Applied and Environmental Microbiology, Dec. 2008, p. 7258–7264 Vol. 74, No. 23
0099-2240/08/$08.00+0 doi:10.1128/AEM.01850-08
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

Generation of *Bradyrhizobium japonicum* Mutants with Increased N_2O Reductase Activity by Selection after Introduction of a Mutated *dnaQ* Gene▽†

Manabu Itakura,[1] Kazufumi Tabata,[2] Shima Eda,[1] Hisayuki Mitsui,[1] Kiriko Murakami,[2] Junichi Yasuda,[2] and Kiwamu Minamisawa[1]*

Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai, Miyagi, Japan,[1] and Neo-Morgan Laboratory, Incorporated, Biotechnology Research Center, Nogawa, Miyamae-ku, Kanagawa 216-0001, Japan[2]

Received 10 August 2008/Accepted 7 October 2008

We obtained two beneficial mutants of *Bradyrhizobium japonicum* USDA110 with increased nitrous oxide (N_2O) reductase (N_2OR) activity by introducing a plasmid containing a mutated *B. japonicum dnaQ* gene (pKQ2) and performing enrichment culture under selection pressure for N_2O respiration. Mutation of *dnaQ*, which encodes the epsilon subunit of DNA polymerase III, gives a strong mutator phenotype in *Escherichia coli*. pKQ2 introduction into *B. japonicum* USDA110 increased the frequency of occurrence of colonies spontaneously resistant to kanamycin. A series of repeated cultivations of USDA110 with and without pKQ2 was conducted in anaerobic conditions under 5% (vol/vol) or 20% (vol/vol) N_2O atmosphere. At the 10th cultivation cycle, cell populations of USDA110(pKQ2) showed higher N_2OR activity than the wild-type strains. Four bacterial mutants lacking pKQ2 obtained by plant passage showed 7 to 12 times the N_2OR activity of the wild-type USDA110. Although two mutants had a weak or null *fix* phenotype for symbiotic nitrogen fixation, the remaining two (5M09 and 5M14) had the same symbiotic nitrogen fixation ability and heterotrophic growth in culture as wild-type USDA110.

Nitrous oxide (N_2O) is a key atmospheric greenhouse gas that contributes to global climate change through radiative warming and depletion of stratospheric ozone (4, 19). Agricultural field soil is a major source of N_2O through the microbial transformation of nitrogen in soil (10, 14, 24) and contributes significantly to the net increase in atmospheric N_2O (4, 25, 27, 36). Soybean fields are also sources of emission of N_2O, probably because of the presence of their nodulated roots in the rhizosphere (18, 40). Several attempts have been made to reduce the emission of N_2O from agricultural systems (24, 25). The use of slow- or controlled-release fertilizers or nitrification inhibitors chemically reduces the emission of N_2O from agricultural systems (2, 36). However, to our knowledge, microorganisms have not been used to biologically reduce emissions of N_2O from agricultural systems.

The complete denitrification of nitrate to dinitrogen (N_2) by bacteria is generally an anaerobic respiratory process wherein the last step is mediated by N_2O reductase (N_2OR) (43, 44). The corresponding structural gene is *nosZ*, which is assembled in the *nosRZDFYL* gene operon (43, 44). *Bradyrhizobium japonicum*, a symbiotic nitrogen-fixing soil bacterium associated with soybeans (7, 15), is capable of denitrification (32, 33, 39). The *B. japonicum nosZ* gene encodes N_2OR, which mediates the reduction of N_2O to N_2 (31, 39). Sameshima-Saito et al. (31) found that the introduction of cosmids carrying *nosRZDFYL* into *B. japonicum* markedly increases N_2OR activity, suggesting that there is room for the enhancement of N_2OR activity in *B. japonicum*.

One molecular breeding procedure in microbes is the use of proofreading-deficient DNA polymerase mutators, which are cells that have higher mutation rates than the wild type (22). In *Escherichia coli*, the epsilon subunit of DNA polymerase III supplies exonuclease activity and is encoded by *dnaQ* (*mutD*) (22). Mutation of the *dnaQ* gene dominantly impairs proofreading activity, resulting in a very strong mutator phenotype via unedited replication errors during growth (9). The availability of proofreading-deficient mutators for the isolation of spontaneous mutants under selective pressure has been reported in antibiotic-resistant mutants of *E. coli* (37) and thermotolerant mutants of *Saccharomyces cerevisiae* (34).

In this work, we aimed to obtain beneficial mutants of *B. japonicum* USDA110 with increased N_2OR activity by introducing a plasmid containing a mutated *B. japonicum dnaQ* gene (pKQ2) and then performing enrichment culture under selection pressure for N_2O respiration. Examination of the genome sequence of *B. japonicum* USDA110 has indicated that the blr0640 gene is a *dnaQ* homolog (15). We therefore constructed a pKQ2 plasmid containing an artificial mutation of blr0640.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Bradyrhizobium japonicum* cells were grown at 30°C in HM salt medium (6) supplemented with 0.1% arabinose and 0.025% (wt/vol) yeast extract (Difco, Detroit, MI). HM medium was further supplemented with trace metals (HMM medium) for the denitrification assay (32). HM liquid media were cultured with reciprocal shaking at 120 rpm (flask) and 300 rpm (test tube). *E. coli* cells were grown at 37°C in Luria-Bertani medium (29). Antibiotics were added to the media in the following concentrations: for *B. japonicum*, 50 or 100

* Corresponding author. Mailing address: Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi 980-8577, Japan. Phone and fax: 81-22-217-5684. E-mail: kiwamu@ige.tohoku.ac.jp.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 10 October 2008.

TABLE 1. Bacterial strains and cosmids used

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Bradyrhizobium japonicum		
USDA110	Wild type; Tc^s Nos^+ Nod^+ Fix^+	15
S2	USDA110 higher-salt-tolerance mutant; Tc^s	This study
S4	USDA110 higher-salt-tolerance mutant; Tc^s	This study
S5	USDA110 higher-salt-tolerance mutant; Tc^s	This study
S9	USDA110 higher-salt-tolerance mutant; Tc^s	This study
5M08	USDA110 higher-N_2OR-activity mutant; Tc^s Nos^{++} Nod^+ Fix^-	This study
5M09	USDA110 higher-N_2OR-activity mutant; Tc^s Nos^{++} Nod^+ Fix^+	This study
5M14	USDA110 higher-N_2OR-activity mutant; Tc^s Nos^{++} Nod^+ Fix^+	This study
20M19	USDA110 higher-N_2OR-activity mutant; Tc^s Nos^{++} Nod^+ $Fix^{+/-}$	This study
Escherichia coli DH5α	Cloning strain; *recA*	Toyobo[b]
Plasmids		
pKS800	Derivative of broad-host-range cosmid pLAFR1; IncP Tc^r	11
pKQ1	pKS800 carrying *aph* promoter and wild-type blr0640; Tc^r	This study
pKQ2	pKS800 carrying *aph* promoter and two-base-pair substitution mutant of blr0640; Tc^r	This study
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km^r	8
pUC4-KIXX	Plasmid carrying 1.6-kb *aph* cassette; Ap^r Km^r	Amersham-Pharmacia[c]

[a] Nos, N_2OR activity; Nos phenotype is expressed as + (N_2OR activity of USDA110) or ++ (N_2OR activity significantly higher than that of USDA110). Fix phenotype (nitrogen fixation) is expressed as + (nitrogen fixation activity similar to that of USDA110), +/− (nitrogen fixation activity significantly lower than that of USDA110), or − (no nitrogen fixation activity). Nod^+, positive for nodule formation on soybeans. blr0640, DNA polymerase III epsilon chain gene homolog; *aph*, aminoglycosidase-3-*O*-phosphotransferase gene.
[b] Toyobo, Inc., Tokyo, Japan.
[c] Amersham-Pharmacia Biotech, Uppsala, Sweden.

μg tetracycline (Tc)/ml, 50 μg polymyxin B/ml, and 100 μg kanamycin (Km)/ml; for *E. coli*, 10 μg Tc/ml and 50 μg Km/ml.

Construction of the mutator plasmid. DNA fragments of wild-type and mutated *dnaQ* genes, $dnaQ^{WT}$ and $dnaQ^{exo-1}$, were amplified with two forward primers ($dnaQ^{WT}$-F, 5′-CCGCTCGAGATGCGCGAAATCGTTCTCGACACCGAAACC, and $dnaQ^{exo-1}$-F, 5′-CCGCTCGAGATGCGCGAAATCGTTCTCGCCACCGCAACC) and a reverse primer (dnaQ-R, 5′-CGCGGATCCCTAACCAGCCGGATTGACGGTAACTG) containing the XhoI and BamHI sites (underlined) from the *B. japonicum* genome. These fragments were digested with XhoI and BamHI and were introduced into the same sites of the cosmid vector pKS800. For *dnaQ* gene expression, the *aph* promoter fragment (U00004) was amplified with a primer pair (aphP-F, 5′-TACATCCTCGAGAAGCCAGTCCGCAGAAACGGTGC, and aphP-R, 5′-TGATGCCTCGAGGATCCTCATCCTGTCTCTTGATCAGA) containing the XhoI site (underlined) from pUC4-KIXX (Amersham-Pharmacia Biotech, Uppsala, Sweden). This fragment was digested with XhoI and introduced into the same site in the wild-type and mutated *dnaQ* plasmids. Finally, we constructed two plasmids named pKQ1 and pKQ2 carrying the wild-type and mutated *dnaQ* genes, respectively (Table 1 and Fig. 1). These two plasmids and the vector pKS800 were transformed into *E. coli* DH5α, and then each was introduced into *B. japonicum* USDA110 by means of triparental mating, using pRK2013 as a helper plasmid, on HM agar plates (26, 31, 35, 41). Transconjugants were selected on HM agar plates containing 100 μg Tc/ml and 50 μg polymyxin B/ml.

Antibiotic resistance. *Bradyrhizobium japonicum* USDA110 cells were cultured at 30°C for 7 days until they had reached a concentration of 10^9 cells/ml. The cultures were then serially diluted with HM broth and inoculated on HM agar plates with and without 100 μg Km/ml. After incubation of the plates at 30°C for 7 days, the frequencies of occurrence of Km-resistant colonies were calculated from CFU on HM agar plates with and without 100 μg Km/ml.

Mean generation time. Precultures (50 μl; 10^9 cells/ml) of USDA110 cells were inoculated into 5 ml of HM broth. The turbidity (A_{660}) of cultures grown aerobically at 30°C was measured every 24 h with a UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). The mean generation time was calculated as described previously (30).

Selection for salt stress. USDA110 carrying pKS800, pKQ1, or pKQ2 (1 ml; 10^9 cells/ml) was inoculated into 100 ml of HM broth in a flask supplemented with 50 μg Tc/ml and 75 mM NaCl and then grown at 30°C for 10 days as the first cultivation. The resultant cultures (1 ml) were reinoculated into 100 ml HM broth medium with 75 mM NaCl and 50 μg Tc/ml and then incubated at 30°C for 14 days. Periodic transfer (1 ml) was repeated at intervals of 10 to 14 days up to

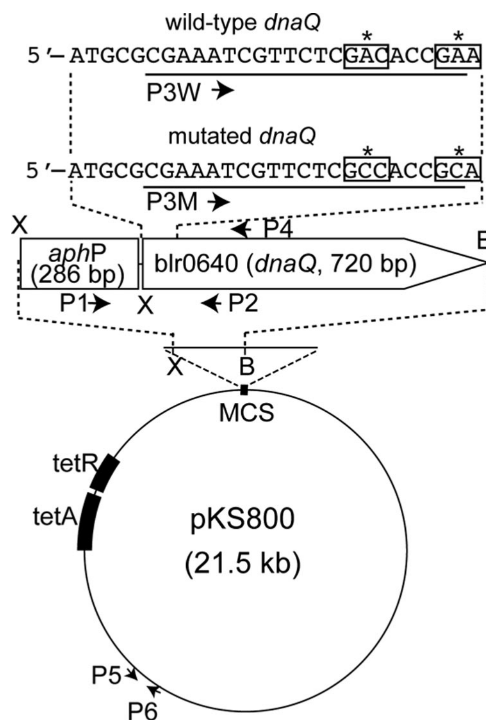

FIG. 1. Construction of plasmids carrying wild-type *dnaQ* (pKQ1) or mutated *dnaQ* (pKQ2). Asterisked nucleotides indicate positions of the two-base-pair substitutions. Boxes indicate wild-type and mutated codons in *B. japonicum dnaQ* homolog for conversion of $_7$Asp (GAC) and $_9$Glu (GAA) into $_7$Ala (GCC) and $_9$Ala (GCA), respectively. *aphP* is the promoter of the Km resistance gene from pUC4-KIXX (Table 1). Arrows with prefix "P" followed by 3W, 3M, 1, 2, 4, 5, or 6 show the positions and directions of PCR primers. DNA sequences of primers 3W and 3M are underlined. "MCS" indicates multicloning sites for pKS800 (Table 1). The resulting plasmids (pKQ1 and pKQ2) were 22.4 kb. X, XhoI; B, BamHI.

TABLE 2. Generation time and frequency of occurrence of spontaneous Km resistance in *B. japonicum* USDA110 carrying pKS800, pKQ1, or pKQ2[a]

Strain	Generation time (h)[b]	Frequency of occurrence of Km^r colonies (ratio relative to value for pKS800)
USDA110(pKS800)	15.1 ± 0.2	$1.6 \times 10^{-7} \pm 0.1 \times 10^{-7}$ (1)*
USDA110(pKQ1)	14.9 ± 0.7	$1.5 \times 10^{-7} \pm 0.2 \times 10^{-7}$ (1)*
USDA110(pKQ2)	15.1 ± 0.5	$1.5 \times 10^{-5} \pm 0.2 \times 10^{-5}$ (95)#

[a] Values are expressed as means ± standard deviations (n = 3). Entries followed by the same symbol (* or #) do not differ significantly by t test (P < 0.01).

[b] Generation times were calculated from growth in HM broth with 50 μg of Tc/ml.

the seventh cultivation cycle. To assay the increase of salt tolerance and the selection for increased NaCl tolerance, cultures (1 ml) were also inoculated into 100 ml HM broth medium with 100 mM NaCl and 50 μg Tc/ml at the first and seventh cultivation cycles.

Selection for N_2O respiration. USDA110 carrying pKS800 or pKQ2 (200 μl; 10^9 cells/ml) was inoculated into 5 ml of HM broth medium with 50 μg Tc/ml. To achieve anaerobic N_2O respiration conditions, N_2O gas was introduced at a final concentration of 5 or 20% (vol/vol) (N_2 balance) into the headspace (29 ml) of a test tube (18-mm diameter by 180-mm height) sealed with a butyl rubber stopper. These cultures were incubated at 30°C for 9 days as a first cultivation. The resultant cultures (50 μl) were reinoculated into 5 ml HM broth medium with 50 μg Tc/ml and then grown for 7 days under conditions of anaerobic N_2O respiration as a 2nd cultivation cycle; periodic transfer (50 μl) was repeated at intervals of 7 to 14 days up to the 10th cultivation cycle.

N_2OR activity. *B. japonicum* cells were washed with HMM broth by centrifugation (5,000 × *g*, 15 min, 4°C) and were suspended at 10^9 cells/ml in the broth. N_2OR activity was determined by using a GC-17A gas chromatograph (Shimadzu) equipped with a ^{63}Ni electron capture detector and a CP-PoraBOND Q-capillary column (internal diameter, 0.32 mm; length, 25 m; Varian, Palo Alto, CA), as described previously (31).

Plant inoculation. Surface-sterilized soybean seeds (*Glycine max* 'Enrei') were germinated in sterile vermiculite for 2 days at 25°C and then transplanted to a Leonard jar pot (20, 38, 42) which contained sterile vermiculite and nitrogen-free nutrient solution (23, 26). The seeds were then inoculated with *Bradyrhizobium japonicum* at 1×10^7 cells per seed. Plants were grown in a growth chamber (LH200; Nippon Medical & Chemical Industries, Tokyo, Japan) for 4 weeks at 25°C with 16 h light and 8 h dark.

Single-colony isolation via plant passage. After soybean cultivation for 4 weeks, nodules excised from the soybean roots were sterilized with NaClO solution (0.5% wt/vol) and washed 10 times with sterilized water. Each sterilized nodule was cut in half, and the bacteroid cells were streaked onto an HM agar plate for isolation of single colonies. After incubation at 30°C for 1 week, single colonies from each nodule were inoculated onto HM agar plates with or without 100 μg Tc/ml to ensure plasmid curing.

Symbiotic phenotype. After soybean cultivation for 4 weeks, the nodulated roots of one plant were introduced into 125-ml vials. Acetylene gas was injected at a final concentration of 10% (vol/vol), and the roots were incubated for 20 min at 20°C. The ethylene concentration was determined with a Shimadzu GC-18A gas chromatograph (Shimadzu) equipped with a flame ionization detector and a Porapack N column (28). Then, the number and weight of the nodules per plant were determined.

PCR verification of plasmid elimination. Total DNA was prepared with an AquaPure genomic DNA isolation kit (Bio-Rad Laboratories, Hercules, CA) as a PCR template. Four primer pairs were designed for PCR amplification of plasmids carrying the *dnaQ* of the mutant or wild type (Fig. 1). The primer sequences were primer 1, 5′-TAGACTGGGCGGTTTTATGG; primer 2, 5′-ATCTCGACACAGCCGATTTC; primer 4, 5′-CTCGGCATTGATGAAGCTGA-3′; primer 5, 5′-ATCGACATCCTCAACGGAAG; and primer 6, 5′-CTTTCCCGCTCACTTTTCAG. Primers 3W and 3M are shown in Fig. 1. Ex*Taq* polymerase (Takara, Osaka, Japan) was used for PCR amplification. For PCR primers 1, 2, 5, and 6, the PCR cycles were 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension cycle of 72°C for 10 min. For PCR primers 3W, 3M, and 4, the PCR cycles were 1 cycle of 94°C for 5 min, 25 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and a final extension cycle of 72°C for 10 min.

RESULTS

Construction of the *B. japonicum* USDA110 mutator. $_7$Asp (GAC) and $_9$Glu (GAA) of blr0640 are amino acid sequence motifs that are conserved among gram-negative bacteria and the exonuclease I motif of the *dnaQ* gene of *E. coli* (9, 13). Therefore, we constructed a two-base-pair substitution mutant of blr0640 by replacing $_7$Asp (GAC) with $_7$Ala (GCC), and $_9$Glu (GAA) with $_9$Ala (GCA) by PCR (Fig. 1). The mutated *dnaQ* gene and *aph* promoter fragment were inserted into the BamHI/XhoI sites of pKS800 (11), resulting in pKQ2 (Fig. 1). pKQ2 was introduced into *B. japonicum* USDA110 by transconjugation. pKS800 and pKS800 carrying the wild-type blr0640(pKQ1) were also introduced into *B. japonicum* USDA110 as controls (Fig. 1).

The generation time of USDA110 carrying pKQ2 was similar to that of USDA110 carrying pKS800 or pKQ1 (Table 2). In contrast, the frequency of occurrence of the spontaneous Km-resistant mutant of USDA110 carrying pKQ2 was significantly higher than that of USDA110 carrying pKS800 or pKQ1 (Table 2), indicating that pKQ2 conferred in *trans* a mutator phenotype on *B. japonicum* USDA110.

Isolation of salt-tolerant mutants. To examine whether the introduction of pKQ2 would produce *B. japonicum* mutants of interest under selective pressure, we tried to isolate mutants with increased salt tolerance. Although USDA110 was unable to grow in medium supplemented with 100 mM NaCl (5), 75 mM NaCl permitted its slow growth (data not shown). Thus, USDA110 cells carrying pKQ2 (as mutator cells) and USDA110 cells carrying pKS800 or pKQ1 (as control cells) were cultured repeatedly in HM medium containing 75 mM NaCl (Fig. 2). The growth of mutator cells [USDA110(pKQ2)]

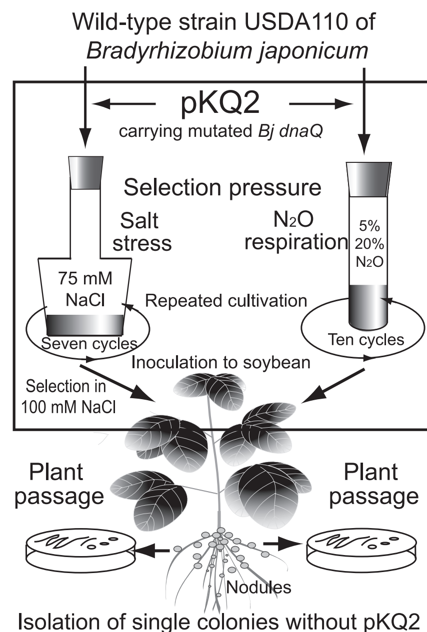

FIG. 2. Strategies for isolation of *Bradyrhizobium japonicum* (*Bj*) mutants with increased NaCl tolerance and increased N_2OR activity by pKQ2 introduction and enrichment culture under selective pressures. Box shows steps by which an increased mutation rate was produced in the cell population of *B. japonicum* USDA110(pKQ2) (see text).

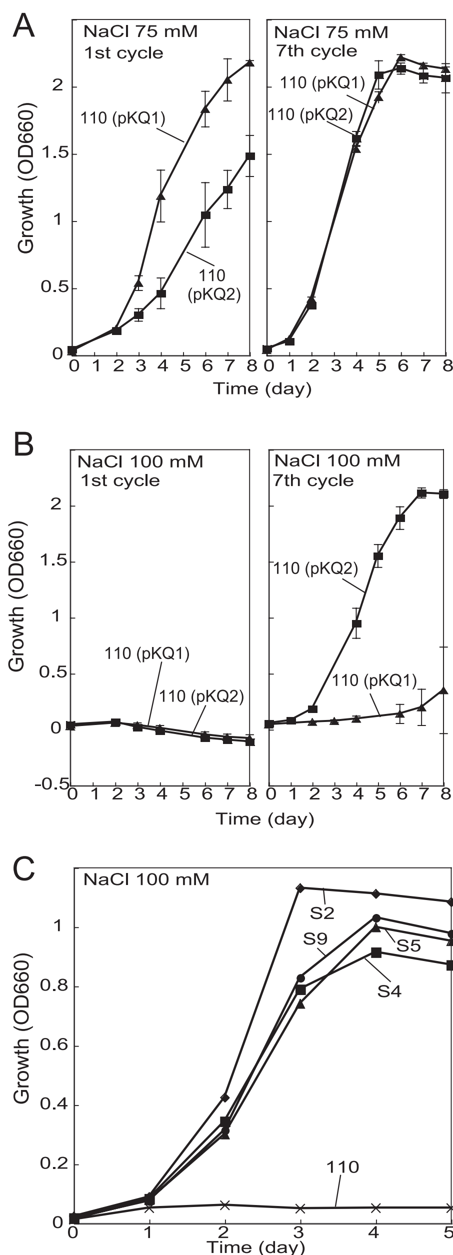

FIG. 3. Growth of cell populations under salt-stress selection and of mutants of *Bradyrhizobium japonicum* USDA110 with increased salt resistance. (A) Cells were repeatedly cultivated in 75 mM NaCl to adapt to salt stress (Fig. 2). Cell growth curves of first and seventh culture cycles are shown. (B) Cells from first and seventh culture cycles were assayed to examine whether the salt tolerance of cell populations increased in 100 mM NaCl. (C) Growth of isolates from seventh culture cycle of USDA110(pKQ2) population in 100 mM NaCl (panel B, right). Error bars indicate standard deviations ($n = 3$). 110, strain USDA110; OD660, optical density at 660 m.

was slower than that of control cells [USDA110(pKQ1)] in the first cultivation cycle (Fig. 3A, left). This was not surprising, because mutation of *dnaQ* generally reduces the growth rate (9, 22). In contrast, in the seventh cultivation cycle, the growth of the mutator cells was similar to that of the control cells (Fig. 3A, right). When we used stronger salt stress (100 mM NaCl), after the seventh cultivation cycle we observed vigorous growth of the mutator cell [USDA110(pKQ2)] population (Fig. 3B, right). In contrast, no growth was observed in control cells [USDA110(pKQ1)] even after the seventh cultivation cycle (Fig. 3B, right).

Mutator cells [USDA110(pKQ2)] from the seventh cultivation cycle were inoculated into soybeans (Fig. 2). Tc-sensitive single colonies from the soybean nodules were then isolated on HM agar plates. Four Tc-sensitive mutants, S2, S4, S5, and S9, were able to grow even in HM broth containing 100 mM NaCl, whereas no growth was observed in wild-type USDA110 (Fig. 3C). This result showed that the mutants isolated still had an increased-salt-tolerance phenotype, although they had probably lost pKQ2.

Isolation of mutants with increased N_2OR activity. In light of the results of the salt-stress experiment, we designed a strategy for isolating mutants with increased N_2OR activity (Fig. 2). A series of cultivations of mutator and control cells were performed in anaerobic conditions under 5% (vol/vol) or 20% (vol/vol) N_2O. The growth of mutator cells [USDA110(pKQ2)] was slower than that of control cells [USDA110(pKS800)] during the first cultivation cycle (Fig. 4A and B, left). At the 10th cultivation cycle, the growth of the mutator cells [USDA110(pKQ2)] was faster than that of the control cells [USDA110(pKS800)] under 5% N_2O and similar to that of the control cells [USDA110(pKS800)] under 20% N_2O (Fig. 4A and B, right).

We therefore determined the N_2OR activity of cells from the 10th cultivation cycle. The mutator populations serially cultivated in 5% or 20% N_2O atmospheres took up N_2O more rapidly than the control populations (Fig. 4C). Based on the reduction rate with time (Fig. 4C), the N_2OR activities of mutator cell populations [USDA110(pKQ2)] from the 5 and 20% N_2O selections were 77 nmol h^{-1} 10^9 $cells^{-1}$ and 65 nmol h^{-1} 10^9 $cells^{-1}$, respectively. On the other hand, the N_2OR activity of the control cell populations was approximately 7 nmol/h per 10^9 cells in USDA110(pKS800) from both the 1st and the 10th cultivation cycles under 5% or 20% N_2O respiration selection.

Mutator cells from the 10th cultivation cycle under selection pressure of N_2O respiration were inoculated into soybeans (Fig. 2). From this plant passage, we obtained 31 Tc-sensitive isolates; we measured their N_2OR activities as a first screening (data not shown) and selected four promising mutants which took up N_2O more rapidly than wild-type USDA110 (Fig. 4D). The N_2OR activities of 5M08, 5M09, 5M14, and 20M19 (85 to 138 nmol h^{-1} 10^9 $cells^{-1}$) were 11, 9, 7, and 9 times those of the wild-type USDA110 (12 nmol h^{-1} 10^9 $cells^{-1}$) (Fig. 4D and Table 3). This result showed that the mutants still had increased N_2OR activity; their activities were similar to that of the pKQ2 mutator population (Fig. 4C).

Verification of pKQ2 elimination. The frequencies of occurrence of spontaneous Km-resistant mutants among the four mutants (20M19, 5M08, 5M09, and 5M14) were of the order of approximately 10^{-7}, the same level as in the wild-type USDA110 (Table 3) and lower than that of USDA110(pKQ2) (Table 2). In addition, PCR fragments were not detected from the four mutants when P1/P2, P3M/P4, and P5/P6 were used as PCR primers, expect for P3W/P4 (see Fig. S1 in the supplemental material). On the other hand, all PCR fragments were detected from USDA110(pKQ2) (see Fig. S1 in the supple-

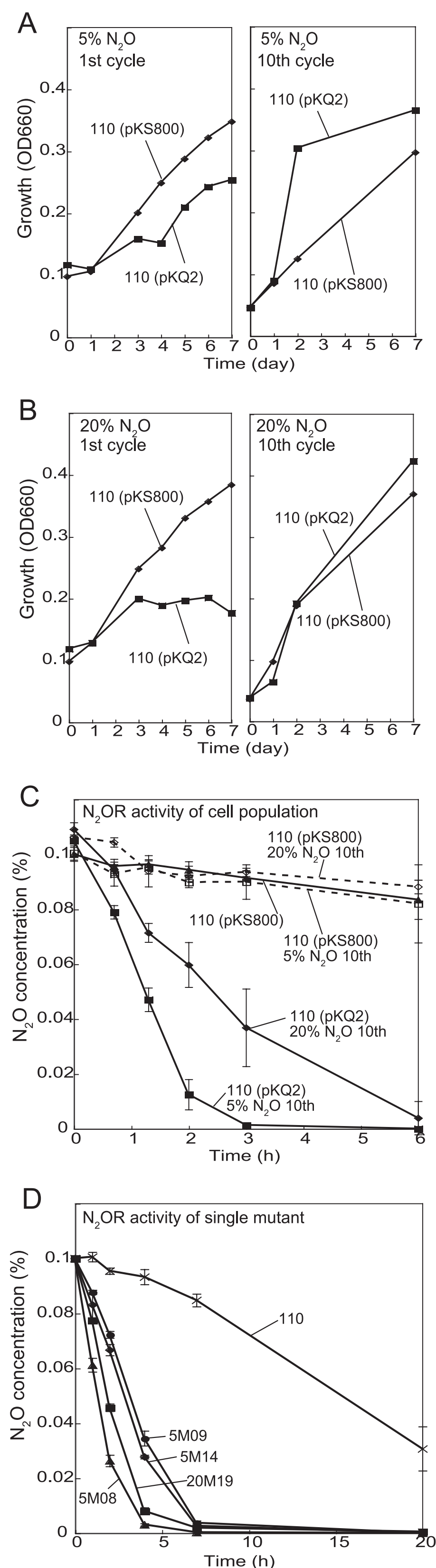

FIG. 4. Growth of cell populations under N_2O respiration and of mutants of *Bradyrhizobium japonicum* USDA110 with increased N_2OR activity. (A and B) Growth of 1st and 10th culture cycles of USDA110(pKS800) and USDA110(pKQ2) populations in HM medium in the presence of 5% (A) and 20% (B) N_2O. (C) N_2OR activity of 10th culture cycles of USDA110(pKS800) and USDA110(pKQ2) populations. (D) N_2OR activity of isolates from 10th culture cycle of USDA110(pKQ2) population. Error bars indicate standard deviations (n = 3). 110, strain USDA110; OD660, optical density at 660 m.

mental material). These results indicate that pKQ2 was genetically and physically eliminated in the mutants.

Growth and symbiotic traits of the mutants isolated. The generation times of the four mutants in HM broth were not significantly different from that of the wild-type USDA110 (Table 3). Mutant 5M08 formed significantly more and smaller nodules than did the wild type (Table 3). The acetylene-reduction activities (ARAs) of the nodules were measured to evaluate the nitrogen-fixing ability of the mutants. 5M09 and 5M14 had ARAs similar to that of the wild-type USDA110, but 5M08 and 20M19 had no ARA or much weaker ARA, respectively, than the wild-type USDA110 (Table 3). Large numbers of tiny nodules like those produced by 5M08 are often produced by Fix^- endosymbionts (16). We therefore considered that 5M08 and 20M19 had been subjected to mutations relevant to symbiotic nitrogen fixation. The remaining two mutants, 5M09 and 5M14, had significantly higher N_2OR activities than the parent strain USDA110, but they had normal symbiotic nitrogen fixation activity.

DISCUSSION

The positions of site-specific mutagenesis of the *B. japonicum dnaQ* gene (Fig. 1) were identical to those of the proofreading-deficient *dnaQ926* mutant in the exonuclease I motif of the *dnaQ* gene of *E. coli* (9). When the mutated *dnaQ* was supplied in *trans* to *B. japonicum* USDA110 by pKQ2, the elevation of the mutation rate depended on the presence of pKQ2, as evaluated from the frequency of occurrence of spontaneous Km-resistant colonies (Tables 2 and 3) and the slower growth in the first cultivation cycle (Fig. 3A, left, and 4A and B, left). These phenotypes were similar to that of the *E. coli dnaQ926* mutant (9, 22). Thus, it is likely that the *dnaQ* gene homolog (blr0640) in USDA110 encodes functional proofreading exonuclease by the epsilon subunit of DNA polymerase in *B. japonicum*.

By transconjugation and plant passage, we manipulated the introduction and elimination of plasmid pKQ2 with a modified *dnaQ* gene of *B. japonicum* USDA110 (Fig. 2). Cell populations showing increased salt stress or N_2OR activity were obtained exclusively from USDA110(pKQ2), indicating the occurrence of adaptive evolution of the mutator populations under the respective selection pressures. This may be due to the simultaneous occurrence of mutagenesis and selection during cell growth (37).

N_2O respiration selection pressure was different from that of salt stress. In the salt-stress experiment, mutant cells were selectively enriched after the seventh cultivation under high-salt conditions (100 mM NaCl) (Fig. 2 and 3B). In contrast, the enrichment processes in N_2O respiration were based on the assumption that cells with higher N_2OR activity grow faster than those with normal N_2OR activity. If so, the population of

TABLE 3. Free-living and symbiotic phenotypes of *B. japonicum* USDA110 mutants with increased N_2OR activity[a]

Strain	N_2OR activity (nmol h^{-1} 10^9 $cells^{-1}$)[b]	Generation time (h)[c]	Frequency of occurrence of Km^r colonies	Number of nodules ($plant^{-1}$)	Nodule wt (mg [fresh wt] $nodule^{-1}$)	Nitrogen-fixing activity (μmol C_2H_4 produced h^{-1} g of nodule [fresh $wt]^{-1}$)[d]
USDA110	12 ± 1¶	10.0 ± 1.2*	$1.9 \times 10^{-7} \pm 0.6 \times 10^{-7}$*	52 ± 7*	13.0 ± 1.7*	12.2 ± 3.8*
5M08	107 ± 0#	9.6 ± 0.0*	$1.3 \times 10^{-7} \pm 0.3 \times 10^{-7}$*	102 ± 8#	5.8 ± 0.9#	0.0 ± 0.0#
5M09	85 ± 4§	9.7 ± 0.6*	$1.1 \times 10^{-7} \pm 0.4 \times 10^{-7}$*	52 ± 14*	11.3 ± 4.2*	14.4 ± 2.8*
5M14	114 ± 2#,§	10.1 ± 0.6*	$2.0 \times 10^{-7} \pm 0.4 \times 10^{-7}$*	48 ± 12*	10.4 ± 3.1*	26.8 ± 6.2*
20M19	138 ± 2*	11.7 ± 0.9*	$3.3 \times 10^{-7} \pm 1.0 \times 10^{-7}$*	49 ± 10*	13.5 ± 3.4*	0.8 ± 0.8#

[a] Values are expressed as means ± standard deviations ($n = 3$). Values in the same column followed by the same symbols (*, #, §, and ¶) do not differ significantly by *t* test ($P < 0.05$).
[b] 5M08, 5M09, and 5M14 were obtained from 5% N_2O selection, while 20M19 was derived from 20% N_2O selection.
[c] Generation times were calculated from growth in HM broth medium.
[d] Nitrogen-fixing activity was evaluated by measuring ARA.

the former cells will dominate with time during selection. This was true for enrichment in an atmosphere of 5% (vol/vol) N_2O (Fig. 4A and Table 3). In contrast, 20% N_2O selection resulted in no significant differences in growth at the final cultivation cycle (Fig. 4). When we adopted selection in a 100% N_2O atmosphere, no growth was observed, even for wild-type USDA110 (data not shown). Thus, a low concentration of N_2O (5% vol/vol) is empirically important for selection based on N_2O respiration.

Among the four mutants with increased N_2OR activity, two (5M08 and 20M19) had weak or null *fix* phenotypes, and the remaining two (5M09 and 5M14) had normal levels of nitrogen fixation that were similar to that of wild-type USDA110 (Table 3). This suggested that mutator cells markedly accumulate unrelated mutations on their genomes.

There are several possible explanations for why the N_2OR activities of the four mutants were higher than that of the wild-type USDA110. The first possibility is that the expression level of the *nos* operon is upregulated by mutations in its promoter or related regulatory networks (21, 39), as has been observed after *nos* plasmid introduction (31). The second possibility is that a mutation in the *nosZ* gene produced an "improved" NosZ protein with increased N_2OR activity. A third explanation is the occurrence of a mutation in the electron carriers relevant to N_2OR activity (12). Because the mutants may accumulate many mutations on their genomes, as described above, simple DNA sequencing could not answer these questions. Genetic and biochemical studies are required to address these questions in relation to the respective mutants.

Soybean fields are sources of the emission of N_2O (18, 40). 5M09 and 5M14 had approximately 10 times the N_2OR activity of the wild-type USDA110 (Fig. 4D and Table 3). Because 5M09 and 5M14 no longer contain pKQ2 and the mutated *dnaQ*, they will not be considered genetically modified organisms. Thus, they could be released into agricultural fields to mitigate global warming (1). By using the pKQ2 system, it would be possible to reprepare *B. japonicum* mutants from indigenous isolates from local field soils. N_2OR activity and denitrification are environmentally important steps, and the mutated *dnaQ* method may be applicable to other environmental bacteria (3, 17).

ACKNOWLEDGMENTS

This work was supported in part by PROBRAIN and in part by a Grant-in-Aid for Scientific Research to K.M. (no. 17658034) from the Japan Society for the Promotion of Science.

We thank M. Sugawara (Tohoku University) and R. Kugimiya (Neo-Morgan Laboratory, Incorporated) for promoter design in the plasmid construction and for valuable discussions, respectively.

REFERENCES

1. **Adl, M. S.** 2008. Setting the tempo in land remediation: short-term and long-term patterns in biodiversity recovery. Microbes Environ. **23:**13–19.
2. **Akiyama, H., H. Tsuruta, and T. Watanabe.** 2000. N_2O and NO emissions from soils after the application of different chemical fertilizers. Global Change Sci. **2:**313–320.
3. **Amano, T., I. Yoshinaga, K. Okada, T. Yamagishi, S. Ueda, A. Obuchi, Y. Sako, and Y. Suwa.** 2007. Detection of anammox activity and diversity of anammox bacteria-related 16S rRNA genes in coastal marine sediment in Japan. Microbes Environ. **22:**232–242.
4. **Banin, A., J. G. Lawless, and R. C. Whitten.** 1984. Global N_2O cycles: terrestrial emissions, atmospheric and biospheric effects. Adv. Space Res. **4:**207–216.
5. **Boncompagni, E., M. Osteras, M. C. Poggi, and D. le Rudulier.** 1999. Occurrence of choline and glycine betaine uptake and metabolism in the family *Rhizobiaceae* and their roles in osmoprotection. Appl. Environ. Microbiol. **65:**2072–2077.
6. **Cole, M. A., and G. H. Elkan.** 1973. Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. Antimicrob. Agents Chemother. **4:**248–253.
7. **Dao, T. V., M. Nomura, R. Hamaguchi, K. Kato, M. Itakura, K. Minamisawa, S. Sinsuwongwat, T. T. Le, T. Kaneko, S. Tabata, and S. Tajima.** 2008. NAD-malic enzyme affects nitrogen-fixing activity of *Bradyrhizobium japonicum* USDA110 bacteroids in soybean nodules. Microbes Environ. **23:**215–220.
8. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
9. **Fijalkowska, I., and R. E. Schaaper.** 1996. Mutants in the Exo I motif of *Escherichia coli dnaQ*: defective proofreading and inviability due to error catastrophe. Proc. Natl. Acad. Sci. USA **93:**2856–2861.
10. **Firestone, M. K., R. B. Firestone, and J. M. Tiedje.** 1980. Nitrous oxide from soil denitrification: factors controlling its biological production. Science **208:**749–751.
11. **Hattori, Y., H. Omori, M. Hanyu, N. Kaseda, E. Mishima, T. Kaneko, S. Tabata, and K. Saeki.** 2002. Ordered cosmid library of the *Mesorhizobium loti* MAFF303099 genome for systematic gene disruption and complementation analysis. Plant Cell Physiol. **43:**1542–1557.
12. **Heikkilä, P. M., U. Honisch, P. Wunsch, and W. G. Zumft.** 2001. Role of the Tat transport system in nitrous oxide reductase translocation and cytochrome cd_1 biosynthesis in *Pseudomonas stutzeri*. J. Bacteriol. **183:**1663–1671.
13. **Huang, Y., D. K. Braithwaite, and J. Ito.** 1997. Evolution of *dnaQ*, the gene encoding the editing 3′ to 5′ exonuclease subunit of DNA polymerase III holoenzyume in gram-negative bacteria. FEBS Lett. **400:**94–98.
14. **Jumadi, O., Y. Hala, A. Muis, A. Ali, M. Palennari, K. Yagi, and K. Inubushi.** 2008. Influences of chemical fertilizers and a nitrification inhibitor on greenhouse gas fluxes in a corn (*Zea mays* L.) field in Indonesia. Microbes Environ. **23:**29–34.

15. **Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada. M. Yamada, and S. Tabata.** 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. **9:**225–256.
16. **Kawaharada, Y., S. Eda, K. Minamisawa, and H. Mitsui.** 2007. A *Mesorhizobium loti* mutant with reduced glucan content shows defective invasion of its host plant *Lotus japonicus*. Microbiology **153:**3983–3993.
17. **Khan, T. S., Y. Horiba, N. Takahashi, and A. Hiraishi.** 2007. Activity and community composition of denitrifying bacteria in poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)-using solid-phase denitrification processes. Microbes Environ. **22:**20–31.
18. **Kim, Y., N. Nakayama, M. Takahashi, S. Shimada, and J. Arihara.** 2005. NO and N_2O emissions from fields in the different nodulated genotypes of soybean. Jpn. J. Crop Sci. **74:**427–430.
19. **Lashof, D. A., and D. R. Ahuja.** 1990. Relative contribution of greenhouse gas emission to global warming. Nature **344:**529–531.
20. **Leonard, L. T.** 1943. A simple assembly for use in the testing of cultures of rhizobia. J. Bacteriol. **45:**523–525.
21. **Mesa, S., E. J. Bedmar, A. Chanfon, H. Hennecke, and H. M. Fischer.** 2003. *Bradyrhizobium japonicum* NnrR, a denitrification regulator, expands the FixLJ-FixK2 regulatory cascade. J. Bacteriol. **185:**3978–3982.
22. **Miller, H. J.** 1996. Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. Annu. Rev. Microbiol. **50:**625–643.
23. **Minamisawa, K., M. Itakura, M. Suzuki, K. Ichige, T. Isawa, K. Yuhashi, and H. Mitsui.** 2002. Horizontal transfer of nodulation genes in soil and microcosms from *Bradyrhizobium japonicum* to *B. elkanii*. Microbes Environ. **17:**82–90.
24. **Mosier, A. R., C. Kroeze, C. Nevison, O. Oenema, S. Seitzinger, and O. van Cleemput.** 1998. Closing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen cycle. Nutr. Cycl. Agroecosys. **52:**225–248.
25. **Mosier, A. R., and C. Kroeze.** 2000. Potential impact on the global atmospheric N_2O budget of the increased nitrogen input required to meet future global food demands. Chemosphere Global Change Sci. **2:**465–473.
26. **Okazaki, S., M. Sugawara, and K. Minamisawa.** 2004. *Bradyrhizobium elkanii rtxC* gene is required for expression of symbiotic phenotypes in the final step of rhizobitoxine biosynthesis. Appl. Environ. Microbiol. **70:**535–541.
27. **Otsuka, S., I. Sudiana, A. Komori, K. Isobe, S. Deguchi, M. Nishiyama, H. Shimizu, and K. Senoo.** 2008. Community structure of soil bacteria in a tropical rainforest several years after fire. Microbes Environ. **23:**49–56.
28. **Saito, A., and K. Minamisawa.** 2006. Evaluation of nitrogen fixation capability of endophytic clostridia by acetylene reduction and reverse transcription-PCR targeted to *nifH* transcript and ribosomal RNA. Microbes Environ. **21:**23–35.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. **Sameshima, R., T. Isawa, M. J. Sadowsky, T. Hamada, H. Kasai, A. Shutsrirung, H. Mitsui, and K. Minamisawa.** 2003. Phylogeny and distribution of extra-slow-growing *Bradyrhizobium japonicum* harboring high copy numbers of RSα, RSβ and IS*1631*. FEMS Microbiol. Ecol. **44:**191–202.
31. **Sameshima-Saito, R., K. Chiba, J. Hirayama, M. Itakura, H. Mitsui, S. Eda, and K. Minamisawa.** 2006. Symbiotic *Bradyrhizobium japonicum* reduces N_2O surrounding the soybean root system via nitrous oxide reductase. Appl. Environ. Microbiol. **72:**2526–2532.
32. **Sameshima-Saito, R., K. Chiba, and K. Minamisawa.** 2004. New method of denitrification analysis of *Bradyrhizobium* field isolates by gas chromatographic determination of ^{15}N-labeled N_2. Appl. Environ. Microbiol. **70:** 2886–2891.
33. **Sameshima-Saito, R., K. Chiba, and K. Minamisawa.** 2006. Correlation of denitrifying capability with the existence of *nap*, *nir*, *nor* and *nos* genes in diverse strains of soybean bradyrhizobia. Microbes Environ. **21:**174–184.
34. **Shimoda, C., A. Itadani, A. Sugino, and M. Furusawa.** 2006. Isolation of thermotolerant mutants by using proofreading-deficient DNA polymerase δ as an effective mutator in *Saccharomyces cerevisiae*. Genes Genet. Syst. **61:**391–397.
35. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. BioTechnology **1:**784–791.
36. **Smith, P., and D. Martino.** 2007. Agriculture, p. 506–507. *In* B. Mets, O. Davidson, P. Bosch, R. Dave, and L. Meyer (ed.), Climate Change 2007: mitigation of climate change. Contribution of working group III to the fourth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, New York, NY.
37. **Tanabe, K., T. Kondo, Y. Onodera, and M. Furusawa.** 1999. A conspicuous adaptability to antibiotics in *Escherichia coli* mutator strain, *dnaQ49*. FEMS Microbiol. Lett. **176:**191–196.
38. **Trung, B. C., and S. Yoshida.** 1983. Improvement of Leonard jar assembly for screening of effective *Rhizobium*. Soil Sci. Plant Nutr. **29:**97–100.
39. **Velasco, L., S. Mesa, X. Chang-ai, M. J. Delgado, and E. J. Bedmar.** 2004. Molecular characterization of *nosRZDFYLX* genes coding for denitrifying nitrous oxide reductase of *Bradyrhizobium japonicum*. Antonie van Leeuwenhoek **85:**229–235.
40. **Yang, L., and Z. Cai.** 2005. The effect of growing soybean (*Glycine max* L.) on N_2O emission from soil. Soil Biol. Biochem. **37:**1205–1209.
41. **Yasuta, T., S. Okazaki, H. Mitsui, K. Yuhashi, H. Ezura, and K. Minamisawa.** 2001. DNA sequence and mutational analysis of rhizobitoxine biosynthesis genes in *Bradyrhizobium elkanii*. Appl. Environ. Microbiol. **67:**4999–5009.
42. **Ye, B., A. Saito, and K. Minamisawa.** 2005. Effect of inoculation with anaerobic nitrogen-fixing consortium on salt tolerance of *Miscanthus sinensis*. Soil Sci. Plant Nutr. **51:**243–249.
43. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.
44. **Zumft, W. G., and P. M. H. Kroneck.** 2007. Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by *Bacteria* and *Archaea*. Adv. Microbial Physiol. **52:**107–227.

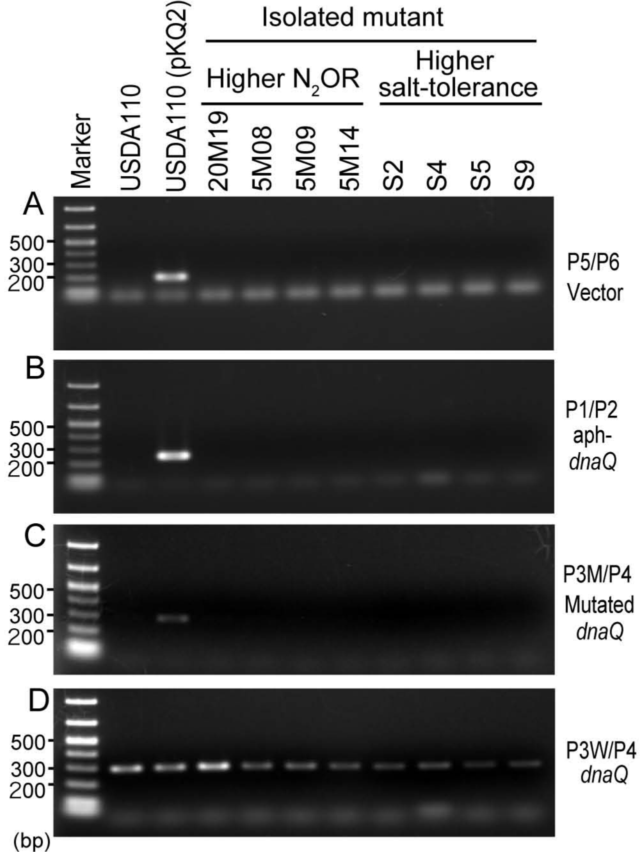

Fig. S1. Verification of pKQ2 elimination by PCR amplification. pKQ2 elimination in the *B. japonicum* mutants was further verified by PCR. In the case of the pKS800 vector (A) and the pKQ2 insert containing the *aph* promoter and *dnaQ* gene (B), both PCR amplicons were observed exclusively in USDA110 (pKQ2), and not in the eight mutants that we isolated or in the wild-type USDA110. We designed the PCR primers P3M and P3W, which can discriminate between wild-type and mutated *dnaQ* genes (Fig. 1). The mutated *dnaQ* gene was detected exclusively in USDA110 (pKQ2) (C). On the other hand, the wild-type *dnaQ* gene was amplified in all samples, including USDA110 (with and without pKQ2) and the mutants (D). These results showed that pKQ2 was eliminated from the eight mutants via plant passage. In addition, mutated *dnaQ* were not detected in the genomes of these mutants.