Construction of *Staphylococcus aureus* Proline Transport Gene Recombinant Plasmids

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ABSTRACT

The bacterial species *Staphylococcus aureus* is responsible for a significant amount of infections and disease in humans each year. The emergence of antibiotic resistance within the species is of great concern. A recent study has suggested that proline transport is important for *S. aureus* survival in mice. This previous study specifically examined the *putP* gene, which codes for a high affinity proline transporter that brings the essential amino acid proline into the bacterial cell. The aims of this study were: 1) to construct a plasmid that replicates in *S. aureus*, containing a full-length copy of the *putP* gene, that will restore a mutation in the *putP* gene and 2) to construct a plasmid for creation of a stable deletion mutation in the *putP* gene of *S. aureus* strain RN6390. A replicating plasmid containing the full-length *putP* gene was constructed and it is in the process of being moved into *S. aureus* strain RN4220 *putP-* cells. The plasmid to be used for generating the deletion mutation has also been constructed and is in the process of being moved into *S. aureus* strain RN4220.

INTRODUCTION

Staphylococcus aureus is a ubiquitous organism responsible for a significant amount of disease in humans, killing hundreds of people each year (2). In recent years, this species has developed high levels of antibiotic resistance. The antibiotic vancomycin used to be a very effective treatment for staphylococcal infections, but recently resistance to this antibiotic has emerged in clinical isolates (6). With the emergence of vancomycin-resistant *S. aureus*, the need for a new class of antibiotics has become increasingly important because of the fear that some infections with *S. aureus* will be rendered untreatable.

A possible method of treating antibiotic resistant staphylococcal infections may eventually be developed through further study of proline transport within *S. aureus*. Proline is an essential amino acid that forms one of the building blocks for proteins in a *S. aureus* cell (3). Previous work by Schwan et al. has suggested that proline transport in *S. aureus* is critical for survival within an animal or human host (8). The proline transport system that was studied involved the high affinity proline transporter encoded by the *putP* gene. It functions to bring proline into the staphylococcal cell when levels of the amino acid are low, such as in many sites in the human body (8). A disruption of the *putP* gene resulted in diminished survival of *S. aureus* cells in mice, and proline uptake assays using radiolabled proline have shown that significantly less proline is transported into the staphylococcal cells that have the mutated proline transporter gene. This previous *putP* mutation is unstable, and it would be useful to create a stable *putP* mutation in *S. aureus* for further study of the proline transport system in this bacterial species.

The purpose of this research was to construct a plasmid that can be used to create a stable deletion mutation in the *putP* gene of *S. aureus* and to construct a second plasmid that can be used for complementation of a *putP* mutation in *S. aureus* (i.e. restoration of the *putP* gene). This was achieved through recombinant DNA technology, cloning, and the polymerase chain reaction. The plasmids have been constructed in *E. coli* strains. It is hoped that the complementing plasmid will eventually be moved into *S. aureus* RN6390, a virulent strain (8). Currently, this plasmid has been successfully transferred into *S. aureus* strain RN4220 for preliminary testing. Moreover, we hope to create the stable *putP* mutation.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *E. coli* strains DH5α and S17λ.*pir* were grown in Luria-Bertani (LB) medium (Gibco BRL, Gaithersburg, MD) overnight at 37°C unless otherwise indicated. Sometimes the media were supplemented with antibiotics (Sigma Chemical Co., St. Louis, MO) at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; chloramphenicol, 10 μg/ml; and erythromycin, 5 μg/ml.

Plasmids. Plasmids used were pLTV-1 (1), pERL350-1/253 (7), pPWC-1 (9), pEX18T (Herb Schweizer, Colorado State University), pUCGM1 (Herb Schweizer), and pCL84 (Chia Lee, University of Kansas Medical Center). All plasmids were isolated from *E. coli* strains using the QIAprep spin mini-prep plasmid isolation kit (Qiagen, Germany).

Gel electrophoresis. DNA gel electrophoresis was performed as described by Sambrook et al. (5). Low-melting point gel electrophoresis along with phenol/chloroform extraction was also performed to isolate some DNA fragments (7). *Hind*IIIλ DNA standard (Boehringer Mannheim, Germany) and supercoiled DNA standard (Gibco BRL, Gaithersburg, MD) were used as size markers.

Polymerase chain reaction (PCR). The oligonucleotide primers used in this study are shown in Table 1. They were synthesized by a commercial source (Sigma-Genosys, The Woodlands, TX). Concentrations of the DNA primers in pmol were determined via spectroscopy, measuring the optical densities at 260 nm and 280 nm. All PCR reactions were cycled in a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). Each reaction mixture contained a total volume of 100 μl, containing 100 pmol of each corresponding primer. PCR was performed using *Taq* polymerase (Promega, Madison, WI), and contained varying template DNA, depending on what was to be amplified. The amplifications of the 5' region and 3' region of the *putP* gene contained pPWC-1 template DNA, and the chloramphenicol resistance (Cm[r]) gene amplification contained pLTV-1 template DNA. The 5' region of the *putP* gene was amplified as follows: initial denaturation at 95°C for 5 min; followed by 32 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min.. The 3' region of the *putP* gene was amplified as follows: initial denaturation at 95°C for 5 min; followed by 32 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min. The Cm[r] gene was amplified as follows: initial denaturation at 95°C for 5 min; followed by 32 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min.

TABLE 1. PCR products and corresponding primers

PCR product	Primer	Sequence
5' region *putP*	SA*putP*3	5' CGATCTAGAGGTGGATATTTAGCTGTATC 3'
	SA*putP*4	5' CGAAAGCTTCGAACTTTGTTTAAGTCAGT 3'
3' region *putP*	SA*putP*1A	5' CACGAGAATTCCAGTCTCTTCAATTGCAT 3'
	SA*putP*2A	5' CGAGGATCCACGAAACCAGAATGTGTATAT 3'
Cm^r	Cm^1	5' TGATGCCGCATAGTTAAGCC 3'
	Cm^2	5' AAGAAAGCAGACAAGTAAGCC 3'

Construction of the *putP* deletion mutation plasmid. The backbone plasmid used for the mutation plasmid was pEX18T. The PCR product of the 5' region of the *putP* gene and the pEX18T DNA were each endonuclease cut with *Xba*I and *Hind*III. All restriction digestions of DNA used specified enzymes with appropriate buffers (New England BioLabs, Beverly, MA). A ligation mixture was set-up with T$_4$ ligase (New England BioLabs) containing the *Hind*III/*Xba*I cut pEX18T DNA and the *Hind*III/*Xba*I cut 5' *putP* gene region. Ligated plasmid DNA was then transformed into *E. coli* DH5α cells (5). Transformed cells were plated onto LB agar plates containing ampicillin, 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), and isopropylthio-β-D-galactoside (IPTG). White colonies that arose were screened for the proper plasmid (4), which was called the pTGputPA-4 plasmid. The 3' region of the *putP* gene and the pTGputPA-4 plasmid were each cut now with the endonucleases *Eco*RI and *Bam*HI. A ligation mixture was set-up containing the *Bam*HI/*Eco*RI cut pTGputPA-4 and the *Bam*HI/*Eco*RI cut 3' *putP* gene region. The ligation mixture was then transformed into *E. coli* S17λ.*pir* cells and plated onto LB agar plates containing ampicillin. This resulted in the construction of the pTGputPAB-2 plasmid. The pUCGM1 plasmid DNA was endonuclease cut with *Sma*I, and the gentamicin resistance gene was separated from the pUC vector DNA by low-melting point gel electrophoresis. The pUC vector DNA was treated with HK™ phosphatase (Epicentre Technologies, Madison, Wis.), and the Cm^r gene PCR product was treated with T$_4$ DNA Polymerase (Boehringer Mannheim, Indianapolis) (5). A ligation mixture was set-up containing phosphatase treated pUC vector DNA and T$_4$ DNA Polymerase treated Cmr gene. The ligation was then transformed into *E. coli* DH5α cells and plated onto LB agar plates containing ampicillin, resulting in construction of pUCCM1. The pUCCM1 plasmid DNA was cut with the restriction endonuclease *Bam*HI, and the Cm^r gene was separated by low-melting point gel electrophoresis. A ligation mixture was set-up containing phosphatase treated/ *Bam*HI cut pTGputPAB-2 and the *Bam*HI cut Cm^r gene, which was then transformed into *E. coli* DH5α cells. The transformed *E. coli* cells were plated onto LB agar plates containing ampicillin, resulting in the construction of the pTGABCM-11 plasmid. The pCL84 plasmid DNA was cut with the restriction endonuclease *Hind*III to cut out a tetracycline resistance gene (Tc^r) gene. This Tc^r gene fragment was then blunted with Klenow fragment (Boehringer Mannheim) (5), isolated with low-melting point agarose, and ligated to pTGABCM-11 plasmid DNA cut with *Sac*II and blunted with T$_4$ DNA polymerase. *E. coli* DH5α cells were transformed and plated onto LB agar plates containing ampicillin and tetracycline, resulting in the construction of the pTGKO5-11 plasmid.

Construction of the *putP* complementing plasmid. The backbone plasmid used for the complementing plasmid was pERL350-1/253, which was cut with the endonuclease *Pst*I. A

ligation reaction was set-up containing the gentamicin resistance gene isolated from pUCGM1 and T$_4$ DNA polymerase blunted/*Pst*I cut pERL350-1/253. The ligation mixture was transformed into *E. coli* S17λ.pir cells and plated onto LB agar plates containing gentamicin, resulting in the construction of pERLGM. The pERLGM was cut with *Eco*RI and *Bam*HI, separated on a low-melting point agarose gel, and the proper fragment to be used was isolated. The pPWC-1 plasmid DNA was cut with *Eco*RI and *Bam*HI and subjected to low-melting point gel electrophoresis to isolate the full-length *putP* gene. A ligation was set-up containing *Eco*RI/*Bam*HI cut pERLGM DNA and the isolated *putP* gene. The ligation mixture was transformed into *E. coli* DH5α cells and plated onto LB agar plates containing gentamicin, resulting in the construction of the pERLGMputP-19 plasmid. The pERLGMputP-19 plasmid DNA was cut with *Xba*I and then blunted using the Klenow fragment. A ligation mixture was set-up containing the Klenow treated/*Xba*I cut pERLGMputP-19 and the Klenow blunted Tc^r gene from pCL84. This ligation mixture was transformed into *E. coli* DH5α cells and plated onto LB agar plates containing gentamicin and tetracycline, resulting in construction of the pTGCOMP plasmid.

Electroporation of plasmid DNA. Transformation of *S. aureus* cells via electroporation was carried out by the method of Iandolo and Kraemer. (4). Trypticase Soy Broth (TSB) was used for the initial growth of the *S. aureus* cells. Eighty microliters of the electrocompetent cells was added to approximately 10 μg of plasmid DNA and electroporated under the following conditions: 2.5 kV, 100 Ω, and 25 μF in a disposable cuvette with a 2.0 mm gap. Immediately after electroporation, the cells were suspended in 1 ml of SMMP (sucrose maleic acid magnesium PAB) broth (4) and were allowed to shake at 37°C for marker expression. Aliquots of the expressed cells were plated onto appropriate agar media containing antibiotics.

RESULTS

As seen in Figure 1, the plasmid needed for creation of the stable deletion mutation of the *putP* gene in *S. aureus* has been constructed and transferred into *E. coli* cells. Also, the plasmid containing the full-length *putP* gene to be used for complementation of the *putP* mutation in *S. aureus* has been constructed and also transferred into *E. coli* cells (Figure 2).
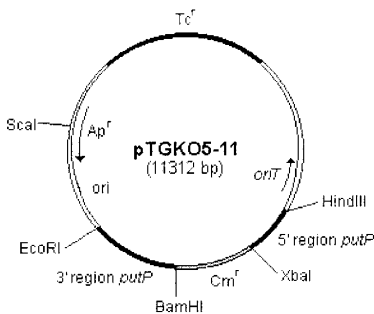

FIG. 1. Plasmid for creation of the stable deletion mutation in the *putP* gene of *S. aureus* containing: an origin of replication for G- bacteria, the 5' region of *putP*, the 3' region of *putP*, a chloramphenicol resistance gene functional in G+ bacteria, an ampicillin resistance gene functional in G- bacteria, and a tetracycline resistance gene that is functional in G+ as well as G- bacteria.
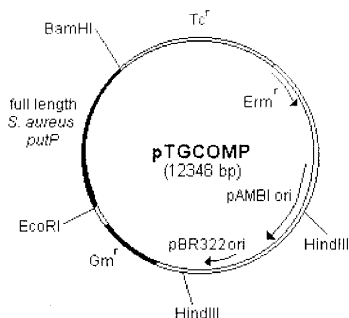
FIG. 2. Plasmid for the complementation of a *putP* mutation in *S. aureus* containing the following: origins of replication for G+ and G- bacteria, the full-length *putP* gene, a gentamicin resistance gene for G-bacteria, an erythromycin resistance gene functional in G+ as well as G- organisms, and a tetracycline gene that is functional in G+ and G- bacteria.

DISCUSSION

A plasmid that can complement the mutation in the *putP* gene of *S. aureus* strains 16F-157 (*putP*::Tn*917*) and RN4220 *putP*⁻ was successfully constructed (Figure 2). This plasmid was designed with origins of replication to allow the plasmid to reproduce itself in *E. coli* cells and Gram-positive bacteria (7). Antibiotic resistance markers for erythromycin and tetracycline were placed into the plasmid to select for the plasmid in *S. aureus* cells. The gentamicin resistance marker was put in to select for the plasmid in *E. coli* cells. A full-length *putP* gene obtained from the pPWC1 plasmid was cloned in to allow the restoration of the defect in the high affinity proline transport gene present in *S. aureus* strains 16F-157 and RN4220 *putP*⁻ (9). This pTGCOMP plasmid has been successfully transformed into both *S. aureus* strains by electroporation (4). Preliminary experiments suggest that the mutation in the *putP* gene has been complemented, restoring proline transport to the level of unmutated *S. aureus* cells (data not shown).

Generation of a plasmid that will hopefully create a stable mutation in the *putP* gene of *S. aureus* was also completed (Figure 1). This plasmid contains an origin of replication for *E. coli* and a tetracycline resistance gene. In addition, the pTGKO5-11 plasmid has an *oriT* site engineered within. The oriT site provides a protein required for successful conjugal transfer between two bacterial cells; however, attempts to conjugally transfer this plasmid from *E. coli* into *S. aureus* have been unsuccessful. We are currently attempting transformation of this plasmid into *S. aureus* RN4220 via electroporation. Transfer of this plasmid into *S. aureus* will cause a recombination event to occur. A successful recombination based on the 5' and 3' regions of *putP* will hopefully integrate the chloramphenicol antibiotic resistance (Cmʳ) marker into the *S. aureus* genome. When a recombination event does occur, the whole plasmid may integrate rather than just the two flanking *putP* DNA regions with the Cmʳ gene in the center. To determine which type of event has occurred, the transformed *S. aureus* strains will be screened for tetracycline resistance. A double crossover mutant will exhibit resistance to chloramphenicol alone, whereas a single crossover mutant (where the whole plasmid has integrated) will show resistance to both tetracycline and chloramphenicol. We desire a double crossover event, and our ultimate goal is to eventually create a stable mutant of *S. aureus* strain RN6390 (8). This mutant could then be used as a possible control for the testing of anti-staphylococcal drugs based on chemicals structurally similar to proline.

ACKNOWLEDGMENTS

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