In Vitro and In Vivo Validation of *ligA* and *tarI* as Essential Targets in *Staphylococcus aureus*[▽]

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A conditional expression system has been developed using the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *Pspac* promoter to validate essential genes of *Staphylococcus aureus* in vivo. The system has been applied to prove the essentiality of *ligA* and to evaluate the function of *tarI*, which was found to be essential in vitro but not in vivo.

The current increase of antimicrobial resistance in pathogenic staphylococci due to the spread of multiply resistant strains has encouraged the search for alternative targets for antimicrobial chemotherapy. Potential target genes should be essential for bacterial viability or pathogenesis (3). However, gene essentiality can vary depending on test conditions, particularly in vitro versus in vivo situations (1). Therefore, the use of in vivo expression systems to validate putative target candidates is critical (12). In this study, a conditional expression system based on a regulatable *Pspac-lacI* promoter element has been evaluated for in vivo use.

This regulatable gene expression system was originally developed by Jana et al. for the analysis of essential genes in vitro but has not been applied for target validation in vivo before (4). The system consists of a *Pspac-lacI* promoter element that regulates the expression of genes of interest depending on the concentration of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG). Repression of the target gene is achieved by production of the repressor LacI, which binds to the *Pspac-lacI* promoter element. The production of sufficient amounts of the repressor LacI is ensured by the introduction of the multicopy plasmid pMJ8426, carrying

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
<i>E. coli</i> DH5α	<i>F⁻ endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 λ⁻ Δ(argF-lac)U169 φ80dlacZΔM15</i>	Bethesda Research Laboratories
<i>S. aureus</i>		
8325	NCTC 8325; laboratory strain	10
RN4220	Restriction minus derivative of NCTC 8325-4	8
Xen29	<i>S. aureus</i> strain ATCC 12600 (a pleural fluid isolate) with a chromosomally integrated <i>lux</i> operon (Tn4001 <i>luxABCDE</i> Km ^r)	6, 7
RN4220 <i>Pspac::ligA</i>	Conditional mutant of <i>ligA</i> ; Cm ^r ; with repressor plasmid pMJ8426 (<i>lacI</i> Tet ^r)	This study
RN4220 <i>Pspac::tarI</i>	Conditional mutant of <i>tarI</i> (SA0245); Cm ^r ; with repressor plasmid pMJ8426 (<i>lacI</i> Tet ^r)	This study
8325 <i>Pspac::tarI</i>	Conditional mutant of <i>tarI</i> (SA0245); Cm ^r ; with repressor plasmid pMJ8426 (<i>lacI</i> Tet ^r)	This study
Xen29 <i>Pspac::tarI</i>	Conditional mutant of <i>tarI</i> (SA0245); Cm ^r ; with repressor plasmid pMJ8426 (<i>lacI</i> Tet ^r)	This study
Plasmids		
pLL30	Shuttle vector; Spc ^r in <i>E. coli</i> ; Tet ^r Cm ^r in <i>S. aureus</i>	4
pMJ8426	Shuttle vector; <i>lacI</i> Spc ^r in <i>E. coli</i> ; Tet ^r in <i>S. aureus</i>	4
pKSL <i>ligA</i>	pLL30 with upstream and 5' regions of the <i>ligA</i> gene for conditional mutation of <i>ligA</i>	This study
pKSL <i>tarI</i>	pLL30 with upstream and 5' regions of the <i>tarI</i> gene for conditional mutation of <i>tarI</i> (SA0245)	This study

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FIG. 1. In vitro growth of *Pspac::ligA* (A) and *Pspac::tarI* (B, C, and D) mutant strains depends on the inducer IPTG. Growth of all conditional mutants can be regulated by IPTG in the presence of the repressor plasmid pMJ8426. The parent strain RN4220 (P) and the *Pspac* mutants (M) in RN4220 were grown in solid medium either supplemented with 300 μ M IPTG (+ IPTG) or without IPTG (– IPTG). Representative growth curves of strains RN4220, 8325, and Xen29 without IPTG (●) or with 0.3 mM IPTG (◆) and of the *Pspac* mutants in strain RN4220 (B), 8325 (C), or Xen29 (D) without IPTG (○) or with IPTG at 0.3 mM (△), 0.03 mM (*), or 0.003 mM (◇) are shown.

lacI behind the constitutively expressed penicillinase (*pcn*) promoter, into the promoter fusion strain.

The strains and plasmids used in this study are listed in Table 1.

In a first step, the in vivo applicability of this conditional expression system was tested. A conditionally lethal mutant expressing the essential gene *ligA*, encoding DNA ligase, in an IPTG-dependent manner was constructed (5). The target gene was placed behind the *Pspac* promoter and integrated into the chromosome by a double-crossover event as described by Jana et al. (4). The upstream fragment was amplified by using primers 5'-CGG AAT TCA AGG AGG ATT AAG GGA TGG C-3' and CGG GAT CCG CTC TTG ATA CAG TTG TAC C-3', and the downstream fragment was amplified by using primers 5'-CCG CTC GAG CCA TAC ACA ATG GTT GGT GG-3' and 5'-ACG CGT CGA CAG CCA TCC CTT AAT CCT CC-3' (restriction sites introduced to facilitate cloning are underlined). In the presence of plasmid pMJ8426 carrying *lacI*, the growth of *Pspac::ligA* depends on the presence of the inducer IPTG (Fig. 1). The conditional mutant strain RN4220

Pspac::ligA was not able to grow in vitro without IPTG in liquid culture and on agar plates. However, in the presence of 0.3 mM IPTG, the mutant strain showed the same growth rate as the parent strain, RN4220 (Fig. 1). These in vitro results confirm the essential function of *ligA* and the conditionally essential phenotype of the constructed mutant. Subsequently, the *Pspac::ligA* fusion strain was used in an experimental *Staphylococcus aureus* mouse abscess model based on collagen Gelfoam implants (Pharmacia & Upjohn, Kalamazoo, MI). The Gelfoam was cut into pieces of 1 cm², incubated overnight in sterile phosphate-buffered saline, and implanted subcutaneously in the backs of female CFW1 mice. One group of mice drank water without and the other group with 25 mM IPTG ad libitum. Three days later, the mice were infected with the *S. aureus* parent strain, RN4220, or with a conditional mutant strain (10⁴ CFU) injected into the implanted Gelfoam. For stable propagation of the plasmid, the animals were treated with tetracycline (due to the corresponding plasmid-encoded antibiotic resistance) immediately and 6 h after the infection of the Gelfoam implants (50 mg/kg of body weight, given intra-

FIG. 2. Determination of CFU of the conditional *S. aureus* RN4220 *Pspac::ligA* strain (A) and the conditional RN4220 *Pspac::tarI* strain (C) 1 day after infection of the murine abscess (Gelfoam) model compared to CFU of strain RN4220 containing the repressor plasmid pMJ8426 (B and D). The number of CFU was determined on agar plates with (+) or without (–) tetracycline. The bacterial load in the Gelfoam homogenate of each individual mouse is indicated by a dot. Each horizontal line indicates the mean for all five mice per group. Asterisks indicate significant differences between the presence and absence of IPTG ($P < 0.05$ by the Mann-Whitney test). The growth of the *Pspac::ligA* mutant strain depends on the addition of IPTG (A), which does not affect the growth of the parent, RN4220 (B). The growth of the conditional *Pspac::tarI* strain is not dependent on the presence or absence of IPTG (C).

peritoneally). One day after infection, Gelfoam implants were removed and homogenized, and bacterial cells were counted on Mueller-Hinton agar containing 0.3 mM IPTG alone or with 10 μ g/ml tetracycline. Statistical analysis was performed using the nonparametric Mann-Whitney U test. For all comparisons, a P value of <0.05 was considered statistically significant. These experiments revealed that the growth of the *Pspac::ligA* mutant in the Gelfoam was dependent on the presence of IPTG, whereas the parent strain, RN4220, grew independently of IPTG (Fig. 2). Bacteria isolated from the Gelfoam were tetracycline resistant, indicating the stability of the repressor plasmid pMJ8426 in vivo under the test conditions.

Moreover, the conditional essentiality of *Pspac::ligA* in vivo has also been confirmed in a second animal model. For this

purpose, a conditional *Pspac::ligA* mutant was constructed in strain Xen29, a clinical isolate that has been used for in vivo imaging of staphylococcal infections. The growth of this strain can be visualized by means of bioluminescent imaging due to expression of *luxABCE* (6, 7). We injected 5×10^7 Xen29 *Pspac::ligA* CFU intramuscularly into the musculus tibialis anterior of female BALB/c mice. Bioluminescence was measured for three consecutive days, as shown in Fig. 3. As in the Gelfoam model, IPTG was given via drinking water (25 mM) to one group from day –4, and tetracycline was injected intraperitoneally at 30 mg/kg daily starting on day zero. These studies showed a significant decline of bioluminescence in mice that drank water without IPTG (Fig. 3, upper panels). Consequently, no bioluminescent signal was detectable after 24 h (day 1) of infection. In contrast, bioluminescent bacteria were

FIG. 3. Bioluminescence of strain Xen29 *Pspac::ligA* (upper panels) and Xen29 *Pspac::tarI* (lower panels) in the muscular infection model in the presence or absence of IPTG. The time course of bioluminescence levels was monitored for 3 consecutive days (day zero to day 3 [d0 to d3]) after intramuscular infection of the musculus tibialis anterior of mice with *S. aureus* Xen29 *Pspac::ligA* or Xen29 *Pspac::tarI*. Images of representative mice are presented. Signal intensity is indicated by a pseudocolor scale superimposed on the grayscale image to produce a composite image of photon emission in relation to the subject. Pixels with low photon counts appear blue, and magenta represents the most intense light emission. The signals were obtained using a Night Owl LB 981 luminograph (Berthold Technologies, Bad Wildbad, Germany) by integrating photons over 1 min.

visible until day 3 postinfection in a control group of mice that received IPTG via drinking water (Fig. 3).

In a different approach, we fused the *tarI* gene behind *Pspac* and integrated the construct into the chromosomes of three *S. aureus* strains: RN4220, 8325, and Xen29. Originally, *tarI* was annotated in strain N315 as *ispD* (SA0245) due to its homology to essential 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferases of *Escherichia coli* and *Bacillus subtilis*, which belong to the 1-deoxy-D-xylulose 5-phosphate pathway (DXP pathway) for isoprenoid biosynthesis (9). However, *S. aureus*—like other gram-positive cocci, such as *Streptococcus pneumoniae* and *Enterococcus faecalis*—does not possess the DXP pathway for isoprenoid biosynthesis. Instead, these species use the mevalonate pathway (13, 14). Meanwhile, there is increasing evidence that *tarI* is involved in teichoic acid synthesis in *S. aureus* (2, 11). Interestingly, *S. aureus* possesses two homologs of the *tarI* gene (SA0241 and SA0245 in strain N315) in the genome that are about 5,000 bp apart and share 75% identity on the amino acid sequence level. Both genes might encode D-ribitol-5-phosphate cytidylyltransferases. Reverse transcriptase PCR analyses revealed that both genes are transcribed in *S. aureus* grown to mid-log phase (data not shown). We could delete the SA0241 homolog, showing that this gene is clearly nonessential in *S. aureus* (data not shown). However, we were not able to delete the SA0245 gene. This observation differs from the results of a previous report by Zalacain et al. (15). Zalacain et al. could make a single mutant of either *tarI* homologous gene (*yacM* according to *Bacillus subtilis* nomenclature) in *S. aureus* RN4220, but they were not able to delete the second gene from either mutant. Interestingly, in a more recent study, the *tarI*-homologous gene SACOL0240 in *S. aureus* strain COL was essential for viability in vitro, as long as the *tarI* homolog *tarO* was not knocked out (2).

To clarify whether or not *tarI* (SA0245) is essential in *S. aureus*, we constructed a conditional mutant of this gene by placing *tarI* behind the conditional *Pspac* promoter using primers 5'-CGG AAT TCG ATA TAG TTG AAT GGA GGA AG-3' and 5'-CGG GAT CCA ATA CGA CCT CAC CAA

CAC C-3' (upstream fragment) and primers 5'-ACG CGT CGA CTT CCT CCA TTC AAC TAT ATC-3' and 5'-CCG CTC GAG TAT CTA GTG TCA CCT AAT CC-3' (downstream fragment). As shown in Fig. 1B, the growth of the conditional *Pspac::tarI* mutant in strain RN4220 was dependent on IPTG in vitro, indicating an essential function of *tarI* in *S. aureus*. In addition to strain RN4220, conditional *Pspac::tarI* mutants were also constructed in strain 8325 and Xen29. Conditional growth experiments using different IPTG concentrations revealed that in the absence of IPTG, growth inhibition was less pronounced in Xen29 and 8325 than in RN4220 (Fig. 1B to D). Consequently, we employed the *Pspac::tarI* mutant of strain RN4220 in the in vivo implant model. For this purpose, both RN4220 and the mutant strain were used in the same manner as that described above for the *ligA* conditional mutant. Surprisingly, these experiments revealed that the mutant strain showed the same capability to grow in vivo as the parent strain (Fig. 2). Obviously, *tarI* (SA0245) is important for the growth of *S. aureus* in vitro, which has been demonstrated in three strains (RN4220, 8325, and Xen29). However, the gene product seems to be dispensable for in vivo growth. To rule out the possibility that the in vivo essentiality of *tarI* is related to the strain background, conditional *tarI* expression was also investigated in the muscle model using the bioluminescent strain Xen29 *Pspac::tarI*. Following challenge with 5×10^7 Xen29 *Pspac::tarI* CFU in the musculus tibialis anterior, no significant difference in bioluminescence between mice that drank water with IPTG and mice that drank water without IPTG could be detected (Fig. 3, lower panels). Furthermore, the musculus tibialis anterior was removed after 3 days of infection and homogenized, and CFU counts were determined, revealing that there was no significant difference between the groups with and without IPTG (data not shown). In addition, to test if the conditional phenotype of the *Pspac::tarI* mutant strain was lost during in vivo passage, in vitro essentiality was reevaluated by selecting representative colonies from the agar plates that had been used for calculation of bacterial loads in infected muscles. Because these colonies maintained their conditionally essential phenotype in vitro, it can be concluded that *tarI* is not essential in vivo.

These data indicate consistently that *tarI* is essential for growth only under specific laboratory conditions. Moreover, the function of the *tarI* (SA0245)-homologous gene SA0241 must be elucidated, since it might complement the functions of *tarI* under certain conditions. Nevertheless, the results of the study highlight the importance of in vivo validation of in vitro data, especially if new targets for antibiotic development are selected.

In summary, the conditional in vivo systems presented here are suitable for testing in vivo essentiality. The application of IPTG via drinking water was a suitable way of providing sufficient concentrations of the inducer in the staphylococcal cells. Thus, the *Pspac* expression system has proven to be applicable as a conditional expression system for investigating the essentiality of a particular target gene in vivo during *S. aureus* infection.

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