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Identification of genomic groups in the genus *Stenotrophomonas* using *gyrB* RFLP analysis

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Abstract

Stenotrophomonas maltophilia isolates have been recovered from various clinical samples, including the respiratory tract of cystic fibrosis (CF) patients, but this organism is also widespread in nature. Previously it has been shown that there is a considerable genomic diversity within *S. maltophilia*. The aims of our study were to determine the taxonomic resolution of restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction-amplified *gyrB* gene for the genus *Stenotrophomonas*. Subsequently, we wanted to use this technique to screen a set of *S. maltophilia* isolates (with emphasis on a specific subset, isolates recovered from CF patients), to assess the genomic diversity within this group. In this study we investigated 191 *Stenotrophomonas* sp. isolates (including 40 isolates recovered from CF patients) by means of *gyrB* RFLP. The taxonomic resolution of *gyrB* RFLP, and hence its potential as an identification tool, was confirmed by comparison with results from published and novel DNA–DNA hybridisation experiments. Our data also indicate that the majority of CF isolates grouped in two clusters. This may indicate that isolates from specific genomic groups have an increased potential for colonisation of the respiratory tract of CF patients.

Keywords: *Stenotrophomonas maltophilia*; Identification; Cystic fibrosis; *gyrB* restriction fragment length polymorphism

1. Introduction

Stenotrophomonas maltophilia is a Gram-negative, non-fermenting and obligately aerobic bacillus. It is increasingly being recognised as an important cause of nosocomial infections in debilitated and immunosuppressed individuals and is resistant to many antimicrobial agents [1]. *S. maltophilia* isolates occupy a wide range of environmental niches [1] and have attracted attention as potential plant growth-promoting and biocontrol organisms [2,3] and as bioremediation agents [4]. *S. maltophilia* was first identified in cystic fibrosis (CF) specimens in the 1970s and its prevalence has been rising since. There are large regional differences in prevalence, which is usually lower in the USA (1.8–10.3%) than in Europe (up to 25%) [1,5,6]. Risk factors for acquisition of *S. maltophilia* by CF patients include decreased pulmonary function, increasing age, and more pulmonary exacerbations, outpatient visits and total duration of hospitalisation [7,8], although in another study the only significant risk factor identified was the use of oral quinolones [9]. At present there is little evidence for extensive patient-to-patient spread of this organism as it appears that, in general, the majority of patients harbour unique isolates or alternatively, a common source of infection (such as sink drains, faucets, and other items frequently in contact with water) can be identified (see for example [10,11]). In a previous study it was shown that there is a remarkable diversity among *S. maltophilia* isolates recovered from human clinical samples and the environment [12]. Using amplified fragment length polymorphism (AFLP) fingerprinting and DNA–DNA hybridisations, Hauben et al. [12] showed that *S. maltophilia* can be subdivided into at least 10 genomic groups. However, considering the high intergroup similarities and the phenotypic similarities between the groups, these groups were not renamed as novel species. Recently, four novel *Stenotrophomonas* species were described: *Stenotrophomonas africana* [13], *Stenotrophomonas nitritireducens* [14], *Stenotrophomonas acidaminiphila* [15] and *Stenotrophomo-*

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nas rhizophila [16]. The relationships of these novel species to the different genomic groups of *S. maltophilia* have however not been investigated yet.

In the present study we assessed the ability of restriction fragment length polymorphism analysis of the gyrase B gene (*gyrB* RFLP) for distinguishing between genomic groups in the genus *Stenotrophomonas*. Subsequently, we used this technique to investigate the genomic diversity of *S. maltophilia*, with special emphasis being put on isolates recovered from CF patients. Our results were compared with novel and published DNA–DNA hybridisation results and with the AFLP results obtained by Hauben et al. [12].

2. Materials and methods

2.1. Bacterial isolates and growth conditions

Ninety-eight *S. maltophilia* reference strains (including the type stain LMG 958^T and representatives of all the genomic groups described by Hauben et al. [12]), six reference strains of the other *Stenotrophomonas* species (*S. africana* LMG 22072^T, *S. nitritireducens* LMG 22074^T, *S. acidaminiphila* LMG 22073^T, *S. rhizophila* LMG 22075^T and two additional *S. rhizophila* strains) and 87 recent isolates (including 40 isolates recovered from CF patients in Belgium, the UK and the USA) were included in this study. Strains were grown aerobically on tryptone soy agar (Oxoid) and incubated overnight at 28°C.

2.2. RFLP analysis of the gyrB gene

Preparation of the DNA, amplification of the *gyrB* gene using conserved primers UP-1 and UP-2r and restriction digests using *Dpn*II, *Hae*III, *Hpa*II, *Mnl*I, *Nla*IV, *Taq*I and *Tru*9I (New England Biolabs) were performed as described previously [17]. Restriction fragments were separated in 10×15-cm 2.5% agarose gels (LSI) in 1×TBE buffer at 80 V for 110 min. A 50-bp DNA ladder (Amersham Biosciences) was included multiple times on all gels to allow standardisation. Gels were stained with ethidium bromide. Densitometric analysis, normalisation and interpolation of the patterns and numerical analysis using the Dice coefficient were performed using the Bionumerics 2.5 (Applied Maths) software package.

2.3. DNA–DNA hybridisations

High-molecular-mass DNA was prepared as described by Pitcher et al. [18] and DNA–DNA hybridisations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki et al. [19] using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridisation temperature was 50°C. Reciprocal experiments were performed for every pair of strains.

2.4. Statistical analysis

The consistence of the cluster analysis of the *gyrB* analysis was determined by calculating the cophenetic correlation coefficient for each cluster, using Bionumerics 2.5. The cophenetic correlation coefficient is the product-moment correlation between all original matrix similarities and all corresponding similarity values derived from the dendrogram. It provides a measure whether or not the matrix is faithfully represented by a bifurcating tree. Two-tailed unpaired *t*-tests were used to compare DNA–DNA hybridisation values within and between *gyrB* RFLP groups, and were performed using SPSS 11.0.1 (SPSS).

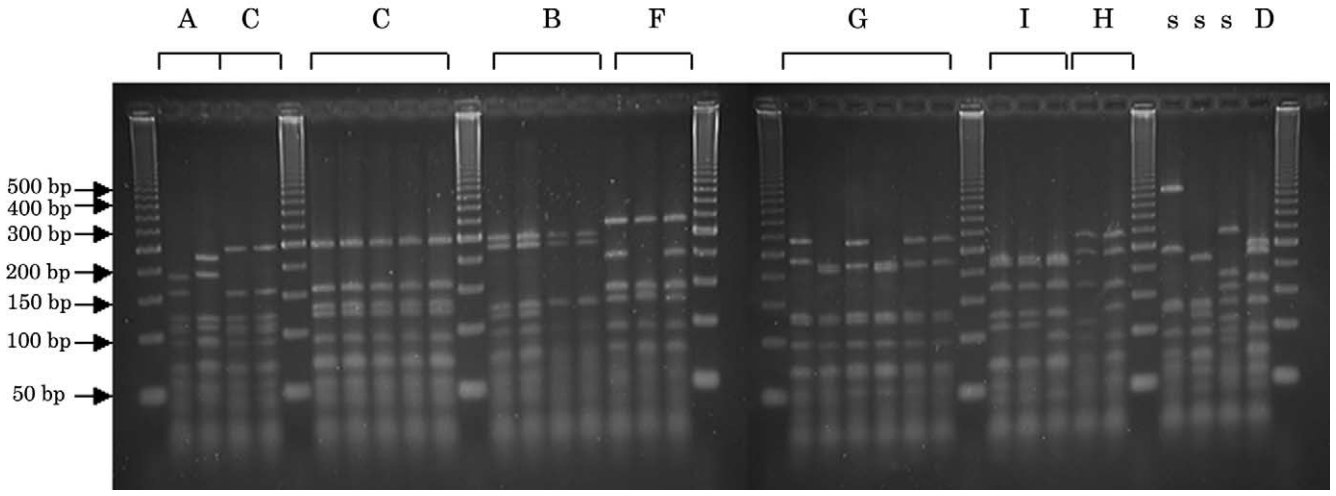

Fig. 1. *Hae*III RFLP analysis of the polymerase chain reaction-amplified *gyrB* gene of selected *Stenotrophomonas* sp. isolates. Cluster names are indicated above patterns. s, separate position in the dendrogram.

3. Results

3.1. RFLP analysis of the gyrB gene

Using primer pair UP-1/UP-2r, the nearly complete (approximately 1200 bp) *gyrB* gene could be amplified from all isolates. We investigated the ability of several restriction enzymes to discriminate amongst *S. maltophilia* isolates. Our experiments revealed that RFLP types generated by digestion with *Hae*III were the most discriminatory among the enzymes tested (data not shown). RFLP analysis with *Hae*III was then performed on all isolates. The resulting RFLP patterns consisted of five to eight bands in the approximate size range of 50–500 bp (Fig. 1). Following numerical analysis, nine clusters (designated A–I) could be delineated, while several strains occupied separate positions in the dendrogram (Fig. 2). The clusters were delineated based on two criteria: (i) internal similarity higher than 75%, and (ii) cophenetic correlation coefficient higher than 70%. The only exception to this was cluster A. This cluster has a cophenetic correlation coefficient of 72% but an internal similarity $<70\%$. Nevertheless, we decided not to split this cluster into subclusters as DNA–DNA hybridisation results indicated that cluster A strains were highly related (see below). The overall cophenetic correlation coefficient was 86%, indicating that the similarity matrix of the *gyrB* RFLP patterns is faithfully represented in the tree. All clusters contained

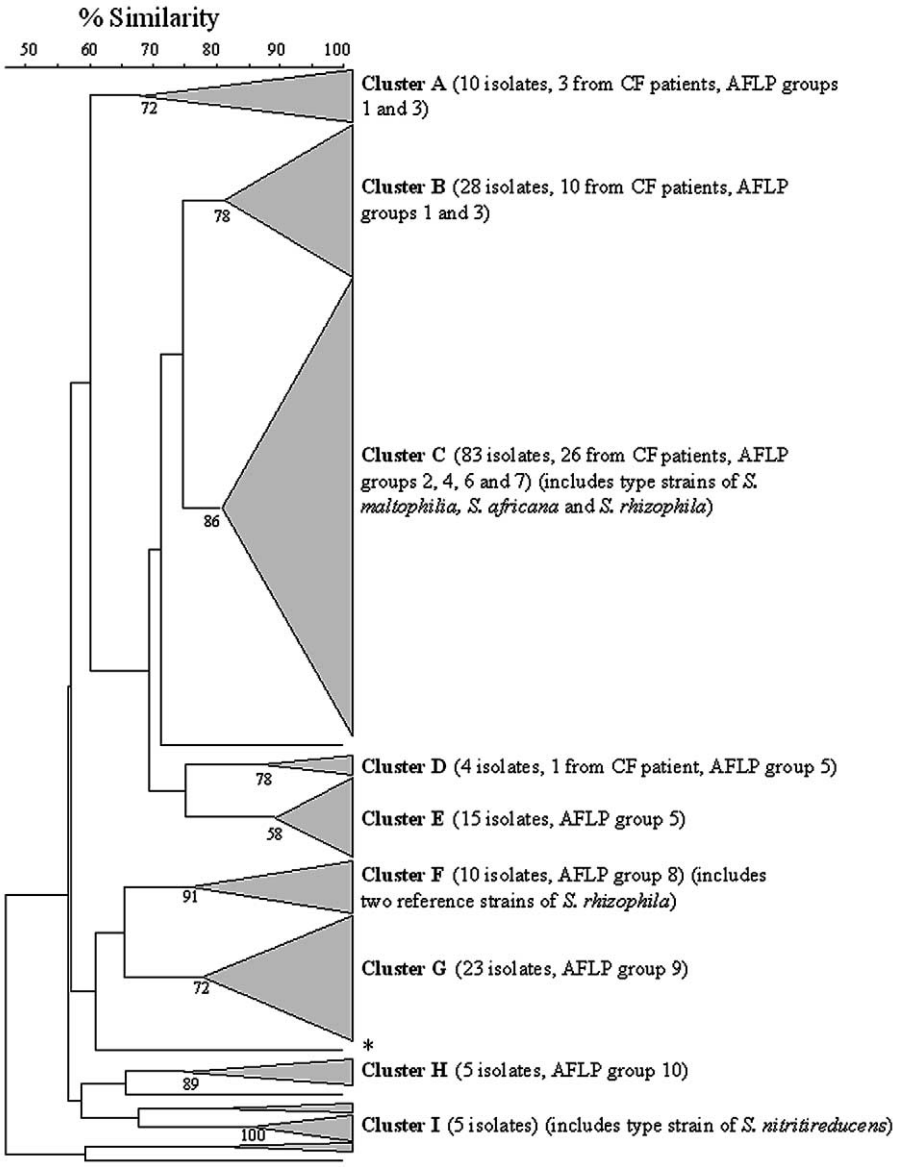

Fig. 2. Dendrogram derived from the UPGMA linkage of Dice coefficients between the *gyrB* RFLP patterns generated by digestion with *Hae*III. Cophenetic correlation coefficients are given for each cluster. The total number of isolates, the number of CF isolates (if present), the representatives of the AFLP groups, and the *Stenotrophomonas* type and reference strains within each of the clusters are given in parentheses. The asterisk indicates the position of the type strain of *S. acidaminiphila*.

both environmental isolates and isolates from clinical origin, except cluster G which only contained environmental isolates. The majority of CF isolates (36/40, 90%) grouped in clusters B and C. Clusters A and D also contained isolates recovered from CF patients (three and one, respectively). No CF isolates were found in clusters E, F, G, H and I. The type strains of *S. maltophilia*, *S. africana* and *S. rhizophila* grouped in cluster C. The patterns of the type strains of *S. maltophilia* and *S. rhizophila* were identical, while the pattern of the type strain of *S. africana* was slightly different (similarity of 87%). Two other *S. rhizophila* strains grouped in cluster F. The type strain of *S. nitritireducens* grouped in cluster I while the type strain of *S. acidaminiphila* occupied a separate position in the dendrogram. Clusters A and B both contain reference isolates from AFLP groups 1 and 3. Cluster C contains all reference isolates from AFLP groups 2, 4, 6 and 7. The AFLP group 5 reference isolates were found in clusters D and E. Clusters F, G and H contain isolates from AFLP groups 8, 9 and 10, respectively. Within clusters that contained isolates from different AFLP groups, there was no subdivision along these AFLP groups. There is no relationship between the geographic origin of the CF isolates and their position in the dendrogram (data not shown).

3.2. DNA–DNA hybridisations

The relationships of the various clusters delineated using *gyrB* RFLP analysis were further investigated by comparing them to previously published and new DNA–DNA hybridisation experiments. The results from these DNA–DNA hybridisation experiments are summarised in Table 1. In general the DNA–DNA binding values confirmed the grouping observed by *gyrB* RFLP. Two-tailed unpaired *t*-test indicated that the average DNA–DNA binding value within the clusters (69.4 ± 9.7%) is significantly higher than the DNA–DNA binding value between the different clusters (34.1 ± 15.3%) ($P < 0.001$). Several DNA–DNA hybridisation experiments previously performed in other studies [12–16] were repeated and the results were generally in good agreement. The only notable exception was observed for two cluster A strains (LMG 10882 and LMG 10883). Hauben et al. reported a DNA–DNA binding value of 6% between both strains [12]; when the experiment was repeated in the present study we obtained a value of 83%. The reason for this discrepancy is at present unclear.

4. Discussion

S. maltophilia isolates are increasingly being recovered from various clinical samples, including the respiratory secretions of CF patients. A previous study has shown that there is a remarkable genomic heterogeneity among *S. maltophilia* isolates, as revealed by AFLP fingerprinting and DNA–DNA hybridisations. The goal of this study was to evaluate the use of RFLP analysis of the *gyrB* gene to distinguish genomic groups within *S. maltophilia* and to assess the genomic diversity of *S. maltophilia* isolates recovered from CF patients.

The results of the *gyrB* RFLP analysis confirmed that there is significant diversity within *S. maltophilia*. Overall there is a good correlation between results previously obtained with AFLP fingerprinting and our results, although fewer groups are discriminated using *gyrB* RFLP analysis. To resolve these apparent differences in grouping, we reanalysed the DNA–DNA hybridisation data reported by Hauben et al. [12] and performed additional DNA–DNA hybridisation experiments (Table 1). Hauben et al. [12] already noted that there are many values in the range of 50–70% and that the differences in DNA–DNA hybridisation levels between AFLP groups and within AFLP groups were not very pronounced. This is corroborated by our findings, but, in contrast to Hauben et al. [12], we found significantly higher average DNA–DNA hybridisation values among representatives of the same *gyrB* RFLP cluster than between representatives of different *gyrB* RFLP clusters. This suggests either that AFLP fingerprinting may be too discriminatory for the separation of genospecies present in *S. maltophilia* or that the cutoff value of 40% used by Hauben et al. [12] to delineate AFLP clusters was too high.

Although *S. maltophilia* is increasingly being recovered from CF patients, its clinical role is still unclear. Our ob-

Table 1
DNA–DNA binding values (%) between the major clusters

	DNA–DNA binding (%) with strains from							
	1	2	3	4	5	6	7	8
1. Cluster A	83 (1)							
2. Cluster B	46.8 ± 26.4 (6)	70.4 ± 21.3 (5)						
3. Cluster C	48.2 ± 14.6 (6)	46.8 ± 13.6 (20)	62.8 ± 13.4 (23)					
4. Cluster D	49.0 ± 10.0 (2)	53.3 ± 7.5 (6)	49.9 ± 5.8 (13)	53.3 ± 14.0 (3)				
5. Cluster E	54.0 ± 2.0 (2)	56.7 ± 3.0 (3)	52.8 ± 5.4 (8)	50.5 ± 11.2 (4)	78.33 ± 10.9 (3)			
6. Cluster F	8.5 ± 3.5 (2)	25.5 ± 10.5 (2)	30.2 ± 10.8 (5)	–	39.0 ± 11.0 (2)	68.5 ± 26.5 (2)		
7. Cluster G	21 (1)	30.7 ± 11.2 (7)	36.3 ± 4.3 (7)	33.0 ± 1.6 (3)	28 (1)	30.2 ± 6.1 (5)	69.5 ± 4.4 (6)	
8. Cluster H	16 (1)	13 (1)	19 (1)	–	17 (1)	19 (1)	12 (1)	–

Average, S.D. and number of experiments (in parentheses) are given. –, no values determined.

servation that the majority of CF isolates group in clusters B and C may indicate that cluster B and C strains have an increased potential for colonisation of the respiratory tract of CF patients. This finding may help further research aiming to elucidate the underlying mechanisms by which *S. maltophilia* colonises the respiratory tract of CF patients.

The exact taxonomic relationships between the species *S. africana*, *S. nitritireducens*, *S. acidaminiphila* and *S. rhizophila* and the genomic groups found in *S. maltophilia* have never been investigated in a systematic way. Our data indicate that *S. africana* may be a subjective synonym of *S. maltophilia* as the type strains of *S. africana* and *S. maltophilia* show high DNA–DNA binding values (70%) and group together in cluster C. The synonymy between *S. maltophilia* and *S. africana* has been reported before [15]. In addition, it appears that *S. rhizophila* is heterogeneous (*S. rhizophila* reference strains are found in clusters C and E) and that cluster I isolates belong to *S. nitritireducens*. Polyphasic-taxonomic studies are required to resolve these issues and to clarify the taxonomic status of isolates belonging to RFLP clusters not containing reference strains. In addition, it would seem wise not to create novel *Stenotrophomonas* species, unless representatives of all genomic groups have been examined. The results from the present study also indicate that taxonomic studies on *Stenotrophomonas* sp. isolates should not only rely on the comparison of type strains.

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