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Isolation of a novel plasmid from *Couchioplanes caeruleus* and construction of two plasmid vectors for gene expression in *Actinoplanes missouriensis*

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ABSTRACT

To date, no plasmid vector has been developed for the rare actinomycete *Actinoplanes missouriensis*. Moreover, no small circular plasmid has been reported to exist in the genus *Actinoplanes*. Here, a novel plasmid, designated pCAZ1, was isolated from *Couchioplanes caeruleus* subsp. *azureus* via screening for small circular plasmids in *Actinoplanes* (57 strains) and *Couchioplanes* (2 strains). Nucleotide sequencing revealed that pCAZ1 is a 5845-bp circular molecule with a G + C content of 67.5%. The pCAZ1 copy number was estimated at 30 per chromosome. pCAZ1 contains seven putative open reading frames, one of which encodes a protein containing three motifs conserved among plasmid-encoded replication proteins that are involved in the rolling-circle mechanism of replication. Detection of single-stranded DNA intermediates in *C. caeruleus* confirmed that pCAZ1 replicates by this mechanism. The ColE1 origin from pBluescript SK(+) and the *oriT* sequence with the apramycin resistance gene *aac(3)IV* from pIJ773 were inserted together into pCAZ1, to construct the *Escherichia coli–A. missouriensis* shuttle vectors, pCAM1 and pCAM2, in which the foreign DNA fragment was inserted into pCAZ1 in opposite directions. pCAM1 and pCAM2 were successfully transferred to *A. missouriensis* through the *E. coli*-mediated conjugative transfer system. The copy numbers of pCAM1 and pCAM2 in *A. missouriensis* were estimated to be one and four per chromosome, respectively. Thus, these vectors can be used as effective genetic tools for homologous and heterologous gene expression studies in *A. missouriensis*.

1. Introduction

Plasmids are extrachromosomal genetic elements with unique copy numbers in host organisms. Because plasmids can be introduced into new hosts by a variety of mechanisms, they are considered capable of mediating genetic exchanges among bacterial populations in natural habitats (Jain and Srivastava, 2013). Plasmids replicate in an autonomous and self-controlled manner using the replication machinery of the host in addition to proteins

Abbreviations: dsDNA, double-stranded DNA; *dso*, double-strand origin; RCR, rolling-circle replication; ssDNA, single-stranded DNA; *sso*, single-strand origin.

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encoded by their own DNA. Circular bacterial plasmids generally replicate by either theta-type replication, strand displacement replication, or rolling-circle replication (RCR) (del Solar et al., 1998). RCR is unidirectional and asymmetric because leading strand synthesis is uncoupled from lagging strand synthesis (del Solar et al., 1993; Khan, 2005). A relevant feature of RCR is that the synthesized leading plus strand remains covalently bound to the parental plus strand. In the current model, RCR is initiated by the plasmid-encoded Rep protein, which binds to a region called the double-strand origin (*dso*) and introduces a site-specific nick into the plus strand. The 3′-hydroxyl end generated by the nick is used as a primer for synthesizing the new leading strand through a process involving host replication proteins such as DNA polymerase III, the single-stranded DNA (ssDNA)-binding protein SSB, and a helicase. Elongation of the leading strand continues until the replisome reaches the reconstituted *dso* and terminates by a DNA strand transfer reaction. Thus, leading strand replication generates a double-stranded DNA (dsDNA) constituted by the parental minus strand and the newly synthesized plus strand, as well as a ssDNA intermediate derived from the parental plus strand. The generation of ssDNA is the hallmark of RCR plasmids (te Riele et al., 1986). The parental plus strand is converted into dsDNA using host proteins that initiate DNA replication at the single-strand origin (*sso*). The *sso* is distant from the *dso* (Espinosa et al., 1995).

Actinomycetes that differ from streptomycetes are called rare actinomycetes because of their low frequency of isolation from soil samples. Rare actinomycetes have attracted much attention as novel sources of pharmaceutically useful natural compounds and several methods for efficient isolation of these microbes have been developed (Couch, 1954; Hayakawa et al., 1991, 2000; Makkar and Cross, 1982; Palleroni, 1980). Some species of actinomycetes have complex morphological development making their cellular differentiation processes very interesting to study (Subramani and Aalbersberg, 2013; Tiwari and Gupta, 2013). However, to date, very few molecular biological studies have been conducted on rare actinomycetes, especially studies on their morphogenesis. *Actinoplanes missouriensis*, a rare actinomycete that lives in aquatic habitats and soil, has a rather complex lifecycle. In solid culture, *A. missouriensis* grows into a substrate mycelium and eventually forms round to globular sporangia on the substrate mycelium surface via a short sporangiophore of 4–5 μm. A sporangium contains a few hundred zoospores (shape, spherical to oval; diameter, 0.8–1.0 μm) with short flagella (Hayakawa et al., 1991; Uchida et al., 2011). Zoospores are released from a sporangium through dehiscence upon contact with water. However, pure water is not sufficient for the onset of dehiscence; therefore, some unknown substance(s) in soil extracts must be required. Once released, zoospores begin to swim immediately at astonishing speed using flagella. Chemotaxis allows a zoospore to move to a preferable environment using its chemotactic properties toward a wide variety of substances including aromatic compounds, sugars, and amino acids (Hayakawa et al., 1991). Finally, the zoospore stops swimming and germinates to form a substrate mycelium. These characteristics make *A. missouriensis* a suitable bacterium for studying morphogenesis and the regulatory mechanisms involved in sensing and responding to environmental changes. The complete genomic sequence of *A. missouriensis* 431^T (NBRC 102363^T) was reported recently (Yamamura et al., 2012). While the linear plasmid pAM1 (approximately 100 kb) was identified in *A. missouriensis* DSM 43046^T (Rose and Fetzner, 2006), no plasmids were found in *A. missouriensis* 431^T (Yamamura et al., 2012). Genetic tools are indispensable for molecular biological studies on *A. missouriensis*, but to date, no plasmid vector has been developed for genetic analysis of this bacterium. Moreover, no small circular plasmid has been reported to exist in the genus *Actinoplanes* or in a closely related genus *Couchioplanes*, although two small circular plasmids, pMZ1 (9.9 kb) and pMR2 (11.0 kb), were found in *Micromonospora zionensis* NRRL5466 and *Micromonospora rosaria* NRRL3718, respectively (Oshida et al., 1986).

Hence, the aim of the present study was to construct plasmid vectors for gene expression in *A. missouriensis*. For this purpose, we investigated 57 *Actinoplanes* and two *Couchioplanes* strains for the presence of small circular plasmids. We identified and characterized a novel RCR plasmid, pCAZ1, from *Couchioplanes caeruleus* subsp. *azureus*. Using pCAZ1, we generated the *Escherichia coli–A. missouriensis* shuttle vectors, pCAM1 and pCAM2, which were introduced successfully into *A. missouriensis* using an *E. coli*-mediated conjugative transfer system.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The *A. missouriensis* 431^T strain (NBRC 102363^T) was obtained from the National Institute of Technology and Evaluation (NITE, http://www.nite.go.jp/index-e.html). *A. missouriensis* was grown in Maltose–Bennett's agar (0.2% meat extract, 0.1% yeast extract, 0.2% NZ amine, 1% maltose monohydrate, pH 7.0) or HAT agar (0.1% sucrose, 0.01% casamino acids, 0.05% K_2HPO_4, 0.2% humic acid, 10 mL trace element solution, pH 7.5) at 30 °C for solid cultures, and grown in PYM medium (0.5% Bacto™ peptone, 0.3% yeast extract, 0.1% $MgSO_4$•$7H_2O$, pH 7.0) at 30 °C for liquid cultures. Agar media contained 2% agar. The trace element solution contained 0.004% $ZnCl_2$, 0.02% $FeCl_3$•$6H_2O$, 0.001% $CuCl_2$•$2H_2O$, 0.001% $MnCl_2$•$4H_2O$, 0.001% $Na_2B_4O_7$•$10H_2O$, 0.001% $(NH_4)_6$ Mo_7O_{24}•$4H_2O$. HAT agar was used for sporangia formation. GYMC medium (0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$, pH 7.2) was used for conjugation with *E. coli* ET12567 (pUZ8002). *E. coli* ET12567 (pUZ8002) and pIJ773 were obtained from the John Innes Centre (Norwich, UK). *E. coli* JM109 used as a routine cloning host was purchased from Takara Biochemicals. The pBluescript SK(+) vector was purchased from Merck. The media and growth conditions for *E. coli* were those described by Maniatis et al. (1982). Apramycin (50 μg/mL), spectinomycin (50 μg/mL) and ampicillin (50 μg/mL) were added when necessary.

2.2. Search for small circular plasmids

Supplementary Table S1 shows the details of the 57 *Actinoplanes* and two *Couchioplanes* strains obtained from