The Regulation of the *Staphylococcus aureus putP* Gene

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ABSTRACT

Staphylococcus aureus is a significant cause of human infections. The species needs to import the amino acid proline from the outside to grow and survive. To accomplish this task, *S. aureus* has two proline transport systems, one of which is encoded by the *putP* gene. In this study, the regulation of the *S. aureus putP* gene was examined. The promoter for the *S. aureus putP* gene was PCR amplified from genomic DNA of strain RN6390 and the product ligated to a promoterless *lacZ* gene. Subsequently, this *putP-lacZ* fusion was then inserted into the plasmid pMOD-1, forming pUB1-1. This pUB1-1 plasmid was electroporated into *S. aureus* strain RN4220 cells and one clone was tested under different growth conditions that had variations in proline concentrations or osmolarities. An increase in osmolarity led to an increase in expression of the *putP* gene in *S. aureus*. Moreover, limiting proline in the medium also caused increased *putP* expression. Our results suggest that osmolarity and low proline concentrations, conditions one might encounter in the human body, appear to activate the *putP* gene in *S. aureus*. This supports the role of *putP* expression in the *in vivo* survival of *S. aureus*.

INTRODUCTION

Staphylococcus aureus infections, including hospital-acquired, are widespread all around the world. The species is the leading cause of post-surgery infections and is responsible for up to one third of all food-borne illness in the United States. Because *S. aureus* is ubiquitous, open wounds are quite common. Unfortunately, many isolates of *S. aureus* have become resistant to the antibiotics that we usually use to treat them (2).

A previous study using a proline uptake mutant strain of *S. aureus* in animal models indicated proline transport is essential for *S. aureus in vivo* survival (7). *S. aureus* uses proline as a carbon and nitrogen source and it must import proline from the environment to survive (4, 6, 8). Two proline transport systems function to do this in *S. aureus*, one of which is a high affinity proline transport system encoded by the *putP* gene (7, 8). Although proline is essential for the organism, factors regulating the *putP* gene are still unknown. Osmolarity and proline concentrations are two conditions that may regulate *putP* activity. Using osmotic conditions that are found in the human body, *putP* expression was measured in *Escherichia coli* and *S. aureus* using a *putP-lacZ* reporter system. Our results indicate that osmolarity is an important environmental signal for the regulation of the *S. aureus putP* gene.

METHODS

Bacterial Strains, Plasmids, and Media

The *E. coli* strain DH5α was used for constructing the *putP-lacZ* reporter system and strain MC4100 was used to test the various osmotic growth conditions in *E. coli* because the strain contains no functional *lacZ* gene. *S. aureus* strain RN4420 was used as the basis to test the effects of osmolarity on *putP* transcription in *S. aureus*. Plasmids pUJ9 and pPP2-6 were used for cloning the *putP* promoter from *S. aureus*. The pUJ9 plasmid possesses a promoterless *lacZ* gene and ampicillin antibiotic resistance and pPP2-6 is a single copy number plasmid with chloramphenicol antibiotic resistance. The pMOD-1 plasmid was used as a vector for transforming the *putP-lacZ* reporter system into *S. aureus*. This plasmid contains a Tn*917* transposon; erythromycin, tetracycline and ampicillin resistance genes; and it retains the ability to replicate in both *S. aureus* and *E. coli*. Luria agar (LA) and broth were used to grow the recombinant *E. coli* cells containing the *putP-lacZ* reporter system. In addition, Brain Heart Infusion (BHI) agar and broth were used to culture the recombinant *S. aureus* cells. M9 minimal media, containing 1% glycerol at pH 7.0, was used to test different osmotic growth conditions in *E. coli*, while a minimal medium for *S. aureus* composed of salts, glucose, amino acids, and vitamins served as the base medium for various osmotic and

proline growth environments in *S. aureus* (5). Osmotic conditions from 0 to 400 mM NaCl and proline conditions ranging from 1.74 mM to 1.74 μM were produced in the *S. aureus* minimal media to assay for β-galactosidase activity.

PCR

Oligonucleotide primers specific for an 1150 bp segment from an area of the *S. aureus putP* gene containing the transcriptional start site were synthesized with *Eco*RI and *BamH*I restriction endonuclease sites flanking the promoter sequence. These primers were named SaputP1A and SaputP2A. PCR amplification using both oligonucleotides was set up as follows: 30 cycles with a denaturing time of 30 sec at 95 ˚C, an annealing time of 90 sec at 55 ˚C and an elongation time of 90 sec at 72 ˚C. Chromosomal DNA from *S. aureus* strain RN4220 served as the template for the PCR amplification. The PCR product was visualized on a 0.8% agarose gel containing ethidium bromide with *Hind*III cut λ phage DNA serving as a standard to verify amplification product length.

Cloning

The PCR amplified 1150 bp *S. aureus putP* promoter fragment was passed through a Microcon 30 filter to concentrate the DNA and then digested with the restriction endonucleases *Eco*RI and *BamH*I. This digested DNA fragment was ligated to pUJ9 cut with *BamH*I and *Eco*RI. The resulting ligated DNA was transformed into DH5α cells, and transformants were selected for on LA containing 100 μg/ml ampicillin and X-Gal. Blue colonies were screened for the appropriate sized plasmid. DNA was purified with a commercial plasmid kit (Qiagen, Valencia, CA).

The resulting plasmid that was obtained was named pLL1-1. This pLL1-1 plasmid DNA was digested with *Not*I and then ligated to *Not*I digested pPP2-6 DNA. Following transformation of the ligated DNA into DH5α, blue colonies were selected for on LA containing 10 μg/ml chloramphenicol and X-Gal. The plasmid containing the *putP-lacZ* reporter system was purified using the commercial plasmid isolation kit mentioned above and transformed into strain MC4100. One plasmid, pLL3-1, was selected to be grown in the various osmotic conditions to test the effect on transcription of *putP* in *E. coli*.

A different vector was utilized to facilitate moving the *putP-lacZ* reporter system into *S. aureus*. A portion of the *Not*I digested plasmid pLL1-1 DNA was blunted using *E. coli* Klenow fragment and ligated to *Sma*I digested pMOD-1 DNA. The DNA ligation product was transformed into DH5α, colonies selected for using LA containing 12.5 μg/ml tetracycline, and screened using 100 μg/ml ampicillin. Plasmid DNA was purified using the same plasmid isolation kit and electroporated into sucrose treated electrocompetent RN4220 cells (1). Transformants were selected for on BHI medium containing 5 μg/ml erythromycin and 5 μg/ml tetracycline. One of the resulting clones was chosen for growth in *S. aureus* minimal media with different osmotic conditions ranging from 0 to 400 mM or proline conditions ranging from 1.74 mM to 1.74 μM.

Beta-galactosidase Assays

Beta-galactosidase assays were carried out on MC4100/pLL3-1 grown in M9 minimal media for *E. coli* (3) and on RN4220/pUB1-1 grown in *S. aureus* minimal media (5). Both sets of assays were set up to have a range of osmotic conditions from 0 to 400 mM. A separate set of assays was performed on RN4220/pUB1-1 cells in *S. aureus* minimal media containing 1.74 mM to 1.74 μM proline. Bacteria were grown to mid-logarithmic phase and β-galactosidase assays performed on SDS and chloroform permeabilized cells according to Miller (3). The mean values were calculated from three separate experiments in both bacterial strains.

RESULTS

As shown in Tables 1 and 2, β-galactosidase activity rose significantly in *E. coli* strain MC4100/pLL3-1 and *S. aureus* strain LL1 as osmolarity rose, suggesting that the *S. aureus putP* gene is activated by high osmotic conditions. Also, assays performed on the *putP-lacZ* fusion in *S. aureus* showed no endpoint of activation in increasing osmotic conditions. Further studies using proline concentrations ranging from 1.74 mM to 1.74 μM revealed that decreasing proline levels in the external environment do not significantly affect the expression of the *S. aureus putP* gene (Table 3). However, the results suggest that an active *put*P gene may be necessary for the overall growth of *S. aureus*.

Table 1. Effect of osmolarity on the *S. aureus putP* gene grown in *E. coli* M9 minimal media

E. coli strain	NaCl (mM)	β-gal activity (Miller Units)
MC4100	0	10 ± 2[a]
MC4100	100	10 ± 2
MC4100	200	10 ± 2
MC4100	400	10 ± 2
MC4100 + pLL3-1	0	405 ± 20
MC4100 + pLL3-1	100	635 ± 17
MC4100 + pLL3-1	200	744 ± 10
MC4100 + pLL3-1	400	589 ± 29

[a] Mean ± standard deviation of at least three separate runs.

Table 2. Effect of osmolarity on the *S. aureus putP* gene compared to RN4220 grown in minimal media

S. aureus strain	NaCl (mM)	β-gal activity (Miller Units)
RN4220	0	0[a]
RN4220	100	0
RN4220	200	0
RN4220	400	0
RN4220	800	0
RN4220	1000	0
LL1	0	11.8±2.2
LL1	100	13.2±1.9
LL1	200	15.8±3.7
LL1	400	24.4±7.1
LL1	800	30.8±7.6
LL1	1000	37.2±8.7

[a] Mean ± standard deviation of at least three separate runs.

Table 3. Effect of proline concentration on *S. aureus* LL1 compared to RN4220 grown in minimal media

S. aureus strain	Proline (□M)	β-gal activity (Miller Units)
RN4220	1740	2±0.9[a]
RN4220	17.4	2±0.9
RN4220	3.48	2±0.9
RN4220	1.74	2±0.9
LL1	1740	24±2.8
LL1	17.4	21.5±3.1
LL1	3.48	15.3±2.6
LL1	1.74	22.5±3.2

[a] Mean ± standard deviation of at least three separate runs.

DISCUSSION

The two proline transport systems in *S. aureus* may be affected by various environmental conditions. High osmotic conditions, similar to those in a human liver or kidney, activate the *S. aureus putP* gene by an unknown mechanism. Once transcribed, the high affinity proline transporter can then be translated into PutP. The PutP protein may then integrate into the membrane and begin binding and transporting proline inside the cell. We observed a plateau at 200 mM NaCl when the *S. aureus putP-lacZ* fusion was tested in *E. coli* showing that increases in the osmolarity affected transcription of the *S. aureus putP* gene even in *E. coli.* However, a three fold increase in expression of the *putP-lacZ* fusion when *S. aureus* grown in 1 M NaCl was compared to 0 mM NaCl with no end point, in terms of activation, being reached. The species *S. aureus* is capable of surviving under high osmotic conditions, more so than *E. coli*. Activation of the *putP* gene under high osmotic conditions appears to aid in the survival and growth of *S. aureus.*

Another environmental factor that may affect the expression of the *S. aureus putP* gene is varying proline concentrations in the environment. A lower abundance of proline outside of the cell may induce an increased expression of the high affinity proline transporter due to an increase in the need for a more efficient transporter. We observed a steady expression rate for the *S. aureus putP* promoter in proline concentrations ranging from 1740 to 1.74 μM. This result suggests that the high affinity proline transporter is essential or important for the survival of *S. aureus* in conditions that mimic those in the human or mouse, and could explain the previous results in animal models of infection (7).

This data helps in the understanding of how *S. aureus* regulates an important gene for survival in environmental conditions that could be found in many areas of the human body that *S. aureus* infects. This information may be used in the future to develop new methods of treating antibiotic resistant *S. aureus* infections by targeting the *S. aureus putP* gene or by regulating a patient's diet.

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