Sequence analysis of domains III and IV of the 23S rRNA gene of verticillate streptomycetes

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Domains III and IV of the 23S rRNA gene of 25 strains of verticillate streptomycetes were sequenced. None of the sequences was identical to any other, with regions of variability being restricted to parts of helices 54 and 64. No relationships were detected between the similarities in the sequence and the assignment to phenetic clusters as defined by the numerical taxonomy studies. Limited agreement was also found between similarity of the sequences and DNA–DNA homology values. However, species (>70% DNA–DNA homology values)-specific diagnostic oligonucleotides generally could be defined, except for *Streptomyces baldacii*. Therefore the determination of the 23S rRNA sequence may be of greater value for fingerprinting individual strains than for taxonomic or identification purposes.

Keywords: *Streptomyces*, *Streptoverticillium*, streptomycetes, 23S rDNA, large-subunit rRNA

INTRODUCTION

Verticillate streptomycetes were grouped by Baldacci (1958) in the genus *Streptoverticillium* with a very short diagnosis. This was enlarged and emended in 1966 (Baldacci *et al.*, 1966) and a full revision of most species belonging to the genus was carried out a few years later (Locci *et al.*, 1969). These were evaluated by numerical methods by Locci and co-workers (Locci *et al.*, 1981; Locci & Schofield, 1989). Probabilistic identification techniques were employed to detect suitable media for their selective isolation (Williams *et al.*, 1985; Cardinali *et al.*, 1989).

Witt & Stackebrandt (1990) proposed the unification of the genera *Streptomyces* and *Streptoverticillium* on the basis of partial sequence- and hybridization analyses of the rRNA of the species previously described as *Streptoverticillium* by Locci & Schofield (1989). More recently, Labeda (1996), on the basis of DNA relatedness, grouped 35 strains of verticillate streptomycetes into 20 clusters, defined at a 70% DNA–DNA homology level. Despite their heterogeneity with regard to DNA relatedness, Stackebrandt *et al.* (1991) reported highly similar 16S rRNA sequences for the

verticillate streptomycetes examined and the failure to develop species-specific oligonucleotide probes useful for their differentiation.

In this paper, we report the sequence analysis of the 23S rRNA of verticillate *Streptomyces* strains, encompassing almost all the phenetic clusters defined by Locci *et al.* (1981), with the aim of assessing the congruity of this molecular marker with the recent systematics of the group and defining species- or group-specific signatures or probe sequences for ecological studies.

METHODS

Strains and cultivation conditions. The strains included in this study are listed in Table 1. They represent 9 out of the 10 major phenotypic clusters of Locci & Schofield (1989). *Streptomyces sapporensis* NCB 261^T, a strain which remained unclustered in the numerical study of Locci *et al.* (1981), '*Streptomyces kitasatoensis*' NCB 846 (which had not been included) and two verticillate streptomycetes (strains NCB 821 and NCB 830) provided by Dr R. W. Attwell, University of Manchester, UK and identified as belonging to cluster III (*S. griseocarneus*) by Cardinali *et al.* (1989) using probabilistic methods (Williams *et al.*, 1985) were also included.

Freeze-dried cultures were rehydrated in nutrient broth (Difco) and then transferred to nutrient agar and Czapek agar (Difco) for morphological examination and to 5 ml nutrient broth for mycelial production. After 10 d, the mycelia were harvested, washed twice in TE (10 mM Tris/HCl, 1 mM EDTA, pH 8) and freeze-dried. The nucleic

Abbreviation: NCB, National Collection of Bacteria, formerly the DPDU, the collection of the Dipartimento di Biologia Applicata alla Difesa delle Piante, Università di Udine, Italy.

The EMBL accession numbers for the 23S rDNA sequences determined in this work are AJ246236–AJ246260 (see Table 1).

Table 1. Strains used in this work

Cluster group*	Species	Strain	Accession no.†
I	<i>Streptomyces roseovorticillatus</i>	NCB 819 ^T = NRRL B-3500 ^T = ATCC 23654 ^T = DSM 40845 ^T = IFO 14693 ^T = JCM 4272 ^T	AJ246254
	<i>Streptomyces netropsis</i>	NCB 70 = NRRL 2886 = DSM 40846 = NCIB 8936 = JCM 4544	AJ246238
	<i>Streptomyces roseovorticillatus</i>	NCB 269 = NRRL 2755 = ATCC 27429 = CBS 644.72 = DSM 40086 = IFO 13343 = JCM 4310 = JCM 4750	AJ246250
	<i>Streptomyces netropsis</i>	NCB 90 = NRRL 2820 = ATCC 19756 = CBS 494.68 = DSM 40093 = IFO 12769 = JCM 4307 = JCM 4370	AJ246259
	<i>Streptomyces griseovorticillatus</i>	NCB 65 ^T = NRRL B-12432 ^T = ATCC 27436 ^T = CBS 721.72 ^T = DSM 40507 ^T = IFO 13420 ^T = RIA1381 ^T = JCM 4202 ^T = JCM 4767 ^T	AJ246237
	<i>Streptomyces netropsis</i>	NCB 749 = NRRL B-1831 = ATCC 12691 = ATCC 23926 = CBS 910.68 = DSM 40052 = IFO 12880 = JCM 4153 = JCM 4647 = NCIB 9624	AJ246260
	<i>Streptomyces netropsis</i>	NCB 266 ^T = NRRL 2268 ^T = ATCC 23940 ^T = CBS 924.68 ^T = DSM 40259 ^T = IFO 12893 ^T = JCM 4063 ^T = JCM 4655 ^T	AJ246251
	<i>Streptomyces rubrovorticillatus</i>	NCB 124 = JCM 4679 = NRRL B-16433	AJ246246
	<i>Streptomyces cinnamoneus</i> subsp. <i>cinnamoneus</i>	NCB 93 ^T = NRRL B-1285 ^T = ATCC 11874 ^T = ATCC 23897 ^T = CBS 293.64 ^T = CBS 683.68 ^T = DSM 40005 ^T = IFO 12852 ^T = JCM 4152 ^T = JCM 4633 ^T = NCIB 8851 ^T	AJ246248
	<i>Streptomyces blastomyceticus</i>	NCB 77 ^T = NRRL B-5480 ^T = ATCC 19731 ^T = CBS 470.68 ^T = DSM 40029 ^T = IFO 12747 ^T = JCM 4184 ^T = JCM 4565 ^T = NCIB 9800 ^T	AJ246255
II	<i>Streptomyces eurocidicus</i>	NCB 264 ^T = NRRL B-1676 ^T = ATCC 27428 ^T = CBS 792.72 ^T = DSM 40604 ^T = IFO 13491 ^T = JCM 4029 ^T = JCM 4749 ^T	AJ246247
	<i>Streptomyces arduus</i>	NCB 99 ^T = NRRL 2817 ^T = ATCC 27417 ^T = CBS 731.72 ^T = DSM 40527 ^T = IFO 13430 ^T = JCM 4543 ^T = JCM 4722 ^T	AJ246256
	<i>Streptomyces cinnamoneus</i> subsp. <i>cinnamoneus</i>	NCB 67 ^T = NRRL B-1699 ^T = ATCC 12686 ^T = CBS 369.58 ^T = IFO 12363 ^T = JCM 4435 ^T	AJ246236
III	<i>Streptomyces mashuensis</i>	NCB 258 ^T = NRRL B-8164 ^T = ATCC 23934 ^T = CBS 279.65 ^T = CBS 918.68 ^T = DSM 40221 ^T = IFO 12888 ^T = JCM 4059 ^T = JCM 4650 ^T	AJ246241
	<i>Streptomyces pentaticus</i> subsp. <i>jenensis</i>	NCB 82 ^T = NRRL B-12088 ^T = DSM 40848 ^T = JCM 4211 ^T	AJ246240
	<i>Streptomyces</i> sp.	NCB 821	AJ246245
	<i>Streptomyces</i> sp.	NCB 830	AJ246249
IV	<i>Streptomyces hachijoensis</i>	NCB 128 ^T = NRRL B-3106 ^T = ATCC 19769 ^T = CBS 507.68 ^T = DSM 40114 ^T = IFO 12782 ^T = JCM 4331 ^T = JCM 4583 ^T	AJ246243
V	<i>Streptomyces salmonis</i>	NCB 98 ^T = NRRL B-1472 ^T = DSM 40895 ^T = IFO 15865 ^T = JCM 4083 ^T	AJ246242
VI	<i>Streptomyces verticillium</i>	NCB 849 = ATCC 15003 = JCM 4924	AJ246253
VII	<i>Streptomyces luteoreticuli</i>	NCB 81 = NRRL B-12435 ^T = ATCC 27446 ^T = CBS 723.72 ^T = DSM 40509 ^T = IFO 13422 ^T = JCM 4788 ^T	AJ246258
IX	<i>Streptomyces abikoensis</i>	NCB 260 = NRRL B-2113 ^T = ATCC 12766 ^T = CBS 487.62 ^T = DSM 40831 ^T = IFO 13860 ^T = JCM 4002 ^T	AJ246239
X	<i>Streptomyces olivoreticuli</i> subsp. <i>cellulophilus</i>	NCB 278 ^T = ATCC 21632 ^T = DSM 41687 ^T	AJ246252
Single-member clusters	<i>Streptomyces sapporensis</i>	NCB 261 ^T = ATCC 12766 ^T	AJ246244
Unclassified	<i>Streptomyces kitasatoensis</i>	NCB 846	AJ246257

* According to Locci & Schofield (1989).

† EMBL accession numbers of the 23S rDNA sequences obtained in this work.

acids were extracted from freeze-dried mycelia using the Doyle & Doyle (1990) protocol.

PCR amplification and sequencing. The nucleic acids were used as the target in standard PCR reactions with the primer pair 23SH54F (5'-AAGCGAGTTGTGGACGGTT-3') and 23SH54R (5'-ATTCGTGCAGGTCGGAAC-3'). These primers were devised from the alignment of complete 23S rRNA sequences of streptomycetes available from public databases (M27245, X61478, U39437 and X62884). Their annealing sites correspond to positions 1337–1345 and 2174–1292, respectively, in the sequence with accession number X61478 (*Streptomyces griseus* subsp. *griseus* 23S rRNA sequence). The amplification products were gel-purified and cycle-sequenced using the internal sequencing primers H54FSQ2 (5'-TCACGAGTGAGAATGCAG-3') and H54RSQ4 (5'-TTCGCTACCTTAGGATGG-3').

Sequence analysis. The sequences obtained from both ends in all strains were aligned using the CLUSTAL W package (Thompson *et al.*, 1994) and edited with the SEQPUP program (Gilbert, 1996).

RESULTS

Domains III and IV of the 23S rRNA gene of 25 strains of verticillate streptomycetes were sequenced and 650 nucleotides (nt), corresponding to positions

1414–2064 of accession number X61478, from each strain were aligned.

None of the sequences was identical to any other, the number of differences ranging from 1 (*S. sapporensis* NCB 261^T vs. either *Streptomyces griseovorticillatus* NCB 65^T or *Streptomyces* sp. NCB 821) to 69 (*Streptomyces luteoreticuli* NCB 81^T vs. *Streptomyces kentuckensis* NCB 749^T). The regions of variability were mostly restricted to parts of helices 54 (positions 1564–1613 in X61478) and 64 (positions 1924–1949 in X61478).

No relationships were detected between the number of differences in the sequences and the assignment to phenetic clusters by the numerical taxonomy study of Locci *et al.* (1981). For instance, the three strains mentioned above with only one nucleotide difference did not share the same cluster, i.e. *S. griseovorticillatus* NCB 65^T was assigned to cluster I, *S. sapporensis* NCB 261^T remained unclustered and *Streptomyces* sp. NCB 821 was identified by probabilistic methods as a member of cluster III by Cardinali *et al.* (1989). In general, the strains assigned by Locci *et al.* (1981) to the same cluster did not share higher sequence simi-

Table 2. Pairwise comparison of partial sequences of the 23S rDNA

The figures in the lower-left triangle refer to the number of nucleotides that differ between the two sequences compared. The DNA relatedness (%) obtained by Labeda (1996) is reported in the upper-right triangle for reference. The numbers indicate the following *Streptomyces* strains: 1, *S. salmonis* NCB 98^T; 2, *S. fervens* NCB 269^T; 3, *S. olivoreticuli* subsp. *cellulophilus* NCB 278; 4, *S. hachijoensis* NCB 128^T; 5, *S. eurocidicus* NCB 264^T; 6, *S. ardens* NCB 99^T; 7, '*S. rubroverticillatus*' NCB 124; 8, *S. blastomyceticus* NCB 77^T; 9, *S. cinnamoneus* subsp. *cinnamoneus* NCB 67; 10, *Streptomyces* sp. NCB 821; 11, *S. griseoverticillatus* NCB 65^T; 12, *S. sapporensis* NCB 261^T; 13, *S. cinnamoneus* subsp. *cinnamoneus* NCB 93^T; 14, *S. netropsis* NCB 70; 15, *S. netropsis* NCB 266^T; 16, *S. netropsis* NCB 90; 17, *S. abikoensis* NCB 260; 18, '*S. kisatoensis*' NCB 846; 19, '*S. pentaticus* subsp. *jenensis*' NCB 82; 20, *S. netropsis* NCB 749; 21, *S. mashuensis* NCB 258^T; 22, '*S. verticillium*' NCB 849; 23, *Streptomyces* sp. NCB 830; 24, *S. roseoverticillatus* NCB 819^T; 25, '*S. luteoreticuli*' NCB 81^T; 26, *S. griseus* subsp. *griseus* [sequence X61478; see Kim *et al.* (1991) for details]. ND, Not determined.

	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
1		36	ND*	22	28	30	24	20	21	ND	19	ND	34	22	39	10	16	ND	47	25	22	ND	ND	52	42	ND
2	5		ND	14	28	35	35	18	15	ND	0	ND	51	7	39	51	0	ND	67	32	10	ND	ND	81	0	ND
3	13	14			ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	9	10	15		20	33	29	35	81	ND	38	ND	56	27	28	0	49	ND	8	50	39	ND	ND	26	31	ND
5	7	10	16	10		23	22	41	28	ND	2	ND	27	29	9	19	0	ND	22	20	9	ND	ND	11	39	ND
6	17	19	26	21	20		42	55	5	ND	0	ND	24	11	12	24	0	ND	27	17	26	ND	ND	34	12	ND
7	15	15	22	16	16	24		19	26	ND	24	ND	49	40	28	11	81	ND	11	14	29	ND	ND	24	35	ND
8	13	11	20	16	14	22	10		49	ND	24	ND	28	23	23	27	0	ND	30	16	12	ND	ND	25	0	ND
9	16	15	21	7	15	28	13	10		ND	26	ND	50	14	23	43	21	ND	18	32	17	ND	ND	23	31	ND
10	17	17	22	15	14	28	20	12	9		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	20	20	24	15	17	29	23	14	9	2		ND	48	25	31	30	10	ND	0	11	25	ND	ND	10	19	ND
12	19	19	23	14	16	29	22	14	8	1	1		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
13	20	20	25	17	19	33	15	19	12	19	20	19		35	56	10	ND	ND	0	27	35	ND	ND	28	57	ND
14	28	28	32	24	26	29	23	22	18	18	19	19	22		70	61	0	ND	40	73	15	ND	ND	0	27	ND
15	25	26	30	20	24	33	18	19	14	20	20	20	17	16		86	6	ND	14	70	16	ND	ND	36	34	ND
16	30	31	35	25	29	36	25	26	21	26	26	26	24	20	5		0	ND	0	99	28	ND	ND	17	30	ND
17	54	50	56	57	56	49	54	50	53	52	52	52	47	47	54	56		ND	0	8	0	ND	ND	0	0	ND
18	33	34	41	36	29	33	43	39	40	40	41	41	45	39	46	47	38		ND	ND	ND	ND	ND	ND	ND	ND
19	22	23	25	25	21	32	32	29	29	28	28	27	32	38	36	39	42	23		11	10	ND	ND	71	19	ND
20	33	35	41	34	33	42	31	29	28	28	29	29	34	26	20	23	44	30	24		9	ND	ND	29	2	ND
21	27	27	35	28	23	39	27	23	22	20	22	21	31	31	31	35	46	24	15	12		ND	ND	15	26	ND
22	36	36	43	34	33	44	36	31	30	25	25	24	39	40	41	43	44	35	19	24	15		ND	ND	ND	ND
23	15	15	23	13	14	26	24	20	18	15	16	15	21	32	30	35	49	42	29	41	34	26		ND	ND	ND
24	21	21	31	21	23	30	18	15	16	16	17	16	24	31	29	35	50	50	32	41	32	22	13		15	ND
25	53	52	57	55	53	56	53	50	53	50	52	51	57	54	60	66	63	68	59	69	61	59	48	47		ND
26	39	37	44	40	35	47	35	34	36	35	37	36	35	47	41	47	47	52	37	44	40	39	34	30	58	

larity among themselves than that shown with strains belonging to other clusters (see Table 2).

Limited correspondence was found between the similarity of the sequences and the DNA–DNA homology values reported by Labeda (1996; see Table 2). On the one hand, both high DNA–DNA homology values and low numbers of sequence differences were detected in the comparisons between *Streptomyces cinnamoneus* subsp. *cinnamoneus* NCB 67^T and *Streptomyces hachijoensis* NCB 128 and between *Streptomyces netropsis* NCB 90 and *S. netropsis* NCB 266^T. On the other hand, in several instances strains shown to share >70% DNA–DNA homology revealed a significant number of sequence differences (*S. netropsis* NCB 749 vs. *Streptomyces distallicus* NCB 70^T, *S. netropsis* NCB 266^T and *S. netropsis* NCB 90; *Streptomyces abikoensis* NCB 260^T vs. *Streptomyces rubroverticillatus* NCB 124; *Streptomyces roseoverticillatus* NCB

819^T vs. *S. roseoverticillatus* NCB 269) and many strains with >98% identical sequences (1–13 different nucleotides) were not found to be related on the basis of DNA–DNA reassociation values (*Streptomyces salmonis* NCB 98, *S. roseoverticillatus* NCB 269, *S. hachijoensis* NCB 128^T and *Streptomyces eurocidicus* NCB 264^T among themselves; *S. griseoverticillatus* NCB 65^T, *S. sapporensis* NCB 261^T and *Streptomyces* sp. NCB 821 among themselves; *Streptomyces blastomyceticus* NCB 77^T, '*S. rubroverticillatus*' NCB 124 and *S. cinnamoneus* subsp. *cinnamoneus* NCB 67^T among themselves).

However, the search for oligonucleotide signatures for the taxon genospecies as defined by Labeda (1996) on the basis of DNA–DNA homology values was successful in most cases. Specific consensus sequences could be detected for most strains included in this work, which clustered at a homology level >70% in

the study of Labeda (1996), as follows. (i) *S. hachijoensis* NCB 128^T and *S. cinnamoneus* subsp. *cinnamoneus* NCB 67^T, a subjective synonym for *S. hachijoensis* according to Labeda (1996), share the oligonucleotide sequence GCCTTCGGGCA (nt positions corresponding to 1574–1585 in X61478). (ii) *S. netropsis* NCB 266^T, *S. netropsis* NCB 90 and *S. netropsis* NCB 749, subjective synonyms for *S. netropsis* according to Labeda (1996), share the oligonucleotide sequence GTGACCCGG (nt positions corresponding to 1606–1614 in X61478). (iii) *S. abikoensis* NCB 260^T and '*S. rubroverticillatus*' NCB 124, subjective synonyms for *S. abikoensis* according to Labeda (1996), share the oligonucleotide sequence TTCYGGTGATSGGAT (nt positions corresponding to 1926–1940 in X61478).

Conversely, no diagnostic oligonucleotides could be found in the sequence of the related strains *S. roseoverticillatus* NCB 819, '*Streptomyces pentaticus* subsp. *jenensis*' NCB 82 and *Streptomyces fervens* NCB 269^T which belonged to the *S. roseoverticillatus* DNA homology cluster.

DISCUSSION

Undoubtedly, the agreement of the results obtained using different approaches in clarifying the systematics of verticillate streptomycetes (in particular, numerical taxonomy versus nucleic acid techniques and also among the latter) is anything but comforting. Nevertheless, the organisms emerge as quite distinct from the other streptomycete species (see Fig. 2 in Witt & Stackebrandt, 1990), they are immediately identifiable by the morphology of their spore-bearing apparatus and by their spore ultrastructure, and they all possess the physiological trait of lysozyme resistance as stated by Attwell *et al.* (1985). Therefore the proposed union of *Streptoverticillium* and *Streptomyces* is still under discussion. Phylogenesis is not always synonymous with classification and the merging of genera simply defers the problem of species identification. At this stage, however, the point is not a purely taxonomic one, but also relates to the finding of suitable means of detecting the organisms in their natural habitats.

One of the interesting results of this and previous studies is the lack of identity between the various species. This should not come as a surprise. The composition of the streptoverticillia is definitely an artificial one. A look at their origins is quite enlightening: most have been isolated in industrial institutions, many produce antimicrobial metabolites and some have been patented. Particularly in the past, the screening practice was simply to discard 'uninteresting' streptoverticillia without bothering to describe unproductive new species. This has led to a completely biased composition of the genus. In fact, most species consist of just a single isolate.

When looking for species-specific oligonucleotide probes for the streptomycetes, Stackebrandt *et al.* (1991) found that 23 out of 31 verticillate strains were

identical in the 16S rRNA variable regions named alpha and beta. Their analysis of the highly variable region named gamma (nt positions 150–200 according to the nomenclature of *Streptomyces ambofaciens*; Pernodet *et al.*, 1989) revealed sequence differences only among some of the verticillate streptomycetes examined. Therefore the authors suggested helix 54 of the 23S rRNA as a more suitable region for the definition of species-specific oligonucleotide probes, supporting this suggestion with the analysis of 119 nt sequences from some streptomycetes, including two related verticillate strains.

Helix 54 appeared to be highly suitable for the development of oligonucleotide probes for actinomycetes since it has been shown to include a relatively long insertion, common only to Gram-positive bacteria with a high DNA G+C content (Roller *et al.*, 1992).

The high variability and suitability for probe definition of helix 54 of the 23S rRNA has since been confirmed by several other studies. Our results show that the variability within verticillate streptomycetes is high even among members of the same taxon genospecies, as defined by DNA–DNA homology studies. Therefore the determination of the 23S rRNA sequence may be of greater value for fingerprinting strains than for taxonomic purposes. In any case, oligonucleotide signatures, of use in defining group- or species-specific oligonucleotide probes, could be defined. The probes, however, should be tested empirically.

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