accordance with previous reports (*2*, *5*), basal extracellular DA concentrations in the DAT-KO mice were higher than those in the wild-type controls: Concentrations of DA in dialysates were 57.3 ± 13.4 fmol/20 μl in the wild-type mice (n = 12) and 354.5 ± 45.9 fmol/20 μl in the DAT-KO mice (n = 15). The data are presented as means and SEMs of the percentage of change from baseline (100%) from a mean of three samples from each mouse before exposure to the novel environment or before drug or saline administration.

16. All assessments of learning and memory processes were conducted in an eight-arm radial maze [E. D. Levin *et al.*, *Environ. Health Perspect.* **105**, 1320 (1997)]. The mice were food-deprived for about 5 hours before testing, and they were tested daily for 21 sessions with a win-shift paradigm. Before a given session, each of the eight arms was baited with slightly less than one-eighth of a piece of Fruit Loops breakfast cereal. At the start of each session, the mouse was placed into a cylinder in the center of the maze. After 10 s, the cylinder was removed and the mouse had full access to all arms of the maze. Only one entry in each arm was reinforced. The session continued until the mouse had entered all eight arms or 300 s had elapsed. Performance was assessed in terms of the numbers of arms entered (out of eight) before making an error (repeating an entry); these data were expressed as the entries to repeat. In addition, the numbers of perseverative errors were calculated. These errors refer to the tendency of a mouse to leave one arm and enter the arm where it had just been previously. Activity in the maze was also measured and expressed in terms of latency (total time in the maze divided by the total number of entries).
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Unresponsiveness to Cannabinoids and Reduced Addictive Effects of Opiates in CB_1 Receptor Knockout Mice

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The function of the central cannabinoid receptor (CB_1) was investigated by invalidating its gene. Mutant mice did not respond to cannabinoid drugs, demonstrating the exclusive role of the CB_1 receptor in mediating analgesia, reinforcement, hypothermia, hypolocomotion, and hypotension. The acute effects of opiates were unaffected, but the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced. These observations suggest that the CB_1 receptor is involved in the motivational properties of opiates and in the development of physical dependence and extend the concept of an interconnected role of CB_1 and opiate receptors in the brain areas mediating addictive behavior.

Marijuana and other derivatives of *Cannabis sativa* have been used for centuries for their therapeutic and mood-altering properties and are the most widely used recreational drugs today (*1*). The active compounds of *Cannabis*, including Δ^9-tetrahydrocannabinol (Δ^9-THC), as well as the endogenous cannabinoid anandamide, act through two G protein–coupled receptor subtypes. The CB_1 receptor is abundant in the central and peripheral nervous systems but is also expressed in several peripheral organs, whereas CB_2 receptor expression is essentially restricted to lymphoid organs (*2*). We investigated the in vivo function of the CB_1 receptor by invalidating its gene in a mouse model (*3*). Northern (RNA) blotting demonstrated the absence of CB_1 transcripts in brain and testis from knockout ($CB_1^{-/-}$) mice, and binding assays confirmed the absence of binding sites for cannabinoid ligands (*4*). Histology of brain and other organs, body weight monitored over a 6-month period, and blood ionogram and cell count appeared to be unaffected by CB_1 gene inactivation.

The consequences of CB_1 receptor inactivation on spontaneous behavior were analyzed. A moderate increase in locomotor activity (*5*) was observed in $CB_1^{-/-}$ mice when

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newly exposed to the arena (119% of controls; $P < 0.001$, unpaired two-tailed Student's t test, $n = 50$), but not after an habituation period. Increased exploratory behavior was also found under the more stressful conditions of an open field ($P < 0.01$, t test, $n = 15$) (*5*) and in the spontaneous alternation test (total number of visits to the arms: $CB_1^{+/+}$, 47.2 ± 1.6; $CB_1^{-/-}$, 58.9 ± 2.2; $P < 0.01$, t test, $n = 15$) (*6*). Both groups of animals exhibited a rapid habituation to the open-field test (*5*). However, the time spent in exploring unknown objects placed in the field was significantly increased for the mutant mice ($CB_1^{+/+}$, 0.66 ± 0.3 s; $CB_1^{-/-}$, 5.33 ± 1.5 s; $P < 0.01$, t test , $n = 15$). Furthermore, a decrease in spontaneous alternation (*6*) was observed for the mutant mice in the Y maze ($CB_1^{+/+}$, 61.4 ± 1.8%; $CB_1^{-/-}$, 53.7 ± 1.9%; $P < 0.01$, t test, $n = 15$). In the elevated plus maze (*5*), the number of entries and time spent in the open arms were unaffected. Taken together, the data suggest that $CB_1^{-/-}$ mice present a mild impairment in the adaptation to new environment that could be related to changes in short-term memory or attention (or both).

The spontaneous nociceptive threshold (*7*) of wild-type and mutant naive mice was similar [not significant (NS), t test] in the hot-plate (jumping behavior: $CB_1^{+/+}$, 52.0 ± 3.8 s; $CB_1^{-/-}$, 46.6 ± 4.5 s; $n = 10$), tail-immersion ($CB_1^{+/+}$, 0.97 ± 0.08 s; $CB_1^{-/-}$, 1.04 ± 0.06 s; $n = 10$), writhing ($CB_1^{+/+}$, 35.2 ± 1.9; $CB_1^{-/-}$, 34.6 ± 2.0; $n = 10$), and tail-pressure tests ($CB_1^{+/+}$, 7.0 ± 0.2 s; $CB_1^{-/-}$, 7.2 ± 0.2 s; $n = 20$). These observations suggest that the endogenous activation of the CB_1 receptor is not crucial for the control of pain or that other endogenous systems might compensate for the absence of this receptor (or both).

The role of the CB_1 receptor in the central effects of cannabinoids was investigated by measuring the response of $CB_1^{+/+}$ and $CB_1^{-/-}$ mice to Δ^9-THC in different assays (Fig. 1). The antinociceptive properties of Δ^9-THC were not observed for mutant mice in the hot-plate test and were strongly reduced in the tail-immersion test, in which a slight antinociceptive effect was observed for the highest dose (Fig. 1, A and B), possibly in line with the recent demonstration that CB_2 receptors may regulate pain initiation at sites of tissue injury (*8*). Other classical effects of Δ^9-THC, namely, the reduction of horizontal locomotor activity (*5*) and the decrease of rectal temperature, were observed in wild-type animals but not in mutant mice (Fig. 1, C and D). In an intravenous self-administration model (*9*), WIN55,212-2 was not self-administered by $CB_1^{-/-}$ mice, in contrast to wild-type animals (Fig. 1E). Dependence induced by Δ^9-THC administration was also investigated in mutant mice (*10*). The selective CB_1 receptor antagonist SR141,716A precipitated behavioral manifestations of abstinence in wild-type mice but not in mutant mice given long-term treatment with Δ^9-THC (Fig. 1F). These results demonstrate that the main pharmacological responses to Δ^9-THC, as well as the addictive properties of cannabinoids, are indeed mediated mostly, if not exclusively, by the CB_1 receptor.

Cannabinoids have been reported to elicit hypotension and bradycardia through peripheral CB_1 receptors (*11*). Basal blood pressure and heart rate were measured in conscious mice (*12*) but were not significantly modified, suggesting that endogenous cannabinoids do not exert a tonic control on these parameters or that other systems may compensate for the absence of the CB_1 receptor. Both anandamide and WIN55,212-2 promoted a sustained decrease in blood pressure and heart rate in $CB_1^{+/+}$ mice, with a biphasic response to anandamide (Fig. 2), in agreement with previous reports (*11*). No significant hypotensive effect of either drug was

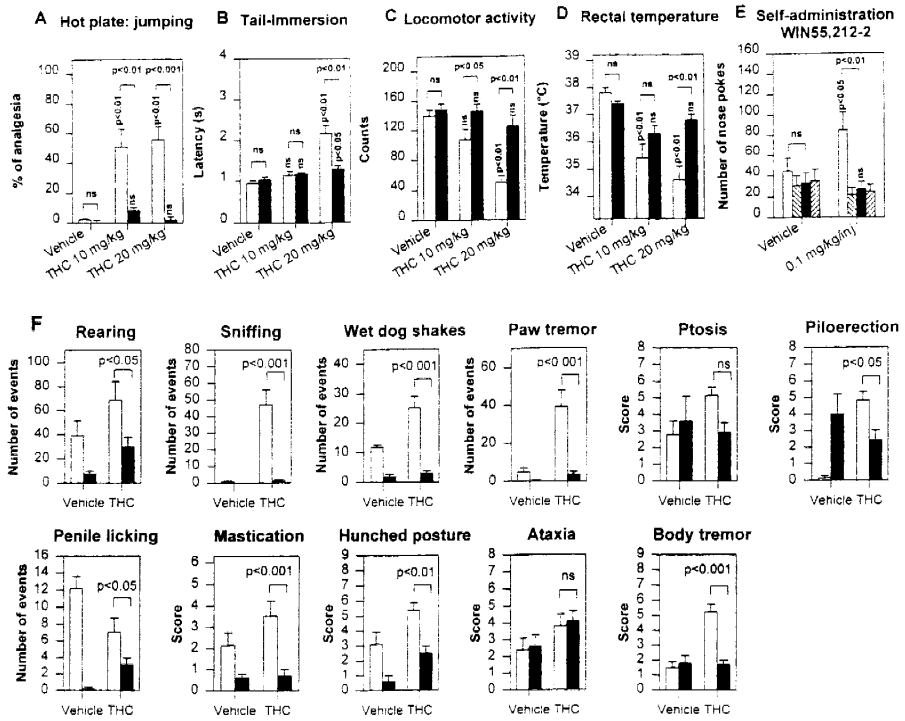

Fig. 1. Central effects of cannabinoids on $CB_1^{+/+}$ (□, ▨) and $CB_1^{-/-}$ (■, ▨) mice. For (A) to (D), an intraperitoneal injection of Δ^9-THC (or vehicle alone) was made 20 min before measurements. (**A**) Latency for escape jumping in the hot-plate test ($n = 10$). (**B**) Latency for tail withdrawal in the tail-immersion test ($n = 10$). (**C**) Spontaneous activity in locomotor activity boxes (number of photocell counts within 10 min; $n = 10$). (**D**) Rectal temperature ($n = 10$). (**E**) Self-administration of WIN55,212-2 (*9*). Injection (inj) of agonist or vehicle to active (□, ■) and passive (▨, ▨) mice was coupled to the nose-poke behavior of the active mouse ($n = 8$ for WIN55,212-2 and 4 for vehicle). (**F**) Signs reflecting Δ^9THC withdrawal (*10*) were monitored ($n = 5$ to 15). The statistical significance [t test for (A) to (D) and (F) and Neuman-Keuls test for (E)] was measured between genotypes and against vehicle for drug-treated groups. Error bars: SEM.

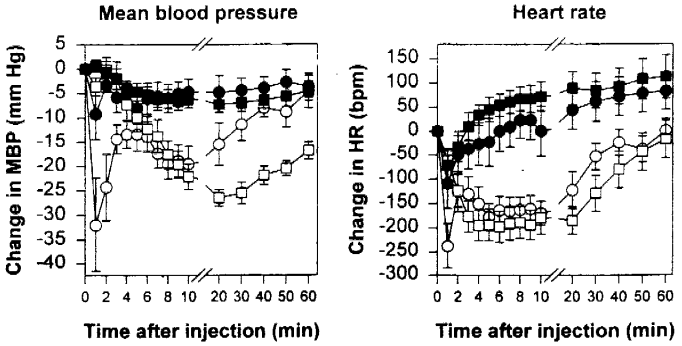

Fig. 2. Cardiovascular effects of cannabinoids on $CB_1^{+/+}$ (○, □) and $CB_1^{-/-}$ (●, ■) mice (*12*). Mean blood pressure (MBP) and heart rate were monitored for 60 min after administration of WIN55,212-2 (0.25 mg/kg; □, ■), anandamide (2 mg/kg; ○, ●), or vehicle (*15*). The transient drop in heart rate after injection in $CB_1^{-/-}$ mice was also observed after vehicle injection only. $n = 9$ to 11 for each group. Error bars: SEM. bpm, beats per minute.

observed after their administration to $CB_1^{-/-}$ mice, demonstrating that the CB_1 receptor is solely responsible for the cardiovascular effects of cannabinoids, including the two components of the response to anandamide.

An interaction between the opioid and cannabinoid systems has been proposed for the control of nociceptive responses (*13*). Opiate antagonists such as naloxone have been reported to inhibit cannabinoid agonist–induced dopamine release in the nucleus accumbens (*14*). Therefore, morphine-induced antinociception and hypothermia, as well as its reinforcing properties and the development of tolerance and physical dependence, were investigated in mutant mice. The antinociceptive effects of morphine in the tail-immersion (*15*) and the hot-plate (Fig. 3A) tests (*7*), as well as its hypothermic effects, were not modified in $CB_1^{-/-}$ mice. Furthermore, long-term morphine treatment (*16*) induced the development of tolerance to morphine antinociceptive effects in the hot-plate (Fig. 3A) and tail-immersion (*15*) tests in both genotypes. In an intravenous self-administration model (*9*, *17*), the number of nose pokes leading to morphine administration was much lower for $CB_1^{-/-}$ mice as compared with $CB_1^{+/+}$ mice (Fig. 3B), suggesting a reduction of the reinforcing effects of the drug. The behavioral expression of naloxone-precipitated morphine withdrawal (*18*), shown to be critically dependent on the μ-opioid receptor (*19*), was also significantly decreased (seven of nine signs evaluated) in mutant mice (Fig. 4), suggesting that CB_1 receptors are required for the development of physical dependence or to obtain a complete manifestation of the somatic signs of opiate withdrawal. These findings are particularly important when one takes into account the proposed interaction between cannabinoids and opioid dependence (*14*, *20*), which could influence the establishment of opiate addiction. Interestingly, our results show a dissociation between the development of opiate tolerance (unchanged) and dependence (decreased) in mutant mice, confirming that these two processes can be independently developed (*21*). The specific interactions between κ-opioid and cannabinoid receptors (*22*) were examined with the selective κ-opioid agonist U-50,488H (*23*). Antinociceptive responses and hypolocomotion induced by short-term U-50,488H administration were similar in mutant and wild-type mice. However, the dysphoric effects of this κ agonist in the conditioning place aversion paradigm (*24*) were observed in wild-type mice but not in mutants (Fig. 3C). Therefore, CB_1 receptors seem to be involved in the behavioral manifestations of morphine physical dependence and the dysphoric properties of κ agonists but not in the acute effects induced by opioids in antinociception, body temperature, and locomotion. Cannabinoid agonists have been considered as therapeutics for their antiemetic, analgesic, anticonvulsant, and intraocular hypotensive effects (*1*). Long-term CB_1 antagonist administration could also be considered for preventing the development of dependence on opiates and possibly other addictive drugs.

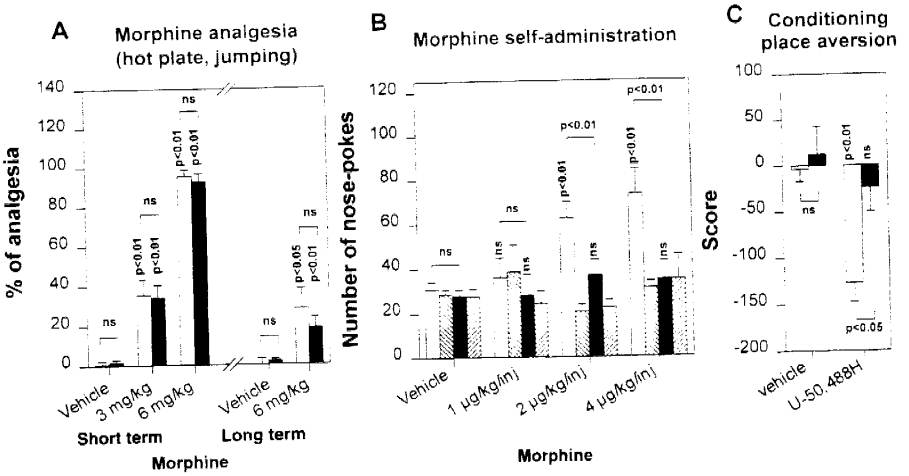

Fig. 3. Central effects of opiates on $CB_1^{+/+}$ (□, ▨) and $CB_1^{-/-}$ mice (■, ▨). (**A**) Hot-plate test (jumping) after injection of morphine (or vehicle) to naïve mice (short term) or mice treated for 6 days with morphine (long term), showing the development of tolerance (n = 8 to 19). Similar effects were obtained for the licking behavior, as well as in the tail-immersion test (*15*). (**B**) Self-administration of morphine (*9*). Injection of morphine or vehicle to active (□, ■) and passive (▨, ▨) mice was controlled by nose pokes of the active mouse, and the number of nose pokes was recorded. n = 6 to 10 per group. (**C**) Place aversion test, with the κ agonist U-50,488H (*24*). n = 10 per group. The statistical significance [t test for (A) and (C) and Newman-Keuls test for (B)] was measured between genotypes and against vehicle for drug-treated groups. Error bars: SEM.

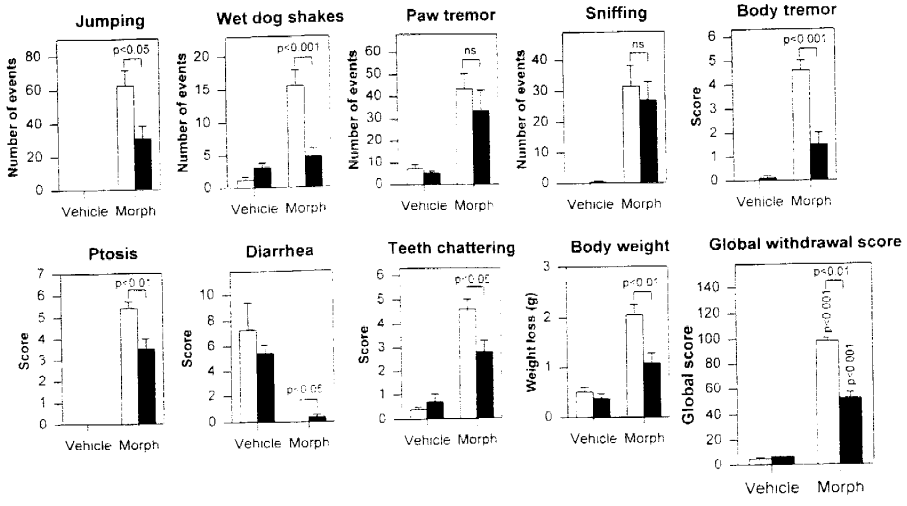

Fig. 4. Morphine withdrawal syndrome on $CB_1^{+/+}$ (□) and $CB_1^{-/-}$ mice (■). Signs reflecting withdrawal were monitored after the long-term administration of morphine (Morph) followed by naloxone injection (*18*). Animals were observed for 30 min and scored. n = 9 to 10 per group. The t test was used. Error bars: SEM.

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3. The CB_1 gene was cloned from a 129/Sv mouse genomic library, and the single coding exon was mapped and sequenced (EMBL/GenBank Y18374). A PGK-Neo cassette was inserted between Avr II and Sfi I sites located 1073 base pairs apart, replacing the first 233 codons of the gene. Homologous recombination in R1 cells and aggregation with CD1 eight–cell stage embryos were performed as described [C. Ledent *et al.*, *Nature* **388**, 674 (1997)]. A recombinant line was used to generate chimeras allowing germ line transmission of the mutant gene. Heterozygous mice were bred for five generations on a CD1 background before generating the $CB_1^{+/+}$ and $CB_1^{-/-}$ mice used in this study.
4. [^{3}H]WIN55,212-2 and [^{3}H]SR141,716A binding as-

says were performed as described [J. E. Kuster, J. I. Stevenson, S. J. Ward, T. E. D'Ambra, D. A. Haycock, *J. Pharmacol. Exp. Ther.* **264**, 1352 (1993)]. Using forebrain membranes from $CB_1^{+/+}$ mice, we found a dissociation constant (K_d) of 0.73 ± 0.12 nM and a maximum binding capacity (B_{max}) of 1.17 ± 0.16 pmol of [^{3}H]WIN55,212-2 per milligram of protein for [^{3}H]WIN55,212-2 and a K_d of 0.74 ± 0.11 nM and a B_{max} of 1.07 ± 0.04 pmol of [^{3}H]SR141,716A per milligram of protein for [^{3}H]SR141,716A. No binding was detected on forebrain or cerebellar membranes from $CB_1^{-/-}$ mice.
5. Animals were housed at 21° ± 1°C with free access to food and water. Experiments were conducted in accordance with local ethical guidelines. The measurement of locomotor activity, and the open-field and elevated plus maze tests were performed as described (*24*). Mice were exposed to the open field for three consecutive days. The number of squares crossed was as follows: wild type: 170 ± 12, 127 ± 10, and 91 ± 11 for first, second, and third days, respectively; knockout: 256 ± 20 ($P < 0.01$), 158 ± 15 (NS), and 120 ± 16 (NS) for first, second, and third days, respectively (t test, n = 15 per group).
6. The spontaneous alternation test was conducted as described (*24*). The percentage of alternation was measured as the number of times the animal visited consecutively all three arms, divided by the total number of visits during a 10-min period.
7. Nociceptive thresholds were monitored by applying thermal (tail-immersion and hot-plate tests), mechanical (tail-pressure test), or chemical stimuli (writhing test) (*24*). For the tail-immersion test, mice were maintained in a cylinder, and their tail was immersed in water at 50°C; latency to tail withdrawal was recorded. In the hot-plate test, mice were placed on a surface heated to 50°C, and the latencies for licking their paws and jumping were recorded. For the tail-pressure test, increasing pressure (tip diameter: 1 mm) was applied to the tails of the mice until a withdrawal response was elicited. In the writhing test, mice received 0.1 ml per 10 g of body weight of a 0.6% acetic acid solution by the intraperitoneal route, and contractions of abdominal musculature (writhes) were counted between 5 and 15 min after the injection.
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10. For Δ^9-THC dependence and withdrawal, mice were injected twice daily for 5 days with Δ^9-THC (20 mg/kg, intraperitoneally) or vehicle (ethanol/chremophor EL/distilled water, 1:1:18). On day 6, they received the morning injection and 4 hours later the cannabinoid antagonist SR141,716A [10 mg/kg, subcutaneously (sc)]. Δ^9-THC withdrawal was evaluated for 35 min as reported [D. M. Hutcheson *et al.*, *Br. J. Pharmacol.* **125**, 1567 (1998)].
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25. We thank A. Nagy for R1 embryonic stem cells, C. Dewolf and M. J. Simons for technical assistance, and D. Penninck for discussions. Supported by the interuniversity Poles of Attraction (Belgian State, Prime Minister's Office, Federal Service for Science, Technology and Culture), the Fonds de la Recherche Scientifique Médicale, the EU BIOMED II programme, and the Fondation Médicale Reine Elisabeth. The scientific responsibility is assumed by the authors. C.L. is Chercheur Qualifié of the Fonds National de la Recherche Scientifique.

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Diminishing Returns from Mutation Supply Rate in Asexual Populations

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Mutator genotypes with increased mutation rates may be especially important in microbial evolution if genetic adaptation is generally limited by the supply of mutations. In experimental populations of the bacterium *Escherichia coli*, the rate of evolutionary adaptation was proportional to the mutation supply rate only in particular circumstances of small or initially well-adapted populations. These experiments also demonstrate a "speed limit" on adaptive evolution in asexual populations, one that is independent of the mutation supply rate.

Surveys of natural populations of pathogenic (*1*) and commensal (*2*) bacteria indicate that more than 1% are dominated by mutator genotypes with increased mutation rates. Such genotypes are even more prevalent among populations of *E. coli* evolving in the laboratory (*3*) and in certain tumors (*4*). Mutators may be favored because they produce rare beneficial mutations more often than do normal genotypes and thereby allow a faster response to selection (*5*). But the actual relation between mutation rates and adaptive evolution may be more complicated, especially in asexual populations that are subject to strong effects of genetic linkage. Indeed, the logic that drives any empirical association between mutators and rapid adaptive evolution can be reversed: Rapid adaptation to a novel or changing environment provides more frequent opportunities for mutators to "hitchhike" to high frequency along with beneficial mutations to which they are genetically linked, even when mutators themselves have little effect on the rate of adaptation (*3*).

Moreover, population genetic models predict that the rate of adaptive evolution in asexual populations will increase proportionately with mutation rate only if populations spend most of their time waiting for beneficial mutations (*6*). Otherwise, two or more beneficial mutations may be simultaneously present in different lineages within a population; they will interfere with one another's spread, and ultimately only the superior mutation prevails while all others are driven extinct (*6*, *7*). Therefore, an increase in the supply rate of beneficial mutations might of-

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Table 1. Estimates of relative mutation rates of the six strains used in the evolution experiment, on the basis of eight separate fluctuation tests for each strain (*14*).

Mutator allele	Relative mutation rate	
	Nonadapted background	Adapted background
Wild type	1	1
mutY	3.3	3.3
mutS	34.9	32.4