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Sec Pathway Influences the Growth of *Deinococcus radiodurans*

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Abstract The release of extracellular DNA molecules (eDNA) contributes to various biological processes, such as biofilm formation, virulence, and stress tolerance. The quantity of eDNA released by bacteria is usually regulated by extracellular nucleases that are secreted by different systems. In this study, we show that high concentrations of eDNA inhibit the growth of two strains of *Deinococcaceae*, *Deinococcus radiodurans,* and *Deinococcus radiopugnans*, but have no effect on other selected organisms, such as *Escherichia coli*. In *D. radiodurans*, an extracellular nuclease was shown to be secreted through the Sec pathway. Disruption of one member of this pathway, SecD/F, inhibited cell growth, suggesting that the Sec pathway plays an important role in growth rate. However, the Sec pathway mutant exhibited a greater deficiency in growth rate compared with the extracellular nuclease mutant, indicating that the pathway not only secretes the extracellular nuclease, but has other unknown functions as well.

Introduction

Both dying and living cells release extracellular DNA molecules (eDNA) all the time. Organisms often benefit from eDNA or its degradation products via their effects on various biological processes, such as biofilm formation, virulence, and stress tolerance [24, 30]. Extracellular nuclease-degraded eDNA molecules are also important nutrient sources for cells [4, 9]. However, if eDNA is not degraded immediately, damaged bases will be reincorporated into the genome, which can increase the mutation rate and lead to cell death [2]. In such instances, extracellular nucleases secreted by cells, as well as their corresponding secretion pathways, play important roles in regulating the degradation of eDNA.

Nearly one-third of cellular proteins are transported across the cytoplasmic membrane and function in the periplasm, outer membrane, or growth medium [25]. Diverse protein secretion systems, which result from the structural differences of the cell wall and cytoplasmic membrane, play roles in biofilm formation, virulence, and stress tolerance [18, 21, 22]. Several routes for protein export and secretion are known, including the general secretion (Sec) pathway and twin-arginine translocation (Tat) pathway. Among them, the Sec pathway was the first secretion pathway to be discovered, and it is conserved across the three domains of life [3, 6]. Proteins secreted through this pathway are immature precursors, and they are non-functional until they properly fold, which occurs after they cross the cytoplasmic membrane [16, 19]. Although there are some differences between Gram-negative and Gram-positive bacteria, all elucidated Sec pathways include similar elements, such as a signal peptide, signal peptidase, and the Sec transport machinery [10, 12].

Deinococcus radiodurans R1 is a red, non-pathogenic, aerobic, Gram-positive bacterium that exhibits strong resistance to ionizing and ultraviolet radiation, hydrogen peroxide, drying, and some chemical mutagens owing to its robust DNA repair and anti-oxidation abilities [2, 15, 17, 23]. After gamma radiation stress, *D. radiodurans* releases

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large amounts of damaged DNA fragments (about 1 kb in length) during the process of genome restoration [26]. An extracellular nuclease degrades these eDNA fragments immediately to avoid their reabsorption into the cells, thereby reducing the mutation rate of its genome.

Our previous studies suggested that dGMP, one of the final products of degraded DNA fragments, could elevate the bacterium's oxidation resistance. Mutation of the only extracellular nuclease, DRB0067, obviously reduced H_2O_2 stress resistance [13]. Here, we show that DRB0067 was secreted through the Sec pathway, and deletion of the *secD/F* homolog *dr1822* strongly represses cell growth, indicating that the Sec pathway plays an important role in the growth of *D. radiodurans*.

Results

High Levels of Extracellular DNA Repress the Growth of *Deinococcaceae*

Our previous study indicated that a high level of eDNA inhibits the growth of *D. radiodurans* while having little effect on *Escherichia coli*. Further investigation suggested that the only extracellular nuclease of *D. radiodurans*, DRB0067, could relieve the inhibition [13]. Here, we selected five other species (*D. radiopugnans*, *Acinetobacter baumannii*, *Enterococcus faecium*, *Lactococcus lactis*, and *Saccharomyces cerevisiae*) and tested whether their growth was also affected by eDNA. As shown in Fig. 1, the growth of *D. radiopugnans* was obviously inhibited by eDNA, while the other four strains were barely affected. Combining with previous result, it seems that excessive eDNA fragments repress the growth of both *D. radiodurans* and *D. radiopugnans*.

The Extracellular Nuclease in *D. radiodurans* is Secreted by the Sec Pathway

Using the online program SignalP (http://www.cbs.dtu.dk/services/SignalP/), we analyzed the signal peptide of extracellular nuclease DRB0067 in *D. radiodurans* and predicted it to be a member of the Sec pathway. The genome of *D. radiodurans* was then searched for homologs of Sec pathway proteins, which revealed that DR0575, DR1822, DR2116, DR2048, and DR1825 are counterparts of SecA, SecD/F, SecY, SecE, and SecG, respectively, in *E. coli*. Usually, SecA acts as a central player in the Sec pathway, associates with the SecYEG channel, and transports preproteins through the cytoplasmic membrane. In some bacteria, SecD/F interacts with YajC to form a complex subunit, which links with SecYEG and stabilizes SecA in its conformation [5, 7, 8]. It has been proved in vivo that *secA*, *secD*, and *secF* are required for the secretion of proteins through the Sec pathway in *E. coli* [1, 20]. We firstly attempted to construct mutant strain that lacks *dr0575*, but failed, even though a variety of different gene deletion methods were employed, suggesting that SecA homolog is essential for the survival of *D. radiodurans*. Because SecYEG is also an essential complex in Sec pathway, we chose *dr1822* for deletion and successfully obtained the corresponding mutant using the deletion–replacement method, indicating that SecD/F is dispensable for the survival of the bacteria.

To confirm that DRB0067 is secreted by the Sec pathway, we tested whether deletion of *dr1822* decreased the secretion of DRB0067. As shown in Fig. 2, no nuclease activity was detected in the supernatant of the △*dr1822* strain. However, the activity was fully restored when the mutant was complemented with the gene *dr1822*. The results demonstrate that inactivation of *dr1822* strongly influenced the secretion of nuclease DRB0067, verifying the above prediction that the extracellular nuclease was secreted through the Sec pathway in *D. radiodurans.*

Disruption of the SecD/F Inhibits the Growth of *D. radiodurans*

It has been proved that deletion of the extracellular nuclease gene *drb0067* does not affect the growth of *D. radiodurans*. Nevertheless, the growth rate of the △*drb0067* strain is greatly suppressed when excessive eDNA fragments exist [13]. Meanwhile, our above-mentioned data showed that DRB0067 was secreted by the Sec pathway. Thus, we measured the growth rate of the *dr1822* mutant strain using the plate streaking method. Figure 3 shows that the △*dr1822* strain exhibited a slower growth rate than the wild-type R1 and △*drb0067* strains under non-eDNA-treated circumstances. However, the growth rate of the △*dr1822* strain was much more dramatically inhibited than the wild-type strain and the △*drb0067* strain in the presence of abundant eDNA. These results demonstrated that the inactivation of the Sec pathway slowed the growth of *D. radiodurans*, which is partly dependent on the deletion of *drb0067* or *dr1822*.

Discussion

In the previous and present studies, we tested the effects of high levels of eDNA on six selected microorganisms, including one eukaryote (*S. cerevisiae*) and five prokaryotes (including the Gram-positive bacteria *D. radiodurans*, *D. radiopugnans*, and *E. faecium,* and Gram-negative bacteria *E. coli*, *A. baumannii*, and *L. lactis*). The results showed that excessive eDNA fragments strongly inhibit the

Fig. 1 Cell growth of five species in response to high levels of eDNA. Five other species (*Deinococcus radiopugnans*, *Acinetobacter baumannii*, *Enterococcus faecium*, *Lactococcus lactis*, and *Saccharomyces cerevisiae*) were added with 0, 1.5, and 3.0 mg/ml eDNA, respectively, to test their growth rates with the plate streaking method

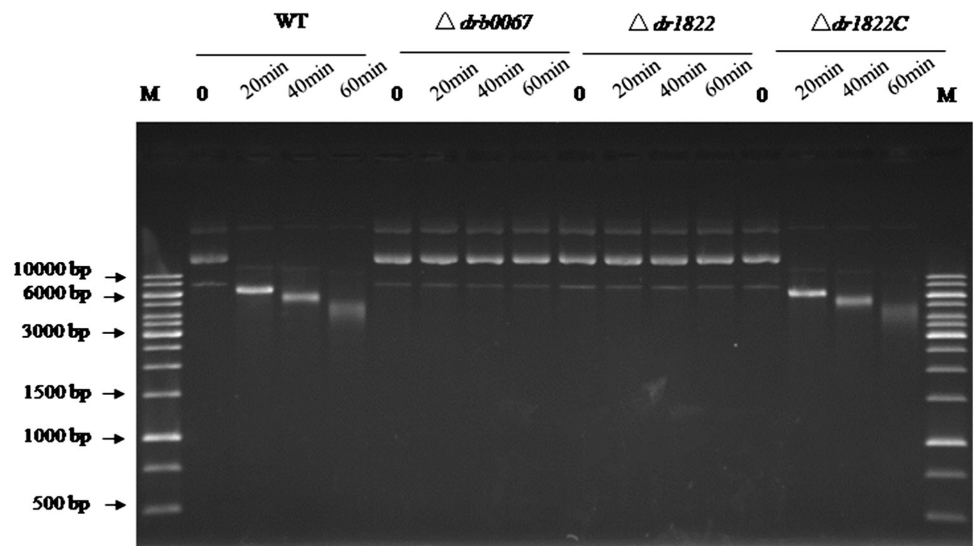

Fig. 2 Measurement of extracellular nuclease activity. Extracellular nuclease activity of the wild-type strain (WT), the nuclease-deletion strain (Δ*drb0067*), *dr1822* mutant strain (Δ*dr1822*), and *dr1822* complementary strain (Δ*dr1822 C*) were measured. At various intervals (0, 15, 30, and 45 min), samples were removed and analyzed

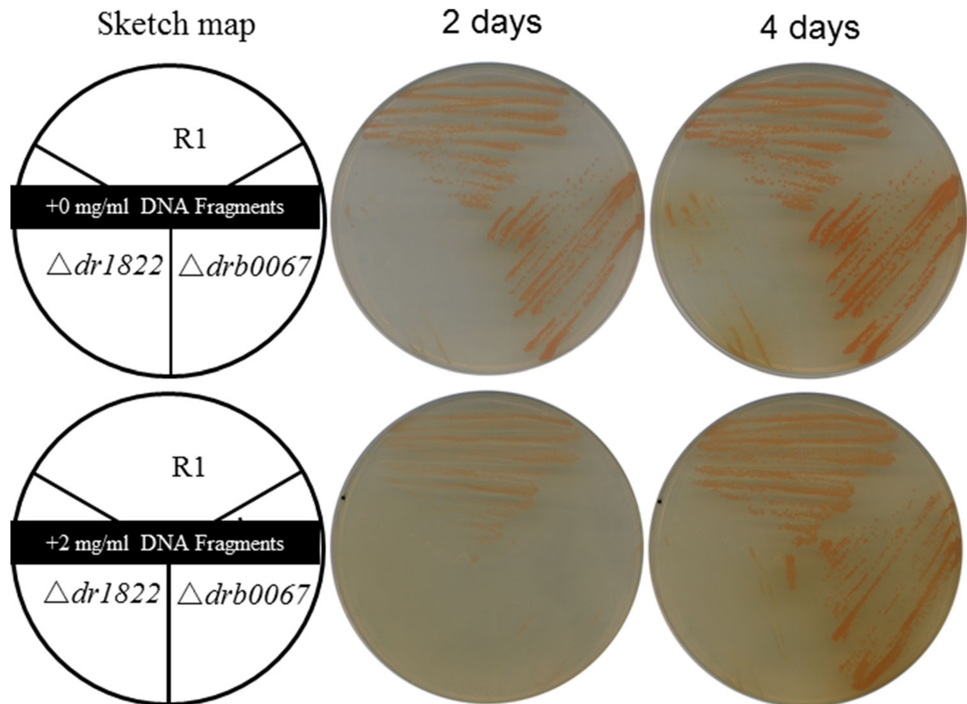

Fig. 3 Measurement of the growth rates of the *drb0067* and *dr1822* mutants

growth of *D. radiodurans* and *D. radiopugnans*, but have little effect on the other selected microorganisms. Both *D. radiodurans* and *D. radiopugnans* belong to the *Deinococcaceae*, which possess relatively strong natural transformation and homologous recombination abilities, as well as extreme stress resistance [11].

Upon exposure to severe stresses, such as gamma radiation, the genome of *D. radiodurans* releases a large amount of damaged DNA fragments. During the subsequent genome recovery, these fragments can be reabsorbed by the cells as a result of their strong transformation ability, which would normally be expected to greatly increase the mutation frequency of the bacteria [14]. However, the genome of this unusual microorganism is consistently maintained in a high-fidelity state [29]. Thus, we investigated how the eDNA is degraded in the bacteria.

As was illustrated, the *drb0067* mutant strain, which lacks the only extracellular nuclease gene, has the same growth rate as the wild-type strain under non-eDNA-treated conditions, whereas the growth of both strains decreased in the presence of excessive DNA fragments [13]. However, this was not the case for the *dr1822* mutant strain, which exhibited a slower growth rate even under non-eDNA-treated condition, suggesting that the Sec pathway is essential for the growth of *D. radiodurans*. The mutant devoid of SecA homolog leads to the death of the bacteria, while disruption of SecD/F homolog decreases the growth rate either in the presence or absence of eDNA, indicating the relationship between growth and the Sec pathway.

Experimental Procedures

Bacterial Strains and Growth Conditions

Deinococcus radiodurans R1 (ATCC13939) was cultured in TGY medium (0.5 % tryptone, 0.1 % glucose, 0.3 % yeast extract; 1.5 % agar was added for solid medium) at 30 °C. *Deinococcus radiopugnans* was also cultured at 30 °C in a medium containing 3 % Peptone-B and 0.3 % tryptone. *Acinetobacter baumannii* was cultured in Luria-Bertani (LB) medium at 37 °C, while *Enterococcus faecium, Lactococcus lactis*, and *Saccharomyces cerevisiae* were cultured at 30 °C in brain–heart infusion (BHI) medium, MRS medium (invented by de Man, Rogosa and Sharp), and YPAD medium (2 % tryptone, 1 % yeast extract, 2 % glucose, and 0.1 mg/ml adenine sulfate), respectively. The final concentration of kanamycin added to the medium for the *D. radiodurans* mutant strains was 30 µg/ml.

Detection of Extracellular DNase Activity Using Methyl Green Agar Plate

Corresponding culture media were added with a certain quality of herring sperm DNA and sterilized at 121 °C for 15 min. When cooling to about 50 °C, 1 ml of 0.5 % methyl green solution was added to 100 ml of medium to form methyl green agar plates. For detection of extracellular DNase activity, a monoclonal strain was selected and cultured to an OD_{600} of 1.0. About 20 µl of culture was

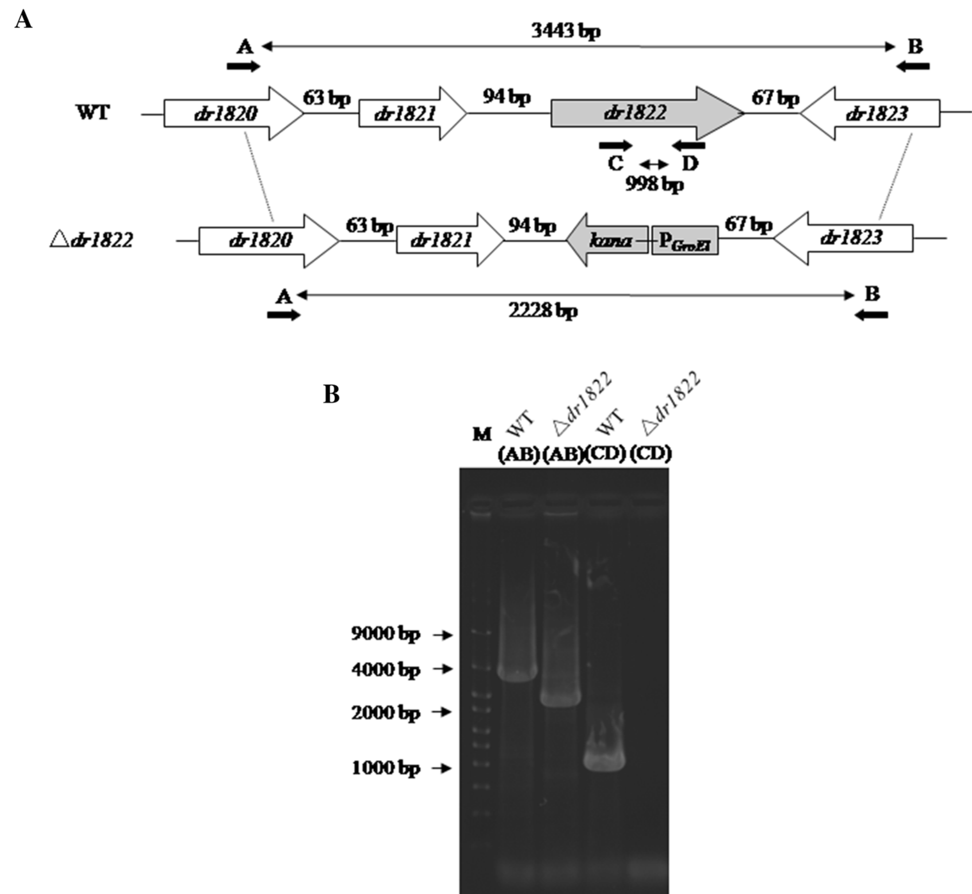

Fig. 4 Construction of the *dr1822* mutant strain. **a** Schematic representation of the *dr1822* gene deletion. A, B, C, and D refers to the primers, respectively. **b** The *dr1822* disruption mutant was confirmed by genomic PCR. AB and CD refer to the primer combinations, respectively

then dotted on the corresponding plate and incubated at 30 or 37 °C for several days before observing the surrounding colonies.

Growth Rate Measurement

The plate streaking method was used to determine the growth rate of bacterial strains. Various strains were streaked onto their corresponding plates and cultured at optimal temperatures for several days to observe their growth rates. Plates containing DNA were prepared as follows: herring sperm DNA was dissolved in water, filtered through a 0.22-μm membrane, and added to the solid medium.

Construction of the Mutants Δ*dr0575* and Δ*dr1822*

These genes were knocked out with a deletion method that takes advantage of the strong homologous recombination ability of *D. radiodurans* [28]. To construct the Δ*dr1822* mutant, pairs of primers upstream and downstream *dr1822* were used to clone DNA fragments upstream and downstream of the gene, using the wild-type R1 genome as a template for the PCRs. Then, the DNA fragments were digested with *Bam*HI and *Hin*dIII, respectively, and ligated to a kanamycin-resistance cassette which was pre-digested with both *Bam*HI and *Hin*dIII (Fig. 4a). Next, a tri-ligation of upstream, kanamycin-resistance, and downstream fragments was performed. The resulting fragment was then amplified and transformed into the wild-type *D. radiodurans* strain. The mutant Δ*dr1822* was screened on TGY plates containing 30 μg/ml kanamycin and verified by PCR and DNA sequencing (Fig. 4b). The complemented plasmid Δ*dr1822* C was constructed as described previously [27].

Measurement of Extracellular Nuclease Activity In Vitro

The wild-type R1 and mutant △*dr1822* strains were cultured to an OD_{600} of about 2.0 and centrifuged for 10 min at 16,000 g. The supernatants were collected for the digestion reaction, using the plasmid pRADK as a substrate. $MgCl_2$ was added to a final concentration of 10 mM, and the reaction was performed at 30 °C. At various intervals, samples were removed and analyzed by 1 % agarose electrophoresis.

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