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Transposon mutagenesis of *Psychrobacter cryohalolentis* PAMC 21807 by tri-parental conjugation

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Abstract Random mutagenesis is commonly used to study gene function. The screening of mutants exhibiting specific phenotypes assists in the identification of phenotype-related genes. In the current study, we isolated Antarctic bacteria, and developed a transposon Tn5 mutagenesis system. A total of 26 strains were isolated from seawater and freshwater near Antarctic King Sejong Research Station, King George Island. Six *Psychrobacter* strains were identified as psychrophilic, with optimal growth temperatures of 10℃ or 15℃ *Psychrobacter cryohalolentis* PAMC 21807 with a high growth rate at 4℃ was selected for transposon mutagenesis. Tri-parental conjugation with a plasmid containing Tn5 produced 13 putative recombinants containing the selectable marker. Genomic Southern hybridization confirmed Tn5 existed as episomes for seven recombinants, and for a single recombinant, Tn5 was integrated into the genome of *Psychrobacter cryohalolentis* PAMC 21807. The result indicates that the mutagenesis method, although successful, has a relatively low rate. The psychrophilic bacteria isolated in this study may be a useful resource for studying cold adaptation mechanisms, and the mutagenesis method can be applied to genetic analysis.

Keywords **cold adaptation, *Psychrobacter*, psychrophilic bacteria, tri-parental conjugation, transposon mutagenesis**

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1 Introduction

Bacterial growth and metabolic processes are generally retarded at low temperatures. At low temperatures, kinetic energy, enzyme activity, and membrane fluidity are reduced, resulting in a lowering of material exchange and signaling. In contrast, psychrophilic bacteria can survive and proliferate below 10℃, sustain metabolic activity at −20℃, and survive at −45℃[1-2]. Psychrophilic bacteria adapt to low temperatures by increasing unsaturated fatty acid content, expressing cold shock and anti-freeze proteins, and regulating resource efficiency and molecular motion[3-9].

Members of genus *Psychrobacter* have been isolated from a variety of polar environments including Arctic permafrost, Antarctic soils and lakes, sea ice, cyanobacterial mats and krill[10-15]. Further to this, some *Psychrobacter* strains have been identified as psychrophilic[15-17]. The genome sequences and proteomic data for several *Psychrobacter* strains have been already been determined[18-20].

Recombinant DNA technology has been used previously to investigate gene function in psychrophilic *Psychrobacter* species. The green fluorescent protein (GFP) was introduced into *Psychrobacter* sp. SW5H using the *npt*-2 promoter and Tn10, whilst *Psychrobacter arcticus* 273-4 knockout mutants were generated using the pJK100 suicide vector and conjugation[9,21-22].

In this study, we isolated bacterial strains from Antarctic seawater and freshwater, selected psychrophilic *Psychrobacter* with high growth rates at 4℃, and developed a

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recombinant system using transposon mutagenesis and tri-parental conjugation. The polar psychrophilic bacteria isolated in the current study are a useful resource for studying cold adaptation mechanisms. In addition, the mutagenesis method can be applied to high-throughput screening of mutants with abnormal phenotypes at low temperature, and the cloning of genes involved in cold adaptation.

2 Materials and methods

2.1 Sample collection

Seawater and freshwater samples were collected from coastal areas and a lake near the Antarctic King Sejong Research Station (King George Island; 62°13′S, 58°47′W) on 20 December, 2005. The temperature of both seawater and freshwater was approximately 3℃. The samples were collected in sterilized cryovial tubes, and transported to the laboratory of the Korea Polar Research Institute (KOPRI) under cold conditions. Water samples were serially diluted 10-fold in either sterilized seawater or distilled water, spread on Nutrient agar, R2A agar and Zobell agar plates, and cultured at 4℃ for 4 weeks. Distinct colonies were selected and sub-cultured repeatedly at 4℃ until a pure colony was obtained. All strains were deposited at PAMC (Polar and Alpine Microbial Collection, http://pamc.kopri.re.kr).

2.2 PCR amplification and sequence analysis of 16S rRNA genes

Almost full-length 16S rRNA gene sequences were amplified by colony PCR using primers 27F (5'-AGA GTT TGA TCN TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). A 50 μL reaction volume was used for all PCR reactions and consisted of 10 μM of each primer, 1×PCR buffer (5 mM KCl, 0.001% gelatin, 1 mM Tris-HCl), 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1 μL template DNA, 2.5 units Taq polymerase, made up to volume with MilliQ water. PCR was performed in a thermal cycler (Biometra, Germany) and the following cycling conditions applied: an initial denaturation at 94℃ for 5 min followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 1 min, with a final extension at 72℃ for 7 min. PCR products were visualized by 1.5% agarose gel electrophoresis and purified with an AccuPrep PCR purification kit (Bioneer, Korea).

The full-length sequences of amplified 16S rRNA genes were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) (KF528714−KF528740). The sequences were submitted to the EzTaxon (http://eztaxon-e.ezbiocloud.net) database, and sequence analyses performed to identify closely related type species[23]. Phylogenetic analyses were performed using PHYLIP[24], and phylogenetic trees constructed using the neighbor-joining method with Kimura's two-parameter model[25]. The closest relatives of the Antarctic strains were determined using the combined data obtained from the EzTaxon and phylogenetic analyses (Table 1).

Table 1 List of Antarctic bacteria identified in this study. Asterisk (*) indicates that the strain failed to form a colony when cultured above 20℃ for 7 d. S: seawater, F: freshwater.

Strain No.	The closest relative (Ac. No.)	Similarity/%	Optimum temp./℃	Sampling site
PAMC 25181	*Albidiferax ferrireducens* T118^T (CP000267)	98.41	15*	S
PAMC 25156	*Arthrobacter scleromae* YH-2001^T (AF330692)	99.28	15-25	F
PAMC 25164	*Chryseobacterium marinum* IMCC3228^T (EF554366)	100	15	S
PAMC 25179	*Chryseobacterium marinum* IMCC3228^T (EF554366)	97.84	15-20	F
PAMC 25160	*Flavobacterium aquatile* ATCC11947^T (M62797)	96.87	15	S
PAMC 25151	*Flavobacterium degerlachei* LMG21915^T (AJ557886)	99.29	20	S
PAMC 25165	*Flavobacterium degerlachei* LMG21915^T (AJ557886)	99.22	15-25	S
PAMC 25178	*Flavobacterium tegetincola* ACAM602^T (U85887)	97.13	15	S
PAMC 25152	*Flavobacterium tiangeerense* 563^T (EU036219)	98.29	20	S
PAMC 25153	*Flavobacterium tiangeerense* 563^T (EU036219)	98.28	15-20	S
PAMC 25148	*Flavobacterium weaverense* AT1042^T (AY581114)	97.00	15	S
PAMC 25149	*Flavobacterium weaverense* AT1042^T (AY581114)	97.57	4-20	S
PAMC 25175	*Pedobacter alluvionis* NWER-II11^T (EU030688)	94.86	4-25	F
PAMC 25183	*Pedobacter boryungensis* BR-9^T (HM640986)	97.88	20	S
PAMC 25174	*Polaromonas naphthalenivorans* CJ2^T (CP000529)	98.78	4-20	F

(To be continued on the next page)

(Continued)

PAMC 25172	*Pseudomonas cannabina* CFBP 2341^T (AJ492827)	99.07	25	F
PAMC 25154	*Pseudomonas meridiana* CMS 38^T (AJ537602)	99.57	20-30	F
PAMC 25180	*Pseudomonas thivervalensis* CFBP 11261^T (AF100323)	99.50	20-30	F
PAMC 25159	*Psychrobacter fozii* NF23^T (AJ430827)	97.73	15*	S
PAMC 25150	*Psychrobacter glacincola* DSM 12194^T (AJ312213)	99.50	20	S
PAMC 25158	*Psychrobacter glacincola* DSM 12194^T (AJ312213)	98.20	10*	S
PAMC 25167	*Psychrobacter glacincola* DSM 12194^T (AJ312213)	98.20	15*	S
PAMC 25168	*Psychrobacter glacincola* DSM 12194^T (AJ312213)	97.98	15*	S
PAMC 25170	*Psychrobacter glacincola* DSM 12194^T (AJ312213)	98.29	15*	S
PAMC 25171	*Psychrobacter glacincola* DSM 12194^T (AJ312213)	98.20	10*	S
PAMC 25176	*Salinibacterium amurskyense* KMM3673^T (AF539697)	99.72	10-20	S

2.3 Temperature characterization of the bacterial strains

Pure colonies were cultured in R2A broth at 15℃. R2A medium was chosen preferentially over Nutrient and Zobell because it supported better colony formation. Further to this, some strains failed to grow on either Nutrient or Zobell agar plates. The culture solutions were diluted 20-fold in R2A broth and inoculated on R2A agar (10×10 cm^2 square dishes) as 35 replicates. Five replicate dishes were cultured at 4, 10, 15, 20, 25, 30 and 37℃ for 7 d. Colony size was measured as colony diameter on days 1, 3, 5 and 7 post-inoculation. The optimum growth temperature for each strain was established from the colony diameter on day 7.

The 37 *Psychrobacter* strains isolated from polar environments and stored in PAMC were investigated for their suitability in mutagenesis studies. Each strain was inoculated on R2A agar at 4, 20 and 37℃ for 7 d.

2.4 Antibiotic resistance profiles of *Psychrobacter* strains

The 37 *Psychrobacter* strains were cultured at 15℃ in R2A broth supplemented with 13 different antibiotics (Table 2). The culture solutions were diluted 20-fold with distilled water, inoculated on R2A agar (10×10 cm^2 square dishes) containing antibiotics, in duplicate, and cultured at 15℃ for 5 d.

2.5 Transposon mutagenesis using tri-parental conjugation

We chose the tri-parental conjugation method for transposon mutagenesis (Figure 1). Optimum culture conditions for the recombination system was determined empirically by incubating *Psychrobacter cryohalolentis* PAMC 21807 and *E. coli* cells on Marine agar 2216 (Difco MA) at different temperatures (15, 25 and 37℃), NaCl concentrations (0, 1, 2, 3, 4 and 5% at 15℃) and kanamycin concentrations (1, 10, 20, 30, 40 and 50 μg·mL^{-1} at 15℃ on Marine agar containing 2% NaCl) for 2 d.

Table 2 Concentrations of antibiotics and sensitivities of *Psychrobacter* strains

Antibiotics	Working solution/ (μg·mL^{-1})	Strains with antibiotics resistance*
Ampicillin (Amp)	10	-
Chloramphenicol (Cm)	100	-
Carbenicillin (Cb)	100	-
Gentamycin (Gm)	30	-
Hygromycin B (Hyg)	100	-
Kanamycin (Km)	30	PAMC 21801
Lincomycin (Lm)	15	All
Neomycin (Nm)	30	-
Penicillin G (Pc)	20	-
Polymyxin B (Pm)	100	PAMC 21852
Streptomycin (Sm)	50	-
Tetracycline (Tet)	30	-
Troleandomycin (Tm)	15	All

*The symbol '-' means there were no strains showing antibiotic resistance.

For tri-parental conjugation, *E. coli* S17-1 (λ pir) pUTKm was used as the transposon donor[26-27], *E. coli* K-12 HB101 pRK2013 as the helper plasmid[28] and *Psychrobacter cryohalolentis* PAMC 21807 as the recipient cell. The tri-parental conjugation system was a modification of previous reports[29-31]. In brief, the *E. coli* strains were cultured at 37℃ on LB agar containing kanamycin (50 μg·mL^{-1}), and the *Psychrobacter* strain cultured at 15℃ on Marine agar containing 2% NaCl. For each strain, a pure single colony was inoculated into 10 mL liquid broth and cultured as per previous experiments. Culture broths (1.5

mL for each strain) were centrifuged at 13 200 rpm for 1 min, the supernatant discarded, and the cell pellets washed three times with 1 mL 0.85% NaCl solution. For tri-parental conjugation, the donor, helper and recipient cells were combined 1:1:6 based on optical density (O.D) values. In total, 3 mL of cell solution was concentrated through a mini filter set[26,32]. The filter membrane was placed on LB agar, and cultured at 25℃ for 22 h. Cells grown on the filter membrane were suspended in 1 mL 0.85% NaCl solution, spread on Marine agar containing NaCl (2%) and kanamycin (50 μg·mL^{-1}), and cultured at 15℃ for 2 d.

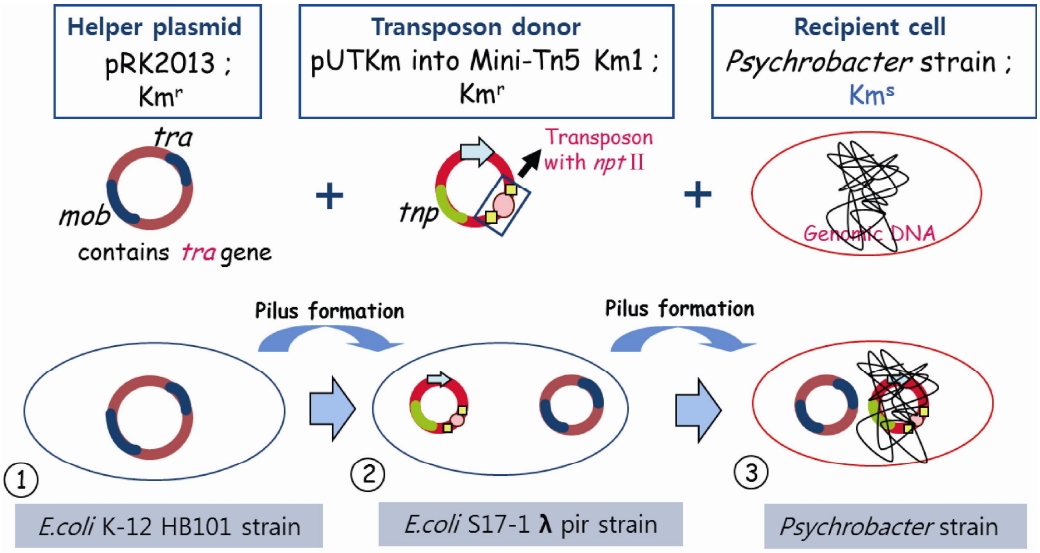

Figure 1 Schematic of the tri-parental conjugation method.

2.6 Confirmation of mutant clones by PCR-RFLP

The presence/absence of the kanamycin resistance gene (*npt*II) in mutant clones was established by colony PCR using *npt*II specific primers: pUTKm_F (5' ATC GAT TGT ATG GGA AGC CC 3') and pUTKm_R (5' GGC AAG ATC CTG GTA TCG GT 3'). The PCR reaction was performed in a thermal cycler (Biometra, Germany) and the cycling conditions consisted of an initial denaturation at 94℃ for 10 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for 15 s, and extension at 72℃ for 1 min. A final extension was performed at 72℃ for 10 min. PCR products were digested using *Hae*III at 37℃ for 3 h, and visualized by 1.5% agarose gel electrophoresis.

2.7 Genomic Southern hybridization

Total genomic DNA was extracted from *Psychrobacter* and *E. coli* strains using an AccuPrep genomic DNA Extraction kit (Bioneer, Korea). Following treatment with Rnase (10 mg·mL^{-1}), genomic DNA was precipitated with cold sodium acetate (pH 5.5) and absolute ethanol, and resuspended in 100 μL Tris buffer. Genomic DNA concentrations were measured on a Nanodrop spectrophotometer.

Five micrograms of genomic DNA was digested with *Eco*RV, and the reaction fractionated on a 1% agarose gel. The gel was submerged in 0.25 M HCl, 1×Denaturing buffer (0.5 M NaOH, 1.5 M NaCl, pH 7.0) and 1×Neutralizing buffer (0.5 M Tris-HCl, 1.5 M NaCl; pH 7.0), in the order as listed, with shaking, at room temperature for 30 min each.

Blotting was performed using the TurboBlotter™ (Schleicher & Schuell, Germany) and Nytran® SuperCharge Nylon membranes (Boehringer Mannheim, Germany). Post-transfer, the membrane containing genomic DNA was rinsed with 2×SSC for 5 min, and DNA fixed to the membrane by baking at 80℃ for 2 h. The membrane was placed in hybridization solution with a *npt*II gene-specific probe, prepared using the DIG High Prime DNA Labeling and Detection starter Kit II (Roche Diagnotics GmbH, Germany). The LAS-3000 imaging system (FUJI, Japan) was used for the chemiluminescence detection of DNA on the membrane.

3 Results and discussion

3.1 Selection of low-temperature adapted strains of *Psychrobacter*

A total of 26 bacterial strains were isolated from Antarctic seawater and freshwater (Table 1). Of these, eight strains belonged to genus *Flavobacterium*, and seven to genus *Psychrobacter*. Two Antarctic strains, PAMC 25160 and PAMC 25175, showed less than 97% sequence similarity with respective type strains, and were regarded as candidates for new species or genus. Seven strains that failed to form colonies at temperatures greater than 20℃ were labeled psychrophiles. Six of these psychrophilic strains belonged to genus *Psychrobacter*, suggesting that *Psychrobacter* species could use cold adaptation mechanisms to grow rapidly at low temperatures. Psychrophilic *Psychrobacter* strains deposited in PAMC were screened to select

strains suitable for further mutagenesis analysis.

Of the 37 *Psychrobacter* strains deposited in PAMC, isolate PAMC 21807 formed the largest colonies at 4℃ (Figure 2), and was therefore selected for transposon mutagenesis analysis.

Strain No.	4℃	20℃	37℃
PAMC 20226			
PAMC 20262			
PAMC 21639			
PAMC 21801			
PAMC 21807			
PAMC 21821			
PAMC 21843			
PAMC 21852			
PAMC 21865			
PAMC 21866			
PAMC 21871			
PAMC 21875			
PAMC 21876			
PAMC 21942			
PAMC 21993			
PAMC 22007			
PAMC 22011			
PAMC 22024			
PAMC 22030			
PAMC 22052			
PAMC 22058			
PAMC 22061			
PAMC 22064			
PAMC 22069			
PAMC 22075			
PAMC 22096			
PAMC 22103			
PAMC 22116			
PAMC 22124			
PAMC 22133			
PAMC 25150			
PAMC 25158			
PAMC 25159			
PAMC 25167			
PAMC 25168			
PAMC 25170			
PAMC 25171			

Figure 2 Growth properties of 37 *Psychrobacter* strains at different temperatures. **a**, 4℃; **b**, 20℃; **c**, 37℃ for 7 d post-inoculation. Scoring standard: 0, 1, 2, 3 and 4 (0, no colony; 1, CD < 2 mm; 2, 2 mm < CD < 4 mm; 3, 4 mm < CD < 8 mm; 4, CD > 8 mm) colony diameter (CD).

3.2 Antibiotic resistance profiles of *Psychrobacter* strains

Antibiotic sensitivity of the 37 *Psychrobacter* strains deposited in PAMC to 13 different antibiotics was investigated (Table 2). All *Psychrobacter* strains were sensitive to the following antibiotics: ampicillin, carbenicillin, chloramphenicol, gentamycin, hygromycin B, neomycin, penicillin G, streptomycin and tetracycline (Table 2). On the other hand, they were all resistant to lincomycin and troleandomycin. With the exception of PAMC 21801, all remaining *Psychrobacter* strains were sensitive to kanamycin. Similarly, all *Psychrobacter* strains except PAMC 21852 were sensitive to polymyxin B. PAMC 21807 was sensitive to kanamycin, a selection maker for Tn5 mutagenesis, and was therefore selected for use as the recipient strain in transposon mutagenesis. PAMC 21807 was isolated from moss growing around Antarctic King Sejong Research Station, and shared 99.4% 16S rRNA gene sequence similarity with *Psychrobacter cryohalolentis* $K5^T$ (Ac. No. CP000323). Isolate PAMC 21807 was therefore identified as *Psychrobacter cryohalolentis* PAMC 21807.

3.3 Transposon mutagenesis using tri-parental conjugation

To perform transposon (Tn5) mutagenesis using the tri-parental conjugation system, optimum growth conditions had to be established for recombinant selection. The optimum growth conditions for recombinant selection was with Marine agar 2216 supplemented with 2% NaCl and 50 $\mu g \cdot mL^{-1}$ kanamycin, and incubated at 15℃ (Figure 3). To select for *Psychrobacter* colonies transformed with Tn5, *Psychrobacter* colonies had to be distinguished from *E. coli*. This was achieved by assessing their growth conditions at 15℃. At 15℃, PAMC 21807 colonies were observed within 24 h, whilst *E. coli* strains S17-1 and K-12 took comparatively longer at 2–3 d (Figure 3a). Hence, plates were grown at 15℃ to select PAMC 21807. Differences in growth rates were also observed for PAMC 21807 and *E. coli* strains on Marine agar with 2% NaCl (Figure 3b). The successful insertion of plasmid pUTKm into the genome of *Psychrobacter* by tri-parental conjugation confers kanamycin resistance to PAMC 21807 (Figure 3c). Thus, the concentration of kanamycin was determined as 50 $\mu g \cdot mL^{-1}$, for which colony formation of non-transformed PAMC 21807 cells was negligible.

A total of 20 putative recombinants were selected and screened for the kanamycin resistant gene *npt*II using PCR (Figure 4a). The *npt*II specific gene was introduced to the recombinants from transformation with *E. coli* S17-1. To confirm whether kanamycin resistant strains were *Psychrobacter cryohalolentis* PAMC 21807, RFLP analysis was applied. When the PCR products of the 16S rRNA gene were digested with *Hae*III, all 20 putative recombinants exhibited the same pattern of DNA fragmentation as PAMC 21807 (Figure 4b). Hence, all 20 putative recombinants

were identified as PAMC 21807, acquiring kanamycin resistance by tri-parental conjugation.

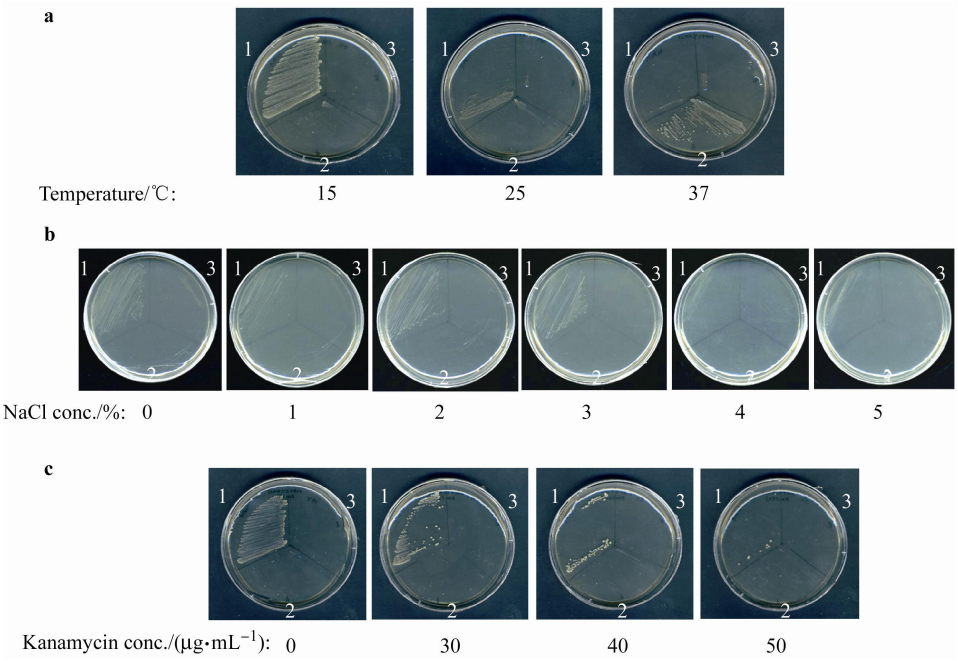

Figure 3 Optimization of growth conditions for selecting recombinant *Psychrobacter* clones. Clones were grown at different temperatures (**a**); on agar plates with varying concentrations of NaCl (**b**); on agar plates with varying concentrations of kanamycin (Km, **c**). 1, *Psychrobacter cryohalolentis* PAMC 21807; 2, *E. coli* containing plasmid pRK2013; 3, *E. coli* containing pUTKm with Mini-Tn5 Km1.

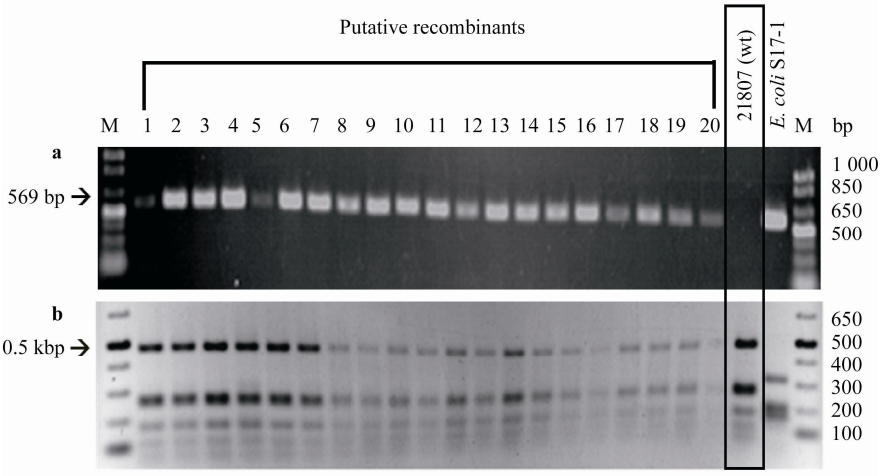

Figure 4 **a**, Screening of colony PCR products obtained for putative recombinants using *npt*II gene-specific primer sets. **b**, Electrophoresis of restricted fragments for the 16S rDNA region. PCR products amplified using 16S rDNA-specific primer sets were digested using *Hae*III. M, 1 kb DNA molecular size marker; lane 1–20, putative recombinants; 21807 (wt), *Psychrobacter cryohalolentis* PAMC 21807.

Southern hybridization with the *npt*II probe was used to establish whether transposons existed as episomes, or if they were integrated into the *Psychrobacter* genome. Of the 20 putative recombinants, seven strains failed to grow in broth. Genomic DNA hybridization was performed on the remaining 13 strains. Two bands consistent in size with Mini-Tn5 Km1 were detected for seven putative recombinants, indicating their existence as episomes (Figure 5). However, one weak band was confirmed for a single recombinant (21807-4), indicating that the transposon was integrated into the chromosome for this recombinant strain. This result revealed that the mutagenesis method developed in the current study could be used successfully, albeit at a relatively low rate.

4 Conclusion

The distinguishing characteristic of psychrophiles is their ability to undergo relatively rapid growth at low temperatures[33]. In this report, we selected 26 Antarctic strains exhibiting good growth at 4℃ across 4 weeks. Six strains belonging to genus *Psychrobacter* were attributed as psy

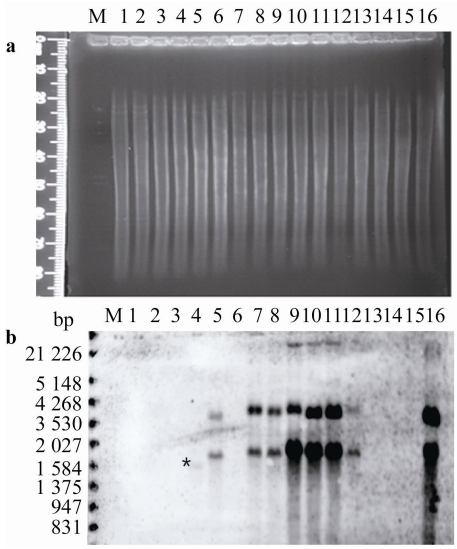

Figure 5 Electrophoretic profile of PAMC 21807 genomic DNA digested with restriction enzymes (**a**), and Genomic Southern hybridization of *npt*II. DNA fragments were transferred to nylon membranes and probed with DIG-labeled *npt*II partial gene sequence (**b**). M, lambda DNA size marker digested with *Eco*RI and *Hind*III. Lane 1, *Psychrobacter cryohalolentis* PAMC 21807 wild type; lane 2–14, putative recombinants; lane15, PAMC 21807 Wild type; lane 16, Mini-Tn5 Km1. Asterisk (*) indicates the transposon that was integrated into the PAMC 21807 chromosome.

chrophilic bacteria, because they failed to grow at temperatures above 20℃. These psychrophilic bacteria may be a useful source for studying cold adaptation mechanisms. *Psychrobacter cryohalolentis* PAMC 21807 demonstrated a high growth rate at 4℃ and as such was selected as a model strain for use in tri-parental conjugation. Tri-parental conjugation with a plasmid containing Tn5 resulted in the selection of 13 putative recombinants, of which Tn5 existed as an episome for seven. Genomic Southern hybridization confirmed a single putative Tn5 chromosomally inserted recombinant was produced by tri-parental conjugation. In future studies, we hope to construct a mutant pool, in which mutants might show abnormal growth patterns at low temperatures. This would be only one of the approaches used to identify cold-related genes, and would further our understanding of the genetic and physiological factors affecting by cold adaptation. High-throughput screening of mutants with abnormal phenotypes at low temperature and the cloning of genes involved in cold adaptation will facilitate a better understanding of cold adaptation mechanisms in microorganisms.

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