

To the Graduate Council:

I am submitting herewith a thesis written by Mary Anne Luzar entitled "An Investigation of Physiological Characteristics and Virulence of Pseudomonas Aeruginosa Cystic Fibrosis Strains." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Thomas C. Montie

Thomas C. Montie, Major Professor

We have read this thesis
and recommend its acceptance:

J. G. Joshi

Raymond W. Beck

W. D. Benis

Accepted for the Council:

C. W. Mink

The Graduate School

AN INVESTIGATION OF PHYSIOLOGICAL CHARACTERISTICS AND VIRULENCE
OF PSEUDOMONAS AERUGINOSA CYSTIC FIBROSIS STRAINS

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Mary Anne Luzar

March 1984

ACKNOWLEDGMENTS

I should like to thank my major professor, Dr. Thomas C. Montie, for his professional guidance and invaluable support throughout the program. Gratitude is extended to my committee members, Dr. Raymond Beck, Dr. David Bemis, and Dr. Jay Joshi, for their helpful collective criticism. I am indebted to Dr. Mary Jane Thomassen (Case Western Reserve) for supplying the strains used in this research. A sincere thank you goes to Dr. Robert Moore for graciously assuming an ad hoc committee member role. David Drake and Janice Allison, my laboratory colleagues, deserve thanks for their assistance and friendship.

A sizable cast of family and friends, here and abroad, contributed to my well-being during these years of study - I am grateful to all. I should especially wish to thank Isabelle and Louis for the dignified constancy of their love and support. I thank Louie, Jane, Denice, Patrick, and James for their sibling comradeship (and for helping me move so many times), as well as the Dr. Arthur Shumaker family for opening its heart and home to me and Dr. John Rickets for his loyalty. Finally, a special "remerciement" is extended to Robert Lagendyk for sharing my labors, aspirations and "joie de vivre" during this past year.

ABSTRACT

Selected physiological parameters of 33 classic and rough Pseudomonas aeruginosa strains from sputum of cystic fibrosis (CF) patients have been examined. An association of a patient's clinical condition (good or poor) with strain physiology has been made. Rough strains from patients in poor clinical condition (P strains) demonstrated severe alterations in motility parameters. Of the 10 rough P strains studied, 70% lacked flagella as determined by electron microscopy. Flagellated P strains demonstrated weak motility in soft agar and in the capillary assay as well as a 40-90% reduction in chemotactic response to three amino acid attractants. A majority of classic and rough strains from patients in good clinical condition (G strains) were highly motile and chemotactic. Characteristics such as auxotrophy and growth rate were not significantly altered in G and P strains; but the absence of protease activity was associated predominantly with P strains.

A series of varied morphological types of CF sputum strains was examined for virulence in an invasive burned mouse model. All 24 strains examined demonstrated reduced virulence when compared to M-2, a virulent burn strain ($LD_{50} = 1.5 \times 10^1$ CFU). Ninety-two percent of the strains tested were avirulent at doses of 10^3 - 10^5 CFU. The LD_{50} of nine representative flagellated CF strains was determined in the same animal model. Five of the strains tested had $LD_{50} > 10^6$ CFU. Reduced virulence of each strain appeared to be associated with loss of one or more physiological

characteristics. Strain 35c resembled M-2 being of classic colonial morphology, protease-positive, O-antigen typable, flagellated and chemotactic. It was the most virulent of the strains ($LD_{50} = 8 \times 10^2$ CFU). A mucoid (G) strain, 412a, intermediate in virulence ($LD_{50} = 10^4$ CFU), polyagglutinated in O antisera and showed reduced motility (60% of M-2). Results suggested that any virulence detected was associated with classic (G) strains prevalent in early, non-chronic infections. It appears that a selection transition occurs favoring P. aeruginosa avirulence (i.e. alterations in factors such as protease production, cell envelope, motility and chemotaxis) as chronic infection is established in the CF lung.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. MATERIALS AND METHODS	14
Bacteria	14
Maintenance of Cultures	14
Media	14
Quantitation of Bacteria	15
O-Antigen Slide Agglutination Assay	16
Motility Assay	16
Electronmicroscopy	16
Preparation of Flagella	17
Polyacrylamide Gel Electrophoresis	17
Capillary Assay for Quantitation of Bacterial Chemotaxis and Motility	18
Growth Experiments	19
Proteolytic Activity	20
Laboratory Animals	20
Experimental Burn Procedure	20
III. RESULTS	22
O Antigen Serotypes	22
Relative Motility and Chemotaxis of CF Strains in Motility Agar	26
Detection of Flagella by Electron Microscopy	31
Polyacrylamide Gel Electrophoresis of Flagella Extracts	37
Growth Comparisons	39

CHAPTER	PAGE
III. (continued)	
Quantitation of Chemotaxis and Motility in the	
Capillary Assay	43
Proteolytic Activity	54
Virulence Comparisons of CF Strains in the Burned	
Mouse Model	54
IV. DISCUSSION	59
BIBLIOGRAPHY	74
APPENDIX	84
VITA	86

LIST OF TABLES

TABLE	PAGE
1. <u>Pseudomonas aeruginosa</u> Cystic Fibrosis Sputum Strains Used in this Study	23
2. O Antigen Serotypes of <u>P. aeruginosa</u> Isolates from Cystic Fibrosis Patients	25
3. Summary of Properties of <u>P. aeruginosa</u> Strains from Cystic Fibrosis Patients	36
4. Summary Chart of <u>P. aeruginosa</u> Strains Grown in MSS, MSG, and Luria Broth	44
5. Motility and Amino Acid Taxis of M-2	46
6. Motility and Amino Acid Taxis of Rough <u>P. aeruginosa</u> Strains from CF Patients in Good Clinical Condition. .	47
7. Motility and Amino Acid Taxis of Rough <u>P. aeruginosa</u> Strains from CF patients in Poor Clinical Condition. .	49
8. Taxis of <u>P. aeruginosa</u> 402c Using Different Concentrations of Amino Acids	50
9. Motility and Amino Acid Taxis of Classic <u>P. aeruginosa</u> Strains from CF Patients in Good Clinical Condition. .	51
10. Motility and Amino Acid Taxis of Classic <u>P. aeruginosa</u> Strains from Patients in Poor Clinical Condition . . .	53
11. Proteolytic Activity of <u>P. aeruginosa</u> CF Strains	55
12. Comparison of Lethality of <u>P. aeruginosa</u> CF Strains in the Burned Mouse Model (HA/ICR Mice)	57

TABLE

PAGE

13. Comparisons of Lethality of <u>P. aeruginosa</u> Strains in the Burned Mouse Model (CF-I Mice)	58
14. Standard Dilution Curves for Quantitation of Bacteria. .	83

LIST OF FIGURES

FIGURE	PAGE
1. Migration of Rough <u>P. aeruginosa</u> Strains in Motility Agar	27
2. Migration in Motility Agar of Rough <u>P. aeruginosa</u> Strains from CF Patients in Good and Poor Clinical Condition	28
3. Migration in Motility Agar of Rough <u>P. aeruginosa</u> Strains from CF Patients in Poor Clinical Condition . .	29
4. Migration in Motility Agar of Rough <u>P. aeruginosa</u> Strains from CF Patients in Good and Poor Clinical Condition. .	30
5. Migration in Motility Agar of Classic <u>P. aeruginosa</u> Strains from Patients in Good Clinical Condition. . . .	32
6. Migration in Motility Agar of Classic <u>P. aeruginosa</u> Strains from Patients in Good and Poor Clinical Condition	33
7. Electron Micrograph of Flagellated Strain 572b from CF Patient in Good Clinical Condition	34
8. Electron Micrograph of 96e, a Non-Flagellated Strain from a CF Patient in Poor Clinical Condition.	35
9. 10% SDS-PAGE (1.5 μ g/protein/well) of <u>P. aeruginosa</u> Flagellin Proteins	38
10. 10% SDS-PAGE (1.5 μ g/protein/well) of <u>P. aeruginosa</u> Flagellin Proteins	40

FIGURE

PAGE

11. Growth Curves of P. aeruginosa Strains 402c and M-2
Grown in MSS. 41
12. Growth Curves of Strains 86f, 66g, and M-2 Grown in MSS . 42

CHAPTER I

INTRODUCTION

Cystic fibrosis is the most common lethal genetic disease in the caucasian population in the United States (4, 42). The US Department of Health and Human Services estimates that twenty to thirty thousand persons are afflicted today by this autosomal recessive gene disorder and that there are as many as ten million symptom-free carriers in the United States. First described by Anderson forty-five years ago, cystic fibrosis (CF) was originally thought to be a pancreatic disorder (42). In the 1940's, discovery of the involvement of mucus-secreting glands complicated efforts to understand the clinical syndrome. It is now known that four organs are affected by this genetic disorder: respiratory, gastro-intestinal, exocrine, and reproductive (23, 25, 26, 42). However, the basic genetic defect remains enigmatic (23, 92). In the absence of identification of a unitary genetic derangement, there is much speculation. One of the more intriguing recent conjectures is that we could be dealing with a genetic aberration which is only briefly expressed during development, programming the individual for self-destruction before receding into the inaccessible background of dormant DNA (23).

Diagnosis and management of the disease are complicated by the fact that in 85% of cases, patients appear normal at birth (53). Since 1959 successful early diagnosis has been accomplished by using one discriminating laboratory determination: the sweat test (23).

This test is based on an observation made in 1953 that 99% of cystic fibrosis patients have high electrolyte levels in sweat (25, 26).

In recent years, prognosis for the disease has changed due to advances in both diagnosis and management. Infant mortality has been rare during the past ten years (42). The single most important factor responsible for decreased infant mortality is the availability of antibiotics which control secondary bacterial infections caused by Staphylococcus aureus which is superseded by Pseudomonas aeruginosa (42, 53).

Aminoglycosides such as gentamicin, tobramycin and amikacin have inhibitory and bactericidal activity against P. aeruginosa which has now emerged as the major pathogen associated with CF lung pathology (25, 38, 56, 59, 79, 84). In the early stages of the disease, patients have a dry, episodic cough. As the disease progresses they develop a persistent, productive cough which is accompanied by weight loss and decreased activity. The condition will become progressively worse if not treated adequately. However, successful prolonged treatment has been hindered in the past ten years by an increase in aminoglycoside-resistant P. aeruginosa strains (24, 41). Recent therapy involves combining an aminoglycoside with a β -lactam. It appears that drug resistance is due to R-plasmids which code for degradative enzymes (16, 22, 43). Other resistance may involve cell envelope barriers and appears to be chromosomally determined. Studies show that, in addition to prophylactic therapy being ineffective in preventing pulmonary disease, it is virtually

impossible to eliminate bacterial pathogens such as P. aeruginosa from the respiratory tracts of patients with CF (37, 80, 85). For this reason, the less encouraging fact remains that the average life expectancy of a CF patient is only twenty years (Dr. R. Wood, personal communication: CF Workshop, Arlington, Va., 1982).

The nature of the disordered biochemistry which predisposes the patient to colonization by P. aeruginosa is not known (23). Acquisition of P. aeruginosa occurs in early stages of the disease. Classic (synonymous with smooth), mucoid, and rough colonial morphology types are found in young adults (79, 85). The mucoid and rough varieties of P. aeruginosa are especially prevalent in older patients with advanced disease (85). Since the lung is normally kept free by mucociliary clearance in the airways and phagocytic activity of macrophages in the peripheral airways and alveoli, an organism capable of persisting in this organ would have to successfully circumvent these two mechanisms. Other secretory and immune factors contributing to lung defense must also be foiled by the invading bacterial species.

High antibody titer to P. aeruginosa in patients with advanced pulmonary involvement prevents developing systemic infections due to the pathogen but cannot protect lungs from progressive tissue damage (77). In fact, high levels of antibody may contribute to hypersensitivity reactions and lung tissue damage. Mucoidy appears to inhibit antibody opsonization and complement-dependent lysis of the bacterial cell (32, 37, 47). Prolonged harboring of the pathogen

in the host causes permanent changes in airways accompanied by pulmonary parenchyma. Elevated levels of cations such as magnesium and sodium in the CF lung cause an increase in extracellular glycocalyx production by P. aeruginosa. The structure and viscosity of lung mucus is also thought to be affected by the altered ionic environment. The resultant clumping of mucus interferes with its normal protective function by allowing greater interaction between the lung surface and inhaled bacteria (31, 32). Bronchial plugging with thick mucus and pulmonary fibrosis are characteristic features of cystic fibrosis pathology.

The complex interaction between the host and pathogen in cystic fibrosis makes it difficult to separate the disease caused by the host and disease caused by the microorganism. Although the pathology of CF lung disease remains unclear, no doubt exists that it is the principal cause of death (92). For this reason much investigation has been directed toward studying the basic mechanisms of pulmonary involvement responsible for the high mortality in this disease.

A voluminous amount of information about the pathogenesis and virulence of P. aeruginosa as a causal agent of a variety of diseases is currently available. The role of this pathogen in acute infections, such as those acquired after thermal injury, has been elucidated using a variety of experimental animal models. One of the most successful models used to study the role of exoproducts and physiological characteristics associated with virulence is the burned mouse model for acute infection following thermal shock. Stieritz and Holder (83) demonstrated that, in this model, the LD₅₀ for P.

aeruginosa was significantly lower in comparison to the LD₅₀ in normal mice. This was not the case for the other bacteria tested. Several extracellular products and physiological characteristics have now been identified as contributors to P. aeruginosa infections in burn wounds (20, 61, 94, 95).

The microenvironment which exists in the lung of the CF patient favors the establishment of a chronic infection rather than an acute one. Three animal models are frequently used to study lung infections. The rat model of chronic respiratory infection developed by Cash et al. (17, 48) introduces the bacteria enmeshed in agar beads via intratracheal inoculation. The enumeration of bacteria recovered from the lung can be compared to the original number in the inoculum; with subsequent histological studies which are semi-quantitatively scored, estimates of lung tissue damage can be made. While this model is purported to be useful in examining the interactions between microbial virulence factors and host defense mechanisms, it may have certain drawbacks when used to study interactions that pertain to the CF patient. While the number of mucoid and classic strains recovered from the lungs was similar, colonization of the kidneys occurred in rats inoculated with the mucoid strain (17). Since in CF there is apparent containment of the organism in the lung, such colonization of a vital organ clouds the issue when evaluations of roles of possible virulence factors are being made. In addition to kidney colonization, the type of inoculation is apt to alter the pathogenicity of the organism. The agar beads in which the bacteria are enmeshed and direct intra-

trachael inoculation may both have subtle albeit important effects on the invading bacteria.

In another recently developed model by Boyd et al. (11), rats are given hexamethylphosphoramide-treated drinking water which denudes the airway epithelium causing microulcerative lesions. Bacteria are then instilled in the trachea of each rat resulting in colonization of the airway. Research using this model supports the hypothesis that colonization by P. aeruginosa may occur in airways where ciliated epithelium has been injured.

Pennington et al. (7) developed an experimental model of chronic lung infection using the guinea pig. Once again the agar bead-enmeshed inoculum is directly instilled into the lower respiratory tract via the trachea. Both host defenses and bacterial virulence factors can be altered in this model to study pathogenesis of Pseudomonas.

As previously mentioned, the interplay between host and microbial factors which contributes to this unusual environment has not yet been clearly determined; but many Pseudomonas exoproducts such as proteases, exotoxins, and phospholipases, known to play an important role in acute infections, are currently being considered as factors which may promote colonization or lung cytopathology. In addition to these exoproducts, certain physiological characteristics such as motility, chemotaxis, and O-antigen composition may shed light on the subject of persistence. Evidence now exists which suggests that certain physiological characteristics of P. aeruginosa

associated with its virulence in acute infections may, in fact, undergo alterations which enhance the state of chronic infection in the CF lung (35, 66, 86).

The identification of typical strains of P. aeruginosa has generally presented no difficulty in the clinical laboratory. However, slight variability in colonial morphology, particularly non-pigment forming strains, was recognized by Wahba (89) in the early 1960's. These atypical strains now appear to be occurring more frequently. It has been hypothesized that use of antibiotics may favor their occurrence, but this has not been substantiated experimentally (M. J. Thomassen, personal communication).

Extensive investigation by Wahba et al. (89) resulted in the identification of six P. aeruginosa colonial types: classic, classic rough, rough, mucoid, gelatinous, and dwarf. Three of these morphological types predominate in the CF lung: classic, rough and mucoid (70, 85, 86, 89). The classic colonial morphology is irregular in shape with a smooth, matt surface. Rough colonies are also irregular in shape, but they have rough, matt surfaces. Mucoid colonies are circular in shape with smooth, shining surfaces (89).

Much has been made of the importance and frequency of P. aeruginosa mucoid strains in cystic fibrosis. The mucoid morphology is due to the biosynthesis of the exopolysaccharide alginate (27, 65). According to Ohman et al. (65), alginate production by P. aeruginosa provides the organism with a survival

advantage in the CF lung. Certain in vitro studies suggest that mucoid strains have advantage over non-mucoid strains against phagocytic cells, antibodies, and antibiotics (32, 37, 54). However, in vivo studies using a guinea pig model of experimental pneumonia were unsuccessful in demonstrating that mucoid coating of P. aeruginosa selectively retards killing of the organism (7).

Research by many investigators (8, 31, 33, 72) indicated that mucoidy of P. aeruginosa is an inherently unstable characteristic which may be strongly influenced by the environment. Studies by Boyce and Miller (8) showed that iron concentration in the culture medium had a profound effect on the selection of non-mucoid cells. Rates of accumulation of non-mucoid cells were lower in media containing lower concentrations of iron. Pugashetti et al. (70) also encountered this instability of mucoid cells and noted a high frequency of reversion to the non-mucoid phenotype which was also a medium-dependent phenomenon. It appears that most mucoid strains revert very rapidly to nonmucoid form on subculture. Although the exact explanation is still elusive, it is hypothesized that one reason may be a rapid growth rate which results when the cells no longer produce large amounts of extracellular polysaccharide (25, 92).

The hypothesis that CF patients are colonized by a non-mucoid strain of P. aeruginosa which converts to a mucoid variant has recently been modified (70, 71). It is now known that many CF patients are colonized by mucoid or non-mucoid strains (79, 85). Simultaneous presence of mucoid and non-mucoid strains of the

organism in the respiratory tract of individual patients has also been reported (70, 71, 85). It was concluded by Rivera et al. (73) in a recent investigation that it is no longer possible to correlate mucoid P. aeruginosa strains in bronchial secretions with presence of cystic fibrosis. Only two of the 31 Pseudomonas-infected patients in their study had CF; the majority had chronic pulmonary disease and common to all was the presence of bronchiectasis.

The basis for serotyping P. aeruginosa is variation in the O antigen side chain composition of the lipopolysaccharide (LPS) of different strains (13, 14, 35). As many as 17 distinct serotypes may be demonstrated depending on the typing system used (18). Recent evidence has been obtained which suggests that some P. aeruginosa strains are deficient in O-antigenic LPS (18, 66, 86, 91). In the cystic fibrosis lung there is a high frequency of non-, self-, or polyagglutinating strains (39, 58, 86). This is in contrast to the high frequency of typable strains associated with acute infection (69).

Studies by Hancock et al. (35) supported these observations about altered LPS. Only six of the 26 CF P. aeruginosa strains they studied were agglutinated by a single typing serum. The majority of the polyagglutinable or nonagglutinable strains were sensitive to normal serum. Hancock observed no major correlation between mucoidy and serum sensitivity, O-antigenic typability, LPS phenotype or outer membrane protein patterns of CF strains. The altered LPS phenotype appears to be a significant property of CF-associated P. aeruginosa.

Serum sensitivity, which appears to be associated with altered LPS may explain why CF patients rarely suffer from bacteremia (35).

Investigations by Thomassen et al. (86) on serum bactericidal effect of CF strains agreed with the work by Hancock. In the Thomassen studies, serum sensitivity could not be correlated with a specific serotype, morphological variety or patient clinical condition. However, the result of a given strain's sensitivity in autologous serum compared to normal serum did correlate with clinical condition suggesting that serum sensitivity is altered as the infection progresses. Patients in poor clinical condition, in contrast to those in good or moderate condition, were more likely to have strains that were serum resistant in autologous serum but sensitive in normal serum.

Penketh et al. (66) reported that the majority of CF strains in their studies had three distinct features: loss of O-antigenic serotype, expression of a new somatic antigen and sensitivity to normal human serum. Patients most severely affected by the disease were colonized by organisms with one or two of these characteristics. Penketh also confirmed the high incidence of polyagglutination by CF strains. In addition, evidence suggests the presence of a new somatic antigen, the polyagglutinating or PA antigen (58, 68). According to these investigators, serum sensitivity was significantly associated with loss of O-type reaction and the concurrent appearance of the PA antigen.

Several extracellular proteolytic enzymes of P. aeruginosa may contribute to pulmonary damage. Of these enzymes, alkaline protease (so named because of its activity at alkaline pH) and elastase (which degrades elastin) are considered important virulence factors (46, 47, 78, 81, 93). In vivo production of elastase in CF patients was measured by Klinger et al. (47) by detection of specific antibodies to this enzyme in the majority of patients. Other in vitro studies demonstrated that alkaline protease is also produced by various CF strains (29, 64). It appears that, even with specific antibodies to the proteases present in the lung, protection against proteolysis may be insufficient. An in vitro study has shown that alkaline proteases can cleave human IgA and elastase can cleave human IgG, IgA, and secretory IgA (28).

Jagger et al. (44) noted that a higher percentage of non-mucoid Pseudomonas strains was proteolytic, although mucoid phenotype did not restrict expression of protease activity. Protease production may be important in the initial colonization of the respiratory tract of CF patients, since data by Jagger revealed that chronically infected patients contained more weakly proteolytic strains than patients newly colonized by P. aeruginosa.

Possession of a single, polar flagellum, which imparts motility to the organism, is one of the stable, physiological traits used to characterize Pseudomonas species (55). Flagellation has been shown to be a stable, strain-dependent characteristic which is not related to other characteristics, such as mucoidy, as was previously thought

(55, 71). It was reported that there is a wide separation of genetic loci for mucoidy and flagellation (72); it is doubtful that a mutation in one would result in a mutation in the other. Motility as a virulence factor has also been demonstrated in the burned mouse model (20, 40, 60). Montie et al. (60) reported that a non-flagellated isogenic mutant of P. aeruginosa strain M-2 showed a loss of virulence in the model by a dosage factor of four logs when compared to the motile parental strain and to a spontaneous, motile revertant.

Various strains of P. aeruginosa are known to be chemotactic to a variety of amino acids, organic acids, and glucose (63). Strain M-2, which is virulent in the burned mouse model, is vigorously motile and highly chemotactic. Conversely, Craven and Montie (20) demonstrated that avirulent strains lacking either proteases or exotoxin A were also deficient in their chemotactic capacity. Since heterogeneity in chemotactic capacity exists among P. aeruginosa strains, this physiological characteristic is now being studied as a possible virulence factor. Preliminary studies using isogenic mutants for chemotaxis or motility indicate that these strains are avirulent in the burned mouse model (D. R. Drake and Dr. T. C. Montie, personal communication). Montie et al. (T. C. Montie, personal communication) have noted in the laboratory that CF P. aeruginosa isolates were less chemotactic than those strains associated with acute infection. However, the role of chemotaxis and motility in establishment of chronic infection in the CF lung has not

been examined. In the realm of speculation, it is reasonable to assume that they may play a significant role in initial invasiveness, but be of less importance in maintaining a persistent infection in the lung.

In order to further elucidate the chronology of events in the CF lung causing establishment of chronic P. aeruginosa infection, we posed the following questions: i) After initial lung invasion by the pathogen, is there progressive host selection against bacterial properties normally associated with virulence? ii) What is the relative virulence of the CF strains compared to an invasive strain in the burned mouse model? iii) If avirulence is characteristic of CF strains, to what may it be attributed?

These questions were answered by pursuing the following specific aims: i) to characterize CF strains (classic and rough colonial morphology) isolated from patients in good and poor clinical condition regarding O-antigenicity, motility, chemotaxis, and proteolytic activity. ii) to test representative strains of varying morphological and physiological traits for virulence using the burned mouse model for acute infection.

CHAPTER II

MATERIALS AND METHODS

Bacteria

All Pseudomonas aeruginosa cystic fibrosis strains were isolated from sputum by M.J. Thomassen (Case Western Reserve School of Medicine, Cleveland, Ohio). P. aeruginosa strain M-2 was originally isolated from the small intestine of a CFI mouse (40). Strain M-2 was obtained from I.A. Holder (Shriner's Burns Institute, Cincinnati, Ohio).

Maintenance of Cultures

Stocks of bacterial cultures were kept at -70°C in Luria broth (various media are described in the following section) and 25% (v/v) glycerol. Bacterial stocks, maintained as dilute suspensions in Luria broth at 4°C , were used to inoculate daily cultures.

Media

The standard growth medium for chemotaxis assays and growth experiments was a mineral salts mixture containing the following: dibasic potassium phosphate 0.04 M, monobasic potassium phosphate 0.02 M, ammonium sulfate 7.6×10^{-3} M, magnesium sulfate 2.0×10^{-4} M and ferric chloride 9.2×10^{-6} M. This medium is referred to in the text as MS. Sodium succinate at 0.4% served as the carbon source except where otherwise noted.

For all other assays bacteria were grown in Luria broth (1% NaCl, 1% tryptone, 0.5% yeast extract, pH 7.0).

Tryptone broth contained 10 g Bacto-tryptone (Difco), 5 g sodium chloride and 3 g yeast extract dissolved in 1 liter distilled water. The medium was dispensed into test tubes and autoclaved.

Tryptone agar contained tryptone broth to which 18 g of agar were added per l of medium before autoclaving. Sterile medium was dispensed 15 ml per 100 mm petri dish. Plates were kept overnight at 37°C to decrease moisture content, then stored covered at 4°C.

Motility medium (synonymous with soft agar) contained 10 g Bacto-tryptone (Difco), 3 g yeast extract, 5 g sodium chloride, 3 g agar and 1 liter distilled water. The final pH was 7.2 before autoclaving. The medium was dispensed into 50 mm petri dishes, stored overnight at 37°C, and inoculated within 24 h.

Quantitation of Bacteria

Bacteria were grown in Luria broth for 14 hours at 37°C with rotary shaking at 175 rpm. Cells were harvested by centrifugation (4000 x g for 15 minutes) and washed twice in a standard diluent (25 mM potassium phosphate, 0.9% NaCl, 0.01% gelatin, 0.2 mM $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ pH 7.0). Cells were diluted in the same diluent. Inocula for the lethality studies were calculated from reference to a predetermined standard dilution curve for each strain tested. For comparison of adsorption versus colony-forming units (CFU), see Table 14, Appendix. Viable cell counts were determined by serial dilutions followed by colony plate counts performed in duplicate on tryptone agar.

O-Antigen Slide Agglutination Assay

Bacteria were grown for 24-30 hours at 37°C on tryptone broth agar. Three loopfuls of bacteria were suspended in 2 ml of sterile saline (0.85% (w/v)). Forty μ l of the bacterial suspension were placed on a glass microscope slide. A 40 μ l drop of antiserum (Difco - American Scientific) diluted 10-fold in saline was added to the cell suspension. The slide was rocked back and forth and observed for two minutes. A positive reaction was visible agglutination occurring within the two minute incubation period (68). Strains were designated as polyagglutinating (PA) if they agglutinated with two or more of the 17 antisera. The strains which did not agglutinate in any of the 17 antisera were designated non-typable (NT).

Motility Assay

Cells were grown for 24 hours on tryptone broth agar. Using a sterile inoculating needle, several colonies were picked and stab-inoculated into the center of a plate of motility medium described previously. For each assay triplicate plates per strain were incubated at 37°C and examined periodically over a one to 24 h period for colony-spreading and the distinctive rings which are characteristic of chemotactic bacteria (1). Colony diameter was measured at various time points. The average of three assays was used to graphically plot the data.

Electronmicroscopy

Bacteria were grown for 14-16 hours in 5 ml of Luria broth at 37°C without shaking. If necessary, the bacterial suspension was

diluted in standard diluent. Cells were stained with 0.5% phospho-tungstic acid, pH 7.0. Specimens were examined using an H-600 Hitachi transmission electron microscope (magnification: 10,000 x).

Preparation of Flagella

Flagella were isolated from CF Pseudomonas strains by a modification of the method of Montie et al. (61). Bacteria were grown overnight in 50 ml MS containing 0.4% succinate in a gyrotary water bath at 30°C. Five ml of cell suspension were reinoculated into 500 ml MS plus 0.4% succinate (4 flasks per strain). These large volume cultures were incubated for 16 h in 30°C water bath with shaking. Luria broth was used as a growth medium for auxotrophs. Cells were harvested by centrifugation for 15 min at 5000 x g, 4°C. Pellets were resuspended in potassium phosphate buffer (0.01 M, pH 7.0) then blended for 30 sec in a Waring commercial blender to shear off the flagella. The suspension was centrifuged for 30 min. at 5000 x g at 4°C; the supernatant was centrifuged again for 15 min. at 16,000 x g, 4°C. The supernatant was then centrifuged for an additional three hours at 40,000 x g, 4°C in a model L5-75 Beckman centrifuge with a 42.1 rotor. The pellets were suspended in 5 ml potassium phosphate buffer, dialyzed against distilled water and rapidly frozen using acetone and dry ice, then lyophilized. The flagellar preparations were stored, desiccated, at -55°C.

Polyacrylamide Gel Electrophoresis

The separating gel consisted of 10% acrylamide and 0.1 SDS in

0.375 M Tris-HCl at pH 8.8. The stacking gel mixture contained 5% acrylamide and 0.1% SDS in 0.175 M Tris-Cl at pH 6.8. Both gels were polymerized with persulfate and TEMED. 1.5 microgram of protein (Biorad assay) per well was used. The gel was run using the method of Fairbanks et al. (30).

The polyacrylamide slabs were stained with Coomassie Blue R250 according to the method of Fairbanks et al. (30). After destaining with 100% acetic acid, gels were dried onto filter paper under vacuum on a slab gel drier (Hoeffer Scientific Instruments).

Capillary Assay for Quantitation of Bacterial Chemotaxis and Motility

For the studies presented in this paper, a modified Adler capillary assay was used (3, 63). All CF P. aeruginosa strains exhibiting motility in motility agar were tested. Bacteria were grown to logarithmic phase in MS plus 0.4% succinate (MSS); 0.4% glucose was used for those few strains unable to utilize succinate as a carbon source. The cultures were chilled on ice for 15 to 60 min then washed twice in chemotaxis (CTX) buffer (potassium phosphate 0.05 M, magnesium chloride 0.05 M, disodium ethylenediamine-tetraacetic acid 2.5×10^{-5} M, pH 7.0). After the final wash, bacteria were resuspended in room temperature CTX chemotaxis buffer to an A₅₉₀ of 0.2. Each chamber was filled with 0.4 ml of suspension. All chambers were prepared in triplicate per attractant and arranged on damp absorbent paper in a 37°C room. Microliter capillaries, flame-sealed at one end, were filled with chemo-attractants dissolved in CTX buffer and filter-sterilized. The

attractants were amino acids at optimum molar concentrations for P. aeruginosa chemotactic response: arginine 10^{-2} M, amino-isobutyrate 10^{-2} M, serine 10^{-3} M (63). For each assay another set of capillaries was filled with CTX buffer for quantitative evaluation of motility. Cells which migrated into a capillary containing an attractant or buffer during a 30 min period were enumerated by viable plate counts after dilution in tryptone broth. The mean accumulation in triplicate assays was expressed in terms of total CFU per capillary. Each day the chemotaxis assay was performed, strain M-2 was also tested as a non-CF strain to be used for comparison. The results for the CF strains were then expressed as % M-2. In addition to % M-2 calculations, all chemotaxis data was statistically evaluated using Student's t-test (15). A difference between a sample mean (CF strain) and a population mean (M-2) existed if the t value was greater than the 0.05 value.

Growth Experiments

A bacterial stock suspension in Luria broth (0.2 ml) was inoculated into 50 ml MS plus 0.4% succinate or 0.4% glucose and grown overnight at 37°C with rotary shaking at 175 rpm. The overnight growth was diluted with fresh medium to give an optical density (A_{590}) of 0.05 to 0.07 on a Bausch and Lomb Spectronic 20 using a 1 cm cuvette. Incubation continued at 37°C with shaking, and optical density readings (A_{590}) were taken periodically for 8 hours or until an OD_{590} of 1.0 was reached. Absorbance readings were plotted on a semilogarithmic graph.

Proteolytic Activity

Proteolytic activity was assayed using a modified method of Sokol et al. (82). Equal volumes of 1.5% skim milk and trypticase soy agar (BBL, Cockeysville, MD) were prepared, autoclaved separately (10 min and 15 min respectively), mixed together after sterilization (at 50°C), then poured into 100 mm plates. The protease plates were kept overnight at 37°C to reduce moisture content. Cells grown for 15 h on tryptone agar were streaked in single lines on the plates. The zone of hydrolysis around bacterial growth was measured at 24 h.

Laboratory Animals

Two different strains of mice were used in the CF P. aeruginosa virulence studies. The first set of virulence tests were performed using female Ha/ICR mice weighing 22-24 g (Cumberland View Farms, Clinton, TN). For LD₅₀ determination, female CF-I mice weighing 19-21 g were used (Charles River, Wilmington, MA). The mice were maintained in groups of 10, given food and water ad libitum, and were used within two weeks after their arrival.

Experimental Burn Procedure

A modification of the burned mouse model of Stieritz and Holder (83) was used to study the virulence of P. aeruginosa CF strains. The backs of mice were shaved one day prior to the burn procedure. The mice were anaesthetized by placing them in a closed container containing the inhalation anaesthetic Methoxyflurane (Metofane, Pitman-Moore, Washington Crossing, NJ) for approximately 2 min. A

teflon board with 35 x 25 mm window was pressed firmly against the shaven back of the mouse. Ethanol (0.5 ml, 95%) was applied to the exposed portion of the back, ignited, allowed to burn for 13 sec, then immediately extinguished. This results in a second degree burn covering approximately 30% of the total body surface. Immediately following the burn, all mice were injected intraperitoneally with 0.5 ml sterile physiological saline for fluid replacement.

Bacteria to be injected into mice were cultured in Luria broth. The actual number of bacteria per inoculum was determined using the plate count method. The mice were challenged immediately post-burn by subcutaneous injection of 0.1 ml of bacterial suspension. Five mice per cage were used in the initial screening for virulence. For LD₅₀ determination, there were 8 mice per cage. Along with each bacterial dose group injected, there were two controls. One group of mice received a subcutaneous injection of 0.1 ml sterile diluent at the burn site. The other control group received 0.1 ml of M-2 at 1×10^2 CFU/0.1 ml (LD₅₀ 1.5×10^1 for Ha/ICR mice and 5×10^0 for CF-I mice: D.R. Drake, personal communication) injected subcutaneously at the burn site. The number of deaths per group of mice was recorded for seven days.

CHAPTER III

RESULTS

The Pseudomonas aeruginosa cystic fibrosis sputum strains and respective patient clinical condition are listed in Table 1. All strains are classified into three colonial morphology types described by Wahba (89). Initially, rough colonial morphology was chosen to avoid mucoidy, a characteristic associated with classical morphology which is unstable in solid medium (8, 66). As the study progressed classic strains and one mucoid strain were obtained for comparison to rough strains.

O Antigen Serotypes

Since colonial morphology may be ambiguous with respect to describing the LPS composition of the isolates, O antigen type was determined. Strains from patients in good clinical condition and those from patients in poor clinical condition were tested. Twenty strains were serotyped using viable cells in a slide agglutination assay (Table 2). Only two of the rough strains were typable, a characteristic associated with intact LPS (35). Of the four rough strains which were non-typable, only one, 572b, was from a patient in good clinical condition. Forty-five per cent of the rough strains polyagglutinated with two or more antisera.

One third of the classic strains tested were typable. Surprisingly, the majority (55%) of the classical strains were

Table 1. Pseudomonas aeruginosa cystic fibrosis sputum strains used in this study.

Strains	Clinical Condition of Patient ^a	Colony Morphology	Isolation Date ^b
572b	good	rough	2-20-80
86f	good	rough	3-11-80
437e ₂	good	rough	10-31-81
412oo	good	rough	1-14-82
423mm	good	rough	1-16-81
320f	good	rough	9-01-82
351g	moderate	rough	3-29-82
402c	poor	rough	11-5-79
409g	poor	rough	10-9-79
96e	poor	rough	10-14-78
66g	poor	rough	10-27-78
155g	poor	rough	6-23-81
415gg	poor	rough	9-05-81
414ii	poor	rough	5-19-81
414mm	poor	rough	8-03-82
503cc	poor	rough	2-02-82
448mm	poor	rough	9-13-82
412a	good	muroid	1-30-80
435c	good	classic	9-1-79
903a	good	classic	10-29-81
35c	good	classic	3-1-79
902c	good	classic	6-29-82
412ee	good	classic	-
423gg	good	classic	-
320e	good	classic	-
414g	poor	classic	-
503g	poor	classic	-
448bb	poor	classic	-
541a	poor	classic	11-6-79
144b	poor	classic	12-16-78
776e	poor	classic	9-13-82
676e	poor	classic	6-22-82
572d	moderate	classic	3-04-82

Table 1 (Continued)

^aThe clinical evaluation of patients is based on a modification of the Schwachman-Kulczycki (85) scoring system in which a maximal score of 25 points is awarded for each of four categories - chest roentgenogram, general activity, physical activity, and nutrition. Patients in consultation with their physicians are then classified into three categories:

1. good: total score 75-100.
2. moderate: total score 60-74.
3. poor: total score less than 60.

^bDate of isolation was not available for strains 412ee - 448bb.

Table 2. O antigen serotypes of P. aeruginosa strains from cystic fibrosis patients.^a

Strain	Clinical condition	Colonial morphology ^b	Serotype ^c
86f	G	R	10
415gg	P	R	11
572b	G	R	NT
414nn	P	R	NT
414ii	P	R	NT
402c	P	R	NT
96e	P	R	PA
409g	P	R	PA
66g	P	R	PA
412oo	G	R	PA
320f	G	R	PA
412a	G	M	PA
35c	G	C	6
435c	G	C	PA
903a	G	C	4
902c	G	C	PA
144b	P	C	6
776e	P	C	PA
676e	P	C	PA
541a	P	C	NT
M-2	Invasive burn strain	C	5

^aSlide agglutination assay using viable cells and O-antisera (Difco-American Scientific) diluted 1:10.

^bC, classic; R, rough; M, mucoid.

^cNT, non-typable, no agglutination in any typing sera; PA, polyagglutinable, agglutinated by two or more typing sera.

polyagglutinable. However, in contrast to rough strains, only one classic strain, 541a, was non-typable.

Of the twenty strains tested, 75% were either polyagglutinable or non-typable; thus indicating that some type of LPS alteration is present in the majority of strains, regardless of patient clinical condition or colonial morphology.

Relative Motility and Chemotaxis of CF Strains in Motility Agar

All CF strains were tested for motility by comparing their colony swarming to M-2 in motility agar. In Figure 1, non-motile rough strains, 155c, 96e and 66g, were easily identified by formation of a non-diffuse colony at the site of inoculation which at 24 h measured only two mm in diameter. Actively motile strains, such as M-2 and 572b, displayed rapid colony spreading over a one to 24 h time period. This spreading required both motility and a chemotactic gradient (1); however, the total phenomenon will be described as "motility" when discussing soft agar assays. The data show that, in Figure 2, strain 86f, from a patient in good clinical condition, was more motile than the control strain. In contrast, the other two strains, 402c, from a patient in poor clinical condition, and 437e₂, an auxotroph from a patient in good clinical condition, exhibited motility less than one half of 86f. All the strains tested in Figure 3 were from patients in poor clinical condition and displayed no motility in soft agar. In Figure 4, 320f and 412oo (from patients in good clinical condition) were similar to M-2 in motility, while 415gg was from a patient in poor clinical condition and was only slightly motile.

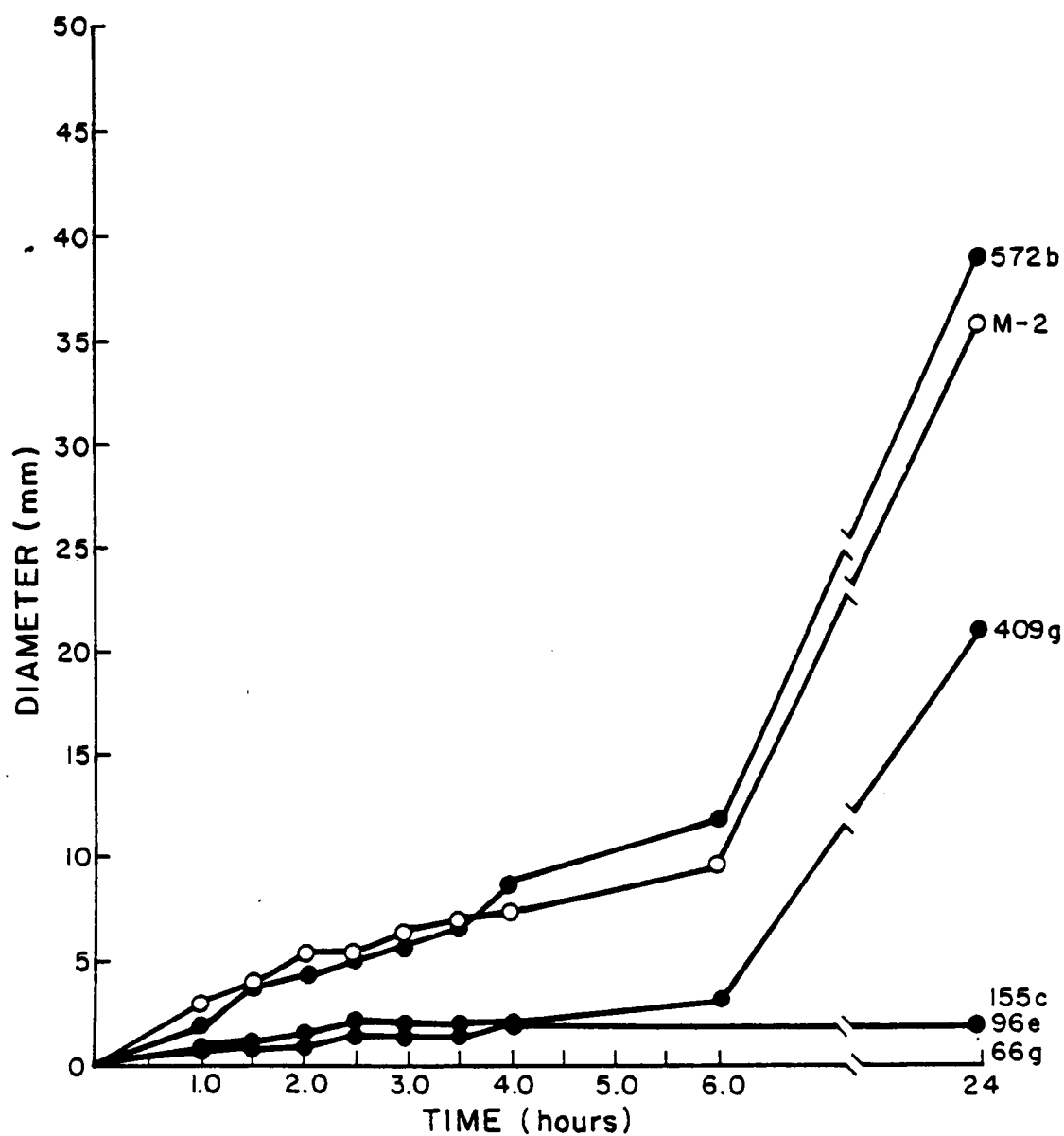


Figure 1. Migration of rough *P. aeruginosa* strains in soft agar. Overnight growth from a tryptone agar plate was stabbed in the center of a plate containing 0.3% yeast extract, 1.0% tryptone, and 0.5% sodium chloride in 0.3% agar. The diameter of the bacterial swarm was measured over a period of 24 h at 37°C.

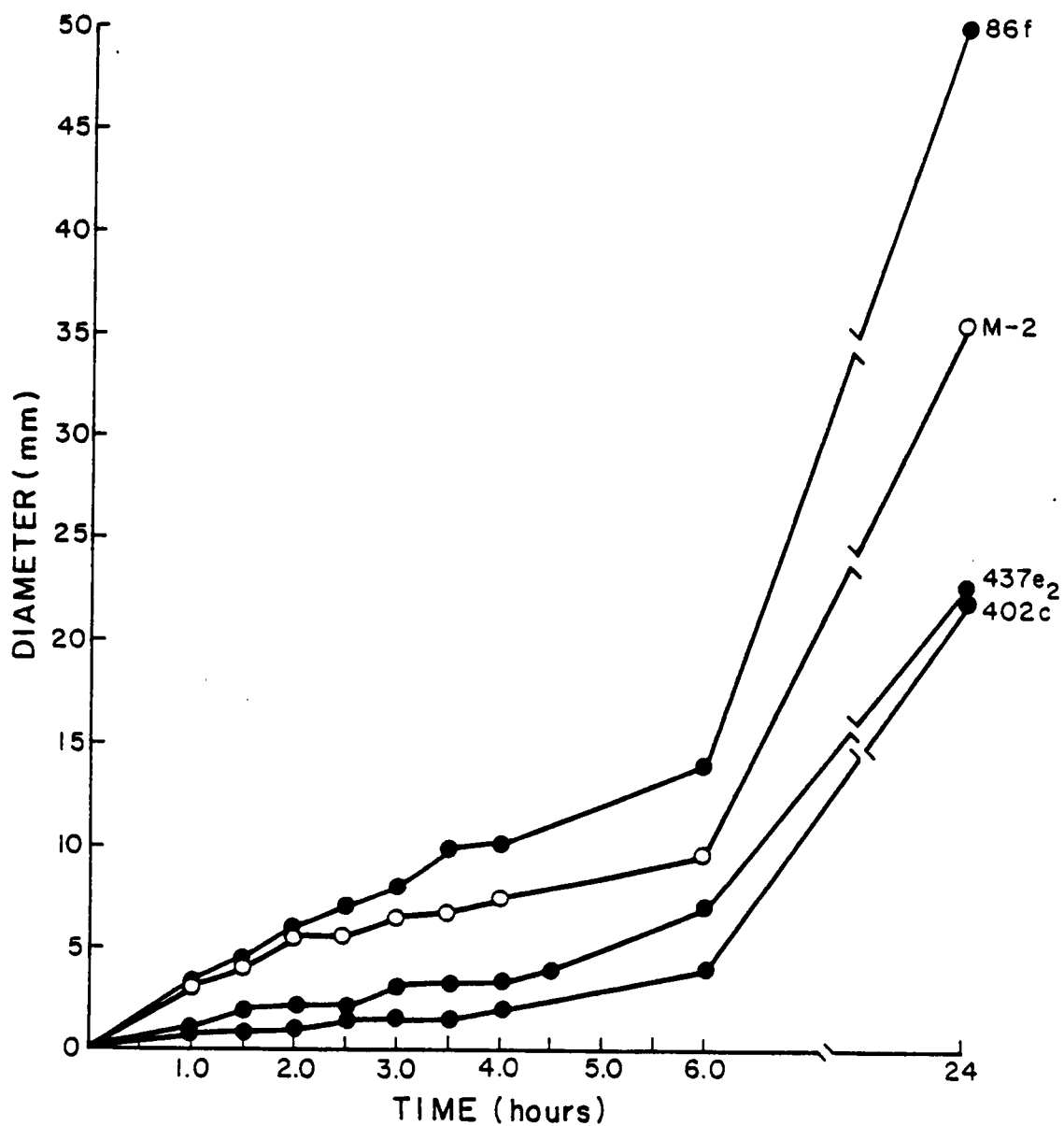


Figure 2. Migration in motility agar of rough *P. aeruginosa* strains from CF patients in good and poor clinical condition. See Fig. 1 for procedure.

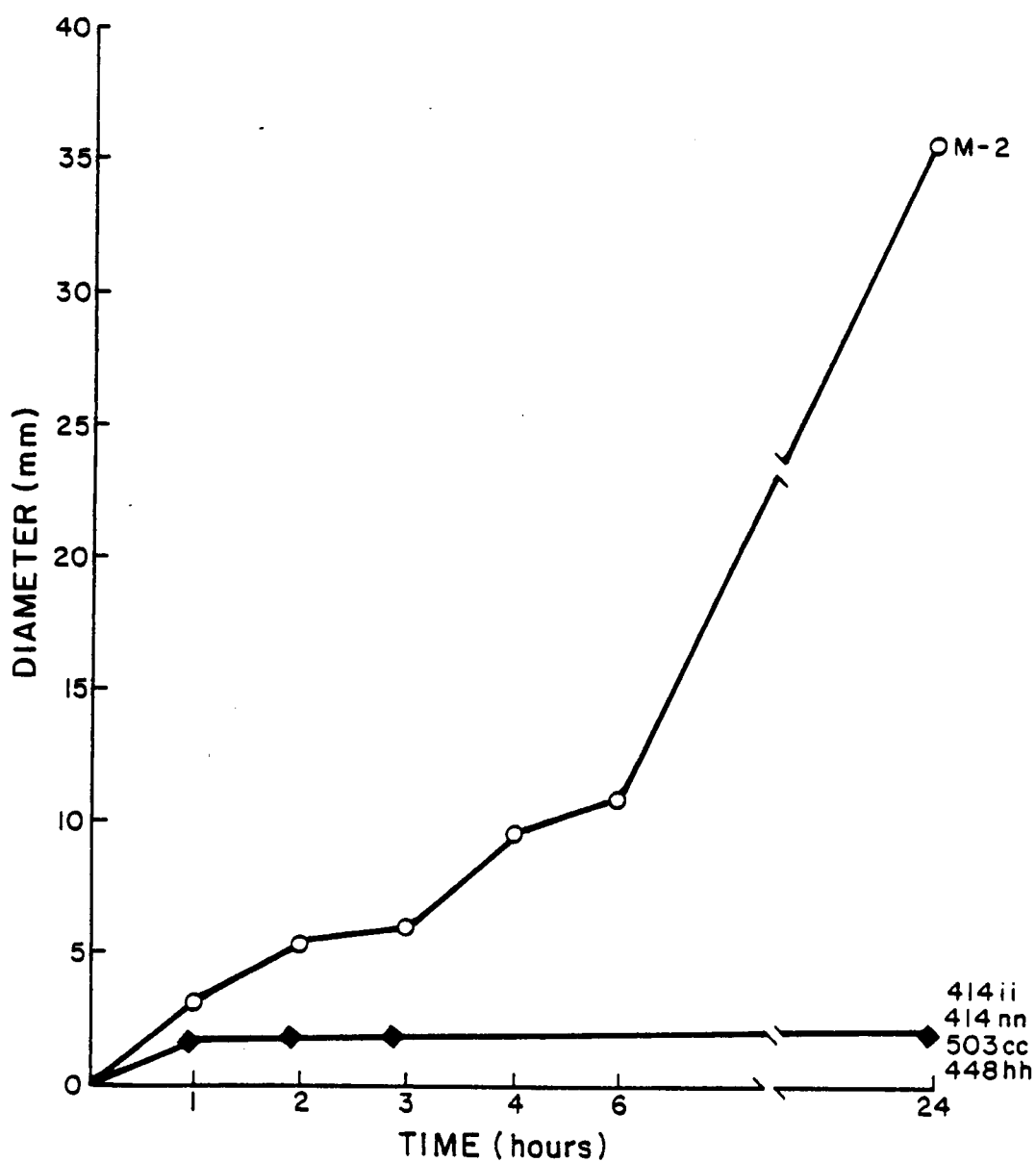


Figure 3. Migration in motility agar of rough *P. aeruginosa* strains from CF patients in poor clinical condition. See Fig. 1 for procedure.

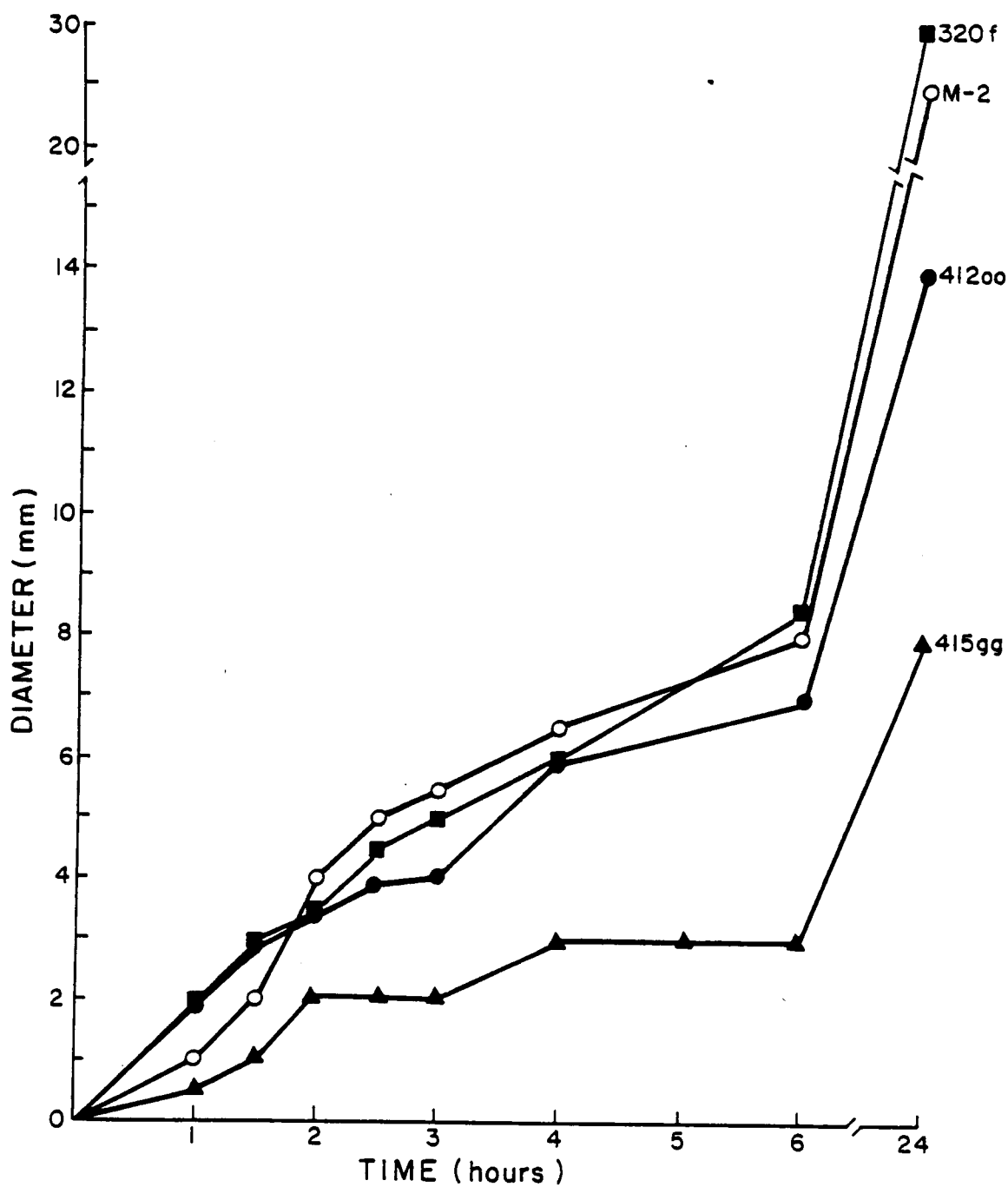


Figure 4. Migration in motility agar of rough *P. aeruginosa* strains from CF patients in good and poor clinical condition. See Fig. 1 for procedure.

Migration in motility agar of classic P. aeruginosa strains used for comparison to rough strains was illustrated in Figure 5. Non-flagellated classic strains were not tested in motility agar, since they were identified prior to the assay by electron microscopy (see below). Two strains, 412a and 35c, from patients in good clinical condition, displayed 24 hour motility similar to M-2 (Figure 5). However, during the first six hours, 35c motility is slightly greater than half that of M-2. Strain 902c, from a CF patient in good clinical condition, displayed colony spread similar to M-2 during the first six hours of the assay (Figure 6). In contrast, both 144b and 541a, strains from patients in poor clinical condition, showed reduced motility as compared to M-2. During the first six hours of the assay, 144b exhibited less than half the motility of M-2, while 541a demonstrated approximately a one-third reduction compared to the control.

Detection of Flagella by Electron Microscopy

Since a large number of strains exhibited no motility in soft agar, the question arose as to whether or not these non-motile organisms possessed flagella. A single, polar flagellum is one of the major taxonomic, phenotypic characteristics of P. aeruginosa and its absence in clinical strains is rarely observed (55). Electron micrographs of a typical flagellated (572b) and non-flagellated (96e) strain are shown in Figures 7 and 8. In Table 3 a summary of electron micrograph data shows that 47% of the strains from patients in poor clinical condition lacked flagella, in contrast to this

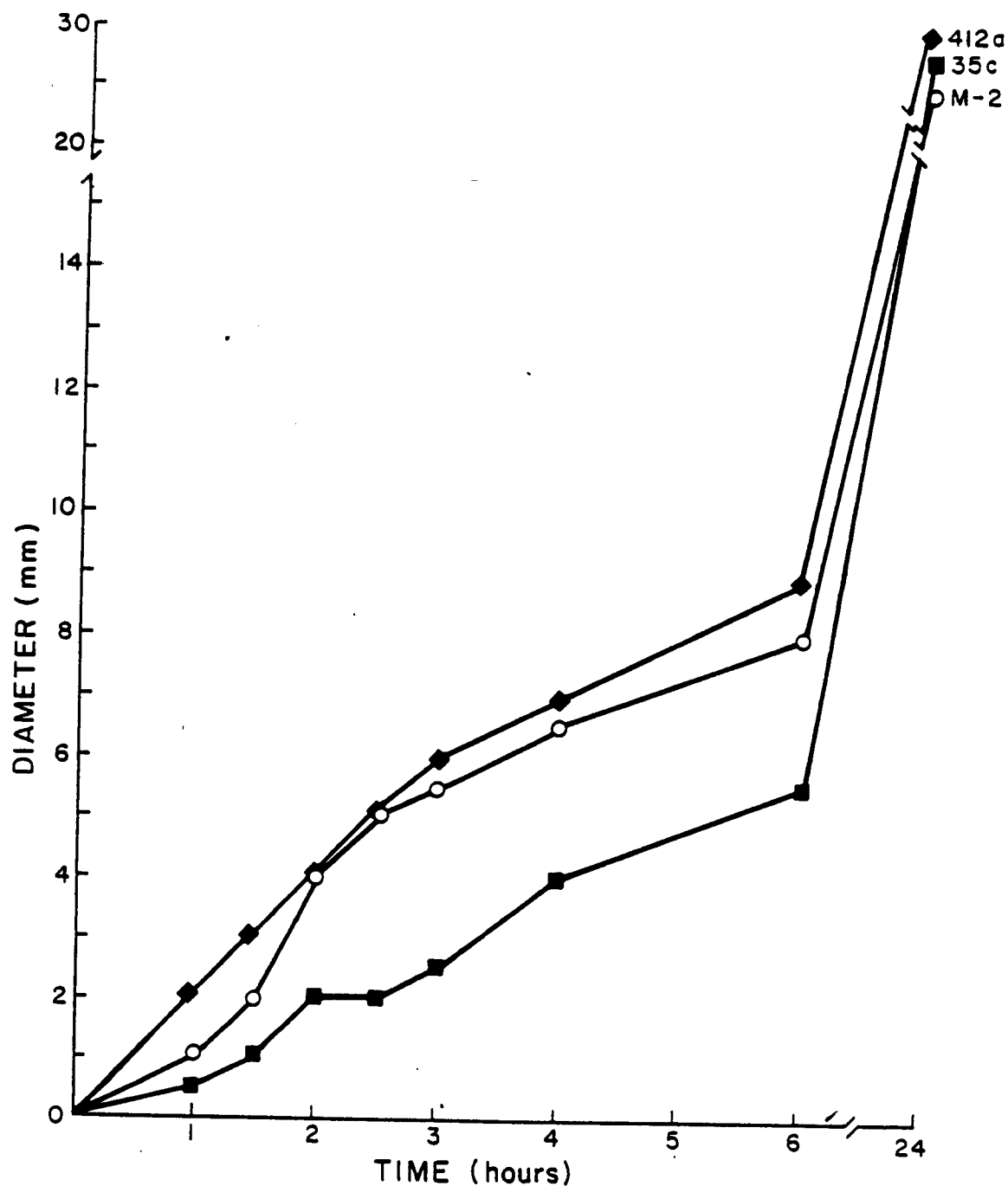


Figure 5. Migration in motility agar of classic *P. aeruginosa* strains from patients in good clinical condition. See Fig. 1 for procedure.

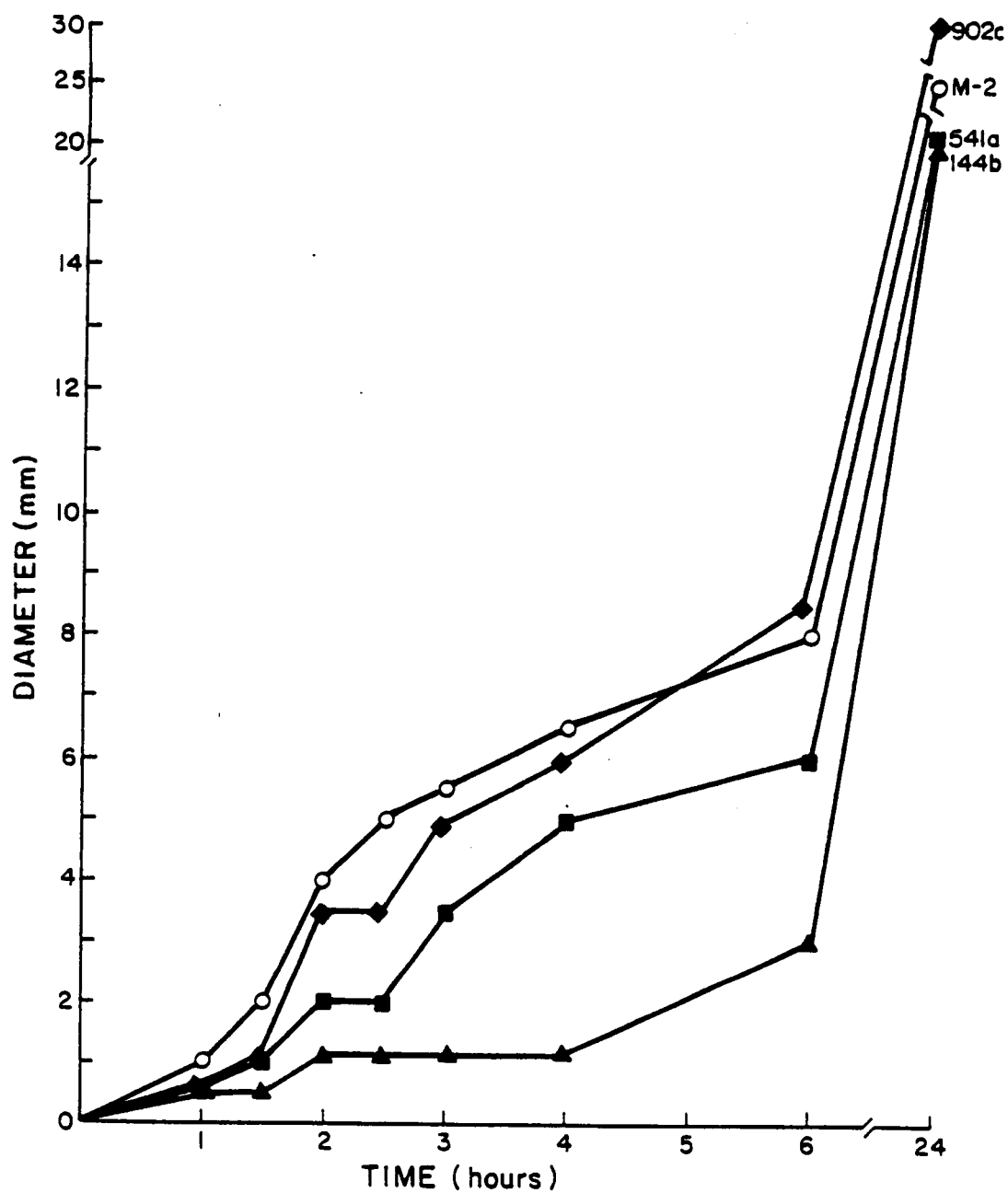


Figure 6. Migration in motility agar of classic *P. aeruginosa* strains from patients in good and poor clinical condition. See Fig. 1 for procedure.

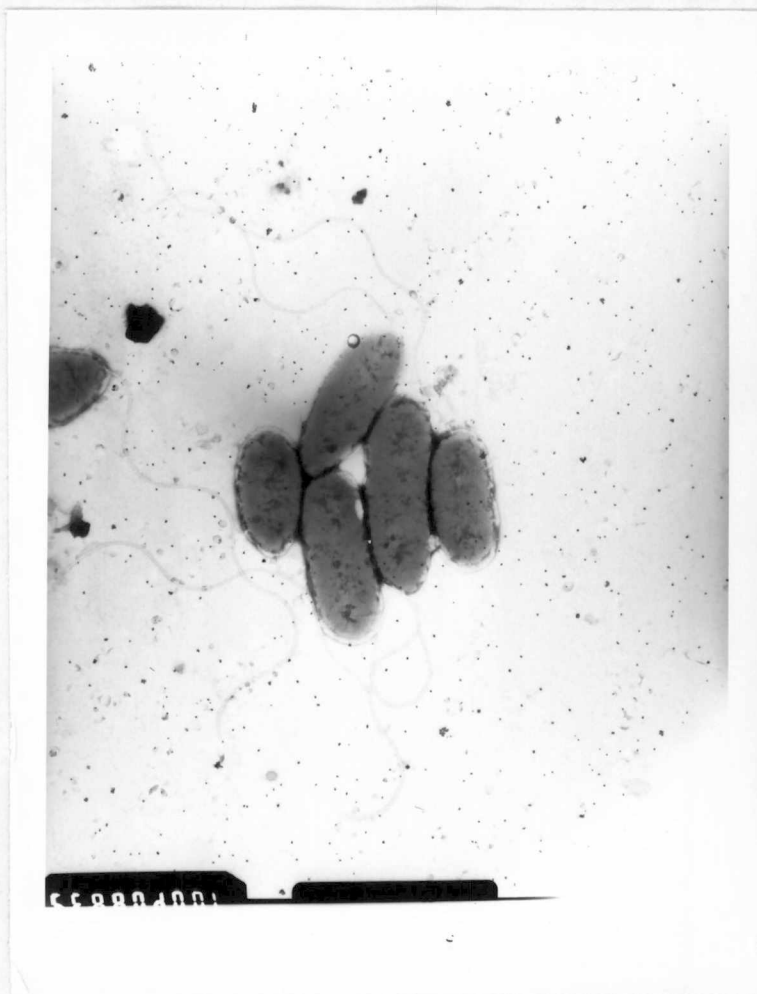


Figure 7. Electron micrograph of flagellated strain 572b from CF patient in good clinical condition. Cells were grown 14-16 h in Luria broth and stained with 0.5% phosphotungstic acid. Magnification: 10,000 x.

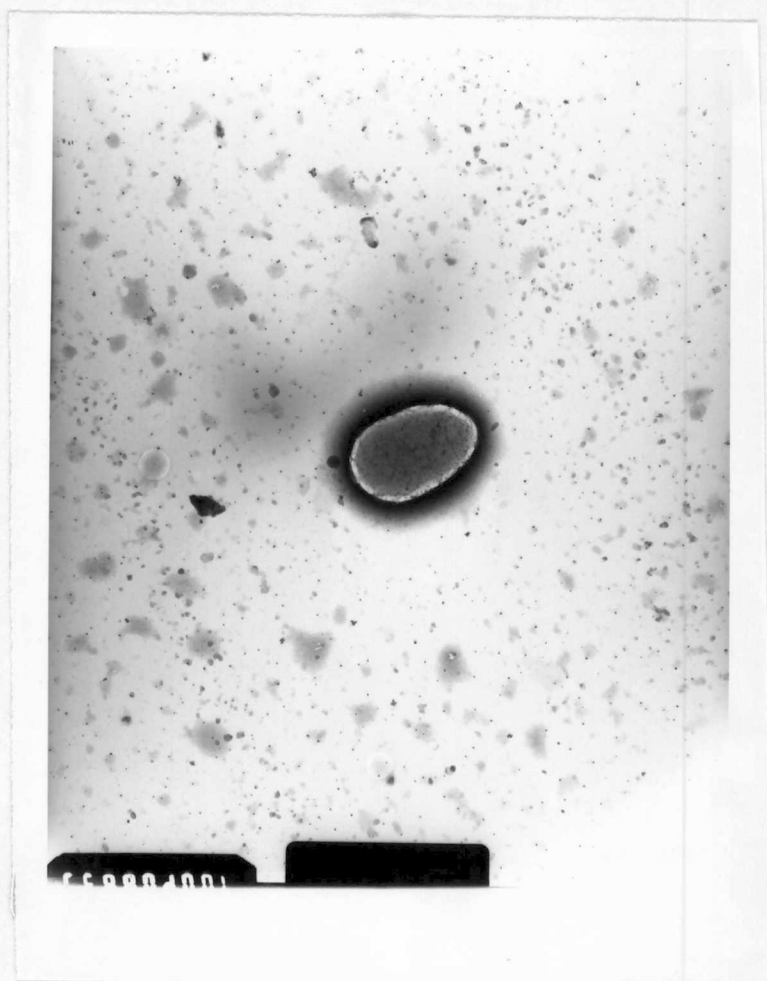


Figure 8. Electron micrograph of 96e, a non-flagellated strain from a CF patient in poor clinical condition. See Fig. 7 for preparation of cells.

Table 3. Summary of properties of *P. aeruginosa* strains from cystic fibrosis patients.

Strain	Clinical condition	Colonial morphology ^a	Flagellum ^b	Motility in soft agar ^c	
				6 h	24 h
M-2	invasive burn strain	C	+	8	37
437e ₂	G	R	+	7	23
572b	G	R	+	12	39
86f	G	R	+	13	50
412oo	G	R	+	9	12
320f	G	R	+	9	30
435c	G	C	+	8	35
903a	G	C	+	5	22
35c	G	C	+	5	28
902c	G	C	+	8	31
320e	G	C	+	-	-
412a	G	M	+	9	31
351g	M	R	+	-	-
572d	M	C	+	8	26
402c	P	R	+	4	20
409g	P	R	+	3	21
415gg	P	R	+	3	8
414g	P	C	+	-	-
503g	P	C	+	-	-
541a	P	C	+	5	21
144b	P	C	+	3	20
776e	P	C	+	-	23
676e	P	C	+	-	15
423mm	G	R	-	2	2
412ee	G	C	-	2	2
423gg	G	C	-	2	2
155c	P	R	-	2	2
96e	P	R	-	2	2
66g	P	R	-	2	2
414ii	P	R	-	2	2
414nn	P	R	-	2	2
503cc	P	R	-	2	2
448hh	P	R	-	2	2
448bb	P	C	-	2	2

^aC, classic; R, rough, M, mucoid.

^bVisible by EM; magnification: 10,000 x.

^cDiameter in millimeters; two mm represents non-spreading colonies.

absence in only 21% of the strains from patients in good clinical condition. It should be emphasized that flagella absence demonstrated by electron microscopy corresponded 100% with the results of the motility assays. Flagella absence was the most striking in those strains of rough colonial morphology. Seventy percent of the rough strains from patients in poor clinical condition lacked flagella, while only one rough strain from a patient in good clinical condition was non-flagellated. In contrast, 80% of all classic strains examined were flagellated; the three non-flagellated strains were from patients in both good and poor condition.

Polyacrylamide Gel Electrophoresis of Flagella Extracts

Previous studies by Ansorg show that there are two major types of flagellin protein among strains of P. aeruginosa (5). Type "a" shows a complex antigen structure with several combinations of subtypes a_0 , a_1 , a_2 , a_3 , a_4 ; type "b" shows a uniform antigenic structure. Type "a" flagellins range in molecular weight from 45,000 to 52,000 (62). Type "b" flagellin have a molecular weight of 53,000 (62).

Flagella were isolated from selected CF P. aeruginosa strains by a modification of the method of Montie et al. (61) and were examined electrophoretically. Two strains, 66g and 96e, characterized as non-flagellated by electronmicroscopy, were treated in an identical manner to the flagellated strains.

The flagellated CF strains possessed the two types of flagellin protein characteristic of P. aeruginosa (Figure 9). The M-2 flagellin band (lane B) corresponds to the 53,000 molecular weight

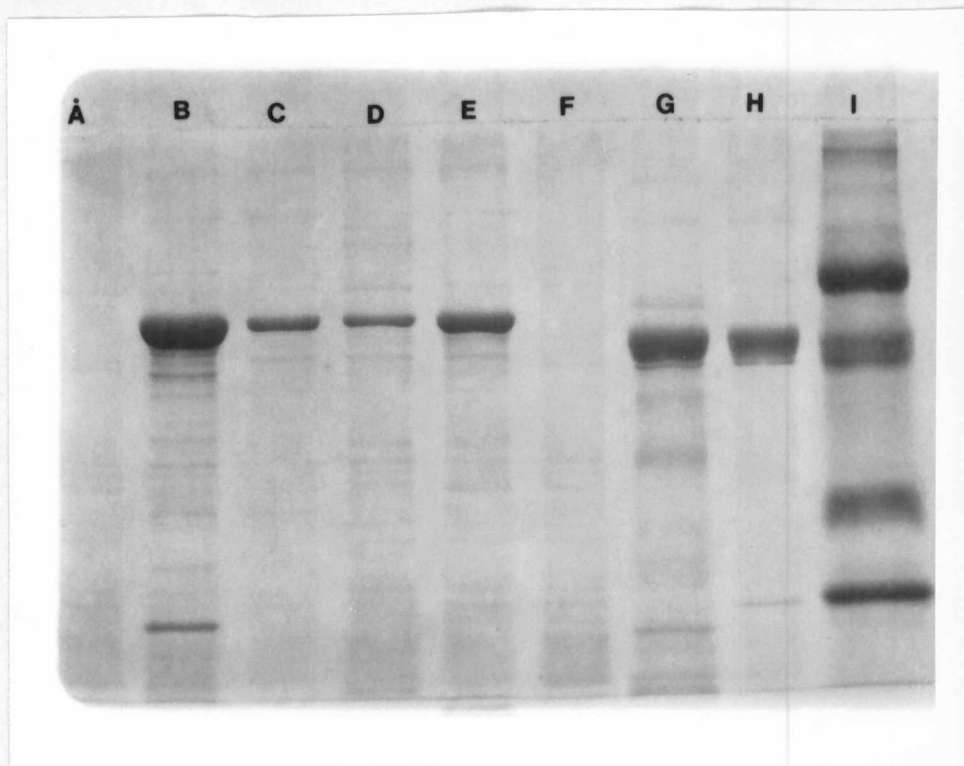


Figure 9. 10% SDS-PAGE (1.5 μ g/protein/well) of *P. aeruginosa* flagellin proteins. Cells were grown in mineral salts plus 0.4% succinate. Flagella were harvested by a modification of the method of Montie et al. (61). Lanes A and F: 66g, a non-flagellated isolate; lane B: M-2; lane C: 409g; lane D: 402c; lane E: 17001; lane G: 572b; lane H: 86f; lane I: standard composed of bovine serum albumin, ovalbumin, and β -lactoglobulin (BSA). Lanes B, C, D and E contain type "b" flagellin antigen; lanes G and H contain type "a" flagellin antigen.

protein characteristic of Ansorg's type b (5). Strains 409g and 402c (lanes C and D, respectively) also possess type b flagella. Lanes A and F show that no flagellin protein is present in strain 66g, thus confirming the results of electron microscopy. Strains 572b and 86f (lanes G and H) possess type "a" flagellin antigens with molecular weights <53,000. SDS-PAGE isolation of flagellin proteins obtained from cells grown in Luria broth (Figure 10) gave the same results as those seen in Figure 9. The flagellin band of M-2 (lanes A, D, E,) is clearly contrasted to the absence of the same protein in lane B (66g) and lane C (96e).

Growth Comparisons

Tactic responses of bacteria toward amino acids might be influenced by the growth medium used in the capillary assays. An organism unable to utilize efficiently the mineral salts medium plus succinate as a carbon source (MSS) may appear non-chemotactic and exhibit reduced motility. Inability to grow well in an enriched medium such as Luria broth could account for reduced virulence in the animal model and reduction of motility in the soft agar assays. Therefore, it was important to establish whether or not CF strains were able to efficiently utilize the growth media required for these experiments. Experiments showed that reduced motility of 402c was not due to an inability to grow in MSS (Figure 11). The generation time of 402c was 93% that of M-2. Both 86f and 66g (a non-flagellated strain) grew as well as M-2 in MSS (Figure 12). In general, no marked differences in growth were observed for the strains tested in the chemotaxis capillary assay.

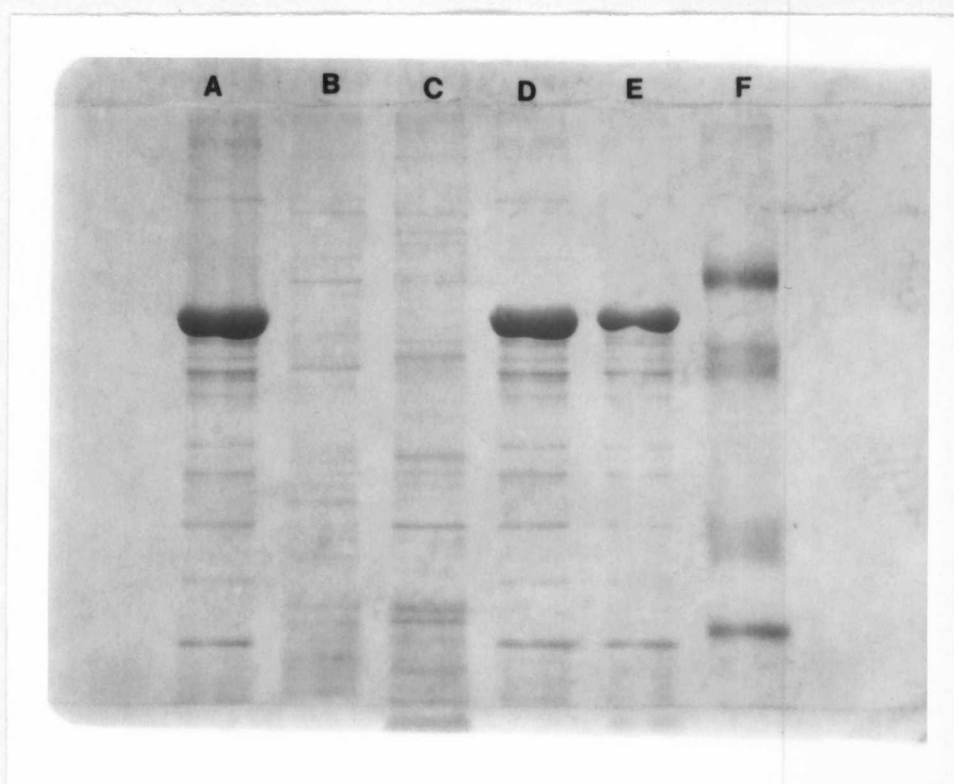


Figure 10. 10% SDS-PAGE (1.5 μ g/protein/well) of *P. aeruginosa* flagellin proteins. Cells were grown in Luria broth. Flagella were harvested by a modification of Montie et al. (61). Lanes A, D, and E: M-2; lane B: 66g (non-flagellated); lane C: 96e (non-flagellated); lane F: standard composed of BSA, ovalbumin and β -lactoglobulin.

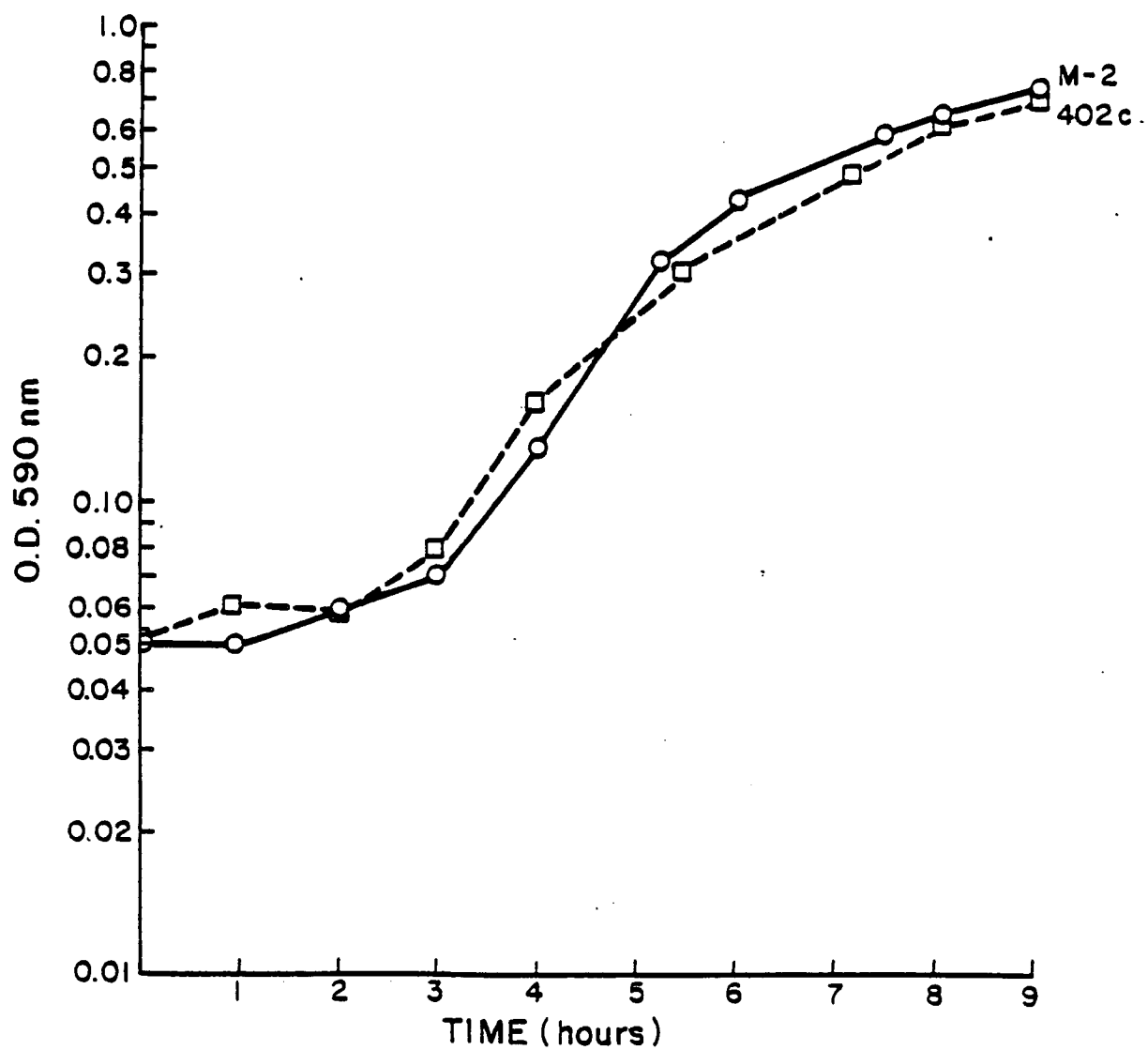


Figure 11. Growth curves of *P. aeruginosa* strains 402c and M-2 grown in MSS.

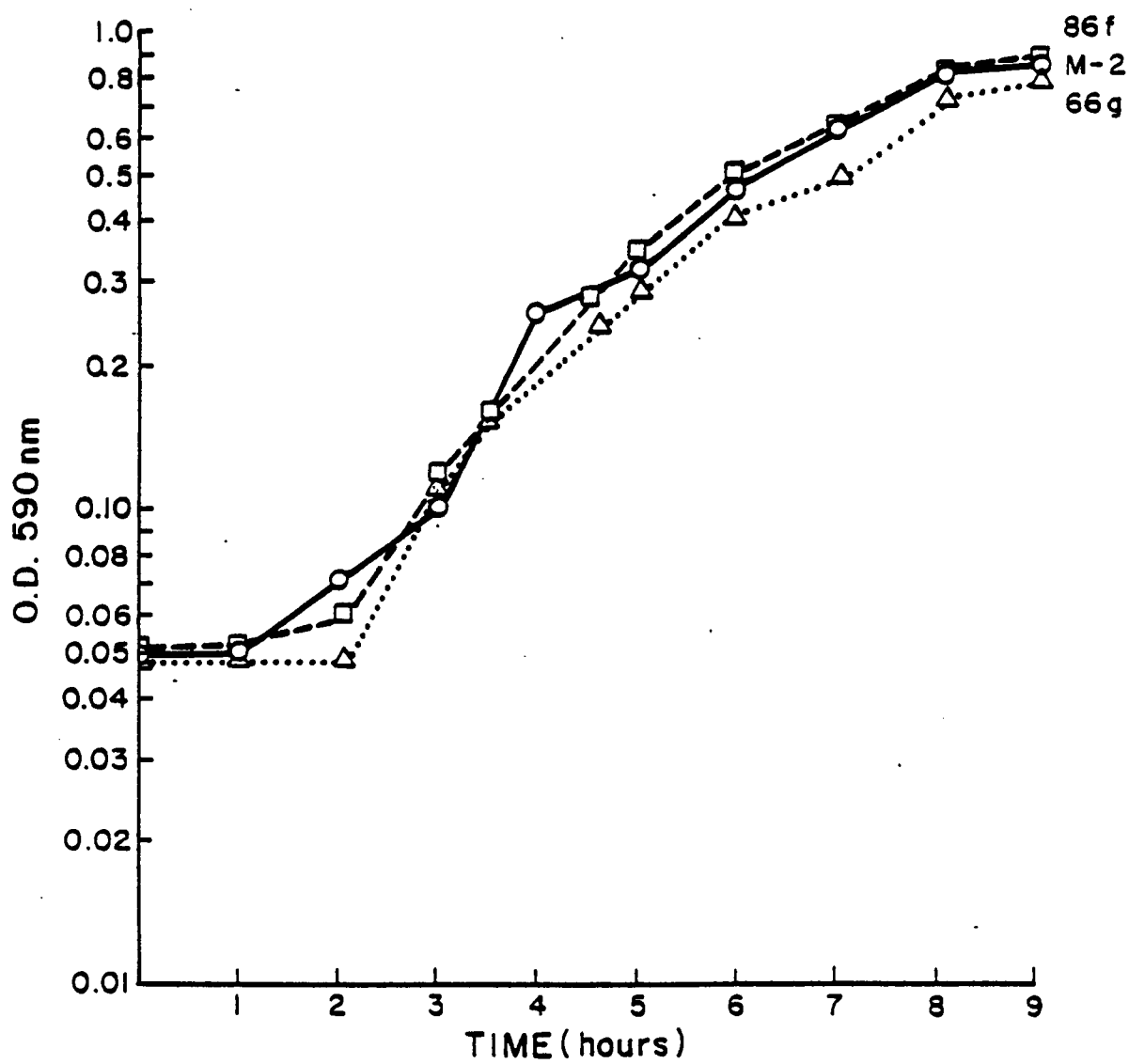


Figure 12. Growth curves of strains 86f, 66g, and M-2 grown in MSS.

One can conclude from the summary growth chart (Table 4) that metabolic deficiencies were not responsible for altered motility or chemotaxis. Auxotrophs such as 437e₂, 351g and 155c are found in patients in all clinical conditions and represent only 23% of the strains tested. It is important to note that all strains grew well in Luria broth, the medium used for virulence studies (see appendix, Table 14)

Quantitation of Chemotaxis and Motility in the Capillary Assay

Recent studies indicate that chemotaxis and motility may be important invasive factors for P. aeruginosa in acute infections following thermal injury (60, 63). In previous research by Craven and Montie (20), strain M-2, which is highly invasive in the burned mouse model, was examined for chemotaxis and motility. In this study, M-2 proved to be highly motile and chemotactic to a number of amino acids and sugars. The mean accumulation of bacteria in buffer capillaries, a quantitative measure of motility, was 1.9×10^4 cells per capillary during a thirty minute assay. Two amino acids, arginine (ARG) and aminoisobutyrate (AIB), were strong attractants for M-2. With M-2, ARG consistently yielded a greater accumulation of cells per capillary than AIB (93.9×10^4 vs 79.0×10^4 respectively). Since AIB is a non-metabolizable analog of glycine, it has an important advantage as an attractant in chemotaxis studies. The chemotactic gradient can be more accurately examined without interference from effects caused by metabolism of the attractant.

Consistent with results of Craven and Montie, the chemotactic response of M-2 in this investigation was greatest for ARG. Results

Table 4. Summary chart of *P. aeruginosa* strains grown in MSS, MSG, and Luria broth.

Strain ^a	Mineral salts + 0.4% succinate	Mineral salts + 0.4% glucose	Luria broth
572b (G)	+	+	+
86f (G)	+	+	+
412a (G)	+	+	+
412oo (G)	+	+	+
320f (G)	+	+	+
435c (G)	+	+	+
903a (G)	+	+	+
35c (G)	+	+	+
902c (G)	+	+	+
423mm ^a (G)	-	+	+
437e ₂ ^a (G)	-	-	+
351g ^a (M)	-	-	+
572d (M)	+	+	+
66g ^a (P)	-	+	+
415gg ^a (P)	-	+	+
155c ^a (P)	-	-	+
402c (P)	+	+	+
409g (P)	+	+	+
96e (P)	+	+	+
414ii (P)	+	+	+
414nn (P)	+	+	+
503cc (P)	+	+	+
448hh (P)	+	+	+
541a (P)	+	+	+
776e (P)	+	+	+
144b (P)	+	+	+
M-2	+	+	+

^aAuxotroph, strain which exhibited no visible growth (O.D.₅₉₀ < 0.05) after 18 h in mineral salts plus glucose or succinate.

from chemotaxis capillary assay of M-2 in Table 5 show its strong response toward all three amino acid attractants. The average accumulation of bacteria per capillary for three assays was 65.1×10^4 for ARG as compared to approximately half that number of cells per capillary for SER and AIB. Although there was day to day variation in M-2 motility, the mean accumulation of cells per buffer capillary (2.5×10^4) was comparable to that reported by Craven and Montie.

Motility and amino acid chemotactic response of flagellated cystic fibrosis strains were compared to M-2. The results for each strain represent an average of repeated assays for each attractant performed on different days. These averages are compared to M-2 results of assays performed on the same days.

Chemotactic capacity and motility of rough strains from patients in good clinical condition are shown in Table 6. Strain 572b was highly chemotactic toward all three amino acids. Its average response to ARG was comparable to that of M-2 (94%). Response exceeded that of M-2 for the other two attractants. The mean accumulation of CFU in the buffer capillary assay was 127% that of M-2. Strains 86f and 41200 were also highly motile and chemotactic. There was a statistically significant difference between 86f and M-2 in response to AIB ($232\% > \text{M-2}$). Of the four rough strains tested, only 320f showed a statistically significant reduction in response to ARG. In addition to reduced chemotactic response, the motility of

Table 5. Motility and amino acid taxis of M-2.^a

Accumulation (10 ⁴ CFU/capillary)			
Serine 10 ⁻³ M	AlB 10 ⁻² M ^b	Arginine 10 ⁻² M	Buffer
29.4	15.6	-	1.3
55.9	41.2	-	3.9
32.3	14.8	-	2.6
34.0	45.0	-	2.0
		75.4	3.2
		43.9	1.7
		75.9	2.7
Ave 37.9	29.15	65.0	2.5

^aCells were grown in mineral salts plus 0.4% succinate.

^bAminoisobutyrate, an amino acid not metabolized by P. aeruginosa.

Table 6. Motility and amino acid taxis of rough *P. aeruginosa* strains from CF patients in good clinical condition.

Strain ^a	Accumulation (10^4 CFU/capillary) (%) ^b			
	Serine 10^{-3} M	AIB 10^{-2} M	Arginine 10^{-2} M	Buffer
572b (G)	65.7 (203)	23.6 (159)	-	3.4 (130)
	75.4 (122)	54.0 (120)	-	.75 (38)
	56.0 (164)	67.0 (146)	-	1.4 (77)
			50.2 (114)	5.2 (300)
			56.5 (74)	2.4 (89)
Ave.	(163)	(142)	(94)	(127)
86f (G)	31.6 (176)	46.8 (238)	-	5.5 (350)
	50.3 (171)	42.6 (273)	-	5.6 (445)
	64.4 (115)	76.8 (186)	-	2.0 (51)
			49.5 (112)	3.3 (192)
			76.7 (101)	1.1 (40)
Ave.	(154)	(232) ^c	(107)	(216) ^c
412oo (G)	-	-	66.4 (120)	5.9 (105)
	56.7 (126)	-	78.9 (110)	2.2 (96)
Ave.	(126)		(115)	(101)
320f (G)	-	-	12.8 (23)	1.4 (24)
	-	-	21.5 (30)	0.8 (33)
	-	-	25.8 (40)	2.6 (79)
Ave.			(31) ^c	(45)
M-2	(100)	(100)	(100)	(100)

^aGrowth medium was mineral salts plus 0.4% succinate.^bCompared to M-2 on the same day.^cResponse was significantly different from M-2 as determined by Student's t-test.

320f was only half that of M-2. These results are generally consistent with results observed in the motility assays (Figures 1-6).

The motility and amino acid taxis of rough strains from patients in poor clinical condition are shown in Table 7. Strain 415gg was not tested since it is a succinate auxotroph. The chemotactic response of 402c to all three attractants was 14% that of M-2. These differences were statistically significant. The chemotactic response of strain 409g was also reduced for all three attractants. This reduction corresponded closely to the results of the buffer capillary assay.

Previous studies by Moulton and Montie (63) found that the concentration response curve for SER showed a peak at 10^{-3} M and 10^{-2} M for ARG and AIB. Since these optimum molar concentrations of attractants were used in the capillary assays, the relative lack of 402c chemotaxis response to the amino acids could be due to the requirement of concentrations other than the ones used. However, results of 402c taxis toward ARG and AIB at different molar concentrations shown in Table 8 indicate that this was not the case. Chemotactic response of 402c was minimal for all three concentrations of ARG and AIB; while M-2, tested on the same day, showed characteristic chemotaxis and motility.

Classic strains from patients in good clinical condition (Table 9) differed in both their taxis toward ARG and AIB and their motility. Strain 902c was significantly more chemotactic than M-2.

Table 7. Motility and amino acid taxis of rough *P. aeruginosa* strains from CF patients in poor clinical condition.

Strain ^a	Accumulation (10^4 CFU/bacteria) (%) ^b			
	Serine $10^{-3}M$	AIB $10^{-2}M$	Arginine $10^{-2}M$	Buffer
402c (P)	2.4 (13)	3.1 (15)	-	1.5 (94)
	1.1 (3)	0.8 (5)	-	0.5 (36)
	-	2.5 (6)	-	0.5 (13)
	8.7 (27)	4.9 (33)	-	2.3 (87)
	2.0 (5)	-	6.2 (8)	0.4 (14)
	-	-	14.0 (18)	1.2 (46)
	Ave. (12) ^c	(15) ^c	(13) ^c	(48)
409g (P)	16.0 (47)	31.0 (69)	-	0.8 (40)
	36.9 (60)	25.6 (56)	-	0.8 (43)
	32.0 (84)	-	34.2 (45)	2.9 (90)
			47.0 (62)	2.2 (81)
	Ave. (64)	(63)	(54)	(64)
M-2	(100)	(100)	(100)	(100)

^aGrowth medium was mineral salts plus 0.4% succinate.

^bCompared to M-2 on the same day.

^cResponse was significantly different from M-2 as determined by Student's t-test.

Table 8. Taxis of P. aeruginosa 402c using different concentrations of amino acids.

Strain ^a	Concentration	Accumulation (10 ⁴ CFU/capillary)		
		AIB	Arginine	Buffer
402c	10 ⁻² M	0.5	0.3	-
	10 ⁻³ M	0.6	0.6	0.3
	10 ⁻⁴ M	0.04	0.3	-
M-2 ^b	10 ⁻² M	16.0	65.0	3.0

^aGrowth medium was mineral salts plus 0.4% succinate.

^bTested on the same day.

Table 9. Motility and amino acid taxis of classic *P. aeruginosa* strains from CF patients in good clinical condition.

Strain ^a	Accumulation (10 ⁴ CFU/capillary) (%) ^b		
	AIB 10 ⁻² M	Arginine 10 ⁻² M	Buffer
35c (G)	12.7 (134)	33.8 (82)	0.9 (56)
	-	64.5 (92)	0.7 (28)
	-	51.7 (86)	0.7 (44)
	57.6 (124)	57.0 (244)	2.1 (102)
Ave.	(129)	(126)	(58)
435c (G)	4.6 (48)	36.7 (89)	0.3 (19)
	-	38.9 (55)	0.6 (21)
	-	11.4 (19)	0.5 (32)
	1.4 (3)	2.5 (11)	0.4 (17)
Ave.	(26) ^c	(43)	(22) ^c
902c (G)	69.3 (404)	72.7 (134)	4.0 (226)
	-	77.6 (130)	1.3 (87)
	84.6 (183)	89.3 (382)	3.6 (176)
Ave.	(294) ^c	(215) ^c	(163)
412a ^d (G)		21.2 (38)	2.9 (52)
	19.8 (132)	51.6 (79)	2.2 (66)
Ave.	(132)	(59)	(59)
M-2	(100)	(100)	(100)

^aGrowth medium was mineral salts plus 0.4% succinate.

^bCompared to M-2 on the same day.

^cResponse was significantly different from M-2 as determined by Student's t-test.

^dMucoid strain.

The response of 435c is reduced for both attractants, but only the taxis reduction toward AIB is statistically significant. This is also the only strain in this group which demonstrated significantly reduced motility in the capillary assay. Strain 35c, although slightly less motile than M-2, was highly chemotactic for both ARG and AIB. The motility and chemotactic responses of 412a, a mucoid strain, were not significantly different from M-2. However, its response to AIB was greater than its response toward ARG.

All four classic strains from patients in poor clinical condition were similar to M-2 in motility buffer capillary cell counts (Table 10). Only strain 541a showed reduced motility, but the reduction was not significant. In contrast, there was a general trend toward reduction in chemotaxis. Strains 676e and 144b gave responses to ARG (12 and 17% of M-2, respectively) similar to 402c, as previously seen. Response to AIB for 144b and 776e were deficient, with that of 144b being significantly reduced to 19% of M-2. Therefore, data in Table 10 shows that two of the four strains exhibited significant reduction in chemotaxis, while only 541a gave a somewhat stronger response toward ARG.

Of the five rough strains (from patients in good condition), only 320f exhibited reduced chemotaxis for ARG. The two rough strains (from patients in poor condition) showed reduction in chemotaxis for all three attractants, although only the response of 402c was significantly different from that of M-2. Of the seven classic strains tested, only 435c exhibited a significant reduction in motility. In contrast to rough strains, four of the seven classic

Table 10. Motility and amino acid taxis of classic *P. aeruginosa* strains from CF patients in poor clinical condition.

Strain ^a	Accumulation (10 ⁴ CFU/capillary) (%) ^b		
	AIB 10 ⁻² M	Arginine 10 ⁻² M	Buffer
776e (P)	4.9 (51)	29.3 (71)	2.1 (133)
	-	14.5 (20)	1.0 (38)
	-	28.2 (47)	1.9 (124)
Ave.	(51)	(46)	(98)
676e ^d (P)	-	7.7 (13)	2.1 (139)
	-	2.7 (11)	1.6 (76)
Ave.		(12) ^c	(108)
144b (P)	5.4 (32)	19.7 (36)	1.4 (73)
	-	6.1 (9)	0.6 (25)
	2.8 (6)	1.3 (6)	2.1 (102)
Ave.	(19) ^c	(17) ^c	(67)
541a (P)	-	86.7 (160)	1.2 (65)
	-	77.6 (110)	0.3 (10)
Ave.	-	(135)	(38)
M-2	(100)	(100)	(100)

^aGrowth medium was mineral salts plus 0.4% succinate.

^bCompared to M-2 on the same day.

^cResponse was significantly different from M-2 as determined by Student's t-test.

^dGrowth medium was mineral salts plus 0.4% glucose.

ones (three from patients in poor clinical condition) showed reduced taxis toward one or both attractants.

Proteolytic Activity

Proteolytic activity is a characteristic of P. aeruginosa strongly associated with invasiveness (36). Extracellular proteases such as elastase and alkaline protease have been implicated in the pathogenicity of P. aeruginosa in acute infections and have been studied in detail (19, 28). A large portion of CF clinical strains are also positive for protease and elastase (47).

Thirty-three strains were screened for proteolytic activity in skim milk plates. This skim milk assay is sensitive for both alkaline protease and elastase (82). Results in Table 11 show that 72% of the strains from patients in good clinical condition demonstrated proteolytic activity. Of the strains from patients in good clinical condition, over half (57% were as proteolytic as M-2, while less than one-third (28%) were non-proteolytic.

In sharp contrast to the above results, only 20% of the strains from patients in poor clinical condition were as proteolytic as M-2, while 60% demonstrated no proteolytic activity. For seven of the 13 strains, absence of proteolytic activity was accompanied by absence of flagellum.

Virulence Comparisons of CF Strains in the Burned Mouse Model

P. aeruginosa is avirulent in normal mice but extremely lethal in those receiving thermal trauma. In the burned mouse model developed by Stieritz et al. (83) the LD₅₀ for M-2 is 1.5×10^1

Table 11. Proteolytic activity of P. aeruginosa CF strains.

Strain	Proteolytic activity ^{a,b}
M-2	+
572b (G)	+
412oo (G)	+
435c (G)	+
903a (G)	+
35c (G)	+
320e (G)	+
412a (G)	+
351g (M)	+
572d (M)	+
776e (P)	+
414g (P)	+
503g (P)	+
86f (G)	+/-
437e ₂ (G)	+/-
902c (G)	+/-
96e (P)	+/-
66g (P)	+/-
541a (P)	+/-
676e (P)	+/-
423mm (G)	-
320f (G)	-
412ee (G)	-
423gg (G)	-
402c (P)	-
409g (P)	-
415gg (P)	-
414ii (P)	-
414nn (P)	-
503cc (P)	-
144b (P)	-
448hh (P)	-
448bb (P)	-

^aZone of clearing (radius) on skim milk plate was measured at 24 h: + > 7 mm, +/- ≤ 3 mm, - = 0 mm.

^bEach isolate was tested at least three times.

CFU following subcutaneous inoculation into the burn site of HA/ICR mice (T. C. Montie, personal communication). A comparison of relative virulence revealed significant differences between M-2 and CF P. aeruginosa strains (Table 12). Injection of 3×10^4 CFU of M-2 caused 100% mortality in HA/ICR mice. Of the twenty-three CF strains examined, only 35c demonstrated virulence comparable to that of M-2 (3.2×10^4 CFU of 35c caused 80% mortality). Injections ranging from 3.5×10^3 CFU to 6.1×10^5 CFU caused 0-20% mortality in 97% of the strains tested.

A series of varied morphological types of CF sputum strains were then examined for virulence in the burned mouse model using CF-1 mice (Table 13). Morphological types tested were smooth and rough strains from patients in good and poor clinical conditions. One mucoid strain was also tested. Of the nine CF strains examined, only 35c resembled M-2, being of classic morphology, proteolytic and O-antigen serotypable. It was the most virulent of the strains with an LD_{50} of 8×10^2 CFU. The mucoid strain, 412a, was intermediate in virulence ($LD_{50} = 1 \times 10^4$ CFU). This strain polyagglutinated in O antisera and demonstrated reduced motility (60% of M-2) in the capillary assay. Avirulence of the other strains appeared to be associated with the loss of one or more important physiological characteristics possibly associated with virulence. Strains such as 402c, in which all physiological properties differ from M-2, caused 0% mortality at 10^6 CFU.

Table 12. Comparison of lethality of P. aeruginosa CF strains in the burned mouse model (HA/ICR mice).

Strain	Dose ^a	% Lethality ^b
M-2(control)	3.1×10^2	100
437e2	3.9×10^5	0
572b	4.1×10^4	20
86f	6.1×10^5	0
412oo	5.5×10^3	0
320f	3.5×10^3	20
435c	1.5×10^4	0
903a	4.6×10^3	20
35c	3.2×10^4	80
902c	5.1×10^4	0
412a	8.2×10^3	40
572d	5.6×10^4	0
402c	2.7×10^4	0
409g	3.8×10^4	0
96e	5.3×10^4	0
66g	6.2×10^4	0
415gg	7.2×10^3	0
414ii	3.8×10^4	20
414nn	4.7×10^4	20
448hh	9.3×10^3	0
541a	3.7×10^4	0
144b	3.8×10^5	20
776e	6.7×10^4	0
676e	4.6×10^4	0

^aSubcutaneous injection of bacteria (in 0.1 ml buffer) into burn site.

^bLethality of a group of five mice.

Table 13. Comparisons of lethality of P. aeruginosa strains in the burned mouse model (CF-I mice)

Strain ^a	Colonial morphology ^b	O-antigen type ^c	Proteolytic activity ^d	% Motility ^e	% Arginine Chemotaxis ^e	LD ₅₀ ^f
M-2	C	5	+	100	100	1.5 x 10 ¹
35c (G)	C	6	+	58	126	8.4 x 10 ²
572b (G)	R	NT	+	127	94	> 7.1 x 10 ⁶
412a (G)	M	PA	+	59	59	3.6 x 10 ⁴
320f (G)	R	PA	-	45	31	1.5 x 10 ⁶
86f (G)	R	10	+/-	216	107	> 4.0 x 10 ⁶
409g (P)	R	NT	-	64	54	> 6.8 x 10 ⁶
144b (P)	C	6	-	67	17	> 6.2 x 10 ⁶
402c (P)	R	NT	-	48	13	> 6.5 x 10 ⁶
776e (P)	C	PA	+	98	46	> 2.4 x 10 ⁶

^aAll strains possess flagella.

^bC, classic; M, mucoid; R, rough.

^cNT, non-typable; PA, polyagglutinable.

^dZone of clearing in skim milk plates measured at 24 h.

^eCompared to M-2 (100%) in capillary assay,

^flethality of a groups of eight mice following subcutaneous injection of bacteria (in 0.1 ml buffer) into burn site; four doses per group of mice (see Table 1, appendix for enumeration of bacteria). The last five isolates listed demonstrated 0% mortality at 10⁶ CFU.

CHAPTER IV

DISCUSSION

It is clear that the majority of CF Pseudomonas aeruginosa strains in this investigation differ markedly from strains associated with acute infections in regard to O-antigenicity, motility, chemotaxis, proteolytic activity, and virulence in the burned mouse model. These phenotypic parameters were stable ones not subject to alteration by environmental conditions. It was hypothesized that, after initial invasion of the lung by the organism, there may be a progressive selection against bacterial properties normally associated with virulence. Strains from patients in good and poor clinical condition were examined to determine if phenotype could be associated with patient clinical condition. An attempt was made to correlate phenotypic heterogeneity of CF strains with colonial morphology. A synthesis of these two methods creates an interesting chronology of events which may shed light on the subject of persistent P. aeruginosa infection in the CF lung.

Serological typing of twenty CF strains yielded results which agreed with those reported by Hancock (35), Penketh (66) and Thomassen (86). In contrast to the 95% of strains from clinical strains other than CF which can be allocated to a specific serotype (66, 69), 75% of the CF strains tested in our investigation were either polyagglutinable or non-typable. Of the eleven rough colonial morphology strains tested, only two were typable. Four of the eleven were non-typable and 45% polyagglutinated with two or more antisera.

In contrast to the rough strains, one third of the eight classic strains were typable, while only one was non-typable. However, as in the case of rough strains, it was found that half the classic strains were polyagglutinable. These results are in agreement with Penketh's study which noted multiple agglutination with O antisera in fifty percent of strains tested and a low frequency (32%) of typable strains.

It is not known whether strains that were non-typable by agglutination possessed O antigen other than those used or lacked O antigen entirely. It is interesting to note that only one classic strain was non-typable. The majority of non-typable strains were of rough colonial morphology and were isolated from patients in poor clinical condition.

The observation that half of both classic and rough strains were polyagglutinable strongly suggests that the cell wall of CF strains is generally altered in a manner not characteristic of Pseudomonas associated with acute infections. As referred to in the introduction, it is postulated that P. aeruginosa clinical isolates may or may not possess the PA antigen as a minor constituent, while it may be an important characteristic associated with CF strains. These strains may possess the major PA antigen reported by Penketh (66) which was present in fifty percent of the strains he studied. It would be helpful to obtain antisera to this PA antigen to see if this is the case. This would also elucidate whether or not determinants

of type specificity are present with the PA antigen or if the two are mutually exclusive.

Our serotyping results are consistent with studies by Thomassen et al., (86) concerning the alteration of bacterial serum sensitivity during the progress of infection. Cystic strains that were serum resistant in autologous serum, but sensitive to normal serum, were primarily from patients in poor clinical condition. The characteristic of serum sensitivity, which appears to be associated with altered LPS may explain why CF patients rarely suffer from bacteremia. The containment of P. aeruginosa in the CF lung may be contrasted to Pseudomonas pneumonia which results in a higher incidence of bacteremia. Phair et al. (67) reported that P. aeruginosa was responsible for over one third of the cases of bacteremia caused by gram negative organisms. Mortality was especially high among patients with pre-existing illness such as neoplastic disease, renal disease or chronic lung obstruction. Of the eleven deaths among the thirty-three patients with P. aeruginosa bacteremia, ten had pneumonia. In contrast, survival among patients with P. aeruginosa infection was 96% if the source of the infection was other than the lungs. Phair's study strongly suggests that the pathogen is especially lethal when infecting the lungs of a compromised host and that important immune response differences exist between the compromised host and the CF patient.

The emergence of the CF P. aeruginosa PA antigen may result in some type of masking phenomenon which protects the pathogen from

bactericidal activity in autologous serum. While it may not be the same as the immunodominant antigenic domain isolated from pilin protein of Neisseria gonorrhoeae for vaccine development (12), the same general type of immunodominance mechanism in P. aeruginosa may be effective in autologous serum but unable to protect the pathogen in normal human serum. Such a masking event may be instrumental in establishing chronic infection and signal a transition in disease progression. Such a transition may be followed by selective pressure for non-typable strains as chronic infection persists. While a precise explanation remains elusive, there is no doubt about the fact that an altered cell envelope is present in most strains associated with the CF lung.

Since a surprisingly large number of CF rough strains were non-motile in the motility assay, we suspected that these organisms lacked flagella. Such a severe physiological aberration would be unusual in P. aeruginosa. The single, polar flagellum is a major taxonomic characteristic of the organism associated with more than 95% of clinical strains (5, 55, 66). In fact, when type-specific thermolabile (flagellar) antigens were studied by Lanyi (55), he reported that only 1 out of 541 strains was non-motile. Detection of flagella by electron microscopy confirmed the absence of flagella in seventy percent of rough strains from patients in poor clinical condition. This severe alteration of phenotype was not limited to rough colonial morphology, since two classic strains from patients in poor clinical condition lacked flagella. However, in contrast to

rough strains, 80% of classic strains from both clinical conditions were flagellated.

Deficiency in flagellation has been previously reported in a Bacillus subtilis mutant by Sasajima and Kumadad (74, 75, 76). A defect in the cell surface structure of the B. subtilis mutant caused altered cell morphology, bacteriophage sensitivity and non-flagellation. According to these investigators, a transketolase mutation may affect the synthesis of polysaccharide which, in turn, could alter cell surface resulting in an inadequate function of membrane proteins. Integration of protein in the outer membrane was also found to be defective in an LPS mutant of Escherichia coli (50). Perhaps a similar mutation event is selected for in the CF lung, thereby favoring non-flagellated P. aeruginosa strains in patients who have been harboring the pathogen for prolonged periods of time. This idea seems plausible, since once established in the lung, the organism would no longer need invasive properties such as motility and chemotaxis.

Since the CF lung is unique in that large amounts of mucus are secreted, especially at later stages of the disease, one would suspect that motile strains would be disadvantaged in such an environment. Any variation in cation elevation of the patient could result in subsequent variation of mucus secretion. Elevation of cations could conceivably alter LPS synthesis as well. Since magnesium is an important requirement for integrity of outer membrane (35), increased cation levels could cause outer membrane stability and select for envelope-deficient phenotypes.

Boyce et al. (9), attempted to correlate mucoidy with non-flagellation by strain selection in the laboratory; however, we found no such correlation in our study. Unlike the transient mucoid phenotype, non-flagellation is not dictated by environment. Non-flagellation of P. aeruginosa is apparently a stable characteristic associated with specific strains. It was not altered by any variation in growth medium, temperature, or aeration. Our findings are supported by those of both Pugashetti (71) and Penketh (66) who found that mucoidy and other physiological characteristics were not related. In addition, one may speculate that, given the high percentage of strains with altered LPS, the more plausible hypothesis is that altered synthesis of LPS, not mucoidy, could create envelope instability which could interfere with the secure anchoring of the flagellum. This line of thinking is supported by the fact that a basal body ring of the flagellum is associated with the LPS-outer membrane layer of the cell (76).

The most significant observation about the chemotaxis of CF strains is the association between patient clinical condition and chemotactic response. Patients in good clinical condition tended to harbor strains similar in chemotaxis to M-2. All rough strains from patients in good clinical condition tested, with one exception, were comparable to M-2 in taxis toward amino acids. Strain 320f was significantly less chemotactic than M-2, a result which did not correlate with the motility assay, in which 320f was more motile and chemotactic during the first two hours. This inconsistency is perhaps explained by the observation in our laboratory that M-2 has

a one hour lag phase when grown in liquid media at 37°C. The difference in lag phase could affect the results in the motility assay. However, this slower initial growth rate would not affect the results observed in the chemotaxis capillary experiments. In the latter, only motility and chemotaxis are measured during the thirty minute assay.

Since rough strains from patients in poor clinical condition were predominantly non-flagellated, only two could be tested for amino acid taxis in the capillary assay. Both strains were less chemotactic than M-2. There was a parallel reduction of motility suggesting that it is directly affected, resulting in reduced taxis. As an alternative explanation, one could suppose that, since the genetic loci for motility and chemotaxis are in close proximity (87, 88), a mutational event in that region of the chromosome could alter both gene products.

Over half of the classic strains displayed reduced chemotaxis toward one or more attractants. As in the case of rough strains, classic strains from patients in poor clinical condition were less chemotactic than those from patients in good clinical condition. While the absence of flagella was less frequently observed in classic strains, the chemotaxis results suggest that the general response is not the same as that observed in motile, chemotactic strains from acute infections. Chemoreceptors and the mechanism for sensory transduction in P. aeruginosa are well studied (2, 21, 51, 52, 90). Several of the gene products involved in motility and chemotaxis are integral components of the bacterial cytoplasmic membrane (10).

Since classic strains also show the trend toward altered LPS (50% polyagglutinated in O antisera), the altered cell membrane could also perturb the chemoreceptors in the membrane.

It was observed in this study that some of the strains from patients in good clinical condition were more motile and chemotactic than the control strain. In particular, 86f, 572b, and 902c were significantly more chemotactic than M-2. While it is perhaps fortuitous, both 86f and 572b possess the type "a" flagellum while M-2 has type "b". The lower molecular weight may result in a more stable flagellum structure with a more efficient wavelength. This could result in swimming capacity superior to that of the higher molecular weight structure. It would be interesting to investigate this further using additional strains.

It may be the case that in the early stages of CF, when the locomotive apparatus is intact and there is less mucus secretion, lung attractants unique to the disease might enhance the chemotactic response of the invading organism. In the chronic stage of the disease, more mucus buildup may impair motility and simultaneously alter or eliminate the attractants. Such changes in the host would select for non-motile, non-chemotactic strains. The lung is a natural site of acute infection such as pneumonia; however, even pneumonia is rare, except in those patients which are immuno-compromised. Therefore, it is difficult to understand how a chronic lung infection is established. This is especially perplexing since most CF patients have good immune response, which under normal conditions would be able to control the infection. Current evidence

suggests that many CF patients have an elevated immune response to P. aeruginosa believed to contribute to lung damage. Hypersensitivity reactions, especially Type I (anaphylactic) and Type III (immuno-complex) have been reported (92).

Two major proteases are associated with P. aeruginosa virulence: alkaline protease and elastase. Hastie et al. (36) found that the majority of CF clinical strains possessed proteolytic activity, while Jagger (44) reported that the more chronically infected patients had more weakly proteolytic strains than patients newly colonized. Our study used the skim milk plate assay which is sensitive for both extracellular products. We concluded that the proteolytic activity of CF strains is primarily associated with the initial, invasive organism. Fifty-two percent of CF strains from patients in good clinical condition were as proteolytic as M-2. Seventy-two percent of all strains from patients in good clinical condition demonstrated proteolytic activity. In sharp contrast, only twenty percent of CF strains from patients in poor clinical condition were as proteolytic as M-2. More striking is the observation that sixty percent of the strains from patients in poor clinical condition demonstrated no proteolytic activity. The loss of this important virulence factor in such a large number of strains from chronically infected patients is not reported in the literature. It is, however, a logical step in the establishment of persistent infection. Proteases have tissue damaging activity and can degrade anatomical barriers to facilitate spread (81, 94). Absence of protease activity in the chronic infection may reflect the fact that the "spreading factor" is no

longer needed once the organism is established in the lung. Protease-positive strains from newly colonized patients may, on the other hand, need this characteristic to invade the lung.

In acute thermal injury, proteases can modify available nutrients by breaking down large protein molecules that cannot penetrate the cell envelope unless converted into smaller nutrients (19). Perhaps with the cell envelope alteration characteristic of strains from patients in poor clinical condition, such degradation of nutrients is not as imperative to the survival of the pathogen. Finally, while a clear explanation is not readily available, it is interesting to note that loss of protease activity is often accompanied by absence of flagellum.

Since many of the CF strains possess phenotypic alterations not manifested in invasive organisms, we suspected that they may also be less virulent when tested in an animal infection model. Several studies support this line of reasoning. A report by Lindberg et al. (57) found that E. coli strains in patients with asymptomatic bacteriuria had altered cell walls compared to those in patients with symptomatic infections. They hypothesized that the cell wall alteration could reduce virulence. Studies by Craven and Montie (20) showed that reduced virulence of P. aeruginosa in the burned mouse model was associated with absence of putative virulence factors such as proteases and exotoxin A.

We decided to examine the relative virulence of the CF strains compared to the virulent burn strain in the burned mouse model for acute infection which is routinely used in our laboratory research.

In a preliminary screening of twenty-three strains, only 35c demonstrated virulence comparable to M-2. For over ninety percent of the strains, injections of up to 10^5 CFU caused 0-20% mortality.

Once the strains were further characterized, we selected a series of nine varied morphological types to test for virulence in the burned mouse model using a different strain of mice. Non-flagellated strains were not included in this second group because of the lack of virulence exhibited by majority of the strains in the initial screening. Another reason for their omission was the severe loss of virulence associated with absence of flagellum in a recent study by Montie et al. (60). The results of the second set of animal studies were consistent with the preliminary screening. Only strain 35c had an LD₅₀ close to M-2. This strain is the CF strain which most resembles M-2 in physiological characteristics studied in this investigation. While the mucoid strain was intermediate in virulence (LD₅₀ = 10^4 CFU), all the other strains tested were avirulent. Five of the strains demonstrated no killing at 10^6 CFU. Avirulence appeared to be associated with loss of one or more physiological characteristics possibly associated with virulence. The weakly proteolytic but highly chemotactic and motile strain, 86f, was avirulent at injections of 10^6 CFU. Strain 402c differed from M-2 in all the parameters under investigation and it, too, was avirulent.

The nine strains used for the virulence studies, as well as M-2, were checked for presence of plasmids using the method of Kado et al. (45). Plasmid-associated virulence has been reported by Kopecko et

al. (49) in a study about Shigella sonnei surface antigens. The virulent form of S. sonnei possesses the surface antigen whose synthesis is mediated by a large plasmid. Some S. sonnei strains spontaneously and irreversibly lose the plasmid and become avirulent. The presence of plasmids in M-2 and 35c could be a possible explanation for their virulence in the animal model, if there were a concurrent absence of plasmids in the other strains. Preliminary screening for the presence of plasmids (data not shown) indicated that all strains, including M-2, possessed no extrachromosomal material. Thus, it is unlikely that a plasmid-mediated virulence factor could account for virulence differences between M-2, 35c and the other strains.

Larger surveys are needed to correlate the phenotypic changes associated with CF strains and their potential role in virulence reduction. It would be valuable to obtain strains from several hospitals to minimize effects of antibiotic therapy, isolation of strains, and maintenance of cultures. It would be interesting to determine whether or not the CF strains produce exotoxin A, an important virulence factor in acute infections such as pneumonia or thermal shock. In vitro studies by Baker et al. (6) using tracheal tissue infected with different P. aeruginosa strains showed that exotoxin A and elastase, but not alkaline protease, may be responsible for much of the destruction of the respiratory tract tissue. Results using an in vivo acute lung infection model found that elastase, but not exotoxin A, was an important virulence factor during acute pneumonia due to P. aeruginosa (7). The in vivo results

appear to depend on the type of model used. Woods et al. (93) determined that both active toxin A and elastase are required for expression of maximum virulence of this pathogen in a chronic pulmonary infection model.

The results of our animal studies indicate that avirulence is manifested by the overwhelming majority of the CF strains tested. It appears to be associated with the loss of any one of the putative virulence factors, as seen by the fact that virulence was expressed only in the strains where all virulence properties investigated in this study were present.

Our investigation culminates with the following hypothesis concerning the establishment of a chronic P. aeruginosa infection in the cystic fibrosis lung. The initial invasive strain would appear to be similar to M-2, the invasive burn strain. Like M-2 it has classic colonial morphology, is vigorously motile, chemotactic, proteolytic and has a single O-antigen serotype. The early invasive strain is virulent in the burned mouse model which indicates that it possesses the physiological factors associated with the expression of full virulence of this pathogen.

We hypothesize that, as the infection is established in the lung, host selective pressures, resulting, for example, from elevated cation levels, result in the emergence of a different phenotype with rough colonial morphology. While it retains its motility, chemotaxis and proteolytic activity, its O-antigen reflects altered LPS and we note a loss of virulence in the animal model.

Once chronic infection is established in the lung, we hypothesize that there is a loss of several characteristics which are associated with virulence in the burn infection. The greater majority of these isolates lack flagella, are protease-negative, non-motile, non-chemotactic and have altered LPS as reflected in the high frequency of polyagglutination and non-typable strains. It is important to note that these are not transient changes, but rather genetically stable physiological characteristics. It would appear that progressive selective pressure in the chronically infected CF lung favors emergence of an avirulent phenotype with an altered cell envelope.

The classic CF strains studied appear to be more resistant to host selective pressures, even during chronic stages of the infection. They show only partial reduction of motility and chemotaxis in some, but not all, strains. Even though fifty percent of the classic strains tested were polyagglutinable, the cell envelope perturbation appears less severe than in the case of rough strains. There was only one non-typable classic strain. However, with the exception of one strain, 35c, the classic strains were also avirulent in the animal model.

While more information must be obtained regarding the adaptation of P. aeruginosa to the special environment of the CF lung, some significant practical applications of this investigation are readily discernible. Observations in this study, particularly the striking absence of flagella and proteolytic activity in strains from patients

in poor clinical condition, offer palpable proof that prophylactic vaccine therapy must be aggressively initiated as soon as cystic fibrosis is detected. Various vaccine preparations based on antigenic subunits, such as flagella and LPS, currently the subject of intense investigation, would be of little use after chronic infections is established in the patient. The observation that cell wall alteration is associated with the majority of CF strains may lessen the efficacy of any envelope-associated immunotherapy and antibiotic therapy. In view of these findings, additional knowledge about host-pathogen interactions and increased efforts aimed at prevention of initial colonization should ultimately ameliorate and prolong the life of the cystic fibrosis patient.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Adler J. 1966. Chemotaxis in bacteria. Science (Washington, D.C.) 153:708-716.
2. Adler, J. 1969. Chemoreceptors in bacteria. Science 166:1588-97.
3. Adler, J. 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by Escherichia coli. J. Gen. Microbiol. 74:77-91.
4. Adler, K. B., W. C. Winn, T. V. Alberghini, and J. E. Craighead. 1983. Stimulatory effect of Pseudomonas aeruginosa on mucin secretion by the respiratory epithelium. JAMA 249:1615-1617.
5. Ansorg, R. 1978. Flagella specific H antigenic schema of Pseudomonas aeruginosa. Zentrabl. Bakteriell. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 242:228-238.
6. Baker, N. R. 1982. Role of exotoxin A and proteases of Pseudomonas aeruginosa in respiratory tract infections. Can. J. Microbiol. 28:248-255.
7. Blackwood, L. L., and J. E. Pennington. 1981. Influence of mucoid coating on clearance of Pseudomonas aeruginosa from lungs. Infect. Immun. 32:443-448.
8. Boyce, J. R., and R. V. Miller. 1982. Selection of nonmucoid derivatives of mucoid Pseudomonas aeruginosa is strongly influenced by the level of iron in the culture medium. Infect. Immun. 37:695-701.
9. Boyce, J. R., and R. V. Miller. 1982. Motility as a selective force in the reversion of cystic fibrosis-associated mucoid Pseudomonas aeruginosa to the nonmucoid phenotype in culture. Infect. Immun. 37:840-844.
10. Boyd, A., G. Mandel, and M. Simon. 1982. Integral membrane proteins required for bacterial motility and chemotaxis. Symposia of the Society of Experimental Biology. Prokaryotic and Eukaryotic Flagella. 35:123-137.
11. Boyd, R. L., R. Ramphal, R. Rice, and J. A. Mangos. 1983. Chronic colonization of rat airways with Pseudomonas aeruginosa. Infect. Immun. 39:1403-1410.
12. Brinton, C. C. 1978. Uses of pili in gonorrhea control: Role of bacterial pili, and progress in the development of a gonococcal pilus vaccine for gonorrhea. p. 155-178. In G. F. Brooks et al. (eds), Immunobiology of Neisseria gonorrhoea. American Society for Microbiology, Washington, D.C.

13. Brokopp, C. D., R. Gomez-Lus, and J. J. Farmer. 1977. Serological typing of Pseudomonas aeruginosa: Use of commercial antisera and live antigens. J. Clin. Microbiol. 5:640-649.
14. Brokopp, C. D., and J. J. Farmer. 1979. Typing methods for Pseudomonas aeruginosa, p. 89-133. In E. G. Dogget (ed.), Pseudomonas aeruginosa: clinical manifestations of infection and current therapy. Academic Press, Inc., New York.
15. Bruning, J. L., and B. L. Kintz. 1968. Computational Handbook of Statistics. Scott, Foresman and Company, Glenville, IL.
16. Bryan, L. E., H. M. Van Den Elzen, and Tseng, J. T. 1972. Transferable drug resistance in Pseudomonas aeruginosa. Antimicrobiol Agents. Chem. 1:22-29.
17. Cash, H. A., D. E. Woods, B. McCollough, W. G. Johanson, and J. A. Bass. 1979. A rat model of chronic respiratory infection with Pseudomonas aeruginosa. Am. Rev. Respir. Dis. 119:453-459.
18. Chester, I. R., P. M. Meadow, and T. L. Pitt. 1973. The relationship between the O-antigenic lipopolysaccharides and serological specificity in strains of Pseudomonas aeruginosa of different O-serotypes. J. Gen. Microbiol. 78:305-318.
19. Cicmanec, J. F., and I. A. Holder. 1979. Growth of Pseudomonas aeruginosa in normal and burned skin extract: Role of extracellular proteases. Infect. Immun. 25:477-483.
20. Craven, R. C., and T. C. Montie. 1981. Motility and chemotaxis of three strains of Pseudomonas aeruginosa used for virulence studies. Can. J. Microbiol. 25:458-460.
21. Craven, R. C., and T. C. Montie. 1983. Chemotaxis of Pseudomonas aeruginosa: Involvement of methylation. J. Bacteriol. 154:780-786.
22. Cross, A. S., S. Opal, and D. J. Kopecko. 1983. Progressive increase in antibiotic resistance of gram-negative bacterial isolates. Arch. Intern. Med. 143:2075-2080.
23. Davis, P., and P. Di Sant' Agnese. 1980. A review. Cystic fibrosis at forty-quo vadis? Pediat. Res. 14:83-87.
24. Day, D. F. 1980. Gentamicin-lipopolysaccharide interactions in Pseudomonas aeruginosa. Cur. Microbiol. 4:277-281.

25. Di Sant' Agnese, P. A., and P. B. Davis. 1976. Research in cystic fibrosis. N. Engl. J. Med. 295:597-602.
26. Di Sant' Agnese, P. A., and P. B. Davis. 1979. Cystic fibrosis in adults: 75 cases and a review of 232 cases in the literature. Am. J. Med. 66:121-132.
27. Dimitracopoulos, G., J. W. Sensakovic, and P. F. Bartell. 1974. Slime of Pseudomonas aeruginosa: In vivo production. Infect. Immun. 10:152-156.
28. Doring, R. G., H.-J. Obernesser, and K. Botzenhart. 1981. Extracellular toxins of Pseudomonas aeruginosa. II. Effect of two proteases on human immunoglobulins IgG, IgA, and secretory IgA. Zentralbl. Bakteriol. Mikrobiol. Hyg. 249:89-98.
29. Doring, R. G., H. J. Obernesser, and K. Botzenhart. 1982. Extracellular toxins of Pseudomonas aeruginosa. II. Effect of two proteases on human immunoglobulins IgG, IgA and secretory IgA. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 249:89-98.
30. Fairbanks, G., T. L. Stech and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606-2617.
31. Govan, J. R. W. 1975. Mucoid strains of Pseudomonas aeruginosa: the influence of culture medium on the stability of mucus production. J. Med. Microb. 8:513-522.
32. Govan, J. R. W., and J. A. M. Fyfe. 1978. Mucoid Pseudomonas aeruginosa and cystic fibrosis: Resistance of the mucoid form to carbenicillin, flucloxacillin, and tobramycin and the isolation of mucoid variants in vitro. J. Antimicrob. Chemother. 4:233-240.
33. Govan, J. R. W., J. A. M. Fyfe, and C. McMillan. 1979. The instability of mucoid Pseudomonas aeruginosa: fluctuation test and improved stability of the mucoid form in shaken culture. J. Gen. Microbiol. 110:229-232.
34. Hammerschlag, M. R., L. Harding, A. Maccone, A. L. Smith, and D. A. Goldmann. 1980. Bacteriology of sputum in cystic fibrosis: Evaluation of dithiothreitol as a mucolytic agent. J. Clin. Microbiol. 11:552-557.
35. Hancock, R. E. W., L. M. Mutharia, L. Chan, R. P. Darveau, D. P. Speert, G. B. Pier. 1983. Pseudomonas aeruginosa isolates from patients with cystic fibrosis: A class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infect. Immun. 42:170-177.

36. Hastie, A. T., S. T. Hingley, F. Kueppers, M. L. Higgins, C. S. Tannenbaum, and G. Weinbaum. 1983. Protease production by Pseudomonas aeruginosa isolates from patients with cystic fibrosis. *Infect. Immun.* 40:506-513.
37. Hoiby, N. 1974. Pseudomonas aeruginosa infection in cystic fibrosis, relationship between mucoid strains of Pseudomonas aeruginosa and the humoral immune response. *Acta. Pathol. Microbiol. Scand. Sect. B*82:551-558.
38. Hoiby, N. 1977. Pseudomonas aeruginosa infection in cystic fibrosis. *Acta. Pathol. Microbiol. Scand.* 262:1-96.
39. Hoiby, N., and K. Rosendal. 1980. Epidemiology of Pseudomonas aeruginosa infection in patients treated at a cystic fibrosis center. *Acta. Pathol. Microbiol. Scand.* 88:125-131.
40. Holder, I. A., R. Wheeler, and T. C. Montie. 1982. Flagellar preparations from Pseudomonas aeruginosa: Animal protection studies. *Infect. Immun.* 35:276-280.
41. Holmes, R. K., B. H. Minshew, K. Gould, and J. P. Sanford. 1974. Resistance of Pseudomonas aeruginosa to Gentamicin and related aminoglycoside antibiotics. *Antimicrobiol. Agents Chemother.* 6:253-262.
42. Huang, N. N., and R. G. Doggett. 1979. Antibiotic therapy of Pseudomonas infection in patients with cystic fibrosis. p. 409-413. In R.G. Doggett (ed.), Pseudomonas aeruginosa: Clinical manifestations of infections and current therapy. Academic Press. New York.
43. Jacoby, G. A. 1974. Properties of R plasmids determining gentamicin resistance by acetylation in Pseudomonas aeruginosa. *Antimicrobiol. Agents Chem.* 6:239-252.
44. Jagger, K. S., D. R. Bahner, and R. L. Warren. 1983. Protease phenotypes of Pseudomonas aeruginosa isolated from patients with cystic fibrosis. *J. Clin. Microbiol.* 17:55-59.
45. Kado, C. I., and S. T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* 145:1365-1373.
46. Kawaharajo, K., Homma, Y. Aoyama, K. Morihara. 1975. In vivo studies on protease and elastase from Pseudomonas aeruginosa. *Jpn. J. Exp. Med.* 45:89-100.

47. Klinger, J. D., D. C. Straus, C. B. Hilton, and J. A. Bass. 1978. Antibodies to proeases and exotoxin A of Pseudomonas aeruginosa in patients with cystic fibrosis: Demonstration of radioimmunoassay. J. Infect. Dis. 138:49-58.
48. Klinger, J. D., H. A. Cash, R. E. Wood, and J. J. Miler. 1983. Protective immunization against chronic Pseudomonas aeruginosa pulmonary infection in rats. Infect. Immun. 39:1377-1384.
49. Kopecko, D. J., O. Washington, and S. B. Formal. 1980. Geneti and physical evidence for plasmid control of Shigella sonnei form I cell surface antigen. Infect. Immun. 29:207-214.
50. Koplow, J. and H. Goldfine. 1974. Alterations in the outer membrane of the cell envelope of heptose-deficient mutants of Escherichia coli. J. Bacteriol. 117:527-543.
51. Koshland, D. E. 1977. A response regulator model in a simple sensory system. Science 196:1055-1063.
52. Koshland, D. E. 1981. Biochemistry of sensing and adaptation in a simple bacterial system. Ann. Rev. Biochem. 50:765-781.
53. Kuzemko, J. 1983. Evolution of lung disease in cystic fibrosis. Lancet 2:448-449.
54. Lam, J., R. Chan, K. Lam, and J. W. Costerton. 1980. Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis. Infect. Immun. 28:546-556.
55. Lanyi, B. 1970. Serological properties of Pseudomonas aeruginosa. II. Type-specific thermolabile (flagellar) antigens. Acta. Microbiol. Acad. Sci. Hung. 17:35-48.
56. Laraya-Cuasay, L. R., K. R. Cundy and N. N. Huang. 1976. Pseudomonas carrier rates of patients with cystic fibrosis and of members of their families. J. Pediatr. 89:23-26.
57. Lindberg, U., L. O. Hanson, U. Jodal, G. Lidin-Janson, K. Lincoln, and S. Olling. 1975. Asymptomatic bacteriuria in schoolgirls. Acta. Paediatr. Scand. 64:432-436.
58. Martin, D. R. 1971. Variation in Pseudomonas aeruginosa. Ph.D. Thesis. London: University of London Library Press.
59. May, J. R., N. C. Herrick, and D. Thompson. 1972. Bacterial infection in cystic fibrosis. Arch. Dis. Child. 47:908-913.

60. Montie, T. C., D. Doyle-Huntzinger, R. C. Craven, and I. A. Holder. 1982. Loss of virulence associated with absence of flagellum in an isogenic mutant of Pseudomonas aeruginosa in the burned-mouse model. *Infect. Immun.* 38:1296-1298.
61. Montie, T. C., R. C. Craven, and I. A. Holder. 1982. Flagellar preparations from Pseudomonas aeruginosa: Isolation and characterization. *Infect. Immun.* 35:281-288.
62. Montie, T. C., J. S. Allison, and M. Dawson. 1983. Electrophoretic separation, identification and molecular weight characterization of Pseudomonas aeruginosa H antigen flagellins. Submitted for publication.
63. Moulton, R. C., and T. C. Montie. 1979. Chemotaxis by Pseudomonas aeruginosa. *J. Bacteriol.* 137:274-280.
64. Obernesser, H.-J., and G. Doring. 1982. Extracellular toxins of Pseudomonas aeruginosa. IV. Radioimmunoassay for detection of elastase. *Zentralbl. Bakteriол. Mikrobiol. Hyg.* 252:248-256.
65. Ohman, D. E., and A. M. Chakrabarty. 1982. Utilization of human respiratory secretions by mucoid Pseudomonas aeruginosa of cystic fibrosis origin. *Infect. Immun.* 37:662-669.
66. Penketh, A., T. Pitt, D. Roberts, M. E. Hodson, and J. C. Batten. 1983. The relationship of phenotype changes in Pseudomonas aeruginosa to the clinical condition of patients with cystic fibrosis. *Am. Rev. Respir. Dis.* 127:605-608.
67. Phair, J. P., H. P. Bassaris, J. E. Williams, E. Metzger. 1983. Bacteremic pneumonia due to gram-negative bacilli. *Arch. Intern. Med.* 143:2147-2149.
68. Pitt, T. L., and J. Y. Erdman. 1978. The specificity of agglutination reactions of Pseudomonas aeruginosa with O antisera. *J. Med. Microbiol.* 11:15-23.
69. Pitt, T. L. 1980. State of the art: Typing Pseudomonas aeruginosa. *J. Hosp. Infect.* 1:193-199.
70. Pugashetti, B. K., H. Metzger, L. Vadas, and D. Feingold. 1982. Phenotypic differences among clinically isolated mucoid Pseudomonas aeruginosa strains. *J. Clin. Microbiol.* 16:686-691.
71. Pugashetti, B. K., H. M. Metzger, J. J. Cardamone, D. E. Feingold. 1983. Flagellation is a strain-dependent characteristic of cystic fibrosis-associated mucoid strains of Pseudomonas aeruginosa. *Eur. J. Clin. Microbiol.* 2:360-361.

72. Reynolds, H. Y., P. A. Di Sant' Agnese, and C. H. Zierdt. 1976. Mucoid Pseudomonas aeruginosa. J. Am. Med. Assoc. 232:2190-2192.
73. Rivera, M., and M. B. Nicotra. 1982. Pseudomonas aeruginosa mucoid strain: Its significance in adult chest diseases. Am. Rev. Respir. Dis. 126:833-836.
74. Sasajima, K., T. Kumada, and A. Yokota. 1977. A pleiotropy in carbohydrate metabolism of Bacillus subtilis mutant lacking transketolase. IFO Res. Comm. 8:69-77.
75. Sasajima, K., and T. Kumada. 1981. Change in the regulation of enzyme synthesis under catabolite repression in Bacillus subtilis pleiotropic mutant lacking transketolase. Agric. Biol. Chem. 45:2005-2012.
76. Sasajima, K. and T. Kumada. 1983. Deficiency of flagellation in Bacillus subtilis pleiotropic mutant lacking transketolase. Agric. Biol. Chem. 47:1375-1376.
77. Schiotz, P. O. and N. Hoiby. 1975. Precipitating antibodies against Pseudomonas aeruginosa in sputum from patients with cystic fibrosis: Specificities and titers determined by means of crossed immunoelectrophoresis with intermediate gel. Acta. Pathol. Microbiol. Scand. [C] 83:469-475.
78. Schultz, D. R. and K. D. Miller. 1974. Elastase of Pseudomonas aeruginosa: Inactivation of complement components and complement-derived chemotactic and phagocytic factors. Infect. Immun. 10:128-135.
79. Seale, T. W., H. Thirkill, M. Tarpay, M. Flux, and O. M. Rennert. 1979. Serotypes and antibiotic susceptibility of Pseudomonas aeruginosa isolates from single sputa of cystic fibrosis patients. J. Clin. Microbiol. 9:72-78.
80. Sheehan, D. J., J. M. Janda, and E. J. Bottone. 1982. Pseudomonas aeruginosa: Changes in antibiotic susceptibility, enzymatic activity, and antigenicity among colonial morphotypes. J. Clin. Microbiol. 15:926-930.
81. Snell, K. I. A. Holder, S. A. Leppla, and C. B. Saelinger. 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with Pseudomonas aeruginosa. Infect. Immun. 19:839-845.
82. Sokol, P. A., D. E. Ohman, and B. H. Iglewski. 1979. A more sensitive plate assay for detection of protease production by Pseudomonas aeruginosa. J. Clin. Microbiol. 9:538-540.

83. Stiertiz, D. D., and I. A. Holder. 1975. Experimental studies of the pathogenesis of infections due to Pseudomonas aeruginosa. Description of a burned mouse model. J. Infect. Dis. 131:688-691.
84. Stratford, B. C., and S. Dixon. 1974. Serum levels of gentamicin and tobramycin after slow intravenous bolus injection. Lancet. 1:378-379.
85. Thomassen, M. J., C. A. Demko, B. Boxer-baum, R. C. Stern, and P. J. Kuchenbrod. 1979. Multiple isolates of Pseudomonas aeruginosa with differing antimicrobiol susceptibility patterns from patients with cystic fibrosis. J. Infect. Dis. 140:873-880.
86. Thomassen, M. J., and C. A. Demko. 1981. Serum bactericidal effect on Pseudomonas aeruginosa isolates from cystic fibrosis. Infect. Immun. 33:512-518.
87. Tsuda, M., and T. Iino. 1983. Ordering of the flagellar genes in Pseudomonas aeruginosa by insertions of mercury transposon T 501. J. Bacteriol. 153:1008-1017.
88. Tsuda, M., and T. Iino. 1983. Transductional analysis of flagellar genes Pseudomonas aeruginosa. J. Bacteriol. 153:1018-1026.
89. Wahba, A. H., and J. H. Darrell. 1965. The identification of atypical strains of Pseudomonas aeruginosa. J. Gen. Microbiol. 38:329-342.
90. Wang, E. and D. E. Koshland, Jr. 1980. Receptor structure in the bacterial sensing system. Proc. Nat'l Acad. Sci. USA 77:7157-7161.
91. Wilkinson, S. G. and L. Galbraith. 1975. Studies of lipopolysaccharides from Pseudomonas aeruginosa. Eur. J. Biochem. 52:331-343.
92. Wood, R. E., T. F. Boat, and T. F. Doershuk. 1976. State of the Art: Cystic fibrosis. Am. Rev. Respir. Dis 113:833.
93. Woods, D. E., S. J. Cryz, R. L. Friedman, B. H. Iglewski. 1982. Contributions of toxin A and elastase to virulence of Pseudomonas aeruginosa in chronic lung infections of rats. Infect. Immun. 36:1223-1228.

94. Wretlind, B., and O. R. Pavlovskis. 1981. The role of proteases and exotoxin A in the pathogenicity of Pseudomonas aeruginosa infections. Scand. J. Infect. Dis., Suppl. 29:13-19.
95. Young, L. L. 1980. Medical prospective. The role of exotoxins in the pathogenesis of Pseudomonas aeruginosa infections. J. Infect. Dis. 142:626-630.

APPENDIX

APPENDIX

Table 14. Standard dilution curves for quantitation of bacteria.

Strain ^a	Adsorption at 590 mm	Log ₁₀ CFU/ml
572b	0.4	8.56
	0.6	8.72
	0.8	8.89
86f	0.4	9.36
	0.6	9.60
	0.8	9.82
402c ^b	0.4	9.53
	0.6	9.83
	0.8	10.13
35c ^c	0.4	8.88
	0.6	9.05
	0.8	9.22
776e	0.4	9.05
	0.6	9.28
	0.8	9.50
144b	0.4	9.06
	0.6	9.24
	0.8	9.41
M-2	0.4	8.69
	0.6	8.85
	0.8	9.02

^aCF strains were grown in Luria broth at 37°C with shaking for 14-15 hours; M-2 was grown for 12 hours.

^b409g quantitation was based on this standard curve.

^c320f, 412a quantitations were based on this standard curve.

VITA

Mary Anne Luzar is from the farming community of Cloverdale, Indiana. While a student in Greencastle Community Schools, she studied classical piano at DePauw University. She attended St. Mary's College of Notre Dame (South Bend, Indiana) for four years and was awarded a Bachelor of Arts degree in French literature and Humanistic Studies. During her sophomore year at St. Mary's, she studied in Angers, France as a member of the Notre Dame foreign study program. Upon graduation, she worked briefly at the National Theatre in San Jose, Costa Rica. She then travelled to Geneva, Switzerland where she was employed for several years at the International Trade Center (UNCTAD-GATT). She returned to the United States and began her studies in biology and chemistry at DePauw University while working as a French instructor. During the course of her initial science studies, she spent summers in Geneva employed as an official, trilingual UN guide.

The author accepted a graduate teaching assistantship at The University of Tennessee, Knoxville, and began study toward a Master of Science degree which was awarded in March 1984. She is a member of Pi Delta Phi and the American Society of Microbiology.