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Decreased Virulence of a *gls24* Mutant of *Enterococcus faecalis* OG1RF in an Experimental Endocarditis Model

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In the current study, the *gls24* disruption mutant TX10100, previously shown to be more sensitive to bile salts and attenuated in a mouse peritonitis model, showed an approximately fivefold higher 50% infective dose than wild-type OG1RF in a rat endocarditis model. When administered as a mixture, TX10100, unlike a downstream *glsB* mutant, was significantly outnumbered by OG1RF in vegetations, organs, and blood, despite being inoculated in greater numbers. These results indicate that *gls24* is important in the pathogenesis of enterococcal endocarditis.

Enterococci account for 5 to 20% of infective endocarditis cases, surpassed in the hospital setting only by *Staphylococcus aureus* strains and in the outpatient setting by viridans group streptococci and *S. aureus* strains (7). While a prior animal study indicated a relatively high propensity for enterococci to adhere to normal and damaged valvular endothelium, comparable to that observed with viridans group streptococci and *S. aureus* strains (5), the mechanisms by which enterococci progress from their usual location in the gastrointestinal tract, how they survive in the bloodstream, and how they attach to valvular surfaces are largely unknown.

Microorganisms, both in their natural environments and when causing infections, have to deal with different stress conditions and may respond in different ways (1, 8, 12). It has been previously shown by Giard et al. that *E. faecalis* (strain JH2-2) responds to glucose starvation through the production of 42 different proteins, called glucose starvation proteins (3). One of these proteins (glucose starvation protein 24) was found to be synthesized continuously during a 24-h period of starvation, and its synthesis was found to be triggered also by other stress conditions, such as exposure to cadmium chloride and bile salts, which led to its being named as a general stress protein (Gls24) (4). A *gls24* mutant had a reduced survival rate in the presence of 0.3% bile salts after starvation compared with wild-type strain, reported by Giard et al. for strain JH2-2 (4) and our group for strain OG1RF (11). We also found that the OG1RF *gls24* disruption mutant TX10100, but not a mutant of the downstream and cotranscribed gene *glsB*, was significantly attenuated in a mouse peritonitis model and that anti-Gls24 rabbit serum protected mice against wild-type OG1RF infection (11).

Endocarditis animal models have long been used to assess virulence factors that may influence the ability of bacteria to adhere to damaged heart valves and/or to survive and multiply in vivo. Endpoint measures have included embolic complications and mortality, the number of bacterial cells recovered from vegetations, 50% infective doses (ID_{50}s), and the comparative ability of a mutant versus wild type strain inoculated as a mixture to cause disease.

In the current study, we first determined the stability of the *gls24* disruption mutant TX10100 (the disrupting fragment confers resistance to 2,000 μg/ml kanamycin) (11) in vitro and in a rat endocarditis model (as described previously [10]). Our results showed that this mutant was stable in vitro, since all colonies tested were kanamycin resistant after TX10100 was grown alone in vitro for 72 h or 120 h. In mixed in vitro cultures, TX10100 (55%) was inoculated with OG1RF (45%), and the percentages of OG1RF versus TX10100 in the culture after 72 and 120 h growth were ca. 45% versus 55% and 42% versus 58%, indicating that the presence of OG1RF in the mixed culture did not affect the growth of TX10100, at least in vitro. When TX10100 was tested alone in the rat endocarditis model and recovered from vegetations 72 or 120 h postinfection, all the colonies tested were highly kanamycin resistant, indicating that this disruption was also stable in vivo.

When administered separately in the rat endocarditis model, TX10100 showed a higher ID_{50} than OG1RF (ID_{50}s for TX10100 and OG1RF were 7.9×10^5 and 1.6×10^5, respectively) (Table 1), although comparison of the individual inocula did not reach statistical significance (relatively few animals were used at each inoculum).

Many factors may influence the ID_{50} results in experimental endocarditis model, such as nonspecific and specific defense mechanisms of individual animals and differences in catheter positioning, in heart valve damage, and in the exact number of bacterial cells inoculated. To overcome these issues, some investigators have used a mixture of bacterial strains (wild types and mutants) which appears more sensitive for detecting virulence differences than the use of separate inocula (6, 13). For example, a *S. aureus* collagen-adhesin (Cna) mutant was outnumbered by the wild type at several time points after initial

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TABLE 1. Infectivity of OG1RF and the *gls24* mutant (TX10100) in a rat endocarditis model

Challenge inoculum (CFU) or parameter	% of rats infected (no. of rats infected/total no. tested)[a] or no. of CFU for infective dose	
	OG1RF	TX10100
10^4	12 (1/8)	14 (1/7)
10^5	61 (11/18)	45 (5/11)
10^6	79 (11/14)	44 (4/9)
ID_{50}	1.6×10^5	7.9×10^5

[a] Not significantly different by Fisher's exact test for OG1RF and TX10100 at each individual inoculum.

valve attachment (6). Therefore, we applied this strategy to OG1RF and TX10100 in the rat endocarditis model.

A mixture of OG1RF and TX10100 was administered to 10 rats, which were sacrificed after 72 h. For six animals, the numbers of CFU of OG1RF and TX10100 in the inoculum were 2.1×10^7 and 3.7×10^7, respectively; thus, the percent of OG1RF in the total inoculum was 36%. For four animals, the numbers of CFU of OG1RF and TX10100 were 7.5×10^7 and 1×10^8, respectively, resulting in OG1RF representing 43% of the total inoculum. At autopsy, vegetations were detected in all rats, weighing a mean of 6.6 mg (range, 5.2 to 11.5 mg). Vegetations, kidneys, and spleens were excised, weighed, and homogenized in 1 ml of normal saline, and 0.1 ml of blood was drawn from the inferior vena cava, followed by serial dilution and plating onto brain heart infusion agar with and without kanamycin. The mean percentages of OG1RF in the total amount of bacteria recovered from vegetations, spleens, kidneys, and blood were 77%, 78%, 80%, and 78%, respectively, demonstrating that the percentage of bacteria that were OG1RF had significantly increased from time zero ($T = 0$) ($P < 0.001$ by chi-square test using data from individual animals). The mean virulence indices of the mutant relative to OG1RF in vegetations, spleen, kidney, and blood were 0.18, 0.15, 0.10, and 0.08, respectively, calculated using the following equation (as previously described for other organisms in mixed infections [2]):

$$\text{Mean virulence index} = \frac{(\text{GM-CFU of OGIRF})_{T=0}/(\text{GM-CFU of the mutant})_{T=0}}{(\text{GM-CFU of OGIRF})_{T=72 \text{ or } 120}/(\text{GM-CFU of the mutant})_{T=72 \text{ or } 120}}$$

where GM-CFU is the geometric mean expressed as CFU.

In a separate experiment, six animals were inoculated as described above with 3.1×10^7 CFU of OG1RF and 4.4×10^7 CFU of TX10100 (the percent of OG1RF in the total inoculum was 41%) and sacrificed after 120 h. The mean percentages of OG1RF in the total population of bacteria recovered from vegetations, spleens, kidneys, and blood were 84%, 91%, 91%, and 92%, respectively, which, again, were significantly greater than the percentages in the initial inoculum ($P < 0.001$), confirming the results obtained at 72 h. The mean virulence index of the mutant relative to OG1RF was 0.09 in this experiment. Results from all 16 rats were combined and are shown in Fig. 1.

Because disruption of *gls24* inactivates *glsB*, the gene cotranscribed with *gls24* (11), we also examined the *glsB* mutant

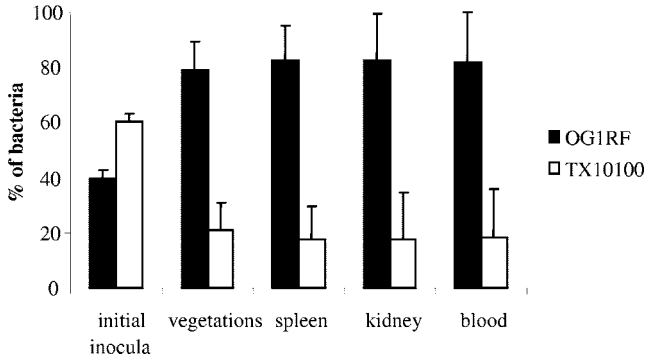

FIG. 1. Percentages of OG1RF and the *gls24* mutant (TX10100) in the total population of bacteria recovered from tissue samples and blood and in the initial inocula. Three different inocula were used, and bacteria were recovered from animals 72 or 120 h after inoculation. All results from 16 rats were combined, and the mean percentages and standard deviations are shown. Statistical comparison was done using the results for individual rats (see the text) ($P < 0.001$ by chi-square test).

TX10200 in the endocarditis model. A mixture of OG1RF and TX10200 (OG1RF represented about 45% of the initial inoculum) was administered to six rats, which were sacrificed after 72 h. The percentages of OG1RF in the total CFU of bacteria recovered from vegetations, spleens, kidneys, or blood at 72 h, unlike in the mixture with the *gls24* mutant, was roughly the same, with a slight increase for OG1RF in the vegetations and blood and a slight decrease for OG1RF in spleen and kidney (Fig. 2). The mean virulence indices of the *glsB* mutant relative to OG1RF in vegetations, spleen, kidney, and blood were 0.43, 1.27, 2.43, and 0.89, respectively, in this experiment. We attempted to obtain results for 120 h postinfection, but rats died at 90 to 100 h, likely due to the full virulence of the *glsB* mutant and OG1RF in the mixed inoculum. These results indicate that *glsB* does not contribute significantly to the in vivo *gls24* mutant phenotype.

In conclusion, our results indicate that *gls24* is important for virulence in the rat endocarditis model. Even though we cannot yet explain how Gls24 functions in this model, we speculate that this protein is crucial for *E. faecalis* survival in vivo. This

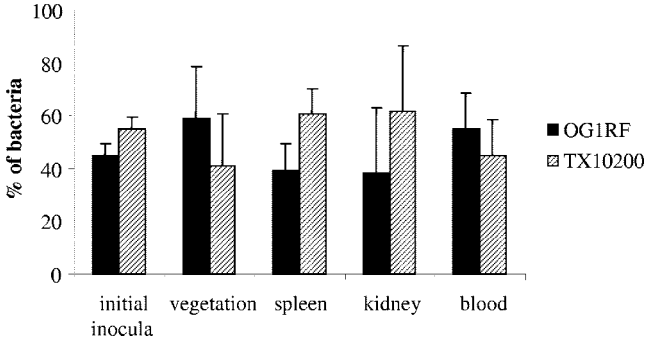

FIG. 2. Percentages of OG1RF and the *glsB* mutant (TX10200) in the total population of bacteria recovered from tissue samples and blood and in the initial inocula. Two different inocula were used and bacteria were recovered from animals 72 h after inoculation. Results from six rats were combined, and the mean percentages and standard deviations are shown.

hypothesis is supported by the observations that *gls24* mRNA in *E. faecalis* grown in serum and urine is increased relative to that in 2× YT medium (9), that *gls24* is important for resistance to bile salts and for *E. faecalis* virulence in both a mouse peritonitis and endocarditis models, and that antibodies against Gls24 were shown to provide protective effects in the same mouse model (11).

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