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Development of a versatile method for targeted gene deletion and insertion by using the *pyrF* gene in the psychrotrophic bacterium, *Shewanella livingstonensis* Ac10

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***Shewanella livingstonensis* Ac10, a psychrotrophic bacterium isolated from Antarctic seawater, grows well at low temperatures close to 0°C. The bacterium is useful as a host in a low-temperature protein expression system. It is also useful as a model microorganism to investigate the mechanisms of microbial cold-adaptation. Versatile genetic manipulation techniques would be useful to investigate the biology of this bacterium and to develop its applications. In this study, we developed a method for targeted gene deletion and insertion by using the gene coding for orotidine-5′-phosphate decarboxylase (*pyrF*), which is involved in pyrimidine synthesis. We found that *S. livingstonensis* Ac10 is sensitive to 5-fluoroorotic acid (5-FOA), which is converted to a highly toxic compound by the product of *pyrF*. A uracil-auxotrophic strain resistant to 5-FOA was constructed by deleting *pyrF*, thus allowing the use of a plasmid-borne copy of *pyrF* for selection of recombinants. We constructed the *pyrF* complementation suicide plasmid pKKP, which contains *pyrF*, the R6K replication origin, the *mob* site of RP4, an antibiotic marker gene, and a multiple cloning site. To demonstrate *pyrF*-based gene replacement, we deleted the internal region of *orf5*, the gene coding for an eicosapentaenoic acid (EPA) synthesis enzyme. We also successfully inserted a His_6-tag-coding sequence into *orf8*, the gene coding for another EPA synthesis enzyme. This system allows the markerless deletion and insertion of desired sequences at specific sites in the genome, which remarkably facilitates genetic manipulation of this bacterium.**

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[**Key words:** Gene deletion; Gene insertion; Homologous recombination; *pyrF*; *Shewanella*]

The genus *Shewanella* is a large group of Gram-negative γ-proteobacteria inhabiting diverse environments including extreme environments such as the polar region and the deep sea (1). Their environmental adaptation mechanisms have been attracting a great deal of attention because information on such mechanisms deepens our understanding on the potential of life. These bacteria are also well known for their ability to metabolize various metal compounds (2). Thus, they are expected to be useful in environmental technologies to remove harmful metal compounds from polluted environments.

Among various *Shewanella* strains, *Shewanella livingstonensis* Ac10 has been intensively studied with particular interest in its marked ability to adapt to low temperatures (3–7). The bacterium was isolated from Antarctic seawater and grows well at low temperatures (4–20°C) (3). Proteomic analysis comprehensively identified the cold-inducible proteins that are supposed to be important for cold adaptation of this bacterium (5,7). The physiological importance of a cold-inducible polyunsaturated fatty acid, eicosapentaenoic acid (EPA), in its cell division was also demonstrated (6,8,9). From the application point of view, we constructed a low-temperature protein expression system using this strain as a host for the production of thermolabile proteins (10). Moreover, Tajima et al. showed that recombinant *S. livingstonensis* Ac10 expressing mesophilic enzymes is useful as a whole cell biocatalyst because the host metabolic enzymes that interfere with the intended bioconversion may be inactivated by moderate heat treatment without significantly affecting the activity of the mesophilic enzymes (11,12). These fundamental and application studies will be significantly facilitated by the development of a versatile genetic manipulation system for this bacterium.

We previously established a method for single-crossover homologous recombination in the genome of *S. livingstonensis* Ac10 to genetically modify the bacterium (6). However, this method has significant drawbacks. One drawback is that the entire sequence of the plasmid used for recombination, including the sequence homologous to the target sequence, is integrated into the genome and is retained in the genome of the recombinant. This makes it difficult to re-use the same system for further genetic engineering. Moreover, the recombinant genome obtained by single-crossover recombination contains two copies of the target sequence, causing the generation of a revertant through recombination between two homologous sequences in the absence of appropriate selection pressure. In order to overcome these problems, it is desirable to develop a new method by which undesired plasmid-

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derived sequences are eliminated from the genome of the recombinant.

To achieve this, we aimed to develop a targeted gene deletion and insertion system in this study by using the gene coding for orotidine 5′-phosphate decarboxylase (*pyrF*) as the selection and counter-selection marker. The *pyrF* gene product is required for uracil biosynthesis (13). On the other hand, the enzyme confers sensitivity to 5-fluoroorotic acid (5-FOA) by catalyzing the conversion of 5-FOA to a highly toxic compound (14,15). Due to these characteristics, the *pyrF*-based selection and counter-selection system has been successfully adapted in various organisms (15–17). By preparing a *ΔpyrF* strain and using it as the parental strain, it is possible to monitor the insertion of plasmid-derived DNA into the genome by using *pyrF* as a selection marker, whereas the elimination of undesired plasmid-derived DNA from the genome can be monitored by using *pyrF* as the counter-selection marker.

Here, we report the successful construction of a *pyrF*-based selection and counter-selection system for *S. livingstonensis* Ac10 and the application of this system for targeted gene deletion and insertion. The developed method makes it possible to generate a completely markerless genome with the desired gene modification, facilitating repeated use of the marker gene. The recombinant strains obtained by this method can be maintained without revertant generation even in the absence of selection pressure because of the absence of homologous sequences in the genome. This offers a systematic gene deletion and insertion strategy for *S. livingstonensis* Ac10 and greatly facilitates fundamental studies of bacterial cold-adaptation mechanisms and functions of poly-unsaturated fatty acids by using this bacterium as a model organism. It will also contribute to application studies such as the development of low-temperature protein expression system.

MATERIALS AND METHODS

Strains and media A spontaneous rifampicin (RIF)-resistant *S. livingstonensis* Ac10 strain (6) was used as the parental strain for genetic manipulation. The strain was routinely cultured in Luria–Bertani medium (LB; tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) containing 50 μg/mL of RIF at 18°C. The synthetic medium for *S. livingstonensis* Ac10 (M95) consisted of 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 1% NaCl, 0.1% NH_4Cl, 0.2% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 × 10^{-5}% thiamin-HCl, 0.5% casamino acid, and 50 μg/mL of RIF (pH 7.0). The *S. livingstonensis* Ac10 *ΔpyrF* strain and its derivatives were also grown in LB or M95 medium. *Escherichia coli* S17-1/*λpir* (18), which was used as the host strain for *pir*-dependent plasmid, was grown in LB medium. Where noted, uracil, 5-FOA, and kanamycin (Km) were added to the medium to a final concentration of 40 μg/mL, 0.1%, and 30 μg/mL, respectively.

Construction of *pyrF*-deleted mutant (*ΔpyrF*) A brief scheme of *ΔpyrF* construction is shown in Fig. 1. In the construction of the *pyrF* deletion cassette, the upstream and downstream regions of *pyrF* were amplified by PCR, fused together, and introduced into pKNOCK-Km^r (19) to obtain pKK-pyrF. The primers used for the amplification of the upstream region [5′-noncoding region (180 bp) and 5′-terminal coding region (7 bp)] were pyrF+200-fw (5′-GACAAGCTTG AATTCTAGTTATCGCTGTACCGCCTG-3′) and pyrF-del2 (5′-CAGTCATTAGGATCCAG CGGC-3′). The downstream region [3′-noncoding region (288 bp)] was amplified with pyrF-del1 (5′-GCCGCTGGATCCTAATGACTGCTAACGGTTAGGTTGACTGCG-3′) and pyrF+200-rv (5′-TTGGTACCACGCGTGCTTGGTTCAGTAATTCTACCGC-3′). The plasmid obtained, pKK-pyrF, was introduced into *E. coli* S17-1/*λpir* and then transferred to RIF-resistant *S. livingstonensis* Ac10 by conjugation as described previously (6). The transformants grown on LB plates containing uracil, Km, and RIF were selected. Single colony re-isolation was performed for the selected colonies by using the same plates. Then, the Km^r colonies were plated onto LB plate containing uracil, RIF, and 5-FOA. After two rounds of selection with 5-FOA, the deletion of *pyrF* from the genome was confirmed by a diagnostic PCR with pyrF-1.1up-fw (5′-GACAAGCTTGAATTCAGCAAGTGCTTAAATGCACCAC-3′) and pyrF-0.8dw-rv (5′-TTGGTACCACGCGTCACTATTGGCCACTCGCTG-3′).

Construction of a plasmid for gene manipulation in *S. livingstonensis* Ac10 The *pyrF*-carrying suicide vector pKKP (Fig. 2) was constructed as follows. A 761-bp DNA fragment containing the entire *pyrF* and a putative ribosome-binding site was amplified by PCR with the genomic DNA of *S. livingstonensis* Ac10 as the template and the primer set of pyrF-fw (5′-GCTATTGCCGCTGGAG TCCTAATG-3′) and pyrF-rv (5′-AAAGGTACCGCAGTCAACCTAACCGTTAGC-3′). A 1.4-kbp fragment from the plasmid pKNOCK-Km^r containing the Km^r gene and its promoter was amplified with Km-fw (5′-GTTGTGGACAACAAGCCAGGGATG-3′) and Km-rv (5′- CATTAGGACTCCAGCGGCAATAGCGGATCCGCGAACCCCAGAGTCCC-3′). These PCR products were connected by crossover PCR using Km-fw and pyrF-rv primers. The DNA fragment containing Km^r and *pyrF* was treated with MluI and KpnI to yield a 1.9-kbp fragment and ligated with a 0.9-kbp fragment bearing R6K γ-*ori* (for replication in the host that produces the replication initiator protein π)

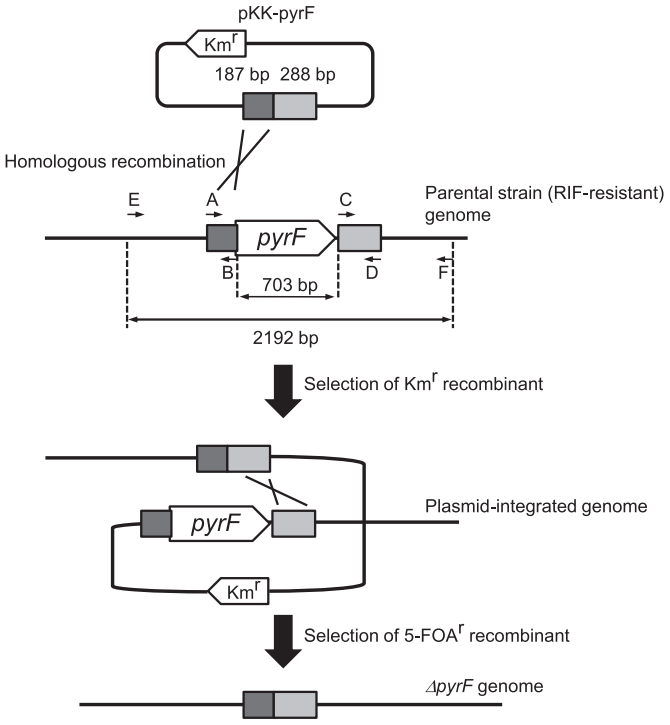

FIG. 1. Construction of *pyrF*-deleted *S. livingstonensis* Ac10 (*ΔpyrF*). The pKNOCK-Km^r-derived plasmid pKK-pyrF harboring the upstream (dark gray) and the downstream (light gray) regions of *pyrF* was introduced into the RIF-resistant strain of *S. livingstonensis* Ac10. By homologous recombination between pKK-pyrF and the genome, a plasmid-integrated Km^r recombinant was obtained. Subsequent homologous recombination between two homologous regions of the genome yielded a *pyrF*-deleted 5-FOA^r recombinant (*ΔpyrF*). Note that only the representative homologous recombination and the resultant genome structures are shown in this figure. Homologous recombination may have occurred between other homologous regions. A–F indicate the primers used for PCR: pyrF+200-fw, pyrF-del2, pyrF-del1, pyrF+200-rv, pyrF-1.1up-fw, and pyrF-0.8dw-rv, respectively. See text for more details.

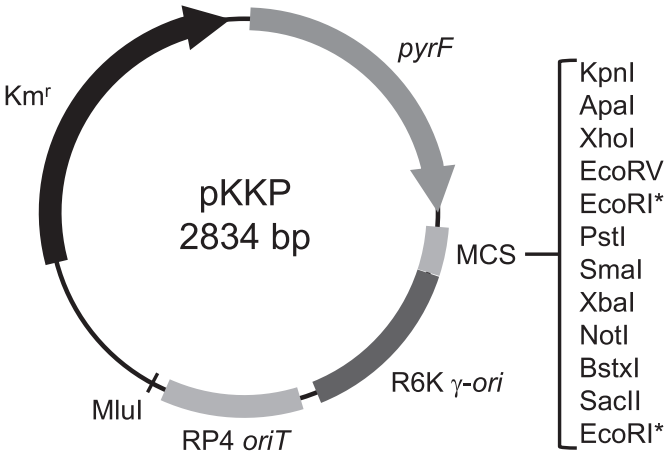

FIG. 2. Schematic of pKKP. The plasmid contains *pyrF* from *S. livingstonensis* Ac10, the Km^r gene, the R6K replication origin (R6K γ-*ori*), the *mob* site of RP4 (RP4 *oriT*), and the multiple cloning sites (MCS). Only the unique restriction sites other than those of EcoRI are shown. See text for construction details.

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