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Development of a Multilocus Sequence Typing Scheme for the Opportunistic Pathogen *Pseudomonas aeruginosa*

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A multilocus sequence typing (MLST) scheme has been developed for *Pseudomonas aeruginosa* which provides molecular typing data that are highly discriminatory and electronically portable between laboratories. MLST data confirm the data from previous studies that suggest that *P. aeruginosa* is best described as nonclonal but as having an epidemic population. The index of association was 0.17, indicating a freely recombining population; however, there was evidence of clusters of closely related strains or clonal complexes among the members of this population. It is apparent that the sequence types (STs) from single isolates, representing each of the present epidemic clones in the United Kingdom from Liverpool, Manchester, and the West Midlands, are not closely related to each other. This suggests distinct evolutionary origins for each of these epidemic clones in the United Kingdom. Furthermore, these clones are distinct from European clone C. Comparison of the results of MLST with those of *toxA* typing and serotyping revealed that strains with identical STs may possess different *toxA* types and diverse serotypes. Given that recombination is important in the population of *P. aeruginosa*, the lack of a linkage between *toxA* type and serotype is not surprising and reveals the strength of the MLST approach for obtaining a better understanding of the epidemiology of *P. aeruginosa*.

Pseudomonas aeruginosa is a gram-negative rod which is reported to be ubiquitous in the natural environment, humans, and animals. The species thrives in moist and wet conditions and is able to utilize a wide range of organic compounds. It can cause severe infections that may be associated with high rates of mortality in immunocompromised patients (3, 8, 24), and it is a frequent cause of infections acquired by patients during hospitalization (4). Almost any type of hospital equipment or utensil has been implicated as a reservoir for *P. aeruginosa*, and these sources may serve as foci for the dissemination of the organism in common-source outbreaks (9).

Infections are most often self-limiting in healthy individuals, such as folliculitis in association with contamination of swimming pools and hot tubs (21). However, occasionally, acute infection of the eyes of contact lens wearers (2) may result in *P. aeruginosa* ocular infections (1).

P. aeruginosa is the most common organism isolated from the lungs of approximately 80% of adult patients with cystic fibrosis (CF). The presence and persistence of the organism correlate with the deterioration of lung function and the clinical decline of the patient (7). Most patients appear to acquire the organism from the natural environment and not from other patients (25), but there is gathering evidence that some clonal lineages are widespread among the CF patient population, apparently contracted through cross infection from other CF patients (12, 15). Indeed, a highly widespread clonal complex, clone C, has been associated with a wide range of different infections in CF and non-CF patients and has been found in the natural environment (6, 20).

The genome size of *P. aeruginosa* varies from 5.2 to 7.1 Mbp (22). This degree of variation has important implications for the methods used to study the evolution and epidemiology of this organism. Recent work suggests that more than 80% of the genome of the sequenced strain (strain PAO1) is shared (with only 0.5% nucleotide divergence) by CF and environmental strains (26). Denamur et al. (5) and Picard et al. (16) considered that the species had a panmictic population structure, but Kiewitz and Tummler (13) proposed a net-like structure characterized by high frequencies of recombination. An epidemic structure was favored by Lomholt et al. (14) and Pirnay et al. (17), who used sequencing-based techniques, such as sequencing of the outer membrane lipoprotein, combined with serotyping and pyoverdine type determination in a polyphasic approach to reveal extensive genetic mosaicism, particularly in the *oprD* gene.

A variety of molecular genetic methods have been used to type *P. aeruginosa* strains (10), but these vary in their discriminatory potentials. Many investigators have considered pulsed-field gel electrophoresis of DNA macrodigests to represent the "gold standard" against which newer methods are measured. However, the lack of a discriminating and portable scheme suitable for population genetics analysis and an exceptionally variable phenotype (18) have hindered epidemiological and population biology studies. We describe the development and use of a multilocus sequence typing (MLST) scheme to characterize a diverse collection of clinical and environmental isolates of *P. aeruginosa*, including representatives of clone C and recently identified epidemic clones from the United Kingdom.

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MATERIALS AND METHODS

***P. aeruginosa* culture collection.** *P. aeruginosa* strains were obtained from the Hajo Grundmann collection (10), deposited at the Health Protection Agency, Colindale, London, United Kingdom. Six isolates were from mushroom compost

at one experimental mushroom farm and were provided by Alun Morgan Horticulture Research International. In addition, ~100 isolates were collected from hospitals across the United Kingdom. See Table 1 for a summary of all 143 isolates. Strains were identified as *P. aeruginosa* as described previously (10), and DNA fingerprinting of *toxA* and serotyping were also performed.

Culture of isolates and preparation of chromosomal DNA. Bacterial strains were maintained at −80°C in 12% (vol/vol) glycerol in brain heart infusion (BHI) broth, streaked to single colonies, and cultured on BHI agar at 37°C under aerobic conditions. Chromosomal DNA was extracted from these purified strains with a DNeasy kit (Qiagen).

Locus selection. Several potential loci were identified by using the *P. aeruginosa* PAO1 genome database (http://www.pseudomonas.com/) (27). Criteria governing locus selection included biological role (e.g., a diverse range of different central housekeeping roles, such as mismatch repair, DNA replication, and amino acid biosynthesis), size (>600 bp), location (i.e., a minimum of 6 kbp upstream or downstream from known virulence factors, lysogenic phage, or insertion sequence elements), and suitability for nested primer design and sequence diversity (ideally, the possession of conserved domains flanking a variable central core). The seven genes finally selected for use with the MLST scheme were *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* (Table 2).

Amplification and sequencing of loci. PCR primers were designed for the loci listed above by using the published *P. aeruginosa* sequences (27). The primers used, all of which had a common melting temperature, are shown in Table 3. The 50-μl amplification reaction mixture comprised ~10 ng of chromosomal DNA, 1 μM each primer, 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 2 mM each deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase (Qiagen). The reaction conditions were denaturation at 96°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles. The amplification product was purified with MinElute UF (Qiagen), according to the protocol of the manufacturer. The nucleotide sequences were determined by using internal nested primers and 2 μl of BigDye Terminator Ready Reaction Mix (version 3.1) with standard sequencing conditions, according to the protocol of the manufacturer. Unincorporated dye terminators were removed by precipitation with 95% alcohol. The reaction products were separated and detected on an ABI PRISM 3100 genetic analyzer by using a standard sequencing module with Performance Optimized Polymer Applera UK, Warrington, United Kingdom) and a 50-cm array.

Allele and ST assignment. An arbitrary number was given to each distinct allele within a locus. Each isolate was therefore given seven numbers that represented its sequence type (ST). Each sequence type was numbered in order of appearance (ST1, ST2, etc.). Allele profiles and STs can be found at http://pubmlst.org/paeruginosa.

Phylogenetic analysis. The number of polymorphic nucleotide sites, calculation of the ratio of the number of nonsynonymous substitutions to the number of synonymous substitutions (the d_N/d_S ratio), and construction of a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) were performed with the START program (http://www.mlst.net) (11).

RESULTS

Allelic variation in *P. aeruginosa*. Among the 143 isolates investigated, the number of housekeeping gene alleles ranged from 21 for *nuoD* to 43 for *acsA* (Table 4). There were between 23 and 37 variable sites within each locus; i.e., 5 to 8% of base pairs represented variable sites.

The d_N/d_S ratio indicates the presence or absence of a selection pressure on the locus. Usually, most nonsynonymous changes would be expected to be eliminated by purifying selection, but under certain conditions Darwinian selection may lead to their retention. Investigation of the number of synonymous and nonsynonymous substitutions may therefore provide information about the degree of selection operating on a system. The low d_N/d_S ratios in Table 4 indicate the absence of a strong positive selective pressure at these loci and the suitability of these loci for population genetic studies.

Relatedness of *P. aeruginosa* isolates. A total of 139 different STs were assigned to the 143 isolates investigated (Table 1). The rank order of isolates within Table 1 was derived from a UPGMA dendrogram of ST allelic profiles. Ten lineages or clonal complexes were identified among these isolates, and these were composed of strains with either identical STs or STs that varied at one or two loci (single- or double-locus variants), with founder strains indicated below with an asterisk. A founder strain has the ST to which all other STs in the clonal group are related (at least for that sample of strains examined). The compositions of these groups were as follows: group 1, 2 isolates; STs 82 and 83; group 2, 12 isolates, STs 7, 11, 15, 27*, 119, 122, 128, and 129; group 3, 5 isolates, STs 14, 17*, 115, 117, and 142; group 4, 6 isolates; STs 53, 97, 102, 111, 113, and 124; group 5, 5 isolates, STs 30, 31, 38, 39, and 46; group 6, 3 isolates, STs 104, 107, and 109; group 7, 2 isolates, STs 61 and 69; group 8, 5 isolates, STs 41, 45, 49, 52, and 57; group 9, 2 isolates, STs 9 and 118; group 10, 2 isolates; STs 5 and 23.

The environmental isolates (from mushroom compost, soil, and an oil-contaminated aquifer) were unrelated to each other but did cluster among the clinical isolates. In fact, mushroom compost isolates 1349 M (ST5, group 10) and 1346 M (ST41, group 8) clustered with clinical isolates from around the United Kingdom; and isolate 2359 (ST7, group 2), which was from a Canadian oil-contaminated aquifer, clustered with 11 clinical isolates from hospitals around the United Kingdom.

Isolates previously identified as members of the Liverpool, Manchester, Midlands, and Melbourne epidemic clones (Table 1) were found to be unrelated, sharing few if any alleles. Previously reported clone C was also unrelated to the United Kingdom epidemic isolates, although two isolates from the United Kingdom, isolates 8277 (ST14) and 8735 (ST17), from Durham and Birmingham, respectively, were identified here by MLST as belonging to clone C. In fact, from this small data set, ST17, the Birmingham isolate, was identified by BURST (based upon related STs) as the founder member of this small group of clone C isolates. BURST is a novel clustering algorithm designed for use with microbial MLST data. The approach specifically examines the relationships within clonal complexes.

The index of association (23) for all 143 isolates was found to be 0.288, and that for the 139 individual STs was found to be 0.17, indicating that *P. aeruginosa* has a nonclonal population structure. This statistical test attempts to measure the extent of linkage equilibrium within a population by quantifying the amount of recombination among a set of sequences and detecting associations between alleles at different loci. Comparisons of the topologies of neighbor-joining trees for the nucleotide sequences of individual loci (data not presented) revealed that there was little, if any, congruence between the trees. This is further evidence of the importance of recombination in the evolution of *P. aeruginosa* and indicates that the long-term phylogenetic inference of interstrain relationships, beyond the closely related groups identified, is relatively meaningless. For this reason we have not presented a dendrogram; however, as mentioned previously, the order of strains in Table 1 is the same as that derived from a UPGMA tree of allelic profiles (STs).

Relationship between ST, serotype, and *toxA* type. The serotypes of 118 of the isolates included in this study had been determined previously, and the *toxA* types had been determined for 38 isolates. Individual serotypes were found to be widely distributed across the dendrogram generated from the

TABLE 1. Properties of the *P. aeruginosa* strains used for validation[a]

Strain	ST	BURST group	Allelic profile	Sero-type	*toxA* type	Hospital, center, or geographic source	Isolation site	Date of isolation (mo-yr)
Soil isolate (n = 2)	145		17, 35, 36, 3, 14, 4, 7	NA		Wellesbourne, UK	Soil	Jan-99
Midlands 1 epidemic	116		17, 20, 1, 3, 13, 6, 7	NA		NA	Sputum	
8623	110		15, 5, 1, 3, 2, 12, 7	NA	07.06.06 T6	NA	NA	
PA0093	83	1	11, 5, 1, 3, 14, 17, 7	PA		Folkestone, UK	Tissue sample	Nov-01
PA0092	82	1	32, 5, 24, 3, 14, 17, 7	O11		Wycombe, UK	Blood	Nov-01
1353 M	100		33, 3, 3, 3, 4, 12, 7	NA		Wellesbourne, UK	Mushroom compost	
7874	129	2	6, 5, 6, 7, 2, 6, 7	O3	07.03.22 T31	West Yorkshire, UK	NA	
7916	119	2	6, 5, 6, 7, 3, 6, 7	O11	10.08.10 T13	Bristol, UK	NA	
8184	27	2	6, 5, 6, 7, 4, 6, 7	O11	04.08.10 T5	Queen Mary's, UK	NA	
8101	27	2	6, 5, 6, 7, 4, 6, 7	O11	10.08.17 T7	Stourbridge, UK	NA	
6954	27	2	6, 5, 6, 7, 4, 6, 7	O4	04.06.11 T10	Glasgow, UK	NA	
6338	27	2	6, 5, 6, 7, 4, 6, 7	O12	02.02.02 T17	NA	NA	
4587	27	2	6, 5, 6, 7, 4, 6, 7	O11	10.08.09 T14	Boston, UK	NA	
7627	11	2	6, 5, 6, 7, 4, 6, 3	O4	01.01.01 T1	Croydon, UK	NA	
2359 Canada	7	2	6, 5, 6, 7, 4, 6, 1	NA		Canada	Oil-contaminated aquifer	Jan-87
8281	15	2	12, 5, 6, 7, 4, 6, 7	NA		NA	NA	
8122	128	2	6, 5, 6, 8, 21, 6, 7	O1	03.10.15. T12	Mid-Glamorgan, UK	NA	
5798	122	2	6, 5, 6, 8, 2, 6, 7	NA		NA	NA	
6563	10		8, 5, 8, 8, 4, 6, 7	O3	01.01.01 T1	Blackburn, UK	NA	
PA0051	44		4, 5, 6, 7, 1, 16, 7	O9		Luton, UK	Sputum	Aug-02
PA0074	66		30, 10, 23, 5, 4, 2, 7	O4		Dorset, UK	Blood	Mar-02
PA0050	43		25, 5, 17, 5, 4, 15, 7	O11		Chelsea, UK	Wound swab	Sep-02
PA0040	33		19, 5, 15, 3, 4, 12, 7	O11		Birmingham, UK	NA	Nov-02
PA0029	25		19, 5, 12, 7, 4, 10, 7	O6		Manchester, UK	Sputum	Jan-03
Clone C	142	3	11, 5, 1, 7, 9, 26, 7	NA	Sputum			
8735	17	3	11, 5, 1, 7, 9, 4, 7	O1	01.01.01 T1	Birmingham, UK	NA	
8277	14	3	11, 5, 1, 7, 9, 8, 7	O1	01.01.01 T1	Durham, UK	NA	
Clone C genotype P10118	117	3	11, 20, 1, 7, 9, 4, 32	NA		NA	Sputum	
Clone C genotype P10119	115	3	11, 20, 1, 7, 9, 4, 7	NA		NA	Sputum	
PA0100	90		39, 25, 9, 7, 9, 20, 7	O1		Northampton, UK	NA	Sep-01
PA0072	64		28, 4, 22, 7, 9, 10, 7	O15		Ormskirk, UK	Urine	Apr-02
Melbourne	141		11, 33, 11, 31, 23, 4, 7	NA		NA	Sputum	
PA0105	95		7, 4, 7, 5, 2, 7, 7	O6		London, UK	NA	Aug-01
PA0049	42		4, 13, 16, 5, 2, 7, 7	O11		London, UK	Bronccoalveolar lavage fluid	Sep-02
PA0075	67		31, 4, 6, 5, 2, 6, 7	O11		Birmingham, UK	NA	Mar-02
PA0057	50		26, 17, 11, 5, 2, 4, 7	O6		Newcastle, UK	Blood	Jul-02
Manchester	143		17, 34, 11, 18, 4, 13, 3	NA		NA	Sputum	
PA0044	37		23, 16, 11, 4, 4, 6, 3	O7		Leeds, UK	Blood	Oct-02
PA0036	124	4	20, 8, 5, 4, 4, 4, 3	O6		Cheshire, UK	NA	Dec-02
8756	113	4	17, 5, 5, 4, 19, 4, 3	O12	06.04.00 T4	France	NA	
8722	111	4	17, 5, 5, 4, 4, 4, 3	O12	06.04.00 T4	Belfast, UK	NA	
6950	102	4	15, 5, 5, 3, 4, 4, 3	O12	06.04.00 T4	Southport, UK	NA	
PA0108	97	4	11, 5, 1, 4, 4, 20, 3	O1		Bristol, UK	NA	Jul-01
PA0061	53	4	11, 5, 19, 4, 4, 4, 3	PA		Brighton, UK	Sputum	Jun-02
1330 M	4		3, 3, 4, 4, 4, 4, 3	NA		Wellesbourne, UK	Mushroom compost	
8103	133		1, 20, 32, 3, 4, 25, 3	O3	07.08.17 T34	St. Mary's, UK	NA	
7272	134		23, 20, 7, 30, 4, 4, 10	O6	04.08.05 T24	Cumbria, UK	NA	
PA0098	88		22, 4, 17, 7, 4, 13, 7	PA		London, UK	Blood	Oct-01
PA0068	60		28, 4, 17, 15, 4, 4, 3	O4		Nottingham, UK	Tissue sample	May-02
8440	132		6, 20, 1, 3, 4, 4, 2	O6	10.08.03 T41	Carmarthen, UK	NA	
PA0094	84		6, 11, 1, 5, 4, 4, 27	O4		Bristol, UK	NA	Nov-01
PA0107	96		6, 5, 1, 11, 17, 7, 2	O6		Leicester, UK	Sputum	Aug-01
PA0020	18		6, 4, 1, 11, 4, 7, 10	PA		Stafford, UK	NA	Feb-03
7306	135		6, 20, 11, 7, 1, 12, 19	6	06.09.13 T25	Queen Elizabeth II	NA	
8113	106		22, 20, 11, 23, 1, 3, 3	O11	10.08.09 T4	Conquest	NA	
PA0076	68		32, 20, 24, 3, 1, 17, 15	O11		Dublin, Ireland	Blood	Mar-02
PA0043	36		11, 15, 11, 3, 1, 14, 15	O1		York, UK	NA	Oct-02
PA0034	28		15, 10, 11, 3, 2, 7, 12	NT		London, UK	Blood	Dec-02
PA0022	20		15, 5, 11, 3, 1, 7, 11	O6		Cheshire, UK	Sputum	Feb-03
Liverpool a	114		11, 20, 11, 3, 4, 23, 1	NA		Liverpool, UK	Sputum	
PA0067	59		11, 4, 11, 11, 4, 12, 20	O11		London, UK	NA	May-02
8364	130		5, 8, 25, 28, 4, 24, 34	O3	01.02.18 T38	University College, UK	NA	
PA0097	87		5, 24, 25, 21, 4, 4, 7	O3		London, UK	Bronccoalveolar lavage fluid	Oct-01
8405	127		43, 30, 31, 26, 4, 24, 32	O8	Atyp 4 T49	Bangor, UK	NA	
5973	131		6, 5, 11, 29, 11, 15, 1	NA		NA	NA	
8734	112		6, 5, 1, 25, 1, 12, 1	O6	06.03.14 T11	Stoke/Trent, UK	NA	
PA0089	125		36, 1, 28, 1, 1, 7, 1	PA		London, UK	NA	Dec-01
NCTC 10662	1		1, 1, 1, 1, 1, 1, 1	NA		NA	NA	Apr-69
7186	103		17, 5, 12, 22, 14, 22, 29	O11	04.08.10 T5	Truro, UK	NA	
PA0084	76		17, 21, 12, 5, 1, 17, 1	O4		London, UK	Blood	Jan-02
PA0024	21		16, 5, 12, 11, 1, 2, 1	O3		Bedford, UK	Skin	Jan-03
PA0026	123		17, 5, 5, 1, 10, 12, 2	O6		Sheffield, UK	NA	Jan-03

Continued on following page

TABLE 1—*Continued*

Strain	ST	BURST group	Allelic profile	Sero-type	*toxA* type	Hospital, center, or geographic source	Isolation site	Date of isolation (mo-yr)
PA0101	91		17, 5, 5, 1, 1, 21, 1	O11		Windsor, UK	Blood	Sep-01
PA0054	47		17, 5, 5, 12, 1, 10, 16	PA		London, UK	Sputum	Aug-02
PA0070	62		2, 5, 5, 16, 3, 1, 19	O11		London, UK	Blood	May-02
PA0062	54		2, 5, 5, 11, 4, 4, 16	O11		Leeds, UK	Hospital sink	Jun-02
8386	121		11, 5, 7, 27, 1, 7, 33	O1	09.11.10 T40	Wexham, UK	NA	
5670	8		7, 5, 7, 3, 7, 1, 6	O11	10.08.04 T16	Scunthorpe, UK	NA	
PA0102	92		40, 5, 30, 3, 1, 4, 8	O9		London, UK	NA	Sep-01
PA0053	46	5	4, 5, 16, 3, 1, 17, 13	O9		Lincoln, UK	Urine	Aug-02
PA0045	38	5	4, 5, 16, 3, 1, 4, 13	O3		Wolverhampton, UK	Blood	Oct-02
PA0046	39	5	18, 5, 5, 3, 1, 6, 13	O11		Folkestone, UK	Blood	Oct-02
PA0038	31	5	21, 5, 5, 3, 1, 4, 13	O14		Ashton under Lyme, UK	NA	Nov-02
PA0037	30	5	15, 12, 5, 3, 1, 4, 13	O10		London, UK	Blood	Nov-02
PA0090	81		37, 5, 29, 3, 4, 4, 26	O6		Folkestone, UK	NA	Nov-01
8318	108		39, 5, 20, 5, 1, 6, 31	O6	01.01.23 T3	Sutton, UK	NA	
PA0081	73		33, 5, 25, 20, 1, 6, 24	O10		London, UK	Blood	Jan-02
PA0104	94		24, 5, 3, 5, 16, 6, 28	O6		Leicester, UK	Hospital floor	Aug-01
PA0111	126		5, 27, 5, 13, 1, 13, 4	O6		Nottingham, UK	Blood	Jul-01
PA0095	85		5, 11, 3, 13, 1, 4, 4	O4		London, UK	Blood	Oct-01
PA0103	93		22, 5, 11, 13, 1, 15, 4	O14		Leeds, UK	Blood	Sep-01
PA0066	58		4, 5, 16, 13, 1, 2, 4	O4		Nottingham, UK	NA	May-02
PA0055	48		6, 5, 4, 13, 1, 4, 4	PA		Newcastle on Tyne, UK	Blood	Jul-02
PA0063	55		27, 5, 20, 13, 1, 2, 8	O6		Bedford, UK	Blood	Jun-02
PA0083	75		17, 5, 26, 13, 1, 17, 25	O6		Brompton, UK	NA	Jan-02
PA0099	89		13, 8, 5, 13, 1, 7, 25	O7		Folkestone, UK	Sputum	Sep-01
8297	16		13, 8, 9, 3, 1, 9, 9	O11	10.08.17 T7	Ports; mouth, UK	NA	
8382	109	6	4, 4, 16, 24, 1, 6, 3	O10	05.03.05 T2	Whippscross, UK	NA	
8202	107	6	4, 4, 16, 21, 1, 6, 1	O10	05.03.05 T2	St. Thomas, UK	NA	
7215	104	6	4, 4, 16, 3, 1, 6, 30	O11	05.03.05 T2	Bath, UK	NA	
8237	13		10, 4, 5, 10, 1, 6, 3	O10	05.03.05 T2	St. Bar, UK	NA	
PA0077	69	7	28, 4, 3, 3, 1, 6, 11	O11		Dudley, UK	Sputum	Mar-02
PA0069	61	7	13, 4, 9, 3, 1, 6, 11	O15		Gloucester, UK	Sputum	May-02
PA0096	86		38, 4, 3, 3, 15, 15, 3	PA		London, UK	Blood	Oct-01
PA0087	79		34, 11, 3, 12, 1, 13, 3	O11		London, UK	Staff hands, intensive care	Dec-01
PA0056	49	8	24, 5, 3, 12, 1, 6, 3	NT		Surrey, UK	NA	Jul-02
1346 M	41	8	24, 3, 3, 12, 1, 7, 3	NA		Wellesbourne, UK	Mushroom compost	
PA0065	57	8	11, 5, 1, 12, 1, 12, 3	O12		London, UK	Blood	May-02
PA0060	52	8	11, 19, 12, 12, 1, 6, 3	O11		Wycombe, UK	Sputum	Jun-02
PA0052	45	8	11, 5, 12, 12, 1, 6, 3	O9		Lincoln, UK	Sputum	Aug-02
PA0085	77		17, 22, 12, 12, 1, 18, 3	O4		Nottingham, UK	Hospital floor	Jan-02
PA0073	65		29, 4, 16, 12, 1, 18, 21	O10		London, UK	Blood	Apr-02
PA0082	74		4, 5, 1, 19, 13, 7, 23	O3		Wycombe, UK	Blood	Jan-02
PA0080	72		4, 4, 16, 19, 13, 10, 23	PA		York, UK	Sputum	Feb-02
PA0047	40		4, 5, 16, 11, 11, 7, 14	O7		Chelsea, UK	Wound swab	Sep-02
PA0041	34		4, 5, 16, 3, 3, 7, 3	O9		Luton, UK	Sputum	Nov-02
PA0078	70		4, 4, 16, 17, 10, 12, 22	PA		London, UK	Sputum	Feb-02
7193	120		17, 22, 5, 3, 4, 14, 3	O4	09.08.11 T22	Milton Keynes, UK	NA	
4785	101		7, 22, 5, 3, 3, 14, 19	O4	04.06.11 T10	Kings College, UK	NA	
PA0064	56		13, 17, 5, 14, 3, 6, 19	O6		Telford, UK	Wound swab	Jun-02
8420	118	9	6, 6, 4, 3, 20, 4, 7	O1	01.13.15 T15	Hope, UK	NA	
5757	9	9	6, 6, 4, 3, 3, 4, 7	O1	01.01.01 T1	Toulouse, France	NA	
7433	105		42, 28, 4, 3, 18, 4, 7	O1	03.10.15 T12	Leicester, UK	NA	
PA0109	98		41, 12, 4, 3, 3, 4, 14	O10		Cheshire, UK	Blood	Jul-01
PA0110	99		17, 26, 5, 3, 3, 4, 7	O11		Brighton, UK	NA	Jul-01
PA0079	71		28, 4, 5, 18, 3, 4, 7	O11		Bristol, UK	NA	Feb-02
PA0086	78		11, 11, 11, 3, 3, 19, 19	PA		Stoke, UK	Catheter	Dec-01
PA0042	35		22, 14, 17, 3, 3, 6, 7	O6		Glasgow, UK	Sputum	Oct-02
PA0035	29		7, 11, 14, 3, 3, 2, 7	O11		London, UK	Blood	Dec-02
PA0030	26		16, 8, 1, 3, 3, 13, 3	PA		Cheshire, UK	NA	Dec-02
PA0025	22		6, 4, 4, 3, 3, 11, 2	O4		Northampton, UK	Tissue sample	Jan-03
1329 M	3		1, 2, 3, 3, 3, 3, 2	NA		Wellesbourne, UK	Mushroom compost	1997
PA0059	51		24, 19, 3, 3, 11, 10, 18	NT		Luton, UK	Eye	Jul-02
PA0088	80		35, 23, 27, 6, 6, 4, 5	O1		London, UK	Trachea	Dec-01
PA0021	19		14, 9, 10, 6, 6, 10, 5	PA		Dudley, UK	Vaginal swab	Feb-03
2351 M	6		5, 4, 5, 6, 6, 5, 5	NA		Wellesbourne, UK	Mushroom compost	
PA0071	63		5, 4, 21, 5, 12, 10, 15	O15		Ormskirk, UK	Urine	Apr-02
PA0027	23	10	18, 4, 5, 5, 5, 4, 4	O15		Tyne and Wear, UK	Urine	Jan-03
1349 M	5	10	4, 3, 5, 5, 5, 4, 4	NA		Wellesbourne, UK	Mushroom compost	
PA0039	32		18, 13, 13, 11, 11, 4, 14	O11		London, UK	Venous line	Nov-02
PA0028	24		18, 5, 13, 11, 2, 6, 2	PA		Tyne and Wear, UK	Urine	Jan-03
8079	12		9, 7, 2, 9, 8, 7, 8	NA		NA	NA	
1327 M	2		2, 2, 2, 2, 2, 2, 2	NA		Wellesbourne, UK	Mushroom compost	

[a] Symbols and abbreviations: NA, not available; NT, nontypeable; UK, United Kingdom; boldface, epidemic clones.

TABLE 2. Functions and genome positions of the seven loci used in the *P. aeruginosa* typing scheme

Locus	Putative function of gene (strain)	Position in PAO1 genome (base pair)
acsA	Acetyl coenzyme A synthetase (PA0887)	969670
aroE	Shikimate dehydrogenase (PA0025)	26711
guaA	GMP synthase (PA3769)	4227237
mutL	DNA mismatch repair protein (PA4946)	5551681
nuoD	NADH dehydrogenase I chain C, D (PA2639)	2983963
ppsA	Phosphoenolpyruvate synthase (PA1770)	1914037
trpE	Anthralite synthetase component I (PA0609)	670980

allelic profiles rather than solely associated with closely related clusters of strains (Table 1). Within the BURST groups of closely related isolates, 7 of 10 BURST groups possessed more than one serotype. Group 2 had five different serotypes among the nine isolates that had previously been serotyped, and the ST27 isolates from group 2 had four different serotypes.

Although fewer data were available for *toxA* types, a picture similar to that for serotypes was also found for *toxA* types. BURST group 2 contained multiple *toxA* types, and each of the five ST27 isolates possessed a different *toxA* type.

Both of these data sets reveal that there is a weak linkage between ST, serotype, and *toxA* type, which could be expected from the population structure. There is evidence that strains possess identical serotypes and *toxA* types but different STs (e.g., ST8277 and ST8735 members of clone C BURST group 2 and ST102, ST111, and ST113 members of BURST group 4), and there are examples of strains with identical STs but different serotypes and *toxA* types (e.g., members of ST27 in BURST group 2).

DISCUSSION

At present there is a great need for a universal technique for *P. aeruginosa* typing that is unambiguous and reproducible and that can be used for epidemiological studies of the organism. It has been shown here that MLST fulfills these criteria and effectively types all strains from a diverse collection of *P. aeruginosa* strains.

Analysis of these data has further confirmed that *P. aeruginosa* has a nonclonal population structure punctuated by highly successful epidemic clones or clonal complexes. Recombination is therefore likely to play an important role in shaping the evolution of *P. aeruginosa*. The weak association between serotypes, *toxA* types, and MLST STs is a probable result of the effect of recombination on the evolution of *P. aeruginosa*. The isolates with identical STs examined usually possessed different serotypes and different *toxA* types. However, further analysis of *toxA* type and serotype stability is required, ideally with a different collection of isolates with a predetermined association in space and time, to better understand the value of the *toxA* type and the serotype for the local epidemiology of *P. aeruginosa* over different periods of time.

Included within the strain collection evaluated in the present study were representative isolates of each of the recently identified clinical epidemic isolates from across the United Kingdom, examples of European clone C, and an epidemic isolate from Melbourne, Australia. It is interesting from the allele profiles in Table 1 that the epidemic clones are not closely

TABLE 3. Dideoxyoligonucleotide primers used for *P. aeruginosa* MLST

Locus and function	Primer sequence (5′ to 3′)		Amplicon size (bp)
	Forward	Reverse	
acsA			
Amplification	ACCTGGTGTACGCCTCGCTGAC	GACATAGATGCCCTGCCCCTTGAT	842
Sequencing	GCCACACCTACATCGTCTAT	GTGGACAACCTCGGCAACCT	390
aroE			
Amplification	TGGGGCTATGACTGGAAACC	TAACCCGGTTTTGTGATTCCTACA	825
Sequencing	ATGTCACCGTGCCGTTCAAG	TGAAGGCAGTCGGTTCCTTG	495
guaA			
Amplification	CGGCCTCGACGTGTGGATGA	GAACGCCTGGCTGGTCTTGTGGTA	940
Sequencing	AGGTCGGTTCCTCCAAGGTC	TCAAGTCGCACCACAACGTC	372
mutL			
Amplification	CCAGATCGCCGCCGGTGAGGTG	CAGGGTGCCATAGAGGAAGTC	940
Sequencing	AGAAGACCGAGTTCGACCAT	ATGACTTCCTCTATGGCACC	441
nuoD			
Amplification	ACCGCCACCCGTACTG	TCTCGCCCATCTTGACCA	1,042
Sequencing	ACGGCGAGAACGAGGACTAC	TTCACCTTCACCGACCGCCA	366
ppsA			
Amplification	GGTCGCTCGGTCAAGGTAGTGG	GGGTTCTCTTCTTCCGGCTCGTAG	989
Sequencing	GGTGACGACGGCAAGCTGTA	TCCTGTGCCGAAGGCGATAC	369
trpE			
Amplification	GCGGCCCAGGGTCGTGAG	CCCGGCGCTTGTTGATGGTT	811
Sequencing	TTCAACTTCGGCGACTTCCA	GGTGTCCATGTTGCCGTTCC	441

TABLE 4. Analysis of the seven loci in the *P. aeruginosa* population sampled

Locus	Fragment size (bp)	No. of alleles	No. of variable sites	% Variable sites	d_N/d_S
acsA	390	43	32	8.2	0.03
aroE	495	35	33	6.7	0.087
guaA	372	36	24	6.5	0.02
mutL	441	31	26	5.9	0.051
nuoD	366	23	25	6.8	0.033
ppsA	369	26	25	6.8	0.06
trpE	441	35	37	8.4	0.017

related to each other, suggesting that they have evolved independently. Furthermore, some of the clone C isolates had different alleles for the *trpE* locus, which lies within a region of the chromosome that has been inverted within some clone C isolates (19). This suggests that the inversions in these isolates were independent events and not that they arose once and were then subsequently transferred between strains. Additional work with large numbers of representatives of each epidemic clone is required to understand better how these clinically important organisms have evolved and to understand more about clonal stability within *P. aeruginosa*.

Finally, environmental isolates from soil, an oil-contaminated aquifer, and mushroom compost did not cluster away from clinical isolates. In fact, some of these environmental isolates were members of clones or clonal complexes that possessed isolates from cases of invasive disease. This corroborates assumptions based on previous studies which found no correlations between habitat and particular clones (17, 20).

The MLST scheme described here shows that *P. aeruginosa* has a nonclonal epidemic population structure. Further work is required to better understand the evolution of epidemic clones; to compare MLST with typing systems that rely upon genome fragment analysis, such as pulsed-field gel electrophoresis and amplified fragment length polymorphism analysis; and to characterize the genetic diversity and assess the risk of environmental reservoirs of *P. aeruginosa*.

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