Genomic diversity of the genus *Stenotrophomonas*

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The clinical and environmental importance of *Stenotrophomonas* bacteria requires thorough, molecular studies on their epidemiology and taxonomy. In order to obtain a complete genomic profile of this genus, over 100 *Stenotrophomonas maltophilia* strains from various origins were investigated by AFLP fingerprinting. A subset of these strains was analysed by DNA hybridization and 16S rDNA sequencing. In contrast to their high phenotypic homogeneity, the strains were found to be very heterogeneous genotypically by AFLP fingerprinting. Nevertheless, ten cores of highly similar strains representing ten genomic groups were observed. The same groups could be retrieved by DNA hybridizations and also, partly, by 16S rDNA sequence analysis. The intergroup DNA similarities were too high to create confident species delineations, neither could the genomic groups be characterized by phenotypic features.

Keywords: *Stenotrophomonas maltophilia*, genomic groups, AFLP fingerprinting

INTRODUCTION

Stenotrophomonas maltophilia (Palleroni & Bradbury, 1993), formerly named *Pseudomonas maltophilia* (Hugh, 1981) and *Xanthomonas maltophilia* (Swings *et al.*, 1983), is a commonly found ubiquitous bacterium. It is increasingly prevalent in hospitals as an opportunistic human pathogen causing nosocomial infections. The numerous recent literature reports, dealing with *S. maltophilia* infections and outbreaks, emphasize the importance of this rapidly emerging clinical pathogen. *S. maltophilia* is a causal agent of infection and colonization of immunocompromised cancer and HIV patients and transplant recipients (Nagai, 1984; Roilides *et al.*, 1992; Spencer, 1995) and has been reported as a cause of bacteraemia and infections of the respiratory tracts (Fisher *et al.*, 1981) and urinary tracts (Vartivarian *et al.*, 1996), post-operative infections (Fisher *et al.*, 1981), ocular infections (Penland & Wilhelmus, 1996) and a variety of other disease syndromes (Swings *et al.*, 1993). The low outer membrane permeability and the ability to produce β -lactamases with broad-spectrum activity are responsible for the high degree of resistance towards most commonly used broad-spectrum antibiotics such as

penicillins and carbapenems (Mett *et al.*, 1988; Lesco-Bornet & Bergogne-Berezin, 1997; Norrby, 1995; Sanders & Sanders, 1992). Retrospective studies have shown that *S. maltophilia* is the second most frequently isolated nosocomial bacterium after *Pseudomonas aeruginosa* (Zuravlev & Yu, 1982) and that its impact as a multidrug-resistant pathogen increases significantly (Heath & Currie, 1995; Suzuki *et al.*, 1995). The growing clinical importance of *S. maltophilia* infections has recently been reviewed by Denton & Kerr (1998).

In the environment, *S. maltophilia* has been isolated as a growth promoting or symbiotic agent from the rhizosphere of plants and crops including chicory, wheat and crucifers (Debette & Blondeau, 1980; Juhnke *et al.*, 1987), sugar beet (Lambert *et al.*, 1990), sunflowers (Fages & Arsac, 1991), maize (Lambert *et al.*, 1987), rice (Iizuka & Komagata, 1963) and orchids (Wilkinson *et al.*, 1994), as well as from soil (Debette & Blondeau, 1977; Boonchan *et al.*, 1998). As a free-living micro-organism, *S. maltophilia* is isolated from a variety of sources, including water and sediment (Aznar *et al.*, 1992), plant tissue cultures (Leifert & Waites, 1992), sewage, frozen fruit, milk, fermenters (this study), and some unusual habitats such as corroded metal surfaces (Wallace *et al.*, 1994), Na₃-decontaminated Sephadex columns (Borowicz *et al.*, 1995) and oil brine (Iizuka & Komagata, 1964).

The remarkable diversity of sources from which *S.*

Abbreviations: AP-PCR arbitrarily primed PCR; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; RAPD, random amplification of polymorphic DNA.

maltophilia strains have been isolated indicates that these bacteria possess a great variety of enzymic functions necessary for adaptation to different habitats. Many special metabolic and enzymic activities have been described for strains of *S. maltophilia* (Iizuka & Komagata, 1963). Both the ecological and metabolic diversity of *S. maltophilia* suggests that this species could be taxonomically heterogeneous. Most of the taxonomic literature about *S. maltophilia* deals with the position of this species within or outside the genus *Xanthomonas*, but little data are available on the genomic relationships among strains of this diverse taxon. Molecular typing studies of *S. maltophilia* have been conducted recently, but these deal only with strains from clinical origin. Laing *et al.* (1995) compared 80 strains isolated from three hospitals by PFGE, and concluded that all but six strains had unique genotypes, regardless of the presence or absence of an epidemiological association. These results suggested that most infections were independently acquired, rather than from resident hospital clones. Similarly, Chatelut *et al.* (1995) found that among 38 *S. maltophilia* strains, all strains from different patients were different in arbitrarily primed (AP) PCR and enterobacterial repetitive intergenic consensus (ERIC) sequence-primed PCR genomic fingerprints. In other studies based on AP-PCR (Van Couwenberghe *et al.*, 1995; Yao *et al.*, 1995) and PFGE (Yao *et al.*, 1995), only a few strains from the same intensive care unit showed identical genomic profiles, thus suggesting nosocomial transmission. The majority of the strains though had distinct unique genomic profiles.

The diversity of genomic patterns obtained by different molecular fingerprinting techniques suggest that a reservoir of *S. maltophilia* strains exists in the environment, from which certain strains adopt as opportunists. These strains are usually multidrug resistant (Spencer, 1995; Zuravleff & Yu, 1982). The question thus arose as to what degree of relationship there exists between strains isolated from nosocomial infections on the one hand and strains isolated from diverse ecological niches on the other hand.

In the present study we have investigated more than 100 presumptive *S. maltophilia* strains collected from various environmental and clinical sources by means of the sensitive genomic AFLP fingerprinting method. The authors who invented the method recommend not to use the name AFLP as an acronym (Vos *et al.*, 1995). The AFLP technique has been previously used with success for taxonomy and fine-typing of bacteria (Huys *et al.*, 1996; Janssen *et al.*, 1996). This method was chosen here because it offers patterns with a large number of distinct bands representing the entire genome, thus being suitable for the detection both of broad (phylogenetic) and narrow (clonal) relationships. A selected set of strains was further analysed by means of DNA hybridization and 16S rDNA sequencing in order to assess the genomic homogeneity and the 16S rRNA gene variability of the species *S. maltophilia*.

METHODS

Bacterial strains. All of the strains used in this study are listed in Table 1. They were obtained from the culture collection of the Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium, or were kindly provided by J. Debette (University of Sciences and Techniques, Lille, France), W. Hansen (University Hospital Brugmann, Brussels, Belgium), H. Goossens (University Hospital St Pieter, Brussels, Belgium) and P. Goullet (Beaujon Hospital, Paris, France). Strains were cultured for 48 h on solid medium containing 3% Trypticase soy broth (BBL) and 2% technical agar. All strains were received as *X. maltophilia* or *P. maltophilia*, which was confirmed using API 20 NE test strips and Biolog GN microtitre plates. Fatty acid analyses by the MIS system of all strains matched the *S. maltophilia* profile as described by Yang *et al.* (1993b).

Isolation and purification of DNA for AFLP fingerprinting. Approximately 10 µg wet cells was suspended in 1 ml resuspension buffer (150 mM NaCl, 10 mM EDTA; pH 8.0) and centrifuged for 10 min at 15000 *g*. The pelleted cells were resuspended in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 7.6) and lysed with 500 µl GES reagent [5 mM guanidium thiocyanate, 100 mM EDTA, 0.5% (v/v) *N*-lauroyl sarcosine]. The DNA was extracted with chloroform/isoamyl alcohol (24:1, v/v), precipitated with 0.54 vol. 2-propanol, washed twice with 150 µl 70% (v/v) ethanol, and dried in sterile air flow for 10 min. DNA pellets were redissolved in 200 µl TE buffer and incubated for 1 h with 50 µl DNase-free RNase (250 µg ml⁻¹). The DNA concentrations were measured spectrophotometrically at 260 nm and the high molecular masses of the DNA preparations were verified by electrophoresing 10 µl of the DNA samples in 1% agarose gels containing 0.5 µg ethidium bromide ml⁻¹.

Preparation of template DNA for PCR. An amount of 1 µg DNA was digested with *Apa*I and *Taq*I (Pharmacia Biotech) and subsequently ligated with T4 ligase (Pharmacia Biotech) to equimolar amounts of oligonucleotide sequence complements 5'-TCGTAGACTGCGTACAGGCC-3' and 5'-TG-TACGCAGTCTAC-3' complementary to the *Apa*I restriction halfsite and 5'-GACGATGAGTCCTGAC-3' and 5'-CGGTCAGGACTCCAT-3' complementary to the *Taq*I restriction halfsite. Thus obtained templates were precipitated with 1.25 M ammonium acetate and 50% (v/v) 2-propanol, washed with 70% (v/v) ethanol and resuspended in 100 µl buffer containing 10 mM Tris, 0.1 mM EDTA (pH 8.0).

Primer labelling and selective PCR amplification. The following selective PCR primers were used: 5'-GACTGCG-TACAGGCCCG-3', complementary to the *Apa*I restriction site and adaptor, with a 3'-terminal guanine as single selective extension base, and 5'-CGATGAGTCCTGACCGAG-3', complementary to the *Taq*I restriction site and adaptor, also with a 3'-terminal guanine as single selective extension base. The *Taq*I primer was radioactively labelled with [γ -³²P]ATP (Amersham International) using bacteriophage T4 polynucleotide kinase (Pharmacia Biotech).

Electrophoresis and visualization of banding patterns. Amplified fragments were electrophoresed on 5% denaturing polyacrylamide gels containing 4.75% (w/v) acrylamide, 0.25% (w/v) methylene bisacrylamide, 8.3 M urea, 100 mM Tris, 100 mM boric acid, 2 mM EDTA; pH 8.3. The gels were vacuum-dried with a gel dryer (model 58; Bio-Rad) for 55 min at 80 °C. The AFLP fingerprints were visualized autoradiographically by exposing the dried gels to Hyper-

Table 1. *S. maltophilia* strains investigated together with their origin and accession numbers of their 16S rRNA gene sequences in the EMBL database

Culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; CIP, Collection Bactérienne de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Microorganismen und Zellkulturen, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Saitama, Japan; LMG, Laboratorium voor Microbiologie Gent Culture Collection, Universiteit Gent Belgium; NCIB, National Collection of Industrial Bacteria, NCIMB, Aberdeen, UK; NCMB, National Collection of Marine Bacteria, NCIMB, Aberdeen, UK; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Hertfordshire, UK.

	LMG no.	Other strain no.	Source	Genomic group	EMBL no.
1	957t1	ATCC 17448	Human blood culture	NG	AJ131114
2	957t2	ATCC 17448	Human blood culture	NG	
3	958 ^T	ATCC 13637	Cortical necrosis, patient with oral cancer	6	X95923
4	959	ATCC 17806	Rice paddies, Japan	9	
5	960	CIP 59.60	Contaminated tissue culture	5	
6	961t1	ATCC 13636	Spinal fluid	6	
7	961t2	ATCC 13636	Spinal fluid	6	
8	978	NCPBP 323	Piper beetle	NG	AJ131919
9	980	NCPBP 1683	<i>Hibiscus rosa-sinensis</i>	NG	AJ131913
10	981	CCM 284	Soil	NG	AJ131116
11	1098	NCMB 200	Skin from <i>Raja batis</i> (fish)	5	
12	1108	NCMB 211	Gills from <i>Raja batis</i> (fish)	5	
13	6606	Leyns 4M11 B 27.1	Rhizosphere	9	
14	6607	Leyns 5A31 A 18.1	Rhizosphere	9	
15	6608	Leyns 6M11 D 1.16	Rhizosphere	9	AJ131918
16	7958	Stevens D1		NG	
17	7959	Stevens D2		4	
18	10841	CIP 54.90	Human blood	1	
19	10848	DSM 50173	Contaminant from streptomycin solution	2	
20	10849	Goossens I	Hospital infection	5	
21	10850	Goossens 1	Human blood culture	3	
22	10851	Goossens 19	Human blood culture	5	AJ131780
23	10852	Goossens 26	Human blood culture	6	
24	10853	Goossens 28	Sputum	3	AJ131914
25	10854	Goossens 29	Human blood culture	2	
26	10855	Goossens 30	Human blood culture	7	
27	10856	Goossens 33	Human blood culture	6	
28	10857	Goossens 34	Human blood culture	10	AJ131117
29	10858	Goossens 35	Human blood culture	3	
30	10859	Goossens 59	Sputum	1	
31	10860	Goossens 62	Human blood culture	5	
32	10871	ATCC 13270	Soil	10	AJ131781
33	10872	ATCC 13843	Contaminated tissue culture	3	
34	10873	ATCC 15099	Case of conjunctivitis	7	AJ131782
35	10874	ATCC 17671	Human blood culture	4	AJ131783
36	10875	ATCC 17672	Sputum	4	
37	10876	ATCC 17674	Cerebrospinal fluid	4	
38	10877	ATCC 17676	Patient with sinusitis, pus	5	AJ131784
39	10878	ATCC 17677	Patient with otitis, pus	4	
40	10879	ATCC 17807	Rice paddy	9	AJ131903
41	10880	ATCC 29510	Female human	7	
42	10881	JCM 1978	Fish	NG	AJ131904
43	10882	JCM 1979	Sewage	NG	AJ131905
44	10883	JCM 1981	Frozen fruit	1	AJ131906
45	10884	JCM 1982	Phenol enrichment culture	4	
46	10885	JCM 1983	Manured oil	9	
47	10886t1	JCM 1985	Manured oil	NG	

Table 1 (cont.)

	LMG no.	Other strain no.	Source	Genomic group	EMBL no.
48	10886t2	JCM 1985	Manured oil	NG	AJ131915
49	10887	JCM 3800	Oil brine	NG	
50	10888	JCM 3801	Oil brine	NG	
51	10889	IFO 13692			
52	10890	IFO 13923	Soil	9	
53	10954	NCIB 9202	Oropharyngeal swab from normal adult	6	AJ131907
54	10955	NCIB 9204	Stagnant water	4	
55	10956	NCIB 11534	Soil	3	
56	10989	Hansen 0230	Gall	NG	
57	10990	Hansen 0294	Urine	6	
58	10991	Hansen 0325	Leg. pus	2	AJ131115
59	10992	Hansen 0362	Expectoration	6	AJ131908
60	10993	Hansen 0408	Pericardial drain	NG	AJ131916
61	10994	Hansen 0422	Tracheal aspiration	6	
62	10995	Hansen 0810	Femoral top	10	
63	10996	Hansen 1132	Leg, ulcer	2	
64	10997	Hansen 1151	Rinsing water from gastrointestinal endoscope	2	
65	10998	Hansen 1213	Eye	2	AJ131909
66	10999	Hansen 2011	Drinking water	6	
67	11000	Hansen 2336	Gall	2	
68	11001	Hansen 2669	Water bed	3	
69	11002	Hansen 2749	Mamilla	3	
70	11003	Hansen 3286	Tracheal aspiration	7	X95924
71	11004	Hansen 3340	Urine	2	
72	11087	C17	Rhizosphere	9	
73	11088	C28	Rhizosphere	9	
74	11089	P1-1	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
75	11090t1	P1-24	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	AJ131910
76	11090t2	P1-24	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
77	11091	P1-25	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
78	11092	P1-34	<i>Cichorium intybus</i> , rhizosphere tuberous roots	9	
79	11093	P1-37	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
80	11094t1	P1-41	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	AJ131911
81	11094t2	P1-41	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
82	11094t3	P1-41	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
83	11095	P1-44	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
84	11096	P1-47	<i>Cichorium intybus</i> , rhizosphere tuberous roots	5	
85	11097	P2-2	<i>Cichorium intybus</i> , rhizosphere tuberous roots	9	AJ131912
86	11098	S-3	<i>Cichorium intybus</i> , rhizosphere tuberous roots	9	
87	11099	S-50	<i>Cichorium intybus</i> , rhizosphere tuberous roots	9	
88	11100	S-51	<i>Cichorium intybus</i> , rhizosphere tuberous roots	9	
89	11101	S-53	<i>Cichorium intybus</i> , rhizosphere tuberous roots	9	
90	11102	S-61	<i>Cichorium intybus</i> , rhizosphere tuberous roots	9	AJ131917
91	11103	E-18	<i>Cichorium intybus</i> , rhizosphere tuberous roots	NG	
92	11104t2	E-41	<i>Cichorium intybus</i> , rhizosphere tuberous roots	NG	
94	11105	B-5	<i>Triticum</i> , rhizosphere	8	
95	11106t1	B-15	<i>Triticum</i> , rhizosphere	8	
96	11106t2	B-15	<i>Triticum</i> , rhizosphere	8	AJ131917
97	11107	B-30	<i>Triticum</i> , rhizosphere	8	
98	11108	RB-42	<i>Triticum</i> , root	8	
99	11109	RB-52	<i>Triticum</i> , root	8	
100	11110t1	RB-76	<i>Triticum</i> , root	NG	
101	11110t2	RB-76	<i>Triticum</i> , root	NG	AJ131917
102	11111t1	90 05 164	Catheter	2	
103	11111t2	90 05 164	Catheter	2	

Table 1 (cont.)

	LMG no.	Other strain no.	Source	Genomic group	EMBL no.
104	11112	90 01 136	Lung, puncture	4	
105	11113	89 06 33	Catheter	6	
106	11114	89 01 88	Human blood culture	1	X95925
107	11115	88 08 116	Bronchae	6	
108	11116t1	88 10 105	Human blood culture	6	

NG, Does not belong to a defined group.

film-MP (Amersham International) for 5–24 h depending on the amount of radiation determined with a regular Geiger-Müller counter. Following exposure, the film was developed with a model RGII X-ray film processor (Fuji).

Densitometric scanning and data processing. Autoradiograms were scanned by using a high-resolution densitometer (model RayVen RSU1; X-Ray Scanner Corp.). Transmission image data were stored as TIFF files and were further processed by using the GELCOMP software, version 4.1 (Applied Maths).

DNA hybridization. DNAs were extracted and purified as described by Marmur (1961). The levels of DNA binding were determined from the initial renaturation rates by using a spectrophotometer (De Ley *et al.*, 1970). The renaturation rates were determined in $2 \times \text{SSC}$ at the optimal renaturation temperature, 80–8 °C, which was calculated from the G+C contents as described by De Ley (1970) ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 sodium citrate).

16S rDNA sequencing. DNA preparation, PCR amplification and sequencing of 16S rRNA genes were done as described previously (Hauben *et al.*, 1997).

16S rDNA sequence comparison and phylogenetic analysis. Alignment, calculation of similarity values and cluster analysis were performed by using the GENECOMP software package (Applied Maths) after discarding all unknown base positions and gaps. The 16S rDNA sequences of *Xanthomonas campestris* LMG 568 (accession no. X95917), *Xanthomonas albilineans* LMG 494 (accession no. X95918), *Xanthomonas sacchari* LMG 471 (accession no. Y10766) and *Xylella fastidiosa* (accession no. M26601) were obtained from EMBL.

RESULTS AND DISCUSSION

The collection of *S. maltophilia* strains we used originated from a wide variety of environments and geographical regions.

Phenotypic typing methods have been applied earlier in an attempt to elucidate the epidemiology of *S. maltophilia* but were often poorly discriminative because of a scarcity of interstrain variability manifested by the bacterium (Schable *et al.*, 1992; Van Nuffel, 1991; Yang *et al.*, 1993a, b). Recently, genotypic typing techniques have received growing attention. Methods evaluated to study the epidemiology of *S. maltophilia* include ribotyping, PFGE, random amplification of polymorphic DNA (RAPD) and ERIC-PCR (Denton & Kerr, 1998). By applying three

genomic techniques with different resolving power for determining taxonomic hierarchies onto a wide collection of strains, the present study revealed a new view on the genomic diversity of the genus *Stenotrophomonas*.

AFLP genomic fingerprinting

The level of reproducibility was determined by using the AFLP pattern of strain LMG 10882 as reference during each electrophoresis run. Following normalization and subtraction of background, we found that the intragel-correlation levels for the reference samples varied from 92 to 97%. Minimal differences in the correlation values between reference lanes were usually introduced by inevitable variations in background intensities and electrophoresis times. Fig. 1 shows normalized AFLP patterns for all strains, sorted according to a UPGMA dendrogram based upon the product-moment correlation between entire densitometric profiles. The combination of *Apal* and *TaqI* as restriction enzymes and guanine as a single extension base for amplification revealed complex AFLP patterns composed of some 45–60 clearly distinguishable bands on average, with sizes between 50 and 600 bp (Janssen *et al.*, 1996). Strains LMG 11116, LMG 957 and LMG 11103 showed significantly less bands, a phenomenon which was reproducible in repeated experiments. Concurrent amplification of large numbers of fragments typically yielded bands with unequal intensities within a single pattern. This fact together with the large numbers of bands made it particularly difficult to assign discrete band positions to the patterns. Therefore, in order to avoid subjective interpretations of band positions, the Pearson product-moment correlation coefficient was applied to measure similarity between normalized densitometric profiles. This coefficient compares curves as a whole and is independent of band definitions. It is largely insensitive to relative concentrations, but sensitive to differences in background and it does not suffer from typical peak/shoulder mismatches (Vauterin & Vauterin, 1992).

The *S. maltophilia* strains showed a remarkable heterogeneity in their AFLP profiles, reflected in the extremely low mean correlation values between the clusters, which range between 15 and 30%. We defined

Fig. 1. For legend see facing page.

ten more or less consistent clusters of highly similar genomes on the dendrogram, showing an internal mean correlation of 40% or higher, and characterized by a number of common bands between the profiles. These clusters are numbered from 1 to 10 on Fig. 1. The genomic heterogeneity revealed by AFLP fingerprinting within *S. maltophilia* was high in comparison with other bacterial groups that have been analysed by this technique. Within the genus *Xanthomonas*, the mean linkage level between species lay between 50 and 60% and within the genus *Aeromonas*, the mean linkage level between hybridization groups lay between 50 and 70% (Huys *et al.*, 1996; Janssen *et al.*, 1996). Two positioned bands or doublets in the AFLP profiles, indicated by an arrow (Fig. 1), were present for most of the *S. maltophilia* strains examined. These bands marked a minimal genomic resemblance within *S. maltophilia*, in spite of the complexity of the genomic fingerprints of the species.

Clusters 1 and 2 consist of clinical isolates only and showed an internal correlation of 61 and 39%, respectively. Strains LMG 980 and LMG 978, two environmental isolates, grouped outside cluster 1. Clusters 2 and 4 consisted of environmental isolates only and showed internal correlations of 45 and 38%, respectively. Strain LMG 7958 and two isolates from oil brine, LMG 10887 and LMG 10888, clustered outside AFLP group 2. The banding patterns of the latter two strains were very similar with a correlation level of 85.2%. Clusters 3 and 6 contain clinical as well as environmental isolates and correlated internally at 34 and 51%, respectively. The type strain of *Stenotrophomonas maltophilia*, LMG 958^T, grouped in cluster 6. Cluster 7 consisted of four clinical isolates and had a high correlation level of 71%. The banding pattern of strain LMG 10993, grouping outside this cluster, was clearly aberrant from the profiles of AFLP group 7. Clusters 8 and 9 consisted of environmental isolates only and correlated at the levels 45 and 25%, respectively. The six strains of group 8 were divided into three pairs, each with very similar banding patterns. Strains LMG 979, LMG 10881, LMG 10886t2, LMG 10886t1, LMG 11116t2 and LMG 10989 did not cluster within any of the AFLP groups and showed very divergent genomic profiles. The banding patterns of LMG 957t1 and LMG 957t2 were identical but showed very few bands. Strains LMG 10882, LMG 981, LMG 11103 and LMG 11104t2 again showed high genomic variations. The last cluster, AFLP group 10, contained two clinical isolates and one environmental one, correlating at a level of 43.1%.

More than one colony type existed within 12 LMG strains. For ten strains, the AFLP patterns of the different types were practically identical with correlation levels of 85% or more. The slight divergences in the banding profiles were due to fluctuating band

intensities and not to different band positions. The different colony types distinguished within LMG 11111, LMG 10997, LMG 11094, LMG 11090, LMG 961, LMG 11106, LMG 10886, LMG 11099, LMG 11088 and LMG 957 could thus be considered as identical. For strain LMG 11110, colony type 2 showed less bands than colony type 1; their correlation level was 82.2%. Colony type 1 and 2 of LMG 11116 were clearly completely different as they correlated at the level of 12.3% and the AFLP profile of type 2 contained only two bands. AFLP seems to be particularly suitable to examine the identity of colony types distinguished within certain strains.

DNA hybridization

A total of 168 DNA hybridization values were determined for 32 strains. All of the values presented below (Table 2) were the means of the values from at least two independent measurements.

At least two strains of AFLP groups 1, 2, 3, 4, 5, 6 and 9 were included in the DNA similarity study. The division of the *S. maltophilia* strains into AFLP groups was being reflected in the DNA hybridization data. AFLP groups 1, 2, 3, 4, 5, 6 and 9 exhibited genomic similarities of at least 58%. A value of 58% occurred between strains LMG 10853 and LMG 11002 of group 3 and between strains LMG 10877 and LMG 10851 of group 5. Between strains LMG 10996 and LMG 11004 of group 2 and strains LMG 6608 and LMG 10890 of group 9, the lowest DNA similarity value observed was 65%. Within AFLP groups 1, 4 and 6, the DNA similarity values were at least 75%.

These values insinuated the possibility of considering AFLP groups 1, 4 and 6 as separate species as a DNA homology value of 70% is the generally accepted minimum value for strains within a species (Wayne *et al.*, 1987). But analysing the DNA hybridization values in more detail proved that the separation of the groups was not very pronounced. The majority of the hybridization values (82%) lay between 10 and 60% with a maximum (29% of the values) between 50 and 60%; the values between the groups were not significantly lower than the values within the groups.

16S rRNA gene sequencing

Direct sequencing of the PCR-amplified 16S rDNA allowed us to determine a continuous stretch of 1298–1502 bases, ranging from positions 28 to 1524 of the 16S rRNA gene sequence of *Escherichia coli*. These

Fig. 1. Dendrogram derived from an unweighted average pair group cluster analysis of the AFLP patterns of all 108 *S. maltophilia* strains included in this study. Levels of linkage are expressed as the Pearson product-moment similarity coefficients.

Table 2. Levels of DNA hybridization (%) between *S. maltophilia* strains

ATLP group...			1			2			3			4			5			6			9			29			30			
Strain...			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1 LMG 11114	100																													
2 LMG 10883	95	100																												
3 LMG 980	19		100																											
4 LMG 978				100																										
5 LMG 10991					100																									
6 LMG 10996	23		53		82	100																								
7 LMG 11004					89	65	100																							
8 LMG 111112					76		83	100																						
9 LMG 11000						71				100																				
10 LMG 10888		15	46								100																			
11 LMG 10887				66		56		60	49	82	53																			
12 LMG 11002			68			51				38	55																			
13 LMG 10853				52							52	100																		
14 LMG 10878			56	49					62	59	61	50	100																	
15 LMG 10874	30	16	55			51				52	54		80	100																
16 LMG 10851			55	33							53		44	61	100															
17 LMG 1108			46			58				62		55		50	87	100														
18 LMG 10877			55						54	56		44	42	44	44	58	64	100												
19 LMG 958			55	54		51			65	52	56	54	41	49	60		53	51	100											
20 LMG 10992				44					37										51	75	100									
21 LMG 95711			55	41						45		57		38	55		47	46	50		100									
22 LMG 10882	6	53							53	41	51	58		52	58	56	52	39	57		59	100								
23 LMG 11108	15	12	17													20														
24 LMG 6608	7			33							36		24	35		28			33		35									
25 LMG 10890					37	38																								
26 LMG 10879								34																						
27 LMG 11087		21		31						42	37		30	37					46											
28 LMG 10881						13																								
29 LMG 10989	19									36																				
30 LMG 981	19	25			43	29		42				30																		100
31 LMG 10857	13	16				31

Fig. 2. Neighbour-joining dendrogram depicting estimated phylogenetic relationships based on pairwise comparisons of 16S rDNA sequences of 29 *Stenotrophomonas* strains, with *X. campestris* LMG 568^T as an outgroup. The distance between two strains is obtained by adding the lengths of the connecting horizontal branches, using the scale at the top.

sequences corresponded to an estimated 85.2–98.6% of the total 16S rDNA primary sequence. The sequence data were confirmed by sequencing the 16S rDNA PCR products, for the most part, in both the forward and reverse directions.

A dendrogram depicting the estimated phylogenetic relationships (Fig. 2) was constructed by the neighbour-joining clustering method (Saitou & Nei, 1987). This dendrogram was based on pairwise comparisons of 30 available 16S rDNA sequences for the genus *Stenotrophomonas*.

Within the genus *Stenotrophomonas*, the mean 16S rDNA sequence similarity was 96.5%, ranging from 91.6 to 99.7%. Five sequence groups were defined (Fig. 2): sequence group I contained strains of AFLP groups 2, 3, 4, 6 and 7 and had a mean 16S rDNA sequence similarity of 98.1%; sequence group II contained strains of AFLP group 5 and showed 98.8% 16S rDNA sequence similarity on the mean; sequence group III contained strains of AFLP group 9 and sequence group IV contained strains of AFLP group 1 with a mean 16S rDNA sequence similarity of 99.0 and 98.4%, respectively. Finally, sequence group V contained strains of AFLP groups 8 and 10 and showed

97.7% 16S rDNA sequence similarity on the mean. Of the ten AFLP groups, only groups 1, 5 and 9 could be distinguished as single groups by 16S rDNA sequencing. The same five groups were defined by 16S rDNA sequence comparisons were found after restriction endonuclease of the 16S rRNA gene of ten *Stenotrophomonas* strains (Nesme *et al.*, 1995). The 16S rDNA sequence diversity within *Stenotrophomonas* was more pronounced than in the related genus *Xanthomonas*, where the mean sequence similarity value was 98.2%. 16S rDNA sequence comparisons between the 20 *Xanthomonas* species resulted in three clusters: one around the type species *X. campestris*, one containing only *X. sacchari* and one around *X. albilineans* (Hauben *et al.*, 1997). One 16S rDNA sequence of each of these clusters was included in Fig. 2.

Sequence group I contained the type strains of the two current species of *Stenotrophomonas*: *S. maltophilia* LMG 958^T and *S. africana* CIP 10-48-54^T. They showed a 16S rDNA sequence similarity of 97.2%, which was higher than the mean 16S rDNA sequence similarity value within *S. maltophilia*. The species *S. africana*, recently found by Drancourt *et al.* (1997), was mainly based on a DNA homology level of 35% with the type strain of *S. maltophilia*. We believe that this value, determined by the S1 nuclease (TCA) method, is not a convincing argument to create a new species for one strain, especially since it is known that different techniques for determining levels of DNA relatedness yield different values. Between strains that are not highly related, the S1 nuclease method is known to produce rather low binding values compared to the nitrocellulose filter method and the spectrophotometric method (Grimont, 1988). In addition, there was only one feature (accumulation of *cis*-aconitate) that discriminated the strain from other *S. maltophilia* strains, of which only the type strain was compared. Moreover, this feature was not known for other *S. maltophilia* strains. The results of this study have now revealed that low DNA hybridization values occur frequently in *S. maltophilia* and this might open some interesting perspectives for taxonomic splitters who can see various new *Stenotrophomonas* species arise. But what would the practical use be of this if there is momentarily no method available to differentiate them at the phenotypic level? A modern polyphasic species definition should be an intermediate one between the species definition by Wayne *et al.* (1987) and a new species definition which will be linked to extensive genotypic and phenotypic databases of strains.

As pointed out by Stackebrandt & Goebel (1994), species possessing 70% or greater DNA similarity usually exhibit more than 97% 16S rDNA sequence similarity. Our sequence data showed that for the genus *Stenotrophomonas* the value was even higher as all of the strains (four in total) that exhibited 70% or more DNA homology exhibited at least 98% sequence similarity. The correspondence between the DNA

Fig. 3. Correlation plot of the DNA homology data versus levels of 16S rDNA sequence similarity for *S. maltophilia* strains.

Fig. 4. Correlation plot of the DNA homology data versus correlation values between densitometric AFLP profiles of *S. maltophilia* strains.

similarity data determined by DNA–DNA hybridization and the 16S rDNA sequence similarity data for the genus *Stenotrophomonas* has been evaluated, and the results are shown in Fig. 3. The percentages of sequence similarity for all of the sequenced *Stenotrophomonas* strains are plotted against the DNA–DNA similarity values in Fig. 3. Within the genus *Stenotrophomonas*, there was a correlation between the two sets of data; organisms that exhibited levels of DNA similarity higher than 40%, always exhibited levels of 16S rDNA similarity higher than 96%. This area contained the correlation data derived from pairwise comparisons of the strains within and between sequence groups I, II and III, and within sequence group IV. Levels of DNA similarity less than 40% corresponded to 16S rDNA sequence similarities between 92 and 99%. The correlation between 16S rDNA similarities and DNA similarities in *Stenotrophomonas* was

more pronounced than for the related genus *Xanthomonas* (Hauben *et al.*, 1997), which could be attributed to the difference in genomic heterogeneity between these two genera.

The correspondence between the DNA similarity data determined by DNA–DNA hybridization and the AFLP fingerprinting data for the genus *Stenotrophomonas* has been evaluated as well, and the results are shown in Fig. 4. Within the genus *Stenotrophomonas*, organisms exhibiting levels of DNA similarity higher than 75% always exhibited levels of AFLP similarity higher than 55%. This area contained similarities measured by the Pearson product–moment correlation coefficient of the strains within AFLP groups 1, 2, 4, 5, 6 and 9. Levels of DNA similarities higher than 70% corresponded to AFLP similarities higher than 50%. Levels of DNA similarity less than 70% corresponded to AFLP similarities between 0 and 50%. The high overall level of correlation between AFLP fingerprinting and DNA hybridization data have indicated that the AFLP method can be considered a relatively fast, reproducible, high-resolution alternative to conventional DNA–DNA hybridization at the subgeneric level (Huys *et al.*, 1996).

Conclusions

With continuing advances in medical technology and the growth of debilitated or immunocompromised populations, the incidence of infection associated with *S. maltophilia* will certainly increase. The data presented in this study are a major contribution to the understanding of the epidemiology of the species *S. maltophilia*. Despite the lack of significant phenotypic interstrain variability within *S. maltophilia*, we demonstrated that their genomes differ a lot. Ten genomic groups could be delineated by AFLP which are largely supported by DNA hybridization data and partly by 16S rDNA sequence data. The relatively high intergroup DNA hybridization data and the lack of characteristic phenotypes for the genomic groups prevented us from describing them as novel species. The conformity of the three genomic techniques applied in this study proved though that the genus *Stenotrophomonas* contains several cores of genomically more related strains which we describe as genomic groups 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 containing the strains as pointed out in Table 1 and Fig. 1.

ACKNOWLEDGEMENTS

L.V. and J.S. acknowledge the Algemeen Bestuur voor de Ontwikkelingssamenwerking. Part of this work was carried out in the framework of Human Capital and Mobility Network grant CHRX CT93-0194 and Concerted Action Exploration of Microbial Diversity grant B102-CT93-0119. We thank A. Krüger and G. Vanderkimpen for excellent technical assistance.

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