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.

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.

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* R1T (ATCC 13939) 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% 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 insertion mutagenesis technique described by Funayaama et al. (1999) with slight modifications. First, a 1.4 kb DNA fragment containing DR1420 was amplified by PCR using the primers 5′-CGAGGGCGGGAACGCGGGAAGG-3′ and 5′-ATCCGCTTCCGTGGGCGAATTCGGG-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 (Funayaama 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 OD₆₀₀ 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′-ACGCGATAACCAGCGATGATA-3′ and 5′-TCTAGGAAACCAAGTCC-3′, which define 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 and the 1.2 kb DNA fragment containing the HincII site at the middle of the fragment would be generated. The PCR primers were 5′-AAGGTAAGTGCTCTCAT-3′ and 5′-GACATGGCAGCAACGGCCC-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 and the DR1420 disruptant to generate a DR1758 disruptant and a DR1420/DR1758 double-disruptant, respectively. Disruption was confirmed by PCR, using primers 5′-GGGGCCGAGTTTTAAGGC-3′ and 5′-ATCATCCGGCGTGAC-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: 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-
acetylaminoadipate kinase catalyses the conversion of N-acetylaminoadipate to N-acetylaminoadipate 6-phosphate. It has been assumed that lysZ encodes this enzyme in *Thermus thermophilus*. A lysZ disruptant of *T. thermophilus* did not grow in minimal medium, but the addition of lysine to the medium restored growth. *Thermus 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 amino adipate 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. thermophilus*, 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 amino adipate pathway and the DAP pathway.

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