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Genomic diversity of pseudomonas aeruginosa in cystic fibrosis

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GENOMIC DIVERSITY OF *Pseudomonas aeruginosa* IN CYSTIC FIBROSIS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CLB	cell lysis buffer
CSB	cell suspension buffer
CTAB	hexadecyltrimethyl ammonium bromide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
F	phenylalanine
LB	Lennox Broth
MEP	mucoid exopolysaccharide
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PIA	*Pseudomonas* Isolation Agar
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
UTI	urinary tract infection

ABSTRACT

Title of Thesis:	Genomic Diversity of *Pseudomonas aeruginosa* in Cystic Fibrosis
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Pseudomonas aeruginosa is a common environmental microorganism. However, it has the genetic capacity to cause diseases in patients with compromised host defense systems. Cystic fibrosis (CF) is one of the major hereditary diseases among Caucasian populations. CF patients are born with a defective chloride channel that is responsible for maintaining fluid and electrolyte balance across the lumen of the lung. This imbalance leads to the production of an abnormal dehydrated viscous mucous, prohibiting the patient from normally clearing the respiratory airway. Chronic pulmonary infections with *P. aeruginosa* are the major causes of high morbidity and mortality in CF. Two of the most discernible phenotypes expressed by *P. aeruginosa* during its chronic presence in CF are mucoidy, the overproduction of the mucoid exopolysaccharide (MEP) alginate and biofilm, the formation of microcolonies, both of which defend the bacterium from host defenses and antibiotic therapy.

Pulsed-field gel electrophoresis (PFGE) was used to analyze the genetic profiles from a collection of 101 clinical CF, 2 burn, 7 laboratory and 4 environmental isolates of *P. aeruginosa*. The goals of this study were to determine the following: i) presence of genetic diversity; ii) correlation of mucoidy with genotype; iii) presence of significant-sized deletions in a set of genes; and iv) presence of CF-specific extra-chromosomal DNA. Seventy-five unique genomic profiles were identified, thus genetic diversity exists throughout a majority of the isolates. Sets of defined CF isolates were also used to track the origin and mode of *Pseudomonas* infection by PFGE. Sequential isolates studied showed conservation of banding pattern throughout the colonization. Also, examples of possible cross-sectional infection between CF patients and common-vehicle environmental infection were identified. No correlation was found between the mucoid phenotype and the overall genomic profiles. Among the 75 unique profiles, one CF isolate, CF32, was found to have identical restriction fragment length polymorphism (RFLP) patterns using 3 different restriction digests (*Spe*I, *Xba*I and *Dpn*I) when compared to the standard reference strain PAO1, originally isolated from a burn patient. This raises the possibility that a burn isolate of *P. aeruginosa* possesses the ability to infect a CF patient, a CF isolate could infect a burn patient, or an environmental PAO1-like strain can infect both CF and burn patients. More importantly, since the entire genome sequence of PAO1 is available, this result indicates that PAO1 can be used as an index strain for genomic comparison to CF clinical isolates.

To establish a link between the RFLP patterns and precise chromosomal alterations, polymerase chain reaction (PCR) was applied to study a panel of 41 CF isolates for the presence of possible significant-sized genomic deletions. Twelve virulence genes were amplified: *algD*, *oprF*, *oprC*, *exoS*, *exoT*, *mucE*, *mutS*, *mutY*, 3 gene cluster *mucBCD* and one undefined gene identified in PA14, a burn isolate. While the burn-specific gene was not present in the CF isolates tested, the results indicated that no significant-sized deletions were seen, suggesting that these genes are not modified during chronic *Pseudomonas* colonizations in CF. Combined PFGE and Southern blot analyses with whole-genome labeling were used to identify CF-specific extra-chromosomal DNA fragments of *P. aeruginosa.* Under the parameters used in this study, no CF-specific DNA was found, yet the procedure is being further developed. Determining a correlation between RFLP banding pattern and inherent virulence properties as well as identification of CF-specific extra-chromosomal DNA and CF-specific genomic deletions will provide basis for the rapid diagnosis of CF lung infections and quick determination of antibiotic resistance of these isolates, which eventually may lead to a more effective treatment for *Pseudomonas* pulmonary infections in CF.

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I am very grateful to my supervisor and mentor, Dr. Hongwei Yu for his undying passion and support during the course of this study. I thank Dr. Terry Fenger for his excellent guidance throughout this study and the Potomac River Project for supplying the PFGE apparatus. I thank Dr. Charles Somerville for his excellent suggestions and enlightening criticisms over my work.

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I would also like to thank the Departments of Forensic Science and Microbiology, Immunology and Molecular Genetics for their understanding adjustment and support throughout my unique opportunity to partake in such a study.

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INTRODUCTION

CYSTIC FIBROSIS

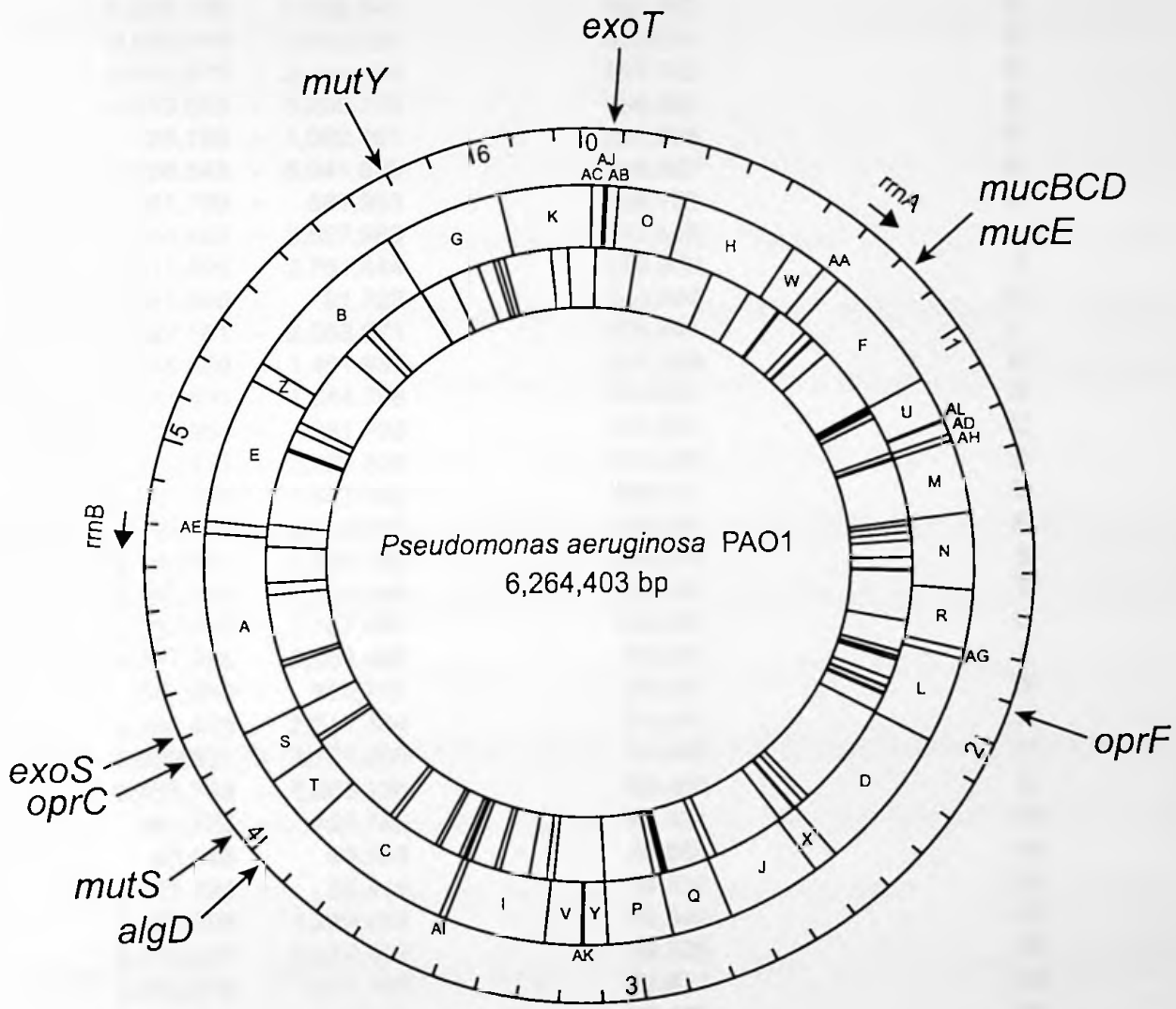
Cystic fibrosis (CF) is among the most common genetic diseases in the Caucasian population. Approximately 30,000 children and adults in the United States have CF, an autosomal recessive disease. One in 31 Americans, more than 10 million people, are carriers of a mutant CF gene. The normal CF gene gives rise to a protein product, CF transmembrane conductance regulator (CFTR). Persons that obtain two copies of the mutant allele of the CF gene are afflicted with the disease from birth (Collins, 1992). The most common mutation, which accounts for 70% of all cases, is a single codon deletion resulting in the missense deletion of the amino acid phenylalanine (F) at position 508. This mutation is also commonly referred to as ΔF508 and an altered protein is produced. CF patients are born with this defective CFTR that is normally responsible for controlling the chloride concentration across the lumen of the lung, thereby maintaining fluid and electrolyte balance (Collins, 1992). In CF individuals, the Cl^- ions cannot adequately leave the lung epithelial cells, Na^+ ions and H_2O are retained in the affected cells and the composition of the secretion is altered which leaves the patient with a characteristic salty sweat. The inability to control the concentration of chloride leads to many problems involving the exocrine glands and the lungs of the patients. This electrolytic imbalance results in production of an abnormal dehydrated viscous mucous within the lung. These conditions leave the CF lung more prone to becoming colonized and infected by

microbial organisms (Govan and Deretic, 1996). Pulmonary infections are associated with chronic colonization by bacterial pathogens and debilitating exacerbations as a result of bacterial and viral infections superimposed upon progressive lung disease. Interestingly, CF is not a systemic disease. The bacterial lung infection is only confined to the respiratory tract. The normal pulmonary defense mechanisms to defend against bacterial infection are mucociliary clearance and phagocytes (macrophages and neutrophils). The build-up of dehydrated mucous within the lung does not allow the host to normally clear the airway, allowing bacterial infection to occur without resistance. Very early in CF, a sequential order of bacteria will colonize the patient, most often beginning with *Staphylococcus aureus* and continuing with *Haemophilus influenzae*, both within the patient's first 3 years (Konstan and Berger, 1996). Since the development of aggressive antibiotic therapy, these organisms can be successfully treated before any long-term damage is inflicted. While both *S. aureus* and *H. influenzae* will re-emerge in the lung throughout the remainder of the disease, *Pseudomonas aeruginosa* ultimately becomes the major inhabitant for the remainder of the CF patient's life. The problem with combating a *P. aeruginosa* infection lies in the organism's ability to alter its phenotype and form a biofilm to circumvent the host's defenses and antibiotic therapy. *P. aeruginosa* is ultimately the major cause of morbidity and mortality in CF patients, which accounts for over 90% of CF deaths.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that has the ability to infiltrate and colonize the lung of CF patients. Recently, the entire genome of *P. aeruginosa* standard strain PAO1 has been sequenced (Stover *et al.*, 2000). The genome of PAO1 consists of 6,264,403 base pairs and 5,570 genes with 1,780 of them having no obvious homologs in other bacteria such as *Escherichia coli* (Figure 1) (Stover *et al.*, 2000). *P. aeruginosa* exists in the environment mainly as a non-mucoid bacterium that flourishes in moist areas. It also has the ability to form biofilms; microcolonies that allow the bacteria to protect itself against environmental stress. When this organism enters the CF lung, emergence of a mucoid variant producing copious amounts of MEP, composed mainly of a sugar-like substance called alginate, is recognized as a poor prognostic indicator (Govan and Deretic, 1996). Bacterial colonization coupled with the CF lung's natural production of a dehydrated mucous prohibits the patient from completely eliminating the organism from the lungs, even with the most aggressive antibiotic therapy. The mucous production also prohibits host phagocytes from combating the bacterium, a phenomenon commonly called "frustrated phagocytosis." (Pedersen *et al.*, 1992). This infiltration of neutrophils to the lung produces excessive inflammation, which causes repeated coughing or episodes of intense breathing problems (Baltimore, 1993). Antibiotic therapy also has little effect on complete removal from the lung because the MEP of the mucoid variant can bind amino-glycosides as well as other antibiotics and

Figure 1. PAO1 genomic map. *Spe*I, *Xba*I restriction digest pattern of the circular chromosome of the standard reference strain *P. aeruginosa* PAO1 showing the location of selected genes *exoT*, *mucE*, *oprF*, *algD*, *mutS*, *oprC*, *exoS*, *mutY* and a gene cluster *mucBCD* examined in this study. The two inner circles show the *Spe*I (fragments labeled; see Table 1) and *Xba*I restriction digest patterns. The *rrn* operons indicate area of reported inversion. The outer circle scale indicates size in megabases. Adapted from Stover *et al.* (Stover *et al.*, 2000).


exoT
mutY
mucBCD
mucE
rrnA
rrnB
oprF
exoS
oprC
mutS
algD
Pseudomonas aeruginosa PAO1
6,264,403 bp
0
1
2
3
4
5
6
AJ
AC AB
AA
AL
AD
AH
AG
AE
AI
AK
A
B
C
D
E
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H
I
J
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N
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R
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T
U
V
W
X
Y
Z

Table 1. *Spe*I digest fragments based on the published PAO1 genomic sequence[*1]

Genome location (bp)	Fragment size (bp)	Fragment name
4,250,766 - 4,790,627	539,862	A
5,266,156 - 5,726,542	460,387	B
3,538,448 - 3,962,321	423,874	C
2,053,672 - 2,444,424	390,753	D
4,819,853 - 5,206,702	386,850	E
725,196 - 1,062,491	337,296	F
5,726,543 - 6,041,639	315,097	G
281,799 - 587,953	306,155	H
3,254,467 - 3,527,969	273,503	I
2,511,495 - 2,761,444	249,950	J
6,041,640 - 21,723	244,487	K
1,827,181 - 2,053,671	226,491	L
1,246,699 - 1,461,937	215,239	M
1,461,938 - 1,644,798	182,861	N
99,954 - 281,798	181,845	O
2,919,511 - 3,089,800	170,290	P
1,644,799 - 1,803,569	158,771	Q
2,761,445 - 2,919,510	158,066	R
4,101,947 - 4,250,765	148,819	S
3,962,322 - 4,101,946	139,625	T
1,062,492 - 1,187,488	124,997	U
3,157,585 - 3,254,466	96,882	V
587,954 - 681,774	93,821	W
2,444,425 - 2,511,494	67,070	X
3,089,801 - 3,151,305	61,505	Y
5,206,703 - 5,266,155	59,453	Z
681,775 - 725,195	43,421	AA
63,068 - 99,953	36,886	AB
21,724 - 56,415	34,692	AC
1,192,398 - 1,226,237	33,840	AD
4,790,628 - 4,819,852	29,225	AE
1,803,570 - 1,827,180	23,611	AG
1,226,238 - 1,246,698	20,461	AH
3,527,970 - 3,538,447	10,478	AI
56,416 - 63,067	6,652	AJ
3,151,306 - 3,157,584	6,279	AK
1,187,489 - 1,192,397	4,909	AL

[*1]Analysis of PAO1 genomic sequence (Stover *et al*., 2000) was performed using the software OMIGA.

decrease their access to the bacteria. The organism is also able to form antibiotic-resistant biofilms. Ultimately, the patient's physical condition continues to deteriorate until death occurs. When *P. aeruginosa* enters the CF lung, it is known that the bacterium undergoes phenotypic changes such as pigment change and level of mucoidy. This change is a defined and predictable characteristic of *P. aeruginosa*.

It is well known that clinical/environmental isolates of *P. aeruginosa* are genetically diverse (Schmidt *et al.*, 1996; Romling and Tummler, 2000). Furthermore, how representative the PAO1 genome is of the genomes of clinical isolates remains largely unknown. Nevertheless, Schmidt *et al.* (Schmidt *et al.*, 1996) noticed that aquatic/CF strain C has a larger genome size than PAO1 (6.5 vs. 6.0 Mb). Very recently, Liang *et al.* (Liang *et al.*, 2001) reported that a clinical urinary tract infection (UTI) isolate *P. aeruginosa* X24509 has a unique genomic island structure of 48.9 kb which is absent from the PAO1 genome. This insert is thought to be acquired through horizontal gene transfer possibly leading to evolution of strains with specific determinants, allowing them to infect different compromised patients (Liang *et al.*, 2001). One of the major virulence mechanisms employed by many bacteria is the acquisition of extra-chromosomal DNA into their genomes in the form of plasmids, transposons and/or bacteriophages. An example of this is the recent publication of *E. coli* O157:H7 genome sequence (Perna *et al.*, 2001). The genome of *E. coli* K12 is 4.6 Mb, whereas O157:H7 is 5.5 Mb. K12 is not pathogenic, whereas O157:H7 is

enterohemmoragic. To investigate the virulence mechanisms associated with these pathogenic bacteria, the ultimate technique is to sequence all the possible isolates: clinical and environmental sources. However, the cost of performing these tasks will be tremendous. In this context, an alternative to this approach is pulsed-field gel electrophoresis (PFGE). PFGE is a technique that can be used to study the genomic variation among CF isolates. This method separates DNA fragments based on size in an agarose gel by producing electrical pulses across differing angles in the electrophoretic field. Changing the voltage directions allows smaller DNA fragments to re-orient more quickly to the electric field than the larger DNA fragments. PFGE is able to resolve DNA fragments up to 10 Mb in length (Schwartz and Cantor, 1984), as opposed to normal gel electrophoresis, which separates fragments up to 20 kb long. When the genome of *P. aeruginosa* is cut with a particular restriction endonuclease, specific fragments of DNA are created and can be separated using PFGE with great distinction. This technique allows the study of the bacterium on a genetic level based on its nucleotide sequence, as opposed to only the phenotypic level.

OBJECTIVES

Rationale: *P. aeruginosa* exists as an aerosol throughout our environment, flourishes in aquatic habitats and colonizes immunocompromised burn patients and CF lungs. The movement from one condition to another must require some adjustment by the bacterium, be it genetic mutation,

rearrangement, or simply altered gene expression. This study investigated the ability of *P. aeruginosa* to move from outside the host into the CF lung. Is the quick adaptation of *P. aeruginosa* to the CF lung due to an intrinsic ability to rapidly alter genome structures and do CF isolates genetically adjust to their environment over the duration of colonization?

The objectives of this study were to determine: 1) the level of genetic variation of PFGE banding pattern among isolates from different CF patients and among sequential isolates from the same CF patient; 2) if any similarities in PFGE banding pattern exist between burn/laboratory/environmental isolates and CF isolates of *P. aeruginosa*; 3) the correlation, if any, of a CF isolate of *P. aeruginosa* PFGE banding pattern and level of mucoid production; 4) the existence of any significant-sized deletions in a set of genes in the chromosome of CF isolates of *P. aeruginosa*; 5) the presence, if any, of CF-specific extra-chromosomal DNA in CF isolates of *P. aeruginosa*.

MATERIALS AND METHODS

GROWTH, ISOLATION AND CLASSIFICATION OF BACTERIA

CF, burn, laboratory, and environmental isolates of *P. aeruginosa* (Table 2) were inoculated on *Pseudomonas* Isolation Agar (PIA; Difco). A single colony was isolated on PIA and incubated for 36 h at 37°C. After incubation, the bacteria were scored by the following phenotypic characteristics: level of mucoidy and pigment (Table 2) before proceeding with the agarose plug preparation.

AGAROSE PLUG PREPARATION

This procedure was adapted from PulseNet, Section 5, Preparation of PFGE Plugs from agar cultures (www.cdc.gov). After incubation on PIA for 18 h at 37°C, the bacteria were suspended in 2 ml cell suspension buffer [CSB; 100 mM Tris; 100mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] to 10% transmittance as indicated by the BioMerieux Vitek colorimeter. When mucoid bacteria are suspended, the large amount of mucous materials can give false bacterial transmittance values. Therefore, to compensate for the production of mucous, the transmittance was adjusted to 5%, thereby allowing more bacteria to be suspended to a comparable concentration to non-mucoid cultures. To prepare the agarose plug, 10 µl proteinase K (20 mg/ml; Gibco BRL, Grand Island, NY) was added to 200 µl of the cell suspension in a 2 ml flat top, round bottom microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) and mixed by

Table 2. Phenotypic classification and summary of PFGE analysis of clinical CF, environmental, burn, and laboratory isolates of *P. aeruginosa* used in this study

Isolate	pigment[*1]	mucoidy[*2]	RE[*3]	pattern[*4]	source[*5]	reference
CF 1	dark green	2	S/X	**1**	SIB	J. Govan
CF 2	green	3	S/X	2		"
CF 3	yellow-green	2	S/X	3		"
CF 4	white-cream	2	S/X	4		"
CF 5	green	2	S/X	5		"
CF 6	white-cream	3	S/X	6		"
CF 7	white-cream	0	S/X	7		"
CF 8	white-cream	0	S/X	8		"
CF 9	white-cream	3	S/X	9		"
CF 10	green	3	S/X	10		"
CF 11	yellow-green	0	S	11		"
CF 12	white-cream	3	S/X	**1**	SIB	"
CF 13	green	1	S	12		"
CF 14	dark cream	0	S	13		"
CF 15	white-cream	3	S	14		"
CF 16	yellow-green	2	S	15		"
CF 17	white-cream	1	S/X	16		"
CF 18	blue-green	3	S	17		"
CF 19	dark cream	0	S/X	18		"
CF 20	white-cream	3	S	19		"
CF 21	yellow-green	2	S/X	20		"
CF 22	white-cream	3	S/X	21		"
CF 23	white-cream	3	S/X	22		"
CF 24	dark cream	3	S/X	23		"
CF 25	blue-green	2	S/X	24		"
CF 26	yellow-green	0	S/X	25		"
CF 27	yellow-green	0	S/X	26		"
CF 28	yellow-green	0	S/X	27		"
CF 29	yellow-green	3	S/X	28		"
CF 30	yellow-green	2	S	29		"
CF 31	white-cream	3	S	30		"
CF 32	white-cream	1	S/X/D	**31**	SS?	"
CF 33	dark cream	2	S	32		"
CF 34	dark cream	0	S	33		"
CF 35	dark cream	2	S	**34**	SIB	"
CF 36	green	3	S	**34**	SIB	"
CF 37	white-cream	3	S	**35**	DP	"
CF 38	dark cream	0	S	36		"
CF 39	white-cream	3	S	37		"
CF 40	dark cream	2	S	**35**	DP	"
CF 41	dark cream	3	S	38		"
CF 42	dark cream	3	S	39		"
CF 43	green	3	S	40		"
CF 44	dark cream	3	S	41		"
CF 45	green	3	S	N/A		"
CF 46	yellow-green	0	S	N/A		"

Table 2 continued. Phenotypic classification and summary of PFGE analysis of clinical CF, environmental, burn, and laboratory isolates of *P. aeruginosa* used in this study

Isolate	pigment[*1]	mucoidy[*2]	RE[*3]	pattern[*4]	source[*5]	reference
CF 47	white-cream	0	S	42		J. Govan
CF 48	green	0	S	43		"
CF 49	yellow-green	0	S	44		"
CF 50	green	0	S	45		"
CF 149	white-cream	0	S/X	46		G. Pier
CF 332	dark cream	3	S/X	47		"
CF 2314	white-cream	3	S/X	48		"
CF 2912	white-cream	2	S/X	49		"
CF 4349	yellow-green	0	S/X	50		"
V209	dark cream	2	S	**51**	SS	P. Phibbs
FRD-1	white-cream	0	S/X	**52**	SS	G. Pier
FRD-1 PGSC	dark cream	3	S	**52**	SS	P. Phibbs
CF 001	white-cream	0	S/X	53		D. Speert
CF 002	brown cream	0	S/X	**54**	SEQ	"
CF 003	yellow-green	0	S/X	55		"
CF 004	white-cream	3	S/X	56		"
CF 005	yellow-green	0	S/X	57		"
CF 006	white-cream	3	S/X	58		"
CF 007	white-cream	2	S/X	59		N. Hoiby
CF 008	green	0	X	N/A		"
CF 009	blue-green	1	X	N/A		"
CF 010	brown cream	3	S	N/A		D. Speert
CF 011	blue-green	3	S	N/A		N. Hoiby
CF 012	green	0	S	60		"
CF 013	white-cream	3	S	**61**	SEQ	"
CF 014	white-cream	0	S	**61**	SEQ	"
CF 015	blue-green	0	S	**62**	SEQ	"
CF 016	blue-green	0	S	**62**	SEQ	"
CF 017	green	3	S	**62**	SEQ	"
CF 018	green	0	S	N/A		"
CF 019	white-cream	3	S	63		"
CF 020	white-cream	0	S	64		D. Speert
CF 021	white-cream	1	S	65		"
CF 022	brown cream	0	S	66		"
CF 023	green	3	S	**54**	SEQ	"
CF 024	white-cream	3	S	67		N. Hoiby
CF 025	white-cream	0	S	68		"
CF 026	white-cream	3	S	69		"
CF 027	white-cream	0	S	N/A		"
CF 028	green	0	S	N/A		"
CF 029	blue-green	0	S	70		"
CF 030	white-cream	0	S	**71**	SEQ	"
CF 031	white-cream	2	S	**71**	SEQ	"
CF 032	white-cream	0	S	**72**	SEQ	"
CF 033	white-cream	3	S	**72**	SEQ	"
CF 034	white-cream	0	S	**73**	SEQ	"

Table 2 continued. Phenotypic classification and summary of PFGE analysis of clinical CF, environmental, burn, and laboratory isolates of *P. aeruginosa* used in this study

Isolate	pigment[*1]	mucoidy[*2]	RE[*3]	pattern[*4]	source[*5]	reference
CF 035	blue-green	0	S	N/A		N.Hoiby
CF 036	dark cream	1	S	**73**	SEQ	"
CF 037	blue-green	1	S	N/A		"
CF 038	white-cream	0	S	74		"
CF 039	blue-green	1	S	N/A		"
CF 040	yellow-green	3	S	**51**	SS	P. Phibbs
CF 041	dark cream	0	S	**75**	SEQ	D. Speert
CF 042	dark cream	3	S	**75**	SEQ	"
CF 043	white-cream	1	S	**75**	SEQ	"
PAM425	white-cream	0	S/X	76		P. Phibbs
PAO1	green	0	S/X/D	**31**	SS?	"
PAO381	green	0	S/X	**31**	DER	"
PAO568	dark cream	3	S/X	**31**	DER	"
PAO578I	yellow-green	3	S/X	**31**	DER	"
PAO578II	white-cream	3	S/X	**31**	DER	"
PAO579	white-cream	3	S/X	**31**	DER	"
PAO581	dark cream	3	S/X	**31**	DER	"
ENV42	dark cream	0	S/X	78		D. Speert
PATIV1	yellow-green	0	S	**79**	SS	C. Somerville
PATIV2	dark cream	0	S	**79**	SS	"
PAOH	green	0	S	80		"
PA14	dark cream	0	S/X/D	81		F. Ausubel

[*1]scored with a set of 7 pigments.

[*2]scored on a numerical scale of 0-3 (0 - non-mucoid, 1 - slightly mucoid, 2 - mucoid and 3 - very mucoid).

[*3]restriction endonuclease used for digestion of each sample (S - *Spe*I, X - *Xba*I, and D - *Dpn*I).

[*4]unique pattern number assigned to a particular isolate.

[*5]DER - derivatives, DP - different patients, SEQ - sequential isolates, SIB - siblings, SS - same source.

inversion. Then, 200 µl of a mixture of 1.6% InCert agarose (FMC BioProducts, Rockland, ME) and 1% sodium dodecyl sulfate (SDS) [1.6% Incert/1% SDS mixture: 0.8 g InCert agarose; 2.5 ml 20% SDS; 46.7 ml Tris-EDTA buffer (TE; 10 mM Tris; 1 mM EDTA, pH 8.0)] was made and mixed with the cell suspension/proteinase K mixture and dispensed in a plug mold (Bio-Rad Laboratories, Hercules, CA). The plug was allowed to solidify at 4°C for 10 min. The plug was then transferred to a 2 ml round bottom tube for cell lysis. Cell lysis buffer (1.5 ml) (CLB; 50 mM Tris; 50 mM EDTA, pH 8.0; 1% N-Lauroylsarcosine) and 40 µl proteinase K were added to the 2 ml round bottom tube. The tube was incubated in a 54°C shaking water bath for 1.5 h, completely immersed with constant and vigorous agitation (75 rpm). After cell lysis, the plug was transferred to a 50 ml centrifuge tube (Corning Inc., Corning, NY) and washed twice for 15 minutes at 48°C with 10 ml pre-heated, sterile, reagent grade H_2O and twice for 15 minutes with 10 ml pre-heated TE buffer. After washing, the plug was stored in 1.5 ml TE buffer at 4°C for up to 1 year until ready for restriction digestion.

RESTRICTION DIGESTION

A 1.5 mm wide slice of gel to be digested was cut from the prepared plug and placed in a 2 ml round bottom tube. A restriction digest mixture was prepared by adding a measured amount of a restriction endonuclease (*Dpn*I, 13.5 U, Gibco BRL; *Spe*I, 5-30 U, Takara Biomedicals, Shiga, Japan; *Xba*I, 20-

100 U, Boehringer Mannheim, Germany) to a 1:10 dilution of manufacturer's buffer in sterile, reagent grade H_2O to a final volume of 100 µl. The mixture was added to the 2 ml round bottom tube, immersing the plug slice, and incubated in a 37°C standing water bath for 1 h.

PULSED-FIELD GEL ELECTROPHORESIS

A 1% Seakem Gold Agarose (BioWhittaker Molecular Applications, Rockland, ME) gel in 0.5X Tris-Borate EDTA (TBE; 10X: 0.9 M Tris; 0.9 M Boric acid; 0.02 M EDTA, pH 8.0) was prepared and poured into a gel mold fitted with a comb (Bio-Rad). After the gel solidified, the comb was removed and the restriction digested plug slice as well as a 1.5 mm wide slice of lambda DNA concatemer size standard agarose plug (Bio-Rad) were placed in the wells within the gel. A volume of 5 ml 1% agarose was poured over the top of the wells to hold the digested plugs in the gel. The electrophoresis unit (Bio-Rad Electrophoresis Chamber) was filled with 2 L 0.5X TBE and cooled to 14°C using a circulating cooler (Bio-Rad Cooling Module). The gel was then mounted inside the electrophoresis unit and run for 18-24 h with interval switch times from 0.22-46.67 s to 5-73.58 s with a ramping factor from linear to 0.35741 at 200 V (Chef Mapper XA Pulsed-Field Electrophoresis System; Bio-Rad). After the completion of the electrophoretic run, the gel was placed in an ethidium bromide solution (100 ng/ml in sterile, reagent grade H_2O) for 30 min. After staining, the gel was photographed utilizing the Bio-Rad Gel Doc 2000 unit and Bio-Rad Quantity

One/The Discovery Series software (Version 4.0.1). Printed images were produced by the Mitsubishi P91W Video Copy Processor.

CHROMOSOMAL DNA EXTRACTION

This protocol was adapted from Quantum Prep AquaPure Genomic DNA Isolation Kit (Bio-Rad). A single colony was removed from a PIA plate and incubated in 1.5 ml Lennox Broth (LB; Difco) for 14-18 h at 37°C. After incubation, 500 µl of cell suspension was transferred to a 1.7 ml microfuge tube (Midwest Scientific, Valley Park, MO) and centrifuged at 16,000 g for 15 s to pellet the cells. The supernatant was removed and the cells were resuspended in 300 µl Genomic DNA Lysis Solution. The mixture was incubated at 80°C for 5 min to lyse the cells. After incubation, 1.5 µl RNase was added to the cell lysate, mixed by inverting the tube 25 times and incubated at 37°C for 45 min. Protein Precipitation Solution (100 µl) was added to the cell lysate, vortexed at high speed (Vortex Genie 2; Fisher Scientific) for 20 s and centrifuged at 16,000 g for 3 min. The supernatant was removed to a clean 1.5 ml microfuge tube containing 300 µl cold 100% isopropanol, mixed by inverting the tube gently 50 times to precipitate the DNA and centrifuged at 16,000 g for 1 min. The supernatant was removed, 300 µl cold 70% ethanol was added, mixed by inverting the tube gently several times and centrifuged at 16,000 g for 1 min. The ethanol was carefully poured off to avoid losing the DNA pellet. The tube was inverted and drained on clean absorbent paper and allowed to dry for 0.5-1 h.

The pellet was hydrated by adding 100 µl TE buffer, vortexed gently and the tube was incubated at 65°C for 1 min. The sample was vortexed gently for 5 s to suspend and pulse centrifuged at 16,000 g for 10 s to collect the DNA at the bottom of the tube. The DNA solution was stored at -20°C.

An alternate procedure was also used (Ausubel *et al.*, 1989). After incubation in 5 ml LB for 14-18 h at 37°C, 1.5 ml cell suspension was transferred to a 2 ml microfuge tube and centrifuged at 16,000 g for 1 min to pellet the cells. The supernatant was removed and the cells were resuspended in 567 µl TE buffer. SDS (30 µl 10%), 3 µl proteinase K and 100 µl 5 M NaCl were added to the microfuge tube and vortexed at high speed for 30 s. Hexadecyltrimethyl ammonium bromide (CTAB; Fisher Scientific)/NaCl solution (80 µl) (2% CTAB; 100 mM Tris-Cl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl) was added to the tube, vortexed at high speed for 30 s and incubated at 65°C for 10 min. Chloroform/isoamyl alcohol (780 µl 24:1) was added to the tube, vortexed at high speed for 30 s and centrifuged at 16,000 g for 5 min. The supernatant was removed to a clean microfuge tube and an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol (Gibco BRL) was added. The tube was vortexed gently for 10 s and centrifuged at 16,000 g for 5 min. The supernatant was removed to a clean microfuge tube and half the tube volume of cold 100% isopropanol was added. The tube was shaken until a stringy, white DNA precipitant became clearly visible. The DNA was centrifuged at 16,000 g for 10 s to collect the DNA at the bottom of the tube. The supernatant was removed and

the DNA pellet was washed twice with cold 70% ethanol. The supernatant was removed and the pellet was resuspended in 100 µl TE buffer at room temperature.

CHROMOSOMAL DNA DIGESTION, DESALTING AND PURIFICATION

To prepare a whole genome digest probe, 8 µl DNA sample (**CHROMOSOMAL DNA EXTRACTION** section) was digested with 4 U *Sau*3A (Gibco BRL) restriction endonuclease in 1X REact 4 buffer (Gibco BRL) at 37°C for 14-18 h.

This protocol was adapted from QIAEX II Gel Extraction Kit (QIAGEN, Germany). The digested DNA sample was resuspended in 100 µl Buffer QX1. After making sure the color of the sample was yellow (for proper pH confirmation), 10 µl QIAEX II resin was added to the sample, vortexed gently for 5 s to mix, and incubated at room temperature for 10 min. The sample was mixed every 2 min. After incubation, the sample was centrifuged at 16,000 g for 30 s and the supernatant was removed. The pellet was washed twice with 500 µl Buffer PE. The tube was inverted and drained on clean absorbent paper and allowed to dry for 20-30 min. The pellet was hydrated with 100 µl sterile, reagent grade H_2O, vortexed gently and the tube was incubated at 50°C for 1 min. After incubation, the tube was centrifuged at 16,000 g for 30 s. The supernatant was removed to a clean microfuge tube. The purified DNA solution was stored at

-20°C.

NON-RADIOACTIVE LABELING OF DNA

The purified DNA solution was diluted 1:200 in sterile, reagent grade H_2O and quantitated using the Perkin Elmer UV/VIS Spectrometer Lambda Bio 20. The concentration of DNA was calculated by multiplying the absorbance at 260 nm by the dilution factor (200) and the extinction coefficient conversion factor of DNA, 50 µg/ml (A_{260} = 1 = 50 µg/ml of double-stranded DNA).

This protocol was adapted from Roche DIG High Prime DNA Labeling and Detection Starter Kit II, Version 1. Template DNA (3 µg) was added to sterile, double distilled H_2O to a final volume of 16 µl in a microfuge tube. The tube was placed in a boiling water bath for 10 min and chilled quickly in an ice bath. DIG-High Prime (4 µl) was added to the denatured DNA, vortexed gently and pulse centrifuged at 16,000 g for 5 s. The DIG-High Prime/DNA mix was incubated at 37°C for 20 h. After incubation, the tube was heated to 65°C for 10 min to stop the labeling reaction. Under optimal conditions, the hypothetical yield for 3 µg of template DNA is 2,650 ng DIG-labeled DNA. The labeled DNA was then diluted to 1 ng/µl with sterile, double distilled H_2O. A dilution series consisting of the diluted original, 1 ng/µl, and DNA Dilution Buffer dilutions of the original: 10, 3, 1, 0.3, 0.1, 0.03, 0.01 pg/µl and a control with only DNA Dilution Buffer were created to determine the labeling efficiency of the probe. A 1 µl spot of each

dilution and the control were placed on a strip of Hybond-N+ membrane (Amersham Pharmacia Biotech Limited, Buckinghamshire, England) and UV-crosslinked (UV Crosslinker UVXL-1000; Fisher Scientific) for 30 s. The diluted original was stored at -20°C. The membrane was incubated at 25°C with agitation for 2 min in 20 ml Maleic acid buffer. The membrane was removed and incubated at 25°C for 30 min in 10 ml Blocking solution then at 25°C for 30 min in 10 ml Antibody solution. The membrane was washed twice at 25°C for 15 min in 10 ml Washing buffer and equilibrated at 25°C for 5 min in 10 ml Detection buffer. A hybridization bag was opened on 3 sides and the damp membrane was placed inside with the labeled surface upright. Chemiluminescent substrate (0.1 ml), CSPD, was applied to the labeled surface and immediately covered by the other side of the bag, spreading the substrate evenly and without air bubbles over the surface. The membrane was incubated at 25°C for 5 min. After incubation, the excess liquid was squeezed out of the bag and the edges were sealed. The membrane was exposed to X-ray film for 15 min at 25°C and the film was developed. The presence of darkened spots at the dilution 0.1 pg/µl and higher indicated the labeled DNA had reached the expected labeling efficiency and could be used at the recommended concentration in the hybridization.

POLYMERASE CHAIN REACTION

Primers selected were prepared by the Joan C. Edwards School of Medicine, Marshall University DNA Core Facility, Huntington, WV. The

polymerase chain reaction (PCR) mixture consisted of 16 µl sterile, double distilled H_2O, 3 µl dimethylsulfoxide (DMSO), 2.5 µl of each primer (5 µM each), 1 µl template DNA (**CHROMOSOMAL DNA EXTRACTION** section), 0.5 µl Taq polymerase (5 U/µl, Perkin-Elmer, Norwalk, CT), in 0.5 ml EasyStart PCR reaction tubes (Molecular Bio-Products, Inc., San Diego, CA). Reaction conditions were 94°C for 2 min and then 33 cycles with 50-59°C for 2 min, 72°C for 2 min, 94°C for 1 min, and 72°C for 10 min in a thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler) with 30-well PCR plates. A 1% agarose product gel in 1X TAE (50X: 242g Tris; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA, pH 8.0) was prepared, 5 µl ethidium bromide (10µg/µl) was added, and poured into an appropriate gel mold fitted with a comb. After the gel solidified, the comb was removed and the gel was placed in an electrophoresis unit (Horizon 11 • 14; Gibco BRL), the wells nearer the cathode, with 900 ml 1X TAE running buffer. PCR product (5 µl) and 2 µl of stain were loaded into a single lane of the gel along with a 1-kb size standard (Gibco BRL) in a separate lane.

SOUTHERN BLOT TRANSFER

After the pulsed-field gel electrophoresis, staining and photographs, the gel was soaked in 0.25 N HCl for 30 min. The gel was rinsed in sterile, distilled H_2O, soaked in 1.5 M NaCl/0.5 M NaOH for 30 min then soaked in 1.5 M NaCl/0.5 M Tris-Cl, pH 8.0 for 30 min. A blotting apparatus was constructed with a filter paper wick, 6 filter paper pieces cut to the size of the gel and 20X SSC

transfer buffer (3 M NaCl; 0.3 M sodium citrate; pH 7.0). The wick and 3 filter paper pieces were prewet in transfer buffer and placed on the blotting apparatus. The gel was briefly soaked in transfer buffer and placed upside down on top of the 3 filter paper pieces. A Hybond-N+ membrane was cut to fit the dimensions of the gel, prewet in sterile, reagent grade H_2O, in transfer buffer and transferred to the gel. The other 3 filter paper pieces were prewet in transfer buffer and placed on top of the membrane. An 8-inch stack of paper towels cut to fit the dimensions of the gel were placed on top of the filter paper pieces with a glass plate and 1.0 kg weight on top of the paper towels. Transfer time was 24 h and when removed, the membrane was briefly placed in fresh transfer buffer and then on fresh filter paper. The damp membrane was UV-crosslinked for 6 X 30 s with 2-min increments in-between each crosslink. The gel was photographed using the BioRad Gel Doc 2000 unit and BioRad Quantity One/The Discovery Series software (Version 4.0.1) to assure proper DNA transfer. If the membrane was not hybridized at that time, it was stored dry at 4°C.

HYBRIDIZATION AND DEVELOPMENT OF SOUTHERN BLOT MEMBRANE

This protocol was adapted from Roche DIG High Prime DNA Labeling and Detection Starter Kit II. 375 ng DIG-labeled DNA was denatured in a boiling water bath for 5 min and chilled quickly in an ice bath. The denatured, labeled DNA was added to 15 ml prewarmed DIG Easy Hyb and mixed gently. A hybridization bag was opened on 3 sides, the membrane was placed inside and

was prehybridized for 30 min in 15 ml prewarmed DIG Easy Hyb at 40°C with gentle agitation after the bubbles were removed and the edges were sealed. The prehybridization solution was removed from the bag and the labeled DNA/DIG Easy Hyb mixture was added to the bag, bubbles were removed and edges were sealed. The prehybridization and hybridization steps were performed with the bag submerged in a 40°C agitating water bath overnight.

After hybridization, the membrane was removed from the bag and washed with the labeled surface upright for 2 X 5 min in 125 ml 2X SSC/0.1% SDS at 25°C and 2 X 15 min in 125 ml 0.5X SSC/0.1% SDS at 68°C, with both sets of washes under constant agitation. After washes, the membrane was incubated with the labeled surface upright for 5 min in 100 ml Washing buffer, 1 h in 150 ml Blocking solution, 30 min in 40 ml Antibody solution, 3 X 15 min in 125 ml Washing buffer and 5 min in 20 ml Detection buffer, with all incubations at 37°C under constant agitation. A hybridization bag was opened on 3 sides, the damp membrane was placed inside with the labeled surface upright and 1.5 ml CSPD was added to the labeled surface. Immediately, the membrane was covered by the other side of the bag, spreading the substrate evenly and removing air bubbles from the surface and incubated for 5 min at 25°C. After incubation, the excess liquid was squeezed out of the bag and the edges were sealed. The membrane was incubated for 10 min at 37°C and exposed to X-ray film for 0.25-11 h at 25°C and developed.

After development, the probe was stripped from the membrane by rinsing it in sterile, reagent grade H_2O, washing for 2 X 15 min in 0.2 M NaOH/0.1% SDS at 37°C and rinsing thoroughly for 5 min in 2X SSC. The membrane was either hybridized again at that time or stored in 2X SSC at 4°C.

RESULTS

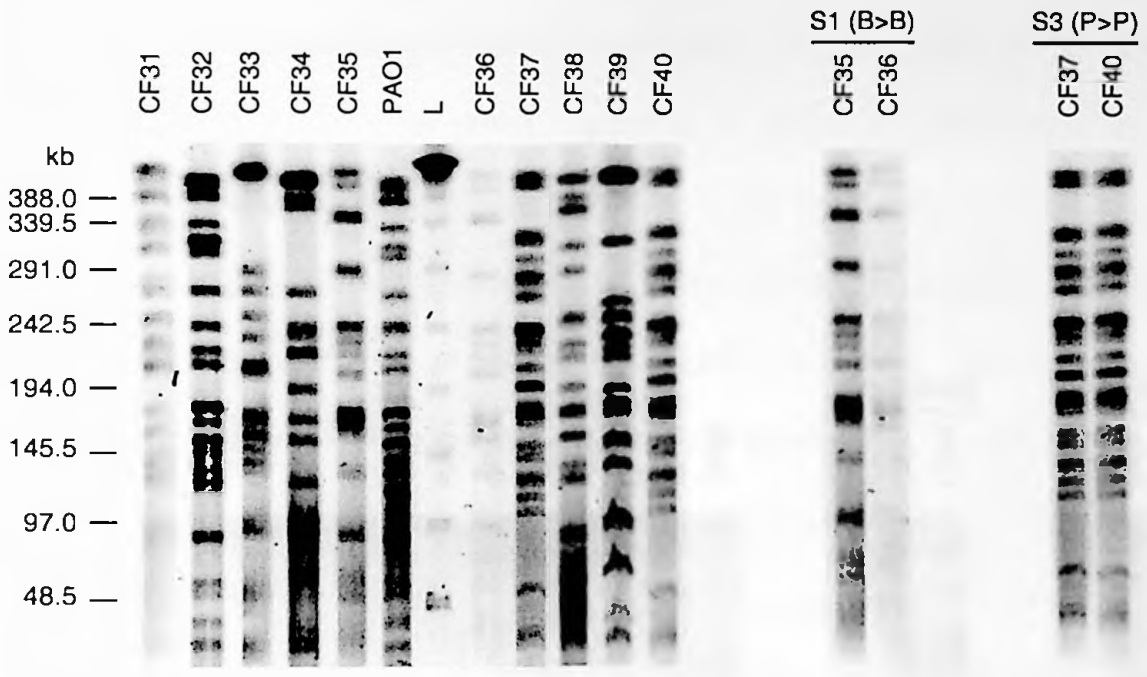
THE GENETIC DIVERSITY AMONG CLINICAL CF ISOLATES OF *P. aeruginosa*

PFGE coupled with restriction digestion of the entire chromosome was used to generate an RFLP pattern representative of each strain listed in Table 2. This procedure was performed on 101 CF strains isolated from various geographical locations (Table 2). Of the 101 isolates tested, 75 unique genomic profiles were established as compared to other patterns based on a) molecular weight markers vs. band position and b) relative distance between bands. Three sets of RFLP patterns were duplicated in different CF patient isolates (SIB and DP in Table 2). Figure 2 shows the RFLP banding pattern after *Spe*I digestion under 3 different separation conditions. Of the 3 sets of RFLP patterns, 2 (S1: CF35/CF36; S2: CF1/CF12, Figure 2) are known to be from sets of brothers and 1 set (S3: CF37/CF40; Figure 2A,B) from 2 different non-related patients cared for in the same hospital. Interestingly, another set of siblings (S4: CF3/CF7, Figure 2C) were not colonized by the same CF isolates.

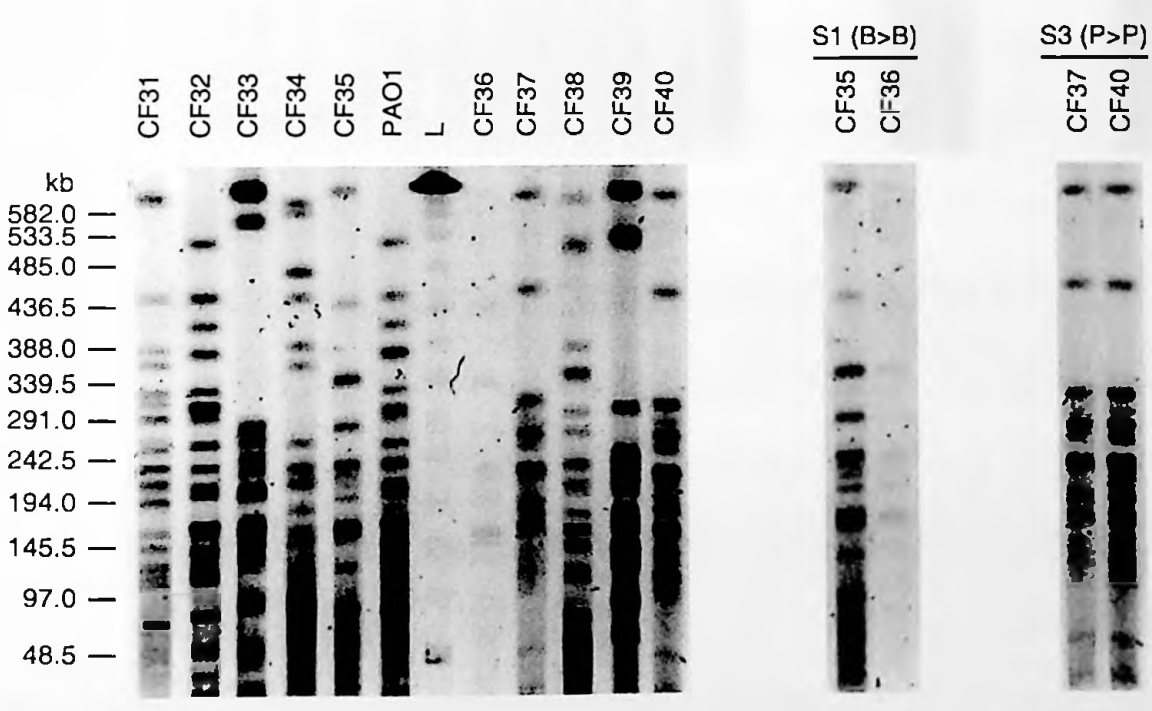
To examine the presence of genetic variability among sequential isolates from the same CF patient, a total of 16 sequential CF strains from 7 patients were tested (SEQ in Table 2; each unique pattern number for SEQ isolates indicates a separate patient). For example, three isolates were tested from a single patient: CF041, isolated in 1990, CF042, isolated in 1994 and CF043,

Figure 2. PFGE patterns of CF isolates from different patients at three different interval switch times. *Spe*I restriction digest PFGE patterns of genomic DNA from clinical CF isolates of *P. aeruginosa.* Lanes CF31-40, isolates from different CF patients; PAO1, standard reference strain. Interval switch times were (A) 3-20 s, (B) 5-35 s, (C) 5-30 s with a ramping factor of 0.35741 at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System (Bio-Rad). Lanes L, λ DNA concatemer size standard (Bio-Rad). S1 and S2 are each sets of similar CF isolate RFLP profiles from 2 sets of brothers, (B>B). S3 is a set of similar CF isolate RFLP profiles from 2 different patients within the same hospital, (P>P). S4 is a set of different CF isolate RFLP profiles from 2 siblings, (S / S). The appropriate lanes are artificially combined for visual comparison.

A


CF31
CF32
CF33
CF34
CF35
PAO1
L
CF36
CF37
CF38
CF39
CF40
S1 (B>B)
CF35
CF36
S3 (P>P)
CF37
CF40
kb
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

B


CF31
CF32
CF33
CF34
CF35
PAO1
L
CF36
CF37
CF38
CF39
CF40
S1 (B>B)
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CF36
S3 (P>P)
CF37
CF40
kb
582.0
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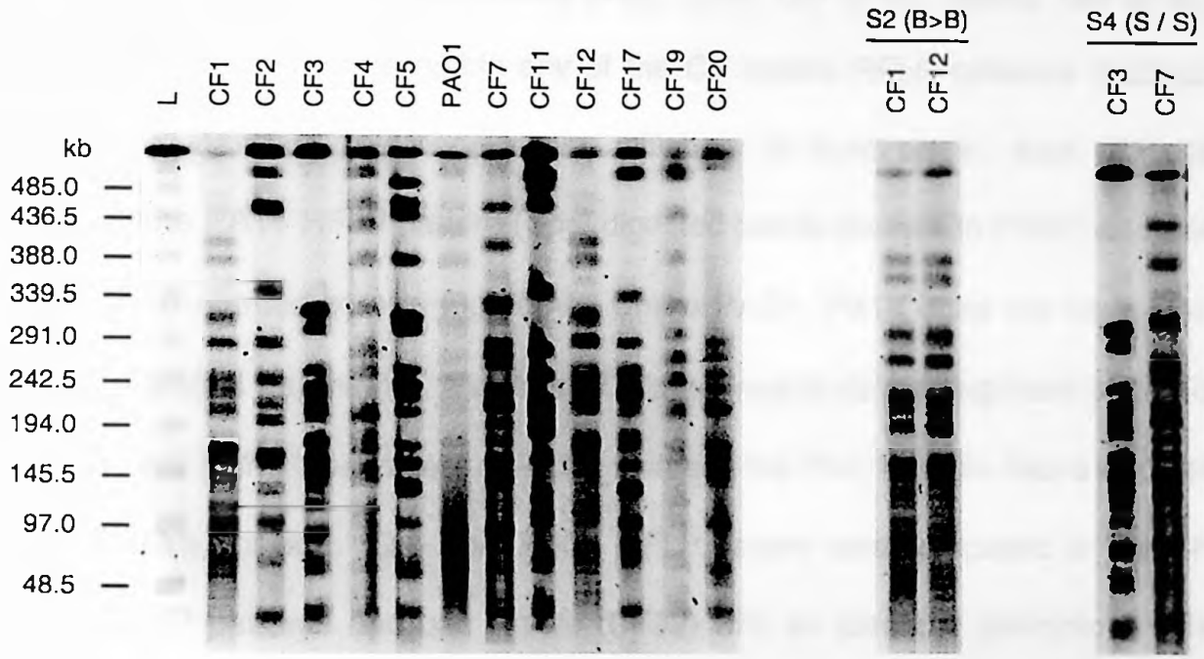
242.5
194.0
145.5
97.0
48.5

C


L
CF1
CF2
CF3
CF4
CF5
PAO1
CF7
CF11
CF12
CF17
CF19
CF20
S2 (B>B)
CF1
CF12
S4 (S / S)
CF3
CF7
kb
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

isolated in 1998. Identical *Spe*I digest PFGE patterns were seen for these 3 isolates (Figure 3).

To study the presence of similarities in RFLP profiles between CF isolates and burn/laboratory/environmental isolates, 2 burn, 7 laboratory and 4 environmental isolates were tested. Of the 4 environmental isolates studied, none resembled the RFLP banding pattern of the CF isolates studied. One of the burn isolates (PA14) (Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999a; Tan *et al.*, 1999b) studied did not resemble any of the CF isolate RFLP patterns studied. PAO1, the standard reference strain, which is of burn origin, also did not resemble the PA14 RFLP pattern (*Spe*I digested bands present in PAO1 and not in PA14 are marked by • in Figure 4). Unlike PAO1, PA14 does not have 540 and 460 kb *Spe*I fragments. A cluster of 4 large fragments ranging from 300-410 kb is present in the *Spe*I digest of PA14, whereas the PAO1 profile has a doublet at the 390 kb position. The *Spe*I PAO1 RFLP pattern was compared to the CF isolate RFLP patterns and one isolate (CF32) with an identical genomic profile was discovered (Figure 5). Given the conditions of the PFGE run that were selected, it was possible to resolve 13 distinct bands (⋆ in Figure 5,6; Table 1) from PAO1 and CF32. Some of the brighter bands in PAO1 represent doublets. For example, the 390 and 310 kb bands are the combination of fragments 391 and 387 kb, and 315 and 306 kb, respectively. These 13 bands appear to be identical between PAO1 and CF 32. According to the Dice equation, which compares the similarities between two RFLP patterns:

Figure 3. PFGE patterns of CF isolates from the same patient over eight years. Comparison of *Spe*I restriction digest PFGE patterns of genomic DNA from clinical CF isolates of *P. aeruginosa*. Lanes CF041-043, sequential isolates from the same CF patient over an 8-year period. Interval switch times were 10-40 s with a ramping factor of 0.35741 at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System. The lanes are artificially combined for visual comparison.

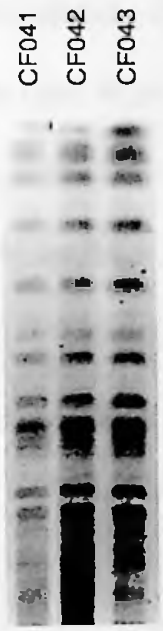

CF041
CF042
CF043

Figure 4. PFGE patterns of burn isolates. Comparison of *Spe*I restriction digest PFGE patterns of genomic DNA from burn isolates of *P. aeruginosa*. Lanes PAO1 and PA14, burn isolates. Interval switch times were 10-40 s with a ramping factor of 0.35741 at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System. Dots (•) indicate bands present in PAO1 and not present in PA14. The lanes are artificially combined for visual comparison.

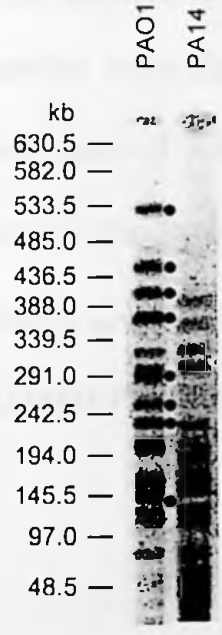

PAO1
PA14
kb
630.5
582.0
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

Figure 5. Comparison of PFGE patterns of CF32 and PAO1. Lane CF32, isolate from a CF patient. Samples were digested with *Spe*I. Interval switch times were 5-35 s with a ramping factor of 0.35741 at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System. Stars (•) indicate similarity of DNA banding pattern between PAO1 and CF32. Arrowheads indicate location of *Spe*I digest fragments A to M of PAO1 (Table 1).

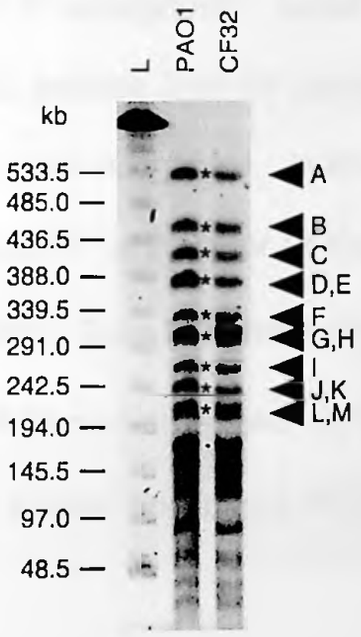
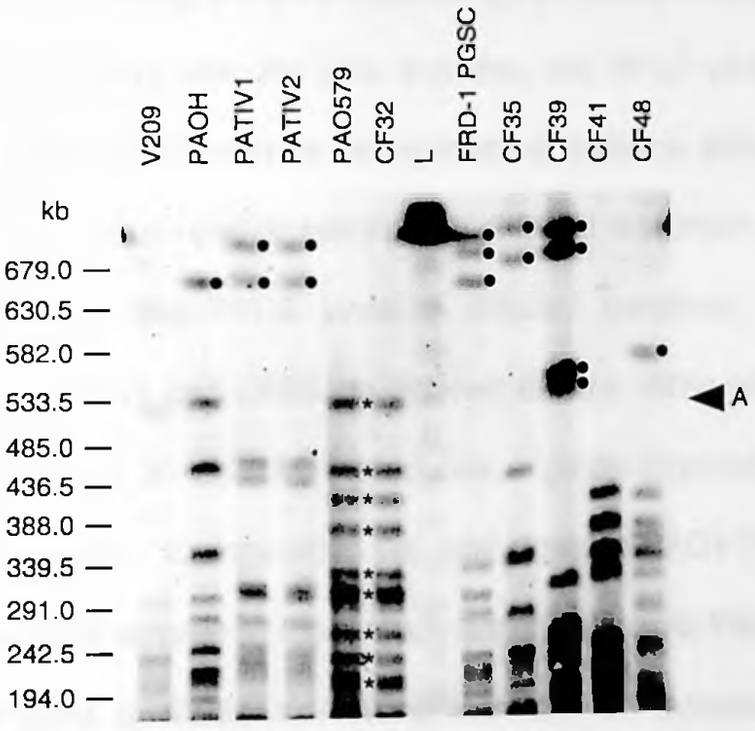

L
PAO1
CF32
kb
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5
A
B
C
D,E
F
G,H
I
J,K
L,M

Figure 6. Genomic diversity among CF and environmental isolates. *Spe*I restriction digest PFGE patterns of genomic DNA from clinical CF and environmental isolates of *P. aeruginosa*. Lanes V209, CF32, FRD-1 PGSC, CF35, CF39, CF41, CF48, isolates from CF patients; PAOH, PATIV1, PATIV2, aquatic habitat; PAO579, laboratory strain. Interval switch times were 15-40 s with a ramping factor of 0.35741 at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System. Stars (⋆) indicate similarity of DNA banding pattern between PAO579, a PAO1-derivative and CF32. Dots (•) indicate DNA fragments larger than the largest *Spe*I digest PAO1 fragment A (540 kb) (Table 1).


V209
PAOH
PATIV1
PATIV2
PAO579
CF32
L
FRD-1 PGSC
CF35
CF39
CF41
CF48
kb
679.0
630.5
582.0
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
A

$(2 \times n)/(a + b)$ = Dice's coefficient

(n represents the number of fragments found to be similar between 2 samples, a represents the number of fragments identified in one sample and b represents the number identified in the comparison sample). If a number greater than or equal to 0.9 is achieved, then the two compared sets of fragments are considered to be identical (Arbeit, 1999). After confirmation of 13 bands from PAO1 and CF32, using the Dice equation [$(2 \times 13)/(13 + 13) = 1$], a value of 1 was reached. Along with the Dice equation, the RFLP profiles of PAO1 and CF32 were further confirmed as having identical patterns with three independent agarose plug preparations, digestions with different restriction enzymes *Xba*I and *Dpn*I and running the PFGE under a different condition. Interestingly, the phenotypes of PAO1 and CF32 are different *in vitro*. After growing them on PIA plates for 36 h at 37°C, PAO1 produces a green pigment, while CF32 is a creamy white color. Compared to the *Spe*I digested PAO1 RFLP pattern, many of the CF isolates appear to have much larger fragments than the largest PAO1 fragment (Figure 6, Table 1). Along with the CF isolates, 3 environmental isolates (PAOH, PATIV1 and PATIV2) also have larger *Spe*I digest fragments than PAO1 fragment A (all bands larger than PAO1 fragment A marked by dots (•) in Figure 6).

CORRELATION BETWEEN MUCOIDY PHENOTYPE AND GENOMIC PROFILES

All of the CF, burn, laboratory and environmental isolates used in this study were first scored for the level of mucoidy on a scale of 0 to 3 with 0 being non-mucoid, 1 being slightly mucoid, 2 being mucoid, and 3 being very mucoid (Table 2). The PFGE results with the mucoid derivatives of PAO1 show that there was little or no genetic changes required for the PAO1 derivatives to become mucoid (Figure 7). As can be seen in Table 2, the mucoid production was scored 3 (very mucoid) for PAO568, PAO578I, PAO578II, PAO579 and PAO581 and scored 0 (non-mucoid) for PAO1 and PAO381. The genomic profiles for these derivatives were identical to that of PAO1 and PAO381 with *Spe*I and *Xba*I digestion (Figure 7). Also, according to Table 2, CF041 was scored 0 (non-mucoid) and CF042 was scored 3 (very mucoid), yet their genomic profiles were identical (Figure 3). Again, there is no apparent correlation between the level of mucoidy and genomic profile.

SEARCHING FOR MAJOR GENOMIC DELETIONS AND CF-SPECIFIC EXTRA-CHROMOSOMAL DNA

A selected group of genes were analyzed in this study by PCR amplification to attempt to locate major deletions in the genome of 43 clinical CF isolates, with 2 environmental and 2 burn isolates as controls (Table 3). After

Figure 7. PFGE patterns of laboratory strains. Restriction digest PFGE patterns of genomic DNA from laboratory strains of *P. aeruginosa.* Lanes PAM425, PAO581, PAO568, PAO579, PAO381, PAO578I, PAO578II, laboratory mucoid PAO1-derivatives. Interval switch times were (A) 3-20 s with a ramping factor of 0.24297, digested with *Spe*I, (B) 5-20 s ramped linear, digested with *Xba*I at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System.

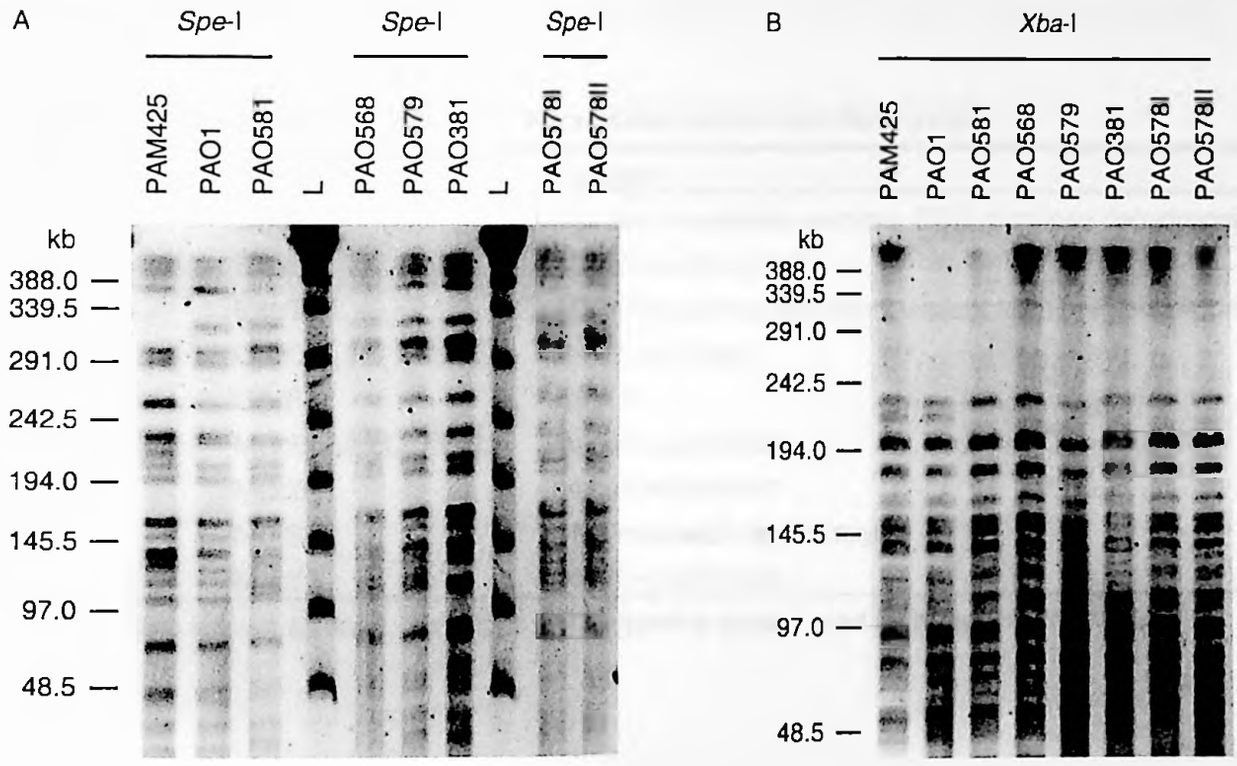

A
Spe-I
Spe-I
Spe-I
PAM425
PAO1
PAO581
L
PAO568
PAO579
PAO381
L
PAO578I
PAO578II
kb
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5
B
Xba-I
PAM425
PAO1
PAO581
PAO568
PAO579
PAO381
PAO578I
PAO578II
kb
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

Table 3. Genes analyzed in this study, the encoded proteins and their function

Gene	Protein(s)	Function
algD	GMDH	alginate biosynthetic enzyme, GDP-mannose dehydrogenase
exoS	ExoS	ADP-ribosylating toxin
exoT	ExoT	ADP-ribosylating toxin (most virulent toxin in *Pseudomonas*)
oprC	OprC	structural protein
oprF	OprF	porin F
mucBCD	MucB, MucC, MucD	mucoid expression
*mucE**	MucE	mucoid expression
mutS	MutS	DNA mismatch repair enzyme
mutY	MutY	DNA N-glycosylase

* - *mucE* also known as *lepA;* produces a GTP-binding protein used for protein secretion and transport.

analyzing the gene products, there were no deletions identified among the group of clinical, burn and environmental isolates studied (Figure 8, Table 4). Based on the limited sequencing results of *mutS* from CF023 and 026, no sequence changes in these 2 strains were identified.

To study the possible presence of CF-specific extra-chromosomal DNA, a method was developed that couples Southern blot with PFGE involving labeling of the entire PAO1 genome and hybridizing to a southern blot transfer. Figure 9 shows whole-genome labeling of the PFGE profile after Southern blot and hybridization. No extra-chromosomal DNA was identified using this procedure.

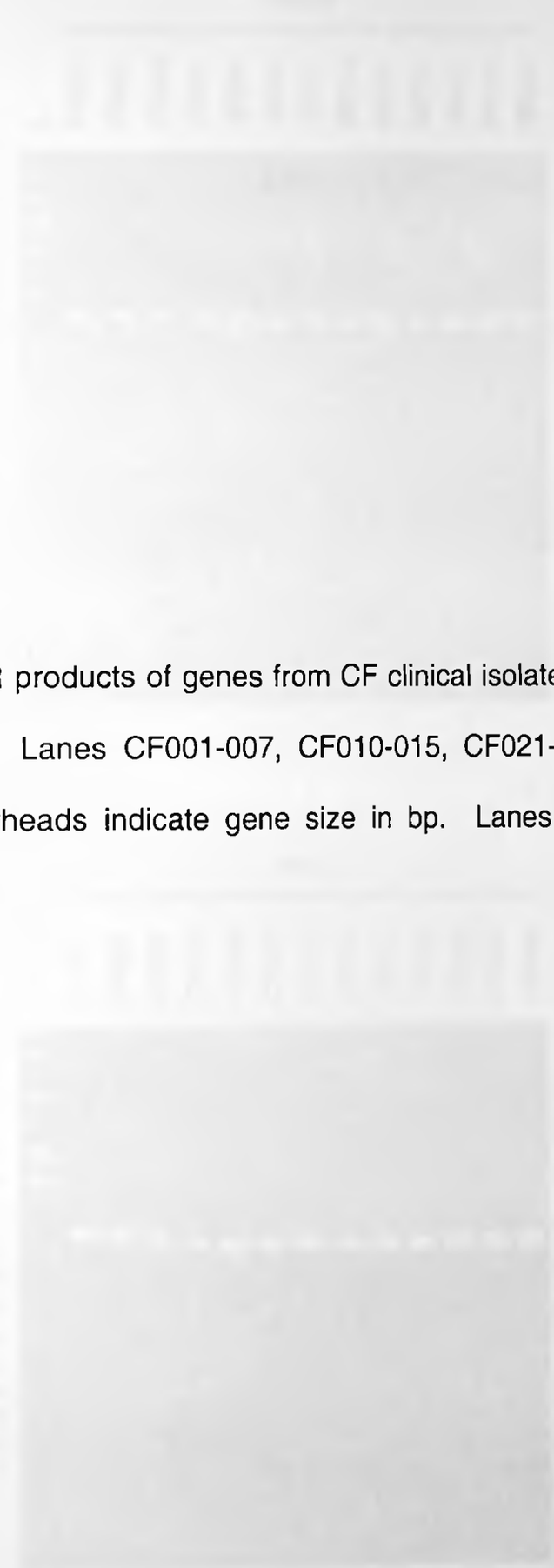
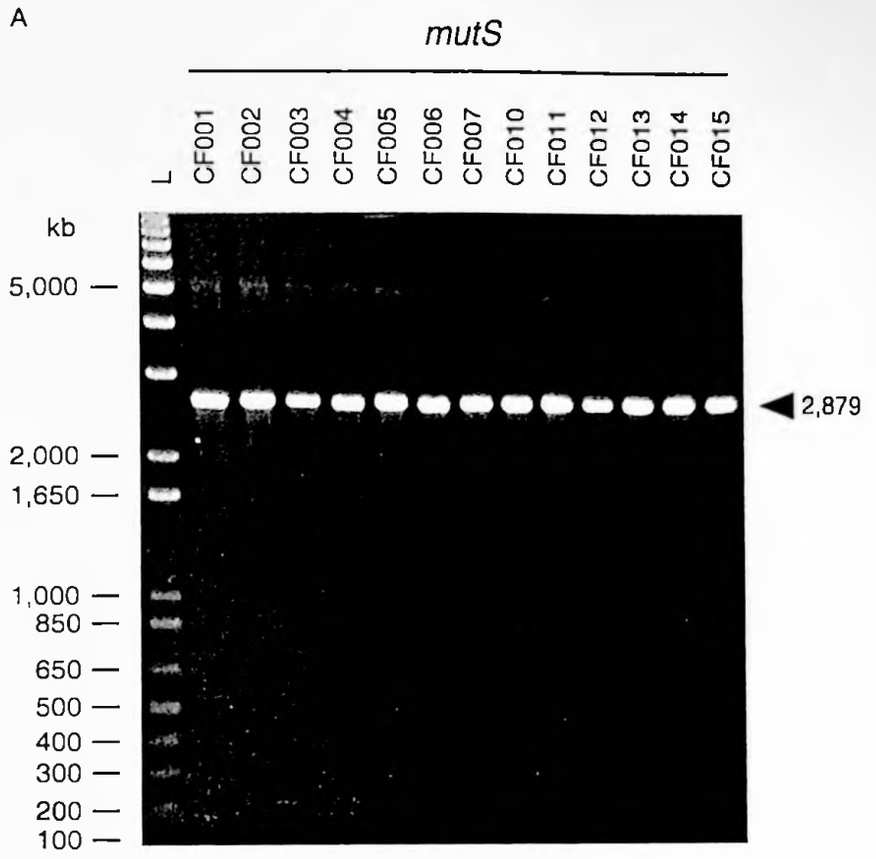
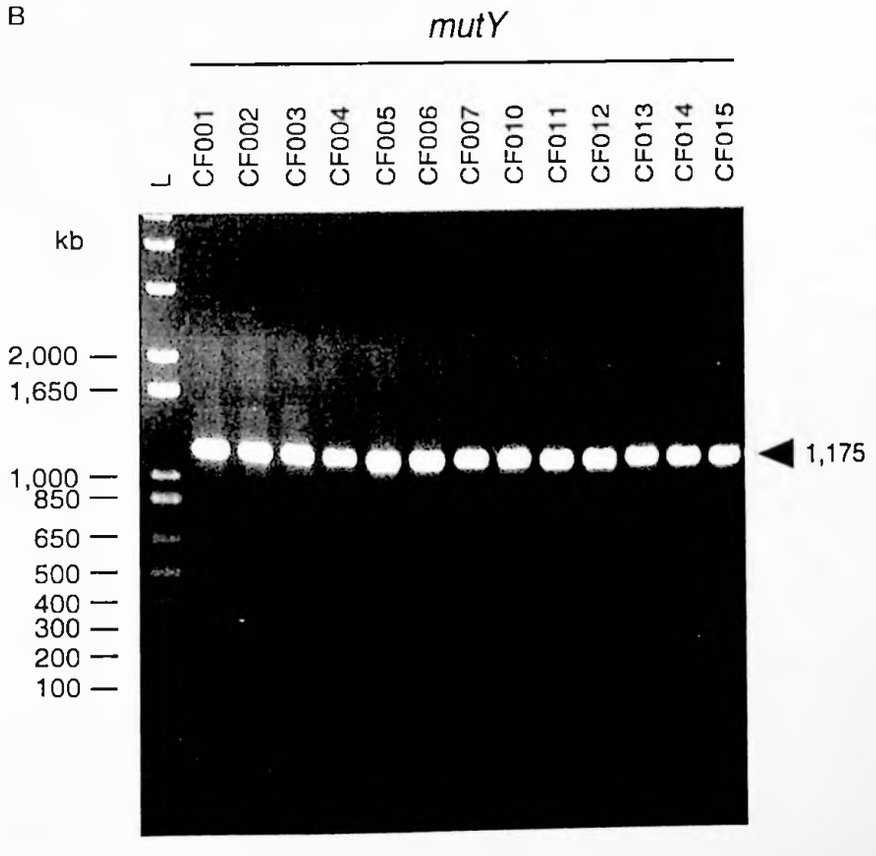


Figure 8. PCR products of genes from CF clinical isolates. *mutS* (A), *mutY* (B) and *algD* (C). Lanes CF001-007, CF010-015, CF021-030, isolates from CF patients. Arrowheads indicate gene size in bp. Lanes L, 1-kb size standard (Gibco BRL).


A
mutS
L
CF001
CF002
CF003
CF004
CF005
CF006
CF007
CF010
CF011
CF012
CF013
CF014
CF015
kb
5,000
2,000
1,650
1,000
850
650
500
400
300
200
100
2,879


B
mutY
L
CF001
CF002
CF003
CF004
CF005
CF006
CF007
CF010
CF011
CF012
CF013
CF014
CF015
kb
2,000
1,650
1,000
850
650
500
400
300
200
100
1,175

C

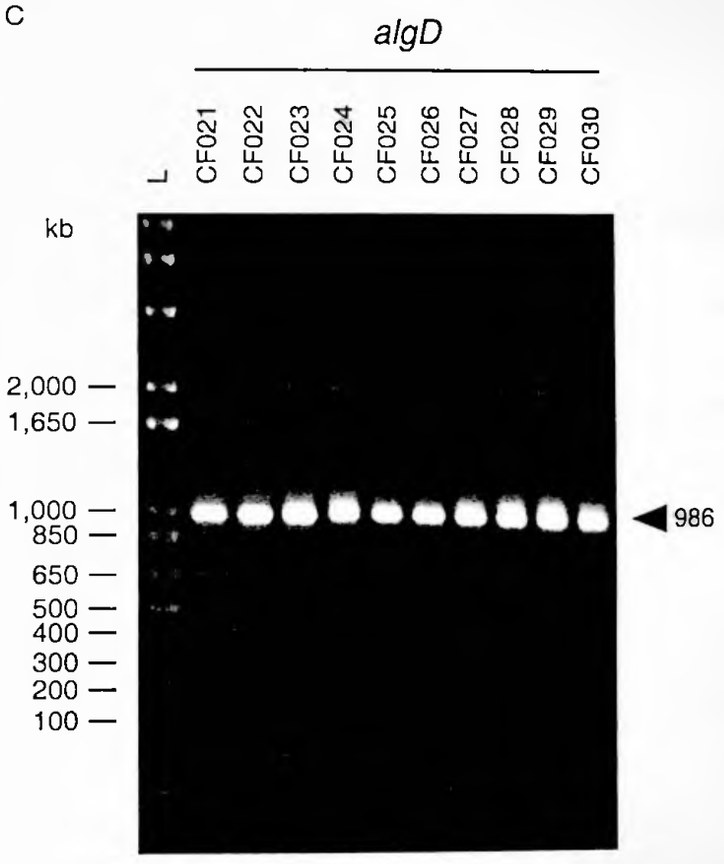

algD
L
CF021
CF022
CF023
CF024
CF025
CF026
CF027
CF028
CF029
CF030
kb
2,000
1,650
1,000
850
650
500
400
300
200
100
986

Table 4. Investigation of the presence of major genomic deletions in selected genes of *P. aeruginosa* by PCR[*1]

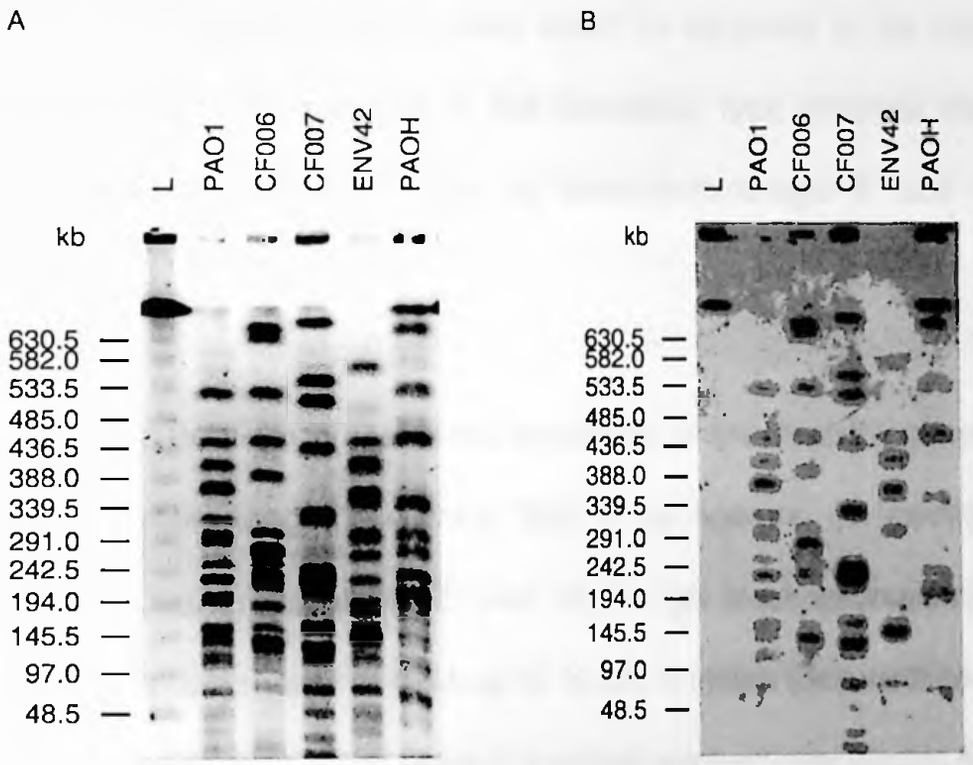
CF Isolate	*algD* (1.3 kb)	*exoS* (1.4 kb)	*exoT* (1.4 kb)	*oprC* (2.2 kb)	*oprF* (1.0 kb)	*mucBCD* (2.9 kb)	*mucE* (1.8 kb)	*mutS* (2.6 kb)	*mutY* (1.1 kb)
CF001	-	-	-	-	-	-	-	-	-
CF002	-	-	-	-	-	-	-	-	-
CF003	-	-	-	-	-	-	-	-	-
CF004	-	-	-	-	-	-	-	-	-
CF005	-	-	-	-	-	-	-	-	-
CF006	-	-	-	-	-	-	-	-	-
CF007	-	-	-	-	-	-	-	-	-
CF010	-	-	-	-	-	-	-	-	-
CF011	-	-	-	-	-	-	-	-	-
CF012	-	-	-	-	-	-	-	-	-
CF013	-	-	-	-	-			-	-
CF014	-	-	-	-	-			-	-
CF015	-	-	-	-	-			-	-
CF016	-	-		-		-*2		-	-
CF017	-	-		-				-	-
CF018	-	-		-				-	-
CF019	-	-		-				-	-
CF020	-	-		-				-	-
CF021	-	-		-				-	-
CF022	-	-		-				-	-
CF023	-	-		-		-*2,3	-*2,3	-	-
CF024	-	-		-				-	-
CF025	-	-		-				-	-
CF026	-	-		-		-*2,3	-*2,3	-	-
CF027	-	-		-				-	-
CF028	-	-		-				-	-
CF029	-							-	-
CF030	-							-	-
CF031	-							-	-
CF032	-							-	-
CF033	-							-	-
CF034	-							-	-
CF035	-							-	-
CF036	-							-	-
CF037	-							-	-
CF038	-							-	-
CF039	-							-	-
CF040	-							-	-
CF041								-*3	-*3
CF042								-*3	-*3
CF043								-*3	-*3
ENV42	-							-	
PAOH	-					-*2	-*2	-	
PA14	-					-*2	-*2	-	
PAO1	-					-*3	-*3	-	-

*1 - Polymerase chain reaction. "-" designates applicable gene did not have a deletion. The blank areas in the table designate data not determined. All annealing temperatures were 55°C unless otherwise noted.

( ) - Parentheses indicate gene size in kilobases (kb). *2 - Samples were run at annealing temperature of 59°C.

*3 - Samples were run at annealing temperature of 50°C.

Figure 9. PFGE pattern whole-genome labeling and Southern blot. (A) *Xba*I restriction digest PFGE patterns of genomic DNA from a standard reference strain, clinical CF isolates and environmental isolates of *P. aeruginosa*. Lanes CF006-007, isolates from CF patients; ENV42, PAOH, aquatic habitat. Interval switch times were 10-40 s with a ramping factor of 0.35741 at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System. (B) PAO1 whole genome DIG-UTP labeling Southern blot of PFGE pattern in (A) exposed for 4 h.


A
L
PAO1
CF006
CF007
ENV42
PAOH
kb
630.5
582.0
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5
B
L
PAO1
CF006
CF007
ENV42
PAOH
kb
630.5
582.0
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

DISCUSSION

Restriction digestion of the entire bacterial chromosome coupled with physical separation of various sizes of genomic DNA fragments in a PFGE system generates a specific RFLP pattern. This pattern is a reflection of genomic structure and can be used as a genetic fingerprint for each strain. The genetic difference between each strain could be attributed to the cause of a particular disease. An example is the potentially fatal intestinal disease of hemolytic uremic syndrome caused by enterohemmoragic *E. coli* O157:H7 (Perna *et al.*, 2001).

PFGE is a useful tool that takes advantage of the resolving power of DNA separation by alternating the electric field in an agarose gel electrophoresis system. The advantages of PFGE over normal gel electrophoresis rest in the ability to separate fragments of DNA up to 10 Mb in length (Schwartz and Cantor, 1984) as opposed to only 20 kb using a constant unidirectional electric field. This property allows estimation of genome size when coupled with infrequent restriction endonuclease digestion and assessment of relevant genomic complexity. Also, the electric field in PFGE may be varied to aid in specific separation of a particular fragment size. The disadvantages of PFGE are the expensive equipment associated with generating the varying electric field as well as the cumbersome and extensive preparation of samples. While PFGE has proven to be an effective and powerful tool to track the epidemiology of a strain,

the molecular basis of the RFLP pattern underlying the onset of a specific disease still remains elusive.

P. aeruginosa is an opportunistic pathogen and can cause fatal pneumonia in CF. The genome of PAO1 has recently been sequenced (Stover *et al.*, 2000). The PAO1 genome digested with *Spe*I generates 37 fragments ranging from 5 to 540 kb (Table 1). These bands can be effectively separated in a PFGE system. However, there are several controversial issues that remain to be resolved. First, PAO1 is a laboratory strain, which was originally isolated from burn wounds (Holloway, 1955). The representative nature of PAO1 in CF is unknown. Second, the genome of *P. aeruginosa* is extremely diversified (Romling and Tummler, 2000). Is it caused mainly by homologous recombination or does horizontal gene transfer also play an important role? What causes the phenotypic changes from nonmucoid to mucoid, homologous recombination and genetic rearrangement (Woods *et al.*, 1991) or adaptive mutation (Mathee *et al.*, 1999)?

THE GENETIC DIVERSITY AMONG CLINICAL CF ISOLATES OF *P. aeruginosa*

In this study, these issues were addressed by subjecting *Spe*I digested chromosomes of 101 clinical CF isolates to PFGE analysis. These samples were isolates from Boston, MA, Greenville, NC, Vancouver, British Columbia,

Copenhagen, Denmark and Edinburgh, Scotland. Of 101 clinical CF strains, 75 (74%) had unique RFLP patterns. Identical patterns (26 total isolates) were due to cross-infection, sequential isolates, or single-source isolation (Table 2). The isolates from sets of siblings demonstrate the ability of *P. aeruginosa* to be passed from one CF patient to another (cross-infection) (Figure 2). Two siblings with CF do not necessarily harbor the same colonizing strain (S4: CF3/CF7, Figure 2C). Also, the identical RFLP patterns of the isolates from 2 different CF patients cared for within the same hospital (S3: CF37/CF40, Figure 2A,B) indicate that CF patients entering a controlled environment that might harbor other CF isolates (either from other patients or environmental reservoirs), such as a CF clinic, are at potential risk of contracting a CF isolate. This example suggests that 2 patients do not have to be in close contact, as is presumed with siblings, to contract the same isolate. Since CF patients have the genetic predisposition to acquire *P. aeruginosa*, the environmental presence of a CF isolate in a clinical environment could, hypothetically, lead to a single strain of bacteria becoming the colonizing isolate at birth in multiple CF patients. Whether this transmission occurs directly (from one patient directly to another) or indirectly (from one patient to another via some environmental reservoir) is yet to be determined.

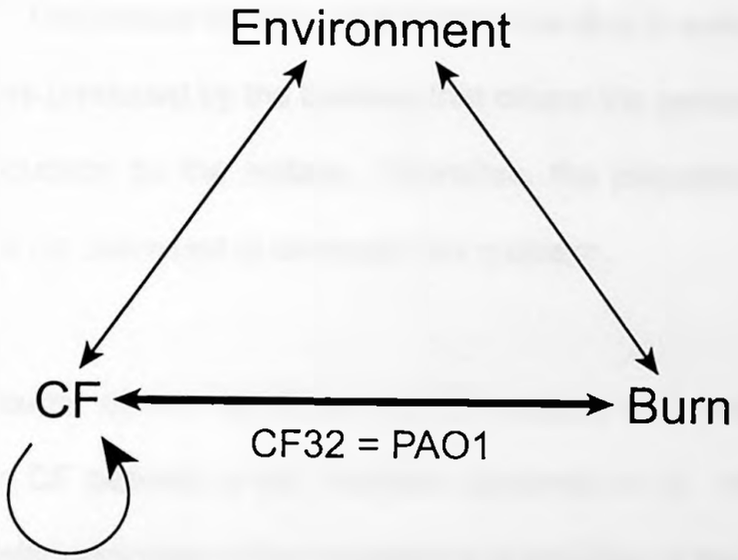
Of the 16 sequential isolates studied, all of the RFLP patterns were conserved from initial to final isolation. There is conservation of RFLP profile over 8 years of colonization (Figure 3) but there is no indication of the slight

genetic adjustments that might take place once inside the CF lung environment. Therefore, it appears that the isolate remained stable in genomic structure once it colonized the CF lung. Nevertheless, some minor mutations may still have occurred, but the PFGE technique may simply fail to detect them. Although CF isolates are variable in genomic profile, they are stable enough to keep a particular genomic pattern during *in vivo* and *in vitro* growth. It should be noted, however, that only 16 sequential isolates were tested and an increased number of sequential isolates need to be tested in order to draw definitive conclusions. However, if it was known what bacterial strain was the initial colonizer in a CF lung, then the genetic profile of that organism could be tracked over the course of the disease. Isolating the initial *P. aeruginosa* strain is difficult in that, once a patient is diagnosed with CF, the colonizing strain has already entered the lung (Konstan and Berger, 1996). It is not known whether genetic changes take place within the first days or weeks of initial colonization or whether the organism undergoes no genetic change in moving from the external environment to the CF host. However, a possible study to be performed could include analyzing RFLP patterns of clinical environmental isolates of *P. aeruginosa* and comparing them to the patterns of clinical CF isolates to determine whether the environment contributes to CF patient infection. A similar study was performed comparing the RFLP pattern of an aquatic isolate, *P. aeruginosa* C, with clinical isolates. It was found that 30% of the CF patients in the study harbored the same environmental strain (Romling, 1994b). This supports the notion that an environmental reservoir of *P. aeruginosa* has the potential to colonize CF patients. Although only 4

environmental isolates were analyzed in this study, variable RFLP patterns were also detected in the environmental isolates, from which the clinical isolates originated. *P. aeruginosa* is a common environmental organism and might have the ability to alter its genome to further accommodate it to a certain living situation (Schmidt *et al.*, 1996). While this study focuses mainly on CF isolates, and the environmental samples were selected to determine general banding characteristics in comparison with CF isolates, an environmental database could be created to compare against the RFLP patterns produced in this study to determine the possible origin of banding pattern variability.

It is known that CF patients have the genetic capacity to acquire environmental strains of *P. aeruginosa* (Romling et al., 1994a) and this study has shown that a CF patient has the possibility of acquiring an isolate from another CF patient. It is also known that burn patients acquire *P. aeruginosa* infections from the environment (Holloway, 1955). This is the first time a burn and CF isolate sharing an identical genetic profile have been identified (Figure 10), which suggests that either PAO1 itself may be able to establish a chronic lung infection in CF, CF32 may be able to infect immunocompromised burn patients or another unidentified strain has given rise to both PAO1 and CF32. Since all the laboratory strains in this study have originated from PAO1 and are identical in RFLP profile to PAO1, except PAM425 (Figure 7), they are also identical in pattern to CF32. PAM425 has a genomic alteration resulting in a different RFLP pattern compared to PAO1 (Figure 7). This alteration results in an extra band at

Figure 10. Schematic diagram of routes of passage of *P. aeruginosa*.

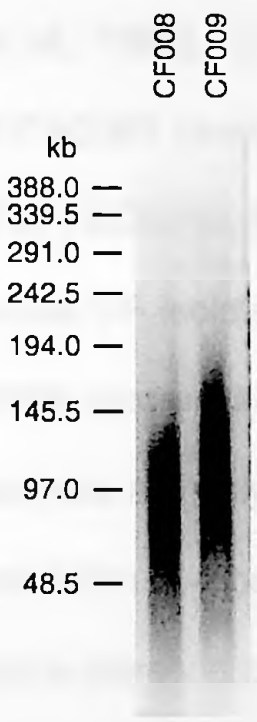

Environment
CF
Burn
CF32 = PAO1

235 kb and a missing band at 337 kb after *Spe*I digestion. Therefore, out of the 101 clinical CF isolates and 75 unique RFLP profiles generated in this study, there is only 1 isolate, CF32, that has an identical genetic pattern as a burn isolate and the standard reference strain, PAO1.

Though the technique of PFGE is highly sensitive and reproducible in profiling the genomes of CF isolates, a significant fraction (12/101 = 12%) of the CF isolates cannot be typed. An example of this non-typable CF isolate is shown in Figure 11. The reason for this is believed to be due to endogenous restriction endonucleases produced by the bacteria that cleave the genomic DNA as well as protease production by the isolate. Therefore, the preparation of the agarose plugs may still be optimized to eliminate this problem.

The source of the variability in RFLP pattern in colonizing strains of *P. aeruginosa* in CF patients is still unknown (Schmidt *et al.*, 1996). Whether the genetic diversity originates in the organism's adaptation to the changing external environment before colonizing the CF host or the variability lies in the bacterium's genetic changes once it enters the CF lung is in debate (Kiewitz and Tummler, 2000). In this study, genetic diversity exists throughout almost all the CF isolates from different patients.

Figure 11. PFGE patterns of non-typable CF isolates. *Spe*I restriction digest PFGE patterns of genomic DNA from CF clinical isolates of *P. aeruginosa*. Lanes CF 008-009, isolates from CF patients. Interval switch times were 12-25 s ramped linear at 200V for 18 h using Chef Mapper XA Pulsed-Field Electrophoresis System.


CF008
CF009
kb
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

CORRELATION BETWEEN MUCOIDY PHENOTYPE AND GENOMIC PROFILES

One of the major virulence factors produced by *P. aeruginosa* during its chronic CF lung infection is mucoidy (Govan and Deretic, 1996). The mucoid phenotype is due to the overproduction of the MEP alginate. This unique phenotype, although unstable, can be reproduced on a selective media (PIA) during *in vitro* growth (Yu *et al.*, 1996). According to Figure 7 and Table 2, the RFLP profiles for PAO1 and PAO381 (scored as non-mucoid) are identical to the profiles for PAO568, PAO578I, PAO578II, PAO579, and PAO581 (scored as very mucoid). Also, the 2 sequential CF isolates, CF041 from 1990 (scored as non-mucoid) and CF042 from 1994 (scored as very mucoid) had identical genomic profiles. While these comparisons are only within the limits of PFGE detection, it appears that the mucoid production is a part of the adaptive response of the CF isolate to its environment and is merely a product of gene expression, which may not be reflected in the genomic profiles.

SEARCHING FOR MAJOR GENOMIC DELETIONS AND CF-SPECIFIC EXTRA-CHROMOSOMAL DNA

A recent study with clinical CF isolates has shown that 46 of 128 (36%) isolates from 30 CF patients were hypermutable (mutator) strains (Oliver *et al.*, 2000). Furthermore, the authors showed that some of the CF isolates had a

deletion in the DNA repair and error-avoidance gene, *mutS* (Oliver *et al.*, 2000). The stable mutations in the CF isolates have also been found to be present in other genes such as *mucA* (Martin *et al.*, 1993). For this reason, the molecular mechanisms of the hypervariable polymorphisms in the genome of the CF isolates were investigated. Even though significant-sized deletions were not detected (Figure 8, Table 4), it is still possible that minor changes, such as point or frame-shift mutations, in these genes have occurred. Based on limited sequencing performed, no such mutations were found.

Liang *et al.* (Liang *et al.*, 2001) demonstrated that a UTI isolate acquired a "genomic island" structure in its genome. This extra-chromosomal DNA is assumed to play an important role in pathogenesis of *P. aeruginosa*. A subtractive hybridization strategy was adapted to detect the presence of CF-specific extra-chromosomal DNA. By labeling the PAO1 genome, the hybridization procedure marks only the PAO1-specific fragments of a CF isolate RFLP profile therefore leaving the unmarked CF-specific fragments. When this CF-specific DNA is located, it can be used for development of a novel treatment strategy. The discrimination parameters are still being developed for the PFGE/Southern blot procedure, however this technique has the potential to allow identification of CF-specific extra-chromosomal DNA that has no sequence homology to PAO1.

CONCLUSION

A PFGE system coupled with restriction digestion was developed in this study to allow assessment of the genomic profiles and to determine the relationships of the clinical isolates with other various sources of *P. aeruginosa*. Genomic diversity exists throughout all of the isolates tested so far, however a relationship of patient-to-patient transmission was established. The sequential isolates retain identical genomic polymorphism patterns *in vitro* and *in vivo*. One CF isolate, CF 32, was found to have an identical genomic profile as a burn isolate, PAO1. Mucoid production appears to be a gene expression function controlled by the organism's adaptation to the environment and was not reflected in RFLP pattern.

Two approaches were taken to search for CF-specific genomic differences in *P. aeruginosa*. One method using PCR allowed investigation into the presence of significant-sized chromosomal deletions. Another approach was PFGE coupled with whole-genome labeling of a Southern blot, which enabled us to examine the existence of CF-specific extra-chromosomal DNA.

This study has established a molecular basis for further investigation of inherent virulence properties of *P. aeruginosa* and how they relate to the chronic infection established in CF patients. Using the procedures developed, further analysis into the genomic profiles and presence of CF-specific deletions and

extra-chromosomal DNA may lead to new treatment for *P. aeruginosa* infections in CF.

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