The search for synonyms among streptomycetes by using SDS-PAGE of whole-cell proteins. Emendation of the species *Streptomyces aurantiacus*, *Streptomyces cacaoi* subsp. *cacaoi*, *Streptomyces caeruleus* and *Streptomyces violaceus*

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A collection of 93 *Streptomyces* reference strains were investigated using SDS-PAGE of whole-cell proteins. Computer-assisted numerical analysis revealed 24 clusters encompassing strains with very similar protein profiles. Five of them grouped several type strains with visually identical patterns. DNA–DNA hybridizations revealed homology values higher than 70% among these type strains. According to the current species concept, it is proposed that *Streptomyces albosporeus* subsp. *albosporeus* LMG 19403T is considered as a subjective synonym of *Streptomyces aurantiacus* LMG 19358T, that *Streptomyces aminophilus* LMG 19319T is considered as a subjective synonym of *Streptomyces cacaoi* subsp. *cacaoi* LMG 19320T, that *Streptomyces niveus* LMG 19395T and *Streptomyces spheroides* LMG 19392T are considered as subjective synonyms of *Streptomyces caeruleus* LMG 19399T, and that *Streptomyces violatus* LMG 19397T is considered as a subjective synonym of *Streptomyces violaceus* LMG 19360T.

**Keywords:** *Streptomyces*, SDS-PAGE of whole-cell proteins, synonyms, taxonomy

**INTRODUCTION**

At the introduction of the genus in 1943 (Waksman & Henrici, 1943) classifications were based solely on morphology and pigmentation. The lack of standardized procedures for describing streptomycetes, together with the unavailability of type strains, led to species which were poorly described. Moreover, antibiotics producing streptomycetes were described on the basis of trivial differences in morphology, for patent purposes. This unsound classification system has been improved by the International *Streptomyces* Project (ISP) in 1964. Standard procedures for describing streptomycetes were developed and applied to 463 *Streptomyces* and (former) *Streptovverticillium* species. Type strains became publicly available when they were deposited in four collections.

No attempts were undertaken by the ISP to determine synonyms among morphologically defined species. With the introduction of the principles of numerical taxonomy by Locci *et al.* (1981) and Williams *et al.* (1983), another view on the taxonomy of this complex genus emerged. Numerical taxonomy of phenotypic features without heavy emphasis on morphology and pigmentation allowed a more reliable classification than those previously defined. The taxonomic study of Williams *et al.* (1983) is therefore considered to be a milestone in *Streptomyces* systematics. An extensive species reduction was proposed, and probability matrices were created to identify isolates (Williams *et al.*, 1989). Kämper & Kroppenstedt (1991) updated the classification system of Williams *et al.* (1983) by extending the numbers of phenotypic traits and by including more taxa.

*Streptomyces* systematics is gradually evolving from a phenotypically dominated taxonomy to a more poly-
Fig. 1. Dendrogram derived from an unweighted pair group method with arithmetic averages (UPGMA) algorithm of $r$ values (expressed as percentages) of protein profiles. Clusters are delineated at a similarity level of 90%. Symbols: *, cultivated for 48 h; †, growth temperature 48 °C.
phasic taxonomy comprising chemotaxonomic and genomic information. The large number of validly described *Streptomyces* species (more than 500) remains a major obstacle in the setting up of large fundamental taxonomic studies, including DNA–DNA hybridizations, and in establishing the link between phenotypic and genomic information. Furthermore, because of the lack of minimal standards for defining *Streptomyces* species (Manfio et al., 1994), the polyphasic approach remains difficult.

The aim of this study was to screen for synonymous taxa in a representative subset of the genus *Streptomyces*, using SDS-PAGE of whole-cell proteins, and to compare the data with those of Williams et al. (1989) and Kämpfer & Kroppenstedt (1991). It has been shown that under highly standardized cultivation conditions, SDS-PAGE of whole-cell proteins is an excellent and generally applicable method for defining relationships at the species level (Kersters, 1985; Manchester, 1989; Williams et al., 1989). Kafer & Kroppenstedt (1990), with some modifications as indicated above. Manchester et al. (1990) has already demonstrated the usefulness of SDS-PAGE of whole-cell proteins in streptomycete systematics.

METHODS

Bacterial strains and cultivation conditions. The strains used in this study can be found in Fig. 1.

All strains were grown on Bennett’s agar slopes (using 0.01 M phosphate buffer, pH 7.0) and incubated for 24 h at 28 °C unless indicated otherwise. Mycelial suspensions were prepared in 2 ml 0.1 M phosphate buffer (pH 7.0) and added to 250 ml flasks containing 80 ml of Bennett’s broth (0.01 M phosphate buffer, pH 7.0). The flasks were shaken for 24 h at 28 °C unless indicated otherwise. The same cultivation method was used for preparing protein or DNA extracts.

To determine the influence of incubation time on the resolution of protein profiles, protein extracts were prepared from *Streptomyces griseus* subsp. *griseus* LMG 5974 and *Streptomyces rimosus* LMG 5984T after 18, 36 and 51 h. Furthermore, the influence of the cultivation method (agar versus broth) was investigated.

SDS-PAGE of whole-cell proteins. Protein extracts were prepared according to the protocol of Manchester et al. (1990), with some modifications as indicated above. SDS-PAGE of the whole-cell proteins, scanning, and normalization of electrophoretic patterns were performed as described by Pot et al. (1994). The similarity between all traces was calculated using the Pearson product moment correlation coefficient with the software package GELCOMPAR version 4.2 (Applied Maths). Out of the obtained similarity matrix, a dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) algorithm.

DNA–DNA hybridizations. DNA of 250–500 mg cells was prepared using the method of Pitcher et al. (1989) supplemented with a lysozyme (Serva) step (10 mg ml⁻¹ in 1 x TE buffer). DNA was finally dissolved in 0.1 x SSC. Only high-molecular-mass DNA was retained for further applications. Microplate hybridizations were performed using the microplate method described by Ezaki et al. (1989), using a model FL-2575 microplate reader (Towa Scientific) for the fluorescence measurements and black Maxisorp FluoroNunc microplates (Nunc A/S). Biotinylated probe DNA was sheared by ultrasonication (30 s, duty cycle 50, output control 1) using a Branson Sonifier (model 250) equipped with microcup horn and was then hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 52 °C in a hybridization solution containing 50% formamide. Salmon-sperm DNA was used as the negative control in all experiments.

**RESULTS AND DISCUSSION**

**SDS-PAGE of whole-cell proteins**

Before routine application of SDS-PAGE of whole-cell proteins, the method was optimized using *Streptomyces* LMG 5974 and LMG 5984T. The extent to which factors such as cultivation time and medium (agar versus broth) influence the resolution of protein profiles was investigated. Our results indicated that high-resolution and reproducible protein patterns were obtained only from cultures grown in liquid medium, whereas cultures grown on solid media yielded a high background (due to proteinase activity) and consequently gave a lower resolution. It was also demonstrated that the growth phase of cells in liquid medium should preferably be within the exponential phase, which means approx. 36 h for fast-growing streptomycetes. Protein patterns of strains harvested at the early stationary phase (51 h), in conditions analogous to those used in the study of Manchester et al. (1990), clearly had a lower resolution. Duplicate protein extracts were prepared in order to verify the reproducibility of the growth conditions and preparation of extracts. A correlation value above 92% was observed between duplicate protein patterns loaded onto separate gels.

Using the above-mentioned growth conditions, protein patterns were generated from 93 *Streptomyces* strains (see Fig. 1) belonging to different phenotypically defined clusters. After processing of the data, a cluster analysis was performed (Fig. 1). A 90% similarity level was used as guideline for delineating 24 clusters (Fig. 1) encompassing strains with very similar protein profiles. A correlation level (r) of 0.90 or higher in protein patterns of bacterial strains has often been used as an indication of possible relatedness at the species level (Kersters, 1985; Manchester et al., 1990).
values are means within cluster 1, the DNA–DNA hybridization results among strains in these clusters. Synonymous names might be found among the species. Experiments were performed to investigate whether or absence of bands. DNA–DNA hybridization patterns (there were minor differences in the presence or absence of bands). DNA–DNA hybridization experiments were performed to investigate whether synonymous names might be found among the species in these clusters. The DNA–DNA hybridization results among strains of the five selected clusters (sharing identical patterns) is presented in Table 1. Within cluster 1, Streptomyces albosporous subsp. albosporous LMG 19403T and Streptomyces aurantiacus LMG 19358T share a DNA homology of 74%. Within cluster 5, Streptomyces cacaoi subsp. cacaoi LMG 19320T and Streptomyces amnophilus LMG 19319T are 100% related. Within cluster 8, Streptomyces endus LMG 19393T shows a relatedness of 92% (data from Labeda & Lyons, 1991) with respect to Streptomyces hygroscopicus subsp. hygroscopicus LMG 19335T. Within cluster 12, Streptomyces violaceus LMG 19360T and Streptomyces violatus LMG 19397T show a DNA relatedness of 90%. Within cluster 19, Streptomyces spheroides LMG 19392T shares DNA relatedness of 77% and 84%, respectively, with Streptomyces niveus LMG 19395T and Streptomyces caeruleus LMG 19399T. S. caeruleus LMG 19399T is highly related to S. niveus LMG 19395T, as they show a DNA homology of 100%. These data confirm that streptomycetes possessing

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DNA–DNA hybridization experiments

In the present study, delineated clusters contain not strains of a single species, but several type strains of different validly described Streptomyces species. Five clusters (1, 5, 8, 12 and 19; Fig. 2) contain type strains with identical profiles (there were only small differences in the intensities of bands). Other type strains grouping in the respective clusters, and all members of the other clusters, show highly similar (though not identical) patterns (there were minor differences in the presence or absence of bands). DNA–DNA hybridization experiments were performed to investigate whether synonymous names might be found among the species in these clusters. The DNA–DNA hybridization results among strains of the five selected clusters (sharing identical patterns) is presented in Table 1. Within cluster 1, Streptomyces albosporous subsp. albosporous LMG 19403T and Streptomyces aurantiacus LMG 19358T share a DNA homology of 74%. Within cluster 5, Streptomyces cacaoi subsp. cacaoi LMG 19320T and Streptomyces amnophilus LMG 19319T are 100% related. Within cluster 8, Streptomyces endus LMG 19393T shows a relatedness of 92% (data from Labeda & Lyons, 1991) with respect to Streptomyces hygroscopicus subsp. hygroscopicus LMG 19335T. Within cluster 12, Streptomyces violaceus LMG 19360T and Streptomyces violatus LMG 19397T show a DNA relatedness of 90%. Within cluster 19, Streptomyces spheroides LMG 19392T shares DNA relatedness of 77% and 84%, respectively, with Streptomyces niveus LMG 19395T and Streptomyces caeruleus LMG 19399T. S. caeruleus LMG 19399T is highly related to S. niveus LMG 19395T, as they show a DNA homology of 100%. These data confirm that streptomycetes possessing

**Table 1. DNA hybridization between several Streptomyces taxa**


**Fig. 2.** All strains found in this study possessing visually identical protein profiles, except for cluster 22, encompassing strains with similar protein profiles. A correlation is made with the numerical phenotypic studies of Williams *et al.* (1989) and Kämpfer & Kroppenstedt (1991). Molecular mass markers (from left to right), in kDa: β-galactosidase (116), bovine albumin (66), egg albumin (45), glyceraldehyde-3-phosphate dehydrogenase (36), carbonic anhydrase (29), trypsin inhibitor (21-1) and lysozyme (14-2).
visually identical protein patterns are genotypically more than 70% related and represent the same genomic species.

In a second step, we investigated the level of DNA homology among the strains showing minor differences in protein profiles (r > 0.90, but differentiated by the presence or the absence of a few bands). Three strains belonging to cluster 22 (r ≥ 0.93), i.e. *S. anulatus* LMG 19301^T^, *S. griseus* subsp. *griseus* LMG 19302^T^ and *S. microflavus* LMG 19327^T^, were studied. As presented in Table 1, *S. anulatus* LMG 19301^T^ shows genotypic relatedness of 61 and 45%, respectively, to *S. griseus* subsp. *griseus* LMG 19302^T^ and *S. microflavus* LMG 19327^T^, *S. griseus* subsp. *griseus* LMG 19302^T^ and *S. microflavus* LMG 19327^T^ show a genotypic relatedness of 43%. These intermediate DNA homology values confirm that strains possessing highly similar protein patterns but showing minor qualitative differences may share DNA homologies below 70% and may be considered as distinct taxa. Numerical analysis of the SDS-PAGE patterns of soluble proteins seems to be a perfect method for screening for highly related strains. Visual identity is indispensable if the relationship at the species level is being determined.

A good correlation exists between the data for SDS-PAGE of whole-cell proteins (the present study) and published DNA-hybridization data (e.g. Labeda & Lyons, 1991; Kim et al., 1999). In the study of Labeda & Lyons (1991), it was proposed that *S. endus* be considered as a subjective synonym of *S. hygroscopicus*. Both strains have identical protein profiles (cluster 8; Fig. 2), confirming their conclusions. In the same study, it was observed that the phenotypically similar species *Streptomyces violaceusniger* and *S. hygroscopicus* show an intermediate DNA hybridization level of 42%. In our study, both strains shared highly similar (r = 0.89), though not identical, protein profiles. Kim et al. (1999) recently clarified the taxonomic status of thermoduric streptomycetes. For instance, it was shown that *Streptomyces thermoviolaceus* and *Streptomyces thermovulgaris* are distinct taxa, as they share a low DNA hybridization value of 10%. This low value is confirmed by different protein patterns (r = 0.83).

**Comparison with phenotypic data**

A low correlation is observed between the protein profiles and the numerical phenotypic data provided by Williams et al. (1989) and Kämpfer & Kroppenstedt (1991). We expected that taxa sharing high DNA–DNA hybridization values would group within the same phenotypic cluster. Only for the type strains of *S. aminophilus*, LMG 19319^T^, and *S. cacaoi* subsp. *cacaoi*, LMG 19320^T^, do the observed high relatedness (100%, Table 1) correlate with their phenotypic cluster assignment (Fig. 2), as both strains are classified in speciesgroup Cat. I-sp. 9 according to Williams et al. (1989), and in cluster 31 according to Kämpfer & Kroppenstedt (1991). A partial correlation with former studies was observed in the following case: *S. hygroscopicus* subsp. *hygroscopicus* LMG 19335^T^ and *S. endus* LMG 19393^T^ (DNA hybridization of 92%) are both grouped in speciesgroup Cat. I-sp. 16 by Williams et al. (1989), but belong to cluster 56 and cluster 41, respectively, according to Kämpfer & Kroppenstedt (1991). *S. niveus* LMG 19395^T^ and *S. spheroides* LMG 19392^T^ (DNA hybridization of 77%) belong to the speciesgroup Cat. I-sp. 2 according to Williams et al. (1989), but are classified in cluster 43 and cluster 40, respectively, according to Kämpfer & Kroppenstedt (1991). *S. caeruleus* LMG 19399^T^, which belongs to the same DNA hybridization group (showing 85 and 100% DNA hybridization, respectively, to *S. spheroides* LMG 19392^T^ and *S. niveus* LMG 19395^T^), is grouped in Cat. IV-sp. 7 by Williams et al. (1989), and in cluster 58 by Kämpfer & Kroppenstedt (1991). *S. violaceus* LMG 19360^T^ and *S. violatus* LMG 19397^T^ (sharing 90% DNA hybridization) are classified in Cat. I-sp. 5 and Cat. I-sp. 11 according to Williams et al. (1989), and in clusters 9 and 50 according to Kämpfer & Kroppenstedt (1991). In one case, no correlation existed between our data and those of other authors, i.e. *S. aurantiacus* LMG 19358^T^ and *S. albosporeus* subsp. *albosporeus* LMG 19403^T^ (having DNA hybridization of 74%), are classified in Cat. II-sp. 13 and Cat. IV-sp. 1 according to Williams et al. (1989), and in clusters 12 and 63 by Kämpfer & Kroppenstedt (1991). These results clearly confirm that the phenotypic groupings provided by Williams et al. (1989) and Kämpfer & Kroppenstedt (1991) do not always reflect the genomic relationships.

In the genus *Streptomyces* (conventional) phenotypic data still dominate the classification system. In other genera containing fewer species (e.g. the genus *Helicobacter*; Dewhirst et al., 2000), this phenotypic classification system has already gradually evolved into a polyphasic approach (in which genomic data are most often used as a basis for delineation of taxa). It is logical that we should pursue an analogous polyphasic taxonomy for the genus *Streptomyces*, and that it is no longer acceptable to use different criteria for speciation in different groups of bacteria. Genotypy should preferably precede phenotypy. Only when genome-based groups are defined can the search for phenotypic traits (which may or may not be successful) begin.

From our study, it is clear that SDS-PAGE of whole-cell proteins, applied under highly standardized conditions, is a valuable phenotypic tool for screening for synonyms within the genus *Streptomyces*, provided that DNA–DNA hybridization experiments are performed to confirm genomic relatedness at the species level.

On the basis of the data from this paper, we propose the following synonyms in the genus *Streptomyces*: *S. albosporeus* subsp. *albosporeus* LMG 19403^T^ is a subjective synonym of *S. aurantiacus* LMG 19358^T^; *S. aminophilus* LMG 19319^T^ is a subjective synonym of *S. cacaoi* subsp. *cacaoi* LMG 19320^T^; *S. niveus* LMG
19395T and S. spheroides LMG 19392T are subjective synonyms of S. caeruleus LMG 19399T; S. violatus LMG 19397T is a subjective synonym of S. violaceus LMG 19360T. Emended descriptions are given below and are based on the species descriptions, as given by Williams et al. (1989), and the original descriptions.

Emended description of Streptomyces cacaoi subsp. cacaoi Waksman in Bunting 1932, 515–517; Waksman and Henrici 1948, 951AL

Spore chains are spirales; the spore surface is smooth. The aerial mass is in the white-yellow colour series. The reverse side of a colony has no distinctive features. The aerial mass is yellow or grey. The reverse side of a colony is dark blue-violet to black. The G + C content of the DNA is 70 mol %. The type strain is INMI 1373T (= LMG 19358T). Subjective synonym: Streptomyces albusporus subsp. albusporus IJSB 30:370AL DSM 40795T (LMG 19403T).

Emended description of Streptomyces violaceus (Rossi-Doria) Waksman and Henrici = Actinomyces violaceus (Rossi-Doria) Gasperini 1894, emend. Krasilnikov 1941; Streptomyces violaceus (Rossi-Doria) Waksman in Waksman and Lechevalier 1953, 43

Spore chains are spirales. The spore surface is spiny. The aerial mass is in the white or red colour series. The reverse side of a colony is reddish or reddish-orange to purple. Reverse mycelium pigment is a pH indicator. Melanoid pigments are formed (sometimes slowly). A red to violet pigment is found in the medium in yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar. This pigment is pH-sensitive. The following carbohydrates are utilized for growth: d-glucose, l-arabinose, d-xylene, d-mannitol and d-fructose. Growth is possible on iso-inositol and raffinose. The G + C content of the DNA is 71 mol %. The type strain is INMI 1T (= LMG 19360T). Subjective synonym: Streptomyces violatus IJSB 30:404AL INMI 1205T (LMG 19397T).

ACKNOWLEDGEMENTS

This study was performed within the framework of the Belgian–Chinese programme ‘Identification and classification of actinomycetes, specifically bioactive Streptomyces strains isolated from Chinese soil’, supported by OSTC Belgium (BL/02/C10) and MOST/NSFC P. R. China. We thank Dirk Dewettinck (RUG, Belgium) for excellent technical assistance in performing SDS-PAGE of whole-cell proteins. The authors are also indebted to Professor R. M. Kroppenstedt (DSMZ, Germany) and Dr T. Kudo (JCM, Japan) for providing cultures.

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