Plasmid 62 (2009) 1–9

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Development of versatile shuttle vectors for *Deinococcus grandis*

Katsuya Satoh, Zhenli Tu [1], Hirofumi Ohba, Issay Narumi *

Gene Resource Research Group and DNA Repair Protein Group, Quantum Beam Science Directorate, Japan Atomic Energy Agency, 1233 Watanuki, Takasaki 370-1292, Japan

ARTICLE INFO

Article history:
Received 24 December 2008
Revised 26 January 2009
Available online 5 February 2009

Communicated by C. Jeffrey Smith

Keywords:
Deinococcus grandis
Deinococcus radiopugnans
Gene expression
Plasmid transformation
Shuttle vector

ABSTRACT

To develop new shuttle vectors for *Deinococcus* species, the nucleotide sequence of the small cryptic plasmid pUE30 from *Deinococcus radiopugnans* ATCC19172 was determined. The 2467-bp plasmid possesses two open reading frames, one encoding 88 amino acid residues (Orf1) and the other encoding 501 amino acid residues (Orf2). The predicted amino acid sequence encoded by Orf1 exhibits similarity to the N-terminal regions of replication proteins encoded by *repABC*-type plasmids of *a*-proteobacteria. On the other hand, the predicted amino acid sequence encoded by Orf2 exhibits similarity to replication proteins encoded by plasmids of *D. radiodurans* SARK and *Thermus* species. Hybrid plasmids consisting of pUE30 and pKatCAT5, which replicates in *E. coli* with a chloramphenicol resistance determinant, were shown to autonomously replicate in *D. grandis* ATCC43672. Deletion analysis revealed that Orf2 was necessary for replication of the plasmids in *D. grandis*. On the other hand, a DNA fragment encompassing the Orf1-coding region was involved in the instability of the plasmid in *D. grandis*. An expression plasmid that possesses the *D. radiodurans* minimal *groE* promoter was constructed, and a firefly luciferase gene was successfully expressed in *D. grandis*. The *D. grandis* host-vector system developed in this study should prove useful in the bioremediation of radioactive waste and for the investigation of DNA repair mechanisms.

1. Introduction

There is a wide diversity of radioresistance in the domain Bacteria, among which *Deinococcus* is the best known genus (Cox and Battista, 2005). The genus *Deinococcus* comprises more than 30 species that exhibit extraordinary resistance to UV or ionizing radiation in the absence of a differentiated resting stage (Asker et al., 2008; Callegan et al., 2008; Im et al., 2008; Kämpfer et al., 2008). Among these, *D. radiodurans* has been intensively studied in an effort to unravel mechanisms pertaining to DNA repair (Cox and Battista, 2005; Blasius et al., 2008). Given their characteristic radioresistance, *D. radiodurans* and *D. geothermalis* possess great potential for use in the bioremediation of radioactive waste mixtures containing organic solvents and heavy metals (Daly, 2000; Brim et al., 2003).

Several shuttle plasmids have been developed for *D. radiodurans*. Smith and Minton (1989) constructed two shuttle plasmids pS28 and pS19 that comprise the *E. coli* vector pS27 and full-length clones of the *D. radiodurans* stain SARK cryptic plasmids pUE10 (37 kb) and pUE11 (45 kb) respectively, and showed that these could replicate in *E. coli* and *D. radiodurans*. Both pS28 and pS19 are large in size (>40 kb). Subsequently, several smaller shuttle plasmids for *D. radiodurans* have been developed. Masters and Minton (1992) constructed pI3, a 16-kb plasmid containing replicons of pUE10 and the *E. coli* vector pKK232-8. Daly et al. (1994) constructed pMD66, a 27-kb plasmid containing replicons of pUE10 and the *E. coli* vector pBR322. The plasmid pMD66 was also used to express functional cloned genes in *D. geothermalis* (Brim et al., 2003). Meima and Lidstrom (2000) constructed pRAD1, a 6.3-kb plasmid containing replicons of pUE10 and the *E. coli* vector pMTL23. Given its small size and versatility, pRAD1 and its deriva-

* Corresponding author. Fax: +81 27 346 9688.
E-mail address: narumi.issei@jaea.go.jp (I. Narumi).
[1] Present address: College of Animal Sciences, Zhejiang University, Kaixuan Rd. 268, Huajiachi Campus, Hangzhou 310029, PR China.

0147-619X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.plasmid.2009.01.005

tives have frequently been used in recent years for the investigation of DNA repair mechanisms in *D. radiodurans* (e.g. Hua et al., 2003; Lecointe et al., 2004; Ohba et al., 2005; Satoh et al., 2006; Jolivet et al., 2006; Gao et al., 2006; Misra et al., 2006). Despite the variety of *Deinococcus* species presently known, genetic engineering investigations have predominantly been applied to *D. radiodurans* and *D. geothermalis*. The development of new host-vector systems is therefore required to expand applications to a broader variety of *Deinococcus* species.

In this study, attention was focused on the small 2.5-kb cryptic plasmid pUE30 from *D. radiopugnans* (Mackay et al., 1985) towards the development of new shuttle vectors for *Deinococcus* species. To accomplish this aim, the complete nucleotide sequence of pUE30 was determined, and hybrid plasmids were constructed by ligating pUE30 to a derivative of pKatCAT, a vector developed for gene disruption studies in *D. radiodurans* (Funayama et al., 1999). We show that the new shuttle vectors constructed in this study autonomously replicated, were stably maintained, and expressed a functional exogenous gene in *D. grandis*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Deinococcus radiopugnans ATCC19172^T, *D. grandis* ATCC43672^T, *D. radiodurans* ATCC13939^T and *D. proteolyticus* ATCC35074^T were purchased from American Type Culture Collection. *Deinococcus geothermalis* DSM11300^T and *D. murrayi* DSM11303^T were purchased from Deutsche Sammlung Von Mikroorganismen Und Zellkulturen. *Escherichia coli* strain JM109 was purchased from Takara Bio. In this study, *D. grandis* and *E. coli* were used as hosts for recombinant plasmids. *D. radiopugnans, D. grandis, D. radiodurans* and *D. proteolyticus* were grown at 30 °C in TGY broth containing 0.5% Bacto Tryptone (Difco), 0.1% glucose and 0.3% Bacto Yeast Extract (Difco), or on TGY agar supplemented with 1.5% Bacto Agar (Difco). *D. geothermalis* and *D. murrayi* were grown at 37 °C in TGY broth or TGY agar. *E. coli* was grown at 37 °C in LB broth-Miller (Difco) or on LB Agar-Lennox (Difco). The following antibiotics were added when necessary: chloramphenicol (3 μg per ml) for *Deinococcus* species; ampicillin (100 μg per ml), chloramphenicol (34 μg per ml) and spectinomycin (80 μg per ml) for *E. coli*.

2.2. Determination of the nucleotide sequence of pUE30

To isolate plasmid pUE30, *D. radiopugnans* cells grown to early stationary phase were harvested, resuspended in buffer P1 (Qiagen) containing lysozyme (1 mg per ml) and incubated at 37 °C for 30 min. Plasmid DNA was isolated using a QIAfilter Plasmid Midi Kit (Qiagen), and further purified by agarose gel electrophoresis and electroelution. Purified pUE30 DNA was digested with HincII or Aor51HI, which cut the plasmid at one site, and either fragment was ligated to the HincII site of the *E. coli* vector pGBM5 (Manen et al., 1997) to yield plasmids pZT1 or pZT5. pZT1 and pZT5 were then processed using a Kilo-sequence Deletion Kit (Takara Bio) to prepare nested deletion clones. The complete nucleotide sequence at the region of the pUE30 insertion was confirmed by DNA sequencing using the nested deletion clones as templates, universal sequencing primers (M4 and RP2) and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI Prism 377 DNA Sequencer.

2.3. Modification of pKatCAT

A NcoI site of pKatCAT (Funayama et al., 1999) was replaced with an NdeI site using the QuikChange Site-directed Mutagenesis Kit (Stratagene) to yield plasmid pKatCAT3. In order to allow for chloramphenicol selection of the recombinant plasmids in *E. coli*, the −35 promoter sequence TGGACA of the *D. radiodurans katA* promoter (*katA*-p) was modified to TTGACA in pKatCAT3 by site-directed mutagenesis to yield plasmid pKatCAT4. Finally, pKatCAT4 was digested with BspHI and EheI, blunted using T4 DNA polymerase and self-ligated to remove an ampicillin resistance determinant. The resulting plasmid pKatCAT5 was used as the source of a replicon required for the replication in *E. coli* and a selection marker in shuttle vectors.

2.4. Construction of shuttle vectors and their deletion derivatives

PCR was performed using *PfuTurbo* HotStart DNA polymerase (Stratagene) with pUE30 and the oligonucleotide primer set, UE30KpF1and UE30KpR1 (Table 1). PCR product was digested with KpnI and ligated to KpnI-digested pKatCAT5 to yield plasmid pZT23. Plasmid pZT23 was digested with AvaI and self-ligated to yield pZT25. Plasmid pZT23 was digested with EcoT14I and BpiI, blunted and self-ligated to yield pZT26. Plasmid pUE30 was amplified by PCR using the oligonucleotide primer set, UE30KpF2 and UE30KpR2 (Table 1). PCR product was digested with KpnI and ligated with KpnI-digested pKatCAT5 to yield pZT27. Plasmid pZT27 was digested with SpeI and self-ligated to yield pZT29. For the construction of pZT30, pZT27 was amplified by PCR using the oligonucleotide primer set, RepDNDF1 and RepDNDR1 (Table 1). PCR product was phosphorylated using T4 polynucleotide kinase and self-ligated. Following propagation in *E. coli*, the DNA sequence of the plasmids was determined to confirm the absence of errors during the construction procedure. Oligonucleotide primers used for DNA sequencing are listed in Table 1.

2.5. Plasmid transformation of Deinococcus species

Transformation of *Deinococcus* species using the calcium chloride method was as follows. Cells (1 ml) grown to early stationary phase were harvested by centrifugation, washed with 1 ml of TGY broth, resuspended in 0.1 ml of TGY broth, and to this was added 40 μl of 0.3 M $CaCl_2$. A 30 μl-aliquot of the cell mixture and 10 μl of plasmid DNA (200–400 μg per μl) were mixed in a new culture tube and incubated at 30 °C for 90 min. To this was then added 2 ml of TGY broth and the mixture was incubated at 30 °C for 24 h. The culture was appropriately diluted with 10 mM sodium phosphate buffer (pH 7.0), spread

Table 1
Oligonucleotide primers used in this study.

Name	Sequence (5′–3′)
For PCR	
UE30KpF1[a]	CGCGGTACCATCTTGCCTCTGAAACTGCGTT
UE30KpR1[a]	GCGGGTACCGCACTTTAGTGCTAATCTTGCG
UE30KpF2[a]	GAAACCGGGCTAGGTACCCCAAATCCTGAC
UE30KpR2[a]	GGTGGTACCCTCAGACATGATCGGGCCTC
RepDNDF1	ATGCTGCTGCCCGAGATCGTCG
RepDNDR1	TTCTCGCGCATTGACCCCTCGAA
Pgro154F[b]	AGACATCGATTGTCAGCTTCGGTCAG
Pgro154R[b]	AAGATCGATGCATGTGGGGTCCTCCT
Pluc+FP	TGGAAGACGCCAAAAACATAAAGAAAG
Pluc+RP	TTACACGGCGATCTTTCCGC
For Sequencing	
UE30F2	ATCTTGCCTCTGAAACTGCGTT
UE30F3	GCCTAGCCGCCCTGCGTGGCG
UE30F4	TCGGTGCCGTGGCTGAGCAAGG
UE30F5	CAGCACCTGTGACGCCTACCTG
RepD-F1	TCGATCATCTCGCACGCCCTGG
UE30F	GATGCCAAGAGCATAGTC
RepD-F2	CAAATCCTGACCGTGCCAAAG
ZT18EheI	CGCCAGCTGGCGTAATAGCG
UC-F1	GGCGATAAGTCGTGTCTTACCG
UC-F2	CCCTGATTCTGTGGATAACCG
RP2	GTGAGCGGATAACAATTTC
pkatCHK3	CGTAACACGCCACATCTTGCG
pkatCHK1	CATTGGGATATATCAACGGTGG
RepD-R1N	GTGTCCACCGGCTCGCCACGC
RepD-R2	CGCCGAGTTGGGGATCAC
RepD-R3	CGCCACTCGTGCTGCCAGTCC
RepD-R4	GCTGGACTCCTGCACATCCGC
UE30R	CATCTTCGTACTCTTCGG
M4	GTTTTCCCAGTCACGAC
ZT18BspH	CAAGCAGCAGATTACGCGCAG
UC-R3	ACTATCGTCTTGAGTCCAACC
UC-R2	ACGGTTATCCACAGAATCAGG
UC-R1	GTCGACCTGCAGGCATGCAAGC
pKatCHK2	CATATATTCGCAAGATGTGGCG
kat-35F1	GACCGAGGGCCCTTGACATTGAGAATG

[a] KpnI site was underlined.
[b] ClaI site was underlined.

onto TGY agar supplemented with chloramphenicol (3 μg per ml), and incubated at 30 °C for 2–3 days. *D. geothermalis* and *D. murrayi* were incubated at 37 °C instead of 30 °C.

Transformation of *D. grandis* using the electroporation method was as follows. Cells (200 ml) cultured in TGY broth to an OD_{600} of 0.6 were harvested by centrifugation, washed twice with 200 ml of cold 10% glycerol and resuspended in 0.5 ml of cold 10% glycerol (1/400 of the original culture volume). The cell suspension was dispensed into 40-μl aliquots and stored at −80 °C until use. For the transformation, an aliquot was thawed on ice, mixed with 1 μl of plasmid DNA (0.05, 0.5 or 1.5 μg per μl) and then transferred to a cold electroporation cuvette with a 0.1-cm inter-electrode gap (Bio-Rad). A Gene Pulser (Bio-Rad) was set at 25 μF, 600 W and 1.8 kV. Immediately following application of an electric pulse, the cell suspension was mixed with 1 ml of TGY broth, transferred into a new culture tube, and incubated at 30 °C for 90 min. The culture was appropriately diluted with 10 mM sodium phosphate buffer (pH 7.0), spread onto TGY agar supplemented with chloramphenicol (3 μg per ml), and incubated at 30 °C for 2 days. The transformation efficiency was calculated as the average number of transformants per μg of plasmid DNA used. Transformations with 0.05 and 0.5 μg of plasmid DNA gave approximately the same transformation frequency. But, transformation frequency was down to approximately 1/50-fold when 1.5 μg of plasmid DNA was used.

2.6. Plasmid isolation from D. grandis transformants

A culture of *D. grandis* transformant (3.5 ml) was harvested by centrifugation, and resuspended in 0.25 ml of buffer P1 (Qiagen) supplemented with lysozyme (10 mg per ml). Following incubation of the suspension at 37 °C for 30 min, plasmid DNA was isolated using a Qiaprep Spin Miniprep Kit (Qiagen) according to the standard protocol described in the manufacturer's instructions.

2.7. Plasmid stability test

Deinococcus grandis transformants were cultivated for 24 h in TGY broth supplemented with chloramphenicol (3 mg per μl). Cultures were diluted 4096-fold in TGY broth without chloramphenicol addition and cultivated for 24 h. Following cell division through 12 generations, cultures were again diluted 4096-fold in TGY broth. This procedure was repeated until cell division reached 96 generations. At each dilution step, aliquots (0.1 ml) of culture were withdrawn, appropriately diluted with 10 mM Tris–HCl (pH 7.0) and spread onto TGY agar supplemented with chloramphenicol (3 μg per ml) and chloramphenicol-free TGY agar to determine the percentage of chloramphenicol-resistant colonies in the total viable counts.

2.8. Construction of an expression plasmid for firefly luciferase gene

A 154-bp DNA fragment containing the *D. radiodurans groE* minimal promoter (*groE*-p) (Meima et al., 2001) was amplified by PCR from the *D. radiodurans* genome using the oligonucleotide primer set, Pgro154F and Pgro154R (Table 1). PCR product was digested with ClaI and ligated to the ClaI site of pZT29 to yield plasmid pZT90. To isolate the firefly luciferase gene, plasmid pSP-luc+ (Promega) was amplified by PCR using *PfuTurbo* HotStart DNA polymerase and the oligonucleotide primer set, Pluc+FP and Pluc+RP (Table 1). PCR product was phosphorylated using T4 polynucleotide kinase and ligated to pZT90 that had been digested with EcoT22I, blunted using T4 DNA polymerase and dephosphorylated using alkaline phosphatase. The resulting plasmid was designated pZTGL93.

2.9. Luciferase assay

Deinococcus grandis transformants carrying pZTGL93 were cultivated as described in Section 2.7. Following every 24 generations, a 10 μl-aliquot was withdrawn from the cultures and assayed for luciferase activity using the Bright-Glo Luciferase Assay System (Promega). Luminescence intensity was measured for 1 min using a Lumi Imager F1 Workstation (Roche Molecular Biomedicals).

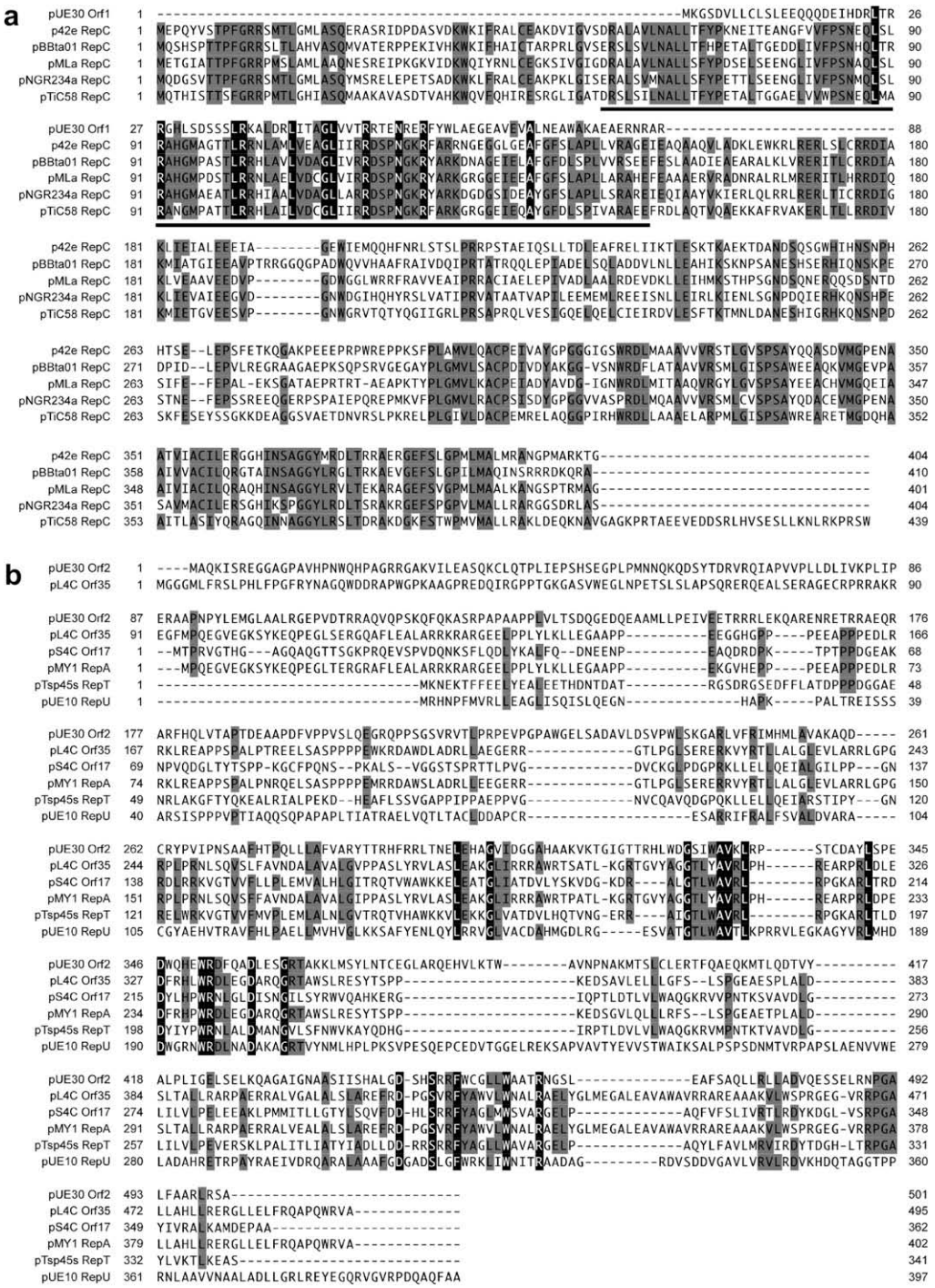

Fig. 1. (a) Multiple amino acid sequence alignment of residues encoded by *D. radiopugnans* ATCC19172 plasmid pUE30 Orf1, *Rhizobium etli* CFN 42 plasmid p42e RepC (González et al., 2006), *Bradyrhizobium* sp. BTAi1 plasmid pBBta01 RepC (Giraud et al., 2007), *Mesorhizobium loti* MAFF303099 plasmid pMLa RepC (Kaneko et al., 2000), *Rhizobium* sp. NGR234 plasmid pNGR234a RepC (Freiberg et al., 1997) and *Agrobacterium tumefaciens* C58 plasmid pTiC58 RepC (Li and Farrand, 2000). Protein sequence motif was analyzed using the MOTIF Search web program (http://motif.genome.jp/). IclR-type helix-turn-helix DNA-binding motif found in pMLa RepC was indicated by an underline. (b) Multiple amino acid sequence alignment of residues encoded by *D. radiopugnans* ATCC19172 plasmid pUE30 Orf2, *Thermus* sp. 4C plasmid pL4C Orf35 (Ruan and Xu, 2007), *Thermus* sp. 4C plasmid pS4C Orf17 (Ruan and Xu, 2007), *Thermus* sp. ATCC 27737 plasmid pMY1 RepA (de Grado et al., 1998), *Thermus* sp. YS45 plasmid pTsp45s RepT (Wayne and Xu, 1997) and *D. radiodurans* SARK plasmid pUE10 RepU (Meima and Lidstrom, 2000). Dashes indicate gaps in the alignment. Numbers are the coordinates of each protein. Reverse letters represent amino acid residues conserved in all proteins. Gray boxes indicate highly conserved amino acid residues. Multiple alignment was employed using the GENETYX-MAC software package version 14 (GENETYX Co., Ltd.).

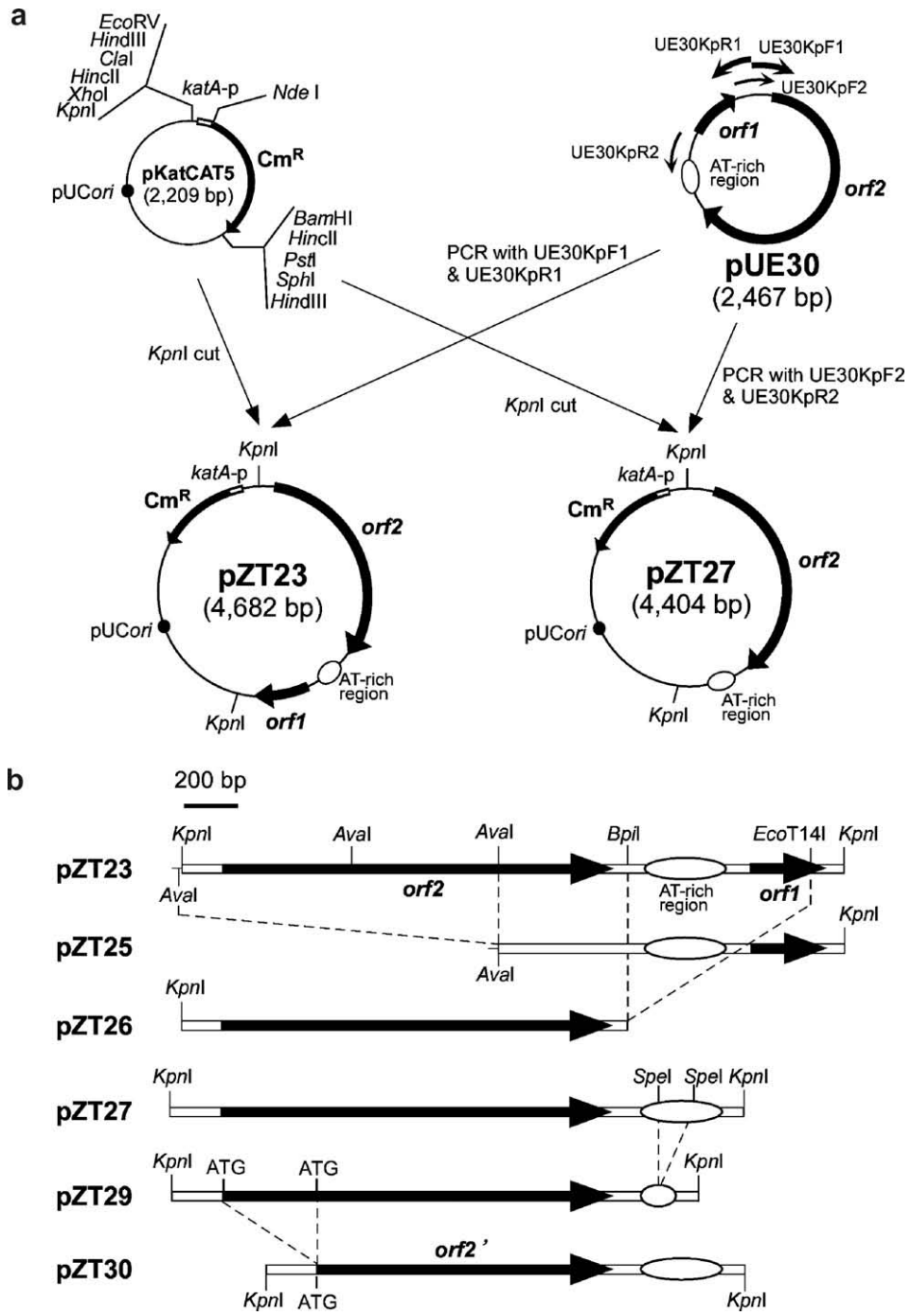

Fig. 2. (a) Construction of shuttle vectors pZT23 and pZT27. (b) Schematic presentation of pUE30-derived regions in pZT23, pZT27 and their deletion derivatives. pKatCAT5-derived regions are not shown. For construction details, see Section 2.4.

3. Results and discussion

3.1. Nucleotide sequence of plasmid pUE30 from D. radiopugnans

Mackay et al. (1985) reported the presence of plasmid pUE30 *D. radiopugnans* with size of ca. 2.5 kb as determined by electron microscopic examination. However, the nucleotide sequence had not yet been determined. The complete nucleotide sequence of pUE30 was determined after the cloning of pUE30 into the *E. coli* vector pGBM5 (Manen et al., 1997). pUE30 comprises 2467 bp and possesses two open reading frames, one encoding 88 amino acid residues (Orf1) and the other encoding 501 amino acid residues (Orf2) (Figs. 1a and b). The predicted amino acid sequence encoded by Orf1 exhibits similarity to the N-terminal re-

gions of replication protein C (RepC) encoded by *repABC*-type plasmids of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Agrobacterium*, and where 11 amino acid residues are perfectly conserved (Fig. 1a) (Freiberg et al., 1997; Li and Farrand, 2000; Kaneko et al., 2000; González et al., 2006; Giraud et al., 2007). Protein sequence motif analysis revealed that the N-terminal regions of RepCs from pBBta01, pMLa and pTiC58 share an IclR-type helix-turn-helix DNA-binding motif (Pfam ID: HTH_IclR), where all conserved residues are located (Fig. 1a). However, the helix-turn-helix DNA-binding motif was not found in Orf1 by protein sequence motif analysis. Therefore, the importance of the conserved amino acid residues between Orf1 and RepCs remains unclear. On the other hand, the predicted amino acid sequence encoded by Orf2 exhibits similarity to those residues encoded by Orf35 of plasmid pL4C and Orf17 of plasmid pS4C from *Thermus* sp. strain 4C (Ruan and Xu, 2007), RepA of plasmid pMY1 from *Thermus* sp. ATCC 27737 (de Grado et al., 1998), RepT of plasmid pTsp45s from *Thermus* sp. strain YS45 (Wayne and Xu, 1997) and RepU of plasmid pUE10 from *D. radiodurans* strain SARK (Meima and Lidstrom, 2000), and where 17 amino acid residues are perfectly conserved (Fig. 1b). Although we tried protein sequence motif analysis, no conserved motif was found between Orf2 and Rep proteins in Fig. 1b. Nevertheless, the fact that Orf2 has similarity to Rep proteins of plasmids from *Deincoccus* and *Thermus* suggests that the conserved amino acid residues might play an important role in the replication of plasmids in *Deinococcus-Thermus* phylum.

The overall G + C content of pUE30 is 58.2%. However, a 370-bp AT-rich region (36.0% G + C) was found in the non-coding region started from 89-bp downstream of *orf2* (Figs. 2a and b). Meima and Lidstrom (2000) showed that the minimal region required for autonomous replication of *D. radiodurans* plasmid pUE10 resides within a 2624-bp fragment containing the *repU* gene and an AT-rich sequence downstream of *repU*. The similarities in amino acid sequence homology and DNA sequence arrangement between pUE10 and pUE30 suggest that Orf1 of pUE30 encodes a predicted replication initiation protein and that the AT-rich region of pUE30 may be involved in replication initiation. Two putative DnaA boxes (TTATC) have been identified in the DNA sequences of *D. radiodurans* plasmid pUE10 (Meima and Lidstrom, 2000) and *Thermus* plasmid pTsp45s (Wayne and Xu, 1997). However, sequences that resemble DnaA boxes were not found in pUE30. The nucleotide sequence of pUE30 has been assigned in the DDBJ/EMBL/GenBank Accession No. AB473550.

3.2. Construction of shuttle vectors

Prior to the construction of shuttle vectors, plasmid pKatCAT, a vector developed for gene disruption studies in *D. radiodurans* (Funayama et al., 1999), was modified as described in Section 2.3. The resulting plasmid pKatCAT5 contains a replicon of the *E. coli* vector pUC19 and a chloramphenicol acetyltransferase gene capable of functioning in *E. coli* and *D. radiodurans* with a *D. radiodurans* catalase promoter (*katA*-p). The hybrid plasmid pZT23 was then constructed by ligating pKatCAT5 to a PCR fragment containing full-length pUE30 (Fig. 2a). Plasmid pZT23 was utilized for the transformation of *D. radiodurans*, *D. radiopugnans*, *D. proteolyticus*, *D. grandis*, *D. geothermalis* and *D. murrayi* using the calcium chloride method. When *D. grandis* was used as a host for the transformation, a few chloramphenicol resistant transformants were obtained. Although *D. radiodurans* and *D. geothermalis* are known to be experimentally transformable with plasmids,

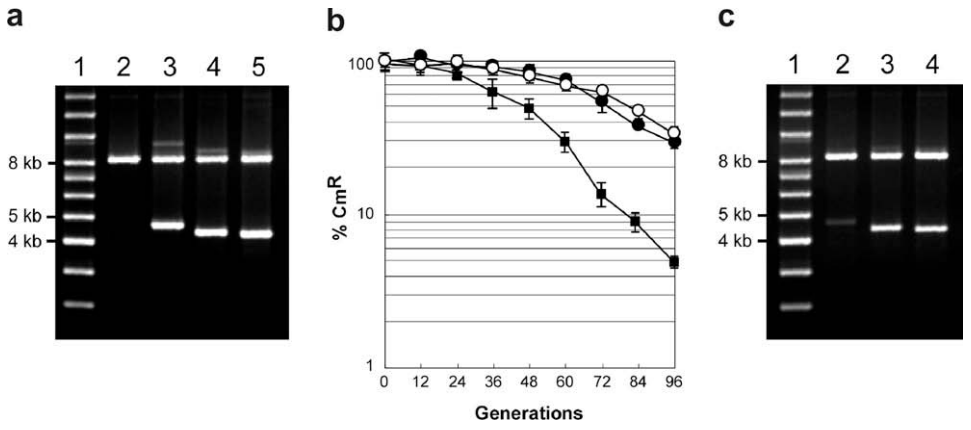

Fig. 3. (a) Plasmid profiles of *D. grandis* and *D. grandis* transformants. Lane 1, Supercoiled DNA Ladder (Invitrogen); lane 2, *D. grandis* (no shuttle vector); lane 3, *D. grandis* transformant carrying pZT23; lane 4, *D. grandis* transformant carrying pZT27; lane 5, *D. grandis* transformant carrying pZT29. Plasmid DNA was isolated from cells cultured in chloramphenicol-enriched broth, except for the DNA in lane 2, which was isolated from cells cultured in chloramphenicol-free broth. (b) Stability of shuttle vectors in *D. grandis*. Transformants were grown in chloramphenicol-free broth until 96 generations. Symbols: closed square, *D. grandis* transformant carrying pZT23; closed circles, *D. grandis* transformant carrying pZT27; open circles, *D. grandis* transformant carrying pZT29. Plotted data are the mean ± standard error of three independent experiments; $n = 9$. (c) Plasmid profiles of *D. grandis* transformants cultivated until 96 generations in chloramphenicol-free broth. Lane 1, Supercoiled DNA Ladder (Invitrogen); lane 2, *D. grandis* transformant carrying pZT23; lane 3, *D. grandis* transformant carrying pZT27; lane 4, *D. grandis* transformant carrying pZT29.

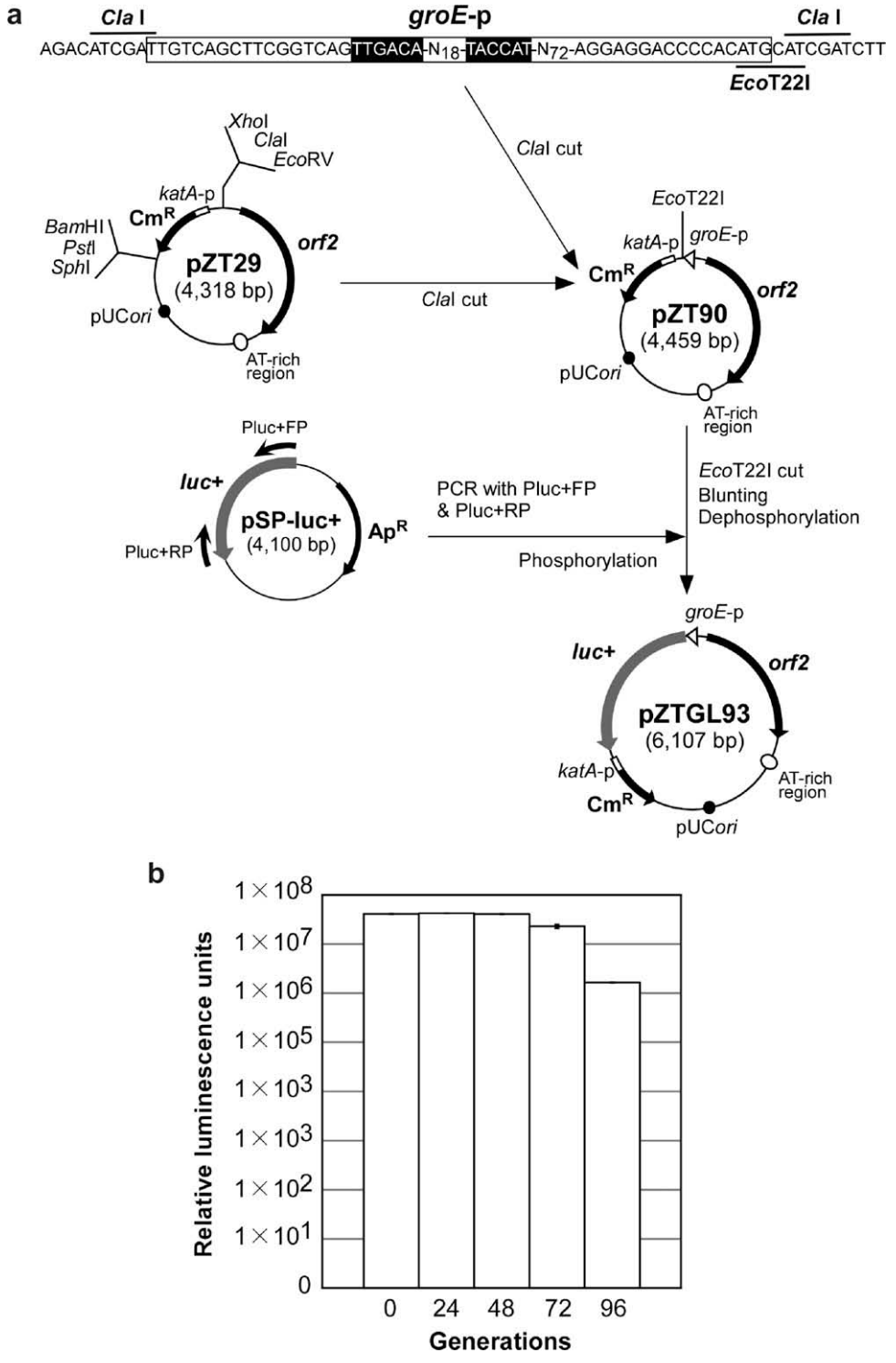

Fig. 4. (a) Construction scheme of the expression plasmid containing the firefly luciferase gene. For construction details, see Section 2.8. The nucleotide sequence of pZT90 has been assigned in the DDBJ/EMBL/GenBank Accession No. AB473551. (b) Luciferase activity of *D. grandis* transformant carrying pZTGL93 and grown in chloramphenicol-free broth. Every 24 generations, 10 μl-aliquots were withdrawn from the cultures and assayed for luciferase activity. The 10 μl-aliquots contained approximately 3.0×10^6 cells. Data represent the mean ± standard deviation; $n = 6$.

transformant was not obtained from *Deinococcus* species other than *D. grandis* in this study. *D. radiopugnans* is the original host of pUE30. However, it is not clear at present whether *D. radiopugnans* is experimentally transformable.

In this study, *D. grandis* was employed as a host for the shuttle vectors in further experiments. To increase the transformation efficiency, a methodology utilizing electroporation was developed for *D. grandis* by optimizing bacterial cell concentration and electric pulse conditions. Plasmid pZT23 could transform *D. grandis* at an efficiency of approximately 6.6×10^3 transformants per μg of DNA using the electroporation method developed.

In an effort to determine the region required for autonomous replication of the shuttle vectors in *D. grandis*, several deletion plasmids were constructed (Fig. 2b). Electrotransformation experiments revealed that pZT27 and pZT29, unlike the other constructs examined, could transform *D. grandis* at efficiencies of approximately 1.1×10^4 and 1.4×10^4 transformants per μg of DNA, respectively. This result indicated that the entire length of the *orf2* region is needed to replicate the shuttle vectors in *D. grandis*, unlike the case with *orf1*. Only part of the AT-rich region downstream of *orf2* is needed, and an 86-bp region surrounded by two SpeI sites in the AT-rich region is dispensable. These results further support the notion that *Orf2* of pUE30 encodes a putative replication initiation protein.

3.3. Stability of shuttle vectors

To confirm whether the introduced shuttle vectors were maintained in the transformants, plasmid DNA was isolated from *D. grandis* cells that were cultured in chloramphenicol-enriched broth and the DNA was analyzed by agarose gel electrophoresis. This revealed that *D. grandis* possesses an endogenous plasmid of 8 kb (Fig. 3a). The shuttle vectors were predominantly present as monomers in the *D. grandis* transformants. The copy number of the shuttle vectors was comparable to that of the endogenous plasmid. To determine whether the shuttle vectors could stably replicate under non-selective conditions, *D. grandis* transformants carrying pZT23, pZT27 and pZT29 were grown in non-selective broth until 96 generations by repeated dilution and incubation, and the percentage of chloramphenicol-resistant colonies in the total viable count was determined. As shown in Fig. 3b, plasmids pZT27 and pZT29 (approximately 30% chloramphenicol-resistant colonies after 96 generations) exhibited higher stability than pZT23 (4.7% chloramphenicol-resistant colonies after 96 generations). The profiles of the plasmids isolated from *D. grandis* that had been cultured until 96 generations in non-selective broth (Fig. 3c) revealed the structural integrity of the plasmids after multiple replications, and reflected the rate of chloramphenicol-resistant colonies shown in Fig. 3b. The DNA sequence of the shuttle vectors following transformation of *E. coli* using pZT23, pZT27 and pZT29 isolates from 96 generations in non-selective broth was determined following isolation of DNA from the *E. coli* transformants. The determined sequences were identical with those of the original plasmid constructs. These results indicate that the instability of pZT23 was not due to a plasmid mutation event. We suggest that the DNA fragment deleted in pZT27 is involved in the instability of the plasmid in *D. grandis*.

3.4. Expression of firefly luciferase gene in D. grandis

In an effort to express a functional exogenous gene in *D. grandis*, an expression vector based on pZT29 was constructed. The strategy underlying the construction is illustrated in Fig. 4a. The expression vector pZT90 possesses the *D. radiodurans groE* minimal promoter (*groE*-p) (Meima et al., 2001) for the expression of an exogenous gene. To express the firefly luciferase gene, a region from the second nucleotide of the start codon to the third nucleotide of the stop codon of the gene was amplified by PCR using a blunt-end producing DNA polymerase and then in-frame inserted into pZT90. The desired construct was easily screened by subjecting *E. coli* transformants to a luciferase assay. The resulting expression plasmid pZTGL93 was then introduced into *D. grandis* and tested for stability under non-selective conditions. As shown in Fig. 4b, *D. grandis* transformants carrying pZTGL93 exhibited constant luminescence intensity until 48 generations, suggesting that the property of pZT29 pertaining to stable maintenance in *D. grandis* was inherited by pZTGL93.

3.5. Concluding remarks

In this study, *E. coli–D. grandis* shuttle vectors were developed based on the small 2.5-kb cryptic plasmid pUE30 from *D. radiopugnans*. The shuttle vectors autonomously replicated, were stably maintained, and expressed a functional firefly luciferase gene in *D. grandis*. Plasmid pZT90 could be used as an expression vector for other exogenous genes by inserting the genes downstream of the *D. radiodurans* minimal *groE* promoter. As *D. grandis* exhibits extreme radioresistance as is the case with *D. radiodurans* and *D. geothermalis*, the *D. grandis* host-vector system developed in this study should prove useful in the bioremediation of radioactive waste and for the investigation of DNA repair mechanisms.

Acknowledgments

We thank all members of our research group for useful discussions. This work was supported in part by a Grant-in-Aid for Scientific Research (B) 19380054 from the Japan Society for the Promotion of Science (to I.N.) and by a Grant-in-Aid for Young Scientists (B) 20780079 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K.S.).

References

Asker, D., Awad, T.S., Beppu, T., Ueda, K., 2008. *Deinococcus misasensis* and *Deinococcus roseus*, novel members of the genus *Deinococcus*, isolated from a radioactive site in Japan. Syst. Appl. Microbiol. 31, 43–49.

Blasius, M., Sommer, S., Hübscher, U., 2008. *Deinococcus radiodurans*: what belongs to the survival kit? Crit. Rev. Biochem. Mol. Biol. 43, 221–238.

Brim, H., Venkateswaran, A., Kostandarithes, H.M., Fredrickson, J.K., Daly, M.J., 2003. Engineering *Deinococcus geothermalis* for bioremediation of high-temperature radioactive waste environments. Appl. Environ. Microbiol. 69, 4575–4582.

Cox, M.M., Battista, J.R., 2005. *Deinococcus radiodurans* – the consummate survivor. Nat. Rev. Microbiol. 3, 882–892.

Callegan, R.P., Nobre, M.F., McTernan, P.M., Battista, J.R., Navarro-González, R., McKay, C.P., da Costa, M.S., Rainey, F.A., 2008.

Description of four novel psychrophilic, ionizing radiation-sensitive *Deinococcus* species from alpine environments. Int. J. Syst. Evol. Microbiol. 58, 1252–1258.

Daly, M.J., 2000. Engineering radiation-resistant bacteria for environmental biotechnology. Curr. Opin. Biotechnol. 11, 280–285.

Daly, M.J., Ouyang, L., Fuchs, P., Minton, K.W., 1994. In vivo damage and *recA*-dependent repair of plasmid and chromosomal DNA in the radiation-resistant bacterium *Deinococcus radiodurans*. J. Bacteriol. 176, 3508–3517.

de Grado, M., Lasa, I., Berenguer, J., 1998. Characterization of a plasmid replicative origin from an extreme thermophile. FEMS Microbiol. Lett. 165, 51–57.

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W.J., Rosenthal, A., Perret, X., 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387, 394–401.

Funayama, T., Narumi, I., Kikuchi, M., Kitayama, S., Watanabe, H., Yamamoto, K., 1999. Identification and disruption analysis of the *recN* gene in the extremely radioresistant bacterium *Deinococcus radiodurans*. Mutat. Res. 435, 151–161.

Gao, G., Le, D., Huang, L., Lu, H., Narumi, I., Hua, Y., 2006. Internal promoter characterization and expression of the *Deinococcus radiodurans pprI-folP* gene cluster. FEMS Microbiol. Lett. 257, 195–201.

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J.C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franck, W.L., Chang, W.S., Saunders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Verméglio, A., Médigue, C., Sadowsky, M., 2007. Legumes symbioses: absence of *Nod* genes in photosynthetic bradyrhizobia. Science 316, 1307–1312.

González, V., Santamaría, R.I., Bustos, P., Hernández-González, I., Medrano-Soto, A., Moreno-Hagelsieb, G., Janga, S.C., Ramírez, M.A., Jiménez-Jacinto, V., Collado-Vides, J., Dávila, G., 2006. The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. Proc. Natl. Acad. Sci. USA 103, 3834–3839.

Hua, Y., Narumi, I., Gao, G., Tian, B., Satoh, K., Kitayama, S., Shen, B., 2003. PprI: a general switch responsible for extreme radioresistance of *Deinococcus radiodurans*. Biochem. Biophys. Res. Commun. 306, 354–360.

Im, W.T., Jung, H.M., Ten, L.N., Kim, M.K., Goodfellow, M., Lim, S., Jung, J., Lee, S.T., 2008. *Deinococcus aquaticus* sp. nov., isolated from fresh water, and *Deinococcus caeni* sp. nov., isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 58, 2348–2353.

Jolivet, E., Lecointe, F., Coste, G., Satoh, K., Narumi, I., Bailone, A., Sommer, S., 2006. Limited concentration of RecA delays DNA double-strand break repair in *Deinococcus radiodurans* R1. Mol. Microbiol. 59, 338–349.

Kämpfer, P., Lodders, N., Huber, B., Falsen, E., Busse, H.J., 2008. *Deinococcus aquatilis* sp. nov., isolated from water. Int. J. Syst. Evol. Microbiol. 58, 2803–2806.

Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., Tabata, S., 2000. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res. 7, 331–338.

Lecointe, F., Coste, G., Sommer, S., Bailone, A., 2004. Vectors for regulated gene expression in the radioresistant bacterium *Deinococcus radiodurans*. Gene 336, 25–35.

Li, P.L., Farrand, S.K., 2000. The replicator of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. J. Bacteriol. 182, 179–188.

Mackay, M.W., Al-Bakri, G.H., Moseley, B.E.B., 1985. The plasmids of *Deinococcus* spp. and the cloning and restriction mapping of the *D. radiophilus* plasmid pUE1. Arch. Microbiol. 141, 91–94.

Manen, D., Pougeon, M., Damay, P., Geiselmann, J., 1997. A sensitive reporter gene system using bacterial luciferase based on a series of plasmid cloning vectors compatible with derivatives of pBR322. Gene 186, 197–200.

Masters, C.I., Minton, K.W., 1992. Promoter probe and shuttle plasmids for *Deinococcus radiodurans*. Plasmid 28, 258–261.

Meima, R., Lidstrom, M.E., 2000. Characterization of the minimal replicon of a cryptic *Deinococcus radiodurans* SARK plasmid and development of versatile *Escherichia coli–D. radiodurans* shuttle vectors. Appl. Environ. Microbiol. 66, 3856–3867.

Meima, R., Rothfuss, H.M., Gewin, L., Lidstrom, M.E., 2001. Promoter cloning in the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. 183, 3169–3175.

Misra, H.S., Khairnar, N.P., Kota, S., Shrivastava, S., Joshi, V.P., Apte, S.K., 2006. An exonuclease I-sensitive DNA repair pathway in *Deinococcus radiodurans*: a major determinant of radiation resistance. Mol. Microbiol. 59, 1308–1316.

Ohba, H., Satoh, K., Yanagisawa, T., Narumi, I., 2005. The radiation responsive promoter of the *Deinococcus radiodurans pprA* gene. Gene 363, 133–141.

Ruan, L., Xu, X., 2007. Sequence analysis and characterizations of two novel plasmids isolated from *Thermus* sp. 4C. Plasmid 58, 84–87.

Satoh, K., Ohba, H., Sghaier, H., Narumi, I., 2006. Down-regulation of radioresistance by LexA2 in *Deinococcus radiodurans*. Microbiology 152, 3217–3226.

Smith, M.D., Minton, K.W., 1989. Shuttle plasmids constructed by the transformation of an *Escherichia coli* cloning vector into two *Deinococcus radiodurans* plasmids. Plasmid 22, 132–142.

Wayne, J., Xu, S.Y., 1997. Identification of a thermophilic plasmid origin and its cloning within a new *Thermus-E. coli* shuttle vector. Gene 195, 321–328.