INFECTION AND IMMUNITY, May 2007, p. 2634–2637 Vol. 75, No. 5
0019-9567/07/$08.00+0 doi:10.1128/IAI.01372-06

Enterococcus faecalis Mutations Affecting Virulence in the *Caenorhabditis elegans* Model Host[▽]

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Received 25 August 2006/Returned for modification 21 September 2006/Accepted 7 February 2007

Enterococcus faecalis **transposon insertion mutants were screened for attenuated killing of the nematode model host *Caenorhabditis elegans*. The genes disrupted in the attenuated mutants encode a variety of factors including transcriptional regulators, transporters, and damage control and repair systems. Five of nine mutants tested were attenuated in a mouse peritonitis model.**

Enterococcus faecalis has emerged over the past few decades as one of the leading causes of hospital-acquired infection, causing diseases such as endocarditis, urinary tract infections, and bloodstream infections (14). *E. faecalis*'s natural ruggedness, which causes intrinsic resistance to many antibiotics, and its versatility in swapping genetic information to gain additional resistance, have played large roles in its advance (12). Several *E. faecalis* virulence factors have been identified by their distinct biochemical properties, by their antigenic phenotypes, or by homology searches using known virulence factors from other bacteria (8). Surprisingly, given the importance of this pathogen, no in vitro or in vivo screens have been performed to isolate new virulence determinants in *E. faecalis* in an unbiased manner. We previously demonstrated that *Caenorhabditis elegans* could be used as a model host to identify potential mammalian virulence determinants (6). Additionally, we built an ordered library of transposon insertion mutants of *E. faecalis* strain OG1RF with approximately 25% of the nonessential genes disrupted (7). In this work, we identified 23 insertion mutants in the ordered library with attenuated killing of *C. elegans*. Five of nine mutants tested were also less virulent in a mouse peritonitis model.

To identify strains of *E. faecalis* mutants from our ordered library of 540 mutants that were deficient in *C. elegans* killing, the following strategy was used. Plates containing a bacterial lawn of each transposon mutant were generated by using growth conditions previously found optimal for killing of *C. elegans* by *E. faecalis* (6). Approximately 30 to 40 worms, strain N2 (the wild type), were placed on each mutant, and survival over time was assayed for 7 days at 24-h intervals. With GraphPad Prism 3.0 or STATA 6.0, survival was plotted by the Kaplan-Meier method and differences between the mutant and parent strain were compared by using the log-rank test. Seventy-two mutants with a difference resulting in a P value of 0.1 or less were tested in a second assay with a larger population of *C. elegans* ($n = 60$ to 80). P values of less than 0.05 were considered statistically significant. Mutants that caused significant attenuation by this criterion in both experiments are listed in Table 1, and an example of a typical killing assay is presented in Fig. 1A. Because mutants with a growth defect could cause a reduction in killing in a nonspecific manner, log-phase growth in liquid brain heart infusion (BHI) medium of all of the mutants was compared to that of the parent strain. All mutants displayed growth similar to that of the wild type, except for mutant 4H12 (Fig. 1B and Table 1). We also assayed growth in 50% serum from human volunteers as described previously (5), with and without heat inactivation. Growth in serum may more closely parallel conditions found in the mammalian host environment, and the comparison with heat-inactivated serum addresses whether or not there is increased sensitivity to complement. None of the mutants displayed a growth defect under either condition, including 4H12 (data not shown). Perhaps the deficiency of 4H12 in BHI is specific to conditions that result in a short doubling time.

The mutants found in the screen were classified by the probable function of the protein encoded by the disrupted gene according to the annotation provided by the V583 genome sequence (19). We subjected the sequences to BLAST analysis and researched the literature for additional information on possible functions. Several putative transcriptional regulators were identified. EF_1302, for example, resembles a *lysR* helix-turn-helix transcriptional regulator involved in virulence and stress response in other bacteria such as *Pseudomonas aeruginosa* (21). EF_1569 encodes Psr, which belongs to a family of negative transcriptional regulators that control cell surface properties such as cell wall and exopolysaccharide composition and synthesis (15). Such a mutant could be affected in adhesion or biofilm formation, properties that affect the infectivity of *E. faecalis*. We also identified EF_1604, which encodes the transcriptional repressor ScrR. In previous work (6) (as well as this study), we found that a mutation in *scrB*, a gene likely regulated by ScrR and encoding sucrose-6-hydrolase, caused attenuation in *C. elegans* killing. Sucrose utilization plays an important role in biofilm formation and pathogenicity in *S. mutans* during caries formation (16) and endocarditis (17), as it is a substrate for the synthesis of the extracellular polymers glucan and fructan (9). We also identified FsrB in

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▽ Published ahead of print on 16 February 2007.

TABLE 1. Attenuated *E. faecalis* mutants identified by screening with *C. elegans*

Type of mutation and mutant	V583 ORF[a]	Mouse model *P* value[b]	Gene name	Gene product, function
Transcriptional regulator				
4G3	EF_0382	NT	None	Helix-turn-helix, Fis type
1C4	EF_1302	0.13	*lysR*	Helix-turn-helix, *lysR* type
1D1	EF_1569[c]	0.14	*psr*	CpsA/LytR/Psr family of transcriptional regulators
5C11	EF_1604	0.19	*scrR-1*	Negative regulator of sucrose operon
4D4	EF_1821	0.001[e]	*fsrB*	Peptide processor of Fsr two-component system
Transporter				
2A1	EF_0243	NT	*brnQ*	Branched-chain amino acid transporter
4C8	EF_1513	0.31	None	Pheromone binding protein
4G6	EF_2598	NT	None	PEP-PTS[f], beta-glucoside-specific IIABC component
Damage control and repair				
6A3	EF_1545	0.005	*recQ-1*	DNA helicase, SOS response
3G8	EF_1598	0.038	*phrB*	Deoxyribodipyrimidine photolyase, DNA repair
4G7	EF_2591	NT	None	*glxI*, glyoxalase, protective against electrophiles
Catabolism				
4G8	EF_0737	NT	None	Putative amidase
6C9	EF_1603	0.01[e]	*scrB-1*	Sucrose-6-phosphate hydrolase
6E9	EF_1623[c]	NT	*pduJ*	Carboxysome protein, metabolism of ethanolamine
Anabolism				
1C9	EF_1576	NT	*thyA*	Thymidylate synthase, deoxyribonucleotide biosynthesis
4H12	EF_2200[d]	NT	*map*	Methionyl aminopeptidase, protein maturation
Other				
4G10	EF_0376	NT	None	Hypothetical protein
6C10	EF_0861[c]	NT	None	GNAT family acetyltransferase
6B3	EF_1542	NT	None	Conserved hypothetical membrane protein
4F8	EF_1590[c]	0.0003	*paiA*	SSAT acetyltransferase, negative regulator of sporulation
6D5	EF_1792	NT	None	Conserved hypothetical
4G9	EF_2675	NT	None	Putative competence protein, CoiA-like family
6D6	EF_2957	NT	None	Hexapeptide repeat acetyltransferase

[a] ORF, open reading frame.
[b] Mouse peritonitis model (6, 22). *P* values are from representative experiments. NT, not tested.
[c] Regulated by Fsr system.
[d] Growth defect.
[e] Previously found to be attenuated in *C. elegans* and mouse peritonitis models (6, 20).
[f] PEP-PTS, phosphoenolpyruvate-dependent phosphotransferase system.

this screen; the Fsr two-component regulatory system is a major regulator of virulence in *E. faecalis*, and mutation of FsrB attenuates virulence in a variety of infection models, including *C. elegans* (6, 11, 18, 20).

Transporters were found, including a pheromone binding protein possibly involved in quorum sensing and a phosphoenolpyruvate-dependent phosphotransferase system beta-glucoside-specific IIABC component. Genes involved in beta-glucoside metabolism were found to be upregulated in *Streptococcus gordonii* on infected heart valves and contributed to biofilm formation (13).

Some of the mutants with attenuated *C. elegans* killing have insertions in genes encoding enzymes that could be involved in damage control and repair (Table 1). For example, *recQ* (EF_1545) and *phrB* (EF_1598) homologs encode DNA repair enzymes. Loss of such enzymes in other pathogens such as *Salmonella* results in attenuated killing of mice (2) and sensitivity to the oxidative burst in macrophages (3).

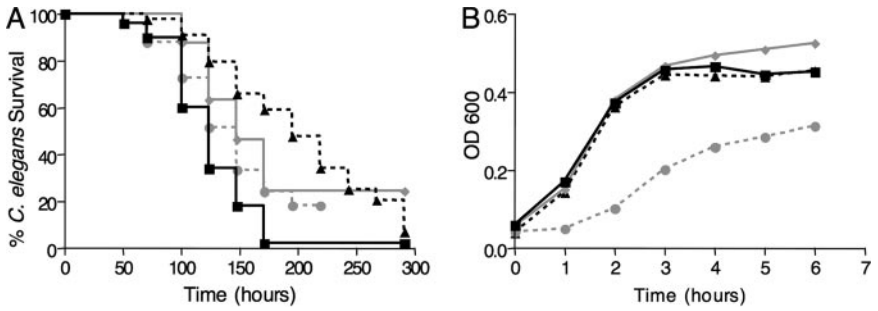

FIG. 1. Example killing assay and growth curve of *E. faecalis* mutants with attenuated *C. elegans* killing. (A) Survival of *C. elegans* on OG1RF (squares) compared to mutants with disruptions in 4D4 (triangles) ($P < 0.0001$), 4G6 (diamonds) ($P < 0.0001$), and 4H12 (circles) ($P < 0.0194$). (B) Growth curves of *E. faecalis* mutants listed in panel A grown in BHI medium. OD 600, optical density at 600 nm.

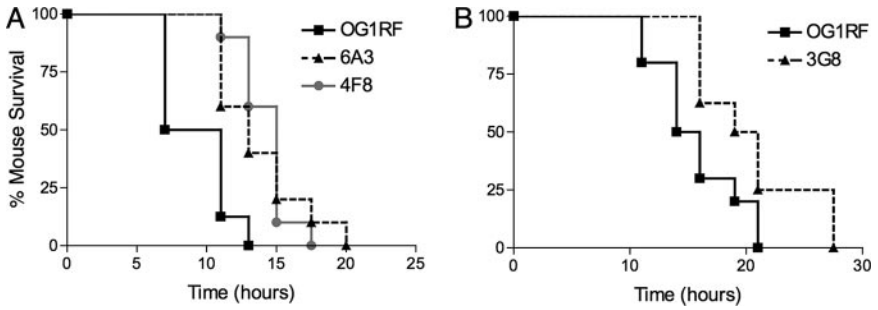

FIG. 2. Survival of mice infected intraperitoneally with *E. faecalis* mutants. Shown are representative experiments with the mutants characterized as attenuated in this model but not previously published (6A3, 4F8, and 3G8). (A) Survival of mice infected with 7.35×10^8 CFU of OG1RF, 7.37×10^8 CFU of 6A3, and 7.41×10^8 CFU of 4F8. (B) Survival of mice infected with 5.55×10^8 CFU of OG1RF and 5.65×10^8 CFU of 3G8.

glxI (EF_2591) has been found to be upregulated in macrophage-engulfed *Salmonella* and may have roles in dealing with oxidative stress (4).

EF_1623 is one of a cluster of genes that are orthologs of the *eut-pdu* operons in *Salmonella*. The *E. faecalis* operon appears to be involved in the use of ethanolamine, a readily available lipid component, as a carbon and nitrogen source. This gene cluster was previously identified as being strongly regulated by the Fsr system (1), and some of the components have been found to be upregulated in *Salmonella* engulfed by macrophages (10).

To determine if any of the newly identified mutants caused loss of infectivity in a mammalian model, seven were tested in a mouse peritonitis model. Mice were inoculated intraperitoneally with *E. faecalis* as previously described (6, 22). The significance of differences in survival time compared to that of the wild type was evaluated in the same manner as described for *C. elegans*. The experiment was repeated twice and carried out under approved protocols. Three mutants, 6A3, 3G8, and 4F8, were attenuated ($P < 0.05$) (Fig. 2 and Table 1). Additionally, two of the mutants identified, 6C9 and 4D4, had insertions in genes characterized as causing attenuation in the mouse model in previous work (6, 20). It is conceivable that use of a different animal model, such as a model of endocarditis, would identify a different subset of attenuated mutants.

In summary, we identified 23 insertion mutants with attenuated *C. elegans* killing, 2 of which were previously known to affect pathogenesis in the worm and the mouse (6, 20). Several orthologs of the genes disrupted by the transposon insertions are known to affect mammalian pathogenesis in other bacterial species, and more than half of those tested were attenuated in a mouse peritonitis model. In conclusion, *C. elegans* is an efficient way to identify potential virulence determinants and screening a complete ordered library would likely uncover additional factors.

We thank Frederick M. Ausubel and Stephen B. Calderwood for early support of this project and Michael C. Lorenz for critical reading of the manuscript.

This work was supported by Public Health Service grants AI603084 (to E.M.) and AI064470 (to D.A.G.) and New Scholar Awards in Global Infectious Diseases from the Ellison Medical Foundation to E.M. and D.A.G. Initial stages were supported by Public Health Service grant AI064332 to Frederick M. Ausubel.

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Editor: A. Camilli