INFECTION AND IMMUNITY, June 2005, p. 3806–3809 Vol. 73, No. 6
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.6.3806–3809.2005

A *rot* Mutation Restores Parental Virulence to an *agr*-Null *Staphylococcus aureus* Strain in a Rabbit Model of Endocarditis

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Received 2 September 2004/Returned for modification 29 November 2004/Accepted 27 January 2005

Mutations in *rot* restore in vitro toxin production to *agr*-negative strains of *Staphylococcus aureus*. We show that a *rot* mutation returns wild-type virulence to an *agr* mutant, as measured in experimental endocarditis infections by target organ bacterial counts. Implications of our data are discussed in terms of *agr* antagonist strategies.

Staphylococcus aureus is an important human pathogen responsible for a broad range of nosocomial and community-acquired infections, gastrointestinal intoxications, and toxin-mediated and systemic syndromes. The prevalence of multiple antibiotic-resistant strains has complicated treatment, further increasing the morbidity and mortality associated with staphylococcal disease. The continuing evolution of resistance against conventional antibiotics (e.g., oxacillin and vancomycin) and new classes of antibiotics (e.g., oxazolidinones) has underscored the critical need for the development of alternative therapeutic strategies (reviewed in references 6, 17, and 21). Since the pathogenesis of *S. aureus* infections is linked to the production of adhesins, toxins, and enzymes, one group of suggested drug targets consists of the regulatory molecules that govern the production of such virulence factors.

A complex network of regulators encoded by *agr*, several two-component signal transduction systems, and at least six transcriptional regulators (SarA and the Sar homologues) function to control virulence factor expression (reviewed in reference 22). In vitro, this regulation is manifest in a temporal orchestration of the transcriptional signals that lead to the postexponential-phase repression of adhesin gene transcription and the parallel increase in the transcription of genes encoding many extracellular toxins and enzymes (23). In general, this transcriptional profile is mirrored by the temporal profiles of adhesin and extracellular protein expressions (5, 19, 23).

An important element in a unified model of virulence factor production is *agr* (22). This locus consists of divergently transcribed messages. One message, RNAII, encodes four proteins (AgrA, AgrB, AgrC, and AgrD), which are involved in an autoinducing pheromone-sensing signal transduction circuit. Two of the proteins, AgrC and AgrA, act as a histidine-kinase sensor and a response regulator, respectively. The autoactivating signal of the *agr* system is a cyclic thiolactone peptide pheromone modified from a prepeptide protein, AgrD. AgrB is the only known enzyme responsible for the modification and secretion of the peptide pheromone. The result of the increase in RNAII is increased activity of the RNAII-encoded activating circuit and the high-level transcription of the divergent *agr* message, a 514-ribonucleotide RNA known as RNAIII. RNAIII is a riboregulator associated with the differential expression of multiple structural genes involved in virulence factor production.

We previously identified *rot* in *S. aureus* strain PM614 (*agr*-null *rot*::Tn*917*) by transposon mutagenesis (16). We showed that a *rot* mutation by itself was not associated with a specific toxin-attenuated phenotype in *S. aureus* RN6390, an *agr*-positive strain. However, in PM614, the same transposon-induced *rot* mutation restored a quantifiable measure of toxin expression, presumably through an *agr*-independent mechanism (9). The *rot* gene product (Rot) is a member of the MarR family of winged-helix transcriptional regulators (pfam01047). In addition, by iterative BlastP searches, Rot has been shown to share homology with 12 proteins of the *S. aureus* genome, at least 6 of which have roles in virulence factor regulation (e.g., SarS, SarT, SarU, SarV, MgrA, and TcaR). Including Rot, these 13 homologous proteins are collectively referred to as "Sar homologues" (reviewed in reference 8).

A comparison of a *rot-agr* double mutant (*rot*::Tn*917*Δ*agr*) to an *agr* (Δ*agr*) mutant by DNA microarray analysis has shown that *rot* is associated with at least twofold negative and positive regulation of 60 and 86 genes, respectively (28). In addition to repressing the virulence factor genes responsible for protease and alpha-toxin activities, wild-type *rot* was inferred to negatively regulate *ebhAB* (genes encoding proteins that are similar to the streptococcal adhesin Emb), *hlb* (the gene encoding β-toxin), *hlgBC* (the gene encoding components of γ-toxin), and *geh* (the gene encoding glycerol ester hydrolase), while upregulating *spa* (the gene encoding staphylococcal protein A). In general, a wild-type allele of *rot* appears to confer a pattern of virulence factor gene regulation opposite to that seen with *agr*.

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TABLE 1. Inoculum-dependent increases in cardiac vegetation and kidney staphylococcal densities

Target organ	Inoculum challenge (CFU)	Strain[a]				*P* value for RN6390, PM783, or PM614 versus PM466[b]
		RN6390 (parent)	PM783 (*rot*::Tn*917*)	PM466 (Δ*agr*)	PM614 (Δ*agr-rot*::Tn*917*)	
Cardiac vegetation	10^4	4.84 ± 1.6 (7)	4.60 ± 1.2 (6)	2.60 ± 0.5 (6)	5.52 ± 2.1 (7)	<0.05
	10^5	5.82 ± 1.7 (8)	5.57 ± 0.8 (6)	4.62 ± 2.15 (8)	5.65 ± 1.9 (7)	<0.05
	10^6	7.19 ± 0.7 (8)	6.52 ± 0.4 (5)	6.85 ± 1.3 (8)	7.45 ± 1.02 (9)	ns
Kidney	10^4	3.32 ± 1.07 (7)	3.98 ± 0.6 (6)	2.17 ± 0.24 (6)	4.03 ± 2.5 (7)	<0.05
	10^5	4.00 ± 1.46 (8)	4.23 ± 0.6 (6)	2.52 ± 0.70 (8)	4.51 ± 1.6 (7)	<0.01
	10^6	4.68 ± 1.44 (8)	4.53 ± 0.9 (5)	4.69 ± 0.95 (8)	5.90 ± 1.5 (9)	ns

[a] Numerical data are reported as the mean $\log_{10}$ CFU/g tissue ± standard deviation. The numbers of animals studied in each group are within parentheses.
[b] ns, not statistically significant.

The present study was designed to determine whether the restored toxin phenotype of the *rot-agr* double mutant seen in vitro correlated with restored in vivo virulence relative to a wild-type strain and to *rot* and *agr* single mutants. For this purpose, we utilized the well-characterized rabbit infective endocarditis model (IE), which represents an acute multisystem infection (30).

We compared the virulences of *S. aureus* strains RN6390 (wild-type parental strain), PM466 (Δ*agr* single mutant), PM783 (*rot*::Tn*917* single mutant), and PM614 (Δ*agr-rot*::Tn*917* double mutant) in the rabbit IE model. Female outbred New Zealand White rabbits (Irish Farms, Corona, CA) underwent carotid-artery-to-left-ventricle catheterization as previously described (12, 24). For comparative virulence assessments, groups of 6 to 9 rabbits each received inocula of 5×10^4, 5×10^5, or 5×10^6 CFU of RN6390, PM466, PM783, or PM614. As established by pilot studies, these inocula encompassed the 95% infective dose for inducing IE for the parental strain used in this study. The challenge inocula were injected intravenously into the marginal ear vein at 48 h postcatheterization. At 48 h postinjection, the animals were sacrificed, and cardiac vegetations and kidneys (major target organs involved in experimental IE) were aseptically removed, homogenized, extensively sonicated, and quantitatively cultured (12, 30). Analysis included only animals with active IE as defined by the proper placement of the transaortic valve catheter and macroscopic, culture-positive vegetations. Data from the different strain groups (i.e., tissue bacterial densities) were analyzed by Kruskal-Wallis ANOVA with Tukey post hoc adjustments for multiple comparisons where appropriate. A *P* value of <0.05 was considered statistically significant.

For all animals, there were inoculum-dependent increases in vegetation and kidney staphylococcal densities (Table 1). At both 10^4 and 10^5 CFU (but not at 10^6 CFU) of challenge inocula, the extent of the staphylococcal proliferation in vegetations and kidneys was significantly less in animals infected with the *agr* mutant compared to those infected with the parental strain (RN6390). With strain PM783 (*rot* knockout mutant with no discernible phenotype distinct from the parental strain, in terms of alpha-toxin and other virulence factor production) (16), there were no significant differences in target tissue counts compared to those for animals infected with the parental strain, RN6390. Of note was the fact that infection with strain PM614 (*rot-agr* double mutant) resulted in the recovery of significantly higher numbers of bacteria from target tissues compared to infection with the *agr* mutant across the same challenge inoculum range. The level of bacterial recovery was similar to that seen with RN6390 and PM783. The *P* values for all pairwise comparisons that used challenge inocula of 10^4 and 10^5 CFU were <0.05.

The fibrinogen-binding adhesins produced by *S. aureus* have been shown to induce bacterial clumping in vivo (14, 15, 20). To rule out the possibility that differences in tissue bacterial densities were artificially caused by a variable capacity of the test organism to clump, we evaluated the in vitro capacities of the study strains to bind to immobilized human fibrinogen, as previously described (29). Briefly, six-well microtiter plates were coated with fibrinogen (50 μg/ml; Sigma Chemicals, St. Louis, MO). Bacteria were grown in Mueller-Hinton broth overnight and then washed, harvested, and diluted to a final concentration of 10^3 CFU/ml (confirmed by quantitative culture). Prior to bacterial seeding, all microtiter plates were overlain with albumin-containing blocking solution for 3 h to prevent nonspecific binding (Boehringer-Mannheim, San Diego, CA). After aspirating off blocking solution, microtiter plates were then seeded with 1 ml of the bacterial inoculum and incubated at 37°C for 1 h, after which all wells were overlain with 2 ml of Mueller-Hinton agar. Plates were then incubated for 18 h at 37°C, and the bacterial colonies were enumerated. Bacterial binding was quantified as the percentage of the initial inoculum bound (mean ± standard deviation of three separate experimental runs). Strains RN6390, PM466, and PM614 bound to fibrinogen at similar low levels (1.8% ± 0.4%, 1.0% ± 1.0%, and 1.7% ± 0.9%, respectively).

The ability of microbial pathogens to initiate endovascular infections such as IE involves complex interactions between the organism and host tissues. The initial site to which endovascular pathogens adhere in order to initiate such infections is the sterile cardiac lesion. This lesion is a composite of endothelial cells, activated platelets, and matrix ligands such as fibrinogen, fibronectin, and fibrin and is relatively devoid of inflammatory cells (3). Thus, pathogens such as *S. aureus* possess a broad array of surface adhesins whose function it is to enable the organism to bind to one or more of these relevant tissues and matrix surfaces (18). In addition, toxins that lyse the major components of the infected vegetation (e.g., alpha-toxin, which causes platelet and endothelial cell lysis) (4) have been viewed as facilitators of the spread of the bacteria within the vegetations, leading to subsequent hematogenous dissemination of the pathogen.

In the context of this latter paradigm, we have previously studied the impact of alpha-toxin on the pathogenesis of experimental IE and demonstrated that inactivation of the alpha-toxin structural gene (*hla*) led to decreased in vivo virulence (3). In the present study, deletion of *agr* (which negatively impacts alpha-toxin production) was also associated with reduced virulence in this model. These latter data parallel those previously confirmed in an investigation on the negative impact of *sarA* and *agr* knockouts upon experimental endocarditis virulence (7). Furthermore, subsequent inactivation of the *rot* locus in the *agr* mutant background (which reconstitutes the capacity of the strain to produce alpha-toxin in vitro) correlated with the reestablishment of parental-level virulence in vivo.

We have shown in several prior virulence studies (in the same experimental model of IE using defined mutations in *S. aureus* RN6390) that at higher levels of challenge inocula (10^6 to 10^8 CFU), virulence differences observed for lower levels of challenge inocula tend to be mitigated (29). This has been hypothesized to relate to the great redundancy of endovascular virulence factors being maximally expressed at high inoculum levels (e.g., adhesins, capsules, etc.) that may well compensate for the genetic inactivation of individual virulence factors that are manifest at lower challenge inoculum levels. It is interesting that in the present study we observed this same phenomenon, by which there were substantive reductions in virulence at the two lower challenge inoculum levels used, which were abrogated at the highest challenge inoculum level (10^6 CFU).

In a larger context, the restoration of virulence of an *agr*-null mutant by a second mutation in *rot*, as seen in the present study, immediately suggests a mechanism for resistance to therapeutic agents designed to disrupt staphylococcal pathogenesis by suppressing *agr* activation and, subsequently, toxin production (reviewed in reference 25). For the suppression of *agr*, two strategies have been pursued. The first is the use of either naturally occurring or synthetic *agr*-inhibitory peptides (10, 13, 25). Such exogenous *agr*-inhibitory peptides appear to function by binding to AgrC without activating the AgrC-AgrA phosphorelay system, thus preventing the initiation of RNAIII synthesis by endogenous autoinducing peptides. The second is the use of antibodies against TRAP (*t*arget for *R*NA-*a*ctivating *p*eptide), a protein implicated in *agr* activation that is encoded outside of *agr*. The mechanism of virulence factor regulation by TRAP remains unclear (1, 11, 22).

Regardless of the mechanism of RNAIII inhibition, because a *rot* mutation is sufficient to restore full virulence to an *agr* mutant, *S. aureus* can be viewed as having a potential intrinsic mechanism for resistance to such strategies (i.e., a spontaneous mutation or downregulation in *rot*). This mechanism remains only theoretical to date. To address this potential adaptive response in vivo, future studies with *agr* mutants should evaluate (i) target organ counts over a longer postinfection period and (ii) in vivo selection of *rot* mutants.

As reviewed by Otto, targeting only *agr* to reduce virulence may have a number of limitations (25). For example, downregulation of *agr* results in the expression of factors that aid in the bacterial colonization that may play a role in persistent *S. aureus* infections. Furthermore, the loss of *agr* activity has been associated with strains possessing an increased capacity for biofilm formation and for attachment to polystyrene. These data suggest that the *agr*-negative strain may have a greater capacity to cause chronic infections than do *agr*-positive strains. It appears that *agr*-inhibiting peptides may best be viewed as an augmentative therapy or that alternative approaches, such as combining inhibitory *agr* pheromones with bactericidal agents or *rot* agonists, may represent better approaches to control *S. aureus* infections (27).

We thank Donna Bates and Richard Proctor for their critical review of the manuscript and for many thoughtful suggestions. We note that all animal experimentation was performed in accordance with the guidelines for animal health and welfare required by the LA Biomedical Research Institute at Harbor-UCLA. We thank Yin Li for excellent technical assistance with the animal studies.

This work was supported by the National Institute of Allergy and Infectious Diseases grants AI053235-01 to P.J.M. and 5R01AI039108-07 to A.S.B.

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Editor: V. J. DiRita