Development of a diagnostic test for the Midlands 1 cystic fibrosis epidemic strain of *Pseudomonas aeruginosa*

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In a previous study of isolates from cystic fibrosis (CF) patients in England and Wales, the Midlands 1 strain of *Pseudomonas aeruginosa* was identified as the second most common clone, representing 10 % of isolates and found in nearly one-third of all CF centres [Scott, F. W. & Pitt, T. L. (2004). *J Med Microbiol* **53**, 609–615]. Using suppression subtractive hybridization, 54 sequences were identified as present in a Midlands 1 strain but were absent from strain PAO1. The distribution of 14 of these sequences amongst representatives of Midlands 1, other CF epidemic strains and unrelated *P. aeruginosa* CF isolates was determined using PCR assays. Using these data, a PCR-based test was developed that was specific for the Midlands 1 clone, which was confirmed using dot-blot hybridization. By applying the test to CF isolates from a CF centre in Liverpool, a Midlands 1 clone was identified. The identity was confirmed using typing by PFGE. The PCR test should facilitate a greater understanding of the distribution of the Midlands 1 strain in the UK and elsewhere.

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INTRODUCTION

Pseudomonas aeruginosa is the most common bacterial pathogen associated with morbidity and mortality in cystic fibrosis (CF), causing chronic lung infections that, once established, are impossible to eradicate. The general view that individual CF patients acquire *P. aeruginosa* infections separately, and thus carry their own unrelated strains, was challenged in 1996 when the spread of a multi-drug-resistant strain of *P. aeruginosa* [named the Liverpool epidemic strain (LES)] amongst patients in a children's CF centre in Liverpool was reported (Cheng *et al.*, 1996). Since then, there have been further reports of CF 'epidemic' strains (Jones *et al.*, 2001; Armstrong *et al.*, 2003; O'Carroll *et al.*, 2004), raising serious questions about whether segregation strategies might be required to counter transmission. A survey of 31 CF centres in England and Wales, in which over 1200 isolates of *P. aeruginosa* were analysed, not only identified LES as the most common clone, being present in 48 % of CF centres and accounting for 11 % of the isolates, but also identified other strains with a wide distribution amongst CF patients in England and Wales (Scott & Pitt,

2004). In particular, the Midlands 1 clone represented 10 % of the isolates and was present in 29 % of the CF centres. Thus, the two most common genotypes (LES and Midlands 1) accounted for 21 % of isolates examined and were found in 58 % of CF centres (Scott & Pitt, 2004). A strain reported as epidemic amongst CF patients in Manchester (Jones *et al.*, 2001) was not widely distributed and represented only 1 % of the isolates in the study (Scott & Pitt, 2004). More recently, the Midlands 1 clone was reported to be present in 30 % of *P. aeruginosa*-positive CF patients in an adult CF unit in the West Midlands (Chambers *et al.*, 2005).

The 'gold-standard' typing method for identification of epidemic strains, macrorestriction analysis using PFGE, is time-consuming and requires specialist equipment. Thus, rapid PCR-based assays for the identification of epidemic strains would be of considerable benefit to CF units. Previously, we have used suppression subtractive hybridization (SSH), a technique that enables the identification of sequences present in one strain but absent from another (Winstanley, 2002), to identify strain-specific sequences. These data were used to develop PCR-based diagnostic tests for the LES (Parsons *et al.*, 2002; Panagea *et al.*, 2003; Smart *et al.*, 2006). In this study, we report the use of SSH to identify sequences unique to the Midlands 1 clone. By this

Abbreviations: CF, cystic fibrosis; LES, Liverpool epidemic strain; SSH, suppression subtractive hybridization.

approach, we were able to gain insight into the accessory genome or mobilome (Ou *et al.*, 2005) and to design a PCR-based test for identification of this CF epidemic strain.

METHODS

Bacterial strains. The Midlands 1 isolate used in SSH (isolate 9245) was originally isolated at Birmingham Heartlands Hospital, UK, and was chosen from isolates identified in a previous study (Scott & Pitt, 2004). The reference strain (driver) for the SSH experiment was strain PAO1, for which the entire genome sequence is known (Stover *et al.*, 2000). For prevalence studies, we used several categories of strain panels as reported previously (Smart *et al.*, 2006). These comprised 12 isolates of the Midlands 1 epidemic strain, 22 LES isolates and 11 isolates of the Manchester epidemic strain, taken mainly from isolates obtained during the survey of CF isolates in England and Wales (Scott & Pitt, 2004). Sixteen genetically distinct non-LES isolates from adult patients in Liverpool were included from our previous SSH studies (Parsons *et al.*, 2002; Smart *et al.*, 2006). In addition, we obtained 43 more-recent, uncharacterized, non-LES isolates (negative for the LES DNA marker PS21) from the Liverpool adult CF unit and 32 uncharacterized non-LES CF isolates from a neighbouring adult CF unit at Clatterbridge Hospital (Smart *et al.*, 2006). We also screened a representative isolate of clone C (Römling *et al.*, 1994) and strain PA14 (Rahme *et al.*, 1995).

Construction and screening of subtraction libraries. Genomic DNA was isolated from the Midlands 1 isolate 9245 and strain PAO1 as described previously (Winstanley & Hart, 2000). SSH was carried out using the Clontech PCR-Select Bacterial Genome Subtraction kit, essentially as recommended by the supplier but with a hybridization temperature of 73 °C. In the hybridizations, DNA from the Midlands 1 strain was used as the tester and DNA from strain PAO1 was used as the driver. PCR amplicons obtained following SSH were cloned into pGEM-T (Invitrogen). The subtraction library of *RsaI* fragments thus constructed was screened by sequencing of plasmid DNA extracted from individual clones (Lark Technologies) using M13 forward and reverse vector primers. BLAST searches of the *P. aeruginosa* genome project website (<http://www.pseudomonas.com>) were used to determine the presence or absence of sequences in the PAO1 genome. Tester-specific sequences were further analysed using BLASTN and BLASTX searches of the database available at <http://www.ncbi.nlm.nih.gov> (last accessed 27 February 2006).

PCR amplification and dot-blot screening of strains. The oligonucleotide primers (Sigma-Genosys) used in PCR assays and for the labelling of probes are listed in Table 1 along with the annealing temperatures used. PCR amplifications were carried out in an Eppendorf MasterCycler thermal cycler as described previously (Smart *et al.*, 2006). Dot-blot hybridization of genomic DNA, extracted using Wizard Genomic DNA Purification kit columns (Promega), was carried out as described previously (Winstanley & Hart, 2000). Digoxigenin-11-2'-dUTP (DIG) (Roche)-labelled probes were made by carrying out PCR amplification in the presence of 60 µM DIG using vector or internal primers. Hybridization and subsequent detection of DIG was carried out following the manufacturer's instructions.

PFGE. PFGE of *SpeI*-digested genomic DNA was carried out using 1% (w/v) agarose gels and interpreted according to the protocol of Tenover *et al.* (1995).

RESULTS AND DISCUSSION

Midlands 1 subtracted library

SSH using the Midlands 1 isolate 9245 as tester and strain PAO1 as driver yielded a library of 54 tester-specific sequences (Table 2), 24% of which either had no significant matches in the database or shared similarity with hypothetical proteins of no known function. Of five sequences sharing similarity with bacteriophage proteins, only one matched a bacteriophage (Pfl; Hill *et al.*, 1991) identified previously in *P. aeruginosa* (Table 2). This sequence (MID-SSH20) matched a Pfl ORF not present in the genome sequence of strain PAO1. The subtraction indicated that Midlands 1 carries genes for the production of type III pyoverdine, which is the least common pyoverdine type amongst isolates of *P. aeruginosa* (de Vos *et al.*, 2001; Parsons *et al.*, 2002), but is shared by the two epidemic strains (LES and Midlands 1). In addition, the SSH data indicated that the Midlands 1 clone had the same serotype as the LES. Both are serotype O6, a widespread serotype (Pirnay *et al.*, 2002). However, the presence of the 16 kb flagellin glycosylation island (Arora *et al.*, 2001, 2004), coupled to our own PCR assays (unpublished data), indicated that the Midlands 1 clone, unlike the LES, is flagellin type a.

There was some evidence that the Midlands 1 clone also contains at least part of the genomic island PAGI-3 (Larbig *et al.*, 2002; Klockgether *et al.*, 2004). In addition, there were several subtracted sequences with similarity to proteins associated with transposition, recombination or DNA modification and genes associated with polyketide synthesis (Table 2).

Distribution of subtracted sequences

From the Midlands 1 subtraction, PCR assays were designed to determine the distribution of 14 sequences amongst Midlands 1 and other *P. aeruginosa* isolates. The sequences chosen had BLASTX similarity to bacteriophage-related proteins (MID1, MID-A8, MID-E8, MID-H5), a putative transposase (MID-SSH14), a siderophore receptor (MID-SSH40), a transport protein (MID-B4), an asparagine synthetase (MID-B5), a type III pyoverdine synthesis protein (MID-C3), restriction-modification proteins (MID4 and MID-C10), an ORF from the flagellin glycosylation island (MID-SSH41) and an acyltransferase (MID-F2). In addition, one sequence with no significant similarity to database sequences was included (MID-B9).

The ten Midlands 1 isolates were all PCR positive for each of the subtracted sequences present (Table 3). All of the LES isolates tested were also PCR positive for sequences MID-B4, MID-B5, MID-C3, MID-F2 and MID-SSH40. Further screening of genetically distinct non-LES isolates indicated that only Midlands 1 isolates were PCR positive for sequences MID1, MID4, MID-A8, MID-C10, MID-E8 or MID-H5

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)	Target
Mid1F	TTGCGCTCCATCGTTTGA	649	56	MID1
Mid1R	CTCCAGATGCCTACGAAA			
Mid4F2	TTCAGGCGCGCTTCAAGA	638	55	MID4
Mid4R2	ACACCTTCGATGGCCAAT			
MidA8F	GAAACGCTGCTACCTGAG	188	56	MID-A8
MidA8R	CGTAGGTAGGACAAAAGG			
MidB4F	CCAGCAAGCGGTTGTTCA	231	56	MID-B4
MidB4R	AAAGACTGCGCGACCAAG			
MidB5F	ATTCCCGTTAGCAGTTTC	498	58	MID-B5
MidB5R	TGATGGCCATGGATACCC			
MidB9F	AGCGTTCGTATGGTTTCG	370	56	MID-B9
MidB9R	AAATGGCCCCAAGCTACC			
MidC3F	GTCAAGGCCATCAAGGAA	326	56	MID-C3
MidC3R	TGTATGGTCTGCTCGTCG			
MidC10F	ATCACCCGGCTGTAATCA	279	58	MID-C10
MidC10R	GCTCACGCTGCTGTTTGT			
MidE8F	TGTTTTGTGCAGGGGGTG	604	56	MID-E8
MidE8R	AGATGATCCAACGCGACC			
MidF2F	TGATGGCCATGGCTACTG	455	56	MID-F2
MidF2R	CCTGAAGGATGGTCTCTG			
MidF8F	CGTATCGCCCATGCTCAT	287	56	MID-F8
MidF8R	CTTCTGTCGTGCCATGGA			
MidH5F	CCAGAGGGTTTCAGTGTG	1024	58	MID-H5
MidH5R	ATTTTCAGGGGCAGCAGC			
MidH6F	CGTCAAGCTCCTGCAACA	526	56	MID-H6
MidH6R	GGTCCTTACAGCCTGATG			
SSH14F	CTTCCTGATATGTGGTTC	265	55	MID-SSH14
SSH14R	TCGTTGCAACACCTTTGG			
SSH40F	TGAGTTTGGTAGCGGGTC	386	55	MID-SSH40
SSH40R	GAAGTACCTGTTTGGAGA			
SSH41F	TGGTCAGGAAACGGCTCT	170	55	MID-SSH41
SSH41R	CTTGCATAGAGTGGCGTG			

(Table 3). Three of these six sequences had best BLASTX matches with bacteriophage sequences (Table 2).

On the basis of experiments using PFGE to compare strains and estimate fragment sizes, we believe that the genome of the Midlands 1 clone is smaller than that of strain PAO1 (data not shown). Previously, we also estimated that the LES genome, which carries approximately 95 % of strain PAO1 genes, is no bigger than that of strain PAO1 (Salunkhe *et al.*, 2005). The SSH analysis identified a number of genes or gene clusters likely to contribute to the accessory genome of the Midlands 1 strain, some shared with the LES. However, multilocus sequence typing suggests that the LES and Midlands 1 lineages are unrelated (Curran *et al.*, 2004).

The MID1 subtracted sequence, matching a P4-like integrase, included at one extremity an exact match to the first 50 bp of a 77 bp tRNA^{Arg} (predicted gene PA0263.1) of strain PAO1. The presence in some strains of insertion

sequence elements in this region has been described previously (Ernst *et al.*, 2003). The insertion of a bacteriophage integrase gene adjacent to this tRNA locus suggests that MID1 (and MID-H5) may form one end of a prophage or genomic island in the Midlands 1 clone. BLASTX matches indicated that SSH sequence MID-A8 may reside near to the putative integrase gene (MID1 and MID-H5) in the Midlands 1 genome. The presence of these and other bacteriophage-related sequences only in Midlands 1 isolates suggests that the Midlands 1 clone contains at least one prophage not commonly carried by strains of *P. aeruginosa*.

Restriction-modification systems often vary among isolates. Our prevalence studies indicated that the restriction-modification system matching five separate subtracted sequences may be unique to the Midlands 1 strain (Table 3).

It is possible that antimicrobial polyketides may play a role in enabling the strain to out-compete other pseudomonads.

Table 2. Midlands 1 SSH summary table

Sequences sharing less than 90 % nucleotide sequence identity with the PAO1 genome were included in the table.

Sequence	Length (bp)	GC content (mol%)	Best BLASTX match/comments [GenBank accession no.]	Identity (%)	Length (aa)	E value
Bacteriophage-related						
MID1*	746	58.2	Site-specific recombinase (N-terminal region), phage integrase family, locus PFL_4977 (<i>Pseudomonas fluorescens</i> Pf-5) [YP_262056]	55	154	2e ⁻⁴²
MID-H5	1404	56.6	Site-specific recombinase (C-terminal region), phage integrase family (<i>P. fluorescens</i> Pf-5) [YP_262056]	54	114	2e ⁻²⁶
MID-A8	349	61.9	Hypothetical protein (<i>Nitrobacter</i> sp.) [ZP_01044916]	48	82	9e ⁻¹⁷
MID-SSH20	357	55.5	Bacteriophage Pf1 ORF90 (<i>P. aeruginosa</i>) [CAA36337]; short predicted ORF not in PAO1	100	90	1e ⁻³⁷
MID-E8	709	60.1	Putative transcriptional regulator, prophage CP4-57 (<i>Azotobacter vinelandii</i> AvOP) [ZP_00415815]	60	104	4e ⁻²⁷
			Putative transcriptional regulator, prophage CP4-57 (<i>A. vinelandii</i> AvOP) [ZP_00415816]	80	63	7e ⁻²²
Siderophore-related						
MID-G1	165	65.0	<i>pvdI</i> (3), type III pyoverdine-related non-ribosomal peptide synthetase (<i>P. aeruginosa</i>) [AY765261]	100	54	7e ⁻²⁶
MID-C3	409	64.3	<i>pvdD</i> (3), type III pyoverdine-related non-ribosomal peptide synthetase (<i>P. aeruginosa</i>) [AY765261]	99	136	6e ⁻⁶³
MID-C5	320	62.8	<i>pvdD</i> (3), type III pyoverdine-related non-ribosomal peptide synthetase (<i>P. aeruginosa</i>) [AY765261]	100	106	3e ⁻⁵⁶
MID-H6	587	63.4	<i>pvdD</i> (3), type III pyoverdine-related non-ribosomal peptide synthetase (<i>P. aeruginosa</i>) [AY765261]	98	195	3e ⁻⁹⁸
MID-E2	598	59.9	<i>pvdD</i> (3), type III pyoverdine-related non-ribosomal peptide synthetase (<i>P. aeruginosa</i>) [AY765261]	100	199	e ⁻¹¹¹
MID-H1	511	61.8	FpvAIII; type III pyoverdine receptor (<i>P. aeruginosa</i>) [AAN62912]	100	155	1e ⁻⁸⁸
MID-SSH40	461	54.9	Outer membrane siderophore receptor (<i>P. aeruginosa</i> C3719) [ZP_00965615]	99	153	6e ⁻⁸⁸
MID-F8	348	60.3	<i>pvdE</i> , ABC transporter, type III pyoverdine-related (<i>P. aeruginosa</i>) [AY765261]	100	115	4e ⁻⁵²
O-serotype						
MID-SSH42	187	52.4	WbpO (<i>P. aeruginosa</i> serotype O6) [AAM27816]	100	62	3e ⁻²⁸
MID-B1	237	58.7	WbpU (<i>P. aeruginosa</i> serotype O6) [AAM27823]	100	78	5e ⁻³⁹
Genomic islands						
MID-H4	179	65.4	Hypothetical protein ORF C/SG116 (<i>P. aeruginosa</i>) [AAN62218]; PAGI-3 genomic island	98	59	5e ⁻²⁸
MID-SSH41	221	58.8	OrfA (<i>P. aeruginosa</i>) [AAP35713]; flagellin glycosylation island	100	73	1e ⁻³⁷
MID-H11	560	57.9	OrfM (<i>P. aeruginosa</i> CF5) [AAP35726]; flagellin glycosylation island	100	130	7e ⁻⁷⁰
MID-C8	478	62.3	OrfM (<i>P. aeruginosa</i>) [AAK15338]; flagellin glycosylation island	100	159	1e ⁻⁸⁷
MID-C9	617	62.7	OrfM (<i>P. aeruginosa</i>) [AAK15338]; flagellin glycosylation island	99	205	e ⁻¹¹⁸
Drug resistance/antimicrobial synthesis						
MID-B4	310	64.8	Outer membrane channel protein PltN, component of ABC transporter (<i>Pseudomonas</i> sp. M18) [AAY59070]	99	103	6e ⁻⁵³
MID-B3	361	61.5	Polyketide synthase type I, PltC, involved in synthesis of pyoluteorin (<i>Pseudomonas</i> sp. M18) [AAQ90174]	98	76	3e ⁻³⁵
MID-C1	362	62.7	2,4-Diacetylphloroglucinol biosynthetic protein (polyketide synthase), PhlD (<i>P. fluorescens</i>) [AAX31636]	78	120	5e ⁻⁴⁵
DNA modification/recombination/transposases						
MID-B10	813	64.2	Possible helicase (<i>Marinobacter aquaeolei</i>) [ZP_00816698]	40	265	1e ⁻³⁷
MID-A12	276	58.7	Putative type IIII restriction enzyme, residues 29–119 (<i>Azoarcus</i> sp. EbN1) [YP_157375]	65	91	5e ⁻²¹
MID-C10	292	58.9	Putative type IIII restriction enzyme, residues 121–216 (<i>Azoarcus</i> sp. EbN1) [YP_157375]	87	96	2e ⁻⁴³

Table 2. cont.

Sequence	Length (bp)	GC content (mol%)	Best BLASTX match/comments [GenBank accession no.]	Identity (%)	Length (aa)	E value
MID-F6	299	59.2	Putative type III restriction enzyme, residues 218–316 (<i>Azoarcus</i> sp. EbN1) [YP_157375]	90	99	2e ⁻⁴⁶
MID4	1138	57.6	Putative type III restriction enzyme, residues 708–1049 (<i>Azoarcus</i> sp. EbN1) [YP_157375]	73	342	3e ⁻¹⁵¹
MID-A6	294	62.6	Putative type III restriction–modification enzyme, methylation subunit (<i>Azoarcus</i> sp. EbN1) [YP_157372]	84	97	3e ⁻⁴⁰
MID-E10	600	59.3	Rhs family protein (<i>P. aeruginosa</i> PA14) [ZP_00348109]	98	89	2e ⁻⁴⁵
MID-SSH3	167	49.1	Rhs family protein (<i>P. aeruginosa</i> C3719) [ZP_00969203]	84	53	3e ⁻²¹
MID-G9*	603	62.0	Rhs family protein (<i>P. aeruginosa</i> 2192) [ZP_00973600]	97	200	2e ⁻¹¹⁰
MID-SSH14	286	58.4	Integrase, catalytic region (<i>Burkholderia cenocepacia</i> HI2424) [ZP_00463497]	78	78	3e ⁻²⁹
Metabolism/enzymes						
MID-A5	931	60.4	Permease (<i>P. aeruginosa</i> PA14) [ZP_00205078]	99	112	3e ⁻⁵⁸
			Alcohol dehydrogenase (<i>P. aeruginosa</i> PA14) [ZP_00137169]	100	28	4e ⁻⁸
MID-B5*	759	59.4	Asparagine synthetase, glutamine-hydrolysing (<i>Psychrobacter</i> sp.) [AAZ18527]	63	210	7e ⁻⁶⁷
MID24*	506	55.9	Catalase (hypothetical protein PA1940) [AAG05328]	100	79	4e ⁻⁴¹
MID-B11	>583	63.0	Oxidoreductase (<i>P. aeruginosa</i> PA14) [ZP_00137167]	100	162	3e ⁻⁸⁹
MID-D6	488	63.5	Probable dipeptidase PvdM (<i>P. aeruginosa</i> ATCCO13) [AAX16336]	100	162	7e ⁻⁸⁵
Acyltransferases						
MID-F2	484	59.9	Acyltransferase (<i>Dechloromonas aromatica</i>) [YP_285087]	51	141	3e ⁻³¹
Regulatory						
MID-B8	739	60.8	AraC-like protein (<i>P. aeruginosa</i> PA14) [ZP_00137168]	100	141	6e ⁻⁷⁶
MID-G4*	>704	50.4	Hypothetical protein PA0821 [AAG04210]	95	68	5e ⁻³¹
			Transcriptional regulator (<i>P. aeruginosa</i> 2192) [ZP_00970851]	100	39	3e ⁻¹³
Hypothetical proteins/unknown function						
MID-A2	497	62.5	Orf74 (<i>Photorhabdus luminescens</i>) [AAO17228]	39	120	1e ⁻¹²
MID-F3	1034	57.4	Orf74 (<i>P. luminescens</i>) [AAO17228] (same protein as A2)	35	262	1e ⁻³¹
MID-A7	464	61.0	Hypothetical protein (<i>P. luminescens</i>) [NP_928266] (also matches the same protein as A2 and F3)	31	142	1e ⁻⁷
MID-B6	>532	62.2	Hypothetical protein PA2403 (<i>P. aeruginosa</i> Rprime) [AAX16316]	100	147	5e ⁻⁸⁴
MID-D5	273	61.5	Hypothetical protein PvdD/PvdJ (<i>P. aeruginosa</i> ATCCO13) [AAX16335]	100	90	6e ⁻⁵²
MID-C4	300	41.0	Hypothetical protein NE0256 (<i>Nitrosomonas europaea</i>) [NP_840346]	76	99	1e ⁻³⁹
MID-D1	347	59.7	Hypothetical protein VV2150 (<i>Vibrio vulnificus</i>) [NP_934943]	45	48	0.003
MID-G7	>656	61.6	Hypothetical protein (<i>P. aeruginosa</i> PA14) [ZP_00140093]	99	218	9e ⁻¹²¹
MID-G12	881	58.5	Hypothetical protein ebA669 (<i>Azoarcus</i> sp. EbN1) [YP_157371]	81	216	1e ⁻⁹²
MID-C11	391	61.4	Hypothetical protein (<i>A. vinelandii</i>) [ZP_00415808]	34	103	2e ⁻⁷
No significant hits (NSH)						
MID-B9	403	58.1	NSH			
MID-F1*	255	60.8	NSH			
MID-G6	382	58.6	NSH			

*Partial match with PAO1 sequence.

Three subtracted sequences matched proteins with probable or possible roles in the production of polyketides, one of which, MID-B4, was carried by both the Midlands 1 clone and the LES.

Interestingly, very few of the subtracted sequences were carried by any of the Manchester epidemic strain isolates, suggesting that the mobilome of the Midlands 1 clone is much more closely related to that of the LES than to the

Manchester strain and that the three epidemic strains may share few novel sequences.

PCR amplification assay for the detection of Midlands 1 isolates

We further tested the MID1 PCR assay on a panel of uncharacterized non-LES isolates from the adult CF unit in Liverpool. We had no previous knowledge of the presence or

Table 3. Distribution of subtracted sequences based on PCR amplification assays

Isolates tested	No. of PCR positives for each MID subtracted sequence														
	1	A8	E8	H5	4	C10	B4	B5	B9	C3	F2	SSH 14	SSH 40	SSH 41	
Midlands 1 (<i>n</i> =12)	12	12	12	12	12	12	12	12	12	12	12	12	12	12	
LES (<i>n</i> =22)	0	0	0	0	0	0	22	22	0	22	22	0	22	0	
Manchester (<i>n</i> =11)	0	0	0	0	0	0	0	0	0	0	0	2	11	0	
Non-LES isolates, genetically different (<i>n</i> =16)	0	0	0	0	0	0	6	4	5	2	7	3	7	11	
Non-LES isolates, Liverpool (<i>n</i> =43)	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
CF isolates, Clatterbridge (<i>n</i> =32)	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
PAO1 (<i>n</i> =1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
PA14 (<i>n</i> =1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Clone C (<i>n</i> =1)	0	0	0	0	0	0	0	0	0	0	0	0	1	1	

ND, Not determined.

absence of the Midlands 1 genotype amongst patients in Liverpool. One of the isolates (59041) was PCR positive for MID1. The isolate was compared with a known Midlands 1 isolate using PFGE and was different by only one band (Fig. 1), confirming it as a Midlands 1 isolate.

Dot-blot hybridization was used to confirm the PCR-based distributions of both MID1 and MID4 sequences amongst five Midlands 1 isolates, strain PAO1, strain PA14, 16 genetically different non-LES isolates and representatives of the LES, the Manchester epidemic strain and clone C (data not shown).

Recently, we and others have identified a small number of non-LES isolates that are positive using the PS21 diagnostic

test (Lewis *et al.*, 2005; Smart *et al.*, 2006). There are other LES-specific PCR tests available for confirmation (Smart *et al.*, 2006) and any positives can be confirmed subsequently with an additional typing method. However, we have observed that sequences identified as unique for LES are often not carried by all LES isolates (Parsons *et al.*, 2002; Smart *et al.*, 2006). This characteristic of LES populations makes it difficult to identify a single PCR test that is 100% specific and sensitive for identification. We did not observe such instability with the Midlands 1 isolates. All six Midlands 1-specific PCR tests identified in this study were positive for each of the 12 Midlands 1 isolates included. In addition, with our collection of CF isolates, representing most of the genotypes present amongst the Liverpool CF population and some beyond, we found no false positives for the Midlands 1 PCR-based test targeting sequence MID1 (phage integrase). Despite the fact that the MID1 sequence is probably associated with a bacteriophage, we found it to be stably maintained amongst our collection of Midlands 1 isolates. However, the unrelated sequence MID4 (restriction-modification enzyme) may serve as an additional, confirmatory PCR-based test. We were previously unaware of the presence of the Midlands 1 genotype amongst the Liverpool CF population, which is dominated by the LES (Panagea *et al.*, 2003). However, having identified the strain in one patient we are now in a position to monitor its progress and take any necessary steps should the strain spread. In addition, this test will also enable others worldwide to assess their CF isolates for the presence of this CF epidemic strain.

Fig. 1. PFGE typing to confirm the Midlands 1 isolate from a CF patient in Liverpool. Lanes: 1, pulse marker, 50–1000 kb (Sigma-Aldrich); 2, known Midlands 1 isolate; 3, isolate 59041. The extra band identified in isolate 59041 is indicated with an arrow.

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