Disruption analysis of *DR1420* and/or *DR1758* in the extremely radioresistant bacterium *Deinococcus radiodurans*

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The extremely radioresistant bacterium *Deinococcus radiodurans* encodes two genes that are homologous to those involved in bacterial lysine biosynthesis. In lysine biosynthesis, these genes are involved in the aminoadipate pathway and the diaminopimelate (DAP) pathway. *DR1420* is homologous to *lysZ*, which is essential for bacterial lysine biosynthesis via the aminoadipate pathway, and *DR1758* is homologous to *lysA*, which is essential for lysine biosynthesis via the DAP pathway. In this study, *DR1420* and/or *DR1758* were disrupted. Each disruptant of *DR1420* and *DR1758*, and of *DR1420* or *DR1758* grew in a minimal medium, as did the wild-type. These results show that *D. radiodurans* performs lysine biosynthesis in a unique way.

Keywords: *Deinococcus radiodurans*, *Thermus thermophilus*, lysine biosynthesis, gene disruption, evolution

INTRODUCTION

Lysine had been believed to be synthesized from aspartic acid through the diaminopimelate (DAP) pathway in all bacteria. However, an extremely thermophilic member of the Bacteria, *Thermus thermophilus*, has been shown to synthesize lysine not through the DAP pathway but through the aminoadipate pathway (Kobashi *et al.*, 1999; Kosuge & Hoshino, 1998). *T. thermophilus* possesses a gene cluster that consists of *lysS*, *lysT*, *lysU*, *lysV*, *lysW*, *lysX*, *lysY* and *lysZ*, which encodes proteins responsible for lysine biosynthesis (Nishida *et al.*, 1999).

Lysine biosynthesis via the aminoadipate pathway has been suggested for an extremely thermophilic anaerobic member of the Archaea, *Thermoproteus neutrophilus*; the presence of this pathway in this organism was inferred from acetate-assimilation patterns (Schäfer *et al.*, 1989). In addition, another extremely thermophilic anaerobic member of the Archaea, *Pyrococcus*, has a gene cluster similar to that responsible for lysine biosynthesis in *Thermus thermophilus* (Nishida *et al.*, 1999). At the present time, no bacterium other than *T. thermophilus* is known to synthesize lysine via the aminoadipate pathway, and no organism is known to synthesize lysine through pathways other than the aminoadipate or DAP pathways.

The complete nucleotide sequence of the *Deinococcus radiodurans* genome has been determined (White *et al.*, 1999). Genome analysis has revealed that approximately half of all of the ORFs identified in *D. radiodurans* encode proteins of unknown function. It has been remarked that the absence of key enzymes for lysine biosynthesis via the DAP pathway in *D. radiodurans* is a puzzling feature, since this organism does not require lysine for growth (Makarova *et al.*, 2001). No gene cluster similar to the *T. thermophilus* *lys* gene cluster is seen within the *D. radiodurans* genome sequence, although genes homologous to the *lys* cluster are found in an unco-ordinated fashion within the genome (Nishida, 2001a; Miyazaki *et al.*, 2001). The existence of this set of homologous genes suggests that *D. radiodurans* synthesizes lysine in the same way as *T. thermophilus*. However, *D. radiodurans* also possesses a homologue of *lysA*, *DR1758*. In almost all bacteria, the product of *lysA* expression catalyses the decarboxylation of DAP in the last step of the typical DAP pathway for lysine biosynthesis (Miyazaki *et al.*, 2001).

The deduced amino-acid sequence of another *D. radiodurans* gene, *DR1420*, shows 68% identity to that of *T. thermophilus* *lysZ*, whose product is assumed to function as an *N*-acetylamino-adipate kinase based on sequence similarity with other proteins and the auxotrophic nature of strains mutated in this gene (Nishida *et al.*, 1999). The deduced amino-acid sequence of *DR1758* shows 32% identity to the DAP decarboxylase (*LysA*) of *Escherichia coli*.

Abbreviation: DAP, diaminopimelate.

The aim of this study was to disrupt *DR1420* (the *lysZ* homologue) and/or *DR1758* (the *lysA* homologue) in *D. radiodurans* and to test the requirement of lysine for growth in mutants carrying the disrupted gene(s).

METHODS

Strain and media. *D. radiodurans* R1^T (ATCC 13939^T) was purchased from the American Type Culture Collection and was used as the parental strain. *D. radiodurans* was grown at 30 °C in TGY broth (0.5% Bacto-tryptone, 0.1% glucose, 0.3% yeast extract) or on TGY agar (TGY broth supplemented with 1.5% agar). Chloramphenicol or kanamycin (both at 3–4 µg ml⁻¹) was added to the medium if necessary. Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen).

Disruption of *DR1420* in *D. radiodurans*. This was performed by using the direct insertional mutagenesis technique described by Funayama *et al.* (1999) with slight modifications. First, a 1.4 kb DNA fragment containing *DR1420* was amplified by PCR using the primers 5'-CGAGGCCGGGGCAACGCGG-GAAGG-3' and 5'-ATGCGGTCCTGCGCGAATTTCGAGG-3'. Since *DR1420* has a single *Eco47III* site (AGC↓GCT), we designed the PCR primers so that the *Eco47III* site was at the middle of the amplified fragment. The amplification was carried out using PCR Beads (Amersham Pharmacia Biotech) and the following protocol: denaturation at 95 °C for 5 min, followed by 30 cycles at 96 °C for 1 min and 60 °C for 1 min, with a final extension at 60 °C for 10 min. The PCR product was then cloned into the pDrive Cloning Vector (Qiagen). Plasmid pDrive was digested with *Eco47III*; the linearized plasmid was then ligated to a 0.9 kb *HincII* fragment containing the chloramphenicol-resistance gene (*cat*) from pKatCAT (Funayama *et al.*, 1999). We then performed a PCR using the resulting plasmid as the template and the same primers as described above. The PCR product was purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech). It was then used to transform *D. radiodurans*.

D. radiodurans was cultured in TGY broth (2 ml) until an OD₆₆₀ value of 0.6 was reached. The cells were collected by centrifugation (7000 r.p.m. for 5 min), washed in 2 ml of TGY broth, resuspended in 200 µl of TGY broth and then mixed with 80 µl of 0.3 M CaCl₂. Aliquots (30 µl) of the suspension were added to 10 µl (0.5 µg) of the PCR product prepared above, and the mixture was incubated at 30 °C. After 90 min incubation, 2 ml of TGY broth was added to the sample and this was incubated for an additional 24 h. Finally, 200 µl of the suspension was spread onto a TGY plate containing chloramphenicol. Colonies that grew on the plate were picked up as candidate strains with a knockout in the *DR1420* gene. Disruption of *DR1420* was confirmed by PCR using the primers 5'-ACGCCATAACCGCCATGATA-3' and 5'-TCT-AGGAAACCACAGTCC-3', the positions of which in the genomic sequence of *D. radiodurans* were just outside those of the primers used for constructing the disruption.

Disruption of *DR1758* in *D. radiodurans*. The *DR1758* gene has a single *HincII* [GT(C,T)↓(A,G)AC] site. We performed the PCR amplification so that a 1.2 kb DNA fragment containing the *HincII* site at the middle of the fragment would be generated. The PCR primers used were 5'-AAGGTAAG-TGCTGCTCAT-3' and 5'-GACATGGCGCACAGCACC-3'; amplification was carried out using the same protocol as described for the disruption of *DR1420*. The PCR product was

cloned into the pCR2.1 Vector (Invitrogen); the plasmid was then digested with *HincII*. The linearized plasmid was ligated with a 1.0 kb *HincII* fragment from pKatAPH2, a kanamycin-resistant version of pKatCAT. Using the resulting plasmid as the template, PCR was performed with the primers described above. The amplified DNA fragment was introduced into *D. radiodurans* ATCC 13939^T and the *DR1420* disruptant to generate a *DR1758* disruptant and a *DR1420/DR1758* double-disruptant, respectively. Disruption was confirmed by PCR, using primers 5'-GGGCCCAGCTTAAAGCGC-3' and 5'-ATCATCGCGCCGATGACC-3'.

Auxotrophic complementation test. The mutants were cultured in TGY broth for 24 h. After harvesting of the cultures by centrifugation, the cell pellets were washed three times with a minimal medium developed for *D. radiodurans* (Venkateswaran *et al.*, 2000) that does not contain lysine. The cells were then cultured in this minimal medium at 27 °C for 2 days. The optical density of the cultures was determined at 660 nm, using a spectrophotometer.

RESULTS AND DISCUSSION

We constructed three mutant strains of *D. radiodurans* ATCC 13939^T: one had a disruption in *DR1420*, one had a disruption in *DR1758* and one had a disruption in both *DR1420* and *DR1758* (see Methods for details). The specific disruption in each mutant strain was confirmed by PCR (Fig. 1). We expected that some of the mutants would not grow in minimal medium because of the discontinuation of the lysine biosynthetic pathway. However, all of the disruptants did grow in the minimal medium (Table 1).

For *T. thermophilus*, the synthesis of lysine via the aminoadipate pathway has been shown to be unique (Kobashi *et al.*, 1999; Kosuge & Hoshino, 1998). This pathway is not similar to the fungal aminoadipate pathway but instead resembles the pathway from glutamine to ornithine involved in bacterial arginine biosynthesis (Miyazaki *et al.*, 2001; Nishida *et al.*, 1999; Nishida & Nishiyama, 2000). In the *T. thermophilus* lysine biosynthetic pathway, the N-

Fig. 1. Confirmation of gene disruption by PCR. Lanes: 1, DNA ladder; 2, product from ATCC 13939^T (wild-type) amplified using primers for *DR1420* disruption; 3, product from ATCC 13939^T amplified using primers for *DR1758* disruption; 4, product from the *DR1420* disruptant amplified using primers for *DR1420* disruption; 5, product from the *DR1420* disruptant amplified using primers for *DR1758* disruption; 6, product from the *DR1758* disruptant amplified using primers for *DR1420* disruption; 7, product from the *DR1758* disruptant amplified using primers for *DR1758* disruption; 8, product from the *DR1420/DR1758* disruptant amplified using primers for *DR1420* disruption; 9, product from the *DR1420/DR1758* disruptant amplified using primers for *DR1758* disruption.

Table 1. Growth of *D. radiodurans* in liquid minimal medium

Strain	OD ₆₆₀ at		
	0 h	24 h	47 h
Wild-type	0.29	0.51	0.56
DR1420 mutant	0.28	0.48	0.48
DR1758 mutant	0.31	0.53	0.56
DR1420/DR1758 mutant	0.33	0.54	0.59

acetylaminoadipate kinase catalyses the conversion of *N*-acetylaminoadipate to *N*-acetylaminoadipate 6-phosphate. It has been assumed that *lysZ* encodes this enzyme in *T. thermophilus*. A *lysZ* disruptant of *T. thermophilus* did not grow in minimal medium, but the addition of lysine to the medium restored growth (Nishida *et al.*, 1999). The DR1420 gene of *D. radiodurans* has the highest similarity with the *T. thermophilus lysZ* gene. In contrast to the *T. thermophilus lysZ* disruptant, a *D. radiodurans* mutant disrupted in DR1420 could grow in minimal medium (Table 1). This result was unexpected, as *D. radiodurans* does not carry another typical *lysZ* homologue; hence, it was suggested that DR1420 was not essential for lysine biosynthesis in *D. radiodurans*. In general, enzymes that show strict substrate specificity are believed to originate from an ancestor that had a broad substrate specificity (Horowitz, 1965; Jensen, 1976; Parsot *et al.*, 1987; Roy, 1999). This is certainly the case for enzymes involved in amino-acid biosynthesis. Recently, it has been shown that the broad substrate specificity of enzymes plays an important role in prokaryotic amino-acid biosynthesis (Nishida, 2001b; Miyazaki *et al.*, 2001). Therefore, we suggest that *D. radiodurans* employs a protein with a broad substrate specificity that functions as an *N*-acetylaminoadipate kinase and which does not have significant similarity to *LysZ*. The difference in the requirement for lysine between *T. thermophilus* and *D. radiodurans* further suggests that the genes organized in a gene cluster in *T. thermophilus* are specialized in their function, whereas the genes scattered in the genome of *D. radiodurans* are not specialized in their function.

It was also shown that the *lysA* homologue DR1758 was not essential for lysine biosynthesis in *D. radiodurans* (Table 1). This result was expected, because other genes encoding key enzymes for lysine biosynthesis via the DAP pathway are absent from the *D. radiodurans* genome. It was also found that DR1758 was not related to genes involved in the aminoadipate pathway for lysine biosynthesis. Thus, at present, it is difficult to infer the reason as to why *D. radiodurans* has a *lysA* homologue. The DR1420/DR1758 double disruptant could also grow in the minimal medium used in this study (Table 1).

We have shown that the system for lysine biosynthesis in *D. radiodurans* is not identical to that of *T. thermo-*

philus, even though the former has homologues of the genes involved in the lysine biosynthetic pathway of the latter. Through further studies, including a combination of gene-disruption and biochemical analyses, we hope to elucidate the nature of the lysine biosynthetic pathway in *D. radiodurans* and to elucidate the evolutionary relationship between the aminoadipate pathway and the DAP pathway.

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