

Low-proline environments impair growth, proline transport and *in vivo* survival of *Staphylococcus aureus* strain-specific *putP* mutants

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Staphylococcus aureus is a common cause of disease in humans, particularly in hospitalized patients. This species needs to import several amino acids to survive, including proline. Previously, it was shown that an insertion mutation in the high-affinity proline uptake gene *putP* in strain RN6390 affected proline uptake by the bacteria as well as reducing their ability to survive *in vivo*. To further delineate the effect of the *putP* mutation on growth of *S. aureus* strain RN6390, a proline uptake assay that spanned less than 1 min was done to measure transport. An eightfold difference in proline levels was observed between the wild-type strain and the high-affinity proline transport mutant strain after 15 s, indicating that the defect was only in proline transport and not a combination of proline transport, metabolism and accumulation that would have been assessed with longer assays. A *putP* mutant of *S. aureus* strain RN4220 was then grown in minimal medium with different concentrations of proline. When compared to the wild-type strain, the *putP* mutant strain was significantly growth impaired when the level of proline was decreased to 1.74 µM. An assessment of proline concentrations in mouse livers and spleens showed proline concentrations of 7.5 µmol per spleen and 88.4 µmol per liver. To verify that the effects on proline transport and bacterial survival were indeed caused solely by a mutation in *putP*, the *putP* mutation was complemented by cloning a full-length *putP* gene on a plasmid that replicates in *S. aureus*. Complementation of the *putP* mutant strains restored proline transport, *in vitro* growth in low-proline medium, and *in vivo* survival within mice. These results show that the mutation in *putP* led to attenuated growth in low-proline media and by corollary low-proline murine organ tissues due to less efficient transport of proline into the bacteria.

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INTRODUCTION

Staphylococcus aureus is a common bacterial species that is responsible for a significant amount of morbidity and mortality in humans. It is of particular concern in hospitals, where complications frequently arise (NNIS, 1996) and because of the high level of antibiotic resistance among clinical isolates (Boyce, 1997; Kauffman & Bradley, 1997). Frontline anti-staphylococcal drugs like methicillin are losing their efficacy in both hospital-acquired and community-acquired strains (Centers for Disease Control and Prevention, 1999; Panlilio *et al.*, 1992). Moreover, the recent emergence of vancomycin-resistant *S. aureus* strains (Centers for Disease Control and Prevention, 1997; Sieradzki *et al.*, 1999; Smith *et al.*, 1999) means there are isolates that are resistant to all available antibiotics.

In a quest to find new potential targets for the development of anti-staphylococcal drugs, a signature-tagged mutagenesis (STM) system was adapted for use in *S. aureus* (Coulter *et al.*, 1998). From this STM system, one of

the *in vivo*-attenuated mutants that were found had a mutation in the high-affinity proline transport gene *putP* (Schwan *et al.*, 1998). An external source of proline is needed by *S. aureus* strain RN6390; thus proline transporters, such as PutP, are critical for the survival of these bacteria. This proline auxotrophy could be a host adaptation by *S. aureus* strains living on warm-blooded animals. To rectify this proline auxotrophy, *S. aureus* has at least two proline transport systems: a low-affinity system presumably linked to a *proP* gene homologue and a high-affinity proline uptake system encoded by the *putP* gene (Bae & Miller, 1992; Pourkomailian & Booth, 1994; Townsend & Wilkinson, 1992; Wengender & Miller, 1995). Of the two proline uptake systems, the high-affinity system encoded by *putP* has been more thoroughly characterized. The *putP* gene of *S. aureus* was first identified by Wengender & Miller (1995) and the STM study has shown the effects of mutating the *putP* gene (Schwan *et al.*, 1998). This *putP* mutant strain of *S. aureus* strain RN6390 was shown to have reduced proline uptake compared to the parental strain. Moreover, marked

in vivo attenuation was noted in several animal models of infection, including murine systemic, wound and abscess models (Schwan *et al.*, 1998), and a rabbit endocarditis model (Bayer *et al.*, 1999; Schwan *et al.*, 1998).

The purpose of this study was to elucidate more thoroughly why the mutation in the high-affinity proline transport gene in *S. aureus* led to the marked attenuation in murine models of infection. By examining growth in low-proline media and analysing proline concentrations in murine organ tissues, we showed that the *putP* mutant is crippled in environments where there are low proline concentrations. Moreover, complementation of the *putP* mutations restored proline uptake as well as virulence.

METHODS

Bacterial strains, media and growth conditions. *Escherichia coli* strains DH5 α and S17.1 λ pir were grown in Luria-Bertani (LB) medium (Gibco-BRL) overnight at 37 °C unless otherwise indicated. *Staphylococcus aureus* strains RN4220 and RN6390 (Novick, 1990), provided by Barry Kreiswirth (Public Health Research Institute Tuberculosis Center, New York, USA), were grown in Brain Heart Infusion (BHI) medium (Difco) overnight at 37 °C. The 16F-157 (*putP*::Tn917) strain derived from RN6390 (Schwan *et al.*, 1998) was provided by PathoGenesis Corp. The minimal medium used for propagation of *S. aureus* was a modified version of the staphylococcal minimal medium described by Rudin *et al.* (1974). This version was modified by the addition of the following components (per litre): L-glycine, 50 mg; L-serine, 30 mg; L-aspartic acid, 90 mg; L-lysine, 50 mg; L-alanine, 60 mg; L-tryptophan, 10 mg; L-methionine, 10 mg; L-histidine, 20 mg; L-isoleucine, 30 mg; L-tyrosine, 50 mg; L-tryptophan, 10 mg; thymine, 20 mg. Proline concentrations were adjusted to be between 1.74 mM and 1.74 μ M. For the growth curves in minimal medium, the *S. aureus* strains were grown overnight in BHI broth with appropriate antibiotics. These overnight cultures were used to inoculate defined minimal medium with various proline levels and again allowed to grow overnight at 37 °C with shaking. From these cultures, aliquots were used to inoculate defined minimal medium with the same proline concentration and the cultures were incubated for 24 h at 37 °C with shaking. Optical density readings at 590 nm were taken every 2 h for the first 12 h of growth and again at 24 h. Sometimes the media were supplemented with antibiotics (Sigma) at the following concentrations: for *E. coli*, ampicillin at 100 μ g ml⁻¹ and gentamicin at 10 μ g ml⁻¹; and for *S. aureus*, erythromycin at 5 μ g ml⁻¹ and tetracycline at 5 μ g ml⁻¹.

Plasmids. The plasmids used were pERL3 501/253 (Schwan *et al.*, 1994), pPWC-1 (provided by Karen Miller, Penn State University; Wengender & Miller, 1995), pUCGM1 (provided by Herbert Schweizer, Colorado State University; Schweizer, 1993) and pCL84 (provided by Chia Lee, University of Kansas Medical Center). All of the plasmids were isolated from *E. coli* or *S. aureus* strains using the QIAprep spin mini-prep plasmid isolation kit (Qiagen). For plasmid isolation from *S. aureus*, the protocol outlined in the kit was modified. After addition of the suspension buffer, the bacterial cells were treated with 100 μ g ml⁻¹ of recombinant lysostaphin (AMBI UK) at 37 °C for 10 min before the addition of the lysis buffer. The lysostaphin was used to break apart the cell wall cross-linking to allow the DNA to more readily exit from the bacterial cells.

Transfer of the *putP*::Tn917 mutation to strain RN4220. Because another background strain besides strain RN6390 was desired, the *putP* mutation in 16F-157 (derived from strain RN6390) was transferred to strain RN4220 via transduction (Kloos & Pattee,

1965) with a lysate of *S. aureus* phage ϕ 80 α (provided by Ambrose Cheung, Dartmouth Medical School) from strain 16F-157 (*putP*::Tn917). Transductants were selected for on trypticase soy agar (Difco) containing 2 mM sodium citrate and erythromycin. Erythromycin-resistant transductants were screened for a disruption of the *putP* gene by PCR. One hundred picomoles of primers SaputP5 (5'-GATGCTACCTAAAGCTAGACG-3') and SaputP6 (5'-TCTTCCGTTAGTGAAGTAGATG-3'), which flank the spot where the Tn917 inserted into the genome of strain 16F-157, were used in the PCR amplifications under the following conditions: an initial denaturation at 95 °C for 5 min followed by 32 cycles of 95 °C, 1 min; 57 °C, 1 min; and 72 °C, 1 min. The absence of a 260 bp band would signify an insertion within the *putP* gene, whereas its presence would signify an uninterrupted gene.

Construction of the *putP*-complementing plasmid. The backbone plasmid used for the complementing plasmid was pERL3 501/253, which contains an erythromycin-resistance gene, a pAM β 1 origin of replication that functions in many Gram-positive bacteria, including *S. aureus* (Luchansky *et al.*, 1988), and a pBR322 origin of replication for *E. coli*. This plasmid DNA was cut with the restriction endonuclease *Pst*I and then blunted using T4 DNA polymerase (Boehringer Mannheim). A gentamicin-resistance gene was isolated from pUCGM1 by cutting the plasmid DNA with *Sma*I, separating the fragments on a low-melting-point agarose gel, cutting out the fragment with the gentamicin-resistance gene, and processing the DNA as previously described (Schwan *et al.*, 1992). A ligation reaction was set up using T4 DNA ligase (New England Biolabs) to ligate the gentamicin-resistance gene isolated from pUCGM1 with the *Pst*I-blunted pERL3 501/253 DNA. The ligation mixture was transformed into *E. coli* S17.1 λ pir (Sambrook *et al.*, 1989) and plated onto LB agar plates containing gentamicin, resulting in the construction of pERLGM. Following isolation of the plasmid DNA, pERLGM was cut with *Eco*RI and *Bam*HI, separated on a low-melting-point agarose gel, and the appropriate fragment to be used was isolated and processed as described above. pPWC-1 DNA was cut with *Eco*RI and *Bam*HI, the fragments separated on a low-melting-point gel, the *putP*-containing fragment cut out, and the full-length *putP* DNA fragment processed as noted above. A ligation was set up between the *Eco*RI/*Bam*HI-cut pERLGM DNA and the isolated *putP* gene. The ligation mixture was transformed into *E. coli* strain DH5 α and cells were plated onto LB agar plates containing gentamicin, resulting in the construction of plasmid pERLGMputP-19. The pERLGMputP-19 DNA was cut with *Xba*I and then blunted by filling in the ends by the use of *E. coli* Klenow fragment (Boehringer Mannheim) (Sambrook *et al.*, 1989). To obtain the tetracycline-resistance gene, pCL84 DNA was cut with the restriction endonuclease *Hind*III. The DNA fragment with the tetracycline-resistance gene was then blunted with Klenow fragment and isolated on a low-melting-point agarose gel. Blunted pERLGMputP-19 DNA was ligated to the blunted tetracycline-resistance gene from pCL84. This ligation mixture was transformed into *E. coli* DH5 α and plated onto LB agar plates containing gentamicin and tetracycline, resulting in construction of pTGCOMP (Fig. 1).

Electroporation of plasmid DNA. Transformation of *S. aureus* strain RN4220 or RN6390 by electroporation was carried out by the method of Iandolo & Kraemer (1990). Trypticase soy broth (TSB) was used for the initial growth of the *S. aureus* cells. Ten micrograms of *putP* recombinant plasmid DNA was initially electroporated into the mutant strain RN4220*putP* under the following conditions: 2.5 kV, 100 Ω and 25 mF in a disposable cuvette with a 2.0 mm gap. Aliquots of the expressed RN4220 cells were plated onto BHI agar containing erythromycin and tetracycline. Once the plasmid had been confirmed to be present in the RN4220*putP* cells, plasmid DNA was isolated from these cells and this DNA was used to electroporate 16F-157 cells.

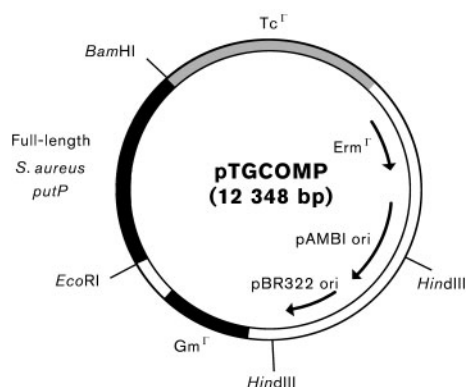


Fig. 1. Map of the *putP* recombinant plasmid pTGCOMP used for the complementation studies of *putP* mutant strains of *S. aureus*.

Proline transport assay. Transport of radioactive proline into the staphylococcal cells was measured using the filtration method described by Bae & Miller (1992). Bacteria were suspended to a final concentration of 25–40 mg total cellular protein ml⁻¹ as determined with the Bio-Rad protein assay kit (Bradford, 1976). The *S. aureus* cells were preincubated at 37 °C for 5 min in the transport buffer, and L-[2,3-³H]proline (Dupont/NEN) was added at a final concentration of 5 µM (specific activity, 40 Ci mmol⁻¹; 1480 GBq mmol⁻¹). The assay was set up as previously noted (Schwan *et al.*, 1998).

Determination of proline concentrations in murine organ tissues. Livers and spleens were obtained from BALB/c mice (Harlan Laboratories) and were immediately frozen until the samples could be dehydrated. Dehydrated tissue samples were weighed and ground to a fine powder with a mortar and pestle. A measured amount of tissue sample (15–50 mg) was hydrolysed with 6 M HCl at 110 °C for 24 h. The HCl-digested samples were neutralized with an equal amount of 6 M NaOH. Analysis for L-proline was performed by Scientific Research Consortium (St Paul, MN, USA). The absorbance at 440 and 570 nm was measured after post-column colour development by ninhydrin reagent at 131 °C using a Beckman amino acid analyser model 7300 and computations calculated with Beckman System Gold 8.10 chromatography software.

LD₅₀ determinations. LD₅₀ determinations (Reed & Meunch, 1938) were performed using BALB/c mice (Harlan Laboratories) inoculated with 10-fold serial dilutions (0.5 ml) of strain RN6390, strain 16F-157 or strain 16F-157/pTGCOMP suspended in TSB containing 1% (w/v) Nutrex administered intraperitoneally. A total of five mice per dilution were used with input titres covering a range of 10² to 10⁸ c.f.u. ml⁻¹. LD₅₀ values were calculated relative to the virulent wild-type parent strain RN6390.

Statistics. Student's *t*-test was used to calculate statistical variation.

RESULTS

Construction of the recombinant *putP*-complementing plasmid

An *E. coli*–*S. aureus* shuttle vector was constructed that contained the full-length *putP* gene for use in complementation studies. Plasmid pERL3 501/253 was chosen as the backbone because of the presence of an origin of

replication for *E. coli* as well as an origin of replication that functions in a variety of Gram-positive bacteria (Luchansky *et al.*, 1988). A gentamicin-resistance gene was added to the plasmid because of the difficulties with using erythromycin as a selectable marker in *E. coli*. Because the Tn917 transposon has genes for resistance to chloramphenicol and erythromycin, we chose to add a tetracycline-resistance gene to the complementing plasmid to allow for easy selection of *S. aureus* cells containing this plasmid. The final recombinant plasmid was labelled pTGCOMP (Fig. 1). Following electroporation of this plasmid into RN4220*putP* cells, plasmid DNA was isolated from these cells and shown to be of the correct size (data not shown). Moreover, PCR amplification with *putP*-specific oligonucleotide primers showed that transformed RN4220*putP* cells had a 260 bp band of the same size as wild-type RN4220 cells, which was missing in the lanes that had lysates from mutants RN4220*putP* or 16F-157 (data not shown). These data indicate that the *putP* recombinant plasmid was successfully transferred into RN4220*putP* cells.

Testing of proline uptake in recombinant RN4220*putP* cells

To determine if the *putP* recombinant plasmid delivered into the RN4220*putP* cells could restore proline uptake in these defective cells, a radioactive proline transport assay was performed. Wild-type strain RN4220 cells were able to transport approximately threefold more radioactive proline than the mutant strain RN4220*putP* ($P < 0.0099$; Fig. 2a). However, the *putP* mutation was overridden by the pTGCOMP plasmid, containing a full-length *putP* gene, which brought the level of proline uptake in the RN4220*putP*/pTGCOMP cells up to the wild-type level through the entire 10 min time-course that was studied. When strain RN6390 and the corresponding *putP* mutant strain 16F-157 were tested over a 10 min period, proline uptake was initially about twofold higher for the wild-type strain RN6390 versus the *putP* mutant strain 16F-157 (although the difference narrowed slightly during the assay period), showing that the same effect was occurring in a different background (Fig. 2b). Complementation of strain 16F-157 also restored the proline uptake to wild-type levels.

Next, a very short time-course (15–30 s) was used to accurately assess the transport kinetics rather than the uptake rate modulated by metabolism of the accumulated proline. In such a short time span, there would not be enough time to demonstrate accumulation of radiolabelled proline. The results show that the *putP* mutant strain RN4220*putP* took up substantially less proline in 15 or 30 s compared to the wild-type strain RN4220 (Table 1), indicating a true defect in proline transport within the *putP* mutant strain and not transport linked with accumulation. Complementation of the *putP* mutation fully restored proline uptake to wild-type levels. Similar results were observed for strain RN6390, where at both 15 and 30 s there was significantly less proline uptake in the *putP* mutant compared to the wild-type. Again, complementation of strain 16F-157 fully restored the

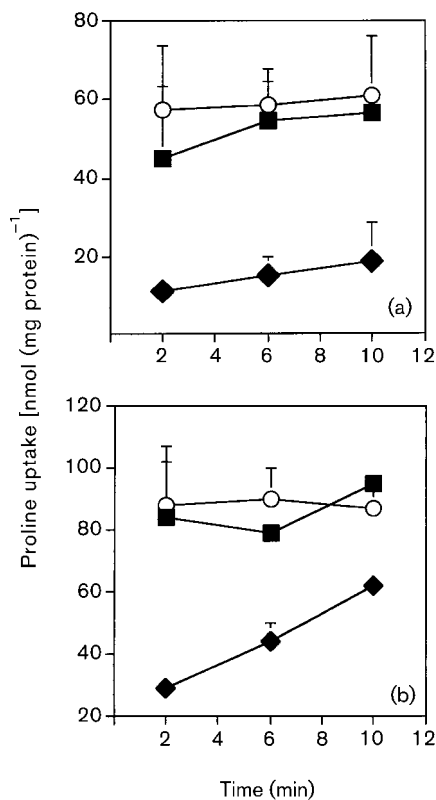


Fig. 2. Proline uptake by the high-affinity system of *S. aureus*. Transport measurements were done after 2, 6 or 10 min by the filtration method of Bae & Miller (1992). Proline was present at 5 µM. Cells were suspended in transport buffer at about 40 µg total cellular protein ml⁻¹. (a) RN4220 and derivatives: ■, RN4220; ◆, RN4220*putP*; ○, RN4220*putP*/pTGCOMP. (b) RN6390 and derivatives: ■, RN6390; ◆, 16F-157; ○, 16F-157/pTGCOMP. The data in both panels represent the mean ± SD of three separate experiments (error bars not shown where smaller than symbols).

level of proline uptake. These results indicate that proline uptake was affected by the *putP* mutation and the mutation could be fully complemented by the recombinant plasmid.

These results also show that the two different *S. aureus* strains used in this study had similar levels of proline uptake.

Growth of the *putP* mutant in low-proline minimal medium

The assays described above showed that *putP* mutants were impaired in proline uptake. To take this one step further, the wild-type strain RN4420, its *putP* mutant, and the *putP* mutant complemented with pTGCOMP were grown in defined staphylococcal minimal medium supplemented with various concentrations of proline, ranging from the standard level of 1.74 mM down to 1.74 µM. In the normal formulation (1.74 mM proline) of defined staphylococcal minimal medium, all three strains grew equally well, and at 17.4 µM proline there were still no differences in the growth curves (Fig. 3). A slight decline in growth was observed for the *putP* mutant compared to the wild-type when the level of proline was decreased to 3.48 µM but this was not significant even after 24 h (*P* < 0.063). However, growth of the mutant was significantly impaired versus both the wild-type and the complemented mutant at a proline level of 1.74 µM for time points at 8 h (*P* < 0.042), 10 h (*P* < 0.038), 12 h (*P* < 0.023) and 24 h (*P* < 0.002). In addition, strain RN6390 had significantly higher growth than its *putP* mutant 16F-157 in medium with 1.74 µM proline (data not shown). Thus, in a proline-deficient environment, the presence of a functional high-affinity proline permease protein appears to be needed for optimal growth of the bacteria.

Proline concentrations in murine tissues

The *in vitro* assays suggested that proline availability and a functional *putP* gene were essential for proper staphylococcal cell growth. To tie this to what might be happening *in vivo*, proline concentrations from two murine organs, liver and spleen, were assayed. Proline levels in spleens averaged 0.20 µmol per mg dry weight of tissue or 7.5 µmol per spleen. Similar proline levels were observed for liver tissue (0.20 µmol per mg dry weight of tissue), although the overall amount of proline per liver was higher (88.4 µmol)

Table 1. Radioactive proline uptake in strains RN4220, RN4220*putP* and RN4220*putP*/pTGCOMP over a very short time-course

Strain	Genotype	Proline uptake [nmol (mg protein) ⁻¹]*	
		15 s	30 s
RN6390	Wild-type	64 ± 15	67 ± 6
16F-157	<i>putP</i> ::Tn917	8 ± 3	18 ± 4
16F-157/pTGCOMP	<i>putP</i> ::Tn917, <i>putP</i> ⁺	65 ± 12	70 ± 4
RN4220	Wild-type	38 ± 4	39 ± 8
RN4220 <i>putP</i>	<i>putP</i> ::Tn917	9 ± 1	10 ± 2
RN4220 <i>putP</i> /pTGCOMP	<i>putP</i> ::Tn917, <i>putP</i> ⁺	51 ± 2	55 ± 4

*The data are the mean ± SD from two or three separate runs.

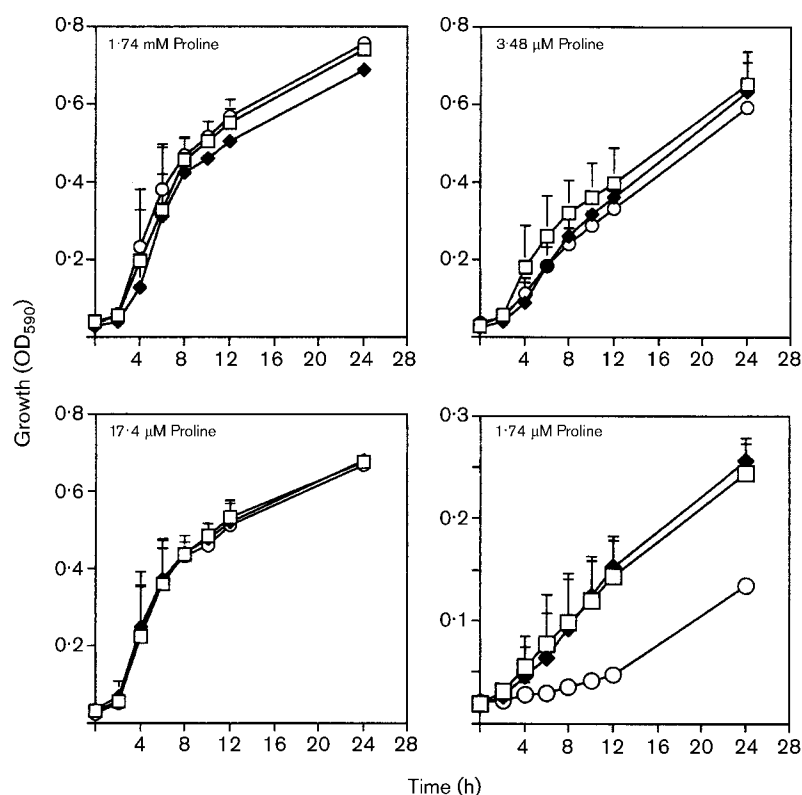


Fig. 3. Growth curves of *S. aureus* RN4220 and derivatives in defined minimal medium with different proline concentrations. ■, RN4220; ○, RN4220*putP*; ◆, RN4220*putP*/pTGCOMP. The data represent the mean \pm SD from at least three separate experiments.

because the liver had a greater mass than the spleen. Thus, *in vivo* proline levels in two key organs are quite low.

Virulence of the *putP* mutant strain restored with the *putP* recombinant plasmid

As shown above, proline transport and accumulation were fully re-established by the *putP* recombinant plasmid. Of more interest though was whether this complementing plasmid could restore virulence to the *S. aureus* cells. Transposon insertions into the genome are known to create polar mutations that affect downstream genes, and these, rather than the point of insertion in one particular gene, may actually cause the phenotype. If a mutation is truly complemented, then virulence will be restored in the mutant strain. To test whether virulence was restored by the addition of the *putP* recombinant plasmid, LD₅₀ determinations were run with the wild-type parental strain RN6390, its Tn917 mutant 16F-157 (*putP*::Tn917) and 16F-157 bearing the *putP* recombinant plasmid pTGCOMP. The results showed an LD₅₀ of 8.13×10^6 bacteria for the wild-type strain RN6390, which was about 10-fold lower than the LD₅₀ of the *putP* mutant strain 16F-157 (7.35×10^7). With the transfer of the *putP* recombinant plasmid into the 16F-157 cells, the LD₅₀ returned to a near wild-type level of 9.23×10^6 . This demonstrated that the *putP* gene alone was needed for both full proline uptake and virulence and that complementation of the *putP* mutation not only restored proline uptake and growth in low-proline medium, but also the virulence of the bacterial cells.

DISCUSSION

Proline is required by a number of strains of *S. aureus* for their survival. The high-affinity proline transporter may be critical in environments when proline concentrations are low, such as the vegetations within the heart tissue (Durack & Beeson, 1972) or the urine of a human host (Inoue *et al.*, 1999). Our previous mutational analysis of the high-affinity proline transport gene (*putP*) has shown that mutation in this gene causes *in vivo* attenuation in several animal models of infection, including an endocarditis model (Bayer *et al.*, 1999; Schwan *et al.*, 1998). Less proline was taken up into staphylococcal cells that possessed a mutation in the *putP* gene (Schwan *et al.*, 1998), so the observation that there was an effect on *in vivo* survival was not unexpected.

In this study, we have demonstrated that the reductions in proline transport and bacterial virulence were indeed due to the mutation of the *putP* gene and not some polar effect caused by the insertion of Tn917. Transformation of both the RN4220*putP* and 16F-157 *S. aureus* mutants with the *putP* recombinant pTGCOMP plasmid re-established optimal proline uptake that matched that of the respective wild-type parental strains, compared to the significantly lower proline uptake levels observed for each *putP* mutant strain. Although proline uptake was diminished in the *putP* mutant strains, a significant amount of proline was still taken up by the staphylococcal cells, presumably due to at least one other proline transport system in *S. aureus* (Bae & Miller, 1992; Townsend & Wilkinson, 1992; Pourkomaillian & Booth, 1994). For the first time, we have

also demonstrated that mutation in *putP* does in fact have an effect on transport in addition to the potential transport and accumulation effects that were previously measured (Bae & Miller, 1992; Wengender & Miller, 1995; Schwan *et al.*, 1998) and also confirmed in this study (Fig. 2). A very short time-course of 15 or 30 s showed that the *putP* mutant strains had significantly less uptake of proline than the wild-type bacteria or the complemented strains. By using this very brief exposure assay, we have conclusively shown that there is an effect on transport because there is not sufficient time to accumulate the radioactive proline during this time-course. After only 15 s, there is an eight-fold difference between the wild-type strain RN4220 and the mutant strain RN4220*putP*, but this ratio decreases to less than a twofold difference by 10 min, probably because of accumulation effects. This is an important distinction for us to fully understand proline transport in *S. aureus*. It is possible that the *putP* mutation may be tied to a deficiency in the transport of another entity in addition to proline. Certainly, sodium levels may be modulated because the PutP protein is thought to be a sodium co-transporter. Another explanation could be the presence of at least one other proline transport system that may be bringing proline into the staphylococcal cells. No genetic analyses have been done to investigate the other system. Nonetheless, when the proline level is low in the environment, the high-affinity proline transporter appears to be very important for bringing proline into the cell. Differences in proline uptake and accumulation within the two staphylococcal strain backgrounds studied were similar for both wild-type strains, but the *putP* mutant strains for each background displayed slightly different kinetic profiles over the time-frame studied.

Of more critical interest is how this defect in proline transport carries through to attenuation of a *putP* mutant in animal models of infection. Since *S. aureus* is an auxotroph for proline (Gladstone, 1937), it must import this key amino acid. The growth studies in minimal medium shed some light on why proline permeases may be a critical part of *S. aureus* survival. In a low-proline environment, the presumption would be that the high-affinity proline transporter would be crucial. Indeed, growth of the *S. aureus putP* mutant in minimal medium with 1.74 µM proline was significantly reduced compared to wild-type bacteria. Low proline levels could be triggering transcriptional activation of the *putP* gene that may be leading to increased PutP protein expression in the *S. aureus* cells growing in proline-depleted environments. When these *in vitro* observations are tied to the level of proline in some murine organs, the attenuation in the *in vivo* survival of the *S. aureus* makes sense. Very few studies have examined the level of amino acids, like proline or proline betaine, in murine and human fluids or tissues (Chambers & Kunin, 1987; Inoue *et al.*, 1996, 1999). Human serum has been observed to have 29.6 mM proline (Inoue *et al.*, 1996) and human urine has been shown to have proline concentrations that range from 6.08 to 17.10 µM (Inoue *et al.*, 1999). Our previous study

indicated a substantial decrease in the bacterial numbers from spleens and livers infected with a *putP* mutant strain compared to a wild-type strain of *S. aureus* (Schwan *et al.*, 1998). Spleens and livers were targeted in this study because of the lower growth by the proline uptake mutant. No information has been previously reported for murine liver and spleen proline levels. In this study, an entire spleen of a mouse was shown to have only 7.5 µmol proline and a murine liver only 88.4 µmol proline. Certainly, proline levels should be higher in areas of the spleen and liver fed directly by the circulatory system, where higher levels of proline prevail (Inoue *et al.*, 1996). However, other areas of both organs should have substantially lower levels of proline that are further from direct contact with the bloodstream. Staphylococcal cells in these organs will have access to only a portion of this proline and are therefore likely to experience low proline concentrations.

The last piece of the puzzle is tying together the concept of growth in a proline-deprived environment and the virulence of the *S. aureus* bacterial cells. When one considers that *S. aureus* can infect virtually every tissue in the human body (Easmon & Adlam, 1983), one can appreciate why the *putP* mutant strain grew poorly in murine livers and spleens (Schwan *et al.*, 1998) if there is limited availability of proline at each of these sites within the mouse. Complementation of the 16F-157 *putP* mutant with the *putP* recombinant plasmid re-established the virulence of the bacteria to a near wild-type level. A study of proline transport in *E. coli* showed that a mutation in the low-affinity proline transport gene caused a 100-fold reduction in *E. coli* recovered from the bladders of the infected mice compared to wild-type *E. coli* with an intact *proP* gene (Culham *et al.*, 1998). These previous findings in *E. coli* combined with our confirmatory study here in *S. aureus* would suggest that proline transport is important for *in vivo* survival in certain niches within the animal host and presumably within the human body because of the low availability of proline in some of the tissues and bodily fluids.

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