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ORIGINAL PAPER

Characterization of a cryptic plasmid from a Greenland ice core *Arthrobacter* isolate and construction of a shuttle vector that replicates in psychrophilic high G+C Gram-positive recipients

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Abstract Over 60 Greenland glacial isolates were screened for plasmids and antibiotic resistance/sensitivity as the first step in establishing a genetic system. Sequence analysis of a small, cryptic, 1,950 bp plasmid, p54, from isolate GIC54, related to *Arthrobacter agilis*, showed a region similar to that found in theta replicating *Rhodococcus* plasmids. A 6,002 bp shuttle vector, pSVJ21, was constructed by ligating p54 and pUC18 and inserting a chloramphenicol acetyl transferase (CAT) cassette conferring chloramphenicol resistance. Candidate Gram-positive recipients were chosen among glacial isolates based on phylogenetic relatedness, relatively short doubling times at low temperatures, sensitivity to antibiotics, and absence of indigenous plasmids. We developed an electroporation protocol and transformed seven isolates related to members of the *Arthrobacter*, *Microbacterium*, *Curtobacterium*, and *Rhodoglobus* genera with pSVJ21. Plasmid stability was demonstrated by successive transformation into *Escherichia coli* and four Gram-positive isolates, growth without antibiotic, and plasmid re-isolation. This shuttle vector and our transformation protocol provide the basis for genetic experiments with different high G+C Gram-positive hosts to study cold adaptation and expression of cold-active enzymes at low temperatures.

Keywords Cryptic plasmids · Glacial isolates · *E. coli* · High G+C Gram-positive shuttle vector

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Introduction

Studies of psychrophilic microorganisms, their genetics, ecology, and ability to survive and maintain cellular functions at low temperatures would be facilitated by the development of specific cloning vectors and transformation protocols. Vectors capable of replicating in cells growing at low temperatures also could be developed as expression systems allowing the proper translation, folding, and large-scale production of cold-active and/or heat-labile proteins. To our knowledge only one vector specific for a psychrophile has been reported, based on a plasmid from an Antarctic Gram-negative isolate *Pseudoalteromonas haloplanktis* (Tutino et al. 2001), and recently Miyake et al. (2007) used a broad host range vector for low temperature expression in a *Shewanella* Antarctic isolate. A similar wide temperature range (4–35°C) expression system for *Rhodococcus erythropolis* was described (Nakashima and Tamura 2004).

Most current vectors were constructed for use in one, well-characterized organism, or have been tested in only a few closely related species. Vectors based on natural plasmids that could be used with a variety of environmental psychrophilic hosts would be particularly useful for examining the genetics and physiology of these organisms. One ubiquitous group contains the high G+C Gram-positive organisms, which also are found in cold and frozen environments. Isolates that have been characterized such as those related to *Arthrobacter* species are metabolically versatile, have morphologically distinct developmental cycles, and may form ultrasmall cells. Although large catabolic plasmids have been found in *Arthrobacter* sp. (Sandu et al. 2005; Overhage et al. 2005) and some vectors based on plasmids from *Brevibacterium* have been used in *Arthrobacter* (Morikawa et al. 1994; Shaw and Hartley

1988), no small vectors have been reported specifically for psychrophilic members of the high G+C Gram-positive group.

Our goal was to establish a cloning system that could be used both with *Escherichia coli* and high G+C Gram-positive psychrophiles by: (a) selecting and characterizing a suitable plasmid, (b) constructing a shuttle vector, (c) selecting suitable Gram-positive recipients, (d) developing an electroporation method useful for several organisms and (e) examining the stability of the vector in different recipients. Here we present the results with one selected cryptic plasmid, p54, from the *Arthrobacter* isolate GIC54 which was used to construct a shuttle vector, pSVJ21, for *E. coli* and Gram-positive organisms. This vector was successfully transformed and maintained in *E. coli* and high G+C Gram-positive psychrophilic recipients related to four different genera from our Greenland ice core isolate collection.

Materials and methods

Plasmids, strains and culture conditions

Characterized new plasmids and vectors used in this study are described in Table 1. The bacterial strains were isolated from a Greenland Ice Sheet Project 2 (GISP2) ice core as described (Miteva et al. 2004; Miteva and Brenchley 2005). Phylogenetic relationships of the isolates were determined via molecular analysis of 16S rDNA sequences (Miteva et al. 2004; this study). The isolates were grown aerobically at 18 or 25°C in trypticase soy broth (TSB) or R2B (Difco Laboratories, Detroit MI) and on similar agar media, TSA or R2A. The isolates' growth temperature range was examined on TSA or R2A at 2, 10, 18, 25, 30 and 37°C and the doubling times of selected isolates determined in TSB or R2B at 5 and 25°C. *E. coli* strain DH5α was grown at 37°C in Luria–Bertani broth (LB). LBA with 100 μg/ml ampicillin (Amp) was the selective medium after transformation of *E. coli*. TSA with 2.5, 10 or 20 μg/ml chloramphenicol (Cm) was used as the selective medium after electroporation of the Gram-positive isolates.

Plasmid isolation and analysis

Plasmid DNA was isolated from TSB or R2B grown cultures using the Wizard SV-plus kit (Promega, Madison, WI). For some isolates showing poor cell lysis, bead-beating was applied with a MiniBeadbeater-8 Cell Disrupter (Biospec Products, Inc.). Plasmids were analyzed on 0.8 or 1% Tris-acetate-EDTA buffer, pH 8.0, agarose gels and sizes were estimated using reference plasmids with known molecular sizes or after digest with restriction enzymes.

Antibiotic sensitivity/resistance screening

Antibiotic sensitivity was determined using the Kirby–Bauer disc diffusion method (Bauer et al. 1966) and the following antibiotic containing Sensi-Discs (Becton Dickinson, MA): ampicillin (10 μg), penicillin (10 units), bacitracin (10 units), chloramphenicol (10 μg), tetracycline (30 μg), erythromycin (15 μg), kanamycin (30 μg), gentamycin (10 μg), nalidixic acid (30 μg), novobiocin (30 μg), ciprofloxacin (5 μg), rifampicin (5 μg), streptomycin (10 μg) and vancomycin (30 μg). One milliliter of TSB broth cultures was spread on each plate and after removing the excess liquid and drying, the antibiotic discs were placed aseptically on the surface. The cultures were incubated and the zones of inhibition measured and evaluated according to the manufacturer's instructions. The sensitivity of some isolates to different concentrations of selected antibiotics ampicillin (10, 20, 50, 100 μg/ml), chloramphenicol (2, 5, 10, 20, 50, 80 μg/ml) and kanamycin (10, 30, 40, 50 μg/ml) was also determined.

Sequencing of a cryptic plasmid from GIC 54 *Arthrobacter* isolate

The plasmid p54 was linearized at a single *Bam*HI site, cloned into pUC18, and sequenced at the Nucleic Acid Facility, Penn State using the M13 Universal primer and synthesized primers from each sequencing step. Two of these primers overlapping the cloning *Bam*HI site were used to sequence the original circular p54 in order to test the uniqueness of the *Bam*HI site. Sequence data were

Table 1 Plasmids used in this study

Plasmids	Relevant characteristics	Source or reference
pUC18	Amp^R, MCS, *rep* (pMB1)	Yanisch-Perron et al. (1985)
p54	Putative replication function for Gram- positive bacteria	This study
pUC18 + p54	Amp^R, Constructed shuttle plasmid	This study
pKSV7	Contains a 1.3-kb *cat* cassette	Smith and Youngman (1992)
pSVJ21 (pUC18 + p54 + *cat*)	Amp^R, Cm^R, Constructed shuttle plasmid	This study

assembled and analyzed using the DNASTAR program. Homology searches were carried out by the BLAST, Open Reading Frame (ORF) Finder and Insertion Sequence (IS) Finder programs of NCBI database and Database of Plasmid Replicons (DPR) (Osborn 1999).

Construction of a shuttle vector

Plasmids p54 and pUC18 were isolated using the Perfectprep® Plasmid Midi kit (Eppendorf, Westbury, NY), purified via gel extraction from a 0.8% agarose gel using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Plasmids p54 and pUC18 were linearized with *Bam*HI. Plasmid pUC18 was treated with calf intestine alkaline phosphatase (CIP) and both linearized plasmids purified via gel extraction. After ligation of the plasmids with the Epicentre Technologies Fast-Link™ DNA Ligation Kit (Madison, WI), the construct was transformed into chemically competent *E. coli* DH5α cells prepared with the Z-Competent kit (Zymo Research, Orange, CA). Recombinant plasmids were purified and examined by restriction analysis.

The chloramphenicol acetyl transferase (CAT) cassette conferring resistance to chloramphenicol was excised from pKSV7 (Smith and Youngman 1992) with *Hinc*II. The digest products were subjected to electrophoresis on a 0.8% TAE agarose gel and the 1.3 kb fragment carrying the CAT cassette was extracted and purified. The construct p54 + pUC18 was linearized with *Ssp*I, treated with CIP and combined with the excised fragment from pKSV7 by blunt end ligation, using the Epicentre Technologies Fast-Link™ DNA Ligation Kit. The ligation mixture was used to transform Z-competent *E. coli* DH5α and transformants selected for ampicillin resistance.

Electroporation of Gram-positive isolates as recipients

Electro-competent cells were obtained by a modified PEG protocol of Desomer et al. (1990) for our selected recipients. Cells were grown in 500 ml of TSB at 18 or 25°C depending on their optimal growth. At mid-log phase cells were harvested by centrifugation (9,000×*g*) and washed with 80 ml of autoclaved de-ionized water. Cells were pelleted again (9,000×*g*) and concentrated 50-fold in 10 ml of autoclaved 30% PEG 1,000. Aliquots of 400 µl were either used immediately for electroporation or frozen at 80°C.

Five micro liter containing 150–500 ng of plasmid DNA was added to fresh or thawed aliquots of electrocompetent cells and the mixture was transferred to the electroporation cuvette (BTX Havard Apparatus) with a gap of 0.2 cm. Electroporation was performed on a BioRad Gene Pulser at 2.5 kV, 400 Ω, 25 µF. After the pulse, cells were tested for viability by plating on non-selective media. The suspensions were immediately transferred to 1 ml SOC broth, containing 5 g yeast extract, 20 g tryptone, 0.5 g NaCl, 10 ml of 250 mM KCl solution, 5 ml of 2 M $MgCl_2$ and 20 mM of glucose per liter and incubated at 25°C with shaking at 200 rpm for 1–2 h to allow recovery and phenotypic expression before cells were plated on selective media. Transformation efficiencies were calculated as number of Cm^R transformants per µg plasmid DNA or as ratio of Cm^R transformants (cells/ml) and total number of viable cells (cells/ml) in each electroporated aliquot.

Plasmid vector stability

The structural stability was assessed by restriction analysis with *Eco*RI, *Rsa*I, and *Hind*III of plasmid DNA from transformants. The segregational stability of the constructed plasmid vector in both Gram-positive and Gram-negative recipients was tested by prolonged cultivation (2–10 days) in broth media without antibiotic. The proportion of antibiotic resistant from 100 randomly selected colonies was determined periodically followed by re-isolation of the plasmid and restriction analysis. Stability percentages were calculated as follows: number of colonies grown on the selective medium/number of colonies grown on non-selective medium × 100.

Results

Screening Greenland ice core isolates for plasmids

Previously, we had screened Greenland ice core isolates for plasmids and antibiotic resistance/sensitivity (Miteva et al. 2004). We examined this collection for those containing plasmids suitable as vectors for genetic manipulation in psychrophilic Gram-positive hosts. Although plasmids were found in about 30% of the isolates, relatively few of the high G+C Gram-positive organisms harbored plasmids (18%). In addition, small plasmids, potentially suitable for vector construction, were detected mostly in proteobacterial isolates. Only one small plasmid, p54 from a rose–red pigmented isolate GIC54 related to *Arthrobacter agilis*, was observed from all the Gram-positive isolates screened.

Characterization of the cryptic plasmid p54 and vector construction

The size of the single cryptic plasmid p54 was first estimated by restriction analysis to be about 2 kb. A construct with p54 ligated to pUC18 was maintained in *E. coli* and used for sequence analysis. The p54 segment, starting at the *Bam*HI site, had 1,950 bp with G+C content of 59.6

mol%, slightly higher than that of the chromosomal 16S rRNA gene sequence (57.3 mol%) found for isolate GIC 54. The uniqueness of the single *Bam*HI site found by restriction analysis was confirmed by sequencing the original circular plasmid p54 with two primers, overlapping the *Bam*HI site used for cloning. BLAST analysis was used to search for replication functions and open reading frames (ORF) on the plasmid. Although there were seven ORFs larger than fifty amino acids, few similarities with known functions were found. One ORF (1538–1235) had 47% similarity to several bacterial exo-nucleases of the Excalibur family, including *Rhodococcus*, and a short sequence (1709–1744) matched part of a hypothetical protein of the *Arthrobacter* FB24 plasmid 3 (EMBL/GenBank/DDBJ Genome sequence database). One region of special interest between nucleotides 1611 and 1950 contained a short sequence (1657–1679) corresponding 100% to sequences from small theta replicating *Rhodococcus* plasmids pB264 (Lessard et al. 2004), pKNRO2 (Na et al. 2005), and pKA22 (Kulakov et al. 1997). Three consecutive directly repeated sequences (1723–1780, 1781–1838, 1839–1856) and one inverted repeat, possibly related to theta replication, were also found in this region (Fig. 1). In addition, sequences were found similar to a number of (insertion sequences (IS), some of which originated from *Leifsonia xyli, Corynebacterium, Rhodococcus* and other high G+C Gram-positive bacteria.

The shuttle plasmid vector was constructed by inserting a CAT gene (conferring chloramphenicol resistance) excised from the *Bacillus* plasmid pKSV7 (Smith and Youngman 1992) into the p54 + pUC18 construct. The resulting new 6,002 bp construct, designated pSVJ21, contained genes for ampicillin and chloramphenicol resistance and replicons for both Gram-positive and Gram-negative bacteria.

Selection of Gram-positive bacterial recipients

Potential recipients were chosen from among 60 high G+C Gram-positive Greenland ice core (GIC) isolates based on their phylogenetic relationships, relatively short doubling times at low temperatures, sensitivity to antibiotics, and absence of indigenous plasmids. Resistance to 1–12 antibiotics was previously detected in 90% of the GIC isolates (Miteva et al. 2004) with multiple antibiotic resistance or sensitivity found within all major phylogenetic groups. In addition, we tested ten of our newer ultrasmall celled high G+C isolates (Miteva and Brenchley 2005) for resistance/sensitivity to chloramphenicol, ampicillin and kanamycin at different concentrations (2.5–100 μg/ml). Most isolates were resistant to kanamycin, including the host strain of p54, and two strains showed resistance to ampicillin (Table 2).

Seven chloramphenicol sensitive isolates that grew at 2°C (the lowest temperature tested), had doubling times of 7–15 h at 5°C and 1.5–4 h at 25°C, and lacked indigenous plasmids were selected as suitable candidates for transformation. Based on the phylogenetic analysis of their 16S rDNA sequences (Fig. 2), these isolates represented four genera and were related to *Arthrobacter agilis, A. oxidans, A. chlorophenolicus, A. sulfonivorans, Curtobacterium flaccumfaciens*, *Microbactetrium aurum* and *Rhodoglobus vestalii* (Table 2).

Development of a transformation method for Gram-positive recipients

Because we were using natural isolates as recipients, it was important to develop an efficient transformation protocol. We tested numerous variations of published electroporation methods for high G+C Gram-positive organisms

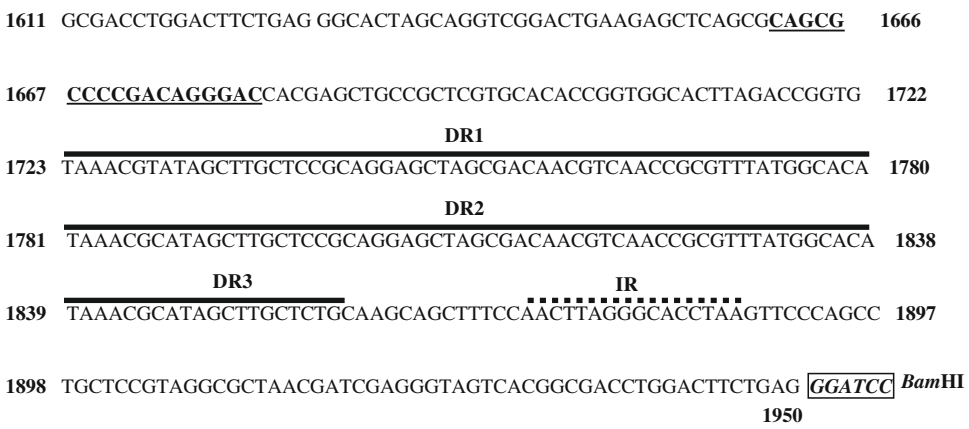

Fig. 1 Nucleotide sequence of the 340 bp region (1611–1950) of plasmid p54 from Arthrobacter isolate GIC54 possibly related to replication. The underlined bolded sequence has homology to several theta replicating plasmids, the three long iteron-like direct repeats are marked with thick lines and are designated DR1, DR2 and DR3, and the inverted repeat is marked with a dotted thick line. Numbering is based on using the BamHI site as the beginning coordinate, i.e. the last nucleotide is adjacent to the BamHI site (in italics)

Table 2 Characteristics of isolate GIC 54 and selected high G+C Gram-positive recipients

Isolate	Closest relative (distance %)	Plasmid	Antibiotic sensitive (S)/resistant (R)			Growth temperature range (°C)[a]	Doubling time (h)	
			Amp	Km	Cm		25°C	5°C
GIC 54	*Arthrobacter agilis* (1%)	p54	S	R	S	2–29	1.5	12
SO3-2	*Microbacterium aurum* (1%)	–	S	R	S	2–37	3	12
SO3-7w	*Arthrobacter sulfonivorans* (1.6%)	–	S	S	S	2–30	2	7
SO3-8	*Arthrobacter globiformis* (0.6%)	–	S	R	S	2–30	2.2	15
UMB24y	*Curtobacterium flaccumfaciens* (0.7%)	–	S	S	S	2–37	4	12
SO3-12	*Arthrobacter chlorophenolicus* (2.2%)	–	S	R	S	2–37	3	10
SO3-14	*Arthrobacter oxydans* (0.7%)	–	R	R	S	2–30	3	10
GIC R18	*Rhodoglobus vestalii* (3.46%)	–	R	R	S	2–25	4[b]	12

[a] Growth was tested at 2, 10, 18, 25, 30 and 37°C

[b] The doubling time for GIC R18 was estimated at 20°C

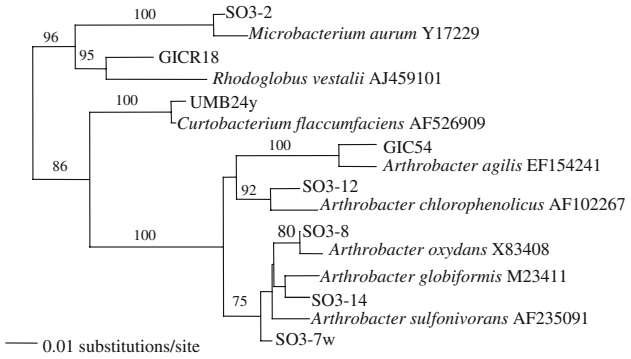

Fig. 2 Phylogenetic relationships based on 16S rRNA gene sequences among the host of p54, *Arthrobacter* GIC54, the seven Greenland ice core isolates used as recipients for plasmid vector pSVJ21 and their closest relative species with corresponding NCBI accession numbers. Sequences were aligned using ClustalX and the tree was generated with the PAUP4.0 Beta 10 program using the neighbor joining method. The values of 100 bootstrap trial replicates are given above nodes

(Brigidi et al. 1990; Desomer et al. 1990; Lessard et al. 2004) by modifying protocols for electro-competent cell preparation, using different gene pulsing equipment, varying the pulse parameters, and including different chloramphenicol concentrations.

Our final protocol was based on polyethylene glycol (PEG 1000) electroporation (Desomer et al. 1990) with the following parameters (2.5 kV, 400 Ω, 25 μF) using a BioRad Gene Pulser. It resulted in cell survival between 0.2 and 1.7% of the initial 10^9–10^{10} cells/ml and allowed successful and reproducible transformation of all selected Gram-positive recipients with pSVJ21. In samples where the survival was below 0.1%, no Cm^R transformants were obtained. Transformants initially only formed colonies on TSA agar with 2.5 μg/ml chloramphenicol, but were able to grow on media containing 5 or 10 μg/ml antibiotic when re-streaked. Transformation was improved by using freshly prepared electro-competent cells and diluting the plasmid DNA to a concentration of 150–500 ng. Transformation frequency in Gram-positive recipients was 10^2–10^3 μg^{-1} DNA or 10^{-5} and 10^{-6} per total cell number surviving the pulse. The efficiency of *E. coli* transformation with plasmid pSVJ21 DNA purified from *E. coli* was usually higher by an order of magnitude (10^3–10^4 μg^{-1} DNA) compared to using pSVJ21 isolated from a Gram-positive host. The plasmid yields were also significantly higher from *E. coli* than from Gram-positive cultures with the same volume and density.

The authenticity of the transformants was demonstrated by microscopy and by showing that the restriction patterns and nucleotide sequences of PCR amplified 16S rDNA from Cm^R colonies corresponded to those found for the original isolates. Additionally, some transformants grown with or without antibiotic were examined using genomic Enterobacterial Repetitive Intragenic Consensus (ERIC) PCR fingerprinting, which gives strain specific profiles of the whole genome, as described earlier (Miteva et al. 2004). All transformants had patterns identical to the original recipient (data not shown).

The initial transformation into different Gram-positive isolates suggested that pSVJ21 has a broad host range. To determine whether the plasmid could be stably replicated and re-transformed into different hosts, we performed successive and cross-transformations into and from *E. coli* and four Gram-positive isolates SO3-2, SO3-7w, SO3-8 and UMB24y and back into *E. coli*. All of the original transformants tested contained the plasmid which was purified and used for the second transformation. New transformants were obtained with all recipients with frequencies of 10^2 μg^{-1} DNA for the Gram-positive recipients and 10^3 μg^{-1} DNA for *E. coli*.

Structural stability of the vector in Gram-negative and Gram-positive recipients

To determine whether pSVJ21 remained structurally stable, *E. coli* cells containing pSVJ21 were grown in liquid media with and without ampicillin and plasmid preparations subjected to restriction analysis with *Eco*RI and *Rsa*I. In every sample, the plasmid migrations and restriction patterns following electrophoresis were identical to the original pSVJ21 vector showing that no major deletions or re-arrangements had occurred.

The structural stability of the vector in Gram-positive hosts was assessed in transformants of all selected recipients receiving plasmids prepared from *E. coli*, in second round transformants of SO3-2, SO3-7w, SO3-8 and UMB24y receiving plasmids prepared from each of these previously transformed isolates, and in cross-transformants between the *Curtobacterium* related isolate UMB24y and the *Arthrobacter* related SO3-8. All plasmids from these Gram-positive transformants, some of which were grown at 2, 10 and 25°C, and after re-transformation into *E. coli*, had identical sizes and restriction patterns as the original pSVJ21 demonstrating its structural stability in these diverse isolates.

Segregational stability of the vector in Gram-negative and Gram-positive recipients

The segregational stability of pSVJ21 was first tested in *E. coli* cells grown in liquid media without ampicillin and sampled after 2, 3, 4, 5, 6, 12 and 32 h. In all samples, essentially 100% of the cells were Amp^R and retained the full-sized plasmid (data not shown). Next, segregational stability was tested in four Gram-positive recipients grown at 25 or 5°C for up to 6 and 12 days, respectively (Fig. 3) to determine whether growth temperature affected plasmid replication. Growth rates and the final density of cultures grown with and without chloramphenicol were similar although cells in the presence of antibiotic sometimes had longer lag phases (Data not shown). The percentage of Cm^R colonies for transformants of SO3-7w, related to the *Arthrobacter* species GIC54, and SO3-2, related to a species in the *Microbacterium* genus, grown at 25°C without chloramphenicol remained high during exponential growth but decreased during late stationary phase (Fig. 3 panels A and B). Interestingly, the same transformants showed much higher plasmid stability when grown at 5°C (Fig. 3 panels E and F).

The segregational stability was also examined in the *Curtobacterium* related UMB24y and *Arthrobacter* related SO3-8 isolates transformed with plasmids prepared from the UMB24y transformants (Fig. 3 panels C, D, G and H). The UMB24y transformants had higher stability at 5°C (Fig. 3 panels C and G). The plasmid appeared least stable at both temperatures in the *Arthrobacter* related SO3-8 transformed with plasmid DNA prepared from UMB24y (Fig. 3 panels D and H). In order to increase the number of generations during segregational stability tests, we also performed four consecutive sub-cultivations of SO3-2, SO3-7w, UMB24y and SO3-8 without chloramphenicol and found similar levels of stability (Data not shown). However, the plasmid was maintained, even in SO3-8 cells, when the selective pressure of chloramphenicol in the growth medium was present.

Discussion

As a first step towards developing an effective genetic system for psychrophilic microorganisms, especially those belonging to the high G+C genera, we screened our Greenland glacier ice isolate collection for potential recipients and plasmids. Interestingly, many of our ice core isolates from different phylogenetic groups were resistant to multiple antibiotics even though they had been trapped in ice for at least 100,000 years and had not been exposed to current antibiotic levels. Although there are numerous physiological effects that can account for antibiotic resistance, some of our closely related GIC isolates with similar properties were resistant to different antibiotics suggesting they may carry specific resistance genes. The intrinsic mechanisms involved in antibiotic resistance in environmental isolates have been reviewed (Hogan and Kolter 2002; D'Costa et al. 2006) and the finding of antibiotic resistant isolates merits further investigation.

The screening of our isolate collection for plasmids showed that although 18 isolates had one or more plasmids, most harbored large plasmids (>50 kb) (Miteva et al. 2004). Few other reports focus on detection of plasmids in isolates from cold and frozen environments. Two early studies found different sized plasmids in diverse Antarctic seawater and soil bacteria (Kobori et al. 1984; Ray et al. 1991). Later, Tutino et al. (2000, 2001) characterized two plasmids from Antarctic isolates related to the Gram-negative genera *Pseudoalteromonas* and *Psychrobacter* and developed an efficient low temperature expression system for *Pseudoalteromonas haloplanktis* (Cusano et al. 2006; Papa et al. 2007). Finally, a large plasmid in an Antarctic *Pseudomonas* isolate that degraded hydrocarbons was recently reported (Ma et al. 2006).

Small plasmids, however, from psychrophilic Gram-positive organisms are less common, making the small size (1,950 bp) of the p54 plasmid from *Arthrobacter* isolate GIC54 valuable. The complete nucleotide sequence of p54 also differs from other known plasmids since no similarity to known genes was found. Although no lengthy region

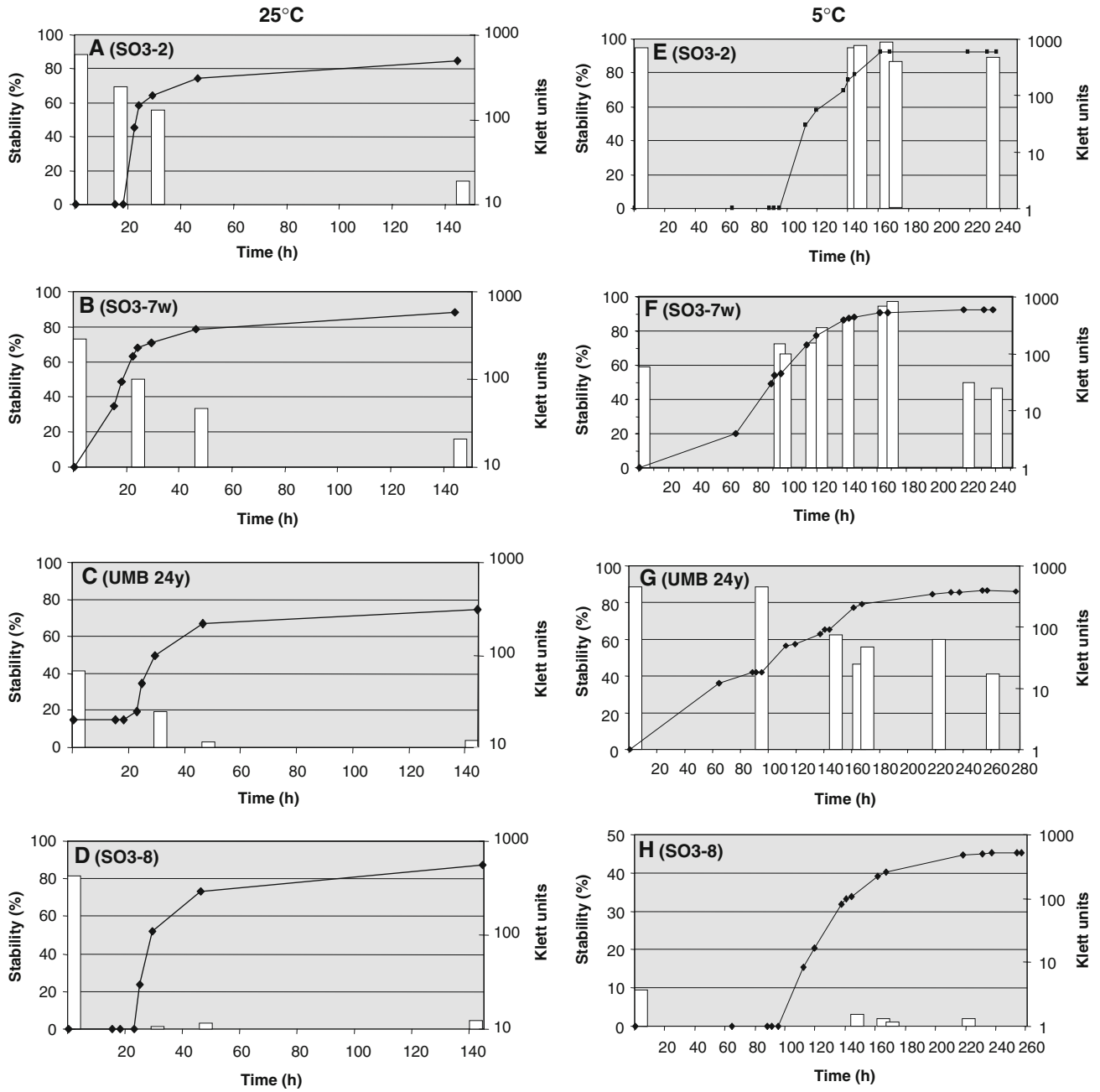

Fig. 3 Segregational stability of pSVJ21 in four high G+C Gram-positive recipients SO3-2, SO3-7w, SO3-8 and UMB24y during cultivation in the absence of antibiotic pressure at 25 and 5°C. Black lines show growth curves (Klett units) and white columns represent percent stability at different time points

with homology to a replication protein was found, a short sequence corresponded to sequences in theta replicating plasmids from *Rhodococcus* species (Kulakov et al. 1997; Lessard et al. 2004; Na et al. 2005). In addition, the iteron-like direct repeats and the adjacent inverted repeat in p54 may be associated with theta replication suggesting this mechanism for p54 (Chattoraj 2000). Theta replicating plasmids have been found in both Gram-negative and Gram-positive bacteria and are considered structurally more stable than those replicating via the rolling circle mechanism (del Solar et al. 1998; Bartoshik et al. 2002; Alpert et al. 2003; Shareck et al. 2004). However, the mechanisms for their stable inheritance have not been well studied and multiple unknown plasmid loci may be involved (Shen et al. 2006). The classification of theta replicating plasmids based on three key components (an initiator rep protein, origin of replication with specific structural organization and a host encoded DNA

polymerase) is also not final (Osborn 1999; Alpert et al. 2003). The differences between p54 and characterized plasmids illustrate our limited knowledge of the potential diversity of plasmids yet to be discovered.

Efficient transformation can be a major bottleneck in developing genetic systems for new organisms. The inability to obtain transformants could be due to numerous factors such as the plasmid not entering the cells, the plasmid not replicating in the cells, or the inability to express the antibiotic resistance gene. After testing several different electroporation protocols and parameters, refinements of one developed for *Rhodococcus fascians* (Desomer et al. 1990) allowed successful transformation of pSVJ21 into a variety of recipients. The successful transformation of isolates related to organisms from four genera indicates a wide host range for pSVJ21 and provides a major advantage for future applications. More importantly, some of our glacier ice isolates used as recipients belong to the group of ultramicrobacteria known for their dominance in many natural habitats, diverse survival strategies, and unique metabolic properties (Cavicchioli and Ostrowski 2003). The ability to transform pSVJ21 into these isolates provides the basis for genetically analyzing the physiology of these ultrasmall-celled organisms.

The pSVJ21 vector had very high structural and segregational stability in all hosts grown with antibiotic, whereas the segregational stability under non-selective conditions differed depending on the host and growth temperature. One observation was that the shuttle vector showed highest stability (above 50%) in SO3-2, related to *Microbacterium aurum*, and lowest (2.5%) in *Arthrobacter* SO3-8 at both 25 and 5°C. Based on the phylogenetic analysis of the recipients and their closest relatives (Fig. 2) SO3-2 was more distantly related to the original host of p54 (GIC54) compared to the more closely related SO3-8. At the same time, SO3-7w was also more closely related to GIC 54 but showed nearly as high stability as SO3-2. These observations indicate that the reduced plasmid stability in some recipients may be due to involvement of specific host-encoded proteins in plasmid replication and maintenance. Our finding that pSVJ21 was more stable in isolates SO3-7w and UMB 24y at 5°C than at 25°C suggests a role of temperature in plasmid stability and makes them good candidates for low temperature protein expression distinct from other wide temperature range expression systems. The combination of this new shuttle vector, based on a small, possibly theta replicating plasmid from a cold-adapted *Arthrobacter* isolate, and the electroporation protocol provide the basis for a new cloning system for low temperature expression of cold-active enzymes in high G+C Gram-positive hosts and for genetic studies of psychrophilic microorganisms.

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