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단 보

Construction of *Pseudoalteromonas* - *Escherichia coli* shuttle vector based on a small plasmid from the marine organism *Pseudoalteromonas*

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극지해양 *Pseudoalteromonas* 유래의 소형 플라스미드에 기반한 *Pseudoalteromonas* - *Escherichia coli* 셔틀벡터 제작

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ABSTRACT: A small plasmid (pDK4) from the Antarctic marine organism *Pseudoalteromonas* sp. PAMC 21150, was purified, sequenced and analyzed. pDK4 was determined to be 3,480 bp in length with a G+C content of 41.64% and contains three open reading frames encoding a replication initiation protein (RepA), a conjugative mobilization protein (Mob) and a hypothetical protein. PCR-amplified pDK4 was cloned in high-copy pUC19 to yield the fusion vector pDOC153. The chloramphenicol resistance gene was inserted into pDOC153 to give an ampicillin and chloramphenicol-resistant, *Pseudoalteromonas* – *Escherichia coli* shuttle vector (7,216 bp; pDOC155). The TonB-dependent receptor (*chi22718_IV*) and exochitinase (*chi22718_III*) genes from Arctic marine *P. issachenkonii* PAMC 22718 were cloned into pDOC155 to produce pDOC158 and pDOC165, respectively. Both vector derivatives were transferred into plasmid-free *Pseudoalteromonas* sp. PAMC 22137 by the triparental mating method. PCR experiments showed that the genes were stably maintained both in *Pseudoalteromonas* sp. PAMC 22137 and *E. coli* DH5α cells, indicating the potential use of pDOC155 as a new gene transfer system into marine *Pseudoalteromonas* spp.

Key words: *Pseudoalteromonas*, cold-active enzyme, gene transfer, shuttle vector

Marine psychrophilic bacteria have successfully developed adaptations that enable them to survive in low temperature environments (Marx *et al*., 2007). The Gram-negative heterotrophic and aerobic *Pseudoalteromonas* is present globally in marine environments, including the sea surface and deep sea bipolar regions, and constitutes 0.5–6.0% of the total bacterioplankton (Wietz *et al*., 2010). *Pseudoalteromonas* is composed of over 30 phenotypically divergent species and is one of the largest genera within the class *Gammaproteobacteria*. Many psychrophilic *Pseudoalteromonas* spp. strains (Feller *et al*., 1999; Mancuso Nichols *et al*., 2004; Médigue *et al*., 2005) have been isolated from the Arctic and Antarctic seawaters. Because of their abundance, high metabolic activities, fast growth and ability to synthesize cold-adapted extracellular enzymes (a key survival element at low temperatures), these bacteria appear to play an important role in cold marine ecosystems (Médigue *et al*., 2005; Al Khudary *et al*., 2008). The production of extracellular hydrolytic enzymes by marine bacteria including *Pseudoalteromonas* is a trait involved in nutrient utilization and turnover of marine microorganisms on a global scale. Therefore, functional studies on genes coding for the distinct features of *Pseudoalteromonas*, such as cold-adaptation and cold-active enzymes, could improve our understanding of the ecological roles of *Pseudoalteromonas*, particularly in cold marine environments.

Because of their importance in marine ecosystems, over 50 genomes of *Pseudoalteromonas* spp. have been completely or

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partially sequenced (Wang *et al*., 2015) and annotated, and this information is available in the NCBI genome database. Since *Pseudoalteromonas* genomes are now accessible, researchers can readily access any isolated gene for functional analysis. Development of DNA transfer systems for heterologous expression, replacement and knockout of target genes is a prerequisite for the study of functionally unknown genes of *Pseudoalteromonas*. However, only a few genetic systems for *Pseudoalteromonas* have been reported and no commercial system for *Pseudoalteromonas* is available (Zhao *et al*., 2011; Yu *et al*., 2014; Wang *et al*., 2015). Since mobile genetic elements such as plasmids can be used in developing genetic systems, we have isolated a native small plasmid from marine *Pseudoalteromonas* sp. and constructed a shuttle vector as a tool for the functional analysis and heterologous expression of genes originating from marine *Pseudoalteromonas*.

From the PAMC (Polar and Alpine Microbial Collection) operated by KOPRI (Korea Polar Research Institute), eighteen Arctic and Antarctic marine *Pseudoalteromonas* spp. strains were screened for the presence of small plasmids (Table 1). The strains were grown in Difco Marine Broth 2216 (MB) at 25°C for 3 days and 1 ml of each cell culture was used for extraction of small plasmids with an AccuPrep Plasmid Mini Extraction Kit (Bioneer). The extracted DNA solutions were mixed with a safe fluorescent dye (Dynebio) and separated on a 1.0% agarose gel in 0.5×TAE at 100 V for 30 min. The gel analysis revealed the presence of a small plasmid in only one strain, Antarctic *Pseudoalteromonas* sp. PAMC 21150 (99% identity with *P. arctica* strain A 37-1-2^T 16S rRNA gene) (Fig. 1). The small plasmid (designated pDK4) was extracted from the gel and purified with the AccuPrep Gel Purification Kit (Bioneer) for sequencing.

Purified pDK4 (0.6 μg) was randomly linearized by the *Sau*3AI restriction enzyme and restriction fragments of different sizes were cloned into the *Bam*HI site of the pBluescript vector and transferred into *E. coli* cells. The insert DNA fragments from ~100 white colonies were sequenced on both strands (Cosmo Genetech). The nucleotide sequences were assembled with the SeqMan program, which is part of the Lasergene software suite (DNASTAR). Sequence gaps between contigs were filled by

Table 1. Summary of *Pseudoalteromonas* spp. strains used in this study

Strain	Closest species[a]	Plasmid[b]	Transformant[c]
PAMC 21150	*Pseudoalteromonas arctica* A 37-1-2(T)	+	ND
PAMC 21322	*Pseudoalteromonas nigrifaciens* NCIMB 8614(T)	-	ND
PAMC 21717	*Pseudoalteromonas arctica* A 37-1-2(T)	-	ND
PAMC 21720	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 21739	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 21745	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 21828	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 22137	*Pseudoalteromonas arctica* A 37-1-2(T)	-	+
PAMC 22159	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 22172	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 22184	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 22193	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 22198	*Pseudoalteromonas arctica* A 37-1-2(T)	-	-
PAMC 22718	*Pseudoalteromonas issachenkonii* KMM 3549(T)	-	ND
PAMC 25442	*Pseudoalteromonas paragorgicola* KMM 3548(T)	-	ND
PAMC 25445	*Pseudoalteromonas nigrifaciens* NCIMB 8614(T)	-	ND
PAMC 25458	*Pseudoalteromonas citrea* NCIMB 1889(T)	-	ND
PAMC 25497	*Pseudoalteromonas issachenkonii* KMM 3549(T)	-	ND

[a] The partial 16S rRNA gene sequence from each *Pseudoalteromonas* PAMC strain was compared with those of type strains available in the EzTaxon database to determine the closet species.
[b] Detection of a small plasmid: +, detected; -, not detected.
[c] Production of Am^R- and Cm^R-transformants possessing shuttle vector pDOC155: +, produced; -, not produced; ND, not determined.

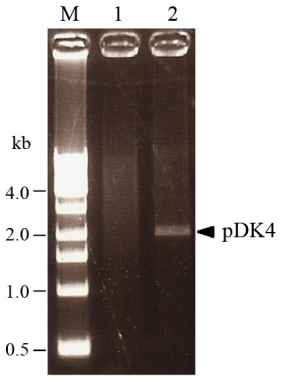

Fig. 1. Agarose gel electrophoresis of genomic DNAs extracted from bipolar marine *Pseudoalteromonas* spp. strains. A small plasmid (pDK4) was separated from the chromosomal DNA on the gel. Lanes: M, 1 kb DNA ladder; 1, *Pseudoalteromonas* sp. PAMC 22137; 2, *Pseudoalteromonas* sp. PAMC 21150.

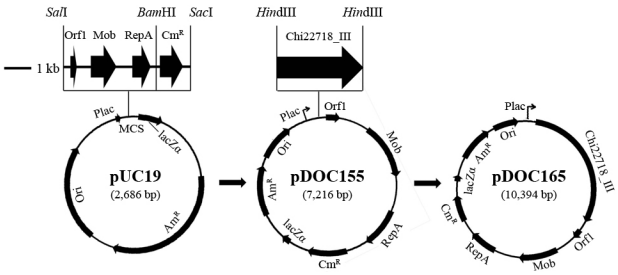

Fig. 2. Schematic representation of *Pseudoalteromonas* – *E. coli* shuttle vector pDOC155 and its derivative pDOC165. Arrows on vectors denote direction and approximate length of the different ORFs.

primer walking. The complete sequence of pDK4 (GenBank accession number KR018806) is 3,480 bp in length with a G+C content of 41.64% and contains three open reading frames (ORFs) that code for proteins > 70 amino acids in length. The amino acid sequences, deduced from pDK4 nucleotide sequences, were analyzed using the BlastP program in NCBI. *Orf2* and *orf3* share significant identity (53% and 73%) with a putative plasmid recombination enzyme (Mob, GenBank Accession No. WP_014293682.1, also known as a conjugative mobilization protein) from the marine halotolerant *Oceanimonas* sp. GK1 and a putative replication initiation protein (RepA, YP_002265403) from the fish pathogen *Aliivibrio salmonicida* LFI1238, respectively. *Orf1* did not match any sequences in the NCBI GenBank database. Generally, genes for mobilization and replication initiation proteins have been identified in plasmids for a wide range of bacteria.

A shuttle vector was constructed based on the method described by Zhao *et al*. (2011) with some modifications as follows. The complete DNA sequence of pDK4 was amplified by PCR with primers pDK4-FP (5′-ACGCGTCGACCGGTCAGCAGAATTAGCG-3′ containing a *Sal*I site) and pDK4-RP (5′-CGGGATCCAGTGGAAAGATGTTGCCTC-3′ containing a *Bam*HI site). The ~3.5-kb linearized pDK4 was digested with *Sal*I and *Bam*HI and cloned into pUC19, which is a small (2,686 bp), high-copy number *E. coli* cloning vector, producing the pUC19/pDK4 fusion vector (designated pDOC153). A DNA fragment containing the chloramphenicol acetyl transferase (CAT) gene and its promoter region was amplified by PCR from pCC1FOS (Epicentre), using primers CAT-FP (5′-cgcggatccCCTGTGACGGAAGATCAC-3′ containing a *Bam*HI site) and CAT-RP (5′-cgagctcgGCTGTTTCCTGTGTGAAATTG-3′ containing a *Sac*I site). The ~1.2-kb product was double-digested with *Bam*HI and *Sac*I, and ligated into pDOC153 treated with the same restriction enzymes. This construct was then transferred into *E. coli* DH5α cells, resulting in a *Pseudoalteromonas* – *Escherichia coli* shuttle vector (designated pDOC155, Fig. 2). Therefore, pDOC155 was expected to replicate in both *Pseudoalteromonas* and *E. coli* because of the presence of the pUC19 ori and pDK4 ori.

To select a proper *Pseudoalteromonas* host strain among marine bacterial strains from the PAMC collection, 10 strains of polar marine *Pseudoalteromonas* spp. (not affiliated into species, Table 1) were grown in MB at 25°C overnight, and 0.4 ml of each cell culture was inoculated into 20 ml of the same medium and grown at 25°C for 4–6 h. The cells were harvested, washed twice with cold sterile-water and resuspended in 100 μl cold water containing 20 mM glucose. Electrocompetent cells (100 μl) and shuttle vector pDOC155 (0.5 μg) were mixed together in a 0.2 cm electrode gap cuvette and electroporated with a Gene Pulser (Bio-Rad) at a voltage of 2.5 kV. After one pulse, 0.5 ml of MB containing 20 mM glucose was added to the cells, which were pre-incubated at 25°C for 17 h without shaking and then incubated on MB plates with ampicillin (50 μg/ml) and chloramphenicol (12.5 μg/ml). Following incubation for 3 days, Am^R- and Cm^R-transformants were obtained only from *Pseudoalteromonas* sp. PAMC 22137 at low frequency. When the DNA solutions from several PAMC 22137 transformants were subjected to PCR with primers CAT-FP and CAT-RP, the CAT cassette was amplified from each of them, indicating the presence of pDOC155. Additionally, the DNA solutions containing

pDOC155 prepared from the transformants could be transformed into *E. coli* DH5α cells, and from the Am^R- and Cm^R-*E. coli* transformants, pDOC155 was again extracted, demonstrating its successful replication in both *Pseudoalteromonas* sp. PAMC 22137 and *E. coli* DH5α cells.

An exochitinase gene (*chi22718_III*; 3,171 bp; KF574005) was amplified by PCR from the genomic DNA of Arctic marine *P. issachenkonii* PAMC 22718 (Kim *et al*., 2014) using primers chi22718_III-FP (5′-tgattacgccAAGCTTATGAGCTCACGTAAAATAATAAC-3′ containing a pUC19 MCS region and the *Hin*dIII site, small letters and underlined, respectively) and chi22718_III-FP (5′-gcaggcatgcAAGCTTTTACAGGCTACAACTTAAGGT-3′). Using the In-Fusion Advantage PCR Cloning Kit (Clontech), the promoter-less *chi22718_III* PCR product (3,203 bp) was cloned into *Hin*dIII-linearized pDOC155 downstream of the *Plac* of pUC19, producing an exochitinase gene-containing vector pDOC165 (Fig. 2). Previously, Zhao *et al*. (2011) demonstrated that the *Plac* promoter was efficient for heterologous expression of erythromycin resistance and cold-adapted cellulose genes from a psychrophilic *Pseudoalteromonas* strain. Additionally, a gene encoding the outer membrane protein, TonB-dependent receptor (designated *chi22718_IV*; 2,856 bp; KT819916) was amplified by PCR from the *P. issachenkonii* PAMC 22718 genome using primers TonB DR-FP (5′-tgattacgccAAGCTTATGTTAAATAACAAAGTTTCAAAAGCA-3′) and TonB DR-RP2 (5′-gcaggcatgcAAGCTTTTAGAACTTAGTAGTAAAGTTCAACT-3′). The *catA* gene for the intracellular soluble protein catechol 1,2-dioxygenase (936 bp; BAA07036) was amplified from a mesophilic soil bacterium *Pseudomonas putida* mt-2 using primers mt-2 catA-FP (5′-tgattacgccAAGCTTATGACCGTGAAAATTTCCCACA-3′) and mt-2 catA-RP (5′-gcaggcatgcAAGCTTTCAGCCCTCCTGCAACG-3′). The resultant amplicons (2,888 bp and 968 bp, respectively) were cloned into *Hin*dIII-linearized pDOC155 following the same methods used for *chi22718_III*, producing pDOC158 (pDOC155 derivative containing *chi22718_IV*) and pDOC159 (pDOC155 derivative containing *catA*).

To transfer pDOC165 into *Pseudoalteromonas* sp. PAMC 22137, the triparental mating method was used with a helper plasmid. *E. coli* DH5α (pDOC165) donor and *E. coli* HB101 (pRK2013) helper cells were grown overnight in LB medium supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml), respectively, at 37°C. PAMC 22137 recipients were grown in MB at 25°C for 24 h. Each 4.0 ml of the donor, helper and recipient was pelleted, washed twice with 2.0 ml 10% glycerol in a microcentrifuge at low speed for 2 min, and suspended in 0.4 ml of 10% glycerol. The cell suspensions were mixed and filtered through a 0.2 μm cellulose filter. The filter was placed on a LB medium plate and incubated at 15°C for 20 h. At the end of the mating period, the filter was transferred into a 1.5 ml microcentrifuge tube containing 1.0 ml of 10% glycerol. The bacterial cells were suspended thoroughly by agitation in a vortex mixer and plated onto MB plates containing ampicillin (50 μg/ml) and chloramphenicol (12.5 μg/ml). The plates were incubated at 15°C until PAMC 22137 transformants appeared (~7 days). The transformation of *Pseudoalteromonas* sp. PAMC 22137 with pDOC158 or pDOC159 was performed according to the same method described above.

To confirm that the cloned genes are stably maintained on the shuttle vector pDOC155 in heterologous *Pseudoalteromonas* sp. PAMC 22137, PCR experiments were performed with DNA solutions extracted from the Am^R- and Cm^R-transformants of *Pseudoalteromonas* sp. PAMC 22137: a transformant containing pDOC159, designated *Pseudoalteromonas* sp. tf24; pDOC158, *Pseudoalteromonas* sp. tf26; and pDOC165, *Pseudoalteromonas* sp. tf31. Each of the transformants was cultivated in MB/ampicillin and chloramphenicol for 48 h at 15°C and then used for recombinant vector extraction with the AccuPrep Plasmid Mini Extraction Kit. PCR reactions were performed with 2 μl of DNA solution and 20 pmol of each primer in an AccuPower HotStart PCR PreMix (Bioneer) with a final volume of 20 μl. The thermocycler program used for the PCR reactions was: 94°C for 5 min, 35 cycles (94°C for 30 sec, 68°C for 45 sec and 72°C for 1–3 min) and finally 72°C for 5 min. The following primers were used to amplify target genes: *catA* gene, mt-2 catA-FP and mt-2 catA-RP; *chi22718_IV* gene, TonB DR-FP and TonB DR-RP2; and *chi22718_III* gene, Chi22718_III-FP and Chi22718_III-FP. As shown in Fig. 3, all of the primer sets generated the PCR products of the expected sizes (*catA*, 968 bp; *chi22718_IV*, 2,888 bp; and *chi22718_III*, 3,203 bp), while no PCR product was detected from pDOC155. At this construction stage of the genetic manipulation system, these results indicate the potential use of the *Pseudoalteromonas* –*Escherichia coli* shuttle vector pDOC155 system for exogenous gene transfer

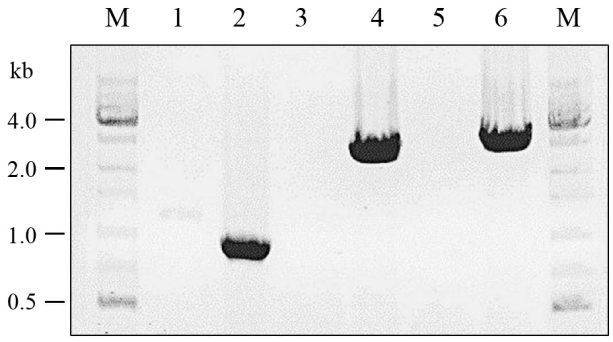

Fig. 3. Agarose gel electrophoresis of PCR amplicons from *Pseudoalteromonas* sp. PAMC 22137 transformants. Lanes: M, DNA ladder; 1, 3 and 5, transformant tf01 (shuttle vector pDOC155); 2, transformant tf24 (pDOC159, pDOC155 derivative containing *catA*); 4, transformant tf26 (pDOC158, pDOC155 derivative containing *chi22718_IV*); 6, transformant tf31 (pDOC165, pDOC155 derivative containing *chi22718_III*).

into marine *Pseudoalteromonas* spp.

Heterologous production of cold-adapted enzymes has been attempted with a variety of *Escherichia coli*-based vector-host systems at different temperatures. However, these systems deliver inert inclusion bodies and, although in active forms, low protein production levels that can be attributed to protein misfolding in *E. coli* cells grown at their optimal temperature (i.e. 37°C) and a low metabolic growth rate of *E. coli* at low temperatures, respectively. Accordingly, there are increasing requirements for the development of efficient over-expression systems for cold-active enzymes originating from psychrophilic microorganisms. We anticipate that this gene-transfer system consisting of marine *Pseudoalteromonas* strains and pDOC155 as host strains and a shuttle vector, respectively, should be potentially suitable for the expression of cold-active enzymes that originate from cold-adapted marine *Pseudoalteromonas* spp.

적 요

남극 해양세균 *Pseudoalteromonas* sp. PAMC 21150에서 분리한 소형 플라스미드(small plasmid, pDK4)의 크기는 3,480 bp이고 G+C 함량은 41.64%이며, 3개의 open reading frames (ORFs)을 포함하고 있다. 3개의 ORF는 replication initiation protein (RepA), conjugative mobilization protein (Mob), 그리고 기능이 밝혀지지 않은 단백질을 코팅하고 있다. PCR 반응으로 증폭한 pDK4를 *Escherichia coli* high-copy pUC19 클로닝 벡터에 삽입하여 fusion vector (pDOC153)를 제작하였고, pDOC153에 chloramphenicol 저항성 유전자를 삽입하여 ampicillin/chloramphenicol 저항성 *Pseudoalteromonas*－*Escherichia coli* 셔틀 벡터(shuttle vector; 7,216 bp 크기; pDOC155)를 제작하였다. 북극 해양세균 *P. issachenkonii* PAMC 22718이 보유한 2개의 유전자(TonB-dependent receptor gene, *chi22718_IV*, and exochitinase gene, *chi22718_III*)를 pDOC155에 삽입하여 두 개의 pDOC155 변형체(pDOC158, pDOC165)를 제작하였다. pDOC158 혹은 pDOC165을 이용하여 triparental mating 방법에 의해 플리스미드 미보유 해양세균인 *Pseudoalteromonas* sp. PAMC 22137를 형질전환하였다. PCR을 이용한 유전자 증폭실험을 통해서, pDOC158와 pDOC165에 삽입된 유전자들은 *Pseudoalteromonas* sp. PAMC 22137와 *E. coli* DH5α 내에서 안정적으로 유지되는 것을 확인하였다. 위의 결과는 셔틀 벡터 pDOC155는 *Pseudoalteromonas* spp. 유래 유전자들을 다른 *Pseudoalteromonas* spp. 세포 안으로 전달할 수 있는 새로운 유전자 전달시스템으로 이용될 수 있음을 보여주었다.

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References

Al Khudary, R., Stösser, N.I., Qoura, F., and Antranikian, G. 2008. *Pseudoalteromonas arctica* sp. nov., an aerobic, psychrotolerant, marine bacterium isolated from Spitzbergen. *Int. J. Syst. Evol. Microbiol*. **58**, 2018–2024.

Feller, G., d'Amico, D., and Gerday, C. 1999. Thermodynamic stability of a cold-active alpha-amylase from the Antarctic bacterium *Alteromonas haloplanctis*. *Biochemistry* **38**, 4613–4619.

Kim, D., Park, H.J., Kim, I.C., and Yim, J.H. 2014. A new approach for discovering cold-active enzymes in a cell mixture of pure-cultured bacteria. *Biotechnol. Lett*. **36**, 567–573.

Mancuso Nichols, C.A., Garon, S., Bowman, J.P., Raguénès, G., and Guézennec, J. 2004. Production of exopolysaccharides by Antarctic marine bacterial isolates. *J. Appl. Microbiol*. **96**, 1057–1066.

Marx, J.C., Collins, T., D'Amico, S., Feller, G., and Gerday, C. 2007. Cold-adapted enzymes from marine Antarctic microorganisms. *Mar. Biotechnol*. **9**, 293–304.

Médigue, C., Krin, E., Pascal, G., Barbe, V., Bernsel, A., Bertin, P.N.,

Cheung, F., Cruveiller, S., D'Amico, S., Duilio, A., *et al.* 2005. Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. *Genome Res*. **15**, 1325–1335.

Wang, P., Yu, Z., Li, B., Cai, X., Zeng, Z., Chen, X., and Wang, X. 2015. Development of an efficient conjugation-based genetic manipulation system for *Pseudoalteromonas*. *Microb. Cell Fact*. **14**, 11.

Wietz, M., Gram, L., Jørgensen, B., and Schramm, A. 2010. Latitudinal patterns in the abundance of major marine bacterioplankton groups. *Aquat. Microb. Ecol*. **61**, 179–189.

Yu, Z.C., Zhao, D.L., Ran, L.Y., Mi, Z.H., Wu, Z.Y., Pang, X., Zhang, X.Y., Su, H.N., Shi, M., Song, X.Y., *et al.* 2014. Development of a genetic system for the deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913. *Microb. Cell Fact*. **13**, 13.

Zhao, D.L., Yu, Z.C., Li, P.Y., Wu, Z.Y., Chen, X.L., Shi, M., Yu, Y., Chen, B., Zhou, B.C., and Zhang, Y.Z. 2011. Characterization of a cryptic plasmid pSM429 and its application for heterologous expression in psychrophilic *Pseudoalteromonas*. *Microb. Cell Fact*. **10**, 30.