

APPLICATION OF MOLECULAR BIOLOGICAL METHODS IN TAXONOMY OF GENUS *STREPTOMYCES*

Krassimira Christova*, Zdravka Sholeva and Valentina Chipeva

National Bank for Industrial Microorganisms and Cell Cultures,
1113 Sofia, P.O.Box 239, Bulgaria

Summary

An extensive literature review concerning the taxonomic status of the species of genus Streptomyces has been made. The classical microbiological and chemo taxonomical methods which form the basis of the present classification of the species of this genus, as well as, the modern molecular biological approaches - analysis of protein patterns, multilocus enzyme electrophoretic (MLEE) analysis, restriction analysis, analysis of the nucleotide sequences of 16S and 23S RNA, RNA/DNA sequencing, DNA fingerprinting with different probes or polymerase chain reaction (PCR) primers were described. The profound analysis showed the advantages of the molecular biological methods for streptomyces taxonomy and indicated that none of these methods applied independently could solve the existing problems in the taxonomy of genus Streptomyces. A wider application of the molecular genetic approaches is necessary in order to specify the taxonomic status of the species included in category III and IV according to Bergey and to find a criterion for distinguishing between the closely related species, as well as, to evaluate the homogeneity of some streptomyces species.

1. Classical and numerical taxonomy of genus *Streptomyces*

Streptomycetes represent a widely spread in nature group of microorganisms; they play an important role in the circle of the organic matter and have an industrial importance as producers of secondary metabolites. In order to protect the patent rights, new species of streptomycetes have been described, in many cases groundlessly (around 3000 patent species in different collections have been deposited) which has resulted in some difficulties in their precise classification.

At present genus *Streptomyces* consists of aerobic, Gram positive actinomycetes, forming substrate mycelium which differentiates in an air one with long chains of conidia. The streptomycetes possess cell wall chemotype I [44].

The earliest attempts for classification of the streptomycetes have been made between 1914 and 1962 and are based on the results obtained from the investigations of limited number of morphological characteristics [61, 63]. Waksman defined the aerobic actinomy-

cetes in genus *Streptomyces* in 1961 [75]. A general revision of the genus was made in 1966 with the International Streptomyces Project (ISP) [62], as a result of which in the approved list streptomycetes species remain just 459 of the initially described 1000. For comparison, the number of the cultures defined with different names in the collection catalogues is much greater: ATCC catalogue (1982) - 673; DSM catalogue (1983) - 473; JCM catalogue (1986) - approx. 600. This classification is based on relatively small number of morphological, cultural and physiological characteristics.

The application of chemical methods influenced the development of the actinomycetes systematics at the genus level and even at higher taxonomic levels [17, 36, 47, 71]. The separation of genus *Streptomyces* from other streptomycetes groups (on the same taxonomic level) is not a problem [17, 65]. The streptomycetes can be distinguished from all the rest actinomycetes in morphological and

chemotaxonomical (aminoacids [67], diaminopimelic acid [67], fatty acids [41], lipids [42, 60], phospholipids [41], menaquinones [1], mycolic acids [47], sugars [67]) characteristics. The use of the above mentioned chemotaxonomic methods leads to more profound and precise characterization of the genera.

Twenty five genera have been described in the last edition of Bergey's Manual [80]: *Actinobispora*, *Actinocineospora*, *Actinoma-dura*, *Actinomyces*, *Actinoplanes*, *Actinocynema*, *Frankia*, *Microbispora*, *Micromonospora*, *Micropolispora*, *Microtetraspora*, *Nocardia*, *Nocardioides*, *Nocardiosis*, *Promicromonospora*, *Pseudomonocardia*, *Intrasporangium*, *Kineospora*, *Kitasatoa*, *Saccharomonospora*, *Saccharopolispora*, *Streptomyces*, *Streptosporangium*, *Streptoverticillium*, *Thermoactinomyces*.

Among the sporoactinomycetes the presence of L-diaminopimelic acid in the cell wall is a fact of diagnostic value for the streptomycetes [17]. The registered differences in some chemotaxonomic characteristics (in the patterns of the fatty and mycolic acids) among the species of genus *Streptomyces*, are of limited importance for their species differentiation [36].

The numerical taxonomy has been applied for streptomycetes since the 60s [16, 64]. Silvestri et al. [64] have studied 200 strains in 100 characteristics, grouped in 25 variation groups. The results of this analysis have shown that many of the characteristics used for classification of the streptomycetes species are strongly variable and hard for interpretation. A considerable step ahead in the taxonomy of the streptomycetes is the numerical classification of Williams, from 1983 [81], which uses 475 strains, among them 394 streptomycetes type cultures from ISP characterized in 139 morphological, cultural, physiological and biochemical characteristics. The type streptomycetes strains are divided into 9 cluster groups that include 23 major (with 6 to 71 strains), 20 minor (with 2 to 5 strains) and 25 single-member (with 1 strain only) clusters. On the basis of this classification the homogenous and well-defined species are treated as minor and single-member clusters, while the major clusters which are with high degree of heterogeneity, are marked as species clusters.

This study has formed the basis of the classification of the species of genus *Streptomyces* in Bergey's manual [80]. The species *S. albidoflavus*, *S. halstedii*, *S. exfoliatus*, *S.*

roshei, *S. lydicus*, *S. fradiae*, *S. laven-dulae*, *S. rimosus* and *S. albus* are accepted as well-defined. The taxonomic status of these species is supported by several other investigations (chemical [46, 59], enzymatic [20, 32], serological [56], RNA/DNA sequencing [48], hybridization studies [38, 39] and rRNA sequence analysis [68, 70, 82]).

However, the status of many taxons including some of the minor and the single-member clusters remains unclear. The species grouped in categories III and IV according to Bergey [80] have to be considered with unclear status, until further genetic information is available.

The species status of the 11 above listed strains is confirmed also by the numerical taxonomic investigations of Kampfer et al. [30] of 821 strains belonging to genera *Streptomyces* and *Streptoverticillium* which are characterized by the use of 329 physiological tests.

The phenetic data of Kampfer et al. [30] confirm the addition by Mordarski et al. [48] of *S. coelicolor*, *S. albidoflavus*, *S. albidus*, *S. alboniger*, *S. canescens*, *S. citreus*, *S. fellus*, *S. limosus*, *S. rutgersensis*, and *S. sampsonii* to one taxon, and *S. alboviridis*, *S. cratifer*, *S. oligocarboophilus* to another genospecies on the basis of DNA-DNA homology. The characterized by Korn et al. [35] strains by phage typing as *S. griseus* are placed in different subclusters and clusters of Kampfer. This confirms the separation of these strains obtained by Okanishi et al. on the basis of DNA homology [51].

The distribution of *S. exfoliatus* and *S. violaceus* in cluster 2 according to Kampfer (*S. exfoliatus*, Williams groups A4 and A6) is in agreement with the standpoint of Williams et al. [81] that these strains have to be grouped in 1 species because of the considerable overlapping of their phenotypes A5 (*S. exfoliatus*) and A6 (*S. violaceus*).

The group of *S. violaceoruber* defined by Korn et al. [35] by phage typing is placed also in cluster 2 according to Kampfer, with the exception of the type strain of *S. violaceoruber* DSM 40049 which shows considerable physiological and biochemical differences and is placed in another cluster.

The greater part of the strains belonging to genus *Streptoverticillium* studied by Kampfer et al. [30] is localized in subcluster 22-1 which fact confirms the standpoint for unification of the 2 genera. The *S. fradiae* strains (Kampfer subcluster 22-5) are closely related to *S. lavendulae* and to the *Strep-*

toverticillium strains which is also in correlation with the results of Williams et al. [81].

The *S. hygroscopicus* species is defined as heterogenous in the work of Kampfer et al. [30]. Four strains of *S. hygroscopicus* which are localized in cluster 25 (*S. lydieus*) are thought to be synonyms of *S. violaceusniger*. These strains differ from the *S. hygroscopicus* strains grouped in clusters 53 to 57. The heterogeneity of the *S. hygroscopicus* strains is confirmed also by the analysis of its fatty acids [36]. Another example for a heterogenous species is *S. violaceusniger*. The strains of this species are localized in closely linked and overlapping clusters according to Kampfer.

The status of the following strains is not confirmed by the numerical taxonomy of Kampfer: *S. griseoruber* ISP 5281T, *S. griseoruber* ISP 5227T (Williams cluster A21), *S. xanthophaeus* ISP 5134T (Williams group F61), *S. amakusaensis* ISP 5219T, *S. fumanus* ISP 5154T, *S. griseochromogenes* ISP 5499T (Williams group A18), although they exhibit very low hybridization values.

The basic data of the numerical taxonomic investigation of Williams are used to create a probabilistic identification matrix [79] which is applied in the identification of unknown streptomycetes from different habitats, as well as, for identification of the species groups defined by the numerical taxonomy [49]. Similar matrix is created and used by Kampfer et al. too [30].

The numerical taxonomy gives more objective criterion for clarification of the intra-group differences in the taxonomy of the streptomycetes but does not help for the clear definition of the species. The similarity level in the groups which is used for that purpose is subjective or is interpreted freely by the investigator.

The data obtained by the numerical taxonomy must be interpreted keeping in mind that the similarity among the strains can be disrupted by numerous factors (see Williams et al. [83]) and that the marker strain is not always the best representative of the species [19]. For instance, the incorporation of *Nocardioides albus*, *N. dassonvillei* and *Saccharopolyspora hirsuta* to genus *Streptomyces* is not made on a solid basis. The strains of *Nocardioides* possess a cell wall chemotype I, they are resistant to virulent phages of streptomycetes [76] and differ from the last in the results of the lipid analysis and DNA/DNA hybridization values [74]. *N. dassonvillei* differs from the streptomycetes in the chemotype of

its cell wall [41] and in the resistance to streptomycetes phages but also in the content of menaquinones [1] and rRNA sequencing [72], while *S. hirsuta* - in its cell wall chemotype, the content of menaquinones and the resistance to virulent phages for streptomycetes [1, 76,].

For more precise characterization of the species of genus *Streptomyces* new methods are applied - serological, physiological, biochemical, etc. [18, 56].

Qualitative enzyme tests for differentiation of the streptomycetes were applied by Goodfellow et al. [18] for 88 streptomycetes strains belonging to 19 different species groups of Williams. Different patterns of extracellular enzyme activities were found in each of the groups using tests for hydrolysis of fluorogenic 4-methylumbelliferyl-linked substrates. In their investigations Kampfer et al. [31] applied enzyme tests with nitrophenyl or nitroanilide-linked substrates and obtained similar results, as well as, some differences. The species *S. albidoflavus* and *S. griseus* (*S. anulatus*) can be distinguished on the basis of the enzyme activity of β -L-fucosidase. But not all strains studied by Kampfer et al. produce β -D-galactosidase and for the β -D-glucuronidase and for sulphatase production, other patterns are obtained. Obviously, these tests are important for characterization of the streptomycetes but they can not be used for their unambiguous identification. These qualitative enzyme tests together with some other physiological and biochemical characteristics are included in the computer assisted classification of Goodfellow et al. [21] in which it is shown that *S. albidoflavus* species-group encompassed taxospecies corresponding to *S. albidoflavus*, *S. anulatus* and *S. halstedii*.

The serotaxonomic analysis of Riddel et al. [56] is important for the taxonomy of streptomycetes because it confirms the view of Williams [81] for unification of 4 closely related strains of cluster 1B (*S. griseus*, *S. griseinus*, *S. griseobrunneus* and *S. globisporus*) in 1 species, as well as, 2 strains from cluster 61 (*S. goshikiensis* and *S. lavendulae*) and 2 from cluster 55 (*Stv. cinnamoneum* and *Stv. griseocarneum*).

The quantitative chemotaxonomic methods are also applied to some species of genus *Streptomyces* [49, 58] but the possibility the species to be differentiated on the basis of data for quantitative determination of fatty acids remains questionable [59].

2. Application of molecular biological approaches in taxonomy

At present the existing classification of the species of genus *Streptomyces* is based only on the similarity of the phenotypic characteristics. The numerical taxonomy is one of the best methods for creation of classification systems for prokaryotes but it does not take into consideration the phylogenetic linkages among the bacteria, and it does not distinguish precisely enough between the closely related species. Great attention has been paid to the application of molecular approach in taxonomic investigations in order to solve this problem. As compared with the phenotypic, chemical, enzymatic and serological characteristics, which cover only 5-10% of the genome, the molecular genetic methods deal directly or indirectly with the genome polymorphism. Such methods as protein patterns, MLEE analysis, plasmid patterns, restriction analysis, analysis of the nucleotide sequences of 16S and 23S RNA, DNA fingerprinting with different probes or PCR primers, give a possibility for estimation of the real genetic similarity among species.

2.1. Indirect analysis of the genome. Some investigators propose ELISA [33], electrophoretic mobility of total protein extracts [10] and computer programmed analysis of radiolabeled protein binding patterns [29] as new approaches in the taxonomy of streptomycetes.

The patterns of the ribosomal proteins obtained by twodimensional PAGE and HPLC, reported by Fierro et al [12] and Ochi [50] provide useful information for the classification and identification of streptomycetes. There is a considerable variability in the ribosomal protein patterns of some species - *S. venazuele*, *S. violaceus*, *S. parvulus*, *S. hygrosopicus*, while the ribosomal protein patterns of *S. lavendulae* and *S. avidinii* are quite similar. This suggests that the two species are closely related (if they do not represent a single taxon [50]).

The multilocus enzyme electrophoresis which has been a standard method in the eucaryotic population genetics and systematic for a long time, is applied more widely in the investigations of genetic heterogeneity in bacterial populations [5, 43, 55] and for clarifying the intraspecies diversity of the prokaryotes. MLEE analysis is not performed for genus *Streptomyces*. As far as the genera of *Actinomycetales* is concerned, this method is ap-

plied only for isolates of genus *Frankia* [13] for aminopeptidases and esterases.

The obtained results showed that the method is applicable for pure cultures and the data coincides in a considerable degree with the results obtained by molecular methods such as restriction fragment length polymorphism (RFLP) and sequencing of amplified rDNA.

2.2. Direct analysis of the genome.

Restriction analysis and DNA/DNA hybridization methods are those suitable for distinguishing of some streptomycetes species but for many others the data are contradictory. For example, unconvincing are the results obtained by DNA/DNA hybridization [39] between the members of the groups F61 (*S. lavendulae*) A21 (*S. griseoruber*) and A18 (*S. cyanneus*) [81]. In many of the investigated strains the hybridization values of the members of one group are lower compared with the members of the different groups of Williams [37, 48].

Phylogenetic relationship has been found out between representatives of: *Streptomyces* cluster-group, the *S. lavendulae* cluster and the *Streptovorticillium* cluster [16].

The RNA/DNA sequencing analysis is a method which registers a phylogenetic relationship. The determination of the full 16S rRNA sequencing of *S. lividans* [73] and *S. coelicolor* [3] has given a possibility the 2 species to be classified as *S. violaceoruber*. The registered partial sequencing of 16S rRNA of 14 species belonging to genera *Streptomyces* and *Streptovorticillium* [82] together with the data of the numerical phenotypic analysis and chemotaxonomic properties give a possibility to include genus *Streptovorticillium* in genus *Streptomyces*.

The more strictly conservative regions of rRNA are in the basis of the phylogenetic analysis and are used for creation of universal oligonucleotide probes and primers for identification of rRNA of the members of higher taxons [68, 70].

The variable rRNA regions (depending upon the degree of variability) allow the incorporation into the isolates to the lower taxons (genus, species, strain) using sequencing analysis [83], oligonucleotide probing [9, 69, 68], or PCR diagnosis [4, 45, 57]. For example, nucleotide 929 of 16S rRNA (*S. ambofaciens* nomenclature [54]) is highly specific

for the streptomycetes and this nucleotide together with its flanking regions allows the construction of genus-specific probe. The regions 158 to 203 of 16S rRNA and 1518 to 1645 of 23S rRNA (helix 54 [54]) are used as diagnostic probes for selection of PCR primers and they possess a great potential for investigation of the species differentiation. The degree of variability in the regions 982 to 998 (α -region), 1102 to 1122 (β -region) and 150 to 200 (γ -region) of 16S rRNA is more weakly represented (in comparison with helix 54 of 23S rRNA) but it is determinative for some species (*S. baldacii*, *S. lavendulae*, *S. albofaciens*, *S. griseus*) [68].

Stackebrandt et al. [60] in their work have used the sequencing information for 16S and 23S rRNA/DNA of 69 strains of 53 species to build phylogenetic trees, which topology is compared with each other and with the clusters of the numerical taxonomy of Williams [81]. For instance:

1. The phylogenetic analysis based on the analysis of 1137 nucleotides of 16S rRNA/DNA sequencing of 15 streptomycetes is important with the clustering of the species of the phenetic groups A and F and with the clustering of non-verticillia forming streptomycetes;

2. In most parts of the phylogenetic tree based on the analysis of 226-nucleotides-long stretch covering the variable regions α and β (*S. ambofaciens* nomenclature) [54] of 59 strains of 53 species, a correlation is found between 16S rRNA and numerical phenetic analysis. This correlation is obvious for the closely related species as *S. albus* and *S. flocculus* [A16] and for the grouping of the species belonging to phenetic clusters A16, A19, A23, A20, A37 and F55 [70].

The sequencing analysis of chosen 16S RNA regions of 77 strains of 55 species also confirms the very high degree of relationship among most of the described streptomycetes species [68]. However, the determination of the species can not be done only on the basis of the homology in 16S rRNA because the differences between the sequences are mainly in the variable regions which are of limited importance for the phylogenetic linkages [68].

Together with 16S and 23S rRNA, 5S rRNA is also used for estimation of the relationship among different procaryotes [8, 40, 53] including the streptomycetes [52]. The data of sequencing analysis of 5S rRNA confirm the view of Goodfellow, 1986 [22, 23, 24,

25] that the genera *Chainia*, *Elitrosporangium*, *Kitasatoa* and *Microelobospora* must be treated as synonyms of genus *Streptomyces*, but more precise molecular-biological investigations are needed to determine the taxonomic status of the 2 streptomycetes sub-clusters which appeared surprisingly in the streptomycetes cluster based on 5S rRNA sequencing analysis [52]. The opinion that the sequencing analysis applied alone can not solve the problems in the taxonomy of streptomycetes is accepted as general.

In spite of the presence of wide base-data and the very high degree of variability of the sequences in the different rRNA regions, according to Stackebrandt et al. [50] there are three main problems in the molecular strategy based on the nucleotide sequences of rRNA for identification of pure cultures:

1. The target region (in 16S rRNA in 23S rRNA or in 16S and 23S rDNA) for PCR primers probably is not unique enough to allow the registration of differences among closely related sequences [66];

2. The application of this strategy is limited to those taxons for which a sequencing information is available;

3. Different taxons can show identical regions, consequently the investigated species will be wrongly referred to one taxon [68].

These problems increase in the taxons which possess a great number of phylogenically closely related members. A typical representative of such a taxon is the genus *Streptomyces*. A possible way of overcoming these problems is applying together different probes and a set of specific PCR primers.

Recently, the use of rRNA or mDNA oligonucleotides for direct hybridization of whole cells by fluorescent probes for strain or species specific genes [2, 27, 28] is applied in the identification of bacterial species. But this method, as well as the above mentioned ones based on the nucleotide sequencing of rRNA and PCR requires availability of sequencing information for respective genes in the genome of the species or the strain and consequently, is not applicable for the taxons for which there is not such an information.

A method by which the above mentioned problems could be overcome is DNA fingerprinting of the whole genome using single primers chosen arbitrary and PCR (AP-PCR - Arbitrary Primed Polymerase Chain Reaction) [77, 78]. AP-PCR does not require a set of specific primers. Primers regardless of the

sequence of their genome are used. Respectively, no knowledge for the biochemistry and molecular biology of the microorganisms under investigation is needed.

Every primer gives a different model of AP-PCR products and by using any primer a polymorphism among the strains can be registered. The data give the possibility even closely related strains of one and the same species to be detected. The registered DNA polymorphism is named by Williams et al [78] Random Amplified Polymorphic DNA Markers (RAPD). The obtained PCR products common only to some individuals, act as polymorphic markers equivalent to the other polymorphic characteristics used in the phylogenetic investigations and genetic mapping.

The AP-PCR method has been firstly applied in 1990 by Welsh and McClelland for testing strains of 5 species *Staphylococcus* and 11 strains *Streptococcus pyogenes*, the relationship between which have been determined by DNA-DNA hybridization [34]. Later it was applied to some eukaryotes [6, 7, 11, 26]. It is shown that AP-PCR gives a quantitative evaluation of the genetic relationship on species and subspecies level which corresponds

to the registered phenotypic schemes [77, 78].

The application of this method will make for distinguishing between the closely related streptomycetes species, for the clarification of the borderlines between the species in the polymorphic III and IV categories [80] and for the obtaining of additional genetic information for a strain of genus *Streptomyces* with unknown taxonomic status.

From the review of the literature concerning the present taxonomic status of genus *Streptomyces* and the methods applied in the bacterial taxonomy it became clear that several important questions have to be solved:

1. Creation of a criterion for differentiation between closely related genera;
2. Evaluation of the homogeneity of some streptomycetes strains;
3. Specification of the taxonomic status of the species included in III and IV categories according to Bergey's manual [80].

Neither of these questions can be solved applying only one of the presently used in the bacterial taxonomy methods.

References

1. Alderson, G., M. Goodfellow, D. E. Minnikin, 1985. *J. Gen. Microbiol.*, **131**, 1671-1679.
2. Amann, R., I. L. Krumholz, A. D. Stahl, 1990. *J. Bacteriol.*, **172**, 762-770.
3. Baylis, H. A., M. J. Bibb, 1987. *Nucleic Acids Res.*, **15**, 7176.
4. Boddington, B., T. Rogall, T. Flahr, H. Blocker, E. C. Bottger, 1990. *J. Clin. Microbiol.*, **28**, 1751-1759.
5. Boerlin, P., J. Rocourt, J.-C. Piffaretti, 1991. *Int. J. Syst. Bacteriol.*, **41**, 59-64.
6. Bostock, A., M. N. Khattak, R. Matthews, J. Buruie, 1993. *J. Gen. Microbiol.*, **139**, 2179-2184.
7. Crowhurst, R. N., B. T. Hawthorne, E. A. Rikkerink, M. D. Templeton, 1991. *Curr. Genetics*, **20**, 391-396.
8. Dekio, S., R. Yamasaki, J. Jidoi, H. Hori, S. Osawa, 1984. *J. Bacteriol.*, **159**, 233-237.
9. DeLong, E. F., S. Wickham, N. R. Pace, 1989. *Science*, **243**, 1360-1363.
10. Dietz, A., 1988. Practical and proposed cooperative investigational criteria for taxonomic studies of the Actinomycetales. In: *Biology of Actinomycetes*. Y. Okami, T. Beppu and H. Ogawara (eds.), Tokyo: Japan Sci. Soc. Press, 203-209.
11. Fegan, M., J. M. Manners, D. J. Maclean, 1993. *J. Gen. Microbiol.*, **139**, 2075-2081.
12. Fierro, J. F., F. Parra, L. M. Quiros, C. Hardisson, J. A. Salas, 1987. *FEMS Microbiol. Lett.*, **41**, 283-287.
13. Gigras, M., J. Schwencke, 1993. *J. Gen. Microbiol.*, **139**, 2225-2232.
14. Gilardi, E., R. L. Hill, M. Turri and L. Silvestri, 1960. *I. Giorn. Microbiol.*, **8**, 203-218.
15. Giovannoni, W. S. J., E. F. DeLong, G. J. Olsen, N. R. Pace, 1988. *J. Bacteriol.*, **179**, 720-726.
16. Gladek, A., M. Mordarski, M. Goodfellow, S. T. Williams, 1985. *FEMS Microbiol. Lett.*, **26**, 175-180.
17. Goodfellow, M., T. Cross, 1984. Classification. In: *Biology of the actinomycetes*. M. Goodfellow, M. Mordarski and S.T. Williams (eds.), London: Acad. Press, 7-164.
18. Goodfellow, M., C. Lonsdale, A. L. James, O. C. MacNamara, 1987. *FEMS Microbiol. Lett.*, **43**, 39-44.
19. Goodfellow, M., C. R. Weaver, D. E. Minnikin, 1982. *J. Gen. Microbiol.*, **128**, 731-745.
20. Goodfellow, M., E. G. Thomas, A. L. James, 1990. *Zbl. Bacteriol.*, **274**, 299-315.

21. Goodfellow, M., E. V. Ferguson, J. J. Sanglier, 1992. *Gene*, **115**, 225-233.
22. Goodfellow, M., S. T. Williams, G. Alderson, 1986. *Syst. Appl. Microbiol.*, **8**, 55-60.
23. Goodfellow, M., S. T. Williams, G. Alderson, 1986. *Syst. Appl. Microbiol.*, **8**, 48-54.
24. Goodfellow, M., S. T. Williams, G. Alderson, 1986. *Syst. Appl. Microbiol.*, **8**, 65-66.
25. Goodfellow, M., S. T. Williams, G. Alderson, 1986. *Syst. Appl. Microbiol.*, **8**, 61-64.
26. Goodwin, P. H., S. L. Annis, 1991. *Appl. Env. Microbiol.*, **57**, 2482-2486.
27. Hahn, D., R. Amann, J. Zeyer, 1993. *Appl. Env. Microbiol.*, **59**, 2753-2757.
28. Hahn, D., R. I. Amann, W. Ludwig, A. D. L. Akkermans, K.-H. Schleifer, 1992. *J. Gen. Microbiol.*, **138**, 879-889.
29. Hook, L. A., P. L. Bioch, R. W. Kohlenberger, P. A. Kinningham, 1987. *Dev. Ind. Microbiol.*, **28**, 149-160.
30. Kampfer, P., R. M. Kroppenstedt, 1991. *J. Gen. Microbiol.*, **137**, 1893-1902.
31. Kampfer, P., R. M. Kroppenstedt, W. Dott, 1991. *J. Gen. Microbiol.*, **137**, 1831-1891.
32. Kilian, M., 1978. *J. Clin. Microbiol.*, **8**, 127-133.
33. Kirby, R., E. P. Rybicki, 1986. *J. Gen. Microbiol.*, **132**, 1891-1894.
34. Kloos, W. E., J. F. Wolfshohl, 1983. *Curr. Microbiol.*, **8**, 115-121.
35. Korn, F., B. Weingartner, H. J. Kutzner, 1978. A study of twenty actinophage: morphology, serology, relationship and host range. In: *Genetics of the Actinomycetales*, E. Freersen, I. Tarnok and J. H. Thumin (eds.), Stuttgart: Gustav Fisher Verlag, 251-270.
36. Kroppenstedt, R.M., 1985. Fatty acid and menaquinone analysis of actinomycetes and related organisms. In: *Chemical methods in bacterial systematics*, M. Goodfellow and D. E. Minnikin. London: Acad. Press, 173-199.
37. Labeda, D. P. and A. J. Lyons, 1988. DNA Relatedness among blue and red-spored Streptomyces. In: *Biology of Actinomycetes*, Y. Okayami, T. Beppu and H. Ogawara (eds.), Tokyo: Japan Sci-entific Press, 241.
38. Labeda, D. P., A. S. Lyons, 1991. *Syst. Appl. Microbiol.*, **14**, 158-164.
39. Labeda, D. P., 1991. *Actinomycetologica*, **5**, 35-37.
40. Lane, D. J., D. A. Stahl, G. J. Olsen, D. J. Heller, N. R. Pace, 1985. *J. Bacteriol.*, **163**, 75-81.
41. Lechevalier, M. P., H. A. Lechevalier, 1980. *Actinomycete taxonomy*, Dietz, A. and D. W. Thayer (eds.), Arlington: SIM Special Publ. 6, Soc. Ind. Microbiol., 225-291.
42. Lechevalier, M. P., H. A. Lechevalier, 1970. *Int. J. Syst. Bacteriol.*, **20**, 435-443
43. Levin, M. H., R. A. Weinstein, R. K. Selander, H. Ochman, S. A. Kabins, 1984. *J. Clin. Microbiol.*, **20**, 758-762.
44. Lechevalier, H., A., M., P. Lechevalier, 1970. A critical evaluation in general of aerobic actinomycetes. In: *The Actinomycetales*, H. Prauser (ed.), Jena: Gustav Fisher Verlag, 393-405
45. Liesack, W., H. Weyland, E. Stackebrandt, 1991. *Microb. Ecol.*, **21**, 191-198.
46. Manchester, L., K. Kersters, M. Goodfellow, 1990. *Syst. Appl. Microbiol.*, **13**, 333-337.
47. Minnikin, D. E., I. G. Hutchinson, A. B. Caldicott, M. Goodfellow, 1980. *J. Chrom.* **188**, 221-233.
48. Mordarski, M., M. Goodfellow, S. T. Williams, P. H. A. Sneath, 1986. Evaluation of species groups in genus Streptomyces. In: *Biological, biochemical and biomedical aspects of Actinomycetes*, G. Szabo, S. Biro and M. Goodfellow (eds.), Budapest: Akad. Kiado, 517-525.
49. O'Donnell, A., 1988. Recognition of novel actinomycetes. In: *Actinomycetes in biotechnology*, M. Goodfellow, S. T. Williams and M. Mordarski, London: Acad. Press, 69-88.
50. Ochi, K., 1989. *J. Gen. Microbiol.*, **135**, 2635-2642.
51. Okanishi, M., H. Agakawa, H. Umezawa, 1972. *J. Gen. Microbiol.*, **72**, 49-58.
52. Park, Y.-H., D.-G. Yim, E. Kim, Y.-H. Kho, T.-I. Mheen, J. Lonsdale, M. Goodfellow, 1991. *J. Gen. Microbiol.*, **137**, 2265-2269.
53. Park, Y.-H., H. Hori, K.-I. Suzuki, S. Osawa, K. Komagata, 1987. *J. Bacteriol.*, **169**, 1801-1806.
54. Pernodet, J., F. Bocard, M.-T. Alegre, J. Guerinneau, 1989. *Gene*, **79**, 33-46.
55. Rattazi, M. C., J. G. Scandalios, G. S. Whitt (Eds.), (1983), *Isoenzymes current topics in biological and medical research*, Genetic and evolution, vol. 10, New York: Alan R. Liss Inc.
56. Ridell, M., S. T. Williams, 1983. *J. Gen. Microbiol.*, **129**, 2857-2861.
57. Rogall, T., J. Wolters, T. Flohr, E. C. Bottger, 1990. *Int. J. Syst. Bacteriol.*, **40**, 323-330.
58. Sacking, M. J., D. Jones, 1992. Computer assisted classification. In: *New bacterial systematics*, M. Goodfellow and A. G. O'Donnell (eds.), London: Acad. Press.
59. Saddler, G. A., A. G. O'Donnell, M. Goodfellow, D. E. Minnikin, 1987. *J. Gen. Microbiol.*, **133**, 1137-1147.
60. Shaw, N. 1974. *Adv. Appl. Microbiol.*, **17**, 63-108.
61. Shirling, E. B., D. Gottlieb, 1966. *Int. J. Syst. Bacteriol.*, **16**, 313-340.
62. Shirling, E. B., D. Gottlieb, 1967. *Int. J. Syst. Bacteriol.*, **17**, 315-322.
63. Shirling, E. B., D. Gottlieb, 1968. *Int. J. Syst. Bacteriol.*, **18**, 69-189.
64. Silvestri, L., M. Turri, L. R. Hill, E. Gilardi, 1962. A quantitative approach to the systematics of Actinomycetes based on overall similarity. In: *Microbial classification*, G.C. Ainsworth and P. H. A. Sneath (eds.), Cambridge: Cambridge University Press, 333-360.
65. Sneath, P. H. A., 1963. The construction of taxonomic groups. In: *Microbial classification*, G. C. Ainsworth and P. H. A. Sneath (eds.), Cam-

- bridge: Cambridge University Press, 289.
66. Sommer, R., D. Tautz, 1989. *Nucleic Acids Res.*, **17**, 6749.
67. Sopot, J., P. M. Lechevalier, H. A. Lechevalier, 1967. *Appl. Microbiol.*, **13**, 1356-1361.
68. Stackebrand, E., D. Witt, C. Kemmerling, R. Kroppenstedt, W. Liesack, 1991. *Appl. Env. Microbiol.*, **57**, 1468-1477.
69. Stackebrandt, E., O. Charfreitag, 1990. *J. Gen. Microbiol.*, **136**, 37-43.
70. Stackebrandt, E., W. Liesack, D. Witt, 1992. *Gene*, **115**, 255.
71. Stackebrandt, E., 1986. The significance of "wall types" in phylogenetically based taxonomic studies on Actinomycetes. In: *Biological, biochemical and biomedical aspects of Actinomycetes*, G. Szabo, S. Biro and M. Goodfellow (eds.), Budapest: Academiai Kiado, Part B, 497-506.
72. Stackebrandt, E., B. Wunner-Fussl, V. J. Fowler, K. H. Schleifer, 1981. *Int. J. Syst. Bacteriol.*, **31**, 420-431.
73. Suzuki, Y., T. Yamada, 1988. *Nucleic Acids Res.*, **16**, 370.
74. Tille, D., H. Prauser, K. Szyba, M. Mordarski, 1978. *Zeitschrift Allg. Mikrobiol.*, **18**, 459-462.
75. Waksman, S. A., 1961. *The Actinomycetes, Vol. II. Classification, identification and descriptions of genera and species*. London: Balliere, Tindall and Cox, 4.
76. Wellington, E. M. H., S. T. Williams, 1981. *Zentral. Bacteriol. Mikrobiol. Hyg.*, **11**, (Abt. I), 93-98.77. Welsh, J., M. McClelland, 1990. *Nucleic Acids Res.*, **18**, 7213-7218.
78. Williams, J., A. Kubelik, K. Livak, 1990. *Nucleic Acids Res.*, **18**, 6531-6535.
79. Williams, S. T., M. Goodfellow, E. M. H. Wellington, J. C. Vickers, G. Alderson, P. H. A. Sneath, M. J. Sackin, A. M. Mortimer, 1983. *J. Gen. Microbiol.*, **129**, 1815-1830.
80. Williams, S. T., M. Goodfellow, G. Alderson, 1989. Genus *Streptomyces* Waksman and Henrici 1943, 339. In: *Bergey's manual of systematic bacteriology*, vol. IV, S. T. Williams, M. E. Sharpe and J. G. Holt (eds.), Baltimore: Williams and Wilkins, 2452-2492.
81. Williams, S. T., M. Goodfellow, G. Alderson, E. M. H. Wellinhton, P. H. A. Sneath, M. J. Sacking, 1983. *J. Gen. Microbiol.*, **129**, 1743-1751.
82. Witt, D., E. Stackebrandt, 1990. *Syst. Appl. Microbiol.*, **13**, 361-367.
83. Woese, C. R., 1987. *Microbiol. Rev.*, **51**, 221-271.