampicillin gene associated with it. It was reasoned that if the Q1550 strain carrying a suppressing R plasmid is lysogenized with P7 cl48ts (selection is for Ap^r), the chance of loss of PlCm bac would be enhanced since the two bacteriophages are incompatible with one another and thus cannot be maintained stably in the same host.

The strains to be lysogenized with P7 cl48ts were grown to exponential phase in LB Broth at 37C. Cultures were spread on LB plates with ampicillin added (30µg/ml), dried at 37C for 15 min, followed by the addition of 0.1 ml of P7 cl48ts (titer; 10⁹ pfu/ml). After an incubation period of 24 hr at 30C, ampicillin-resistant lysogens were further streaked to give single colonies and replica plated onto supplemented minimal agar plates with either chloramphenicol (Cm), ampicillin (Ap) or tetracycline added. These selective plates enabled colonies to be screened for loss of PlCm bac, P7 cl48ts and the respective R plasmid. The two schemes that were employed are shown diagrammatically in Fig III-1.

Figure III-1.

Scheme used for the construction of dnaBam266 sup⁺ strains carrying various R plasmids with ban genes associated with them.

Frequency of lysogenization by P7

The procedure is similar to the one used by Rosner (1972) for bacteriophage P1. Overnight cultures of the test strains in LB Mg at 37C were diluted 20-fold and regrown to approximately 2×10^8 cells/ml in a similar medium. Cultures were kept in the cold for 10 min and CaCl_2 was added to a final concentration of 0.01 M. Phage P7 cl48ts was diluted (in the range of 10^{-1} to 10^{-5}) with LB Mg and 0.1 ml of each P7 cl48ts dilution was added to 1 ml of bacterial culture and was incubated at 30C for 30 min. 0.1 ml of the mixture was plated on LB+Ap (30 μ g/ml) plates and incubation was at 30C for 24 hr. Viable counts of the test strains were determined for the calculation of frequency of lysogenization.

Tests for the absence of amber suppressor (informational suppressor) in dnaBam266 sup⁺ (su-) strains carrying an R plasmid that was inferred to carry a ban gene

- (a) Efficiency of suppression of an R plasmid carrying an amber mutation in its beta-lactamase gene (R1-bla₃₁).
See "Materials and Methods" of Appendix II
- (b) Ability to plaque two amber mutants of bacteriophages T4 (T4am B22 and T4am E509). See "Materials and Methods" of Appendix II
- (c) Ability to plaque amber mutant of λ (λ am). Exponentially growing cultures in LB Broth at 37C were spread on λ plates and were allowed to dry at 37C. A drop of λ am

(approximately 0.1 ml; titer, 10^8 pfu/ml) was added and the plates were incubated at 37C for 10-12 hr. A zone of clearing indicated the ability of the test strain to support the growth of λ am.

- (d) Efficiency of suppression of two chromosomal amber mutations (trp am and lac am). Strain Q125 (dnaB⁺ sup⁺) carries two amber mutations in its tryptophan and lactose operons (trp am & lac am). An R plasmid that codes for an amber suppressor is expected to suppress both of these two mutations. R plasmids that were inferred to carry a ban gene were introduced into a dnaB⁺ sup⁺ recipient (strain Q125) by liquid mating. Transconjugants were purified, and were grown in NB at 37C to exponential phase, diluted and plated on supplemented minimal agar plates without the addition of tryptophan. Cells were also plated on EMB agar plates with lactose added as a carbon source.

DNA-DNA Hybridization

Preparation of high titer of P1 lysates and isolation of P1 DNA

P1 phage was prepared from plate lysates with a total of 100 plates (Rosner, 1972). P1 DNA was extracted and purified by the procedure described by Yun and Vapnek (1977). After purification by a CsCl step gradient (Yun and Vapnek, 1977), P1 DNA was alkali denatured, diluted with 2X SSC (0.15 M NaCl; 0.015 M sodium citrate, pH 7) to a concentration of 1 μ g/ml and filtered through nitrocellulose membranes (Sartorius; porosity, 0.45 μ) and the membranes were dried at 42C for 1 hr

(Kourilsky, Leidner & Tremblay, 1971). The Pl-charged filters were kept in a dessicator at room temperature for further use.

(³H)thymine labeling and DNA extraction from test strains

Overnight cultures of the test strains in MB broth supplemented with thymine (2µg/ml), thiamine (1µg/ml) and L-amino acids (40µg/ml) were diluted 10-fold into the same medium; but with (³H)thymine (10µCi/2µg/ml) substituted for cold thymine. The cells were allowed to incorporate the label for 3-4 hours. The total DNA was extracted as described by Louarn, Funderburgh and Bird (1974). Cells were washed twice with TES buffer (0.01 M tris hydroxymethyl aminomethane; 0.05M EDTA, pH 7.6) and resuspended in 1 ml of TES. Lysozyme was added to a final concentration of 50µg/ml and the mixture was incubated in ice for 10 min. followed by Sarkosyl to a final conc. of 0.1%. The lysate was incubated with pancreatic RNase (50 µg/ml) for 30/min at 37C, followed by Pronase for 1 hr at 37C. The lysate was further incubated with RNase (50µg/ml) for 30 min. Lysates were purified by two-step CsCl centrifugation as described by Brunk and Leick (1969). The lower portion of the CsCl step-gradient consisted of 5g CsCl dissolved in 3ml of TES ~~buffer~~ and the upper portion contained 2.4g CsCl dissolved in 3ml of TES. Centrifugation was carried out in a Beckman L2-65B (rotor type; 50Ti) ultracentrifuge for 44 hr at 15C. 1 ml-fractions were collected in small vials after locating the DNA peak (this was achieved by the addition of 10µl sample onto Whatman 3MM discs, precipitating with 10% TCA and count-

ing); fractions containing total DNA were pooled, dialyzed against SSC (0.15M NaCl; 0.015M Na citrate) to remove CsCl and kept in the cold.

Hybridization

Purified DNA prepared as described above was sonicated to yield DNA fragments of a M.W. of approximately 1.6×10^6 daltons, denatured with NaOH, by bringing the DNA solution to pH 12 and after standing for 5 min., the solution was neutralized with HCl. The DNA was made up to 4X SSC and diluted 1:1 with formamide. 5 mm microfilters were punched out from Pl-charged filters, incubated with 0.5 ml of (^3H)-labeled DNA at 42C for 45-48 hrs. Filters were washed in 2X SSC-50% formamide, followed by two washings in 2X SSC. They were then dried, covered with a Scintillation fluid and counted.

RESULTS

Construction of sup^+ (su^-) strains carrying the amber mutation dnaB266 and an additional suppressing R plasmid

Strain Q1550 carries an amber mutation in the dnaB gene but does not carry any known amber suppressor. However, this strain is viable because it maintains a bacteriophage, PlCm bac . PlCm bac is a mutant of PlCm and it produces at derepressed levels a Ban protein specified by the ban gene of the phage. The symbol bac stands for "Ban analog constitutive (D'Ari *et al.*, 1975)". Suppressing R plasmids were introduced individually into the dnaBam266 sup^+ PlCm bac strain (strain Q1550) by filter mating. Those plasmids that were known to specify informational amber suppressors (see Appendix II to Thesis; p. 139-161) were excluded. Chloramphenicol-resistance and tetracycline-resistance (or kanamycin-resistance in the case for R144-3) served as markers for the presence of PlCm bac and the R plasmid respectively. Such transconjugants were found to maintain both PlCm bac and the R plasmid stably (PlCm bac and the R plasmid are mutually compatible). A total of 11 transconjugants carrying dnaBam266 sup^+ PlCm bac and an additional R plasmid were isolated.

From these transconjugants (dnaBam266 sup^+ / PlCm bac/R^+) attempts were made to select for the loss of PlCm bac . This was achieved by (a) selection for spontaneous loss of chloramphenicol-resistance (b) by lysogenization with bacteriophage P7 cl48ts followed by thermal induction of this phage. P7 cl48ts

is a derivative of P7 (ϕ amp) carrying an ampicillin-resistance gene, and is also incompatible with bacteriophage P1. Furthermore, the temperature-sensitive mutation on this phage would have enabled the selection for loss of such phage by heat induction. Successful selection for ampicillin-resistance indicated lysogenization by P7 cl48ts. Since these two bacteriophages are incompatible with one another and the selection was for the P7-associated marker, this favors the loss of PlCm bac. Ampicillin-resistance and chloramphenicol-sensitive strains were scored by replica plating. These strains carried only P7 cl48ts and further heat induction would select for the subsequent loss of P7 cl48ts. The scheme used in these experiments is summarized diagrammatically in Fig. III-1.

From a total of 11 dnaBam266 sup⁺/PlCm bac/R⁺ strains constructed in this manner (Table III-3), five segregants were isolated (either by spontaneous loss or by P7 cl48ts-induced loss of PlCm bac (Table III-3)). These strains were labeled PW2, PW4, PW6, PW8 and PW10. The strains from which they were derived were designated PW1, PW3, PW5, PW7, and PW9 respectively (see Table III-3). Evidence indicating that the PW odd number strains (PW1, PW3, PW5, PW7 and PW9) carried both PlCm bac and an additional R plasmid was as follows: (a) these strains were efficient donors of the R plasmids to R⁻ recipients (b) they showed a reduced efficiency of lysogenization by P7 cl48ts (Table III-4).

The frequency of P7 cl48ts-induced loss of PlCm bac was

TABLE III-3
Frequency of Spontaneous or P7-Induced Loss of P1 from
Strain Carrying An R Plasmid in Addition to P1

Strain	relavent chromosomal markers	R plasmid	Frequency of occurrence of clones that have lost P1	
			spontaneous	P7-induced
Q125/P1Cm <u>bac</u>	<u>dnaB</u> ⁺ <u>sup</u> ⁺	nil	$<10^{-3}$ ^a	$<10^{-2}$ ^b
Q1550	<u>dnaBam266</u> <u>sup</u> ⁺	nil	$<10^{-3}$	$<10^{-2}$
Q1550/RA1	<u>dnaBam266</u> <u>sup</u> ⁺	RA1	$<10^{-3}$	$<10^{-2}$
Q1550/R40a	<u>dnaBam266</u> <u>sup</u> ⁺	R40a	$<10^{-3}$	$<10^{-2}$
Q1550/R386 (=PW5)	<u>dnaBam266</u> <u>sup</u> ⁺	R386	0.5×10^{-2}	0.1
Q1550/R100-1	<u>dnaBam266</u> <u>sup</u> ⁺	R100-1	$<10^{-3}$	$<10^{-2}$
Q1550/R124	<u>dnaBam266</u> <u>sup</u> ⁺	R124	$<10^{-4}$	$<10^{-2}$
Q1550/R27	<u>dnaBam266</u> <u>sup</u> ⁺	R27	$<10^{-3}$	$<10^{-2}$
Q1550/R621a (=PW9)	<u>dnaBam266</u> <u>sup</u> ⁺	R621a	10^{-3}	1.3×10^{-1}
Q1550/R64-11(=PW1)	<u>dnaBam266</u> <u>sup</u> ⁺	R64-11	0.3×10^{-2}	0.25
Q1550/R144-3(=PW3)	<u>dnaBam266</u> <u>sup</u> ⁺	R144-3	0.5×10^{-2}	0.1
Q1550/R112	<u>dnaBam266</u> <u>sup</u> ⁺	R112	$<10^{-3}$	$<10^{-2}$
Q1550/RP4 (=PW7)	<u>dnaBam266</u> <u>sup</u> ⁺	RP4	0.5×10^{-2}	0.7×10^{-1}

N.B. Although RP4 carries an ap^r gene, P7 lysogens were observed to produce larger colonies on ampicillin agar.

^a $<10^{-3}$ refers to the fact that 1000 colonies were replica plated and none was found to have lost the P1Cm bac-associated Cm^r marker.

^b $<10^{-2}$ refers to the fact that 100 colonies were replica plated and none was found to have lost the P1Cm bac-associated Cm^r marker.

much higher than that of the spontaneous loss of PlCm bac. This was to be expected (Table III-3). Chloramphenicol-sensitive segregants were not detected in either the parental strain dnaB⁺ sup⁺ (strain Q125) or dnaBam266 sup⁺ PlCm bac (strain Q1550) (< 1 in 1000 and < 1 in 1800 respectively; see Table III-3). The frequency of spontaneous loss of PlCm bac from these strains (column 4) although less than upon P7-induction, was still much higher than we expected because it has been reported by Rosner (1972) that loss of PlCm from wild type strains is as low as $< 10^{-4}$. It is the spontaneous Cm^S-derivatives that were chosen for further studies (PW even number strains).

From the results shown in Table III-3, it can be seen that within the limits set by the number of colonies examined in these experiments (1000 for spontaneous loss and 100 for P7-induced loss), PlCm bac could not be eliminated from 6 of the 11 strains. These R plasmids, RA1, R40a, R100-1, R124, R27 and R112 presumably do not specify a Ban protein. The results with R100-1 are particularly interesting as an example and deserve further study. From Chapter I, Table I-3, it can be seen that R100-1 suppressed all 4 dnaB(ts) mutations efficiently and yet no evidence could be obtained that R100-1 could rescue the strain carrying dnaBam266 in the absence of PlCm bac. Similar observations have been made by introduction of R100-1 into strain PW4 and thus constructing a dnaBam266 sup⁺ strain that stably maintained R100-1 and R144-3, a plasmid which like PlCm bac also carried a ban gene. Once again, R144-3 could not be eliminated spon-

taneously from this strain (800 colonies tested) and a dnaBam266 sup⁺ strain carrying only R100-1 could not be constructed. This confirmed that R100-1 is unable to rescue the strain carrying dnaBam266 sup⁺ and therefore provides no evidence for the presence of ban gene associated with this plasmid.

That the PW even number strains were free from PlCm bac was confirmed by the following experiments: (a) these strains showed a higher frequency of lysogenization by P7 cl48ts than their corresponding PW odd number strains (strains PW1, PW3, PW5, PW7 and PW9) and their parental strain Q1550 (Table III-4). (b) since P1 and P7 are incompatible with one another, strain PW1 was found to be unable to maintain these two phages stably, whereas strain PW2 was able to maintain this phage (P7) stably. Strain Q1550 was also found to be unable to maintain both phages stably. After lysogenization of the P1-carrying cells by P7, P7 lysogens were selected on LA + ampicillin (the P7-associated marker). Under these conditions and under normal circumstances, selection was for the loss of P1. Nevertheless, P1 was never found to be lost and upon subsequent transfer to an antibiotic-free medium, it was P7 that was lost. (c) DNA-DNA hybridization experiments showed that strain Q1550 and the PW odd number strains (PW1, PW3, PW5, PW7 and PW9) had nucleotide sequences complementary to P1 DNA, while strain Q125 and the PW even number strains (PW2, PW4, PW6, PW8 and PW10) showed little or no detectable complementary nucleotide sequences (Table III-5).

TABLE III-4

Frequency of Lysogenization by P7 cl48ts

Strain	Plasmids in strain	Frequency of lysogenization by P7 <u>cl48ts</u> ^a
Q1550	P1Cm <u>bac</u>	7.8×10^{-3}
PW1	P1Cm <u>bac</u> ; R64-11	3.8×10^{-2}
PW2	R64-11	7.9×10^{-1}
PW3	P1Cm <u>bac</u> ; R144-3	1.2×10^{-2}
PW4	R144-3	1.7×10^{-1}
PW5	P1Cm <u>bac</u> R386	7.5×10^{-3}
PW6	R386	1.1×10^{-1}
PW9	P1Cm <u>bac</u> ; R621a	4.2×10^{-3}
PW10	R621a	2×10^{-1}

^a Frequency of lysogenization was calculated as the number of (amp^r colonies/number of viable cells) X 100.

TABLE III-5
Hybridization of ^3H -labeled DNA Extracted from Various
Strains with P1 DNA on Filters

Source of ^3H -labeled DNA	P1-associated genetic markers (Cm ^r)	cpm bound to ^a uncharged filter	cpm bound ^b to P1 DNA charges filter	Difference ^c between col-umn (2) and (3)
Q1550	+	5470	31985	26515
Q125	-	6395	8230	1385
PW1	+	4825	26735	21910
PW2	-	3770	5610	1840
PW3	+	7735	39425	31690
PW4	-	5280	7312	2082
PW5	+	1165	12575	11410
PW6	-	4335	6420	2085
PW7	+	1935	14180	12245
PW8	-	5835	7421	1585
PW9	+	1725	18735	17010
PW10	-	2100	4256	2155

^a cpm retained by uncharged filters.

^b cpm retained by P1-charged filters.

^c cpm bound to filters charged with P1 DNA minus cpm bound to plain filters.

Absence of amber suppressors associated with R plasmids
R64-11, R144-3, R386, RP4 and R621a

As indicated in the Appendix II of this Thesis, some R plasmids do code for efficient amber suppressors (informational suppressors) that suppress amber mutations. It was therefore necessary to demonstrate that the R plasmids that rescued the dnaBam266 sup⁺ strain did not do so because they carried an amber-suppressor gene. The following experiments demonstrate that none of the five plasmids tested code for an efficient amber suppressor:

- (a) the R plasmids R64-11, R144-3 and R386 did not suppress the amber mutation in the beta-lactamase gene of the R plasmid R1-bla31 (Table I of Appendix II).
- (b) none of the test R plasmids suppressed the amber mutation of two different T4~~amber~~ mutants (T4am B22 and T4am E509) (Table 2 of Appendix II).
- (c) none of the R plasmids was able to suppress the amber mutation of λ am; that is, the presence of the individual R plasmid in a sup⁺ host did not lead to the plaquing of λ am.
- (d) none of the five R plasmids suppressed amber mutations in the trp and lac genes (Table III-6).

The results of the above experiments, thus argue against the possibility that R plasmids R64-11, R144-3, R386, RP4 and R621a specify an informational suppressor that suppresses amber mutations. The reason why dnaBam266 sup⁺ Pl-free strains carrying an additional R plasmid are viable is probably be-

TABLE III-6

Ability of R⁺ dnaB266 sup⁺ Strains to Suppress
Amber Mutations in the trp and lac Genes

Strain	Relevant characteristics	Ability of strain to grow on	
		min plate with no trp added	lactose min plate
Q125	<u>dnaB⁺</u> <u>sup⁺</u>	- a	-
Q126	<u>dnaB⁺</u> <u>supE</u>	+ b	+
PW2	<u>dnaBam266</u> <u>sup⁺</u>	-	-
PW4	<u>dnaBam266</u> <u>sup⁺</u>	-	-
PW6	<u>dnaBam266</u> <u>sup⁺</u>	-	-
PW8	<u>dnaBam266</u> <u>sup⁺</u>	-	-
PW10	<u>dnaBam266</u> <u>sup⁺</u>	-	-

a no growth

b indicates growth

6

cause of a DnaB analog (Ban) protein specified by these R plasmids and synthesized in these strains. Such an R-specified Ban protein can substitute for the missing DnaB protein to fulfil its role in chromosomal DNA replication. Alternatively, the dnaBam266 mutation (in the presence of sup⁺) leads to the synthesis of an incomplete protein which is sufficiently stable to interact functionally with the R-specified Ban protein.

SPECIFIC DISCUSSION AND CONCLUSIONS

In the presence of the bacteriophage PlCm bac, a dnaBam266 sup⁺ strain (strain Q1550) could be constructed. It was inferred that this occurs because of the production of a DnaB-analog or Ban protein which was sufficiently similar to the DnaB protein to restore cellular viability. That is, the Ban protein coded by PlCm bac can substitute for the function of the product of the dnaB gene (D'Ari et al., 1975). The success in constructing dnaBam266 sup⁺ strains carrying R plasmids and the demonstration that such strains are free from the Pl genome as well as from any informational amber-suppressor gene indicate that such R plasmids do carry ban genes that code for Ban protein(s). Since mutation dnaB266 is an amber mutation, the observation that in the absence of any amber suppressor, such strains are viable can best be explained by the presence of a Ban protein coded by these R plasmids. Each of these Ban proteins can substitute for the function(s) of the product of the dnaB gene.

A protein believed to be the dnaB gene product has been purified (Wickner et al., 1974; Ueda et al., 1978). This DnaB-protein is an oligomer with a native molecular weight of 250,000. Assay for dnaB protein is based on an absolute requirement for it, along with 12 other essential factors to reconstitute in vitro conversion of phage φX174 single-stranded DNA to a duplex replicative form. Since the DnaB protein is made up of more than one polypeptide, and the amber mutation dnaBam266 can still

code for incomplete polypeptides, the possibility that interaction between the Ban protein coded by an R plasmid and an incomplete DnaB protein chain cannot be ruled out at the present time.

Some R plasmids suppress different mutations in the dnaB gene with high efficiency, but they were not found to rescue the strain carrying dnaBam266. For example, R100-1 suppressed all dnaB(ts) mutations tested with high efficiency (Chapter I, Table I-3), but after the introduction of this plasmid into a dnaBam266 sup⁺ PlCm bac strain (strain Q1550), it was not found possible to cure PlCm bac spontaneously or by the P7-induction technique. Such a result suggests that either in the presence of R100-1, the strain can maintain PlCm bac more stably or R100-1 does not specify a Ban protein that is able to substitute for the product of dnaB. The fact that R100-1 cannot be cured even when a strain derived from PWA carried another plasmid (i.e. strain dnaBam266 sup⁺/R144-3/R100-1) makes the first possibility less likely. Strain dnaBam266 sup⁺/R100-1 is probably not viable because R100-1 does not code for an efficient Ban protein that can substitute for the DnaB protein. However, this plasmid (R100-1) does suppress dnaB(ts) mutations indicating that other mechanisms such as an interaction between a DnaB protein and a product of this plasmid (this product is not a Ban protein) is possible. In other words, the possibility is that some plasmids like R100-1 specify a protein that can functionally interact with a DnaB protein but which cannot functionally substitute for it. This situation is discussed further in Chapter VI.

The frequency of spontaneous loss of prophage PlCm bac from the PW odd number strains (PW1, PW3, PW5, PW7 and PW9) was higher than that reported by Rosner (1972) (Table III-3). Such a result can be explained best by the instability of PlCm bac caused by the presence of an R plasmid and the dnaBam266 mutation in the PW odd number strains. PlCm bac was found to be stable in a dnaB⁺ sup⁺ R⁻ strain (strain Q125/PlCm bac) (frequency of loss: $<10^{-4}$; Table III-3).

After lysogenization of strain Q1550 by phage P7, no loss of PlCm bac could be detected (Table IPI-3). Since these two prophages are incompatible with one another (Yun & Vapnek, 1977; Rosner, 1972), either one of these two phages could theoretically be lost. However, the result of such an experiment showed that it was always the incoming plasmid (P7) that was lost and the resident plasmid (PlCm bac) retained. Such a result also suggests that wild-type P7 does not make a Ban protein or makes very little of it, so that in this strain, selection will be for the preferential retention of PlCm bac.

The PW even strains (PW2, PW4, PW6, PW8 and PW10) were free from PlCm bac. This was confirmed by the following experiments. The presumptive PlCm bac-free strains showed a higher frequency of lysogenization by bacteriophage P7, whereas lysogenization by the same phage (P7) of the corresponding strains carrying both PlCm bac and an additional R plasmid (PW odd number strains) was much reduced (Table IPI-4). Such a result

was not surprising because PlCm bac is in the same Incompatibility Group as P7 (Rosner, 1972; Yun & Vapnek, 1977). Hybridization of total DNA from PW strains with DNA isolated from phage P1 indicated that the PW even number strains did not have detectable nucleotide sequences complementary to that of P1. As expected, DNA from the PW odd number strains carrying both PlCm bac and an additional R plasmid showed a higher degree of binding to P1 DNA. This indicates that the entire P1 prophage was lost from the PW even number strains, and not only the gene conferring resistance to Cm.

Some R plasmids such as R478 and R446b do carry efficient suppressors of amber mutations (see Appendix II to Thesis). Therefore, it was very important to test whether R plasmids that specify Ban proteins carry any informational amber suppressor. No amber suppressors were found to be associated with plasmids R64-11, R144-3, R386, RP4 and R621a. This conclusion was based on the fact that these plasmids were unable to suppress the amber mutations of the R plasmid R1-bla31, two T4 amber mutants, λ am, trp am and lac am. The conclusion that each of these five R plasmids specify Ban proteins thus rests on reasonably firm grounds.

CHAPTER IV

Evidence Suggesting Interactions Between the Product of ban Genes Specified by Different Plasmids

Introduction.....	69-70
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INTRODUCTION

Evidence indicating that each of the R plasmids R64-11, R144-3, R386, RP4 and R621a do have a ban gene associated with them was presented in Chapter III. The production of a Ban protein specified by the respective genes on these R plasmids could have led to the restoration of cell viability in dnaBam266 sup⁺/R⁺ strains (PW even number strains). Since the suppression or enhancement of dnaB(ts) mutations could be the result of interaction between a dnaB(ts) protein and the Ban protein specified by the R plasmid (see Discussion; Chapter I), it could be postulated that interaction would also occur between the Ban protein of PlCm bac and the one specified by the R plasmids in the PW odd number strains (PW1, PW3, PW5, PW7 and PW9) carrying both of these plasmids (PlCm bac and R). D'Ari et al., (1975) reported that strain Q1550 (dnaBam266 sup⁺ PlCm bac) is viable but cryosensitive. This implies that although the Ban protein specified by PlCm bac can function normally at 37C and 41C, and restore cell viability, this Ban protein is probably cold sensitive. If interaction does occur between the Ban protein of PlCm bac and a Ban protein coded by any one of the five R plasmids, changes in phenotype with respect to cryosensitivity of DNA replication or colony-forming ability may occur. An additional test that could be used is the ability of strains supplying a particular DnaB

protein or a particular Ban protein to support the growth of bacteriophage λ . This becomes possible because of a previous observation (D'Ari et al., 1975) that although the Ban protein specified by PlCm bac was able to restore cell viability to E. coli K-12, it was unable to support the replication of bacteriophage λ even at a temperature that is permissive for cell growth (30C). This phenotype is known in the literature as the GroP phenotype and in fact some E. coli dnaB(ts) mutations (i.e. a subclass of mutations mapping within the dnaB gene of E. coli K-12) show this phenotype (Georgopoulos & Herskowitz, 1971). The dnaB function is known to be essential for the growth of bacteriophage λ . The observation of Georgopoulos and Herskowitz (1971) had indicated that it was possible to genetically modify the DnaB protein (by mutation) so that it would no longer support λ replication at any temperature although it could support bacterial DNA replication at a permissive temperature. Two relevant questions could be asked: are there differences among Ban proteins with respect to their ability to support λ replication? In a situation where two Ban proteins could be produced by a cell, could evidence be obtained for an in vivo interaction between such proteins at the level of chromosomal DNA replication or λ replication? The results in this Chapter provide evidence that such functional interactions between Ban proteins do occur.

MATERIALS AND METHODS

Bacterial Strains and Bacteriophages

The bacterial strains used are described under "Materials and Methods" of Chapter III (page 45). Wild type phage λ was used to test the ability of the PW strains to plate this phage.

Media and Chemicals

The composition of Nutrient Broth (NB), Nutrient Agar (NA) and Davis minimal agar (MA) are described under "Materials and Methods" in Chapter I. R top agar contained per liter of distilled water; Bacto tryptone; 10g, yeast extract; 1g; NaCl; 8g and 0.8% agar. After autoclaving, 2 ml of 1 M CaCl_2 and 5 ml of 20% glucose were added. The recipe for R bottom agar is the same as that of R top agar except that 2% agar was used.

Colony-forming ability at different temperatures

The procedure used to determine colony-forming ability at different temperatures is described under "Materials and Methods" in Chapter I.

Measurement of the relative increase of (^3H)thymine incorporation into DNA at different temperatures

DNA synthesis (relative increase) was measured by the incorporation of (^3H)thymine into TCA precipitable counts. The procedure followed was as described under "Materials and

Methods in Chapter I:

Test of the ability of strains to plaque wild-type λ

Overnight cultures of the test strains were diluted in LB Broth and regrown at 37C to approximately 2×10^8 cells/ml. Wild-type λ phage lysates were first harvested from dnaB⁺ PlCm bac strain (strain Q125/PlCm bac) to prevent any DNA restriction by the test strains carrying PlCm bac. The lysate of phage λ was appropriately diluted and 0.1 ml added to 0.1 ml of bacterial cultures in test tubes. This mixture was incubated at 37C for 30 min. 3 ml of R top agar was added and overlaid onto R bottom agar plates. Plates were incubated at 37C for 10-12 hr and the number of plaques counted.

RESULTS

Differences in temperature-sensitivity of strains carrying one or two *ban* genes specified by different plasmids

DNA synthesis and colony-forming ability in *dnaBam266 sup*⁺ strains carrying either R plasmids alone (PW even number strains) or both PlCm *bac* and an additional R plasmid (PW odd number strains) were tested. The results are shown in Table IV-1 and Figures IV-1-IV-5. D'Ari *et al* (1975) have reported that strain Q1550 is viable but cryosensitive. This suggested that although the Ban protein coded by PlCm *bac* can function normally at 37C, this protein is probably cold sensitive. The degree of cryosensitivity of strain Q1550 was found to be somewhat less than that reported by D'Ari *et al* (1975). Strain PW2 carrying only R64-11 was neither cryosensitive nor heat-sensitive. This indicates that the Ban protein coded by R64-11 behaves quite differently from that coded by PlCm *bac*. However, when both plasmids PlCm *bac* and R64-11 were present in PW2, there was an intermediate level of cryosensitivity. This behaviour is not inconsistent with the suggestion that when both plasmids were present in this strain (PW2), the cold sensitive Ban protein of PlCm *bac* and the cold resistant Ban protein specified by R64-11 are both synthesized and they cooperate or interact *in vivo*, resulting in an intermediate level of cryosensitivity. It should be noted that essentially all the cells in PW2 population carry both plasmids and the population is not heterogeneous.

When DNA synthesis and colony-forming ability were followed in

TABLE IV-1

Colony-forming ability of *dnaBam266 sup*⁺ strains
carrying either PlCm *bac* or PlCm *bac* and an additional R plasmid

Strain	Plasmid(s) in strain	Colonies at temperature relative to those at 37C		
		26C	30C	41C
Q125	nil	6.3×10^{-1}	1	7.8×10^{-1}
Q1550	PlCm <i>bac</i>	1.4×10^{-4}	1×10^{-2}	7.8×10^{-1}
PW1	PlCm <i>bac</i> ; R64-11	8.2×10^{-4}	6.6×10^{-1}	1.2×10^{-1}
PW2	R64-11	1.2×10^{-1}	6.5×10^{-1}	1.3×10^{-1}
PW3	PlCm <i>bac</i> ; R144-3	3×10^{-2}	7×10^{-2}	5×10^{-2}
PW4	R144-3	1.7×10^{-5}	1.3×10^{-3}	2×10^{-2}
PW5	PlCm <i>bac</i> ; R386	5.4×10^{-1}	7.7×10^{-1}	5×10^{-1}
PW6	R386	5.7×10^{-1}	4.4×10^{-1}	4.6×10^{-1}
PW7	PlCm <i>bac</i> ; RP4	1.3×10^{-5}	8.8×10^{-1}	2.2×10^{-3}
PW8	RP4	1.9×10^{-5}	9×10^{-1}	2.1×10^{-3}
PW9	PlCm <i>bac</i> ; R621a	2×10^{-2}	7×10^{-2}	2×10^{-2}
PW10	R621a	1.5×10^{-3}	3×10^{-3}	4.2×10^{-3}

Figure IV-1.

DNA synthesis at three different temperatures (37C, 26C and 42C) in strains carrying the functional dnaB⁺ gene or ban genes associated with PlCm bac and/or R64-11.

Q125 (wild type dnaB, plasmid⁻), (O—O)

Q1550 (dnaBam266 sup⁺, plasmids PlCm bac), (●—●)

PW1 (dnaBam266 sup⁺, plasmids PlCm bac & R64-11), (□—□)

PW2 (dnaBam266 sup⁺, plasmid R64-11), (■—■)

Figure IV-2.

DNA synthesis at three different temperatures (37C, 26C and 42C) in strains carrying the functional dnaB⁺ gene or ban genes associated with PlCm bac and/or R144-3.

Q125 (wild type dnaB, plasmid⁻), (O—O)

Q1550 (dnaBam266 sup⁺, plasmid PlCm bac), (●—●)

PW3 (dnaBam266 sup⁺, plasmids PlCm bac & R64-11), (□—□)

PW4 (dnaBam266 sup⁺, plasmid R144-3), (■—■)

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Figure IV-3.

DNA synthesis at three different temperatures (37C, 26C and 42C) in strains carrying the functional dnaB⁺ gene or ban genes associated with PlCm bac and/or R386.

Q125 (wild type dnaB, plasmid⁻), (O—O)

Q1550 (dnaBam266 sup⁺, plasmid PlCm bac), (●—●)

PW5 (dnaBam266 sup⁺, plasmids PlCm bac & R386), (□—□)

PW6 (dnaBam266 sup⁺, plasmid R386), (■—■)

Figure IV-4.

DNA synthesis at three different temperatures (37C, 26C and 42C) in strains carrying the functional dnaB⁺ gene or ban genes associated with PlCm bac and/or RP4.

Q125 (wild type dnaB, plasmid⁻), (O—O)

Q1550 (dnaBam266 sup⁺, plasmid PlCm bac), (●—●)

PW7 (dnaBam266 sup⁺, plasmids PlCm bac & RP4), (□—□)

PW8 (dnaBam266 sup⁺, plasmid RP4), (■—■)

Figure IV-5.

DNA synthesis at three different temperatures (37C, 26C and 42C) in strains carrying the functional dnaB⁺ gene or ban genes associated with PlCm bac and/or R621a.

Q125 (wild type dnaB, plasmid⁻), (O—O)

Q1550 (dnaBam266 sup⁺, plasmid PlCm bac), (●—●)

PW9 (dnaBam266 sup⁺, plasmids PlCm bac & R621a), (□—□)

PW10 (dnaBam266 sup⁺, plasmid R621a), (■—■)

strain PW5 (dnaBam266 sup⁺ PlCm bac/R386) and PW6 (dnaBam266 sup⁺/R386), there was no difference with respect to growth and DNA synthesis (Table IV-1 and Fig. IV-3). At high temperature (42C), the two strains behaved very much like their parental strain Q1550. DNA synthesis and colony-forming ability at 26C showed that both of these strains were more cold resistant than Q1550. Such a result suggests that the Ban protein coded by R386 is relatively more cold resistant than the one coded by Pl. Since no difference could be detected with respect to cryosensitivity in PW5 and PW6, the implication would be that the Ban protein coded by R386 either does not interact with the Ban protein of PlCm bac or that the product of such an interaction is similar to R386 Ban in temperature sensitivity. An alternative explanation for this and similar results is considered in Chapter VI. In the present context, this would state that at low temperature, it is the production of the PlCm bac-specified protein that is temperature sensitive. It will be clear from the rest of this thesis that there are reasons for favouring the first explanation. Both PW7 (dnaBam266 sup⁺ PlCm bac/RP4) and PW8 (dnaBam266 sup⁺/RP4) were found to be cold sensitive, and no difference with respect to cryosensitivity could be detected between these two strains. This result is similar to that obtained with R386.

Both strains PW4 (dnaBam266 sup⁺/R144-3) and PW10 (dnaBam266 sup⁺/R621a) showed an increased level of cold-sensitivity as compared to their Cm^r derivatives (strains PW3 and PW9) (Table IV-1, Fig. IV-2 and IV-5). This result suggests that both of the Ban

proteins of R144-3 and R621a are cryosensitive. It appears that "interaction" between the Ban protein coded by PlCm bac and the one coded by either R144-3 or R621a resulted in an increased level of resistance to cold.

Since these results suggested that in vitro "interaction" between the Ban protein of PlCm bac and the Ban protein specified by an R plasmid may occur, it was necessary to test whether the Ban protein specified by R plasmids would interact with the wild-type DnaB protein in a functionally detectable manner. To test this possibility, the five test R plasmids (R64-11, R144-3, R386, RP4 and R621a) were introduced individually into strain Q125 (dnaB⁺ sup⁺) and colony-forming ability was followed in the transconjugants after they had been purified. The result of such an experiment is shown in Table IV-2. None of the five R plasmids tested would change the phenotype of strain Q125 indicating that all of these Ban proteins specified by R plasmids were unable to alter the phenotype determined by the wild type DnaB protein in a detectable manner.

Although the Ban protein coded by PlCm bac was able to restore cellular viability to strain Q1550 (dnaBam 266 sup⁺), this Ban protein does not support the replication of wild type bacteriophage λ in the absence of an effective DnaB product (D'Ari et al, 1975 and Table IV-3). In other words, the Pl specified Ban protein (unlike the E. coli K-12 wild type DnaB protein) is unable to support the growth of bacteriophage λ at any temperature. To test whether the Ban protein specified by R plasmids have the ability to support the growth of wild type λ , the PW strains and their parental strain Q1550 were tested for their ability to

TABLE IV-2

Colony-forming ability of the dnaB^+ sup^+ strain (strain Q125)
and its derivatives carrying an additional R plasmid
that specifies a Ban protein

Strain	R plasmid in strain	Colonies at temperature relative to those at 37C	
		26C	42C
Q125	nil	6.3×10^{-1}	7.8×10^{-1}
Q125/R64-11	R64-11	1.4×10^{-1}	6.7×10^{-1}
Q125/R144-3	R144-3	6.7×10^{-1}	6.7×10^{-1}
Q125/R386	R386	4.6×10^{-1}	7.8×10^{-1}
Q125/RP4	RP4	8.3×10^{-1}	8×10^{-1}
Q125/R621a	R621a	4.1×10^{-1}	6.4×10^{-1}

TABLE IV-3

Efficiency of plating wild type λ by
PW strains and their parental strain Q1550

Strain	Plasmid in strain	Frequency of plating ^a wild type λ at 37C
Q125	nil	0.69
Q1550	PlCm <u>bac</u>	$3/1 \times 10^{-6}$
PW1	PlCm <u>bac</u> ; R64-11	4×10^{-6}
PW2	R64-11	3.1×10^{-6}
PW3	PlCm <u>bac</u> ; R144-3	3.2×10^{-6}
PW4	R144-3	2.3×10^{-6}
PW5	PlCm <u>bac</u> ; R386	3.1×10^{-6}
PW6	R386	0.73
PW7	PlCm <u>bac</u> ; RP4	1.9×10^{-6}
PW8	RP4	1.7×10^{-6}
PW9	PlCm <u>bac</u> ; R621a	2.4×10^{-6}
PW10	R621a	0.76

^a Frequency of plating = number of plaques/ml divided by number of cells/ml; the latter (cells/ml) was approximately the same for all strains.

plate λ (Table IV-3). The results showed that the Ban proteins specified by two R plasmids, R386 and R621a were able to support the replication of wild type λ . Interestingly, the combination of PlCm bac and either R386 or R621a was unable to do so (Table IV-3), a result which is again consistent with the postulate that interactions occur in vivo between the two Ban proteins, one specified by Pl and the other by the respective R plasmid. It is hypothesized from these observations that the Ban protein specified by a plasmid like R386 is able to support the growth of bacteriophage λ in vivo but upon interaction with the Pl Ban protein, it is no longer able to do so.

CONCLUSIONS

The overall results obtained from the interaction experiments are summarized below:

<u>Plasmid-associated ban genes from</u>	<u>Temperature sensitivity of cells (DNA synthesis and colony-formation)</u>	<u>Ability to support λ replication</u>	<u>Evidence for interaction between two Ban proteins</u>
P1	cryosensitive	-	
R64-11	not cryosensitive	-	
P1 R64-11	cryosensitive; intermediate between P1 or R64-11 alone	-	+
R386	less cryosensitive than P1	+	
P1 R386	less cryosensitive than P1	-	+
RP4	cryosensitive and heat sensitive	-	
P1 RP4	cryosensitive and heat sensitive	-	-
R144-3	cryosensitive	-	
P1 R144-3	less cryosensitive than P1 or R144-3 alone	-	+
R621a	cryosensitive and slightly heat sensitive	+	
P1 R621a	not cryosensitive and less heat sensitive than R621a alone	-	+

Thus, using alterations in cryosensitivity or ability to support the replication of bacteriophage λ as criteria for in vivo interactions between ~~ban~~ proteins, it can be concluded that such interactions occur in four of the five cases tested. This is consistent with previous arguments and tentative conclusions (Chapter I to III) that in vivo interactions between E. coli DnaB(ts) proteins and Ban proteins occur. In this Chapter, it has been shown that interactions between different Ban proteins may also occur with functional consequences. This conclusion is supported by recent observations (Ueda et al., 1978) that a ~~protein~~ believed to be the product of the dnaB gene of E. coli is an oligomer with a molecular weight of 250,000 which can be dissociated into monomers of 55,000. The P1-specified Ban protein also appears to be an oligomer and in vitro studies have indicated that heteromultimers between P1-Ban and DnaB proteins can be isolated (Schuster, personal communication).

CHAPTER V

Mutant Plasmids of R64-11; Mutations Affecting dnaB(ts) Suppression and Related Effects

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INTRODUCTION

From the results presented in previous Chapters, it was concluded that several R plasmids have ban genes associated with them and that suppression (or enhancement) of the temperature sensitivity of dnaB(ts) mutations arises by an interaction between DnaB(ts) proteins and Ban proteins. If this is a valid conclusion, one might expect that it may be possible to isolate mutants of any of these plasmids that would be affected in suppressing (or enhancing) ability. Plasmid R64-11 was chosen for this purpose and to test for this expectation.

Two independent mutants of R64-11 were isolated following mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid. NTG is known to cause missense mutations while nitrous acid could lead to a deletion (Miller, 1972). The use of these mutagens might thus allow the isolation of mutants of R64-11 that carry missense and deletion mutations in the ban gene. However, the isolation procedure left open the possibility that the induced mutations were not on the plasmid but on the chromosome. This made it necessary to do further tests to determine that the mutations were in fact associated with the plasmid. In this Chapter, the isolation of two mutant plasmids pCU1 and pCU2 is described. Plasmid pCU1 was isolated by G. Selvaraj in this laboratory for a different purpose and I have utilized it in this study after confirming it to be a mutant derivative of R64-11. As will be shown, the mutant plasmid pCU2 that I have isolated has properties that are different

from that of pCU1. The properties of both mutations are consistent with expectations for mutations affecting the ban gene of R64-11.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains used are listed in Table V-1 and Table V-2 lists the bacterial plasmids.

Media and Chemicals

Recipes for NB, NA, and MB are similar to that as described under "Materials and Methods" in Chapter I. The composition of LB is the same as that described under "Materials and Methods" in Chapter III. The concentration of antibiotics used were the same as that described in "Materials and Methods" in Chapter I.

Isolation of a mutant of plasmid R64-11 using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis: mutant plasmid pCU2

An overnight 37C culture of the strain to be mutagenized (strain CO3/R64-11) in LB plus tetracycline (20 µg/ml) was diluted 20-fold and regrown in the same medium to approximately 2×10^8 cells/ml. The cells were washed twice with citrate buffer (0.1 M citric acid, 4.7 volumes; 0.1 M sodium citrate, 15.4 volumes; pH 5.5) and resuspended in 4 ml of citrate buffer. 0.2 ml of a 1 mg/ml solution of NTG in citrate buffer (pH 5.5) was added and the mixture was incubated at 37C for 30 min. The culture treated with NTG was then washed once with phosphate buffer (contained equal molar solutions of dibasic sodium phosphate, 61 ml; monobasic sodium phosphate, 39 ml; pH 7) and the washed pellet was resuspended in 20 ml of LB and this was allowed to grow overnight at 37C. This overnight culture was diluted 20-fold in LB and regrown to exponential phase at 37C.

TABLE V-1

Bacterial Strains

Strain	Genetic markers ^a	Reference
Q125	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺	D'Ari <u>et al</u> , 1975.
Q1502	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB8</u>	D'Ari <u>et al</u> , 1975.
Q1506	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB43</u>	D'Ari <u>et al</u> , 1975.
Q1508	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB70</u>	D'Ari <u>et al</u> , 1975.
Q1610	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>supE</u> <u>dnaB266</u>	D'Ari <u>et al</u> , 1975.
I279 ^b	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺ <u>dnaB279</u>	This study
Q1516	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺ <u>groPB558</u>	D'Ari <u>et al</u> , 1975.
Q1550	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺ <u>dnaB266</u>	D'Ari <u>et al</u> , 1975.
Q1550/pCU1 ^c	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺ <u>dnaB266</u>	This study
Q1550/pCU2 ^c	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺ <u>dnaB266</u>	This study
PW13 ^d	<u>trp</u> <u>thy</u> <u>thi</u> <u>lac</u> <u>mal</u> <u>str</u> <u>sup</u> ⁺ <u>dnaB266</u>	This study
CO3	<u>thi</u> <u>pro</u>	A. Ahmad
E279	<u>thr</u> <u>leu</u> <u>thy</u> <u>thi</u> <u>str</u> <u>supE</u> <u>dnaB279</u>	Wechsler & Gross, 1975
WD266II	<u>thy</u> <u>mal</u> <u>gal</u> <u>str</u> <u>supE</u> <u>dnaB266</u>	Ogawa, 1975.

^a trp, tryptophan; thy, thymine; thi, thiamine; pro, proline; thr, threonine; leu, leucine; str, streptomycin; lac, lactose; mal, maltose; gal, galactose; sup, suppression; dna, deoxyribonucleic acid.

^b This strain was constructed by P1 transduction by growing P1-vir on strain E279 and subsequently transducing Q125. Selection was for mal⁺ and inability of transductant to grow at non-permissive temperature.

^c pCU1 and pCU2 are mutant plasmids of R64-11. See also Table V-2.

^d This strain is the Cm^S derivative of strain Q1550/pCU2 and is free from P1Cm bac.

TABLE V-2

Plasmids

Plasmid	Relevant markers ^a	Reference
R64-11	<u>sul</u> <u>tet</u> <u>str</u> <u>drd11</u>	Meynell & Datta, 1967.
pCU1 (=R64-11a) ^b	<u>sul</u> <u>tet</u> <u>str</u> <u>drd11</u>	This study
pCU2 (=R64-11d) ^c	<u>sul</u> <u>tet</u> <u>str</u> <u>drd11</u>	This study

^a sul, sulphonamide; tet, tetracycline; str, streptomycin; drd, derepressed.

^b This plasmid was isolated from R64-11 using nitrous acid mutagenesis. This plasmid was found not to suppress any of the dnaB(ts) mutations used in this study. It was also inferred that this plasmid does not have any functional ban gene associated with it (see "Results" to this Chapter).

^c Isolated from R64-11 using NTG mutagenesis; this plasmid suppressed the mutations dnaB279 with a very high efficiency. This plasmid also enhanced the temperature sensitivity of the wild-type dnaB⁺ strain (strain Q125). It was inferred that this plasmid has ban associated with it. (see "Results" to this Chapter).

This exponential culture was used as donor in a subsequent liquid mating. The recipient strain carried dnaB279 (strain E279) and was grown to exponential phase (approximately $2-5 \times 10^8$ cells/ml) at 30C. Liquid mating was performed at 30C for 45 min with a donor to recipient ratio of 5 : 1. The mating mixture was then diluted 100-fold into fresh LB plus tetracycline (20 µg/ml) and regrown to late exponential phase (approximately 10^9 cells/ml). The mixture was then appropriately diluted and plated on sets of supplemented minimal agar plates with streptomycin and tetracycline added. Plates were incubated at 30C and 42C. Those colonies arising from supplemented minimal agar plates at 42C were purified twice by single colony streaking. The presumptive mutant plasmid of R64-11 obtained in this manner was designated pCU2.

A second mutant of plasmid R64-11 designated pCU1 had been isolated using nitrous acid mutagenesis.

Dominance Test

It should be pointed out that genetic dominance tests with isogenic or nearly-isogenic bacterial plasmids is complicated by the phenomenon of plasmid incompatibility. Because two such plasmids cannot be maintained stably by a cell, dominance test can at best be done only in transient diploids. Nevertheless, such a test was attempted and its results proved to be informative. The test was done by introducing R64-11 into a DnaB(ts) strain carrying pCU1 and testing for temperature sensitivity soon after. The recipient strain (WD26611/pCU1)

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was grown in NB at 30C to stationary phase (approximately $2-5 \times 10^9$ cells/ml), and the donor strain carrying R64-11 was grown to exponential phase at 37C. Growth of an R^+ cell into stationary phase is a common procedure for converting them into R^- phenocopies. Donor and recipient were mixed in a ratio of 10 : 1 and conjugation was allowed for 30 min at 30C. The conjugation mixture was then diluted 10^{-4} with prewarmed NB (at 39C) and incubation was continued at 39C. For controls, cultures of WD266/pCU2 and WD26611/R64-11 were treated in the same manner, but the step for conjugation was omitted. At intervals of 1 hr, samples were withdrawn, appropriately diluted and plated on supplemented minimal agar plates. Plates were incubated at 30C for 48 hrs. and the number of colonies were counted.

Construction of a dnaBam266 sup^+ strain carrying pCU2

The rationale of the procedure and the various steps used for the construction of a dnaBam266 sup^+ strain carrying the mutant plasmid pCU2 were the same as that described under "Materials and Methods" in Chapter III for wild-type plasmids (see also Fig. III-1)

Colony forming ability and DNA synthesis

Colony-forming ability and DNA synthesis of R^- strains and their derivatives carrying either pCU1 or pCU2 were studied using the methods described in "Materials and Methods" in Chapter 1.

RESULTS AND DISCUSSION

Mutants of R64-11 (pCU1 and pCU2) with altered suppressing ability

Two independent mutants of R64-11 have been isolated following NTG and nitrous acid mutagenesis. These two mutants of R64-11 were designated pCU1 and pCU2 respectively. The selection for the strain carrying pCU2 favored the isolation of a more temperature-resistant strain than the one carrying R64-11. Indeed, the initial isolate E279/pCU2 was able to form colonies at 42C while E279/R64-11 was not. The strain carrying pCU1 (strain WD26611/pCU1) was selected as one that had lost the temperature-resistance conferred by R64-11 while still retaining the other plasmid-associated markers. It was necessary to show conclusively that both of these mutations induced in the strain carrying plasmid R64-11 were in fact plasmid-associated.

Transfer of "mutant" plasmid to other strains and properties of their derivatives

Mutant plasmid pCU2 was observed to suppress the dnaB279 mutation when this same mutation was present in two different host strains. That is, the presence of pCU2 in either the parental strain E279/pCU2 or in the derived strain I279/pCU2 (isogenic to the Q strains and carrying the same mutation dnaB279) resulted in an increase in the efficiency of suppression (Table V-3; Fig. V-1A). Mutant plasmid pCU2 was also observed to suppress the temperature-sensitivity of the Q strains

TABLE V-3

Mutational alterations in suppressing ability of R64-11

Strain	<u>dnaB</u> allele	Mutant R64-11 plasmid	Colonies at 41C rela- tive to those at 30C *
Q125	<u>dnaB</u> ⁺	nil	5.5×10^{-1}
Q125/R64-11	<u>dnaB</u> ⁺	R64-11	1.2×10^{-1}
Q125/pCU1	<u>dnaB</u> ⁺	pCU1	4.7×10^{-1}
Q125/pCU2	<u>dnaB</u> ⁺	pCU2	4×10^{-3}
Q1502	<u>dnaB6</u>	nil	$< 1 \times 10^{-6}$
Q1502/R64-11	<u>dnaB6</u>	R64-11	2.3×10^{-5}
Q1502/pCU1	<u>dnaB6</u>	pCU1	3.2×10^{-6}
Q1502/pCU2	<u>dnaB6</u>	pCU2	6.3×10^{-5}
Q1506	<u>dnaB43</u>	nil	$< 1.3 \times 10^{-6}$
Q1506/R64-11	<u>dnaB43</u>	R64-11	1.1×10^{-5}
Q1506/pCU1	<u>dnaB43</u>	pCU1	6.8×10^{-6}
Q1506/pCU2	<u>dnaB43</u>	pCU2	6.6×10^{-5}
Q1508	<u>dnaB70</u>	nil	$< 5.3 \times 10^{-6}$
Q1508/R64-11	<u>dnaB70</u>	R64-11	1.6×10^{-5}
Q1508/pCU1	<u>dnaB70</u>	pCU1	2.7×10^{-6}
Q1508/pCU2	<u>dnaB70</u>	pCU2	1.3×10^{-2}
Q1610	<u>dnaB266</u>	nil	$< 3 \times 10^{-7}$
Q1610/R64-11	<u>dnaB266</u>	R64-11	1.3×10^{-6}
Q1610/pCU1	<u>dnaB266</u>	pCU1	7×10^{-7}
Q1610/pCU2	<u>dnaB266</u>	pCU2	6.7×10^{-6}
I279	<u>dnaB279</u>	nil	1.2×10^{-5}
I279/R64-11	<u>dnaB279</u>	R64-11	5×10^{-3}
I279/pCU1	<u>dnaB279</u>	pCU1	2.2×10^{-5}
I279/pCU2	<u>dnaB279</u>	pCU2	1.3×10^{-2}

* At 30C, there were no significant differences in colony-forming ability between these strains.

Figure V-1.

DNA synthesis at 42C in three plasmid-negative strains and corresponding derivative strains carrying either R64-11 or pCU2.

Fig. A: I279 (dnaB279 supE, plasmid⁻), (O—O)
I279/R64-11 (dnaB279 supE, plasmid R64-11), (Δ—Δ)
I279/pCU2 (dnaB279 supE, plasmid pCU2), (□—□)

Fig. B: Q1508 (dnaB70 supE, plasmid⁻), (O—O)
Q1508/R64-11 (dnaB70 supE, plasmid R64-11), (Δ—Δ)
Q1508/pCU2 (dnaB70 supE, plasmid pCU2), (□—□)

Fig. C: Q125 (wild type dnaB, plasmid⁻), (O—O)
Q125/R64-11 (wild type dnaB, plasmid R64-11), (Δ—Δ)
Q125/pCU2 (wild type dnaB, plasmid pCU2), (□—□)

carrying four other different dnaB allele (dnaB6, dnaB43, dnaB70 and dnaB266). The efficiency of suppression of dnaB6, dnaB43 and dnaB266 was slightly higher than that observed with R64-11 (Table V-3). However, when pCU2 was present in strain Q1508, it suppressed the temperature-sensitivity of dnaB70 with an efficiency much higher than that of R64-11 (Table V-3; Fig. V-1B). Interestingly, when pCU2 was introduced into strain Q125 (dnaB⁺ sup⁺) the resulting strain Q125/pCU2 had become heat-sensitive both in DNA synthesis and colony-formation (Table V-3; Fig. V-1C). From this, it was inferred that this mutant plasmid (pCU2) enhanced the temperature-sensitivity of strain Q125 carrying the dnaB⁺ allele. On the other hand, mutant plasmid pCU1 was not observed to suppress or enhance significantly the temperature-sensitivity of any of the DnaB(ts) mutant strains tested (Table V-3).

To indicate that in conjugation reactions, the mutant plasmids pCU1 and pCU2 are transferred with the same efficiency as the parent plasmid R64-11, conjugation experiments were carried out. Table V-4 shows that although both mutant plasmids pCU1 and pCU2 behaved quite differently from their parental plasmid R64-11 with respect to their ability to suppress dnaB(ts) mutations, these two plasmids showed a conjugal transfer frequency similar to that of R64-11 (Table V-4).

A dominance test was attempted by introducing R64-11 into a DnaB(ts) strain carrying the mutant plasmid pCU1 (strain WD266II/pCU1) and testing the temperature-sensitivity soon after. The results in Figure V-2 suggest that although one or the other of the two

TABLE V-4

Conjugal transfer efficiency of R64-11
and its mutant plasmids pCU1 and pCU2^a

Donor strain	Recipient	Conjugal efficiency ^b
CO3/R64-11	Q125	4.2×10^{-2}
CO3/pCU1	Q125	4.9×10^{-2}
CO3/pCU2	Q125	2.7×10^{-2}
Q1506/R64-11	WD266II	2×10^{-3}
Q1506/pCU1	WD266II	1×10^{-3}
Q1506/pCU2	WD266II	1.8×10^{-3}

^a Mating was performed at 37C for dnaB⁺ strains and for DnaB(ts) strains, mating was at 30C.

^b Conjugation frequency was calculated as the number of transconjugants per 100 donor cells

Figure V-2.

Dominance test as measured by the percentage survival after incubating the test strains at 39C. For details, see "Materials and Methods" in Chapter V.

WD266/pCU1 (dnaB266 supE, plasmid pCU1), (Δ — Δ)

WD266/R64-11 (dnaB266 supE, plasmid R64-11), ($\bigcirc$ — $\bigcirc$)

CO3/R64-11 (donor) X WD266/pCU1 (recipient), ($\square$ — $\square$)

Plasmids is eventually lost from these strains, in the transient diploid, pCU1 is recessive to a wild-type allele in R64-11.

Attempts to construct sup^+ (su^-) strains carrying either pCU1 or pCU2

Using a procedure similar to that described under "Materials and Methods" in Chapter III, attempts were made to construct sup^+ dnaBam266 strains carrying only pCU1 or pCU2. The results of these experiments are shown in Table V-5. It can be seen that from strain Q1550 (dnaBam266 sup^+ PlCm bac) carrying pCU1, it was not possible to cure PlCm bac spontaneously or by P7-induction. On the other hand, when pCU2 was present in strain Q1550, PlCm bac could be cured by either one of the two methods (either spontaneously or by P7-induction). Thus, a dnaBam266 sup^+ strain carrying only pCU2 could be constructed. This shows that pCU2, a mutant derivative of R64-11 still contains a functional ban gene.

Colony-forming ability and DNA synthesis

It has been reported that strain Q1550 (dnaBam266 sup^+ /PlCm bac) is viable but cryosensitive and gives rise to fewer colonies at a temperature of 26C (D'Ari et al., 1975; see also Table IV-1). DNA synthesis and colony-forming ability in strain Q1550 carrying either pCU1 or pCU2 and the Cm^S derivative carrying only pCU2 (strain PW13= dnaBam266 sup^+ /pCU2) were followed. The results of these experiments are presented in Table V-6 and Figure V-3. It can be seen that the strain carrying both PlCm bac and pCU1 behaved very much like

TABLE V-5

Frequency of spontaneous or P7-induced loss of P1 from strains carrying mutant plasmid of R64-11 in addition to P1

Strain	Relevant chromosome Markers	Mutant plasmid of R64-11	Frequency of occurrence of clones that have lost P1	
			Spontaneous	P7-induced
Q1550	<u>dnaBam266</u> <u>sup</u> ⁺	nil	$< 10^{-3}$ *	$< 10^{-2}$
Q1550/pCU1	<u>dnaBam266</u> <u>sup</u> ⁺	pCU1	$< 5 \times 10^{-4}$	$< 10^{-2}$
Q1550/pCU2	<u>dnaBam266</u> <u>sup</u> ⁺	pCU2	1.7×10^{-3}	8×10^{-1}

$< 10^{-3}$ refers to the fact that 1000 colonies were replica-plated and none was found to have lost the PlCm bac-associated Cm^r marker.

TABLE V-6

Colony-forming ability of dnaBam266 sup⁺ strains
carrying either PlCm bac or PlCm bac and an additional R plasmid

Strain	Plasmid in strain	Colonies at temperature relative to those at 37C	
		26C	41C
Q1550	PlCm <u>bac</u>	3.1×10^{-4}	5.6×10^{-1}
PW1	PlCm <u>bac</u> ; R64-11	4.4×10^{-4}	3.6×10^{-1}
PW2	R64-11	7×10^{-2}	6.9×10^{-1}
Q1550/pCU1	PlCm <u>bac</u> ; pCU1	2.8×10^{-4}	1.9×10^{-1}
Q1550/pCU2	PlCm <u>bac</u> ; pCU2	8×10^{-2}	3×10^{-1}
PW13 ^a	pCU2	4.5×10^{-4}	4.3×10^{-1}

^a This strain is the Cm^S derivative of Q1550/pCU2 and it does not carry PlCm bac.

Figure V-3.

DNA synthesis at three temperatures (37C, 26C and 42C) in dnaBam266 sup⁺ strains carrying ban genes associated with PlCm bac, pCU2, the combination of PlCm bac and pCU1, and the combination of PlCm bac and pCU2.

Q1550 (dnaBam266 sup⁺, plasmid PlCm bac), (○—○)

Q1550/pCU1 (dnaBam266 sup⁺, plasmids PlCm bac & pCU1), (●—●)

Q1550/pCU2 (dnaBam266 sup⁺, plasmids PlCm bac & pCU2), (△—△)

PW13 (dnaBam266 sup⁺, plasmid pCU2), (▲—▲)

(note: the combination of PlCm bac and the wild type R64-11 yielded a level of cold sensitivity (26C) that was intermediate between strains carrying PlCm bac or R64-11 alone: see Fig. IV-1. This is unlike the situation seen here for the combination of PlCm bac and the mutant derivative pCU2).

the strain Q1550, both in DNA synthesis and colony formation. It was found that in similar experiments, all of the several strains carrying pCU1 behave as if they do not carry any functional ban gene. That is, pCU1 does not alter the temperature-sensitivity of any of these strains. Taken together with the fact that the mutation was induced by nitrous acid, this behaviour is consistent with the mutation being a deletion. Strains carrying pCU2 could also be distinguished from strains carrying the wild-type R64-11 in several ways:

- (a) pCU2 confers heat-sensitivity to a strain carrying the wild-type dnaB gene, R64-11 does not (Table V-3; Fig. V-1C).
- (b) a strain carrying only pCU2 but no functional dnaB gene is cryosensitive and a strain carrying R64-11 instead of pCU2 is not (Table V-6; Fig. V-3)
- (c) a strain carrying PlCm bac and pCU2 (each of the plasmids by itself confers cryosensitivity) is not cryosensitive in the absence of a functional dnaB gene (Table V-6; Fig. V-3). A similar strain carrying PlCm bac and R64-11 shows a level of cryosensitivity intermediate between isogenic strains carrying either PlCm bac or R64-11 alone (Fig. IV-1, p. 75).
- (d) pCU2 suppresses the temperature-sensitivity of all dnaB(ts) mutations to a slightly greater extent than does R64-11, but in two cases (dnaB70 and dnaB279), this increased suppression is greater than in the case of others (Table V-3; Fig. V-1).

pCU2 was induced by NTG mutagenesis and is probably a missense mutation. The properties of strains carrying this mutant plasmid are consistent with a change in the structure of the plasmid-

specified Ban protein which affects its temperature-sensitivity (to cold or heat) either when it functions by itself (cold sensitive) or in combination with other proteins with a similar function such as the E. coli DnaB protein or the Pl-Ban protein.

Growth of bacteriophage λ

Bacteriophage λ requires the functional dnaB gene for its own replication. Thus, λ will not replicate in all DnaB(ts) mutants of E. coli at a temperature that is non-permissive for host DNA replication. A subclass of DnaB(ts) mutants have been found recently in which wild-type λ will not replicate even at a temperature permissive for host DNA replication. These mutations are called groP or dnaB(groP) because they do map within the dnaB gene, and mutations (called π mutations) in one of the DNA replication genes of λ (gene P) reverse the effect of groP. These and other observations have been interpreted to imply that to drive λ DNA replication, a cooperation or interaction occurs between the E. coli DnaB protein, the λ -P protein and other proteins (Georgopoulos and Herskowitz, 1971; McMacken et al., 1977). λ and $\lambda\pi$ mutants can therefore be used to some advantage in studying dnaB genes and ban genes specified by various plasmids. In strains that were inferred to produce the Pl-Ban protein but no functional DnaB protein (strain Q1550; dnaBam266 sup⁺/PlCm bac) we confirmed a previous observation (D'Ari et al., 1975) that λ will not grow (Table V-7). With respect to λ growth, the strain producing only the

Pl-Ban protein thus behaved like the E. coli DnaB(GroP) strains rather than DnaB⁺ or DnaBts(GroP⁺) strains. However, in strains capable of producing both the Pl-Ban protein and the DnaB(GroP)-protein, λ will replicate. $\lambda\pi$ mutants which carry mutations in the replication gene P of λ and which can grow on GroP strains fail to grow on strain Q1550, the strain producing only the Pl-Ban protein. These results are shown in Table V-7. It appears that the product specified by the ban genes of either PlCm bac, R64-11 or pCU2 cannot support the growth of λ although each of these genes were found to support bacterial DNA synthesis. These analog genes thus allow discrimination between the requirements for bacterial, and λ growth just as the dnaB(groP) class of dnaB(ts) mutations do. However, the behaviour of the three plasmid-associated ban genes is not identical to the dnaB(groP) gene or to one another for the following reasons: (a) although neither of the strains that produce the DnaB(GroP) protein alone nor the Pl-Ban protein alone plate wild-type λ , strains that can produce both of these proteins (PlCm bac lysogens of DnaB(GroP) strains) do plate λ (Table V-7, last line); a favoured interpretation of this observation is that λ replication is restored through an interaction between subunits of the DnaB(GroP) protein and the Pl-Ban protein. Thus the Pl-Ban protein though similar cannot be identical. The $\lambda\pi$ mutants that were isolated as those that can grow on a DnaB(GroP) strain (mutation in the replication gene P of λ) cannot grow on strains producing only the Pl-Ban protein. Furthermore, in Chapter IV, it was shown that strain Q1550 (the PlCm bac strain producing only the

TABLE V-7

Ability of some strains carrying R64-11 and
its mutant plasmid pCU1 or pCU2 to plate bacteriophage λ

Strain	<u>dnaB</u> allele and any other rele- vant allele	Plasmid in strain	Ability to plate λ at 30C
Q125	<u>dnaB</u> ⁺	nil	+
Q125/PlCm <u>bac</u>	<u>dnaB</u> ⁺	PlCm <u>bac</u>	+
Q125/R64-11	<u>dnaB</u> ⁺	R64-11	+
Q125/pCU1	<u>dnaB</u> ⁺	pCU1	+
Q125/pCU2	<u>dnaB</u> ⁺	pCU2	+
Q1550	<u>dnaBam266</u>	PlCm <u>bac</u>	-
Q1550/R64-11	<u>dnaBam266</u>	PlCm <u>bac</u> ; R64-11	-
Q1550/pCU1	<u>dnaBam266</u>	PlCm <u>bac</u> ; pCU1	-
Q1550/pCU2	<u>dnaBam266</u>	PlCm <u>bac</u> ; pCU2	-
PW13	<u>dnaBam266</u>	pCU2	-
Q1506	<u>dnaB43</u>	nil	+
Q1506/R64-11	<u>dnaB43</u>	R64-11	+
Q1506/pCU1	<u>dnaB43</u>	pCU1	+
Q1506/pCU2	<u>dnaB43</u>	pCU2	+
Q1516	<u>dnaB(groPB558)</u>	nil	-
Q1516/PlCm <u>bac</u>	<u>dnaB(groPB558)</u>	PlCm <u>bac</u>	+
Q1516/R64-11	<u>dnaB(groPB558)</u>	R64-11	-
Q1516/pCU1	<u>dnaB(groPB558)</u>	pCU1	-
Q1516/pCU2	<u>dnaB(groPB558)</u>	pCU2	-

Pl-Ban protein) was cold-sensitive. The DnaB(GroP) strains are heat-sensitive. (b) Like the strains producing only the Pl-Ban protein, the two strains producing only the R64-11-Ban or pCU2-Ban proteins are also unable to plate λ (Table V-7). λ replication is however not restored in strains producing both the DnaB(GroP)-Ban and the R64-11-Ban (or pCU2-Ban) proteins. This latter behaviour discriminates between strains producing the Pl-Ban protein and those producing the R64-11-Ban protein. In Chapter IV, it was also shown that unlike strains producing only the Pl-Ban protein, the strains producing only the R64-11-Ban protein are not cold-sensitive. Thus, by these in vivo experiments not only can the dnaB or the dnaB(groP) gene products be distinguished from the products specified by the plasmid-associated ban genes but one plasmid-associated ban can be distinguished from another plasmid-associated ban.

CONCLUSIONS

Mutant plasmid pCU2 was originally isolated as a plasmid that suppressed dnaB279 (strain E279) with a high efficiency. After the introduction of this plasmid into strain I279 (isogenic to the Q strains) carrying the same dnaB allele (dnaB279), it was observed that pCU2 also suppressed this mutation in the new genetic background with a higher efficiency than that of the wild-type plasmid R64-11. pCU2 also suppressed mutations dnaB6, dnaB43 and dnaBam266 with a slightly higher efficiency than that of R64-11. The suppression efficiency of dnaB70 by this mutant plasmid (pCU2) was comparable to the suppression of dnaB279 and greater than the efficiency of the remaining three dnaB(ts) mutations. The fact that like R64-11, plasmid pCU2 rescues the dnaB(ts) amber mutation, provides evidence that pCU2 specifies a Ban protein. However, the behaviour of strains carrying R64-11 is different from strains carrying pCU2. These differences are summarized in Table V-8. As in the case of the mutant plasmid pCU2 suppression of the dnaB(ts) mutations could be the result of an interaction between the DnaB(ts) protein and a Ban protein specified by mutant plasmid pCU2 (pCU2-Ban). The observation that the introduction of pCU2 into a dnaB⁺ strain (strain Q125) resulted in an increased level of temperature-sensitivity provides further evidence for the hypothesis of DnaB-Ban interaction. In such a case, the interaction is between wild-type DnaB protein of E. coli and the Ban protein specified by pCU2, and the effect is a negative one.

The implication from the results presented in Table V-3 was

TABLE V-8

Summary of differences between strains carrying
R64-11 and mutant plasmid pCU1 or pCU2

Strain	<u>dnaB</u> allele	Plasmid in strain	Relevant phenotype (DNA syn- thesis and colony-formation)
Q1550	<u>dnaBam266</u>	PlCm <u>bac</u>	Heat resistant; cold sensitive.
Q1550/pCU1	<u>dnaBam266</u>	PlCm <u>bac</u> ; pCU1	Heat resistant; cold sensitive.
Q1550/pCU2	<u>dnaBam266</u>	PlCm <u>bac</u> ; pCU2	Heat and cold resistant.
PW2	<u>dnaBam266</u>	R64-11	Heat and cold resistant.
PW13	<u>dnaBam266</u>	pCU2	Heat resistant; cold sensitive.
Q125	<u>dnaB</u> ⁺	nil	Heat and cold resistant.
Q125/R64-11	<u>dnaB</u> ⁺	R64-11	Heat and cold resistant.
Q125/pCU1	<u>dnaB</u> ⁺	pCU1	Heat and cold resistant.
Q125/pCU2	<u>dnaB</u> ⁺	pCU2	Heat sensitive; cold resistant.
I279	<u>dnaB279</u>	nil	Heat sensitive; cold resistant.
I279/R64-11	<u>dnaB279</u>	R64-11	Less heat sensitive than R ⁻ strain (I279) and cold resistant.
I279/pCU1	<u>dnaB279</u>	pCU1	Heat sensitive; cold resistant.
I279/pCU2	<u>dnaB279</u>	pCU2	Heat and cold resistant.

that either the amount of the Ban protein specified by pCU2 or that its structure is different from the Ban protein specified by R64-11. That is, the mutation in pCU2 is either in the structural gene for the Ban protein or in some genes which regulate the amount of this protein which in turn affects the temperature-sensitivity of DNA synthesis and colony-formation. For example, it could be considered that in the mutant, the production of R64-11-Ban is cold sensitive rather than the mutant protein being cold sensitive. The latter hypothesis is favoured because of the following observations: (a) although strains producing either Pl-Ban or pCU2-Ban alone are both cold sensitive, the combination of both of them in one strain results in the restoration of cold resistance (Table V-6; Fig V-3). The simplest interpretation of this is that the Pl-Ban strain (Q1550) and the pCU2-Ban strain (strain PW13) are cold sensitive for different reasons and that cold resistance in the strain carrying both plasmids is a consequence of a positive interaction between the subunits of the Ban proteins specified separately by each plasmid. Since strain PW2 (the relevant strain carrying the parental plasmid R64-11) was not cold sensitive and pCU2 which was derived from R64-11 confers cold sensitivity in a similar situation, the mutation must have a qualitative effect and probably in the structural ban gene of R64-11. Unlike pCU2, no evidence could be obtained for the presence of a ban gene associated with the mutant plasmid pCU1. Furthermore, it was found neither to suppress nor enhance the temperature-sensitivity of any of several strains carrying dnaB(ts) mutations or ban genes specified by other plasmids (Tables V-3 and V-6). From these observations and the fact that a deletion

promoting mutagen was used for its isolation, it is inferred that pCU1 has a deletion in the ban gene region of R64-11. A more direct demonstration of the location of these two mutations must await the devising of methods for accurately mapping this plasmid. The isolation and the properties of pCU1 and pCU2, the two mutant derivatives of R64-11, suggest that this approach could be a rewarding one in the future.

CHAPTER VI

Further Discussion

FURTHER DISCUSSION

In the past decade, much work has been done on the bacterium Escherichia coli with respect to its mode of chromosomal DNA replication. The mechanism of replication of the E. coli chromosome is a very complex process involving many gene products and other factors. Based on available information, a brief summary is first presented on the steps believed to be involved in chromosomal DNA replication. It is emphasized that at the present time, one can be confident only about the overall scheme of replication and many detailed aspects are either poorly understood or completely speculative. Following this description, there will be a detailed discussion of the dnaB and the dnaB analog gene including the ones that were discovered and described in the earlier Chapters.

The replication of the chromosome of E. coli is a semi-conservative process (Meselson and Stahl, 1958). Replication starts at a fixed origin and finishes at a fixed terminus. Both of these regions are genetically defined (Bird et al., 1972). It was first proposed that chromosome growth was unidirectional (Cairns, 1963). With improved genetical, biochemical and autoradiography studies, a bidirectional mode of chromosome growth is now well documented for E. coli (Bird et al., 1972; Prescott and Kuempel, 1972). Since the E. coli chromosome is such a compact structure with a molecular weight of 2.5×10^9 daltons (about 4×10^6 base pairs), it has seemed reasonable to suppose that for DNA synthesis to commence, the double helix must first unwind. Unwinding proteins

in E. coli have been postulated and sought for many years. Proteins that were thought to be and called unwinding proteins have been isolated from E. coli, e.g. T4 gene 32 protein (Epstein et al., 1963; Huberman et al., 1972), the gene 5 protein of phage M13 (Alberts et al., 1972; Oey and Knippers, 1972), and a protein isolated from uninfected E. coli (Siegal et al., 1972). Subsequent studies on the properties of these proteins (Sinha and Snustad, 1971; Oey and Knippers, 1972) have however suggested that they act cooperatively to stabilize the DNA in a single-stranded form. In the more recent literature, they are therefore referred to as either "DNA binding" protein or "stabilizing protein", the idea being that they act in vivo to stabilize or to "hold open" the replication fork in such a manner that other relevant proteins and precursors can gain access to this region and act. Details of how this is coordinated with other steps are unknown. In addition to this class of proteins, at least one protein has been found in E. coli that untwists or relaxes a supercoil: protein ω (Wang, 1971). This protein, also referred to sometimes as the rep protein (McMacken et al., 1977) is a candidate for the unwinding function which presumably precedes stabilization. Since every round of chromosomal DNA replication begins at a fixed replication origin, there must be one or more special events that occur in this region that are distinct from the actual polymerization of DNA strands. These events have sometimes been called "initiation" events as distinct from "elongation" events, the latter signifying events associated with the propagation or transit of a replication fork.

However, a considerable amount of evidence has now accumulated all of which indicate that the transit of a replication fork involves repeated initiation and synthesis of short pieces of DNA fragments (1000-2000 nucleotides) which are subsequently joined (reviewed by Kornberg, 1974; Geftter, 1975). Thus it may be necessary to think in terms of two kinds of initiation events; a general one occurring at regular spaced intervals throughout the chromosome and a special one, which occurs at a unique region of the chromosome origin. Genetic studies are likely to be useful in understanding the distinctive features of these two types of events because some temperature-sensitive mutants have been shown to affect only the events at the chromosome origin and other events at the replication fork (see Table VI-1 for a summary of the genes and gene products that are known currently).

Although the polymerases of E. coli (polI, polII and polIII specified respectively by polA, polB and polC(dnaE) can all extend a DNA chain from a free 3'OH terminus, none of them has the ability to initiate DNA synthesis de novo. Therefore some other primers have seemed necessary to initiate DNA synthesis. Since RNA polymerase can initiate transcription without a primer, one model of initiation of DNA synthesis is that RNA polymerase synthesizes a short fragment of RNA using the DNA template. The 3'OH end of this RNA can then be covalently extended by an enzyme such as DNA polymerase III. That the primer is a short fragment of RNA is supported by the following observations. The replication of the single-stranded circular DNA molecule of phage M13 to its replicative

TABLE VI-1.

Genes of E. coli K-12 that are involved in DNA synthesis

Note: In addition to these genes and their products, several protein factors have been isolated and found to be necessary for the in vitro conversion of single-stranded ϕ X174 DNA (or DNA of other small phage) to its replicative form. It is possible that some of these factors are also necessary for the in vivo synthesis of the E. coli chromosome. Of these factors, it is believed that proteins i and n operate during chain elongation. However, genes that correspond to these proteins have not yet been recognized. There must also be one or more sequences on the DNA such as the chromosome origin that serve as recognition sequences for the products of other genes.

TABLE VI-1

Genes of E. coli K-12 that are involved in DNA synthesis

Locus	Function in DNA synthesis (or gene product)	Reference
<u>dnaA</u>	initiation of DNA synthesis	Wechsler & Gross, 1971.
<u>dnaB</u>	DNA chain elongation (DNA initiation ?)	Wechsler & Gross, 1971; Ueda <u>et al.</u> , 1978.
<u>dnaC</u> (D)	initiation of DNA synthesis	Wechsler & Gross, 1971; Carl, 1970.
<u>dnaE</u> (<u>polC</u>)	DNA polymerase III	Wechsler & Gross, 1971; Gefter <u>et al.</u> , 1971.
<u>dnaF</u>	ribonucleoside reductase (renamed <u>nrd</u>)	Wechsler & Gross, 1971; Fuchs <u>et al.</u> , 1971.
<u>dnaG</u>	DNA chain elongation; primase, synthesis of RNA primer	Carl, 1970; Chen & Carl, 1975; Rowen & Kornberg, 1978.
<u>dnaH</u>	initiation of DNA synthesis	Sakai <u>et al.</u> , 1974.
<u>dnaI</u>	initiation of DNA synthesis	Beyersmann <u>et al.</u> , 1974.
<u>dnaP</u>	membrane defect	Wada & Yura, 1974.
<u>dnaS</u>	accumulation of very short DNA	Konrad & Lehman, 1975.
<u>dnaZ</u>	DNA chain elongation; <u>dnaH</u> revised	Filip <u>et al.</u> , 1974; Truitt & Walker, 1974.
<u>lig</u>	DNA ligase	Gottesman <u>et al.</u> , 1973.
<u>polA</u>	DNA polymerase I; DNA repair	Gross & Gross, 1969; De Lucia & Cairns, 1969.
<u>polB</u>	DNA polymerase II	Campbell <u>et al.</u> , 1972; Hirota <u>et al.</u> , 1972.
<u>polC</u> (<u>dnaE</u>)	DNA polymerase III	Wechsler & Gross, 1971; Gefter <u>et al.</u> , 1971.
<u>rif</u>	Beta subunit of RNA poly- merase	Taylor & Trotter, 1972; Heil & Zillig, 1970.

form (RF) does not require the expression of any viral gene, but rather uses host enzymes. If one of these enzymes is an RNA polymerase, the addition of rifampicin (an inhibitor of RNA synthesis) will inhibit transcription by RNA polymerase and should then prevent initiation of M13 DNA replication. In fact, this has been observed (Brutlag et al., 1971). An additional evidence is that the addition of rifampicin to a mutant with RNA polymerase resistant to this drug did not lead to inhibition of DNA replication in M13.

Once DNA replication has been initiated, it occurs in a short discontinuous manner. Short segments of DNA called Okazaki fragments which sediment at 10 S (1000-2000 nucleotide long) are then joined to the main body of the DNA chain by ligase. In E. coli mutants deficient in ligase and/or DNA polymerase I, the Okazaki fragments accumulate and they comprise the bulk of the newly synthesized DNA (Sugimoto et al., 1968; Newman and Hanawalt, 1968). Before the joining of the Okazaki fragments can occur, the RNA primers are excised and the gaps between short segments of DNA filled in or repaired by DNA polymerase I and DNA ligase activity.

The above summary is a simplified one and each of the steps outlined is probably by itself a complicated process. Table VI-1 lists all the genes and gene products that are now known or suspected to be required for the replication of the E. coli genome. Of these, the dnaB gene is of particular relevance to the subject of this thesis. This gene and its product are continuously required

for E. coli chromosomal DNA replication and for the replication of some plasmids (e.g. ColE1) and bacteriophages such as λ and ϕ X174. For example, a DnaB(ts) mutant of E. coli with an amber mutation in its dnaB gene will not be viable if such a strain does not carry any known amber suppressor (D'Ari et al., 1975, Ogawa, 1975). Since most DnaB(ts) mutants exhibit immediate cessation of DNA synthesis at a non-permissive temperature, it implies that the dnaB gene product is required in DNA chain elongation. There is however some recent evidence that the dnaB gene product could also be involved in DNA initiation at the chromosome origin. This will be discussed in a later section. A brief summary on the isolation and the characterization of the dnaB gene product and speculations on the role it plays in DNA chain elongation is presented below.

Extensive work has been done on the isolation and characterization of the dnaB gene product during the past few years (Wickner et al., 1973; Wright, Wickner and Hurwitz, 1973; Schekman et al., 1975; Wickner and Hurwitz, 1974; McMacken et al., 1977; Ueda et al., 1978). The discovery that the replication of the bacteriophage ϕ X174 DNA requires some host functions including that specified by dnaB allowed the use of the in vitro system for converting ϕ X174 single-stranded DNA to its duplex form as an assay for the purification of the DnaB protein. This protein has been purified from both wild-type E. coli and DnaB(ts) strains (Wickner et al., 1974). The DnaB protein has a molecular weight of 250,000 (Wickner et al., 1974) and is an oligomer of 55,000-dalton subunits (Ueda et al., 1978). The DnaB protein is temperature-sensitive when isolated

from DnaB(ts) mutants (Wright et al., 1973; Schekman et al., 1975). The purified DnaB protein has also associated with it a ribonucleoside triphosphatase activity which is stimulated 10-fold by single-stranded DNA (Wickner et al., 1974). Based on the fact that ϕ X174 will not replicate in DnaB(ts) mutants at a non-permissive temperature and that in vitro replication of ϕ X174 single-stranded DNA to its duplex form requires the product of the dnaB gene together with some other proteins (Wickner and Hurwitz, 1974), it has been inferred that the replication of ϕ X174 DNA could be similar at least in parts to the replication of the E. coli chromosome. Thus, using the in vitro system for the replication of ϕ X174 DNA, a general though speculative model for the role that the DnaB protein plays in DNA replication has been proposed by McMacken et al. (1977).

The first step in the conversion of ϕ X174 single-stranded DNA to its duplex form is the synthesis of a nucleoprotein intermediate (Weiner et al., 1976). It has been suggested that the DnaB protein is an essential component of the intermediate and that it retains its ATPase activity. The DNA binding or stabilizing protein that was mentioned earlier is also required for the initial events occurring at or close to the replication fork. It binds to single-stranded DNA at regular intervals, thus exposing some gaps or "open" regions on the DNA for the binding of the DnaB protein. DnaB protein is first transferred to the site close to the DNA replication fork by the action of dnaC(D) gene product, proteins i and n, ATP and Mg^{2+} , forming the replication-intermediate. Once

DnaB protein is bound, it is suggested that it can propel itself along the DNA strand. The synthesis of RNA primers depends on the product of dnaG (primase) (Rowen and Kornberg, 1978). This occurs only in the DNA that has been converted previously to the replication-intermediate form. The DnaB protein thus participates in the initiation of RNA primers which appear to be aligned at regular intervals along the template strand. If DnaB protein can migrate along the DNA strand utilizing energy from hydrolysis of ATP for propulsion, it can act as a "mobile promotor" for DnaG protein to produce RNA primers. In this manner, many primers along the DNA strand can be synthesized close to one replication fork by the function of only one DnaB protein. After the synthesis of RNA primers, DNA chain growth by the action of DNA polymerase III will thus lead to the synthesis of short Okazaki fragments. The synthesis of the RNA primers by DnaG protein is thus promoted by the binding of the DnaB protein to the DNA template. If, as proposed, the transfer of a DnaB protein to a site on the replication fork requires the product of dnaC(D) and other factors, a mutation in the dnaC(D) could lead to the inability to transfer DnaB protein. Since the dnaC(D) gene product is required to initiate DNA synthesis at the chromosome origin and based on the above assumption, the DnaB protein could be indirectly involved in DNA initiation at the chromosome origin. Such a hypothesis is supported by the following observations. The replication protein P of bacteriophage λ interacts with the DnaB protein (Georgopoulos and Herskowitz, 1971). By doing this, it may specifically transfer

the DnaB protein to the replication origin of λ DNA so that replication of the λ chromosome may commence at its origin. If a mutation should occur in the dnaB so that it cannot interact with the P protein of λ , no transfer of DnaB protein to the replication origin of λ DNA would occur and thus there will be no initiation of λ DNA. A further expectation of the hypothesis that the DnaB protein can interact or cooperate with the λ replication protein is that some mutations in the λ P gene will restore to λ the ability to grow in DnaB(GroP) mutants. This expectation has been met by the isolation of the λ T mutants which carry mutations that are localized in the gene P of λ . Whether the λ -P protein interacts with the DnaB or DnaB(GroP) proteins directly or indirectly when the DnaB protein is part of the postulated replication-intermediate is not known. As discussed in Chapter V, this does not occur with the dnaB(groP) subclass of dnaB(ts) mutations.

The model proposed by McMacken et al. (1977) for the overall role that the DnaB protein plays in DNA synthesis is speculative but there is some experimental evidence indicating that the binding of the DnaB protein to the DNA strand precedes the synthesis of RNA primers by the function of the DnaG protein. Also the idea that the transfer of the DnaB protein to the DNA template requires the product of dnaC(D) gains support from the observation that the DnaB protein can interact physically in vitro with the product of dnaC(D) (Wickner and Hurwitz, 1974). A dnaC(D) mutation has also been found (Wechsler, 1973) whose properties are consistent with

the loss of the ability of its product to interact with the DnaB protein.

This study was concerned with the effect of bacterial R plasmids on strains carrying dnaB(ts) mutations. At the beginning of this study and from the published literature, it could not have been suspected that so many plasmids would modify the DnaB phenotype. The published literature recorded a contrary belief that most large plasmids that are maintained at relatively low copy numbers are dependent on the functional dnaB gene of E. coli. The direction of the studies described in this thesis was determined to a large extent by the contrary observation (Chapter I) that so many naturally occurring R plasmids could alter the temperature-sensitivity of DnaB(ts) mutants. In most cases, the effect observed was a phenotypic suppression of temperature-sensitivity. This led to a study of the mechanism of suppression. There was a precedent for plasmid-induced suppression of dna(ts) mutations in E. coli in the phenomenon of integrative suppression of dnaA(ts) mutations. In this phenomenon, suppression has been found to be always accompanied by the insertion or integration of the plasmid into the E. coli chromosome. In contrast, we have not observed plasmid-induced suppression of dnaB(ts) mutations to be accompanied by plasmid integration (Chapter II). Plasmid-induced suppression of temperature-sensitivity is an example of intergenic suppression (Gorini and Beckwith 1966; Hartman et al., 1971) and an important class of this type of suppression is "informational suppressor", that is, mutations or alterations in some component of the code-reading machinery (e.g. transfer,

RNA) so that a triplet code on a messenger RNA (m-RNA) is genetically misread. If this mechanism was the major one and since R plasmids were found to suppress several missense mutations, this would imply the presence on a plasmid of several missense suppressors. As a general mechanism for dnaB-suppression we did not find this model to be attractive enough to pursue although the existence of missense suppressors on plasmids is not excluded. Instead, the hypothesis that was examined experimentally was one which required R plasmids to specify a protein that was sufficiently similar to the E. coli DnaB protein so as to functionally substitute for it if the latter could be engineered to be absent. A test of this hypothesis showed that 5 out of 11 plasmids examined specified such proteins (Chapter III). These proteins are referred to as dnaB analog or Ban proteins and the structural genes specifying them as ban genes. These studies also revealed differences between the different Ban proteins with respect to degree of temperature-sensitivity (heat or cold) or ability to support λ replication. It should thus be made clear that we are concerned here with a series of different ban genes associated with different R plasmids rather than a single ban gene associated with different plasmids.

Suppression of DnaB(ts) mutants by wild-type bacteriophage P1 was first suspected by Lanka and Schuster (1970). They showed that lysogenization of DnaB(ts) mutants by P1 not only led to some phage synthesis at 41C, but that it also led to a transient recovery of chromosomal DNA replication. It should be recalled that in the lysogenic state, P1 exists in cells as a low copy-number plasmid

(Rosner, 1972; Yun and Vapnek, 1977). Recent studies have shown that P1 codes for a DnaB analog (Ban) which is repressed in the wild-type phage, but expressed constitutively in mutant of P1 variously called Pl**bac** (D'Ari *et al.*, 1975) or Pl**mcb** (Ogawa, 1975). The terminology of **bac** and **ban** have gained acceptance in the literature (Schuster *et al.*, 1975; Yarmolinsky, 1977) and therefore I have adopted it in this thesis. A derivative of Pl**bac** was also isolated by D'Ari *et al.* (1975). They called this Pl**bac ban** and in strains carrying this plasmid, the expression of the Ban protein was prevented. As mentioned earlier, the replication of ϕ X174 DNA both *in vivo* and *in vitro* requires the DnaB protein of *E. coli* in addition to several other proteins (Wright *et al.*, 1973; Wickner and Hurwitz, 1974; McMacken *et al.*, 1977). Schuster *et al.* (1975) demonstrated that ϕ X174 DNA replication is restored at a non-permissive temperature in DnaB(ts) mutants carrying Pl**bac**, but not in the same mutants carrying Pl**bac ban**. Using a soluble enzyme fraction from DnaB(ts) lysogens, single-stranded ϕ X174 DNA was also converted to duplex form, whereas the corresponding fraction from DnaB(ts) mutants or mutants carrying Pl**bac ban** were completely inactive. Although Pl**cm bac** specifies a Ban protein which can substitute for the function of **dnaB** in a situation where both the Pl-Ban and *E. coli* DnaB(ts) proteins can be made, the exact molecular mechanism of suppression of **dnaB(ts)** mutations is still unclear at the present time. Based on our observations and those of D'Ari *et al.* (1975), one mechanism that we favour is that heteromultimeric proteins are formed between the different subunits of

DnaB(ts) and Ban proteins. These heteromultimers may have altered temperature-sensitivity or altered ability to promote λ replication. Although DnaB(ts) mutants are affected in DNA synthesis at non-permissive temperatures, thermolabile DnaB(ts) protein can still be produced (Wickner and Hurwitz, 1974; Wright et al., 1973). The different thermolabile DnaB(ts) proteins produced by DnaB(ts) mutants with different dnaB allele could differ from one another in their thermo-sensitivity or their ability to interact with other proteins. It is therefore not surprising that there exists evidence for some specificity of protein-protein (DnaB-Ban) interaction. That is, a product specified by one particular R plasmid may only interact with the DnaB(ts) protein specified by a particular dnaB allele. The result of such an interaction will thus lead to the observed variability in the degree of suppression or enhancement.

In Chapter III, the construction of strains producing no functional DnaB protein but producing instead a Ban protein specified by each of the six different plasmids (PlCm bac and five R plasmids) was described. These strains showed differences in either temperature-sensitivity of DNA replication (and colony formation) or in their ability to support the growth of bacteriophage λ . The latter difference was ascribed to the inability of the Ban protein to interact or cooperate with an essential λ replication gene such as the gene P of λ . An extension of the postulate that Pl-Ban can interact and form heteromultimers with DnaB(ts) proteins is that the Ban proteins specified by R plasmids may also do so. In Chapter I, it was shown that R plasmids that specify Ban

proteins suppress the temperature-sensitivity of different dnaB(ts) mutations but they do so with different efficiencies. This variability in suppression efficiency of different dnaB(ts) mutations is consistent with the hypothesis of Ban-DnaB subunit interactions. Ban-Ban interactions, that is, interactions between Ban proteins specified by two different ban genes may also be expected. In Chapter IV, it was shown that such interactions may occur with functional consequences. Finally, from the results presented in Chapter V, it was inferred that mutations can be produced in a plasmid that affect the ability of its Ban protein to interact with DnaB or other Ban proteins. For convenience, these observations on differences between Ban and DnaB proteins and on interactions that were presented in detail elsewhere (Chapters I, III, IV and V) are summarized in Table VI-2. Inferences drawn from the observations are presented in abridged form in the last column. A few points should be borne in mind when this tabulated summary is inspected and they are emphasized here:

- (a) interactions are considered specifically for each pair of dnaB or ban allele and their respective proteins, i.e. if it is inferred that there is evidence for an interaction between a particular Ban protein and a particular DnaB protein, there is no implication that such an interaction will occur between the same Ban protein and all DnaB proteins (or other Ban proteins).
- (b) in each of the possible interactions, the alternative hypothesis that was considered was whether the attributes of the

TABLE VI-2

Summary of data and inferences implying in vivo interactions

DnaB or Ban protein(s) specified by	Phenotype of strain: DNA synthesis and colony-formation		λ Growth at
PlCm <u>bac</u>	CS	HR	-
R64-11	CR	HR	-
pCU2	CS	HR	-
R144-3	CS	HR	-
R386	CS	HR	+
RP4	CS	HS	-
R621a	CS	HS	+
DnaB(GroP) (<u>E. coli</u>)	CR	HS	-
DnaB(ts)* (<u>E. coli</u>)	CR	HS	+
PlCm <u>bac</u> + DnaB(ts)	CR	HR	+
PlCm <u>bac</u> + DnaB(GroP)	CR	HR	+
R64-11 + DnaB(ts)	CR	HR	+
+ DnaB(GroP)	CR	HR	+
+ PlCm <u>bac</u>	intermed. level to cold	HR	+
pCU2 + DnaB(ts)	CR	more HR	+
+ DnaB(GroP)	CR	HR	-
+ PlCm <u>bac</u>	CR	HR	-
R144-3 + DnaB(ts)	CR	HR	+
+ DnaB(groP)	CR	HR	-
+ PlCm <u>bac</u>	CR	HR	-

TABLE VI-2

g in vivo interactions ~~between~~ DnaB and Ban or different Ban proteins

h: DNA
 formation λ Growth at 30°C Comments relevant to the postulate of interaction

		Following phenotypic class are recognized	
	-	CS	HR
	-	CS	HR
	-	CS	HS
	-	CS	HS
	+	CR	HR
	-	CR	HS
	+	CR	HS
	+	providing a convenient way of discrimination	
	+	PlCm <u>bac</u> suppresses all <u>dnaB(ts)</u> mutations with approx. equal efficiency but by <u>itself</u> confers cold sensitivity. Pl <u>bac</u> <u>ban</u> does not suppress any <u>dnaB(ts)</u> mutation.	
	+	neither DnaB(GroP) nor PlCm <u>bac</u> permit λ replication, the combination does.	
	+	<u>dnaB279</u> suppressed more efficiently than other mutations.	
	+	no observable effect	
	+	no observable effect	
HR	+	suppresses different <u>dnaB(ts)</u> mutations with different efficiency; furthermore, the specificity changes as a result of the mutation (<u>dnaB70</u> suppressed more efficiently by pCU2)	
HR	-	no observable effect	
HR	-	PlCm <u>bac</u> and pCU2 alone confer cold sensitivity. The combination of the two confer cold resistance.	
HR	+	suppresses different <u>dnaB(ts)</u> mutations with different efficiency.	
HR	-	no observable effect	
HR	-	PlCm <u>bac</u> and R144-3 by themselves confer cold sensitivity, the combination confers cold resistance.	

| 2 or 2 |

TABLE VI-2 (continued)

DnaB or Ban protein(s) specified by	Phenotype of strain: DNA synthesis and colony-formation	Growth of λ at 30C	Comments
R386 + DnaB(ts)	CR HR	+	suppresses d
+ DnaB(GroP)	CR HR	+	no discrimi tial utiliza
+ PlCm <u>bac</u>	CR HR	-	PlCm <u>bac</u> and combination
RP4 + DnaB(ts)	CR HR	+	suppresses d
+ DnaB(GroP)	CR HR	-	dnaB(groP) a the combinat
+ PlCm <u>bac</u>	CS HS	+	no discrimi zation of R
R621a + DnaB(ts)	CR HR	+	suppresses d
+ DnaB(GroP)	CR HR	+	no discrimi zation of R
+ PlCm <u>bac</u>	CR HR	-	PlCm <u>bac</u> and the combinat

Abbreviations: CS, cold sensitive at 26C; CR, cold resistant at 26C;
HS, heat sensitive at 41C; HR, heat resistant at 41C.

* for convenience in summarizing, all four dnaB(ts) muta

rowth of λ
at 30C

Comments relevant to the postulate of interaction

- + suppresses different dnaB(ts) mutations with different efficiencies.
- + no discrimination can be made between interaction and preferential utilization of R144-3-Ban.
- PlCm bac and R386 by themselves confer cold sensitivity, the combination confers cold resistant.
- + suppresses different dnaB(ts) mutations with different efficiencies.
- dnaB(groP) and RP4 by themselves confer heat sensitivity, the combination of the two confers heat resistant.
- + no discrimination between interaction and preferential utilization of RP4-Ban.
- + suppresses different dnaB(ts) mutations with different efficiencies.
- + no discrimination between interaction and preferential utilization of R621a-Ban.
- PlCm bac and R621a by themselves confer cold sensitivity, the combination confers cold resistance.

cold resistant at 26C;

heat resistant at 41C.

, all four dnaB(ts) mutations are considered together.

| 2 or 2 |

- strain carrying two plasmids could be explained by postulating that only one of the two possible Ban proteins is functional.
- (c) in the case of the mutant plasmid pCU2 (derived from R64-11), an attempt was made to discriminate between a "qualitative" change in the R64-11-Ban and a "quantitative" change. The strain carrying pCU2 was cold-sensitive relative to the strain carrying R64-11. The question raised was: did the mutation render the R64-11-Ban protein cold-sensitive or its production cold-sensitive?
- (d) although the term "interaction" has been used here for convenience, the molecular mechanism of interaction cannot be specified in detail. Association between DnaB-Ban or Ban-Ban subunits to form heteromultimers is favoured only because it appears to be the simplest explanation.

An earlier study by Lark and Wechsler (1975) supports the suggestions of DnaB-Ban protein interaction. By using F' merodiploid strains carrying two different dnaB alleles, they presented evidence that DnaB proteins may interact in vivo with each other to increase or reduce the temperature-sensitivity of DNA replication in these merodiploids. Interactions between wild-type DnaB protein and DnaB(ts) protein were also suggested. This was based on the observation that after the introduction of an F' plasmid carrying the dnaB(ts) mutation dnaB107 into a dnaB⁺ strain, such a strain showed an increased level of heat sensitivity. Such an observation is very similar to the result obtained in this study when mutant plasmid pCU2 was introduced into a dnaB⁺ strain (strain Q125).

The results summarized in Table VI-2 show how different ban genes and their products can be discriminated in vivo. This type of in vivo discrimination could be extended if necessary because dnaB mutations (and therefore presumably wild-type ban genes and ban mutations) have been shown to have other primary effects (Palchoudhury and Iyer, 1971; Bezanson and Iyer, 1975). Some R plasmids used in the present study were able to substitute for the E. coli DnaB protein in such a way as to allow for cell survival. However, these same R plasmids were able to suppress dnaB(ts) mutations efficiently. This groups of plasmids deserve to be studied further. One possibility is that they specify a kind of protein that is sufficiently similar to the DnaB(ts) proteins so as to modify them but one that is by itself inadequate to allow E. coli DNA replication.

Although most of the DnaB(ts) mutants when first isolated were thought to be elongation mutants because of an immediate arrest of DNA replication at non-permissive temperatures, a DnaB(ts) mutant carrying the allele dnaB252 has been isolated recently. Its gene product appeared to be required during the initiation of DNA replication (Zyskind and Smith, 1978). This raises the possibility that the dnaB gene product can participate in both DNA chain elongation and DNA initiation at the chromosome origin. Further studies would be necessary to confirm and clarify this phenomenon. If found to be true, this introduces a new level of complexity to processes in which the DnaB protein can be involved. A purpose of this discussion was to suggest that a study of genes associated with bacterial

plasmids may prove useful in understanding variety and subtlety in dna genes and the dnaB gene in particular.

APPENDIX I

Isolation and Characterization of a Cryptic Plasmid (pCU3) in Some Laboratory Strains of E. coli K-12

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INTRODUCTION

During the study of the effect of suppression or enhancement of the temperature-sensitivity of dnaB(ts) mutations by conjugative R plasmids, it was necessary to analyze the total DNA isolated from DnaB(ts) strains carrying suppressing R plasmids in ethidium bromide-cesium chloride density gradients. As controls, the total DNA from the R⁻ strains were also radioactively labeled and analyzed in similar density gradients. Unexpectedly, this revealed the presence of supercoiled DNA (plasmid DNA) in some of the DnaB(ts) strains (strains E107 and E279) received from other laboratories. Because of this alarming observation, in the main part of the experiments described in this thesis, we used an isogenic set of strains that were constructed and found to be plasmid-free, that is, plasmid-free strains into which each dnaB(ts) mutation had been introduced genetically by P1-transduction (the Q and the WD series of strains). However, we went on to examine the strains carrying the cryptic plasmid (designated as pCU3 in our laboratory). Its properties are described in this Appendix as they are not directly relevant to the subject of dnaB analogs or suppression except that it is clear that pCU3 does not suppress or enhance the temperature-sensitivity of those DnaB(ts) mutants in which it was detected.

MATERIALS AND METHODS

Preparation of plasmid DNA for electron microscopy

An overnight culture in LB broth at 30C was diluted 20-fold into 500 ml of fresh LB broth and regrown with good aeration to exponential phase (approximately 2×10^8 cells/ml). The following lysis procedure is a slight modification of that of Bazaral and Helinski (1968). Cells were washed 2X with TES buffer (0.05 M NaCl, 0.05 M Tris, 0.005 M EDTA, pH 7.8) and resuspended in 25 ml of a 20% sucrose solution in 0.25 M Tris. The suspension was quickly frozen and thawed at room temperature and 5 ml of lysozyme added (5 mg/ml in 0.25 M Tris, pH 7.8). 10 ml of a 0.2 M EDTA solution in water (pH 7.8) was added and the mixture was incubated at 37C for 1 hr. 40 ml of a 2% Sarkosyl solution in 0.05 M Tris plus 1.5 M NaCl (pH 7.8) was added and the mixture was sheared by passing through a syringe (18 gauge needle) until the viscosity was reduced. The mixture was kept in ice for 1 hr and centrifuged at 18,000 rpm for 20 min. This low speed of centrifugation removes chromosomal DNA to the extent of about 95% and is called "cleared lysate" (Clewell and Helinski, 1969). The cleared-lysate (in the supernate) was titrated to pH 12 with NaOH and the solution was neutralized with Tris-HCl. The rationale for this procedure is that by titration to pH 12 and neutralization, linear and open circular DNA are denatured, but supercoiled DNA will remain native. Excess denatured DNA was removed by a bulk nitrocellulose procedure. This involved a centrifugation step at 5,000 rpm for 10 min. The supernate

(approximately 80 ml) was removed and 25 ml of a saturated cesium chloride (CsCl) solution was added and the sample centrifuged at 15,000 rpm at 2°C for 15 hr using a type-30 fixed-angle rotor. The top 25 ml was removed gently and added to 2 ml of an ethidium bromide solution (10 µg/ml in phosphate buffer, pH 8). Solid cesium chloride was added to bring the refractive index to 1.3890-1.3895. The sample was centrifuged at 40,000 rpm at 15°C for 40 hr using a 50 Ti fixed-angle rotor in a Beckman L2-65B ultracentrifuge. After this centrifugation step, two bands could be seen. The lower band containing supercoiled DNA was removed using a syringe and puncturing the side of the centrifuge tube. It was rebanded by repeating the centrifugation and the ethidium bromide then removed by dialyzing against 1 M Tris Buffer (pH 8). Plasmid DNA prepared in this manner could be stored in the dark at 4°C for up to several weeks before using it for electron microscopy (E.M.).

Electron microscopy

Plasmid DNA isolated using the above method was prepared for EM study using the well-known technique (aqueous technique) of Kleinschmidt (1968) with a slight modification. The hyperphase (spreading solution) contained 0.5 µg/ml DNA, 0.1 mg/ml cytochrome-C (last to be added), 1 mM EDTA (pH 7.5) and 0.25 M ammonium acetate (pH 7.5). 50 µl of a spreading solution was spread using a capillary tube at about 1 cm above the surface of the hypophase and was at once picked up with a grid (copper grid; 200 mesh) previously coated with Parlodion (purified Pyroxylin). The grid was

immediately washed with 0.05 M uranyl acetate (0.05 M uranyl acetate in 50 m mol HCl) for 30 sec at room temperature followed by washing in 2-methyl butane for 20 sec, and was then dried. The grid was then shadowed with platinum at an angle of 7° and 10 cm away from the grid. Negatives bearing photographs of intact open circular molecules were projected at a suitable magnification and the projections traced on paper. The contour lengths of the tracing were measured with a map reader. From these measurements and the magnification factors, the mass of each molecule was calculated using the formula:

$$\frac{\text{contour length (cm)} \times 10^4}{\text{EM magnification} \times \text{projector mag.}} \times 2 \times 10^6 \text{ daltons}$$

About 30 open circular molecules of each observed size class was measured.

RESULTS

Those DnaB(ts) strains that were found to carry plasmid pCU3 (cryptic plasmid) were isolated from a strain called CR34. We called this strain CR34G to distinguish it from strain CR34 originated from other laboratories. Since plasmid pCU3 was observed to be present in strain E107 and E279, it seemed necessary to check for the presence of this plasmid (pCU3) in their parental strain CR34G. Indeed, supercoiled plasmid DNA was also found in strain CR34G.

To further characterize plasmid pCU3, supercoiled DNA from strains CR34G, E279/R64-11 and CO3/R64-11 (no supercoiled DNA was observed to be present in the R⁻ strain of CO3) was isolated and analyzed by electron microscopy as described. The contour lengths of 30 open circular molecules of each size class was determined and it was found that pCU3 is much smaller than plasmid R64-11 and has a mass of $12 \pm 2 \times 10^6$ daltons. The mass of plasmid DNA isolated from strain CO3/R64-11 was $64 \pm 5 \times 10^6$ daltons. When the plasmid DNA was isolated from strain E279/R64-11, it was observed that it had the above two size classes of plasmid DNA and in addition, a third size class with a mass of $75 \pm 5 \times 10^6$ daltons which is approximately the sum of the sizes of pCU3 ($12 \pm 2 \times 10^6$ daltons) and R64-11 ($64 \pm 5 \times 10^6$ daltons). A single selected print of an electron micrograph displaying all 3 size classes of molecules is shown in Fig. Ap I-1.

Since plasmid DNA prepared from strain E279/R64-11 contained 3 different size classes, it was possible to measure the proportion

Figure Ap I-1

An electron-micrograph of DNA molecules from total
plasmid DNA isolated from strain E279/R64-11.

Magnification: 17,000 X

FIGURE Ap I-1

of the 3 classes of supercoiled DNA (pCU3, R64-11 and the combination of pCU3 and R64-11). Such measurement may not however reflect the in vivo proportion of these molecules. For example, during the isolation procedure for plasmid DNA, the smallest molecules of pCU3 may be nicked less frequently so that fewer of them assumed the open circular form. On the other hand, the larger molecules may be nicked more than once and thus became linear. With this reservation in mind, the proportion of the three different size classes was found to be 4:5:5. This suggests that fusion or recombination between plasmids pCU3 and R64-11 is a frequent event within the cells.

In addition to its ability to fuse or recombine with plasmid R64-11, Jan Relf in our laboratory has observed that no antibiotic or chemotherapeutic-resistance markers was associated with pCU3. However, in strains carrying this plasmid (pCU3), there was an increase in sensitivity to mercuric chloride and cobalt chloride.

APPENDIX II

Informational Amber Suppressor Genes Associated with Some R plasmids

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Results.....142-145

INTRODUCTION

Many R plasmids were capable of altering the temperature-sensitivity of different dnaB(ts) mutations (see Table I-3; Chapter I). It was demonstrated that the mode of dnaB(ts) suppression was not the result of the insertion of the suppressing plasmid into the host chromosome (see Discussion to Chapter II) and that in some cases suppression was mediated by interactions between E. coli DnaB(ts) proteins and plasmid-specified Ban proteins (Chapter III). A second possible mechanism of suppression of dnaB(ts) mutations by conjugative R plasmids could be mediated through efficient amber suppressors specified by some of the suppressing R plasmids. Experimental results described in this Appendix indicated that some R plasmids do carry gene(s) that specify informational suppressors. The known informational amber suppressor genes all specified an altered t-RNA that recognizes amber codons on mRNA. The presence of these suppressor genes on some R plasmids will explain the suppression of the amber dnaB(ts) mutation dnaBam266, but will not, however explain the suppression of other missense mutations (dnaB8, dnaB43 and dnaB70). Thus, the fact that some R plasmids have informational amber suppressor genes associated with them, does not exclude the possibility that they also have ban genes associated with them.

Since informational-suppression is not central to this thesis, and may yet be useful information to plasmid biologists, these experiments and their results are presented as an Appendix to the thesis.

MATERIALS AND METHODS

Bacterial strains, Plasmids and Bacteriophages

The bacterial strains and plasmids used were the same as those described in Tables I-1 and I-II. The T4 amber mutants used were T4am B22 and T4am E509.

Media and Chemicals

The composition of NB is described under "Materials and Methods" in Chapter I. T4 top agar contained per liter of distilled water; Bacto tryptone, 13 g; sodium chloride, 8 g; sodium citrate, 2 g; Difco agar, 6.5 g; glucose, 3 g. The pH was adjusted to 7.4 before autoclaving. T4 bottom agar was the same as T4 top agar except that 10 g agar and 1.3 g glucose were used.

Test of the presence of amber suppressors associated with some suppressing R plasmids

(a) Test of the penicillin resistant level in sup⁺ strains carrying R1-bla31 and an additional suppressing plasmid

The plasmid, R1-bla31 (R1-am), carries an amber mutation affecting penicillin resistance (Kennedy and Crowlesmith, 1975). The amber mutation in this plasmid affects β -lactamase activity and the activity of this enzyme is correlated with the degree of penicillin resistance. To test if dnaB(ts) suppression is mediated through informational suppressors specified by some plasmids, plasmids that suppress the mutation dnaBam266 were introduced into the

sup⁺ (su⁻) strain bearing the plasmid R1-bla31. After ascertaining that both plasmids were maintained stably in the strains, penicillin resistance was then compared. This was achieved by plating appropriately diluted exponential cultures on NA plates containing penicillin G in the range of 20 µg/ml to 75 µg/ml.

(b) Test of the ability of some suppressing plasmids to support the growth of two T4 amber mutants

Plasmids that suppressed dnaB(ts) mutations (see Table I-3; Chapter I) were introduced into a sup⁺ strain (strain WD00). The ability of the resulting strains to plaque two T4 amber mutants (T4am B22 and T4am E509) was determined by mixing 0.1 ml of late exponential culture with 0.1 ml of T4 lysate (titer of approximately 10^{10} pfu/ml). This mixture was then incubated at 37 C for 30 min and was added to 3 ml of T4 top agar which was then overlaid onto T4 bottom agar plates.

RESULTS

The ability of some suppressing R plasmids to suppress the amber mutation of the penicillin resistance gene of R1-bla31 is presented in Table Ap II-1. The presence of R1-bla31 in a sup⁺ (su⁻) strain (strain WDOO) resulted in a low level of resistance to penicillin, but the resistance to penicillin was greatly increased when the same plasmid (R1-bla31) was present in a host strain carrying supF. The transfer RNA (t-RNA) suppressor, supF is a more efficient suppressor than either supD or supE (D'Ari et al., 1975; Ogawa, 1975). This was confirmed by testing the penicillin resistance level in a strain carrying R1-bla31 and either one of the three suppressors (supD, supE and supF). The introduction of two plasmids, either R446b or R478 into strain WDOO carrying R1-bla31 resulted in a high level of resistance to penicillin. This indicates that these two plasmids carry efficient suppressors of the amber mutation on R1-bla31. This was confirmed by their ability to suppress two amber mutants of bacteriophage T4 (Table Ap II-2).

The ability of sup⁺ strains carrying suppressing R plasmids to plaque two amber mutants of T4 (T4am B22 and T4am E509) was also tested. The result of such an experiment is shown in Table Ap II-2. Strain WDOO (sup⁺) was unable to plate T4am while WDOO3 (supF) plaqued T4am with a high efficiency (Table Ap II-2). Strain WDOO/R478 also plaqued T4am with a high efficiency. In addition, strain WDOO/R16 was also found to plate T4am mutants with a high

TABLE Ap II-1

Alterations in Penicillin Resistance Conferred by R1-bla31
when either Known Amber Suppressors or Other
Plasmids are also Present in the Strain

Strain	chromoso- mal amber suppressor in the strain	addit- ional plasmid in the strain	Percentage survival in the pre- sence of varying concentrations of Penicillin G added to NA plates			
			20µg/ml	25µg/ml	50µg/ml	75µg/ml
WD00/R1- <u>bla31</u> ^a	nil	nil	3×10^{-3}	3×10^{-4}	5×10^{-5}	1×10^{-6}
WD001/R1- <u>bla31</u>	<u>supD</u>	nil	8×10^{-5}	7×10^{-2}	7×10^{-3}	3×10^{-4}
WD002/R1- <u>bla31</u>	<u>supE</u>	nil	3×10^{-1}	1×10^{-2}	4×10^{-5}	4×10^{-5}
WD003/R1- <u>bla31</u>	<u>SupF</u>	nil	9×10^{-1}	6×10^{-1}	2×10^{-1}	7×10^{-3}
WD00/R1- <u>bla31</u> /RA1	nil	RA1	2×10^{-2}	4×10^{-3}	7×10^{-6}	3×10^{-6}
WD00/R1- <u>bla31</u> /R386	nil	R386	2×10^{-2}	4×10^{-3}	3×10^{-5}	4×10^{-7}
WD00/R1- <u>bla31</u> /R64-11 ^b	nil	R64-11	5×10^{-4}	1×10^{-4}	6×10^{-6}	5×10^{-7}
WD00/R1- <u>bla31</u> /R144-3 ^b	nil	R144-3	9×10^{-3}	4×10^{-3}	2×10^{-5}	1×10^{-6}
WD00/R1- <u>bla31</u> /R446b ^c	nil	R446b	2×10^{-1}	2×10^{-1}	9×10^{-4}	3×10^{-4}
WD00/R1- <u>bla31</u> /R478 ^c	nil	R478	4×10^{-1}	3×10^{-1}	9×10^{-2}	4×10^{-3}

^a The plasmid R1-bla31 (formerly R1-am) has been shown to carry an amber mutation in its penicillin-resistance gene and there is a direct relationship between the degree of penicillin resistance and the amount of β -lactamase produced by the strain (Kennedy and Crowlesmith, 1975). All other plasmids indicated do not carry penicillin resistance gene and are maintained stably with R1-bla31.

^b Similar results were obtained with R64 and R144-3.

^c The presence of amber suppressors in association with these plasmids has been further confirmed by using two different amber mutants of T4 (see Table 2 of Appendix).

TABLE Ap II-2

Efficiency of sup⁺ Strains Carrying a Suppressing
Plasmid to Plaque Amber Mutants of T4

Strain	Chromosomal amber suppressor	R plasmid	Plaquing efficiency of T4am B22 ^a (pfu/ml)
WD00	nil	nil	1.2×10^3
WD003	<u>supF</u>	nil	3.8×10^8
WD00/RA1	nil	RA1	5.4×10^3
WD00/R40a	nil	R40a	2×10^5
WD00/R386	nil	R386	6.5×10^3
WD00/R100-1	nil	R100-1	2×10^2
WD00/R64-11 ^b	nil	R64-11	2×10^3
WD00/R144-3 ^b	nil	R144-3	1.5×10^3
WD00/R621a	nil	R621a	8×10^2
WD00/R471a	nil	R471a	8×10^2
WD00/R446b	nil	R446b	4.4×10^8
WD00/R16	nil	R16	2.5×10^8
WD00/RP4	nil	RP4	1.6×10^3
WD00/R478	nil	R478	5×10^8
WD00/S-a	nil	S-a	1.8×10^3

^a Similar results were obtained with T4am E509

^b Similar results were obtained with R64 and R144

Note: none of the above plasmids showed restriction to T4am B22 or T4am E509. This was confirmed by introducing each plasmid into strain WD003 and the resulting strains were tested for their ability to plaque T4am B22 and T4am E509. All of the above strains plate these two T4 amber mutants with an efficiency similar to that of the R⁻ strain (strain WD003).

efficiency, While WDOO/R40a supported the growth of T4am to a slight extent. The rest of the WDOO/R⁺ strains plaqued T4am mutants with an efficiency similar to that of the R⁻ strains (strain WDOO). These plasmids were inferred not to carry any informational suppressors that would suppress the amber mutations of T4 and R1-bla31.

Although some plasmids are shown to have amber suppressors associated with them, it is emphasized that the five plasmids shown to specify Ban proteins do not specify amber suppressors.

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