Differential Growth Characteristics and Immunogenicity of Tight and Coasting Temperature-Sensitive Mutants of *Pseudomonas aeruginosa*

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Genetically attenuated vaccines capable of limited replication in the vaccinate may elicit stronger, longer-lasting immunity than that induced by component, killed whole-cell, or nonreplicating live vaccines. We have isolated and partially characterized temperature-sensitive *Pseudomonas aeruginosa* mutants of two different phenotypes: a tight mutant, which ceases all growth immediately after its transfer to 36°C, and a coaster, which continues to replicate for five generations at 36°C. The growth profiles of the two temperature-sensitive phenotypes were compared both in vitro and in vivo; maintenance of the coasting phenotype in vivo was confirmed. The immunogenicity of the two phenotypes was compared in two models. In model 1, ICR mice were immunized intraperitoneally (i.p.) with graded doses of either mutant and challenged 3 weeks later i.p. with lethal doses of the wild-type strain. In model 2, DBA/2J mice were immunized intranasally with either mutant and subsequently challenged with an aerosolized inoculum of the wild-type strain, and lung clearance was measured over 4 h. In both models, the coaster demonstrated slightly higher immunogenic potential and, in addition, induced significantly higher levels of immunotype-specific serum immunoglobulin G after i.p. immunization.

Approaches to vaccine development for the prevention of *Pseudomonas aeruginosa* infection have included the use of lipopolysaccharide, common endotoxin protein, toxoids, cross-reacting mutant toxins or nontoxic materials, flagella, pili, high-molecular-weight polysaccharides, ribosomes, and outer membrane proteins (2). Theoretically, genetically attenuated, live bacterial vaccines capable of limited replication in the vaccinate might induce levels of immunity similar to those seen after natural infection (7). This hypothesis is based upon the rationale that residual growth (coasting) in the host allows the use of lower doses, thereby minimizing reactogenicity, and provides natural amplification of antigenicity, thus enhancing immunogenicity. Perhaps an equally important feature of this approach is that vaccine strains undergoing limited replication after administration could also express antigens that might be significant in inducing protective antibodies.

The present study was designed to compare the immunogenicity of two temperature-sensitive (ts) mutants of *P. aeruginosa*: one with a tight phenotype (i.e., it ceases replication immediately after transfer to the nonpermissive temperature) and the other (a coaster) which continues to divide for five generations at 36°C.

MATERIALS AND METHODS

Bacteria and growth media. *P. aeruginosa* Fisher-Devlin-Gnabasik immunotype 1 (originally obtained from Carl Heifetz, Parke, Davis & Co., Detroit, Mich.) and two ts derivatives, D/1/8 (tight phenotype) and E/9/9 (coaster), isolated after mutagenesis with nitrosoguanidine (6), were used throughout this work. A streptomycin-resistant mutant of E/9/9 was derived by plating >10^{10} CFU on medium

containing 300 μg of streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml and used in the experiments to determine the growth rate in vivo. The streptomycin-resistant mutation had no effect on the growth rate of E/9/9 in vitro. The reversion rates of the two ts mutants are 10^{-7} and 10^{-6} for D/1/8 and E/9/9, respectively. Fisher-Devlin-Gnabasik immunotypes 4 and 5 were the gift of Jerald Sadoff (Walter Reed Army Institute of Research, Washington, D.C.). All strains were cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 29°C in a G-24 environmental incubator (New Brunswick Scientific Co., Inc., Edison, N.J.) at 300 rpm unless otherwise indicated. All platings were on tryptic soy agar (England Laboratories, Rockville, Md.).

Growth profiles in vitro. The tight mutant (D/1/8) and the coaster (E/9/9) were cultured in Trypticase soy broth at 29°C until they reached mid-log phase. The temperature was then shifted to 36°C. The cultures were monitored spectrophotometrically and sampled during the growth period for counts of viable cells.

Growth profiles in vivo. The tight mutant (D/1/8) was inoculated intraperitoneally (i.p.) into mice, and at various times thereafter, groups of three to four mice were sacrificed by cervical dislocation, their peritoneal cavities were lavaged with warm saline, and samples were diluted and plated for quantitation of CFU. For quantitation of the coasting of E/9/9 in vivo, mixtures of D/1/8 and the streptomycin-resistant mutant of E/9/9 at ratios of 7:1 and total doses of 1 × 10^9 to 2 × 10^9 CFU were inoculated i.p. into mice. At various times thereafter, groups of three to four mice were sacrificed and the peritoneal lavage fluid was diluted and plated on tryptic soy agar with and without streptomycin for quantitation of the total CFU and the CFU of E/9/9, respectively. The ratios of the two mutants were determined for
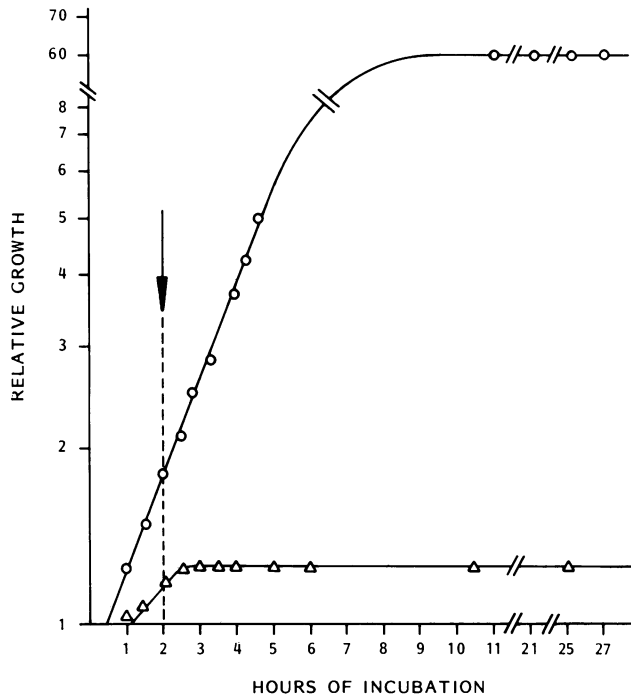
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FIG. 1. Growth curves of the tight and coasting ts phenotypes of *P. aeruginosa*. The tight mutant D/1/8 (△) and the coaster E/9/9 (○) were grown in Trypticase soy broth at 29°C and, at the time indicated by the arrow, the cultures were shifted to 36°C. Samples were plated for quantitation of CFU (E/9/9) per ml, or the culture was monitored spectrophotometrically (D/1/8).

each sampling time, and the number of generations of E/9/9 was calculated from the formula $n = \log (r_0/r_t)/\log 2$, for which n is the number of generations and r_0 and r_t are the ratios of the two mutants at time zero and at the times sampled throughout the experiment, respectively. The derivation of the formula, the assumptions upon which it is based, and the arguments for their validity have been described in detail recently (8).

Animals. We purchased 3- to 5-week-old male ICR and 7- to 8-week-old DBA/2J mice from Harlan Farms, Rockville, Md., and Jackson Laboratory, Bar Harbor, Maine, respectively, and maintained them in the Research Resources Facility of Georgetown University according to standard protocol.

Immunization schedule. Groups of 6 ICR mice were immunized i.p. with various doses of the two phenotypes. Two weeks later, the animals were challenged with lethal doses of the parental wild-type (wt) strain and mortality was recorded. The DBA/2J mice (12 in each group) were immunized intranasally with 3×10^6 to 4×10^6 CFU of D/1/8 or E/9/9, 10^9 Formalin-killed wt cells, or saline in 10 microliters at weekly intervals for 3 weeks.

Aerosol challenge. DBA/2J mice immunized intranasally with D/1/8, E/9/9, Formalin-killed wt cells, or saline were challenged 1 week after immunization 3 with an aerosol of the virulent immunotype 1 by exposure of the animals for 30 min in a chamber built in our laboratory to specifications described previously (9). Lung clearance of the aerosolized challenge was measured after sacrificing groups of mice immediately and 4 h after exposure and aseptically removing the lungs for homogenization in 5 ml of sterile distilled water in Potter-Elvehjem glass grinders (A. H. Thomas, Philadel-

phia, Pa.). Appropriately diluted samples of the lung homogenates were plated and incubated at 36°C for the quantitation of viable cells.

ELISA. Blood samples were taken from the retro-orbital sinuses of mice immunized either i.p. or intranasally with the tight or coasting mutants. Serum samples were kept at −20°C until tested for *P. aeruginosa*-specific antibody by the enzyme-linked-immunosorbent assay (ELISA). The details of the procedure have been described previously (10), and sera from unimmunized animals served as controls.

RESULTS

Growth profiles in vitro. Both phenotypes grew well in trypticase soy broth at 29°C, although E/9/9 grew slightly faster than did the tight mutant (D/1/8). When the cultures were shifted to 36°C, however, marked differences in their growth profiles were revealed. The curves in Fig. 1 show that after the temperature shift to 36°C, D/1/8 stopped all replication almost immediately. The very slight increase in growth observed during the first 30 min after the shift to 36°C probably reflects the completion of a cycle of replication by a small percentage of cells which had already passed the point when the block occurs. On the other hand, the coaster (E/9/9) continued to divide for five generations after transfer to the nonpermissive temperature. The behavior of both mutants at 36°C is in marked contrast to the wt growth rate, which characteristically increases at 36°C, with mean generation times of 20 to 30 min both in vitro and in vivo (8).

Growth profiles of the mutants in vivo. It was important to establish that the coasting mutant did indeed continue replication after its inoculation into animals. To ascertain whether replication was occurring in vivo, it was necessary to overcome the problems of concomitant host clearance, so
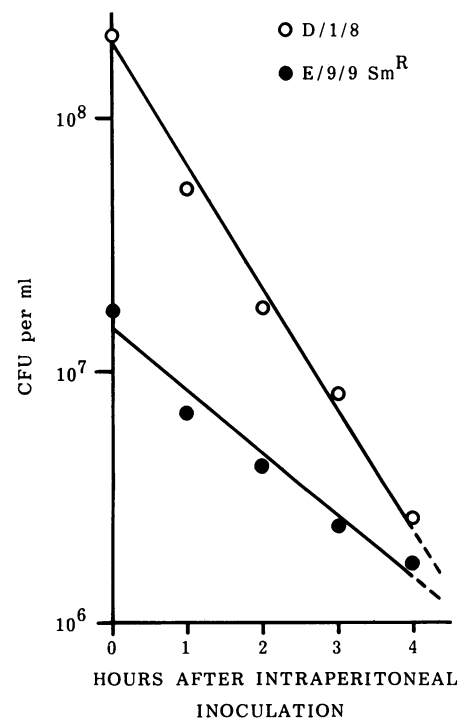

FIG. 2. Clearance of D/1/8 and E/9/9 from the murine peritoneal cavity. Mixtures of the tight mutant (D/1/8) and streptomycin-resistant coaster (E/9/9) were inoculated i.p. into mice, and samples taken over time as described in Materials and Methods.

we developed a model for measuring bacterial replication under those conditions (8). The clearance curves in Fig. 2 indicate that both mutants were apparently being cleared by the mice but at different rates. This apparent difference in the rates could be attributed to the residual replication of the coasting mutant. E/9/9 did continue dividing after inoculation into the peritoneal cavity of the mice and, furthermore, did so at the same coasting rate as at the nonpermissive temperature in vitro (90 min) (Fig. 3). In contrast, the tight mutant apparently did not replicate at all in the peritoneal cavity and was cleared rapidly by the mice (Fig. 2) (8).

Immunization and challenge (i.p.). ICR mice were immunized i.p. with graded doses of the two mutants, and 2 weeks later they were challenged i.p. with 20 median lethal doses of the virulent parental strain. At equivalent doses (1.8×10^8 to 2×10^8 CFU), the coasting mutant induced significantly higher protection than did the tight mutant ($P = 0.0080$ versus $P = 0.7909$, respectively, when compared with the control values) (Table 1). It should be noted that at high immunizing doses (10^9 CFU), the coaster did not induce good immunity when the animals were challenged only 2 weeks later—a phenomenon that has been observed and discussed before (6). In an attempt to correlate these results with the persistence of the coasting strain, we inoculated similar groups of mice i.p. with 5×10^8 CFU and their spleens, livers, and peritoneal cavities were sampled for 10 days for the presence of the immunizing strains. No differences were observed (data not shown) over the time studied; both mutants were cleared from the peritoneal cavity by day
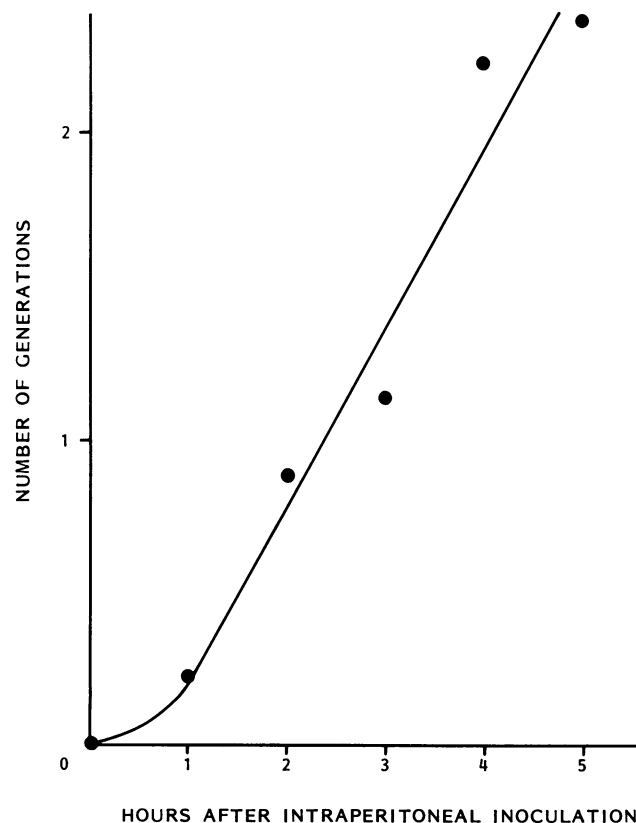
TABLE 1. Survival of mice after i.p. immunization and i.p. challenge[a]

Strain and immunizing dose (CFU)	No. of mice surviving/ no. tested	P[b]
Tight mutant (D/1/8)		
2.0×10^9	4/6	0.0407
8.0×10^8	4/6	0.0407
2.0×10^8	1/6	0.7909
Coasting mutant (E/9/9)		
1.0×10^9	4/6	0.0407
1.8×10^8	5/6	0.0080
2.8×10^7	1/6	0.7909
Control (saline)	1/12	

[a] Mice were challenged 2 weeks postimmunization with 20 median lethal doses (4×10^9 total CFU) of the virulent wt parent.

[b] Significance values were determined by the CHISQR program in the EPISTAT package developed by Tracy L. Gustafson, Round Rock, Tex., on PC computer (IBM Corp., White Plains, N.Y.). P values less than 0.05 were considered significant.

10 but could still be cultured in similar numbers from the spleen and liver. Significantly, only one revertant (of D/1/8) was detected among the CFU recovered from the animals (up to 50 isolates from each sampling were tested, depending upon the number recovered), so the persistence of the organisms in the animals was apparently not due to continued replication of revertants.

Enhancement of lung clearance of wt *P. aeruginosa* cells. DBA/2J mice, immunized intranasally three times at weekly intervals with D/1/8, E/9/9, or Formalin-killed wt cells, were challenged with aerosols containing the parental immunotype 1 organisms, and their ability to clear these organisms from the lungs over 4 h was compared with that of
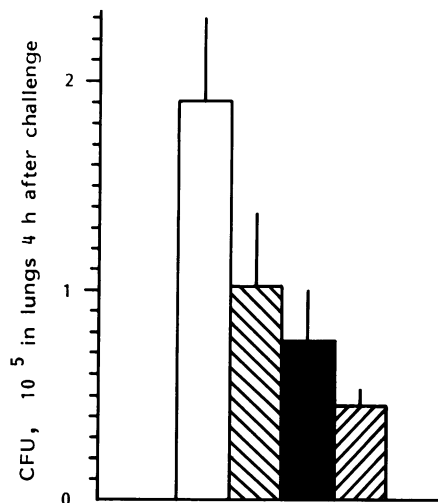

HOURS AFTER INTRAPERITONEAL INOCULATION

FIG. 3. Growth of the coasting mutant in vivo. Mixtures of the tight mutant (D/1/8) and streptomycin-resistant coaster (E/9/9) were inoculated i.p. into mice, and samples were taken over time as described in Materials and Methods.

FIG. 4. Enhancement of lung clearance after intranasal immunization with ts mutants. Groups of DBA/2J mice were immunized with 3×10^6 to 4×10^6 CFU of D/1/8 or E/9/9, 10^9 Formalin-killed wt cells, or saline in 10 microliters at weekly intervals for 3 weeks. One week after immunization 3, the mice were challenged with aerosol doses of the wt parental strain and the clearance of the challenge dose was measured after 4 h. Results were compared for statistical significance by the rank sum test (RANKTEST program, EPISTAT statistical package by Tracy L. Gustafson, Round Rock, Tex.). ▢, Saline; ▧, Formalin-killed *P. aeruginosa*; ■, D/1/8; ▨, E/9/9.
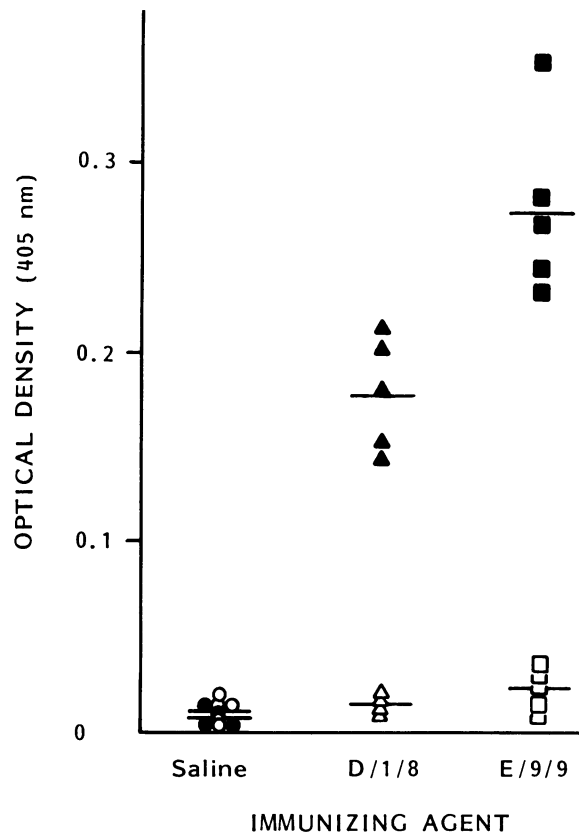
FIG. 5. Induction of ELISA antibody in mice immunized with the tight and coasting ts mutants of *P. aeruginosa*. ICR mice were immunized i.p. or intranasally with D/1/8 or E/9/9 as described in Materials and Methods. Open symbols, serum immunoglobulin G from intranasally immunized animals; solid symbols, serum immunoglobulin G from i.p. immunized mice.

saline-immunized mice. The data in Fig. 4 show that all three immunized groups cleared their lungs more efficiently than did the control animals, but the difference between the D/1/8- and E/9/9-immunized animals, although suggestive, was not significant. Enhanced clearance of the wt cells by the animals immunized with mutant E/9/9 was, however, significant ($P < 0.05$) when compared with that of mice immunized with Formalin-killed wt organisms. Moreover, the doses of D/1/8 and E/9/9 were more than 2 logs lower than that used to immunize with the Formalin-killed wt cells.

Induction of ELISA antibody by the mutants. Groups of mice were immunized once i.p. with 8×10^7 CFU of either the tight mutant (D/1/8) or the coaster (E/9/9). Sera obtained from the animals before and 3 weeks after immunization were tested for the presence of immunoglobulin G specific for the immunizing immunotype 1 and the unrelated immunotypes 4 and 5. The sera of animals immunized with E/9/9 contained significantly higher levels of immunoglobulin G specific for the surface antigens of immunotype 1 than did the sera from mice immunized with the same dose of mutant D/1/8 (Fig. 5). No ELISA antibody to the antigens presented by antibiotic-killed immunotypes 4 and 5 could be demonstrated (data not shown).

The sera of animals immunized intranasally with the two mutants according to the regimen described above contained no detectable antibody to *P. aeruginosa*. No attempts were made to measure antibody in nasal secretions because the

volumes would necessarily be too small for practical consideration. Studies with concentrated, pooled nasal washes are planned to answer this question.

DISCUSSION

The major cause of morbidity and ultimate mortality in patients with cystic fibrosis is chronic pulmonary colonization with *P. aeruginosa* organisms (11). Attempts to treat the infection with antibiotics fail to eradicate the organism, and the patient eventually succumbs to the irreversible damage caused by both the *P. aeruginosa* and the host cells responding to the pathogen (11). Approaches to the development of a vaccine to protect against *P. aeruginosa* infection in the lungs have encompassed all the traditional methods of vaccine preparation (2) and some genetic approaches (1), but none has yet proved successful for the cystic fibrosis patient population. We have chosen to explore the feasibility of developing genetically attenuated *P. aeruginosa* strains for ultimate use as vaccines which could be administered to cystic fibrosis patients before they become colonized with the pathogen. The goal is to stimulate local immunity in the upper respiratory tract to produce antibodies which would prevent adherence and subsequent colonization and infection by *P. aeruginosa*.

Live, attenuated vaccines offer several advantages over chemically or physically treated whole cells or extracts of cell or membrane constituents. First, all the surface antigens, uncompromised by inactivation or purification procedures, are present in their natural conformation. Second, amplification of the antigenic mass occurs during replication in the vaccinate, maximizing immunogenicity. Third, replication in the vaccinate allows the expression of genes coding for antigens that may only be synthesized in vivo. Fourth, such vaccines can be administered at the natural portal of entry and thus induce immunity at the first line of defense. Finally, live vaccines usually induce higher levels of longer-lasting immunity, reducing the need for repeated immunizations. Temperature sensitivity offers further advantages: mutants with lesions in genes encoding essential proteins cannot sustain replication in the vaccinate under any circumstances and mutants capable of limited replication in the vaccinate can be easily isolated and used to amplify the antigenic mass presented to the immune system.

Temperature-sensitive strains of *Salmonella enteritidis* were first developed in 1970 (3), and Robert Chanock and co-workers (4, 5) have experimented with *Mycoplasma pneumoniae* and *Streptococcus pneumoniae* mutants. An inherent problem with this approach is that there could be significant numbers of virulent cells which arise as revertants of the mutant strain during batch culture for vaccine preparation. On the surface this appears to be an insoluble problem, for mutations which produce the desired phenotype must still permit culture of the cells at low temperature, and only single base change mutations, as opposed to deletions or frameshifts, will produce this phenotype. By their nature, base change mutations have significant reversion frequencies (ca. 10^{-7}). We have addressed this problem by combining three ts mutations in one strain of *Haemophilus influenzae* (7). Because the reversion rate of a strain containing three ts mutations, each of identical phenotype, is the product of the rates of the individual strains, mutations with reversion frequencies as high as 10^{-7} can be combined to make a strain with a reversion frequency of 10^{-21}. This would place the vaccine strain within the same safety limits as those set down for recombinant-DNA strains. Such a live

vaccine should possess high immunogenic potential and negligible risk and be easily produced in bulk at minimal cost.

Before beginning the task of combining multiple mutations of identical phenotype into one strain of *P. aeruginosa*, we thought it prudent to test our hypothesis that coasting strains induce better immunity than do the tight phenotype. In the work reported here, the coaster E/9/9 did appear to be more immunogenic than the tight mutant (D/1/8) was at equivalent doses. In the i.p. model of immunization and challenge, low doses of E/9/9 induced protection levels of 83% compared with 17% induced by the same dose of D/1/8. Intranasal immunization with either the coaster or the tight mutant induced significant enhancement by the lung clearance model, and although the enhancement induced by immunization with the coaster was not significantly better than that induced by the tight mutant in individual experiments, the trend was identical in each of five separate experiments. The coaster induced the formation of significantly higher levels of serum antibody to whole cells as antigen, as measured by ELISA, than did the tight mutant when injected i.p. Finally, we were able to show that the coaster maintained its coasting phenotype in vivo for at least two divisions, allowing it to express antigens which might only be synthesized in vivo and perhaps explaining the superior immunogenic potential observed with E/9/9.

ACKNOWLEDGMENT

LITERATURE CITED

1. Cryz, S. J., R. L. Friedman, and B. H. Iglewski. 1980. Isolation and characterization of a *Pseudomonas aeruginosa* mutant producing a nontoxic, immunologically crossreactive toxin A protein. Proc. Natl. Acad. Sci. USA 77:7199–7203.
2. Cryz, S. J., Jr. 1984. *Pseudomonas aeruginosa* infections, p. 317–351. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., Orlando, Fla.
3. Fahey, K. J., and G. N. Cooper. 1970. Oral immunization against experimental salmonellosis. I. Development of temperature-sensitive mutant vaccines. Infect. Immun. 1:263–270.
4. Greenberg, H., C. M. Helms, H. Brunner, and R. M. Chanock. 1974. Asymptomatic infection of adult volunteers with a temperature-sensitive mutant of *Mycoplasma pneumoniae*. Proc. Natl. Acad. Sci. USA 71:4015–4019.
5. Helms, C. M., M. B. Grizzard, B. Prescott, L. Senterfit, S. Urmacher, G. Schiffman, and R. M. Chanock. 1977. Temperature-sensitive mutants of *Streptococcus pneumoniae*. I. Preparation and characterization *in vitro* of temperature-sensitive mutants of type I *S. pneumoniae*. J. Infect. Dis. 135:582–592.
6. Morris Hooke, A., P. J. Arroyo, M. P. Oeschger, and J. A. Bellanti. 1982. Temperature-sensitive mutants of *Pseudomonas aeruginosa*: isolation and preliminary immunological evaluation. Infect. Immun. 38:136–140.
7. Morris Hooke, A., J. A. Bellanti, and M. P. Oeschger. 1985. Live bacterial vaccines: new approaches for safety and efficacy. Lancet i:1472–1474.
8. Morris Hooke, A., D. O. Sordelli, M. C. Cerquetti, and A. J. Vogt. 1985. Quantitative determination of bacterial replication in vivo. Infect. Immun. 49:424–427.
9. Sordelli, D. O., M. C. Cerquetti, A. Morris Hooke, and J. A. Bellanti. 1983. Enhancement of *Pseudomonas aeruginosa* lung clearance after local immunization with a temperature-sensitive mutant. Infect. Immun. 39:1275–1279.
10. Sordelli, D. O., R. A. Rojas, M. C. Cerquetti, A. Morris Hooke, P. J. Degnan, and J. A. Bellanti. 1985. Enzyme-linked immunosorbent assay antibody responses to a temperature-sensitive mutant of *Pseudomonas aeruginosa*. Infect. Immun. 50:324–327.
11. Wood, R. E., T. F. Boat, and C. F. Doershuk. 1976. State of the art: cystic fibrosis. Am. Rev. Respir. Dis. 113:833–878.