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Isolation, characterization, and utilization of a temperature-sensitive allele of a *Pseudomonas* replicon

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Abstract

In order to facilitate genetic study of the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, we isolated a conditional, temperature-sensitive plasmid origin of replication. We mutagenized the popular *Pseudomonas* stabilizing fragment from pRO1610 *in vitro* using the *Taq* thermostable DNA polymerase in a polymerase chain reaction (PCR). Out of approximately 23,000 potential clones, 48 temperature-sensitive mutants were isolated. One mutant was further characterized and the origin of replication was designated as mSF^{ts1}. The mutations that resulted in a temperature-sensitive phenotype in mSF^{ts1} were localized to the 1.2 kb of minimum sequence required for replication in *P. aeruginosa*. The DNA sequence analysis revealed two mutations within the coding sequence of the Replication control (Rep) protein. Growth of *P. aeruginosa* carrying the temperature-sensitive plasmid at the non-permissive temperature of 42°C resulted in loss of the plasmid by greater than 99.9999% of the cells after 16 hours of growth. In order to facilitate its utilization, the mSF^{ts1} was converted into a genetic cassette flanked by mirrored restriction endonuclease digestion sites of a pUC1918 derivative. We demonstrate utilization of the mSF^{ts1} for genetic studies involving complementation and regeneration of a mutant in *P. aeruginosa* research.

Keywords

stabilizing fragment; conditional replicon; plasmid cure; PCR mutagenesis; DNA sequencing; genetic cassette

1. Introduction

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that primarily infects patients who are immunocompromised or suffer from the genetic disorder cystic fibrosis (CF). This gram-negative bacterium is found almost ubiquitously in diverse environments including soil, in the hospital environment, and in association with various eukaryotic organisms.

P. aeruginosa has been the subject of intense research for the past several years as the model system for study of biofilms and quorum sensing mechanisms (Miller and Bassler, 2001, O'Toole et al., 2000). However, even with the advances in molecular technology such as DNA array and proteomic approaches, many of the studies on *P. aeruginosa* that highlight biofilms and quorum sensing are still performed using classical genetic tools, including transposons (Brint and Ohman, 1995, Lindsey et al., 2008, O'Toole and Kolter, 1998). Thus, it is important

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to continue developing better genetic tools to facilitate the study of *P. aeruginosa*. This is especially true for plasmid replicons. The two most commonly used plasmid replicons for *P. aeruginosa* research are pRK2 (pRP4) in the pLAFR series of vectors (Friedman et al., 1982, McIver et al., 1993, Vanbleu et al., 2004) and the pRO1610 replicon that is designated as the *Pseudomonas* stabilizing fragment (SF) (Olsen et al., 1982). The SF has been incorporated into the pUC series of *E. coli* plasmid cloning vectors to generate *P. aeruginosa*-*E. coli* shuttle vectors designated as the pUCP series of vectors (Schweizer, 1991, West et al., 1994). In addition, the 1.9 kb of SF, which carries the 3'-end of the *bla* gene has been incorporated into the unique *Pst*I site of the *bla* gene of pBR322 plasmid to allow replication in *P. aeruginosa* without affecting the *bla* mediated resistance to β -lactam antibiotics (Olsen, et al., 1982). In 1994, it was determined that approximately 1.2 kb of the original 1.9 kb of SF was necessary to mediate replication in *P. aeruginosa* (West, et al., 1994). This 1.2 kb fragment carries the origin of replication and the replication protein. During our studies to elucidate environmental stress mechanisms of *P. aeruginosa*, we sought to develop genetic tools that facilitate manipulation of the bacterium, including a conditional replicon of *P. aeruginosa* that can be cured relatively easily from the cell. Such a replicon could be used in a variety of studies in this bacterium. In this communication, we present the isolation, characterization, and utilization of a temperature-sensitive replicon SF that facilitates genetic manipulation of *P. aeruginosa*.

2. Materials and Methods

2.1. Bacterial strains, media, growth conditions

Bacteria were grown in L broth (LB) or LB supplemented with appropriate antibiotics at 37°C with aeration, unless otherwise indicated. The following antibiotic concentrations were used in this study (per milliliter): ampicillin (Ap^r), 100 μ g for *Escherichia coli*; carbenicillin (Cb^r), 250 μ g for *P. aeruginosa*. The bacterial strains and plasmids used in this study are listed in Table 1.

2.2. DNA manipulations, transformations, and conjugations

For cloning, *E. coli* strain DH10B was routinely used as the host. Plasmids were purified with QIAprep Miniprep columns by Qiagen (Santa Clarita, CA) or the FastPlasmid Mini columns by Eppendorf AG (Hamburg, Germany). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction system (Qiagen) according to the manufacturer's instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). The high-fidelity thermostable DNA polymerase *Pfu* from Stratagene (La Jolla, CA) was used to amplify DNA fragments for cloning, and Amplitaq thermostable DNA polymerase from Perkin Elmer (Foster City, CA) or *Taq* from NEB was used to screen for appropriate clones. Oligonucleotide linkers for *Sac*I were purchased from NEB and oligonucleotide primers for PCR amplification were purchased from Operon, Inc. (Alameda, CA). DNA was introduced into *E. coli* via by electroporation. A standard electroporation procedure was used for *E. coli* using the *E. coli* Gene Pulser by Bio-Rad (Hercules, CA). Electroporation of plasmids into *P. aeruginosa* were performed as previously described (Suh et al., 1999). For transfer of plasmids into *P. aeruginosa* via conjugation, tri-parental conjugation was performed as previously described (Suh, et al., 1999) with the following modifications. Briefly, the *E. coli* donor and the helper strains were grown to mid-log phase of growth at 37°C with aeration, while the recipient *P. aeruginosa* strain in stationary phase of growth was mixed 1:1 in LB containing 40 mM of NaNO₃ and incubated for at least 2 hours at 42°C without aeration. Then the donor, helper, and recipient cells were mixed at 7:7:1 ratio in 1 ml of saline, centrifuged, and resuspended in 25-50 μ l of saline. The cell suspension was spotted on a LB plate and incubated overnight at 30°C. Following incubation, cells were scraped with a sterile cotton swab, resuspended in 2 ml of saline, and plated on selective plates.

2.3. Isolation of a temperature-sensitive allele of the *Pseudomonas* Stabilizing Fragment (SF^{ts})

Two different PCR amplifications were performed to isolate temperature-sensitive alleles of the 1.9 kb *Pseudomonas* SF: a standard amplification approach that relied on the relatively low fidelity of the *Taq* polymerase, and a mutagenic amplification approach as described by Leung et al. (1989). In the mutagenic approach, the reaction mix contained Mn²⁺ in addition to Mg²⁺ and contained a 5-fold lower concentration of dCTP relative to the other three dNTPs in the reaction. Amplitaq (Perkin-Elmer, Foster City, CA) was used as the thermostable polymerase in both amplifications. The SF was amplified with the following oligonucleotide primers containing *Pst*I sites as noted by italics: SSO-62 (5'-AACGCTGCAGCAATGGCAACAACGTTGCGC-3') and SSO-63 (5'-AACGCTGCAGAAAGGCAGGCCGGGCCGTGG-3'). Briefly, the SF was amplified from pLJR0 (Runyen-Janecky et al., 1997) via either standard or mutagenic conditions, digested with *Pst*I, and ligated into the unique *Pst*I site within the *bla* gene of pSS191, a pBR322 derivative that carries the 230 bp *moriT* of RP4 at the *Hind*III site. The resulting ligation mixtures were electroporated into *E. coli* DH10B and approximately 30,000 Ap^r transformants were pooled. The pooled mixture was conjugated into *P. aeruginosa*, and then plated on selective medium (PIA Cb) to obtain 200-300 Cb^r colonies per plate. Following growth at 30°C, the Cb^r colonies were replica plated onto fresh PIA Cb plates and growth of the colonies at 30°C and 42°C were compared to identify putative SF^{ts} alleles.

2.4. Localization of the SF^{ts} mutation and construction of a genetic cassette

To map the SF^{ts} mutation within the minimal 1.2 kb sequence necessary for replicon function (West, et al., 1994), the SF^{ts} was PCR amplified from pSS227 with SSO-47 (AAG GCA TGC TGT CCA ACC GCT CTG TAG GC) and SSO-48 (AAG GCA TGC CTG CAG AAA GGC AGG CCG GG) as a *Sph*I fragment. Subsequent cloning of the 1.2-kb fragment into the unique *Sph*I site of pSS191 generated pSS254, which exhibited the same temperature-sensitive phenotype as pSS191. This confirmed that the SF^{ts} mutation(s) was localized within the mSF sequence.

A genetic cassette of SF^{ts} was constructed by attaching a *Sac*I linker (NEB) to the 1.2 kb mSF^{ts} and the resulting fragment was cloned into the unique *Sac*I site of pUC1918 (Schweizer, 1993). This generated pSS255, which carries the mSF^{ts} as a cassette that can be liberated with various restriction enzymes common to multiple cloning sites of plasmids.

2.5. Curing SF^(ts) from *Pseudomonas aeruginosa*

To measure the frequency by which plasmids carrying either the mSF or the mSF^{ts} are cured from *P. aeruginosa*, strain PAO1 carrying plasmids with each of the replicons was grown overnight at 30°C in LB Cb. The cultures were then diluted 1:2000 in LB and grown at either 30°C, 37°C, or 42°C for 16 hours and plated on LB and LB Cb plates to obtain colony forming units (cfu). Presence (Cb^r) or absence (Cb^s) of the plasmid was verified by PCR amplification of the mSF.

2.6. Construction of *rhIR* complementing plasmids

To complement the *rhIR* mutation in SS165 (PAO1 *rhIR201::aacCI*) (Chen et al., 2005), *rhIR* was PCR amplified from PAO1 with *Pfu* and cloned into pUC19 as a 1.3 kb *Eco*RI-*Xba*I fragment. The resulting plasmid, pSS10, was converted to a mobilizable plasmid, pSS440, by the addition of a *moriT* to the *Eco*RI site (Suh et al., 2004). Then either 1.2 kb mSF or mSF^{ts} was introduced as a *Hind*III fragment to pSS440 to generate pSS447 and pSS441, respectively. These plasmids were then introduced into SS165 to generate SS1673 and SS1921, respectively, for complementation tests.

2.7. DNA sequencing

The 1.9 kb *Pst*I fragment carrying the *SF^{ts1}* was sequenced with specific internal primers that corresponded to the SF. The plasmid clone that carried the *SF^{ts1}* allele (pSS227) was used as the template. Each sequence of the *SF^{ts1}* was covered four times to ensure accuracy. DNA sequencing was performed by the ACGT, Inc. (Northbrook, IL). DNA sequences were aligned and analyzed with the Lasergene software (DNA Star, Madison, WI).

2.8. Elastase assays and pyocyanin measurements

P. aeruginosa *rhlR201::aacCI* mutant (SS165) complemented with either pSS447 (mSF) or pSS441 (mSF^{ts1}) was initially streaked and grown at 30°C with Cb selection to ensure the presence of plasmids. The next day, individual colonies were restreaked onto L-agar plates and grown at 42°C overnight without Cb selection to promote plasmid curing. Individual colonies were then subcultured into LB and grown at 37°C for 15-18 hrs. From these cultures, the number of viable cells (colony forming units) was measured and supernatants were collected to assay for elastase and pyocyanin productions. Elastase activity was determined via the elastin congo-red hydrolysis assay using ~20-30 µg of supernatant protein as previously described (Ohman et al., 1980). The elastase activity was calculated as increase in $A_{495} \text{ min}^{-1} \text{ g}^{-1}$ of protein. Pyocyanin levels were determined as previously described (Suh, et al., 1999). Both elastase activity and pyocyanin productions were normalized to the number of viable cells.

3. Results

3.1. Isolation of a temperature-sensitive SF

We isolated temperature-sensitive alleles of a popular *Pseudomonas* replicon to provide a means for efficient elimination of plasmids. The temperature-sensitive Stabilizing Fragment (SF^{ts}) was isolated by random PCR mutagenesis of the 1.9 kb *Pst*I fragment that contains the necessary functions for plasmid maintenance in *P. aeruginosa* (Olsen, et al., 1982). Two different approaches were taken to introduce random mutations in the SF during DNA amplification. In the first approach, we used the standard PCR amplification protocol and relied on the relatively low fidelity of the *Taq* polymerase. In the second approach, we followed the procedure of Leung et al. (Leung et al., 1989) to increase the potential error rate of *Taq* polymerase. The amplified fragments were then cloned into the unique *Pst*I site located within the *bla* gene of a mobilizable pBR322 derivative, pSS191 and electroporated into *E. coli*. Approximately 30,000 Ap^r clones were initially isolated and pooled. To screen for temperature-sensitive mutants, the SF library was conjugated into PAO1 by triparental mating. Approximately 23,000 *P. aeruginosa* colonies from this conjugation were genetically screened for carbenicillin sensitivity following growth at 42°C indicating loss of the plasmid. From the genetic analysis, we identified 48 putative mutants that were unable to replicate at 42°C. On initial analysis, all 48 putative SF mutants behaved similarly in their Cb sensitivity at 42°C. Therefore, one of the constructs was characterized further and designated as SF^{ts1}. Subcloning of SF^{ts1} and DNA sequencing revealed that the temperature-sensitive phenotype of the replicon was located within the 1.2 kb mSF sequence between *Stu*I and *Pst*I. This is the minimum replication sequence determined by West et al. (1994) to be required for function. DNA sequence analysis revealed two mutations that resulted in temperature-sensitive phenotype: a G:C→T:A transversion that changed Gly to Cys at residue 100 and a C:G→A:T transversion that changed Ser to Arg at residue 204 (Figure 1). Both of these mutations are located within the ORF that encodes for the replication protein. The mechanism by which the mutations cause the temperature-sensitive phenotype is unclear.

3.2. Temperature-sensitive phenotype mSF^{ts1}

We measured efficiency of the loss of plasmids harboring the mSF^{ts1} at 42°C in order to determine the usefulness of the mSF^{ts1} carrying plasmids for genetic manipulation of *P. aeruginosa*. We found that when mSF^{ts1} harboring cells were grown at 42°C for 16 hours without antibiotic selection, greater than 99.9999 % of the cells had lost the plasmid while only 2.7778% of the non-temperature-sensitive SF carrying plasmids were lost under the same condition (Table 2). At 37°C, the frequency of loss was similar at 99.9997% for mSF^{ts1} and 3.0457% for mSF (data not shown). The loss of mSF^{ts1} in these cells was verified via PCR (data not shown). Thus, the loss of plasmids that replicate via mSF^{ts1} is very efficient in the absence of a selection. To cure the mSF^{ts1} carrying plasmid from a cell, we routinely grow cells overnight at the non-permissive temperature of 42°C, dilute the culture (10^{-6} - 10^{-8}) and plate the cells on L-agar to obtain individual colonies.

3.3. Construction of a genetic cassette and utilization of the mSF^{ts1}

In order to facilitate use of the SF^{ts1}, we subcloned the 1.2 kb mSF^{ts1} into the pUC1918 derivative as a *SacI* fragment to convert it into a genetic cassette that is flanked by mirrored restriction endonuclease sites of pUC19 with the exception of *EcoRI* (Figure 2). Therefore, the mSF^{ts1} can be liberated as a cassette and cloned into any *E. coli* plasmid carrying *P. aeruginosa* genes. This will convert the plasmid into a conditional *P. aeruginosa* plasmid that can be utilized for complementation and then subsequent curing from the strain.

To demonstrate the utility of mSF^{ts1} in genetic analysis of *P. aeruginosa*, we used the conditional replicon to first complement and then restore the original mutant phenotype of a *rhlR* mutant. RhlR is a global regulator of gene expression that is involved in cell-cell communication for optimal production of various virulence factors, including elastase and pyocyanin in *P. aeruginosa* (Brint and Ohman, 1995, Ochsner et al., 1994). To complement the PAO1 *rhlR201::aacCI* mutant (SS165) (Chen, et al., 2005), we cloned the mSF and mSF^{ts1} into pSS440, a plasmid that carries a wild-type copy of *rhlR* for complementation and *oriT* of RP4 for conjugation, to generate pSS447 and pSS441, respectively. The plasmids were introduced into SS165 via triparental mating and selected for Cb^r. The presence or either plasmids, pSS447 and pSS441, were verified by extraction and analysis of plasmids from the respective strains (data not shown). We then grew the bacterial cells at 42°C to promote plasmid curing as described in Materials and Methods, and determined the genetic complementation of *rhlR* mutant phenotype by measuring elastase activity and pyocyanin. As demonstrated in Figure 3, pSS447 which carries the mSF, was not cured during growth at 42°C as indicated by complementation of the *rhlR* mutation. In contrast, pSS441, which carries the mSF^{ts1}, was efficiently cured from the bacterium during growth at 42°C and no longer complemented the *rhlR* mutation. This resulted in restoration of the original SS165 (PAO1 *rhlR201::aacCI*) mutant phenotype.

4. Discussion

Plasmids with temperature-sensitive origins of replication are useful tools for a variety of genetic manipulation of bacteria. One of the most popular utilizations of temperature-sensitive replicons is as a delivery vehicle for transposons (Buchan et al., 2008, Menendez et al., 2006). At non-permissive temperatures, the plasmids are unable to replicate. Thus, transposons that remain on the plasmid and do not integrate into the host genetic material are lost. This facilitates selection of transposon insertion mutants that have acquired the element from the delivery plasmid. In addition, temperature-sensitive replicons have been utilized for temporary production of proteins (Cherepanov and Wackernagel, 1995). Furthermore, temperature-sensitive replicons have been used for conditional expression of essential genes (Jasin and Schimmel, 1984, Pyne and Bognar, 1992). Finally, temperature-sensitive replicons can be used

to generate mutations at specific regions of the chromosome by homologous recombination (Choi, 2008; Link, 1997; Wang, 2005). In this study we isolated a temperature-sensitive derivative of pRO1610 replicon, mSF^{ts1}, and expanded the potential utilization of conditional replicons by demonstrating temporary genetic complementation of a mutant.

Plasmids containing the pRO1610 replicon replicate in many Gram-negative bacteria, including *Burkholderia* spp. (Hassett et al., 1996). Unexpectedly, however, it was recently shown that a plasmid containing the mSF^{ts1} replicon was non-replicative in *Burkholderia thailandensis*, even at the permissive temperature of 30°C (Choi et al., 2008). Furthermore, a plasmid containing a pRO1610 temperature-sensitive replicon selected in *B. thailandensis* replicated in *P. aeruginosa* at 30°C but did not exhibit a temperature-sensitive phenotype in this bacterium. These data demonstrate that molecular mechanism that governs the function of pRO1610 replicon in various Gram-negative bacteria is complex and a comprehensive structure and function study is needed to elucidate its function.

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Figure 1.

DNA sequence of *mSF^{ts1}*. The inferred amino acid sequence below the DNA sequence is the replication (*rep*) protein ORF. A comparison of the 1.2 kb *mSF* sequence between the wt and the *SF^{ts1}* revealed the presence of two mutations within the coding region of the replication protein that resulted in amino acid alterations: a G→T (Gly₁₀₀ → Cys₁₀₀) and a C→A (Ser₂₀₄ → Arg₂₀₄). The mutated bases are shown in capital letters. → = location of the altered amino acids. Each nucleotide was sequenced four times to ensure accuracy.

Figure 2.

Genetic Cassette of mSF^{ts1}. A genetic cassette carrying the mSF^{ts1} can be isolated with any of the restriction enzymes present in the MCS of pUC1918. There is an additional *SphI* site internal to the *SacI* site.

Figure 3.

Curing of mSF^{ts1} plasmid for *rhlR* complementation. SS1673 and SS1971 were grown at 42°C overnight to promote curing of pSS447 or pSS441 that carry mSF and mSF^{ts1} respectively. The bacterial cultures were then subcultured and grown at 37°C to promote elastase and pyocyanin productions. *P. aeruginosa* strains PA01 and SS165 were used as positive and negative controls. Shown are the values of elastase activity and pyocyanin production normalized to the number of viable cells in each culture. Each assay was performed independently twice in duplicate.

Table 1**Bacterial strains and plasmids**

Strain	Genotype, relevant characteristics	Source
PAO1	Wound isolate, nonmucoid	(Holloway <i>et al.</i> , 1979)
SS165	PAO1 <i>rhIR201::aacCI</i>	(Chen <i>et al.</i> , 2005)
SS1673	PAO1 <i>rhIR201::aacCI</i> / pSS441	This study
SS1921	PAO1 <i>rhIR201::aacCI</i> / pSS447	This study
Plasmid		
pLJR0	<i>lacZ</i> transcriptional fusion vector carrying a 1.9-kb <i>Pst</i> I SF from pRO1614	(Runyen-Janecky <i>et al.</i> , 1997)
pSS191	pBR322 with <i>moriT</i>	This study
pSS124	mSF replicon cassette in pUC1918	This study
pSS227	SF ^{tsI} replicon in pSS191	This study
pSS254	mSF ^{tsI} replicon in pSS191	This study
pSS255	mSF ^{tsI} replicon in pUC1918	This study
pSS440	<i>rhIR</i> and <i>moriT</i> in pUC19	This study
pSS441	mSF ^{tsI} in pSS440	This study
pSS447	mSF in pSS440	This study

Frequency of curing mSF^{tsI}.

	SF ^{wt}		SF ^{tsI}	
	30°C	42°C	30°C	42°C
T				
Cb	+	-	+	-
CFU	2.83×10 ⁹	2.45×10 ⁹	2.52×10 ⁹	2.67×10 ⁹
%			2.69×10 ⁹	1.44×10 ⁹
Loss	0.7018	2.7778	0.7435	99.9999

Cells were grown over-night in LB at 30°C, 37°C or 42°C with aeration, serially diluted and plated on either LB or LB Cb plates. Following over-night incubation at 30°C, number of colonies were enumerated and compared.

Table 2