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Genetics

Bleomycin-resistance gene derived from the transposon Tn*5* confers selective advantage to *Escherichia coli* K-12

(death rate/phase of decline/fitness/DNA repair)

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ABSTRACT **The plasmid pRAB2 contains a silent operon derived from the transposon Tn*5* and carrying the gene *neo* for neomycin–kanamycin resistance and a truncated *ble* gene (*ble333*) for bleomycin resistance. Spontaneous mutants that express the two resistances provide *Escherichia coli* cells an improved fitness during the phase of decline in the absence of the antibiotics. It is shown that the *ble333* gene product is responsible for this better fitness. These results can explain a previously described selective advantage attributed to the presence of Tn*5*. The improved fitness of bleomycin-resistant bacteria is proposed to relate to DNA repair by the *ble* gene product. The consequences of the presence of an accessory gene improving fitness are discussed in terms of evolutionary stable strategy of a transposon in populations of *E. coli*.**

Bacterial transposable elements are defined as DNA segments that can insert into several sites in a genome (1). A conceptual debate arose in the early 1980s about the biological significance of transposable elements. According to one hypothesis, a mobile genetic element represents "selfish-DNA" or "the ultimate parasite" (2, 3), which is maintained solely by overreplication and can be considered as "a not-too-harmful parasite within its host" (3). An alternative view, based on mathematical models (4), argues that long-term selection would most probably eliminate an accessory DNA maintained only by overreplication. Rather, a mobile element "earns its keep" in the host (5). Hence, an evolutionary role was assigned to some mobile genetic elements to explain their maintenance in bacterial populations in the long term.

In this paper, we report the identification of a gene, the product of which increases the fitness of *Escherichia coli* cells. This fortuitous discovery was made after an attempt to measure mutation rates of plasmid pRAB2 under different physiological conditions. This plasmid, which contains a silent kanamycin-resistance (Km^r) marker (*neo*; cloned from Tn*5*), had been designed to allow easy detection of spontaneous mutations leading to Km^r. Such mutants originated mostly from insertions of insertion sequence (IS) elements and from deletions in the λ*c*I region of the plasmid (6). During the phase of decline, in resting bacterial cultures, the mutants increased in proportion (Km^r/total number of cells) >10-fold (7). We now show that the increase in the proportion of mutants is due to a difference of fitness between *E. coli* containing pRAB2 and those carrying a mutant plasmid. The improved fitness of the host bacteria depends on the expression of another gene located after *neo* on the transposon Tn*5* and coding for bleomycin resistance (Bm^r). The possible involvement of this gene product in DNA repair is discussed as well as evolutionary consequences for the long-term maintenance of the transposon in bacterial genomes.

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MATERIALS AND METHODS

Bacterial Strains. Two strains of *E. coli* K-12 were used in this study: 431 (F^+, *tonA24*, *spoT1*, *metB1*, *gal*^{c}) (8) and JM109 [*recA*, *thi*, *hsdR17*, *supE44*, Δ(*lac-proAB*), (F′, *traD36*, *proAB*, *lacI*^{9}ΔM15)] (9).

Plasmids. The plasmid pRAB2 and some of its Km^r derivatives (Fig. 1) have been described (6). It is composed of the *cI*–P_R region of bacteriophage λ cloned on a vector plasmid pUC7 containing the *bla* gene for ampicillin resistance (Ap^r). It also contains a *Bgl* II/*Pst* I fragment from Tn*5* with the promoterless neomycin–Km^r gene *neo* followed in the same operon by a large portion [291 of 387 base pairs (bp)] of the N-terminal part of the Bm^r gene *ble* (10). Ligation of the truncated *ble* gene to the pUC7 sequence extended the reading frame by 42 bp and resulted in the 333-bp gene *ble333*. The genes *neo* and *ble333* are silent in pRAB2. Spontaneous Km^rBm^r mutants are either affected in the *c*I function so that P_R gets derepressed or are the result of integration in front of *neo* of an IS element with an outreading promoter. Bm^r is also observed (Table 1) in the Km^r mutants described in ref. 6.

Deletion derivatives carrying only one functional *neo* or *ble333* reading frame were constructed as follows (11): The 491-bp deletions in *neo* of pRAB2 and pRAB2-1 were obtained by *Bss*HII/*Eag* I digestion followed by filling-in and ligation, which resulted in pAW550 and pAW551, respectively (Table 1). The 194-bp deletions in *ble333* of pRAB2, pRAB2-3, and pRAB2-32 were created by *Bsu*36I/*Bst*BI digestion, followed by filling-in and ligation resulting in plasmids pAW552, pAW554, and pAW553, respectively (Table 1).

Media and Growth Conditions. Standard growth media were Luria broth (LB) and Luria broth agar (LA). Where appropriate, antibiotic concentrations were the following: Ap, 200 μg·ml^{-1}; Km, 100 μg·ml^{-1}; Bm, 10 μg·ml^{-1} for JM109 and 30 μg·ml^{-1} for 431.

For each batch culture, an Erlenmeyer flask with 40 ml of LB/Ap was inoculated with ≈10^4 cells and gently shaken at 37°C for 250 h. Cultures were always made in triplicate and three independent daily aliquots were collected from each flask. Aliquots were diluted and plated on LA/Ap to give the number of colony-forming units containing the plasmid (CFU·ml^{-1}), on LA/ApKm for the frequency of Km^r mutants and on LA/ApBm for the frequency of Bm^r mutants.

Data Analysis. If $A_{(t)}$ defines the number of viable cells measured as CFU·ml^{-1} in a culture, the death rate (k_A, h^{-1}) is taken as the slope coefficient of the linear regression of $\ln[A_{(t)}]$ against time during the phase of decline. We define the beginning of the phase of decline when the number of bacteria starts to decrease. The reference of cell death during the

Abbreviations: Ap, ampicillin; Bm, bleomycin; Km, kanamycin; EMS, ethyl methanesulfonate; CFU, colony-forming unit(s); superscripts r and s, resistant and sensitive; IS, insertion sequence.

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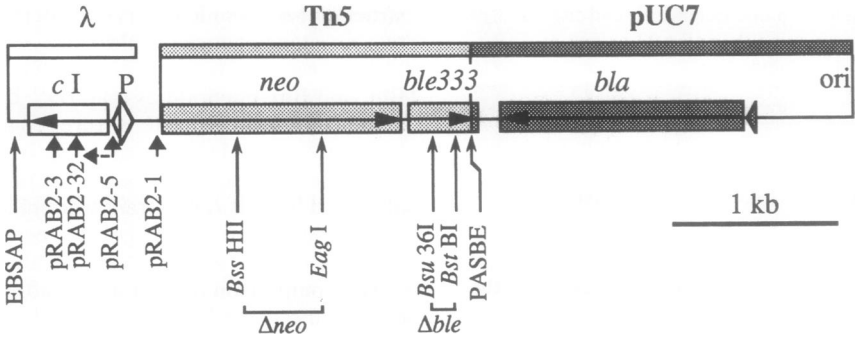

FIG. 1. Genetic map of the plasmid pRAB2 (6). The plasmid is composed of a cartridge cloned in a pUC7 vector (dark stippled box in the top line). The cartridge contains a fragment (open box) from the bacteriophage λ, then ≈350 bp of pBR322 (not marked), and a fragment (light stippled box) from the transposon Tn*5*. Promoters are indicated by triangles, and the directions of the reading frames are shown by arrows in the boxes. *c*I, λ repressor gene; P, P_{RM} and P_R promoters from λ; *neo*, neomycin–Km^r gene; *ble333*, reading frame for a functional product providing Bm^r. This 333-bp sequence consists of the 97 5′-terminal codons of the wild-type *ble* gene fused to 14 codons from pUC7; *bla*, gene for Ap^r. PASBE, polycloning site corresponding to P (*Pst* I), A (*Ava* I), S (*Sal* I), B (*Bam*HI), E (*Eco*RI). The locations of the four Km^{r}Bm^r mutants of pRAB2 listed in Table 1 are indicated, as well as the locations of the two fragments deleted (Δ*neo* and Δ*ble*) in the constructs pAW550 to -554 (see Table 1). kb, Kilobase.

phase of decline is defined with the bacterial culture harboring the original plasmid pRAB2. The difference between the death rate of the reference (k_R) and another (k_A) gives the selection coefficient: $s = k_R - k_A$. The strain A is favored, neutral, or disfavored according to whether $s > 0$, $s = 0$, or $s < 0$ (12).

Resistance to DNA-Damaging Agents. LA/Ap plates spread with host bacteria carrying either the plasmid pAW551 (Bm^r) or pAW553 [Bm sensitive (Bm^s)] were subjected to UV irradiation (Steril-Lamp, Westinghouse) at a distance of 0.6 m, for 15 s with the *recA*$^-$ strain JM109 and for 70 s with the *recA*$^+$ strain 431. After overnight incubation at 37°C in the dark, the survival frequency was determined by comparison to nonirradiated control plates.

Lesions in the DNA were promoted with ethyl methanesulfonate (EMS), an alkylating agent used at a concentration of 0.5% (vol/vol) with strain JM109 and 3% with strain 431. Overnight cultures of strains carrying either pAW551 (Bm^r) or pAW553 (Bm^s) were treated with EMS for 2 h at 37°C and spread on LA/Ap. After incubation in the dark, the survival frequency was determined by comparison to nontreated cultures.

RESULTS

Measurement of Death Rate During Phase of Decline. The observation pertaining to a differential fitness between cells of strain 431 harboring either pRAB2 or one of its Km^r mutants is illustrated in Fig. 2 with the mutant pRAB2-32. The death rate remains nearly constant between 50 and 250 h of incubation. During this period, the death rate of 431(pRAB2) is 0.0118 h^{-1} while that of 431(pRAB2-32) is 0.0088 h^{-1}. The selection coefficient is thus positive for the mutant ($s = 0.0030$), which indicates that 431(pRAB2-32) has a better fitness during the phase of decline than the reference. Similar selection coefficients are obtained with other Km^r mutants of pRAB2 (Table 2). This suggests that the effect is relatively independent of the nature of the mutation. The fact that, in the mutant pRAB2-1, IS*2* is inserted only 30 bp upstream of the *neo* initiation codon (6) suggests that the effect is due to expressed sequences downstream of this location.

Table 1. Phenotype in *E. coli*, genotype, and source of plasmids used

Plasmid	Phenotype	Relevant genotype	Ref.
pRAB2	Ap^r	(*neo*), (*ble333*)	6
pRAB2-1	Ap^r, Km^r, Bm^r	pRAB2::IS*2*	6
pRAB2-32	Ap^r, Km^r, Bm^r	pRAB2::IS*3*	T. Raabe*
pRAB2-3	Ap^r, Km^r, Bm^r	pRAB2::IS*5*	6
pRAB2-5	Ap^r, Km^r, Bm^r	pRAB2, Δ*c*I	6
pAW550	Ap^r	pRAB2, Δ*neo*, (*ble333*)	This study
pAW551	Ap^r, Bm^r	pRAB2-1, Δ*neo*	This study
pAW552	Ap^r	pRAB2, (*neo*), Δ*ble333*	This study
pAW553	Ap^r, Km^r	pRAB2-32, Δ*ble333*	This study
pAW554	Ap^r, Km^r	pRAB2-3, Δ*ble333*	This study

Genotypes in parentheses indicate that the open reading frame is intact but the gene is not expressed.
*Personal communication.

Explanation for the Increase in the Fraction of Km^r Mutants. The observed plasmid dependence of fitness might either entirely or only partially be the cause of the previously described 10-fold increase in the proportion of Km^r mutants in bacterial cultures resting over a period of 10 days (7). To test the consequences of a differential fitness between 431(pRAB2) and its mutants, we have used a mathematical model. It predicts the fraction of Km^r cells during the phase of decline depending on the death rate related to the Km phenotype. At any period of time, four factors may affect the number of mutants [$m_{(t)}$]: the population size $N_{(t)}$, the mutation rate (μ), the number of mutants that appeared previously

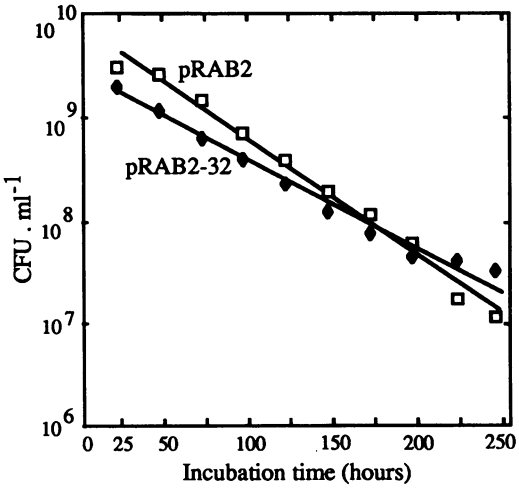

FIG. 2. Survival of *E. coli* K-12 strains 431(pRAB2) and 431(pRAB2-32) during 250 h of incubation. The death rates are 0.0118 and 0.0088 h^{-1}, respectively.

Table 2. Death rate (k), selection coefficient (s), and fitness effect of *E. coli* strain 431 carrying either pRAB2 or one of its Km^r mutants during 250 h of incubation

Plasmid	k, h^{-1}	$s \times 1000$	Fitness effect
pRAB2	0.0118	0	Reference
pRAB2-1	0.0084	+3.4	Favored
pRAB2-32	0.0088	+3.0	Favored
pRAB2-3	0.0092	+2.6	Favored
pRAB2-5	0.0089	+2.9	Favored

Correlation coefficients of the linear regressions used for the calculation of k range between 0.93 and 0.97.

$[m_{(t-1)}]$, and the death rate of mutants (k_A). Therefore, during a constant phase of decline,

$$m(t) = [N(t) \times \mu + m_{(t-1)}] \times [1 - k_A],$$

and

$$N(t) = N_{(t-1)} \times [1 - k_R].$$

In the simulations, two mutation rates are used: (*i*) $\mu = 0$ mutation per cell per h, no mutation occurs; (*ii*) $\mu = 1.3 \times 10^{-9}$ mutation per cell per h, this is the value measured previously with a fluctuation test for strain 431(pRAB2) during its growth phase (7). The death rate of mutants k_A is taken as 0.0088 h^{-1} and k_R is 0.0118 h^{-1}. The origin of the phase of decline ($t = 0$) is arbitrarily taken at 24 h after inoculation when the following experimental values are observed: $N_{(0)} = 3.3 \times 10^9$ CFU·ml^{-1} and $m_{(0)} = 240$ CFU·ml^{-1}.

The expected fraction of mutants $m_{(t)}/N_{(t)}$ for the two assumed values of μ are plotted in Fig. 3 against the time of incubation. The two simulations do not differ significantly and indicate that mutation has a minor effect on the increase of the proportion of mutants. The two simulations lead to values in the same range as the experimental data, which indicates that the fitness effect—i.e., the difference of death rate—is sufficient to explain an observed 10-fold increase in the fraction of mutants. However, the observed curve does not refute the possibility that the mutation rate might show some increase during the phase of decline.

Identification of a Gene Responsible for a Better Fitness of Mutants. The DNA sequence downstream of the IS*2* insertion site of pRAB2-1 contains only two extended open reading frames that could depend on the λ P_R promoter (see Fig. 1). These are the genes *neo* and *ble333*, which are both of Tn*5* origin. Therefore, the expression of one or both of these genes might be responsible for the better fitness of the Km^{r}Bm^r mutants. To explore these possibilities we constructed five plasmids carrying a deletion in either one of the two resistance genes (Table 1).

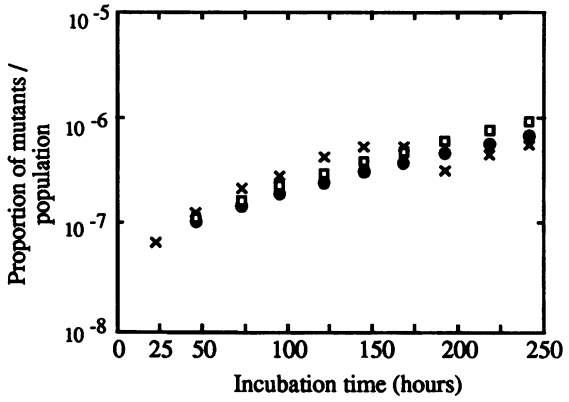

FIG. 3. Proportion of Km^r mutants observed (×) during 250 h of incubation of a culture of 431(pRAB2) and simulations with no new mutation (●) or mutation rate equivalent to that in the growth phase (□). Simulations start at 24 h with the first point taken as observed.

Since *recA* bacteria provide better discrimination between Bm^s and Bm^r phenotypes than *rec*$^+$ bacteria, in which some of the DNA lesions due to Bm are repaired by the SOS system (13), the strain JM109 was used instead of 431 for these experiments. The death rates and the selection coefficients observed for this *recA* strain were higher than for 431 (Table 3) and suggested a linkage between cell death during the phase of decline and DNA repair abilities.

The comparison of selection coefficients among the different strains (Table 3) distinguishes the strains expressing Bm^r (pRAB2-1, -32, -3, -5, and pAW551) with positive values from the others (pAW553 and pAW554) with negative values. Thus, the expression of Bm^r gives a selective advantage. On the contrary, no significant differences can be observed for the expression of Km^r. To illustrate this result, Fig. 4 gives the examples of JM109(pRAB2-32), JM109(pAW551), and JM109(pAW553) that have death rates of 0.0307, 0.0262, and 0.0583 h^{-1}, respectively.

Moreover the strain JM109(pAW553) seems to also have a disadvantage during the growth phase compared with the Bm^r strains because it takes longer to exhaust the same quantity of medium. Thus, in Table 3, its death rate was calculated for only the phase of decline corresponding to the period 48–175 h.

Mutations, Fitness Effect, and Bm^r. In an independent approach to demonstrate the effect of the *ble333* gene product on fitness, we measured the death rate of Km^{r}Bm^r, Km^{s}Bm^r, or Km^{r}Bm^s mutants spontaneously appearing in cultures of JM109(pRAB2), JM109(pAW550), or JM109(pAW552), respectively, and thus forming heterogeneous populations of mutants of different nature (Table 4). Only the mutant populations that could express resistance to Bm showed a better fitness than that of the reference and thus increased their proportion during 175 h of incubation. In contrast, the mutant population derived from pAW552, which did not express Bm^r, did not differ from the reference—i.e., it did not increase in proportion during the phase of decline.

***In Vivo* Function of the Bm^r Gene Product.** During the phase of decline, cells die slowly. For a proportion of the cells, death is likely due to natural DNA damage. The improved fitness of Bm^r cells could then relate to repair of DNA. This hypothesis finds support in the notion that Bm cleaves DNA (14). By its postulated involvement in DNA repair, expression of the *ble333* function might thus not only provide resistance to the drug but it might also provide protection against other sources of DNA damage. As shown in Table 5, this is the case for both the *recA*$^-$ strain JM109 and the *rec*$^+$ strain 431 when treated with EMS, an alkylating agent. EMS survival is 100-fold higher for Bm^r than for Bm^s bacteria. In contrast, no protection effect of the Bm^r phenotype can be

Table 3. Death rate (k), selection coefficient (s), and fitness effect of *E. coli* K-12 strain JM109 carrying either pRAB2, one of its Bm^{r}Km^r mutants, or a deletion derivative during 175 h of incubation

Plasmid	k, h^{-1}	$s \times 1000$	Fitness effect
pRAB2	0.0499	0	Reference
pRAB2-1	0.0303	+19.6	Favored
pRAB2-32	0.0307	+19.2	Favored
pRAB2-3	0.0324	+17.5	Favored
pRAB2-5	0.0305	+19.4	Favored
pAW551	0.0262	+23.7	Favored
pAW553	0.0583*	−8.4*	Disfavored
pAW554	0.0561*	−6.2*	Disfavored

Correlation coefficients of the linear regressions used for the calculation of k range between 0.88 and 0.95.
*Calculations performed only for the period 48–175 h.

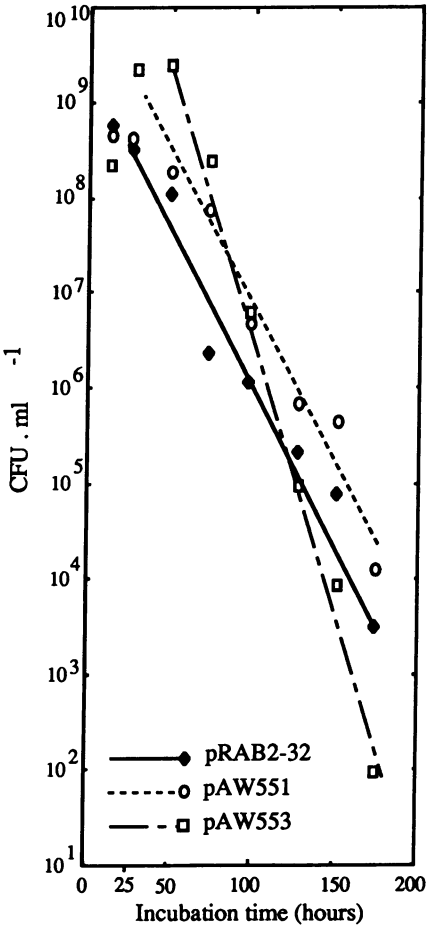

FIG. 4. Survival of the Bm^rKm^r strain JM109(pRAB2-32), the Bm^rKm^s strain JM109(pAW551), and the Bm^sKm^r strain JM109(pAW553) during 175 h of incubation. The death rates of the two Bm^r strains are practically identical (mean death rate, 0.0285 h^{-1}) and differ significantly from that of the Bm^s strain. For strain JM109(pAW553), which enters late into the phase of decline, only values observed during the period 48–175 h were considered for the linear regression.

seen after UV irradiation of either the *recA*$^-$ or the *recA*$^+$ strain. These results indicate that expression of the *ble333* gene provides protection against certain kinds of DNA damage but not against others.

DISCUSSION

The experiments presented reveal that the expression of a Bm^r gene derived from Tn*5* provides a selective advantage to *E. coli* K-12 bacteria during their phase of decline. The advantage is substantial, with selection coefficients of $\approx 3 \times 10^{-3}$ for the *rec*$^+$ strain 431 and 2×10^{-2} for the *recA* strain

Table 4. Death rate (k), selection coefficient (s), and fitness effect of populations of mutants appearing spontaneously in strain JM109 carrying either pRAB2 (Km^rBm^r), pAW550 (Km^sBm^r),or pAW552 (Km^rBm^s) during 175 h of incubation

Plasmid	k, h^{-1}	$s \times 1000$
pRAB2 reference	0.0499	0
pRAB2 mutant	0.0240	+25.9
pAW550 mutant	0.0195	+30.4
pAW552 mutant	0.0518	−1.9

Correlation coefficients of the linear regressions used for the calculation of k range between 0.89 and 0.98. Values for the reference pRAB2 are taken from Table 3.

Table 5. Survival frequency (viable cells/total cells) of Bm^r and Bm^s strains after UV irradiation or treatment with EMS

	Phenotype	Survival	
		To UV	To EMS
recA$^-$ strains			
JM109(pRAB2-32)	Bm^r	2.3×10^{-5}	2.5×10^{-4}
JM109(pAW553)	Bm^s	4.8×10^{-5}	2.6×10^{-6}
recA$^+$ strains			
431(pRAB2-32)	Bm^r	9.0×10^{-4}	1.7×10^{-5}
431(pAW553)	Bm^s	6.6×10^{-4}	1.8×10^{-7}

For JM109, cells were UV-irradiated for 15 s or treated with EMS at a concentration of 0.5%. For 431, treatment was a 60-s irradiation or EMS at 3%.

JM109. During the phase of decline and in the absence of Bm, the positive fitness effect consists of a lower death rate for bacteria expressing Bm^r. A differential cryptic growth on the expense of lysing cells is insufficient to account for the observed effect (M.B., unpublished results).

In 1983 an increase of fitness for cells carrying the complete or a partial transposon Tn*5* on their chromosome was reported (12, 15). These studies established that the advantage was independent of the transposition function and of the Km^r but took its origin in the region between the gene *neo* and the IS*50R* outside end. More precisely, mutant Tn*5-112* (16) had 3 kilobases deleted from the right end of the transposon and did not show the positive fitness effect. It was only 2 years later that the *ble* gene was discovered located in the same region of the transposon (10). We can therefore assume that the findings reported in 1983 and our results are manifestations of the same phenomenon related to the expression of Bm^r.

The glycopeptide Bm is used as an antitumor agent. It induces extensive DNA strand scissions that cause cell death (17). Three types of possible Bm^r might be distinguished: (*i*) mechanisms related to permeability modifications involving genes mostly encoded on the chromosome (18); (*ii*) binding of the Bm^r gene product to Bm and inactivation of the antibiotic (19); (*iii*) involvement in the repair of DNA damage (20, 21). The mechanism of Tn*5* resistance to Bm has not been elucidated *in vivo*. However, our finding that survival to EMS treatment is improved for bacteria expressing the *ble333* gene indicates that this Bm^r gene provides for DNA repair. This repair is ineffective on UV-induced DNA lesions, although the SOS repair system was shown to repair some Bm lesions (13). However, the different components of the DNA repair pathway responsible for the better fitness effect of Bm^r remain to be identified.

One can assume that the Tn*5* *ble* gene product is involved *in vivo* in the repair of naturally occurring DNA lesions independent of the presence of Bm. Thus, our results during the phase of decline can be interpreted by a *ble*-dependent improvement of DNA repair of damages occurring in senescent bacteria. The improved fitness of Tn*5*-bearing strains grown in the chemostat under carbon source limitations (12, 15) could also be explained with the same molecular mechanism by the repair of naturally DNA-damaged cells. This would lead to a decrease of the proportion of dying bacteria and therefore to a higher growth rate than the Tn*5*-free strains. This view is supported by our observation that Bm^r cells seem also to show an advantage during the growth phase.

Although discovered in a plasmid, the positive fitness effect due to the expression of *ble333* provides insight into the role of this gene, in the absence of Bm, on the life style of the transposon Tn*5* in the genome of *E. coli* K-12. The positive fitness effect indicates that this transposon is not selfish-DNA in the sense of ref. 2, but it cooperates with its *E. coli*

host for their common survival and should be considered more as a symbiotic DNA. Our results give an experimental confirmation and they offer a mechanistic explanation to a population dynamics model for the transposon Tn*5* (22), predicting the long-term maintenance of Tn*5* by providing a positive fitness to its bacterial host. Considering the evolutionary stable strategy of Tn*5*, we present here an adaptive mechanism that produces a means for a transposon to increase its frequency in the host population without transposition and, thus, to be able to balance the genetic load due to lethal transpositions (see ref. 23 for discussion).

We are aware that the description of a particular case in which a composite transposon carries a gene whose product provides to the host a net advantage cannot be used as a generally valid argument against the hypotheses that transposable elements and in particular IS elements are parasitic DNA. Rather, we believe that such arguments are found in the fact that transposable elements, by their DNA rearrangement activities, contribute to the steady generation of spontaneous mutations representing an essential substrate for biological evolution (24). Such advantage would not be a short-term adaptation of fitted individuals but more a mutator effect at the population level leading to a provision of polymorphisms affecting the entire genome. This is possibly also the way by which the *ble* gene originally became associated with IS*50* to form Tn*5* in a fortuitous DNA rearrangement.

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