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Evolution

Mutation, recombination, and incipient speciation of bacteria in the laboratory

MARIN VULIĆ*†, RICHARD E. LENSKI‡, AND MIROSLAV RADMAN*§

*Laboratoire de Mutagenèse, Institut Jacques Monod, 2 place Jussieu, 75251 Paris Cedex 05, France; and ‡Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824

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ABSTRACT **Mutations in the DNA mismatch repair system increase mutation and recombination. They may thereby promote the genetic divergence that underlies speciation, after which the reacquisition of a functional repair system may sustain that divergence by creating a barrier to recombination. We tested several lines of *Escherichia coli*, derived from a common ancestor and evolved for 20,000 generations, for their recombination ability. Some lines, but not others, had become mismatch repair-defective mutators during experimental evolution, providing different opportunities for DNA sequence divergence. We knocked out the repair system in lines that had retained this function, and we restored function to those lines that had become defective. We then estimated recombination rates in various crosses between these repair-deficient and -proficient strains. The effect of the mismatch repair system on recombination was greatest in those lines that had evolved nonfunctional repair, indicating they had undergone more sequence divergence and, consequently, were more sensitive to the recombination-inhibiting effect of a functional repair system. These results demonstrate the establishment of an incipient genetic barrier between formerly identical lines, and they support a model in which the mismatch repair system can influence speciation dynamics through its simultaneous effects on mutation and recombination.**

The processes leading to the origin of new species have long been of interest and often a source of debate, e.g., whether ecological barriers to gene flow are essential for populations to diverge into distinct species (1–3). The biological species concept emphasizes the roles of sex and recombination in maintaining evolutionary cohesion (1, 3). This concept is often problematic for organisms, such as bacteria, that do not reproduce sexually. Indeed, some may question the validity of bacterial species given their lack of regularized sex and recombination. Nonetheless, bacteria do undergo genetic exchange via plasmid-mediated conjugation, virus-mediated transduction, and (in some groups) transformation (4, 5). Therefore, the potential for recombination may be one useful metric, among others, for delimiting bacterial species, even if it is not sufficient in all cases (6–8).

Recent attention has focused on the genetic basis of species differences in both eukaryotes (9, 10) and bacteria (11). To date, this work has been largely statistical and has not considered the special importance of genetic factors that can modulate recombination. In this study, we test the role of one such class of genetic factors in promoting speciation in bacteria. In particular, we examine the effect of the methyl-directed mismatch repair (MMR) pathway, in which genetic defects simultaneously increase mutation and recombination rates (12–16). Because MMR influences the rate of mutation as well as recombination, this pathway may be important with respect to speciation - defined broadly as the process of genetic divergence of populations - whether or not one favors a species definition that is based on recombination potential. For example, mathematical models indicate that the low rates of recombination thought to be typical of bacteria may be unable to prevent population divergence in certain circumstances (8). Even in that case, the rate of genetic divergence will be influenced by MMR through its effect on mutation rate even if recombination is inconsequential.

In addition to their possible relevance to speciation, defective repair genes may sometime promote more rapid adaptive evolution in bacteria (17–19). Such rapid evolution can be a serious public health problem as new bacterial pathogens emerge and both old and new pathogens evolve resistance to antibiotics (21–22).

MATERIALS AND METHODS

Bacteria. The strain derivations are shown schematically in Fig. 1. Four populations of *Escherichia coli* B were propagated for 20,000 generations under culture conditions described previously (23). Two of the populations, A−2 and A+3, evolved defects in methyl-directed MMR (24) at generations 2,400 and 3,200, respectively, whereas the other two, A−1 and A+2, retained functional MMR systems throughout 20,000 generations. Donors (MVTP72, MVT63, MVT75, MVT135, MVT145) were constructed by introducing F′ from K−12 strain SS14 (carrying F42*finP301lacI42*::Tn*10lacZ*$^+$ episome) into *ara*$^+$ *rif*^{r} derivatives of the ancestral and 20,000-generation-evolved clones. During construction of recipients, *nal*^{r} genotypes were derived from the *ara*$^-$ ancestral and evolved clones, and they were made MMR$^-$ (if necessary) by introducing the *mutL218*::Tn*10* allele (25) by using P1 transduction. These constructs were then converted into isogenic recipient pairs, MMR$^+$ (MVBP51, MVB63, MVB75) and MMR$^-$ (MVBP52, MVB64, MVB76), by transforming them with either plasmids pBA40 and pMQ339 overproducing wild-type MutS and MutL respectively (26), or with corresponding vectors lacking *mut* gene inserts.

Crosses. The base medium was M63 plus 30 μg/ml thiamin for both liquid and solid media. Exponentially growing cells were harvested from liquid M63 supplemented with 0.4% glucose and appropriate antibiotics at ≈3 × 10^8 cells per ml, washed, mixed at a ratio of ≈1:2 F′/F$^-$, put on a 0.45-μm pore size filter (Schleicher & Schuell), and incubated on prewarmed M63 agar plates supplemented with 0.4% glucose. After 60

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Abbreviation: MMR, mismatch repair.
†To whom reprint requests should be addressed at present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115. e-mail: mvulic@hms.harvard.edu.
§Present address: Faculté de Médicine Necker–Enfants Malades INSERM E9916, Université de Paris-V, 156 rue de Vaugirard, 75730 Paris Cedex 15, France.

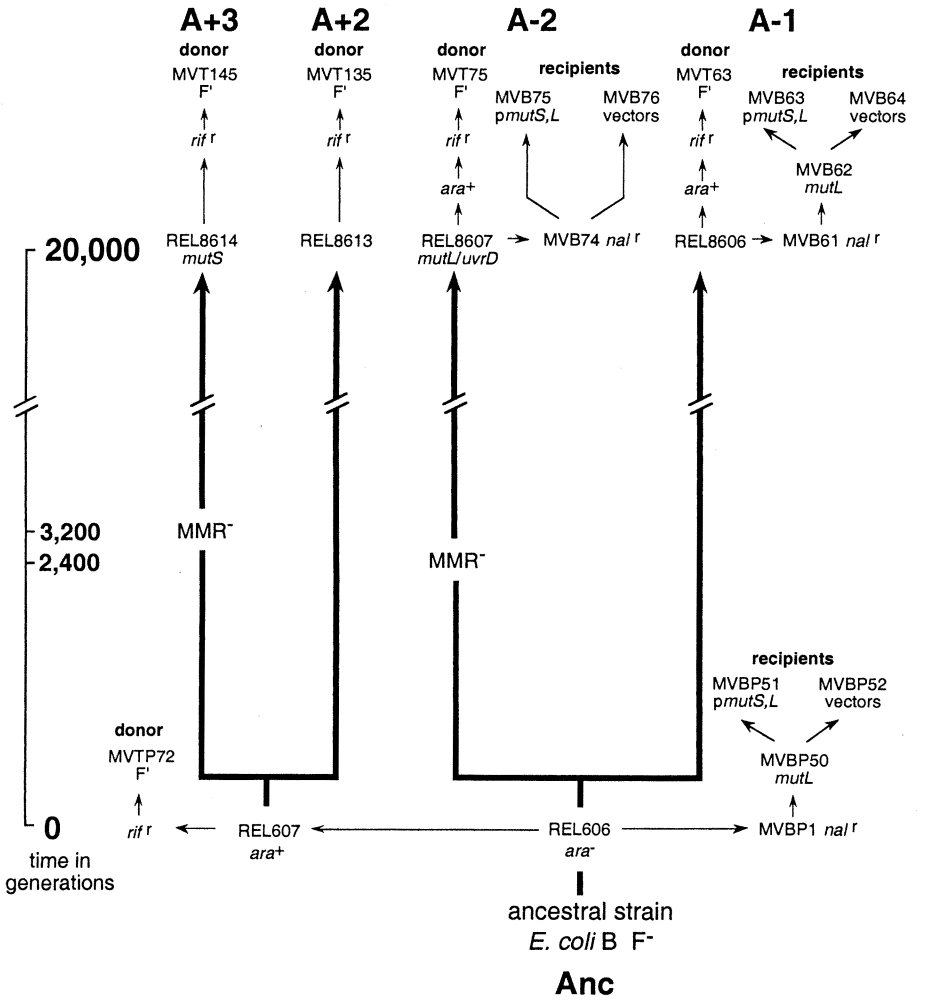

FIG. 1. Strain derivations. Lines A−1, A−2, A+2, and A+3 were derived from the ancestral strain by serial propagation for 20,000 generations (thick arrows). Mutator genotypes defective in methyl-directed MMR (MMR$^-$) overtook lines A−2 and A+3 at the generations indicated, whereas lines A−1 and A+2 remained MMR$^+$ throughout. Thin arrows indicate modifications of ancestral or derived clones by spontaneous mutation (*ara*$^+$, *nal*^{r}, and *rif*^{r}), P1 transduction (*mutL*), plasmid electroporation (p*mutS, L* and vectors), and conjugation (F′).

min at 37°C, conjugants were resuspended in 10 mM $MgSO_4$ and separated by swirling with a Vortex mixer. Exconjugants were then spread on M63 agar plates supplemented with 0.4% arabinose (to counterselect recipients) and 30 μg/ml nalidixic acid (to counterselect donors). Antibiotics assuring plasmid maintenance were included in the medium. Recombinants were scored after 60 hr at 37°C. Recombination rates are expressed per donor, and they were calculated after subtracting unmated *ara*$^+$ revertants.

RESULTS AND DISCUSSION

A long-term study (23) of *E. coli* populations adapting to a new environment provides a unique opportunity to examine the consequence of mutations that disrupt MMR for genetic divergence. The evolving populations were founded from a single asexual clone, and mutation was their only source of genetic variability. They were propagated for some 20,000 generations in a simple, defined environment. The ancestral strain, and most derived lines, have functional MMR pathways, but some lines spontaneously evolved mutator phenotypes caused by defects in MMR (24). In this study, we examine two lines (designated A−1 and A+2) that retained functional MMR throughout the 20,000 generations of experimental evolution, two other lines (designated A−2 and A+3) that became defective for methyl-directed MMR around generation 3,000 and remained mutators throughout the subsequent 17,000 generations, and their common ancestor (designated Anc). Fig. 1 provides an overview of this evolutionary history as well as the derivation of all the genotypes used in our experiments.

From each of the ancestral and derived lines, we constructed donor genotypes that had useful markers for measuring recombination rates in conjugative mating experiments (Fig. 2). We also constructed pairs of genotypes for use as recipients that had functional (MMR$^+$) and nonfunctional (MMR$^-$) MMR systems, but which were otherwise isogenic. We made the following four predictions. (*i*) When a line is crossed to itself, the rate of recombination should be unaffected by the functionality of MMR. This prediction is based on the fact that MMR impedes recombination as a consequence of sequence divergence between donor and recipient, and there is no divergence in self-crosses. This prediction is independent of a line's evolutionary history (ancestral, derived nonmutator, or derived mutator). (*ii*) When the two independently derived nonmutator lines are crossed, the effect of MMR on the rate of recombination should be imperceptible. This reflects the fact that sequence divergence during 20,000 generations in nonmutator populations should be very small. Assuming a simple model of neutral divergence for 20,000 generations and a mutation rate of 5×10^{-10} per base pair per generation (27), one expects two independently derived lines to differ in their

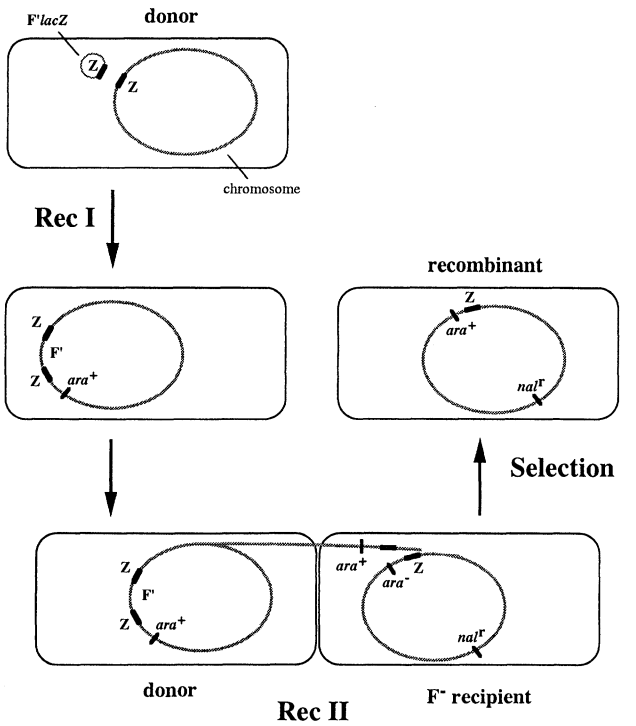

FIG. 2. The scheme of genetic recombination experiments. The net recombination rate reflects two homologous recombination events: Rec I, integration of F′ plasmid into bacterial chromosome via recombination between *lacZ* sequences found within both replicons, which produces an Hfr (*H*igh *f*requency of *r*ecombination) donor; and Rec II, subsequent gene exchange between the incoming Hfr DNA (mobilized by F′ conjugative functions) and the F⁻-recipient's chromosome. If the recipient has a functional MMR system, then the second event is inhibited by sequence divergence between the donor and recipient (12–14, 16).

DNA sequences by only 0.002%, which is probably too small to detect from the effect of MMR on the recombination rate (16). (*iii*) When the two independently derived mutator lines are crossed, the effect of MMR on the recombination rate should be much greater, as a consequence of the ≈100-fold higher mutation rate that prevailed during most of their evolutionary divergence. Given the same assumptions as above, except using a 100-fold higher base pair mutation rate, one expects the mutator lines to have diverged by about 0.17%. Such divergence might be detected by the inhibitory effect of MMR on the recombination rate (16). (*iv*) Finally, when each of the derived mutator lines is crossed with the ancestral strain, the inhibitory effect of the MMR system on recombination should be about half that observed when the two mutator lines are crossed to one another. This prediction reflects the fact that the mutator lines should have about twice the genetic distance relative to one another as to their common ancestor.

We now present the results of experiments to test these predictions. Before doing so, we emphasize an important aspect of the experimental design: all recombination rates were measured in a strictly paired fashion, such that crosses between a donor and the MMR⁺ version of a given recipient strain were performed simultaneously with crosses between that same donor and the isogenic MMR⁻ version of the recipient. This design has the important benefit of taking into account any temporal fluctuations in assay conditions as well as any variation among strains in their capacity for conjugation.

Fig. 3 summarizes the results, in which the data are shown as the log-transformed ratio of recombination rates for otherwise isogenic MMR⁻ and MMR⁺ recipients, for various pairs of donors and recipients that differ in their opportunity for sequence divergence. The ratio of recombination rates

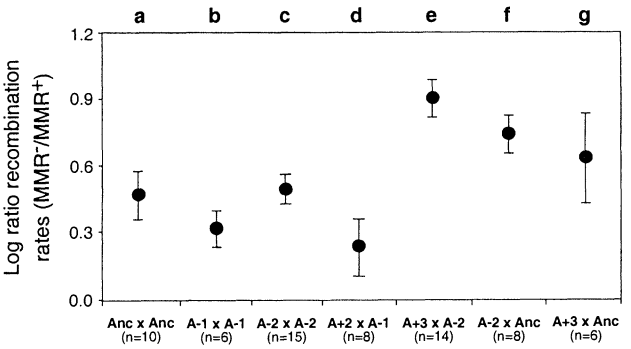

FIG. 3. Effect of MMR on recombination rates for different types of crosses. The *y* axis shows the log-transformed ratio of recombination rates measured for isogenic MMR⁻ and MMR⁺ recipients during paired crosses with the same donor genotype. Higher values of the log-transformed recombination ratio indicate stronger inhibition of genetic recombination caused by functional MMR in recipient. Values are therefore expected to increase as a function of DNA sequence divergence between donor and recipient. (*a–c*) Three different self-crosses. (*d*) A cross between two lines that diverged for 20,000 generations as nonmutators. (*e*) A cross between two lines that diverged as mutators for most of 20,000 generations. (*f–g*) Two different crosses between lines that diverged as mutators and their common ancestor. Error bars are standard errors; n = number of replicate assays for each cross, where each replicate assay involves two paired crosses.

measures the extent of the genetic barrier created by the MMR system (16). Higher values of this ratio indicate stronger inhibition of recombination in the presence of functional MMR in the recipient. Stronger inhibition, in turn, implies more sequence divergence between the donor and recipient strains because MMR operates on differences in sequence. Fig. 3 *a–c* show three self-crosses: Anc × Anc (ancestor), A−1 × A−1 (derived nonmutator), and A−2 × A−2 (derived mutator). In each case, the log-transformed ratio is slightly and significantly positive ($P < 0.02$ for all three t tests). It is unclear why these values are positive, because MMR is not expected to influence recombination rate in the absence of sequence divergence, although an effect of the same magnitude has been reported previously (15). In any case, this effect does not vary significantly among the self-crosses (ANOVA: $F_{2,28} = 0.8899$, $P = 0.4220$). Fig. 3*d* shows the cross between two independently derived nonmutator lines, A+2 × A−1. The effect of the recipient's MMR status is no greater than was seen in the self-crosses; in fact, it is slightly smaller than the effects observed for the self-crosses, although not significantly so ($P > 0.05$ for all three t tests). These data indicate that there is not enough sequence divergence between the two nonmutator lines to be detected by this test, consistent with our second prediction.

Fig. 3*e* shows the results of the cross between two independently evolved mutator lines, A+3 × A−2. Confirming our critical third prediction, the ratio of recombination rates in MMR⁻ and MMR⁺ recipients is substantially higher than the ratios observed in the previous crosses, indicating that functional MMR inhibits recombination because of the greater sequence divergence in the mutator lines. The average log-transformed ratio for this cross between the two mutator lines is significantly greater than the corresponding averages for any of the preceding crosses ($P < 0.005$ for all four t tests). Fig. 3 *f–g* show that this effect is not an artifact of some peculiar interaction between these two particular lines. Separate crosses between each of the derived mutator lines and the ancestor, A+3 × Anc and A−2 × Anc, show intermediate effects of MMR on the recombination rate. These data indicate that each mutator line independently diverged from the ancestor to an extent that was sufficient to create a barrier

to recombination in the presence of functional MMR. Finally, Fig. 4 shows there is a highly significant overall effect of inferred DNA sequence divergence on the log-transformed ratio of recombination rates for MMR^- and MMR^+ recipients ($r = 0.9132$, $n = 7$, $P = 0.0041$).

In conclusion, these data support all of our predictions concerning the effects of MMR on recombination rate, with the minor exception that MMR slightly impedes recombination even among self-crosses; this last effect has been observed previously in yeast (15). These findings thus support three key components of a recent model (16, 28) that postulates an important role for MMR in speciation: (*i*) populations that evolve defects in MMR undergo rapid genetic divergence from other populations because of their mutator phenotypes; (*ii*) this accelerated sequence divergence does not impede recombination as long as the MMR system remains defective; but (*iii*) on reacquisition of a functional MMR system, the accumulated sequence divergence presents a genetic barrier to further recombination. Of course, the magnitude of the barrier to recombination in our experiments is much smaller than the barrier between such clearly distinct species as *E. coli* and *Salmonella enterica* (13, 14, 16), which have diverged from a common ancestor for ≈130 million years (29). Nonetheless, our findings indicate that an incipient barrier can evolve rapidly, during only 20,000 generations (less than 10 years under the experimental conditions).

We have demonstrated experimentally the plausibility of this model in which speciation is promoted by mutations that destroy MMR (accelerating sequence divergence) followed by reacquisition of functional MMR (reducing subsequent recombination). There is a high incidence of MMR deficient mutators among natural isolates of *E. coli* and *Salmonella* (20, 21). Ongoing retrospective (phylogenetic) studies, aimed to determine how often functional MMR genes have been reacquired by gene transfer, will be critical to assess the importance of these events in nature.

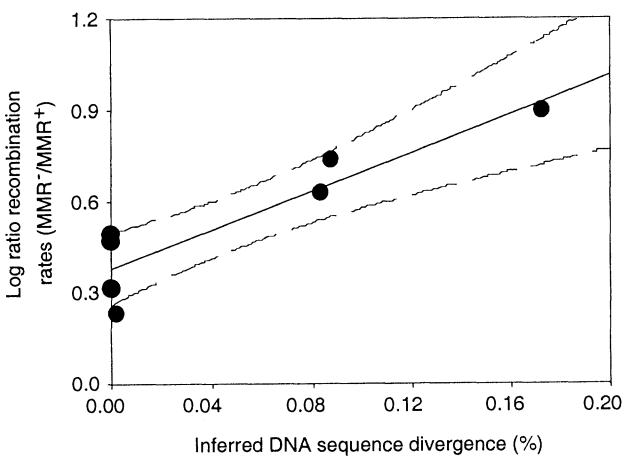

FIG. 4. Inhibition of recombination because of MMR increases with inferred sequence divergence. The inferred divergence between each of the donor–recipient pairs in Fig. 2 was calculated by using a neutral model of evolution, assuming a mutation rate of 5×10^{-10} per base pair per generation (27) for nonmutators and a 100-fold higher rate for mutators. For example, A−2 diverged from the ancestor for 2,400 generations at the nonmutator rate and 17,600 generations at the mutator rate. The inferred divergence is $1 - (1 - 5 \times 10^{-10})^{2400} \times (1 - 5 \times 10^{-8})^{17,600} = 0.00088 = 0.088\%$. The line is the regression of the log-transformed ratio of recombination rates for isogenic MMR^- and MMR^+ recipients crossed with the same donor vs. the inferred sequence divergence between donor and recipient ($r = 0.9132$, $n = 7$, $P = 0.0041$). The dashed curves show 95% confidence interval around the regression.

Note. The highly mosaic stucture of MMR gene sequences of the C collection of natural isolates of *E. coli* (30) may be evidence that the loss and reacquisition of MMR functions have been frequent events in the evolutionary past of *E. coli* (E. Denamur, G. Lecointre, F. Taddei, P. Darlu, C. Acquaviva, C. Sayada, J. Elion, M.R., and I. Matic, unpublished work).

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