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Transformation of crustacean pathogenic bacterium *Spiroplasma eriocheiris* and expression of yellow fluorescent protein

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ABSTRACT

Spiroplasma eriocheiris, the cause of crab trembling disease, is a wall-less bacterium, related to *Mycoplasmas*, measuring 2.0–10.0 μm long. It features a helical cell shape and a unique swimming mechanism that does not use flagella; instead, it moves by switching the cell helicity at a kink traveling from the front to the tail. *S. eriocheiris* seems to use a novel chemotactic system that is based on the frequency of reversal swimming behaviors rather than the conventional two-component system, which is generally essential for bacterial chemotaxis. To identify the genes involved in these novel mechanisms, we developed a transformation system by using *oriC* plasmid harboring the tetracycline resistant gene, *tetM,* which is under the control of a strong promoter for an abundant protein, elongation factor-Tu. The transformation efficiency achieved was 1.6×10^{-5} colony forming unit (CFU) for 1 μg DNA, enabling the expression of the enhanced yellow fluorescent protein (EYFP).

1. Introduction

Spiroplasma is a genus of bacteria belonging to the class *Mollicutes*, which includes animal and plant pathogens such as the *Mycoplasma* and *Phytoplasma* species and are characterized by small genomes and the lack of a peptidoglycan layer [1]. *Spiroplasma* species, pathogenic for higher plants and arthropods, form a helical cell shape and swim in viscous media by propagating kink pairs along their cell body from front to back, a mechanism that is not related to other motility systems (Fig. 1A and Video S1) [2–5].

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.03.144.

Spiroplasma eriocheiris has been isolated from Chinese mitten crab, *Eriocheir sinensis,* and has had disastrous effects on aquaculture in China in recent years [6]. It grows faster than other *Spiroplasma* species and swims up to five μm per second in a viscous medium, a speed that is advantageous for studying the swimming mechanism. The distinct morphology of *Spiroplasmas* is defined by an internal structure and a dumbbell-shaped structure at the front end connected by a flat ribbon and surrounded by a spiral filament [7,8]. The ribbon structure lining the inside of the helix should be linked to the cell helicity and the kink traveling with their conformational changes. As the change in helicity occurs at the front end, the dumbbell structure may be involved in this switch, possibly through torque generation at the connecting part. The flat ribbon is composed of an abundant internal protein, Fib, and at least four classes of MreB, a bacterial homolog of eukaryotic actin [8–10]. Sixteen proteins also have been identified as the components of internal structure. *S. eriocheiris* exhibits chemotactic behaviors, such as changing the reversal frequency of movements, that do not use the conventional two-component system (TCS) that most bacteria use [11]. The chemotaxis of *S. eriocheiris* is also characterized by the change in the reversal frequency of swimming direction, which is a chemotactic behavior similar to that of other motile bacteria, although genes for TCS have not been found in the genome of *Spiroplasmas* [8,12].

Gene manipulation is a powerful tool for identification of proteins in microbiological studies. *oriC*-based plasmids are considered a promising tool for genetic studies of bacteria limited by the scarcity of suitable genetic vectors and have already been applied successfully in studies of many *Mycoplasma* and *Spiroplasma* species [13–15]. The *oriC* region is comprised of the *dnaA* gene encoding a helicase responsible for the initiation of DNA replication and the flanking regions containing 7 to 10 putative DnaA boxes

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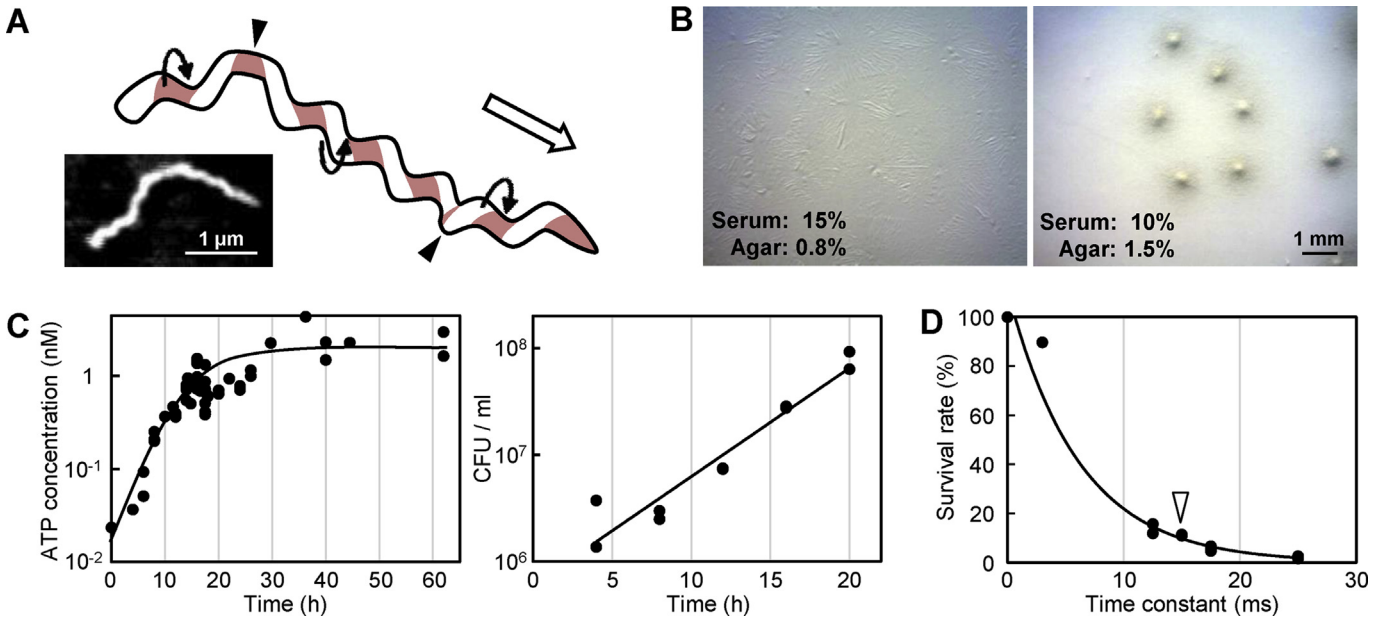

Fig. 1. *S. eriocheiris* cell observation and optimization of conditions for transformation. (A) Schematic of *S. eriocheiris* helical cell. The cell swims in the direction of tapered end, as indicated by an open arrow. Black arrows and black triangles indicate the direction of cell helicity and kinks between left- and right-handed helical regions, respectively. The small panel shows a cell observed using dark-field microscopy. (B) Formation of film and spots under different concentrations of serum and agar. Film and spots cover the medium surface in the left panel. The surface and colonies of the solid medium were observed after 5 days of incubation. (C) Cell growth monitored by ATP concentration (left) and CFU (right). The doubling time was estimated at around 150 min. (D) The survival rate is represented by CFU. The time constant for electroporation was calculated from resistance multiplied by capacitance. The 10% survival is indicated by an open triangle.

(5′-TTATCCACA-3′), which are the binding sites for the DnaA proteins [16]. A transformation system using *oriC*-based plasmids has been developed for *Spiroplasma citri*, a pathogen for citrus and arthropods [15], but the slow growth rate of this species is disadvantageous for advanced gene manipulations.

In the present study, we developed a transformation system for *S. eriocheiris* by using *oriC* plasmids, which enabled the expression of fluorescent protein in the cells.

2. Materials and methods

2.1. Strains and culture conditions

The type strain, TDA-040725-5^T (=CCTCC M 207170^T = DSM 21848^T) of *S. eriocheiris* was cultured in R2 medium at 30 °C [6,17]. The *Escherichia coli* strain, stellar cells from In-fusion PCR EcoDry Cloning Kit (TaKaRa Bio, Shiga, Japan), and DH5α were used for DNA manipulation.

2.2. Plasmid construction

S. eriocheiris genomic DNA was prepared using the Genomic-tip System (Qiagen, Hilden, Germany). Primers and plasmids used in the present study are listed in Table S1. The *oriC* region of *S. eriocheiris* was amplified from isolated genomic DNA via PCR using the *Bam*HI-*oriC*-upstream-F and *Bam*HI-*oriC*-downstream-R primers. The *oriC* sequence of *Mycoplasma pulmonis* in pMPO1 between the *Bam*HI sites was replaced with that of *S. eriocheiris*, resulting in a plasmid, pTSeO [13]. The predicted promoter regions and the ORF sequences coding the *N*-terminal eleven amino acids of elongation factor-Tu (*tufA*), Fib protein (*fib*), and DnaK (*dnaK*) were amplified from isolated genomic DNA by PCR using TufP-F and TufP-R, FibP-F and FibP-R, and DnaKP-F and DnaKP-R primers, respectively. The pTSeO was linearized by inverse PCR using pTSeO-TetM-F and pTSeO-promoter-upstream-R primers, and the 320-bp region including spiralin promoter and the initial codon of *tetM* was replaced with the amplified fragments, by using the In-Fusion EcoDry PCR Cloning Kit (TaKaRa Bio), producing plasmids pTSeT, pTSeF, and pTSedK, respectively. The codon-optimized gene of enhanced yellow fluorescent protein (*opt-eyfp*) was amplified from pMobopt via PCR using optEYFP-infusion-F and optEYFP-infusion-R primers [18–20]. The pTSeT was linearized via inverse PCR using pTSeO-TetM-F and pTSeT-tufP-inverse-R primers, and the *opt-eyfp* gene was inserted into a position between the *tuf* promoter and *tetM* in pTSeT by using the In-Fusion EcoDry PCR Cloning Kit, resulting in a plasmid, pTopt.

2.3. Transformation of S. eriocheiris

The transformation method used was modified from the method generally used for *Mycoplasma* and *S. citri* [20–22]. The major modifications were optimization of the solid medium and the use of stronger conditions for electroporation. *S. eriocheiris* was grown in liquid R2 medium until the late-exponential phase (× 10^8 CFU/ml). The cells were collected by centrifugation at 11,000×*g* at 4 °C for 10 min and then washed twice in an equivalent volume of electroporation buffer [272 mM sucrose and 8 mM HEPES (pH 7.4)]. The cells were suspended in 100 μl of electroporation buffer at a concentration of approximately 10^9 cells per ml, were kept on ice with 0.5 μg of plasmid DNA for 30 min, and were transferred to a 2 mm prechilled electroporation cuvette. Electroporation was performed using a Gene Pulser II electroporation system and Pulse Controller II module (Bio-Rad Laboratories, Hercules, CA). Optimized electroporation conditions of 2.5 kV, 25 μF and 600 Ω were used. Immediately after the electroporation, 900 μl of prechilled R2 medium was added, and the cells were kept for 3 h at 30 °C for outgrowth. Aliquots of 200 μl were plated onto a solid R2 medium containing 10% horse serum, 1.5% agar and appropriate antibiotics, and were cultured at 30 °C for 18–20 days. Based on a drug-resistant test of *S. eriocheiris*, 0.7 μg/ml tetracycline