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Role of Alginate O Acetylation in Resistance of Mucoid *Pseudomonas aeruginosa* to Opsonic Phagocytosis

GERALD B. PIER,[1]* FADIE COLEMAN,[1] MARTHA GROUT,[1] MICHAEL FRANKLIN,[2] AND DENNIS E. OHMAN[3,4]

Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115-5804[1]; Department of Microbiology, Montana State University, Bozeman, Montana 59717[2]; Department of Microbiology and Immunology, Medical College of Virginia Campus of Virginia Commonwealth University, Richmond, Virginia 23298-0678[3]; and McGuire Veterans Affairs Medical Center, Richmond, Virginia 23249[4]

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Establishment and maintenance of chronic lung infections with mucoid *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF) require that the bacteria avoid host defenses. Elaboration of the extracellular, O-acetylated mucoid exopolysaccharide, or alginate, is a major microbial factor in resistance to immune effectors. Here we show that O acetylation of alginate maximizes the resistance of mucoid *P. aeruginosa* to antibody-independent opsonic killing and is the molecular basis for the resistance of mucoid *P. aeruginosa* to normally nonopsonic but alginate-specific antibodies found in normal human sera and sera of infected CF patients. O acetylation of alginate appears to be critical for *P. aeruginosa* resistance to host immune effectors in CF patients.

The predominant bacterial pathogen in chronic pulmonary infection in cystic fibrosis (CF) patients is the mucoid variant of *Pseudomonas aeruginosa*, which is encapsulated by and overproduces mucoid exopolysaccharide (MEP), or alginate. That alginate is the major virulence factor of *P. aeruginosa* in CF lung infection is evident from the epidemiology of this disease. The pulmonary function of patients with CF declines only when mucoid *P. aeruginosa* is isolated and associated lung pathology develops (9, 32, 33). The growth of mucoid *P. aeruginosa* as a biofilm in the lungs of CF patients has been suggested to be a major factor in long-term bacterium survival. Biofilm formation by *P. aeruginosa* has been linked to genes involved in quorum sensing (7) and motility (31), with a recent demonstration that the acyl-homoserine lactone molecules involved in the quorum-sensing system (8) can be detected in the sputa of CF patients (42). However, the genes controlling alginate production appear to be independent of control by the known quorum-sensing genes of *P. aeruginosa*, including *lasR* and *rhlR* (8, 44, 45). Therefore, the question of whether there is a regulator or environmental cue common to both alginate production and quorum-sensing systems has not yet been answered.

The conversion of *P. aeruginosa* to the mucoid state in CF patients is often associated with mutations at the *mucA* locus (23). MucA and MucB (also called AlgN) act as anti-sigma factors for the alternative sigma factor σE (47), encoded by *algT* (25), also known as *algU* (22). Increased activity of this sigma factor results in hyperexpression of the alginate biosynthetic operon located at 34 min on the *P. aeruginosa* genome (25). Conversion of *P. aeruginosa* to the mucoid state is often associated with the loss of production of the lipopolysaccharide (LPS) O side chains that normally render strains serum resistant (18, 30, 34).

MEP/alginate is a high-molecular-weight polysaccharide of β1–4-linked residues of mannuronic and guluronic acids (40, 41). The ratio of mannuronic acid to guluronic acid varies from strain to strain, on the order of 10:1 to 1:1 (40, 41). Acetylation occurs on the C-2 and C-3 hydroxyl groups of the mannuronic acid residues. The products of *algI*, *algJ*, and *algF*, located on the alginate biosynthetic operon, are required for the O acetylation of alginate (14, 15). Much research has been published on the biosynthesis of alginate (5, 6, 26, 46, 48) as well as on the control of synthesis by both genetic (2–4, 11, 13, 29) and environmental (10–12, 24, 25) factors. Despite this wealth of information, the exact molecular mechanisms by which alginate promotes the survival of bacteria in the lungs of otherwise immunocompetent hosts for years to decades have not been fully elucidated.

Defining the molecular properties of alginate that mediate the resistance of mucoid *P. aeruginosa* to host immune effectors is key to understanding the role of this material in pathogenesis. A property of MEP/alginate previously reported to be involved in the inability of CF patients to clear mucoid *P. aeruginosa* from their lungs is its elicitation during chronic infection of specific antibodies that fail to mediate the opsonic killing of mucoid *P. aeruginosa* growing either in suspension (32, 38) or in biofilms (27). Another characteristic of MEP/alginate that may confer bacterial resistance to host phagocytes and complement, particularly in the presence of the loss of production of the LPS O side chains that normally render strains serum resistant (18, 30, 34), is the presence of acetate substituents. Acetate residues are bound via ester linkages to hydroxyl groups that, when unsubstituted, can serve as acceptors for covalent linkage of the complement opsonins C3b and C4b to the bacterial surface (19). In addition, the presence of acetate residues may affect the activation of complement in an

* Corresponding author. Mailing address: Channing Laboratory, 181 Longwood Ave., Boston, MA 02115-5804. Phone: (617) 525 2269. Fax: (617) 731-1541. E-mail: gpier@channing.harvard.edu.

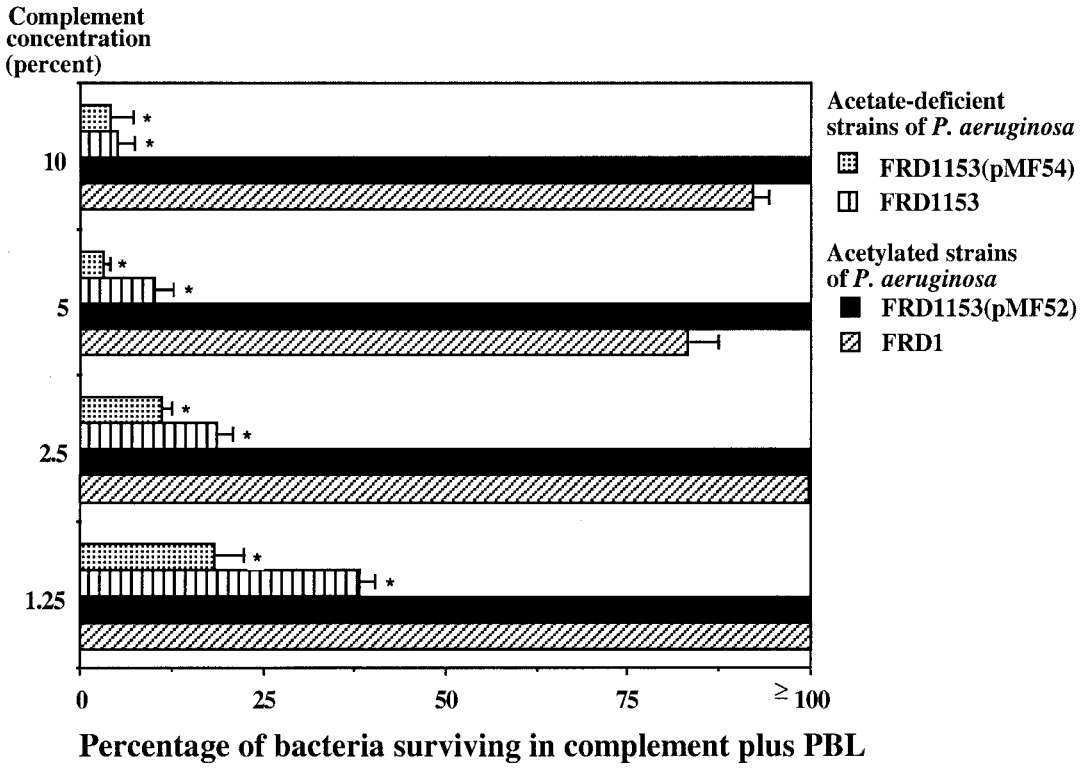

FIG. 1. Susceptibility of variously acetylated mucoid *P. aeruginosa* strains to opsonic killing by human peripheral blood leukocytes (PBL) and complement at the indicated concentrations. Strain FRD1153 is an *algJ* mutant strain, unable to O acetylate alginate. Strain FRD1 is the parental strain of FRD1153. Plasmid pMF52 contains the *algI*, *algJ*, and *algF* genes under the control of the P*trc* promoter and restores the O-acetylation phenotype to FRD1153. Plasmid pMF54 is the cloning vector. Bars represent the mean CFU surviving, and error bars indicate the standard deviation. Asterisks indicate that at all complement concentrations tested, the percentage of bacteria surviving was significantly lower in both of the *O*-acetyl-deficient strains than in the *O*-acetyl-sufficient strains ($P < 0.001$, as determined by ANOVA and Fisher's PLSD test for pairwise comparisons).

antibody-independent fashion. Thus, by linking acetate to hydroxyl groups, mucoid *P. aeruginosa* may be able to escape phagocytic killing by dampening the activation of complement. We therefore evaluated the susceptibility of *P. aeruginosa* FRD1153 (14, 15), an *algJ* mutant derived from mucoid *P. aeruginosa* strain FRD1, to opsonic killing by antibody-free human complement and by human complement with added MEP-specific opsonic and nonopsonic antibodies. These studies were performed to define further the role of acetate substituents in the long-term persistence of mucoid *P. aeruginosa* in the lungs of CF patients.

Comparative susceptibilities of strains to opsonic killing mediated by complement and leukocytes only. We initially assessed whether two components of the innate immune system—phagocytes and complement—could mediate the opsonic killing of parental, O-acetylation-deficient, and *trans*-complemented mucoid *P. aeruginosa* strains in the absence of antibody by using a well-established opsonophagocytic assay (1). The strains used were mucoid *P. aeruginosa* FRD1, a clinical isolate that has been extensively studied (2, 4, 5, 17, 29); mucoid *P. aeruginosa* FRD1153, which contains a point mutation generated in *algJ* as described previously (14, 15) and which produces only 7% of the parental level of O acetylation on alginate; strain FRD1153 complemented with plasmid pMF52 (15), which contains the *algI*, *algJ*, and *algF* genes under the control of the P*trc* promoter and which provides full restoration of parental levels of alginate acetylation in strain FRD1153; and strain FRD1153 complemented with plasmid pMF54, the vector control (15).

Strains with plasmid pMF52 or pMF54 were routinely cultured in Trypticase soy broth or on Trypticase soy agar plates containing 300 μg of carbenicillin/ml. Human serum was used as a source of complement; the serum was diluted 1:10 in RPMI medium–15% fetal calf serum and adsorbed twice with 1 to 2 mg of lyophilized *P. aeruginosa* strain FRD1 cells at 4°C for 30 min to remove specific antibody. Bacterial cells were removed by centrifugation, and the serum was filter sterilized and then diluted further for studies involving different concentrations of complement.

As shown in Fig. 1, 80 to 100% of cells of the parental strain, FRD1, and the fully complemented strain, FRD1153(pMF52), survived when up to 100 μl of a 10% concentration of adsorbed normal human serum was added to an opsonic killing assay with a final volume of 400 μl. Higher concentrations of human serum could not be used because mucoid strains from CF patients produce rough LPS (18), rendering the organisms sensitive to killing by complement alone at higher serum concentrations (35). In contrast, a maximum of 37% of cells of acetate-deficient strains FRD1153 and FRD1153(pMF54) survived when 100 μl of a 1.25% concentration of adsorbed nor-

mal human serum was added to the assay (Fig. 1). The survival of FRD1153 was reduced at higher concentrations of serum, with less than 10% survival at a 10% serum concentration.

Complementation in *trans* of FRD1153 with *algJ* from plasmid pMF52 restored the parental level of resistance to complement-mediated opsonic killing, whereas FRD1153 with the vector control, pMF54, was susceptible to complement-mediated opsonic killing. At all concentrations of human complement tested, the rate of survival of the strains producing acetylated MEP/alginate was significantly greater than that of strains with deficient levels of acetylation of MEP/alginate ($P < 0.001$, as determined by analysis of variance [ANOVA] and Fisher's probable least-significant-difference [PLSD] test). The data shown in Fig. 1 were reproduced four additional times with different adsorbed normal human sera as sources of complement (data not shown), with essentially identical results.

The above results indicate that acetylation of MEP/alginate is likely essential to the development of resistance of mucoid *P. aeruginosa* to basic host defenses. The very low level of complement that effectively opsonized the acetylation-deficient derivatives would make such strains prone to elimination by host defenses in the lungs. However, it is difficult to know what the actual level of complement activity is in CF lungs. Since most mucoid *P. aeruginosa* cells isolated from the lungs of CF patients produce rough LPS (35) and are susceptible to bactericidal killing at serum concentrations of ≥10%, it can be assumed that the levels of complement needed in chronically infected CF lungs to form the membrane attack complex capable of killing *P. aeruginosa* cells are <10% the levels in serum. It is not known whether the observed phagocytic killing of strains deficient in acetylation of alginate in vitro with complement concentrations as low as 1.25% is indicative of an inability of such strains to survive in CF lungs. Nonetheless, the ability of very small amounts of human serum to mediate opsonic killing of nonacetylated mucoid *P. aeruginosa* suggests that acetylation of MEP may be critical for bacterial resistance to host defenses during chronic lung infection in CF.

Effect of acetate substituents on antibody-independent complement activation. To determine the effect of acetate substituents on complement activation at serum concentrations lower than 10% that mediate phagocyte-dependent opsonic killing of the nonacetylated mutant, we compared the consumption of the activity of the alternative pathway of complement by the *P. aeruginosa* acetylase-deficient strains and by strains with wild-type levels of acetate. This goal was accomplished by dilution of human sera 1:10 in Veronal-buffered saline, adsorption as described above with lyophilized cells of *P. aeruginosa* strain FRD1 to remove specific antibody, and incubation with 10^7 CFU of the various strains for 30 min at 37°C. Bacteria were removed by centrifugation, and 10^8 rabbit red blood cells were added to the residual sera. After 30 min at 37°C, the samples were centrifuged to remove intact red blood cells, 100 μl of the supernatant was added to 96-well plates, and the amount of hemoglobin released into the supernatant was measured at 405 nm. Controls included serum samples treated with zymosan to consume all of the alternative pathway components and samples with no bacteria, whose hemoglobin release value represented 100% of the complement activity. The percentage of residual activity of the alternative pathway of complement left after incubation with each strain was calculated as follows: 100 × (optical density at 405 nm of test sample/optical density at 405 nm of sample showing 100% lysis of red blood cells). At a concentration of adsorbed, intact human serum of 6.25%, 76% of the alternative pathway activity remained after incubation with O-acetylated mucoid *P. aeruginosa* strain FRD1 (Fig. 2). In contrast, the poorly O-acetylated strain, FRD1153, consumed essentially all of the alternative pathway activity at this serum concentration; 96% of the alternative pathway activity remained when serum was incubated with FRD1153 containing *algJ* in *trans*, but 100% of the activity was consumed by incubation with strain FRD1153 containing only the vector control. These results are indicative of a role for the acetate substituents in abrogating the activation of the alternative pathway, an event that could lead to deposition of opsonic fragments of C3 and C4 and phagocytic killing. Therefore, at the molecular level, it appears that acetylation of alginate, particularly when it is expressed on a rough-LPS *P. aeruginosa* strain, dampens complement activation, leading to resistance to antibody-independent phagocytic killing.

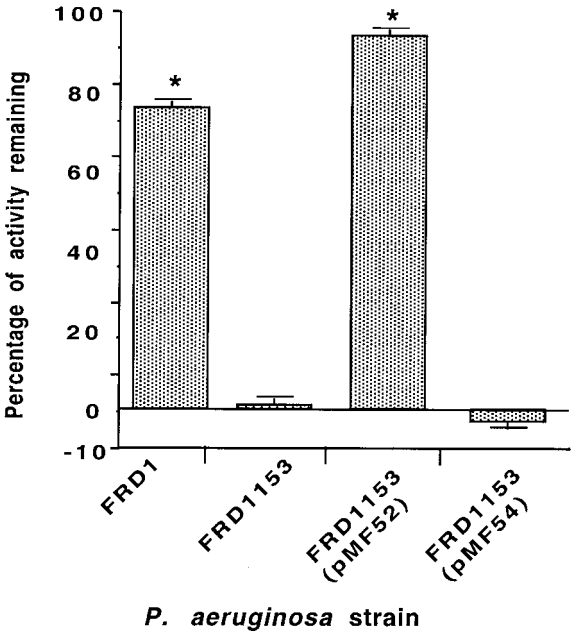

FIG. 2. Acetate residues on MEP/alginate inhibit activation of the alternative pathway of complement. Exposure of 6.25% adsorbed normal human serum to 10^7 CFU of each mucoid *P. aeruginosa* strain for 30 min followed by the removal of the bacteria and the evaluation of the residual complement-activating capacity of the serum showed that the fully O-acetylated strains, FRD1 and FRD1153(pMF52), activated <25% of the available complement, whereas the poorly O-acetylated strains, FRD1153 and FRD1153(pMF54), activated >98% of the available complement. Values of residual activity below 0% are due to experimental variation. Data are reported as mean and standard deviation. Asterisks indicate a significant difference between the value shown and that obtained with 100% residual activity (i.e., 100% red blood cell lysis; $P < 0.0001$, as determined by ANOVA and Fisher's PLSD test).

Effect of acetate substituents on the functional activity of MEP-specific opsonic and nonopsonic antibodies. Another factor involved in the pathogenesis of chronic mucoid *P. aeruginosa* infection in CF lungs is the lack of elicitation of an

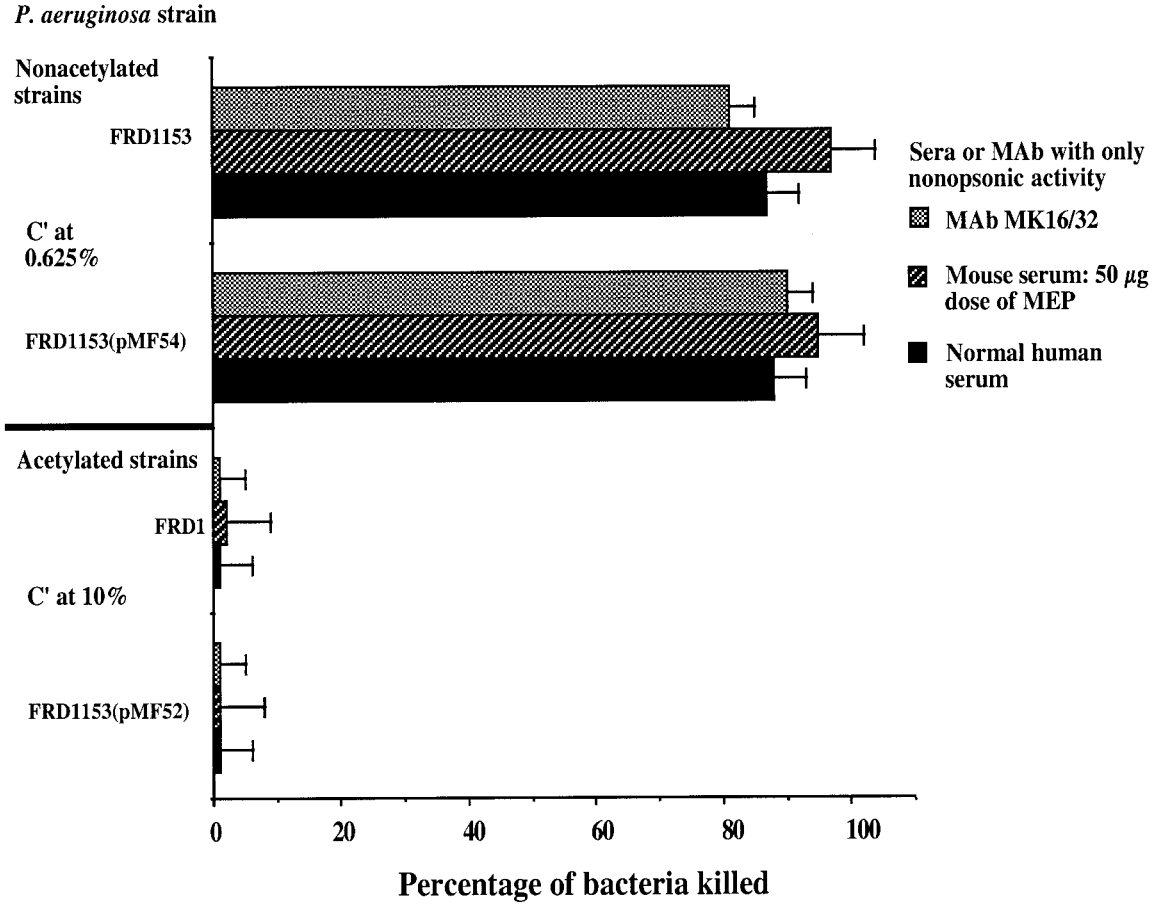

FIG. 3. Opsonic killing of nonacetylated or acetylated mucoid *P. aeruginosa* strains in the presence of a nonopsonic monoclonal antibody (MAb), nonopsonic antibody to MEP in a normal human serum sample, and nonopsonic antibody in pooled sera from mice immunized with a high dose (50 μg) of MEP. Bars represent mean CFU killed, and error bars show the standard deviation. The percentage of the two nonacetylated strains killed by each of the three antibodies was significantly higher than the percentage of the acetylated strains killed by the corresponding antibodies ($P < 0.001$, as determined by ANOVA and Fisher's PLSD test for pairwise comparisons). C′, complement.

immune response that is effective at controlling infection. In other studies, we attributed this situation, in part, to the production of MEP-specific antibodies incapable of mediating opsonic killing of either suspended or biofilm-grown *P. aeruginosa* (27, 32, 38). Immunogenicity studies using MEP and mice have indicated that in the presence of preexisting nonopsonic antibodies to MEP, opsonic antibodies cannot be readily elicited, even with doses of MEP that do elicit opsonic antibodies in naive mice (16). Nonopsonic antibodies to MEP occur naturally in all human sera examined to date and are present in the sera of young CF patients prior to colonization with *P. aeruginosa* (38). These nonopsonic MEP-specific antibodies mediate high levels of complement activation in the presence of mucoid *P. aeruginosa* (37), but opsonic complement fragments derived following activation fail to bind efficiently to mucoid *P. aeruginosa* cells (37). In contrast, opsonic MEP-specific antibodies of the same immunoglobulin isotypes both activate complement and deposit opsonically active C3b and C3bi fragments onto the bacterial surface (37).

To determine whether the acetate substituents on MEP/alginate form the molecular basis for the lack of opsonic killing by nonopsonic antibodies, we carried out phagocytic assays using complement along with the following: (i) normal human serum containing naturally occurring antibodies to MEP that fail to mediate opsonic killing (36, 38), (ii) immunization-induced nonopsonic mouse antibodies obtained from mice immunized three times at 5-day intervals with purified MEP at a high dose (50 μg/dose), or (iii) an MEP-specific murine immunoglobulin G2a (IgG2a) monoclonal antibody that does not mediate opsonic killing of mucoid *P. aeruginosa* (37, 43). To determine if MEP-specific opsonic antibodies recognized acetylated epitopes, we used the following: (i) human sera with MEP-specific opsonic antibodies obtained from individuals vaccinated with purified MEP (36) (which already contained preexisting nonopsonic antibodies); (ii) sera from mice immunized three times at 5-day intervals with purified MEP at a low dose (10 μg), which elicits both opsonic and nonopsonic antibodies (16); or (iii) an opsonic IgG2a monoclonal antibody (43). To avoid phagocytic killing of the poorly acetylated strains in an antibody-independent manner, we used human serum at a concentration of 0.625%, which does not on its own opsonize nonacetylated mucoid *P. aeruginosa* strains for phagocytic killing. For the fully acetylated strains, we used human serum at a concentration of 10% as a source of complement. As shown in Fig. 3, even at a very low

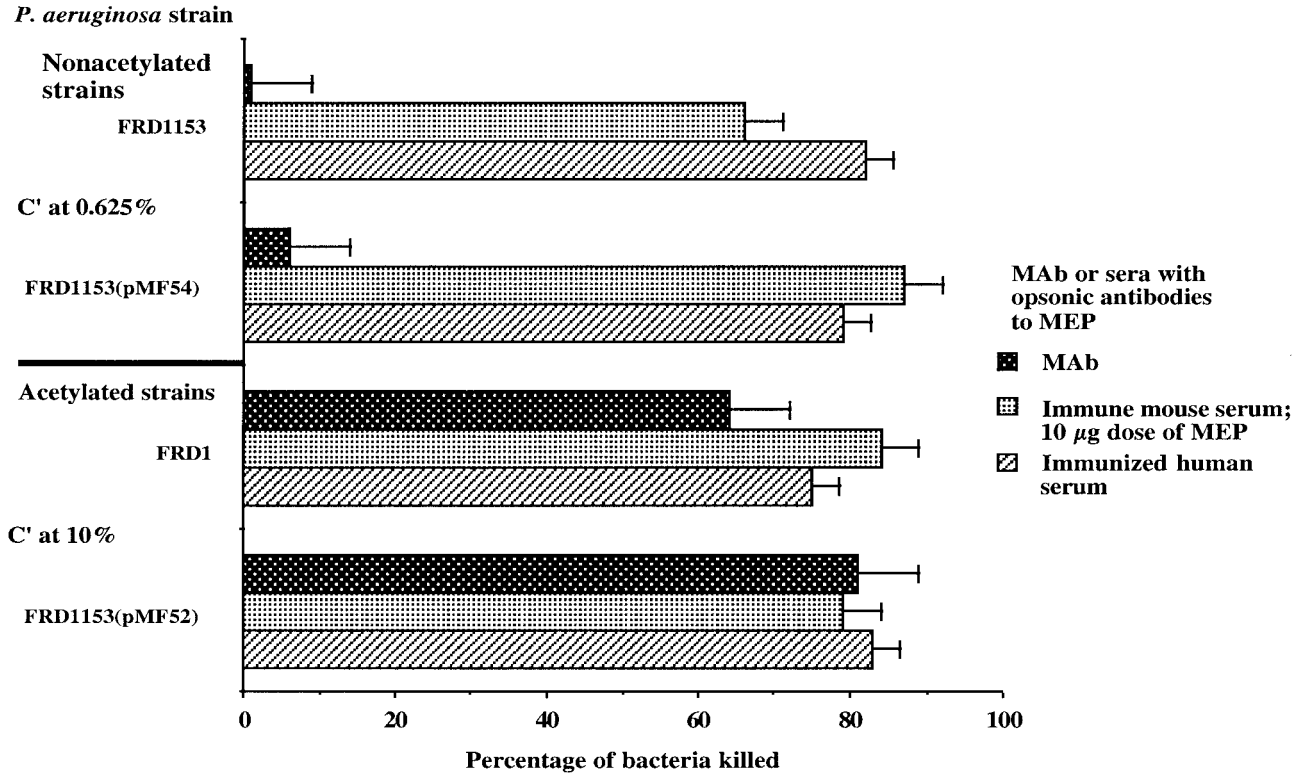

FIG. 4. Opsonic killing of nonacetylated or acetylated mucoid *P. aeruginosa* strains in the presence of opsonic monoclonal antibody (MAb 9/5/23) to MEP, opsonic antibody in serum from a person immunized with MEP, or opsonic antibody in sera pooled from mice immunized with a low dose (10 μg) of MEP. Bars represent the mean CFU killed, and error bars show the standard deviation. The percentage of the two nonacetylated strains killed by the opsonic MAb was significantly lower than the percentage killed by the other antibody preparations ($P < 0.01$, as determined by ANOVA and Fisher's PLSD test for pairwise comparisons). The immune mouse and human sera killed the nonacetylated strains because of the concomitant presence of nonopsonic antibody induced by immunization in mice (a 10-μg dose of MEP elicits both opsonic and nonopsonic antibodies) or occurring naturally in humans.

complement concentration, the usually nonopsonic antibodies readily mediated phagocytic killing of the poorly O-acetylated strains, FRD1153 and FRD1153(pMF54). In contrast, at a complement concentration of 10%, the fully O-acetylated mucoid *P. aeruginosa* strains, FRD1 and FRD1153(pMF42), were resistant to phagocytic killing by the nonopsonic antibodies. Data are shown for sera pooled from five immunized mice and for one normal human serum sample. The assays were repeated with four other normal human serum samples, with essentially identical results (data not shown).

Effect of *O*-acetyl substituents on the functional activity of MEP-specific opsonic antibodies. Sera obtained from mice or humans vaccinated with MEP and developing specific opsonic antibodies killed both poorly and fully acetylated mucoid *P. aeruginosa* strains in the phagocytic assay (Fig. 4). Again, data are shown for sera pooled from five immunized mice and for one immunized human serum sample, but the assays were repeated with four other immunized human serum samples, with essentially identical results (data not shown). The poorly acetylated strains were phagocytosed due to the concomitant presence of MEP-specific nonopsonic antibodies in the sera from the vaccinated mice and humans. However, when a murine IgG2a monoclonal antibody with opsonic killing activity was used, only the fully acetylated strains were opsonized for phagocytic killing (Fig. 4), a result indicating that acetate substituents form the epitope recognized by opsonic antibodies in vaccinated mouse and human sera and by the murine opsonic monoclonal antibody.

Overall, our results indicate one potential molecular basis for the pathogenesis of chronic mucoid *P. aeruginosa* infection in CF lungs. O acetylation of MEP/alginate prevents activation of the alternative pathway of complement; the result is resistance to antibody-independent phagocytosis. In addition, acetylation of MEP/alginate precludes phagocytic killing by the nonopsonic antibodies to MEP found both in normal human sera and at high titers in the sera of infected CF patients (38). Acetate residues appear to be key factors in the epitope that is recognized by protective human and murine opsonic antibodies (39), and complement activation by these antibodies leads to phagocytic killing via deposition of C3b and C3bi (37). Phagocytic killing of mucoid *P. aeruginosa* correlates with the resistance of older, uninfected CF patients to infection with mucoid *P. aeruginosa* (32, 38) as well as with protective efficacy in rodent models of endobronchial infection with mucoid *P. aeruginosa* (39). In addition, acetate residues on MEP/alginate have been shown to be able to scavenge hypochlorite produced by activated phagocytes, potentially protecting mucoid *P. aeruginosa* from this host defense (20), as well as to suppress neutrophil and lymphocyte antibacterial functions and responses (21). Thus, *O*-acetyl substituents are likely one of the

important components of MEP/alginate protecting mucoid *P. aeruginosa* from host defenses.

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