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Conjugation of Different Immunogenic Enterococcal Vaccine Target Antigens Leads to Extended Strain Coverage

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Enterococci have emerged as important nosocomial pathogens due to their resistance to the most commonly used antibiotics. Alternative treatments or prevention options are aimed at polysaccharides and surface-related proteins that play important roles in pathogenesis. Previously, we have shown that 2 *Enterococcus faecium* proteins, the secreted antigen A and the peptidyl-prolyl cis-trans isomerase, as well as the *Enterococcus faecalis* polysaccharide diheteroglycan, are able to induce opsonic and cross-protective antibodies. Here, we evaluate the use of glycoconjugates consisting of these proteins and an enterococcal polysaccharide to develop a vaccine with broader strain coverage. Diheteroglycan was conjugated to these 2 enterococcal proteins. Rabbit sera raised against these glycoconjugates showed Immunoglobulin G titers against the corresponding conjugate, as well as against the respective protein and carbohydrate antigens. Effective opsonophagocytic killing for the 2 sera was observed against different *E. faecalis* and *E. faecium* strains. Enzyme-linked immunosorbent assays against whole bacterial cells showed immune recognition of 22 enterococcal strains by the sera. Moreover, the sera conferred protection against *E. faecalis* and *E. faecium* strains in a mouse infection model. Our results suggest that these glycoconjugates are promising candidates for vaccine formulations with a broader coverage against these nosocomial pathogens and that the evaluated proteins are potential carrier proteins.

Keywords. Vaccine; glycoconjugate; carrier protein; capsular polysaccharide; diheteroglycan; enterococcal proteins; *Enterococcus faecalis*; *Enterococcus faecium*; opsonophagocytic assay; mouse infection model.

The increasing prevalence of pathogens exhibiting antimicrobial resistance has encouraged the identification of novel vaccine targets [1]. In addition to preventing infections, vaccination can help to reduce the use of broad-spectrum antibiotics by providing protection to individuals at risk [2, 3].

Enterococcus faecalis and *Enterococcus faecium*, respectively, are the third and fourth most commonly isolated nosocomial pathogens worldwide [4, 5]. The increased prevalence of enterococci as nosocomial pathogens has been mainly attributed to their antimicrobial resistances, their ability to acquire virulence factors, and to form biofilms on indwelling devices [6]. Several cell-surface polysaccharides and protein structures have been proposed as potential vaccine candidates to prevent and/or treat enterococcal infections [7]. Among capsular polysaccharides, diheteroglycan (DHG) has been demonstrated to elicit opsonic and protective antibodies against *E. faecalis* and is therefore an attractive immunogenic antigen for vaccine development [8]. DHG, present in *E. faecalis* CPS-C and CPS-D strains, seems to contribute to enterococcal pathogenicity by conferring resistance to opsonophagocytosis and masking antigens from detection by the host's immune system [9]. This highlights the importance of capsular DHG in pathogenic interactions and supports its use as an antigen for vaccine development [10]. For *E. faecium*, several cell-surface–associated protein antigens have been proposed as vaccine candidates [11–13]. Cell wall–associated proteins have been proven to play important roles in adhesion and invasion of the host cells [14]. The secreted antigen A protein (SagA) is well conserved among *E. faecium* strains and has been demonstrated to be associated with biofilm formation, stress response, and adhesion to extracellular matrix proteins [11, 15]. The peptidyl-prolyl cis-trans isomerase protein (PpiC) is involved in β-lactam antibiotic resistance and has been shown to confer resistance to high salt concentrations [16]. In previous studies, we have demonstrated that both SagA and PpiC are able to induce opsonic and cross-protective antibodies that target enterococci [11, 12].

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Owing to the normally T-cell–independent immune response against polysaccharides, conjugation to a carrier protein is necessary to activate T cells to induce an effective immune humoral response [17]. Currently, several licensed glycoconjugate vaccines have shown to be safe and successful in the prevention of infectious diseases [18]. However, the increasing number of conjugate vaccines relying on the same carrier proteins could cause a reduced immune response against polysaccharide antigens, resulting in vaccine interferences [19]. Therefore, in this study we evaluated the use of SagA and PpiC from *E. faecium* as carrier proteins conjugated to DHG from *E. faecalis*, to develop a cross-species vaccine with broad coverage against the 2 clinically most important enterococcal species.

METHODS

Bacterial Strains and Culture Conditions

E. faecalis type 2 was used to evaluate the polysaccharide component of the conjugates. To study the protein component in the conjugate we used the vancomycin-resistant clinical isolate *E. faecium* 11236/1. For cross-reactivity tests, the enterococcal strains used are listed in Figure 1. Strains were grown at 37°C in tryptic soy agar (Carl Roth). For polysaccharide purification *E. faecalis* type 2 was grown in Columbia broth (Becton-Dickinson) with 2% glucose at 37°C until an OD_{600} of 0.8 was reached. For production of recombinant proteins, *Escherichia coli* M15/pQE30 protein-gene strains were cultured under shaking at 37°C in Luria/Miller medium (Carl Roth) supplemented with 100 µg/mL ampicillin and 25 µg/mL kanamycin.

Semisynthesis of Glycoconjugates

Antigens DHG, SagA, and PpiC were produced and purified as described previously (Supplementary Materials) [8, 11, 12]. After the purification procedure, rSagA and rPpiC were used for conjugation at 5 mg/mL. DHG was covalently coupled to the proteins as described by Lees et al, using the cyanylating reagent 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP; Sigma-Aldrich) at 100 mg/mL in acetonitrile [29]. A solution of 1 mg of DHG in 100 µL of ultrapure water was slowly mixed with 10 µL of CDAP. After 30 seconds, 15 µL of 0.2 M trimethylamine was added. Final coupling was done by adding 1 mg of protein to the mixture, and the reaction was incubated overnight at room temperature. The glycoconjugates (DHG-SagA and DHG-PpiC) were cleaned up with an Amicon ultrafiltration device with a 100-kDa membrane (Merck-Millipore). The correct conjugation process was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (Supplementary Materials). Sugar and protein content were determined by hexose and Bradford assays to establish the polysaccharide to protein ratio in the glycoconjugates (Supplementary Materials).

Strain	CPS Serotype(s)	Source (Specimen)	Van Resistant	Opsonophagocytic Assay	ELISA Immunoreactivity, Geometric Mean Absorbance (Immune/Preimmune) ± SD					Reference
					α-DHG-PpiC	α-DHG-SagA	α-DHG	α-Ppic	α-SagA	
E. faecalis										
Type 2	C	Clinical (urine)	No	OPA	60.78 ± 2.23	56.98 ± 3.56	50.18 ± 1.13	1.09 ± 0.02	1.16 ± 0.16	[20]
V583	C	Clinical (blood)	Yes	OPA	3.66 ± 0.18	3.14 ± 0.06	9.09 ± 0.02	1.93 ± 0.04	1.59 ± 0.04	[21]
FA2-2	C	Clinical	No	OPA	3.31 ± 0.03	4.52 ± 0.10	5.24 ± 0.45	2.24 ± 0.03	1.76 ± 0.01	[22]
Type 14	C	Clinical	No	SRPA	3.30 ± 0.051	3.28 ± 0.02	5.23 ± 0.13	2.85 ± 0.26	1.34 ± 0.02	[20]
Type 21	C	Clinical (feces)	No	CMP	9.39 ± 0.09	6.86 ± 0.27	10.37 ± 0.08	1.83 ± 0.04	1.55 ± 0.05	[20]
Type 6	C, D	Clinical	No	CMP	1.64 ± 0.02	1.73 ± 0.10	5.94 ± 0.03	2.09 ± 0.12	1.60 ± 0.07	[20]
Type 5	D	Clinical (urine)	No	OPA	2.26 ± 0.05	1.73 ± 0.03	3.54 ± 0.08	1.19 ± 0.01	1.04 ± 0.02	[20]
Type 18	D	NCTC 8730	No	OPA	3.61 ± 0.35	2.70 ± 0.42	8.55 ± 0.03	1.92 ± 0.02	1.46 ± 0.01	[20]
68114	D	Clinical	No	CS	2.26 ± 0.04	2.40 ± 0.13	5.77 ± 0.06	1.75 ± 0.06	1.72 ± 0.05	[23]
12030	A	Clinical	No	SRPA	3.05 ± 0.18	2.18 ± 0.06	2.03 ± 0.07	2.60 ± 0.03	1.84 ± 0.02	[24]
Type 7	A	Clinical	No	SRPA	2.01 ± 0.11	2.14 ± 0.11	1.87 ± 0.03	2.74 ± 0.11	1.63 ± 0.04	[20]
12107	B	Clinical	No	CMP	4.08 ± 0.11	5.44 ± 0.21	7.15 ± 0.09	2.56 ± 0.14	1.77 ± 0.01	[25]
OG1RF	B	Oral (human)	No	CS	2.63 ± 0.01	4.82 ± 0.14	6.73 ± 0.17	2.11 ± 0.05	1.66 ± 0.02	[26]
E. faecium										
11236/1	NA	Clinical (feces)	Yes	OPA	2.22 ± 0.08	1.93 ± 0.03	1.24 ± 0.06	4.28 ± 0.05	2.80 ± 0.10	This study
1230933	NA	Clinical (blood)	Yes	OPA	2.11 ± 0.12	2.71 ± 0.07	1.13 ± 0.14	2.54 ± 0.03	3.38 ± 0.20	[27]
1231408	NA	Clinical (blood)	No	SRPA	1.99 ± 0.06	2.62 ± 0.07	1.20 ± 0.08	2.25 ± 0.06	2.90 ± 0.053	[27]
1231410	NA	Clinical (SSTI site)	Yes	CMP	2.07 ± 0.03	6.76 ± 0.12	1.16 ± 0.04	3.00 ± 0.03	9.99 ± 0.43	[27]
757875	NA	Clinical	Yes	OPA	1.97 ± 0.01	3.13 ± 0.11	1.18 ± 0.03	1.86 ± 0.06	2.77 ± 0.04	[24]
E1162	NA	Clinical (blood)	No	SN	2.28 ± 0.048	9.43 ± 0.47	1.13 ± 0.09	3.78 ± 0.06	16.04 ± 0.14	[27]
E155	NA	Clinical (feces)	Yes	SRPA	1.92 ± 0.14	3.18 ± 0.10	1.26 ± 0.04	2.66 ± 0.03	5.45 ± 0.17	[28]
E980	NA	Community (feces)	No	CS	1.58 ± 0.06	2.87 ± 0.04	1.17 ± 0.07	2.14 ± 0.01	5.53 ± 0.07	[27]
U0317	NA	Clinical (urine)	No	OPA	2.19 ± 0.10	2.46 ± 0.15	1.36 ± 0.14	1.98 ± 0.03	2.58 ± 0.05	[27]

Ratio: <1.4 | 1.4 ≤X<2 | 2 ≤X<4 | 4 ≤X<8 | 8 ≤X<15 | X>15

Figure 1. Immunoreactivity detected by whole-bacterial-cell enzyme-linked immunosorbent assays (ELISAs) for α-DHG-PpiC, α-DHG-SagA, and unconjugated sera against diverse *Enterococcus faecalis* and *Enterococcus faecium* strains. CMP, complement-mediated phagocytosis in the opsonophagocytic assay; CPS, capsular polysaccharide; CS, susceptible to complement in the opsonophagocytic assay; NA, not applicable; OPA, possible to test by the opsonophagocytic assay; SRPA, susceptible to rabbit preimmune antibodies in the opsonophagocytic assay; SSTI, skin and soft-tissue infection; Van, vancomycin.

Rabbit Immunzations

Rabbit immune sera raised against DHG from *E. faecalis* type 2 (α-DHG), SagA (α-SagA), and PpiC (α-PpiC) have been previously described [8, 11, 12]. For DHG-PpiC and DHG-SagA, New Zealand white rabbits were vaccinated with 2 subcutaneous injections of 10 μg of conjugate in incomplete Freund's adjuvant given 2 weeks apart. An intravenous injection of 5 μg of the conjugate was given 7 days later, followed by 2 more intravenous injections of 5 μg delivered 2 days apart from each other. On day 35, a test serum was collected, and 7 and 14 days later intravenous boosts with 5 μg of antigen were administered. On day 56, a terminal immune serum was collected from each rabbit. All sera were heat inactivated at 56°C for 30 minutes. Terminal immune sera raised against the conjugates were designated α-DHG-PpiC and α-DHG-SagA. The antibodies were purified using an rProtein A GraviTrap column (GE Healthcare) in accordance with the manufacturers' instructions. Immunoglobulin G (IgG) contents were measured by enzyme-linked immunosorbent assays (ELISAs) in purified and unpurified sera (Supplementary Materials).

IgG Titer Measurement

The antigens DHG-SagA, DHG-PpiC, DHG, SagA, and PpiC (prepared at 1 μg/mL in 0.2 M sodium carbonate/bicarbonate buffer) were used to coat Nunc-immuno Maxisorp 96-well plates (Thermo-Fisher Scientific) overnight at 4°C. Wells were washed 3 times with 200 μL of PTB (phosphate-buffered saline [PBS] with 0.05% Tween 20 at pH 7.4). Wells were blocked with 200 μL of blocking buffer (3% bovine serum albumin in PBS) at 37°C for 1 hour and washed 3 times with 200 μL of PTB. Sera were adjusted to 50 μg/mL IgG in blocking buffer, and 2-fold serial dilutions were made in the same buffer. After that, 100 μL serum dilutions were added in triplicate, and plates were incubated for 1 hour. Wells were washed 3 times with 200 μL of PTB. Afterward, 100 μL of alkaline phosphatase–conjugated anti-rabbit IgG produced in goat (Sigma-Aldrich) at 1:1000 were added to each well, and plates were incubated for 1 hour. Finally, wells were washed 4 times with 200 μL of PTB, and 100 μL of the p-nitrophenyl phosphate substrate (Sigma-Aldrich) at 1 mg/mL in glycine buffer were added to each well before incubation for 30 minutes. To stop the reaction, 50 μL/well of 3 M NaOH were added, and the absorbance was measured at 405 nm in an ELISA reader (Synergy H1 Hybrid reader, BioTek). Titers were calculated as follows: for each serum sample, the linear relationship between the OD and the $\log_{10}$[dilution factor] was used to extrapolate the intercept of an absorbance of 0.3 for each test, and this was taken as the ELISA end point titer.

Opsonophagocytic Assay (OPA)

OPAs were performed as previously described [11]. The bacterial strain, complement, white blood cells and rabbit sera were prepared individually prior the assay. The bacterial suspension was diluted in RPMIF (Roswell Park Memorial Institute 1640 medium with 15% fetal bovine serum) to yield a final concentration of 2×10^6 cells/mL. Lyophilized baby rabbit complement (Cedarlane) was dissolved in RPMIF at 6.7%, absorbed for 60 minutes at 4°C with the target bacterial strain, and sterilized by filtration before use. Rabbit sera or purified antibodies were diluted in RPMIF at the indicated concentration. White blood cells were freshly prepared from human blood specimens and adjusted to a final concentration of 2×10^6 cells/mL. For opsonophagocytic inhibition assays, purified antibodies were inhibited with the corresponding DHG-protein conjugate, protein alone, or DHG alone. Concentrations ranging from 0.08 to 200μg/mL of inhibitor were incubated overnight at 4°C with an equal volume of a purified antibody at the indicated concentration. After incubation, the mixture of inhibitor/serum was used as a source of antibodies in the OPA as described above.

Whole-Bacterial-Cell ELISA

Immunoreactivity against the enterococcal strains listed in Figure 1 was measured by whole-cell ELISAs with α-DHG, α-protein, and α-DHG-protein sera. Bacteria were grown on tryptic soy agar plates and incubated overnight at 37°C. Colonies were collected from the plate and inoculated in 50 mL of tryptic soy broth at an OD_{650} of 0.1. Cultures were grown to an OD_{650} of 0.4 and harvested by centrifugation (at 7450 xg and 4°C for 10 minutes). Cell pellets were washed twice with 50 mL of PBS, resuspended in 25 mL of 8% paraformaldehyde (Sigma-Aldrich) in PBS, and incubated at 4°C for 30 minutes under gentle shaking. Then, cells were washed twice with 25 mL of PBS and finally resuspended in 10 mL of 0.2 M sodium carbonate/bicarbonate buffer. Nunc-immuno Maxisorp 96-well plates were coated with 100 μL/well of cell suspension and incubated overnight at 4°C. ELISAs were performed as described above to measure the IgG titer. The immunoreactivity with a specific serum sample was calculated as the ratio of absorbance of the terminal immune serum to the absorbance of the preimmune serum.

Intravenous Mouse Infection Model

The mouse sepsis infection model was performed as described previously with some modifications [13, 30, 31]. Briefly, male BALB/c mice (weight range, 20–25 g; Harlan, Italy) were randomly separated into 4 groups of 6 and intraperitoneally injected 3 times with 200 μL of either normal rabbit serum, α-DHG-PpiC, or α-DHG-SagA 48 hours before, 24 hours before, and 4 hours after the bacterial challenge. Overnight cultures of *E. faecalis* type 2 and vancomycin-resistant *E. faecium* 11236/1 grown in brain heart infusion broth (Sigma-Aldrich) supplemented with 40% heat-inactivated horse serum (Sigma-Aldrich) were centrifuged, and the resulting pellets were resuspended in sterile PBS to achieve final concentrations of 10^9 bacteria/mL. Mice were first anaesthetized with 100 mg/g ketamine (Merial) and 12 mg/g xylazine (Bayer) via intraperitoneal

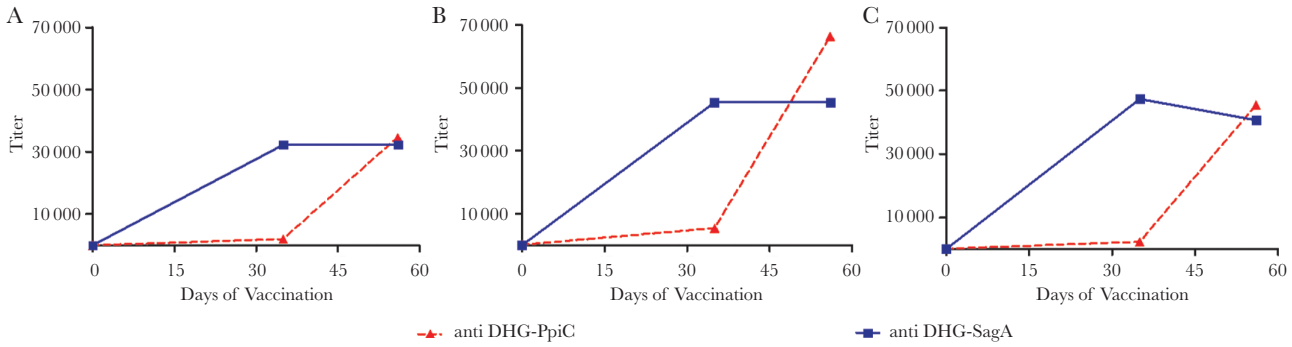

Figure 2. Immunoglobulin G (IgG) titer curves of sera raised against different DHG-protein glycoconjugates. Rabbit sera α-DHG-PpiC (solid line) or α-DHG-SagA (dotted line) were examined during the immunization schedule for specificity toward the native DHG (*A*), the respective carrier proteins SagA and PpiC (*B*), and the respective different DHG-protein glycoconjugates (*C*). IgG titers were measured by enzyme-linked immunosorbent assays, using 1 µg per well of antigen. Rabbit sera were plated in 2-fold serial dilutions, starting at an IgG concentration of 50 µg/mL.

injection. Aliquots of 100 µL from each strain suspension were injected intravenously into the corresponding group of mice. The animals were monitored twice per day before being euthanized by cervical dislocation 48 hours after the bacterial challenge. Kidneys and livers were aseptically removed, weighed, and homogenized in PBS for 120 seconds at high speed in a Stomacher (Pbi International). Serial dilutions were plated onto Enterococcus Selective Agar (Fluka Analytical) to determinate the number of colony-forming units.

Statistical Analysis

For statistical analysis, Prism, version 7.00 (GraphPad), was used. The percentage of opsonophagocytic killing and absorbance detected by whole-cell ELISA was expressed as the geometrical mean and standard error of the mean. For OPAs and opsonophagocytic inhibition assays, statistical significance was determined by the nonparametric Kruskal-Wallis test, followed by the Dunn post hoc test for multiple comparisons. Results of in vivo experiments were subjected to statistical analysis by using 1-way analysis of variance with a Dunnett multiple comparison test. P values of $<.05$ were considered statistically significant.

Ethics Statement

Rabbits were housed, immunized, and had serum samples collected by Biogenes (Berlin, Germany), in accordance with national and international animal welfare regulations. Rabbit immunizations were performed under approval and with assurance from the National Institutes of Health Office of Laboratory Animal Welfare (identifier A5755-01). Mouse experiments were conducted under a protocol approved by the Institutional Animal Use and Care Committee at Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, and authorized by the Italian Ministry of Health (protocol 1F295.37, 11/05/2017; authorization 903/2017-PR, 11/05/2017) according to Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy.

RESULTS

DHG-Protein Conjugate Synthesis

After purifying the conjugates, protein and sugar amounts in the conjugates were determined. The ratios of protein to polysaccharide in the DHG-PpiC and DHG-SagA conjugates were 1:8.7 and 1:7.5, respectively. To verify the conjugation, gels used in SDS-PAGE were either stained with InstantBlue or Stains-All or were blotted into a membrane for Western blot analysis (Supplementary Materials). All glycoconjugates showed broad bands, with staining showing greater molecular weights than those of the unconjugated polysaccharide and protein. Western blot analysis using sera raised against the unconjugated DHG and proteins showed that the conjugates were recognized by these antibodies. This demonstrates that, in the polysaccharide and protein parts of the conjugate, the important immunogenic epitopes remained intact after the conjugation process.

Specific Antibodies Were Generated During Immunization

To determine antibody titers raised against DHG, carrier proteins, or DHG-protein conjugates during immunization, sera collected at different time points of the immunization regimen were analyzed against the unconjugated molecules and glycoconjugates. Figure 2 shows that increasing amounts of IgG antibodies against all evaluated molecules were generated during the immunization procedure. The IgG titer against DHG-PpiC with the terminal immune serum α-DHG-PpiC was 11% higher than the titer against DHG-SagA exhibited by α-DHG-SagA terminal immune serum (Figure 2C). Thereafter, in all subsequent experiments, terminal immune serum α-DHG-SagA was used 1.1 times more concentrated than that from α-DHG-PpiC terminal immune serum, and preimmune serum were used at the same IgG concentration as the terminal immune serum.

Raised Antibodies Mediate Opsonophagocytosis

Antibodies targeting DHG were tested using *E. faecalis* type 2 because this strain was previously demonstrated to possess this antigen and to be targeted by α-DHG opsonic antibodies [8]. Vancomycin-resistant clinical isolate *E. faecium* 11236/1 was used as target strain for anti-protein antibodies, since most *E. faecium* strains encode and expresses the 2 immunogenic proteins SagA and PpiC [11, 12]. Percentages of opsonophagocytic killing against *E. faecalis* Type 2 and *E. faecium* 11236/1 elicited by α-DHG-PpiC and α-DHG-SagA were comparable and concentration dependent (Figure 3). Control preimmune sera from α-DHG-protein did not significantly mediate opsonophagocytic killing.

Antibodies Are Specific Against DHG or Protein Antigens

Specificity was evaluated by incubating the antibodies with different amounts of unconjugated molecules. As observed in Figure 4A, opsonophagocytic killing of *E. faecalis* type 2 by α-DHG, α-DHG-PpiC, and α-DHG-SagA sera was inhibited by high amounts of DHG and restored at lower amounts of antigen. For vancomycin-resistant *E. faecium* 11236/1, activities of α-PpiC, α-SagA, α-DHG-PpiC, and α-DHG-SagA sera were inhibited in a dose-dependent manner with increasing amounts of rPpiC or rSagA proteins (Figure 4B).

Antibodies Against Conjugates Are Cross-reactive Against Other *E. faecium* and *E. faecalis* Strains

Cross-reactivity of antibodies targeting DHG was evaluated against *E. faecalis* from serotypes CPS-C and CPS-D, ie *E. faecalis* V583 and *E. faecalis* type 5, respectively, strains that are susceptible to α-DHG sera in OPA (Figure 5A and 5B) [8]. Antibodies targeting proteins in the α-DHG-protein sera were evaluated against *E. faecium* E155 and *E. faecium* 757875 (Figure 5C and 5D), previously reported to be effectively phagocytosed by α-SagA and α-PpiC sera [11, 12]. α-SagA, α-PpiC, and α-DHG-protein mediated opsonophagocytic killing of the 4 enterococcal strains tested, while α-DHG only mediated the opsonophagocytosis of *E. faecalis* strains. Nevertheless, higher opsonophagocytic killing activities against the *E. faecalis* strains were observed for α-DHG-protein sera when compared to α-protein sera. To further examine the cross-reactivity and to determine surface availability of our antigenic determinants in a broader bacterial collection, we performed whole cell ELISAs (Figure 1). α-DHG-protein sera recognized all enterococcal strains tested, whereas α-DHG sera only recognized *E. faecalis* strains. Even though some immunoreactivity with the *E. faecalis* strains was observed for α-protein sera, it was much lower than the one observed with α-DHG-protein sera. As expected, *E. faecium* strains immunoreacted only with α-protein

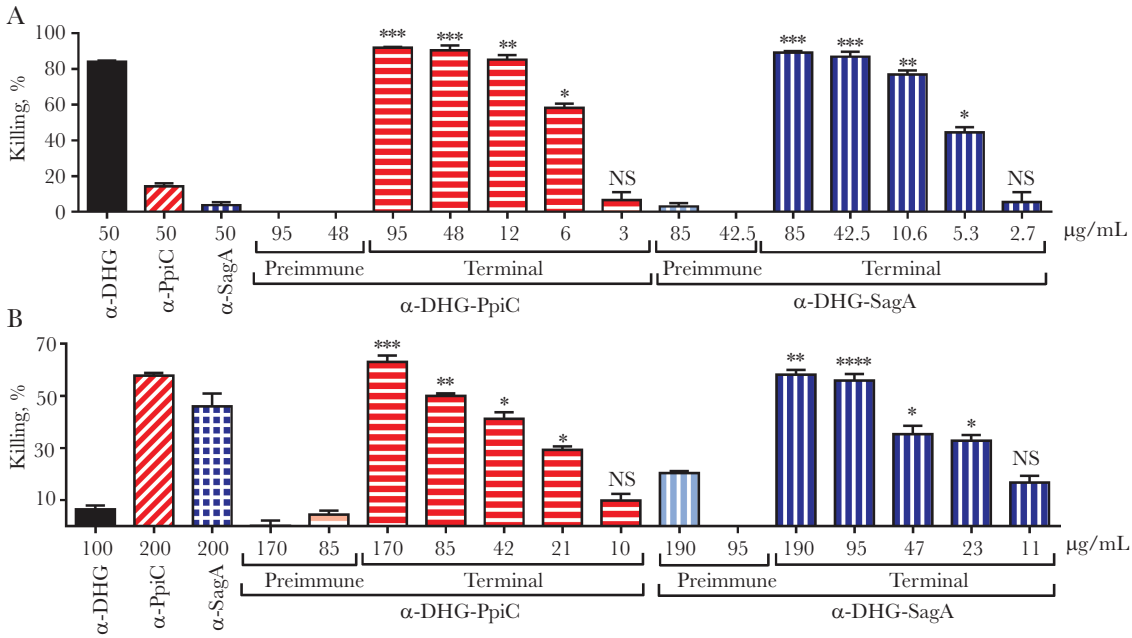

Figure 3. Opsonophagocytic killing activity of anti glycoconjugate rabbit sera against prototype enterococcal strains. *Enterococcus faecalis* type 2 (*A*) and vancomycin-resistant *Enterococcus faecium* 11236/1 (*B*) were tested with antibodies raised against the conjugates DHG-PpiC (horizontal stripes) and DHG-SagA (vertical stripes), used at the same titer concentration ratio. The absolute immunoglobulin G (IgG) concentration of the sera is shown in the *x*-axis. Sera raised against the native DHG (black bar) and the recombinant proteins PpiC (diagonal stripes) and SagA (square grids) were used as controls. The effectiveness of opsonophagocytic killing by the anti-conjugate rabbit sera obtained from terminal immune sera (terminal) was compared to that by the preimmune rabbit sera (preimmune; lighter color). Comparisons of preimmune and terminal immune sera at the same concentration were made by the nonparametric Kruskal-Wallis test, followed by the Dunn multiple comparisons post hoc test. Bars and whiskers denote mean values ± standard errors of the mean. NS, not significant ($P \geq .05$). *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$.

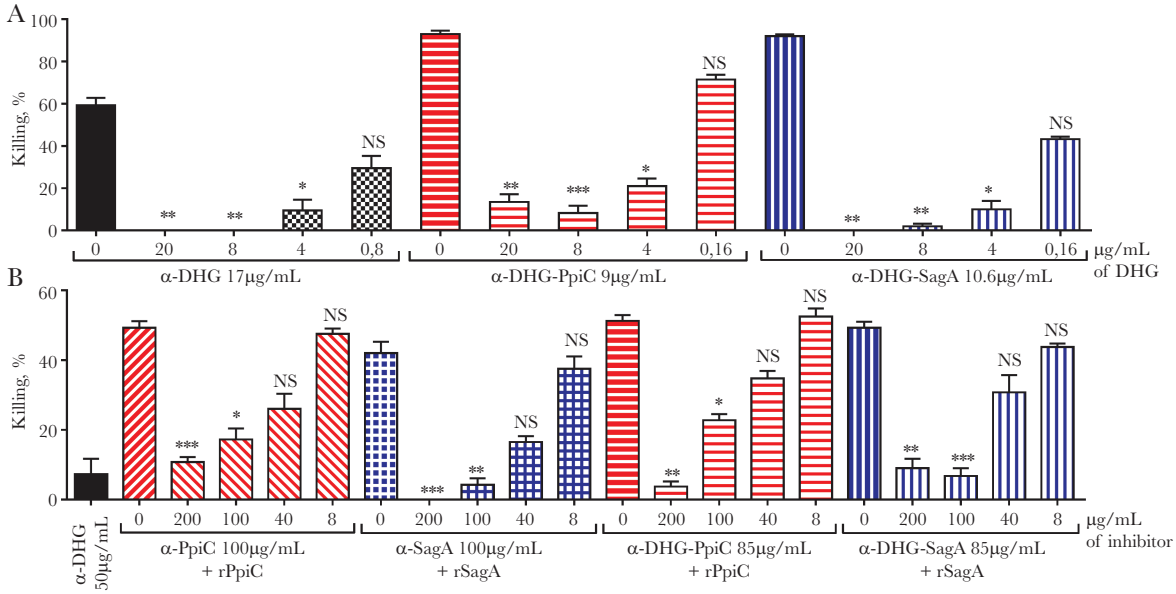

Figure 4. Inhibition of opsonophagocytic killing activity of α-DHG-protein rabbit sera by polysaccharide and protein components. The purified antibodies against the conjugates DHG-PpiC (horizontal stripes) and DHG-SagA (vertical stripes) were preincubated with different inhibitors. *A*, The different α-DHG-protein antibodies at a concentration yielding opsonic killing activities between 60% and 90% against *Enterococcus faecalis* type 2 were incubated with different amounts of native DHG. *B*, The antibodies at a concentration yielding opsonic killing activities ranging from 40% to 60% against vancomycin-resistant *Enterococcus faecium* 11236/1 were incubated with different amounts of the corresponding protein. As controls, α-DHG serum (black bars) was incubated with native DHG, whereas α-PpiC serum (diagonal stripes) and α-SagA serum (square grids) were incubated with recombinant PpiC and SagA proteins, respectively. The final inhibitor concentration is shown below the *x*-axis, as well as the sera concentration used for each set of samples. Statistical significance was performed by the nonparametric Kruskal-Wallis test, followed by the Dunn multiple comparisons post hoc test. Bars and whiskers denote mean values ± standard errors of the mean. NS, not significant ($P \geq .05$). *$P < .05$, **$P < .01$, and ***$P < .001$ for comparison of the mean killing by control serum without inhibitor to that of the serum sample incubated with the corresponding inhibitor.

and α-DHG-protein antibodies. For *E. faecalis* strains, some opsonophagocytic killing activity was observed by α-protein, as previously described [11, 12].

Antibodies Against Conjugates Promotes Clearance of Bacteria in Mice

To evaluate the protective efficacy of antibodies raised against the DHG-protein conjugates, we used a mouse infection

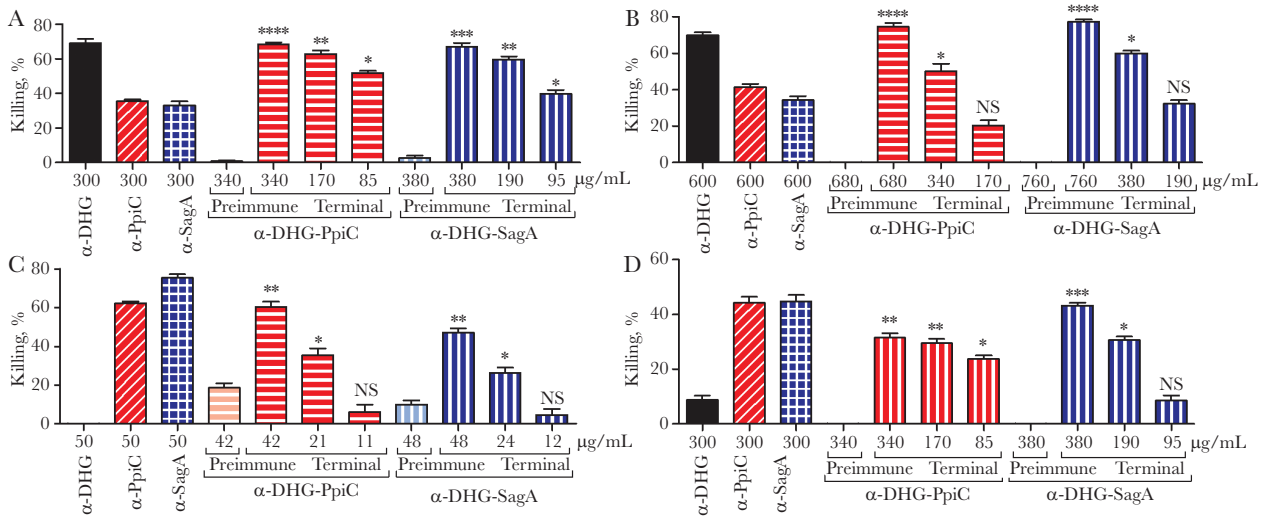

Figure 5. Opsonophagocytic assay of anti-glycoconjugate rabbit sera against different enterococcal strains. The opsonophagocytic activity was tested against *Enterococcus faecalis* V583 (*A*), *E. faecalis* type 5 (*B*), *Enterococcus faecium* E155 (*C*), and *E. faecium* 757875 (*D*). The antibodies raised against DHG-PpiC (horizontal stripes) and DHG-SagA (vertical stripes) were used at the same titer concentration ratio. Serum raised against the native DHG (black bars) and the recombinant proteins PpiC (diagonal stripes) and SagA (square grids) were used as controls. Effective opsonophagocytic killing in the α-DHG-protein rabbit sera from terminal immune sera (terminal) was compared to preimmune rabbit sera (preimmune; lighter color) by the nonparametric Kruskal-Wallis test, followed by the Dunn multiple comparisons post hoc test. Bars and whiskers denote mean values ± standard errors of the mean. NS, not significant ($P \geq .05$). *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$.

model, in which we challenged mice with *E. faecalis* type 2 and vancomycin-resistant *E. faecium* 11236/1. For *E. faecalis* type 2, the protection conferred by the sera raised against the different DHG-protein conjugates was comparable in the livers and kidneys (Figure 6A and 6B) and significantly better than that conferred by control sera (normal rabbit sera). Mice challenged with *E. faecium* 11236/1 were protected in the livers (Figure 6C) when passively vaccinated with the α-DHG-protein sera. However, no protection in mouse kidneys against the *E. faecium* 11236/1 was observed for any of the sera (data not shown).

DISCUSSION

Enterococci are the second most common cause of nosocomial infections [32]. The pathogenicity of enterococci is greatly enhanced by their genetic versatility and ability to acquire antimicrobial resistance and virulence determinants [33]. Immunoprophylactic approaches are of great importance and could provide protection against infections caused by antibiotic-resistant bacteria [3]. Therefore, the development of a vaccine against enterococci would be a valuable approach to fight this opportunistic pathogen [7].

Key points in the development of an antibacterial vaccine have been already pursued in enterococci, such as serotyping and elucidation of carbohydrates responsible of serodiversity. For *E. faecalis,* 4 different serotypes (CPS A–D) have been previously described, and 2 polysaccharides responsible for most of this serodiversity have been proposed as antigens for the development of an enterococcal vaccine (ie, lipoteichoic acid for CPS-A/B and DHG for CPS-C/D) [8, 24]. A study evaluated the genetic diversity of *E. faecalis* strains and showed that half or more CPS-C strains appear to be more virulent than CPS-A/B strains [10]. On the other hand, for *E. faecium* several cell-surface associated proteins have been studied as antigens for vaccine development [11–13]. However, none of them have been evaluated for their dual role as a carrier protein for conjugation with a polysaccharide and as vaccine antigen.

In this work, we semisynthetized 2 enterococcal conjugates consisting of the immunogenic *E. faecalis* polysaccharide DHG and either PpiC or SagA, 2 immunogenic proteins of *E. faecium*. We demonstrated that DHG-PpiC and DHG-SagA not only induced polysaccharide specific antibodies toward DHG but also elicited antibodies against the protein immunogens. The resulting sera, α-DHG-PpiC and α-DHG-SagA, showed good and specific opsonophagocytic killing against both clinically relevant enterococcal species. We observed that the α-DHG-protein sera had cross-reactive opsonophagocytic activities against *E. faecalis* strains expressing the DHG polysaccharide and *E. faecium* strains expressing PpiC and SagA proteins.

Some bacterial strains cannot be evaluated by OPA because they can be susceptible to rabbit preimmune antibodies or to complement alone, while others undergo complement-mediated phagocytosis. To further analyze the sera coverage, we performed whole-cell ELISA with a collection of 13 *E. faecalis* and 9 *E. faecium* strains. Similar immunoreactivities toward *E. faecalis* strains were observed with α-DHG-protein sera when compared to α-DHG sera. For the *E. faecium* strains, sera raised against the conjugates showed reduced immunoreactivities in comparison to α-protein sera, which may be caused by the fact that the concentration was adjusted to the conjugate titers and not to protein titers. Lower immune responses have been also observed for protein carriers, since the conjugation process may affect immunogenic protein epitopes involved in the linkage of protein-polysaccharide molecules [34]. Nevertheless, α-DHG-protein sera had a broader immunoreactivity toward

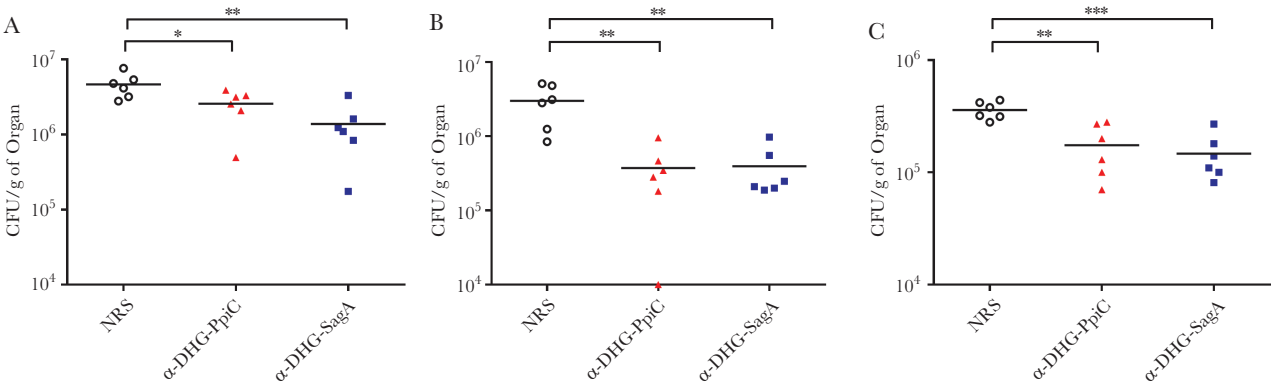

Figure 6. Intravenous mouse infection model. Mice were passively immunized with the sera raised against DHG-PpiC (triangles) and DHG-SagA (squares) conjugates and challenged with *Enterococcus faecalis* type 2 and *Enterococcus faecium* 11236/1. After 48 hours of challenge, mice were euthanized, and their kidneys and livers were removed to assess viable counts. *A* and *B*, Viable counts in mice livers and kidneys challenged with *E. faecalis* type 2, respectively. *C*, Viable counts in mice livers challenged with *E. faecium* 11236/1. Each point represents the bacterial counts from a single mouse. Bars indicate the median number of colony-forming units (CFU) per gram of organ for the group. Statistical analysis was done by 1-way analysis of variance with the Dunnett post hoc test for comparison of the animals immunized with the antibodies raised against the DHG-protein conjugates to the control animals immunized with normal rabbit serum (NRS; circles). Horizontal bars represent geometric means. *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$.

the enterococcal collection than the sera raised against the unconjugated antigens. Although immunoreactivity does not completely correlate with phagocytic killing, whole-cell ELISA can be used to determine whether the antigen/pathogen is recognized by antibodies [35].

There is renewed interest in studying the dual role (carrier/protective antigen) of protein moieties in glycoconjugate vaccines to reduce vaccine formulation complexity, increase vaccine coverage, and simplify vaccination schedules [34, 36]. Dual-role carrier proteins have been studied in several bacterial pathogens, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Clostridium difficile*, *Neisseria meningitidis*, group B *Streptococcus*, *Salmonella enterica*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [37–45]. In most of these studies, protein and polysaccharide from the same pathogen have been conjugated to increase either the protection and/or coverage against same bacterial species [37–45]. However, most of these studies have not assessed the potential cross-reactive coverage that this kind of glycoconjugates may have, since they evaluate 1–2 strains for each part of the glycoconjugate but no larger strain collections. Cross-immunoreactivity was assessed only for *N. meningitidis*, *K. pneumoniae*, and *P. aeruginosa*, for 12, 11, and 3 different bacterial strains, respectively [41, 45]. Only 1 study has conjugated 2 antigens from different bacterial pathogens (ie, O-antigen from *K. pneumoniae* and flagellin proteins from *P. aeruginosa*) to increase the coverage of the potential vaccine against gram-negative bacterial infections [45]. In our work, we conjugated antigens from the 2 most clinically relevant enterococcal species and immunized rabbits with the resulting glycoconjugates. The sera were evaluated by in vitro assays against 6 strains, whereas their cross-immunorecognition was tested against 23 enterococcal strains. Also, an in vivo infection model showed that the α-DHG-protein sera promoted clearance of bacteria in mice livers (*E. faecalis* type 2 and *E. faecium* 11236/1) and kidneys (*E. faecalis* type 2), comparable to previous experiments with unconjugated antigens [8, 12, 13]. Nevertheless, further studies should assess whether these conjugates are more potent than the single antigens in the same animal models or if they offer protection in others (ie, rat endocarditis or urinary tract infection). Additionally, different conjugation strategies between DHG, SagA, and PpiC (ie, selective conjugation and noncovalent association), as well as the physicochemical and immunological characterization of our carrier proteins, should be used to improve the immunogenicity of the glycoconjugates and to ensure their controlled production [34].

In summary, the glycoconjugates evaluated here are potential vaccine candidates that offer a broad coverage against infections caused by the 2 most clinically relevant enterococcal species. Furthermore, the SagA and PpiC proteins are promising immunogenic carrier proteins that could be used either conjugated to DHG or to other polysaccharides to develop a multivalent vaccine against enterococci.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. F. R.-S., D. L., C. M., R. T., E. K., and D. M.-M. performed the experiments. F. R.-S., D. L., C. M., R. T., M. S., and J. H. analyzed the results. F. R.-S., D. L., and J. H. wrote the manuscript. All authors read and approved the final manuscript.

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