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Naturally Acquired Antibodies against Four *Enterococcus faecalis* Capsular Polysaccharides in Healthy Human Sera

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Healthy human sera (HHS) contain naturally acquired enterococcal antibodies which promote neutrophil-mediated killing. The target antigens remain unknown. The present study used a capsular polysaccharide (CPS)–enzyme-linked immunosorbent assay (ELISA) to investigate whether the HHS antibodies of 12 healthy donors bound to the CPS of four *E. faecalis* serotypes (CPS-A to CPS-D) and then employed an opsonic-killing assay to determine if these antibodies mediated phagocyte-dependent killing. All HHS contained immunoglobulin G (IgG) and IgM antibodies directed against capsular polysaccharides of the four serotypes. Absorption of the sera with homologous and heterologous strains showed a majority of antibodies to be cross-reactive among the prototype strains. The susceptibility of the four prototype strains to opsonic killing varied. Opsonic killing of CPS-A and CPS-B strains was significantly higher than killing of CPS-C and CPS-D strains. Absorption studies revealed that the opsonic killing of HHS was only partially type specific, with cross-reactivity between CPS-A and CPS-B strains and between CPS-C and CPS-D strains. These data indicate that healthy individuals possess opsonic antibodies specific for CPS-A and CPS-B but only low titers of opsonic antibodies against CPS-C and CPS-D. Titers of opsonic antibodies did not correlate with antibody titers measured by ELISA. Whether this lack of correlation is due to the low frequency of opsonic antibodies or to increased resistance to the opsonophagocytic killing of some serotypes remains to be determined.

Enterococci are commonly found in the intestinal tracts of humans (up to 10^8 CFU/g stool) and animals (12, 19). In smaller numbers, they are also detectable in the human genital tract and oral cavity. Considered facultative pathogens, enterococci cause a variety of infections (e.g., urinary tract, intra-abdominal, pelvic, and soft tissue infections; bacteremia; and endocarditis) (17). In immunocompromised patients, infections with enterococci can be life-threatening (16). Due to the increasing resistance of enterococci to virtually all clinically available antibiotics, new treatment options are urgently needed (17).

In previous experiments, we and others have found antibodies directed against enterococcal antigens in healthy volunteers and in nonimmunized laboratory animals (1, 4, 5, 9). Enterococcus-specific antibodies potentially enhance the neutrophil-mediated killing of *Enterococcus faecalis* and *Enterococcus faecium* (1, 4, 5). Although the target antigens of these opsonic enterococcus-specific antibodies have not been fully described, it has been speculated that carbohydrate-dependent moieties are involved (2, 23).

In 1999, Huebner et al. identified and structurally characterized a capsular polysaccharide (CPS) in a clinical *E. faecalis* isolate and a vancomycin-resistant *E. faecium* strain. This antigen is a teichoic acid and constitutes the target of opsonic antibodies (9). Antibodies raised against this antigen protected mice in an experimental model of systemic enterococcal infection. This suggests new possibilities for future treatment and prevention (8).

Hufnagel et al. (10) studied the diversity of CPS in a collection of *E. faecalis* strains by using four type-specific immune sera raised to polysaccharide antigens which had been extracted from the cell walls of four *E. faecalis* prototype strains (CPS-A to CPS-D). Of the 29 strains tested, 55% could be assigned to one of four serotypes using a CPS-specific enzyme-linked immunosorbent assay (ELISA). These findings suggested that only a limited number of *E. faecalis* capsular serotypes would need to be incorporated into a broadly active immunotherapeutic agent (10).

The exact nature and function of naturally acquired antibodies against enterococcal antigens in healthy human sera (HHS) have not been extensively investigated. In the present study, we tested HHS from healthy adult volunteers for their ability to bind to enterococcal capsular polysaccharide antigens as well as for their opsonic-killing activity against the four *E. faecalis* prototype strains, representing the serogroups CPS-A to CPS-D (10).

MATERIALS AND METHODS

Bacterial strains and cultures. Four bacterial strains were used in this study. Strain CPS-A (originally named EFS 12030 [9]) and strain CPS-B (originally named EFS 12107 [8]) are clinical isolates from the United States. CPS-A is the only *E. faecalis* strain for which the structure of the capsular polysaccharide has been described (9, 25). CPS-C and CPS-D are part of the collection of 21 prototype *E. faecalis* strains isolated in Japan and have been described by Maekawa et al. (14, 15). These four strains were used as capsular prototype

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strains, and bacteria reactive with sera raised against these strains represent two-thirds of typeable *E. faecalis* isolates described to date (10). All *E. faecalis* strains were grown without agitation at 37°C either in Todd-Hewitt broth (THB; Becton Dickinson, Sparks, MD) or, for the CPS-ELISA, in Columbia broth (CB; Difco Laboratories, Detroit, MI), with the addition of 0.5% glucose.

Sera. Healthy human sera were drawn from healthy adult volunteers and immediately stored at −80°C. For opsonophagocytic assays, HHS was heated at 56°C for 30 min to inactivate the complement.

For the serum absorptions, bacteria were grown in THB to mid-log phase at 37°C. Bacteria were harvested at 12,000 rpm for 10 min (Hettich Micro 20 instrument) and then suspended in HHS (approximately 2×10^9 to 3×10^9 bacteria per ml). The sera were incubated with end-over-end rotation at 4°C for 1 h. After incubation, the absorbed sera were centrifuged at 12,000 rpm for 10 min. The absorption procedure was repeated twice. After the third incubation step, HHS was either used the same day or else stored at −80°C.

Purification of crude polysaccharide extracts for CPS-ELISA. Crude polysaccharide extracts were prepared from all enterococcal strains as described previously, with minor modifications (10). Bacteria were grown overnight at 37°C to stationary phase in 5 ml of CB supplemented with 0.5% glucose. The next day, bacteria were added to 50 ml fresh CB with 0.5% glucose and incubated for 2 h to reach mid-log phase. The culture was centrifuged at room temperature for 10 min at 12,000 rpm. The cell pellet was then dissolved in 500 μl of 10% trichloroacetic acid (TCA) and incubated on a rotor rack at 4°C for 18 to 24 h. The suspension was again centrifuged for 10 min at 12,000 rpm and the supernatant precipitated with 1.25 ml of ethanol and left for 18 to 24 h at −20°C. After centrifugation for 10 min at 12,000 rpm, the supernatant was discarded and the pellet was dissolved in 1 ml of distilled water. The carbohydrate content was measured by a hexose assay (6).

Quantitative determination of capsular polysaccharide-specific antibodies by ELISA. Ninety-six-well microtiter plates (Immulon 2HB; Dynex Technologies, Chantilly, VA) were coated with 1 μg antigen per well in 100 μl of 50 mM phosphate buffer, pH 7.0, and incubated overnight at 4°C. For calculation of an immunoglobulin G (IgG) or IgM standard, the microtiter plates were also coated with either an anti-IgG (γ-chain-specific 1:250 dilution in goat; Sigma Chemicals, St. Louis, MO) or an anti-IgM (μ-chain-specific 1:1,600 dilution) capture antibody. Plates were washed three times with phosphate-buffered saline with 0.025% Tween 20 and blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C. Human sera were applied (unabsorbed or absorbed with the homologous *E. faecalis* strain) in dilutions of 1:100, 1:1,000, and 1:5,000 and incubated for 1 h at 37°C. In a row parallel to the primary antibodies, an IgG or IgM standard was added in different concentrations (from 0 ng/ml to 250 ng/ml). The secondary antibody (i.e., goat anti-human IgG whole molecule or IgM whole molecule, alkaline phosphatase conjugated; Sigma Chemicals, St. Louis, MO) was applied at a dilution of 1:1,000. For the detection of IgG subclasses, mouse anti-human IgG1, IgG2, IgG3, or IgG4 antibodies (ICN Biomedicals, Aurora, OH) were employed. Finally, the detection reagent *p*-nitrophenyl phosphate (1 mg/ml) was added (Sigma Chemicals, St. Louis, MO). Plates were read at 405 nm after 1 h in a Bio-Tek EL 309 ELISA reader (Bio-Tek Instruments, Winooski, VT). Each strain was tested at least in duplicate. Both positive (i.e., immune rabbit sera) and negative controls (i.e., no human sera) were included on all plates.

Resulting titers at an optical density at 405 nm were normalized against the negative controls. Antibody concentrations were calculated by linear regression using the respective IgG and IgM standards. Antibody concentration was classified as either high (>75th percentile), medium (25th to 75th percentile), or low (<25th percentile). Total amounts of human IgG and IgM were accordingly determined using a capture ELISA.

In vitro opsonophagocytosis assay. Human sera were tested for their opsonic-killing activity against the four *E. faecalis* prototype strains. The opsonophagocytic assay was performed as described elsewhere with some modifications (9–11). Polymorphonuclear neutrophils (PMN) were freshly prepared from human blood collected from healthy adult volunteers; then, 20 to 30 ml was mixed with an equal volume of dextran-heparin buffer and incubated at 37°C for 45 min. The upper layer containing the PMN was collected, and remaining erythrocytes were lysed with 1% NH_4Cl buffer and subsequent washing steps with RPMI (Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum. Trypan blue exclusion was used to determine the number of viable cells, and the final PMN count was adjusted to 2×10^7 neutrophils/ml. Serum used as the complement source was obtained from baby rabbits (Zymed, South San Francisco, CA) and absorbed with the homologous *E. faecalis* strain (2×10^7/ml) at 4°C for 1 h before use.

For the opsonophagocytic assay, the following components were mixed: 100 μl of PMN, 100 μl of bacteria (2×10^7/ml, confirmed by viability counts, grown to mid-log phase in THB, PMN/bacteria ratio of 1:1), 100 μl of human serum (1:1,000 dilution) either unabsorbed or absorbed with the homologous *E. faecalis* strain, and 100 μl of baby rabbit serum (absorbed with the homologous *E. faecalis* strain) at a dilution of 1:20 as the complement source. The mixture was then incubated on a rotor rack at 37°C for 90 min, and samples were plated at time zero and after 90 min. Percent killing was calculated by comparing the colony counts in the inoculum (i.e., viable counts at time zero [T_0]) to the colony counts after a 90-min incubation at 37°C (T_{90}), using the following formula: (mean CFU at T_0) − (mean CFU at T_{90})/(mean CFU at T_0) × 100. The killing activity of human sera in combination with complement and PMN was compared to control samples with human sera and complement but without PMN. Values less than zero indicated growth during the incubation period.

Statistical analysis. For statistical analysis, Microsoft Excel and the software program GraphPad PRISM version 3 were used. Correlations were calculated by linear regression. Comparison of antibody concentrations between two groups employed Student's *t* test. Those conducted among three or more groups used analysis of variance, followed by Tukey's multiple-comparison test for pairwise comparisons. For nonparametric analysis of variance, the Kruskal-Wallis test was employed, followed by Dunn's multiple-comparison test for pairwise comparisons. A P value of <0.05 was considered statistically significant.

RESULTS

Human sera contain IgG and IgM antibodies directed against capsular polysaccharides of four *E. faecalis* prototype strains. Using the CPS-ELISA, IgG and IgM antibodies against the CPS of all four *E. faecalis* prototype strains were detected in all 12 human sera (Fig. 1A and 1B). IgG concentrations against the four prototype strains were highly variable, with a 10-fold difference between the lowest (0.89 μg/ml) and the highest (8.9 μg/ml) antibody concentrations. The IgM concentrations showed a similar pattern: a 14-fold difference between the lowest (2.4 μg/ml) and the highest (33.8 μg/ml) antibody concentrations. IgM concentrations were 2 to 3.2 times higher than IgG concentrations. The mean concentrations of IgG or IgM specific to the four prototype antigens showed no statistical difference ($P = 0.273$ for IgG and $P = 0.676$ for IgM). If a serum contained high IgG or IgM concentrations against one prototype strain, it also contained high IgG or IgM concentrations against the other three prototype strains. Similarly, if a serum had low IgG or IgM concentrations against one prototype strain, it also displayed low IgG or IgM concentrations against the other three prototype strains (data not shown). To test for the type specificity of the IgG antibodies, sera absorbed with homologous and heterologous *E. faecalis* strains were used. The resulting titers for all human sera were compared with data from unabsorbed sera, and the percentage of absorption was calculated on this basis. The amount of antibody removed by homologous absorption of the four *E. faecalis* strains was comparable for all human sera (average, 89.0%; 95% confidence interval, 86.5 to 91.4%). Absorptions with the respective heterologous strains also inhibited binding in the CPS-ELISA but did so to a lesser degree (average, 61.8%; 95% confidence interval, 59.2 to 64.4%). These data indicate that IgG antibodies, measured by CPS-ELISA in healthy volunteers, are composed of populations cross-reactive among the four prototype strains, as well as antibodies more narrowly directed at specific epitopes. As seen with the IgG antibodies, binding of IgM antibodies could be inhibited by absorption of the human sera with the homologous *E. faecalis* strain (average, 91.4%; 95% confidence interval, 86.4 to 93.3%). IgM concentrations against the four prototype antigens were not correlated with IgG concentrations

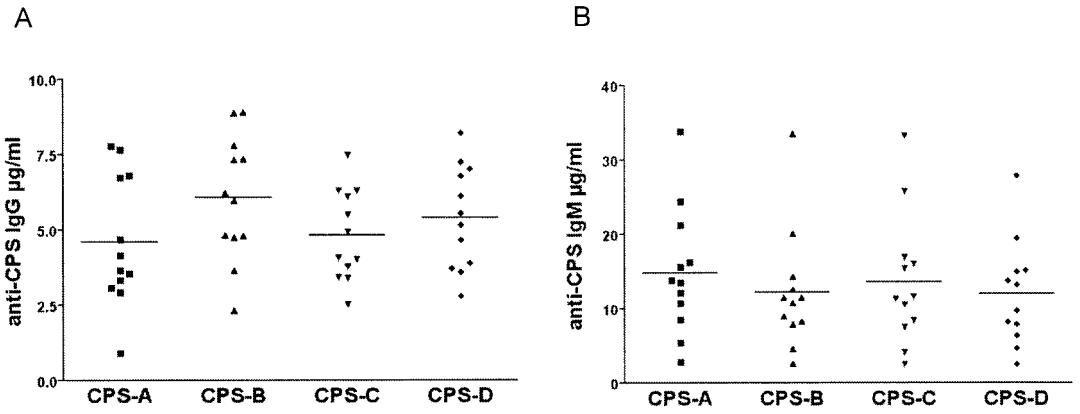

FIG. 1. IgG (A) and IgM (B) concentrations (CPS-ELISA) against the capsular polysaccharides of four *E. faecalis* prototype strains in the sera of 12 healthy humans. Horizontal lines show the average antibody concentrations. The average concentrations for both anti-CPS IgG and anti-CPS IgM among the four capsular serotypes are not statistically different ($P > 0.05$).

(data not shown). Using the CPS-ELISA, IgG1, IgG2, IgG3, and IgG4 subclass antibodies were determined separately and then compared with total IgG antibodies against CPS isolated from all four prototype strains. IgG2 subclass antibodies were more prevalent than other IgG subclasses (data not shown).

Human sera contain opsonic antibodies against all four *E. faecalis* capsular prototypes. The four prototype strains showed variable susceptibility to opsonic killing (Fig. 2). All human sera tested showed good killing against CPS-A and CPS-B strains (>50% killing in a serum dilution of 1:1,000 by five out of six and by four out of six sera against CPS-A and CPS-B, respectively). CPS-C and CPS-D strains were less susceptible to opsonic killing by human sera (>50% killing by one out of six sera against CPS-C, while no serum against CPS-D showed killing of more than 28%). Pairwise comparison of the mean opsonic killing of each of the four prototype strains showed less killing of CPS-C and CPS-D than of CPS-A and CPS-B ($P < 0.01$). No significant differences in the killing activity of the human sera were found between CPS-A versus CPS-B and CPS-C versus CPS-D.

To test for the type specificity of the opsonic killing, all 12 human sera were tested following absorption. Killing activity against any of the four prototype strains present in a given serum was reduced by absorption with the respective homologous strain (Table 1, data shown for a human serum with high CPS-ELISA antibody concentrations).

In addition, the opsonic killing activities of three human sera (one serum with high, one with medium, and one with low IgG or IgM antibody concentrations in the CPS-ELISA) were determined after absorption with their respective homologous strains and compared with absorption with each of the three heterologous strains. Absorption with heterologous strains reduced killing activity less efficiently than absorption with the homologous bacterial strain. A pairwise comparison was performed by examining the killing activity of unabsorbed human sera versus the killing after absorption with the respective homologous strain or absorption with each of the three heterologous prototype strains. Absorption of human sera with either CPS-A ($P < 0.001$ versus that for unabsorbed sera) or, to a lesser degree, CPS-B ($P < 0.05$ versus that for unabsorbed sera) reduced the killing activity of human sera against strain CPS-A. The median killing activities (and standard deviations) of unabsorbed serum against prototype strain CPS-A versus serum absorbed with the three heterologous strains were as follows: for unabsorbed serum, 68.7 (9.4%); for serum absorbed with the heterologous strain CPS-B, 33.6 (11.3%); for serum absorbed with the heterologous strain CPS-C, 56.7 (7.5%); and for serum absorbed with the heterologous strain

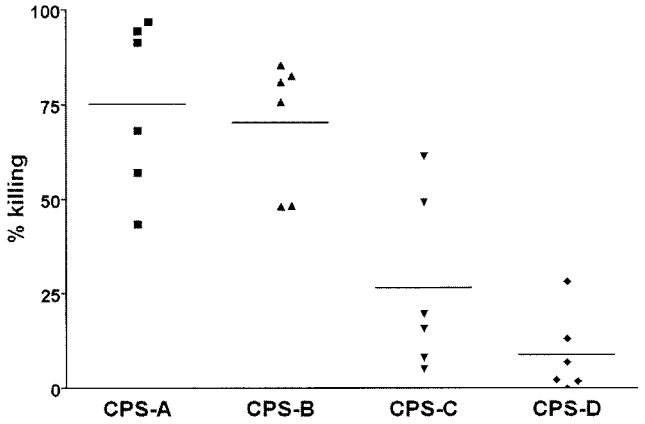

FIG. 2. Opsonophagocytic killing of six representative human sera (serum dilution of 1:1,000) of healthy volunteers against the four *E. faecalis* prototype strains. Horizontal lines show the average percent killing.

TABLE 1. Opsonophagocytic killing activity of a human serum with high CPS-ELISA antibody concentrations against four *E. faecalis* capsular prototype strains

Target strain	Median killing activity (SD [% killing])	
	Unabsorbed serum against target strain	Serum absorbed with the homologous strain against target strain
CPS-A	68.7 (9.4)	5.1 (9.3)
CPS-B	88.1 (1.2)	−47.6 (30.8)
CPS-C	52.0 (1.9)	−366.1 (66.5)
CPS-D	16.9 (2.7)	−115.3 (18.8)

CPS-D, 73.5 (4.5%). Thus, absorption with CPS-C and CPS-D resulted in only a modest reduction of killing activity. A similar pattern was observed for strain CPS-B (data not shown). Opsonophagocytic killing of CPS-C and CPS-D, on the other hand, was reduced by absorption with either of the two strains but only minimally by absorption with CPS-A or CPS-B (data not shown).

The killing activity of human sera showed no statistically significant correlation to their IgG or IgM concentrations (data not shown). High (or low) antibody concentrations in the CPS-ELISA did not predict a high (or low) killing activity in the opsonophagocytic assay.

DISCUSSION

The rapid development and spread of antibiotic resistance among enterococci has spurred research for new treatment options. One promising therapeutic approach is the development of a vaccine based on capsular polysaccharides (8, 9, 13). We and others have shown that healthy human sera contain specific antibodies against enterococcal antigens (1, 4, 5, 9) and that the complement system is an essential part of the innate immune response to enterococci (1, 4). Inactivation of complement, particularly of the alternative pathway, abolishes the killing of enterococci (1, 4, 5). The essential role of the alternative pathway could be demonstrated by the inability of MgEDTA, a compound that inhibits the classical complement pathway, to reduce antibody-mediated opsonic killing (1). Under normal conditions, the addition of antibodies is required to mediate the opsonic killing of encapsulated bacteria (1, 11).

In the present study, we demonstrate that IgG and IgM antibodies in HHS can be found against enterococcal capsular polysaccharide antigens. Carbohydrate-specific antibodies were measured using capsular material, which was extracted with 10% TCA. The TCA extracts contain probably a mixture of carbohydrate antigens with protein levels below the detection limit of a modified Bradford assay (10). We have shown previously that treatment of TCA extracts with sodium periodate reduces the carbohydrate content of the extract and the reactivity of type-specific rabbit antiserum against the extract (10). Using the simplified TCA extraction method to prepare crude polysaccharide antigens from enterococcal strains, we demonstrated that specific antibodies against these antigens are detectable in human and animal sera (10), thus confirming findings by Arduino et al. (1) and Rakita et al. (23). These authors also detected antibodies directed against enterococcal antigens in HHS and in convalescent-phase sera from patients after systemic enterococcal infections. Arduino et al. (2) treated phagocytosis-resistant *E. faecium* with sodium periodate, a substance that destroys carbohydrate moieties by oxidation. In the absence of antibodies, this treatment eliminated resistance to neutrophil-mediated internalization. Rakita et al. (23) absorbed immune rabbit sera with a carbohydrate fraction of phagocytosis-resistant *E. faecium* and thereby reduced the killing activity observed with unabsorbed immune sera.

The capsular polysaccharide-specific antibodies detected in the sera of the 12 human volunteers by the CPS-ELISA measured both type-specific and cross-reactive polysaccharide antigens. Cross-absorption studies showed a significant degree of inhibition of binding in the CPS-ELISA by heterologous strains for all four of the prototype strains studied. The majority of antibodies, however, are likely to be reactive against common cell wall antigens, and only a smaller proportion is directed against a type-specific capsular polysaccharide antigen. A similar phenomenon involving antibody reactivity with common bacterial antigens was discovered for secretory IgA antibodies against *E. faecalis* and other oral streptococci detected in the saliva of infants (3). The type-specific CPS antigen in CPS-A has been shown to be a teichoic acid-like molecule with a glycerol-phosphate backbone and two glucose residues (9, 25). While preparing this teichoic acid from CPS-A for structural analysis, we were able to detect a second CPS antigen. This fraction showed no immunoreactivity with the immune serum raised against the teichoic acid (13). This antigen was composed of a glucose, galactose, rhamnose, *N*-acetylgalactosamine, and phosphate, and it may be identical to the tetraheteroglycan first described by Pazur (20) and another polysaccharide described by Xu et al. (26–28). The preparation method used for the CPS-ELISA in our studies (i.e., extraction of CPS by trichloroacetic acid) is not highly specific for teichoic acids; it may also extract significant amounts of other cell wall polysaccharides. We believe that the antibodies detected in HHS react primarily with common CPS antigens coextracted by TCA. This may explain the reduced type specificity and the observed cross-reactivity in the CPS-ELISA.

The antibody concentrations against enterococcal polysaccharide antigens found in this study are within the same range as the concentrations of natural antibodies against the capsular polysaccharides of other gram-positive bacteria, such as pneumococci (18, 24). The development of antibodies to polysaccharides of many pathogenic bacteria is a well-established phenomenon, even when contact with these pathogens is unknown (7).

The opsonophagocytosis assay is the best in vitro surrogate for a biological function of an antiserum (i.e., opsonic killing [21, 22]). The susceptibility of the four strains to opsonic killing varied considerably. Two strains, CPS-A and CPS-B, were highly susceptible to opsonic killing, whereas strains CPS-C and CPS-D proved more resistant. Absorption studies showed that the killing activity of the sera distinguished between CPS-A and CPS-B versus CPS-C and CPS-D but did not do so within these two groups, indicating that opsonic antibodies induced in healthy individuals are only partially type specific. This finding confirms previously published serological and genetic data with respect to the cross-reactivity of prototype strains (10). The killing activity was not correlated with IgG or IgM concentrations against capsular polysaccharides in the CPS-ELISA. This lack of correlation suggests that the antibodies mediating the opsonic killing make up only a minor part of antibodies measured by the CPS-ELISA.

Our results suggest that environmental exposure is an ineffective mode of induction of opsonic antibodies. By contrast, the majority of antibodies induced after vaccination in animal models (e.g., rabbits) are type specific, regardless of whether they are measured by CPS-ELISA or by opsonophagocytosis assay (10). From our data, it is not clear whether the poor opsonophagocytic killing mediated by HHS against serogroups CPS-C and CPS-D is due to low induction of these antibodies or caused by intrinsic resistance of these serotypes to opsonophagocytic killing. The differences in immune response

after environmental exposure and vaccination may explain why natural enterococcal antibodies are inefficient in preventing disease despite their presence in healthy volunteers. Our results also underscore the need for the development of an immunization that induces type-specific opsonic antibodies like these to be directed against the teichoic acid-like molecule described by Huebner et al. (9) and Wang et al. (25).

From the present study, we conclude that opsonic antibodies induced after environmental exposure are only partially type specific. It appears that the majority of antibodies found in HHS are directed against the capsular polysaccharides shared between serotypes and that these antibodies are neither type specific nor opsonic. These findings may explain why naturally acquired antibodies against enterococci do not protect from infection.

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