NUMERICAL TAXONOMY AND SELECTIVE ISOLATION OF
INDUSTRIALLY IMPORTANT ACTINOMYCETES

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INTRODUCTION

Isolation and screening programmes are rate limiting steps in the development of new bioactive compounds, notably antibiotics, from actinomycetes. Modern screening programmes are still dependant on the isolation or generation of large numbers of microorganisms¹⁾ though sophisticated ways are used to detect the presence of very small quantities of natural products²⁾. Objective isolation strategies are needed if better quality biological material is to be screened for new products and the costly rediscovery of known compounds reduced. The importance of including novel and rare non-streptomycete actinomycetes in industrial screening programmes is abundantly clear (Table 1) though it has to be stressed that streptomycetes are still the source of the largest number and greatest variety of new antibiotics.

Table 1. Number of actinomycetes produced by selected actinomycete taxa*

Genus	1974	1984	Genus	1974	1984
<i>Streptomyces</i>	1974	3477	<i>Streptosporangium</i>	7	26
<i>Micromonospora</i>	41	269	<i>Actinosynnema</i>	0	25
<i>Nocardia</i>	45	107	<i>Dactylosporangium</i>	0	19
<i>Actinomadura</i>	0	51	<i>Streptoalloteichus</i>	0	14
<i>Actinoplanes</i>	6	95	<i>Faenia</i>	2	7
<i>Streptoverticillium</i>	19	64	<i>Microbispora</i>	4	6
<i>Saccharopolyspora</i>	0	33	<i>Thermomonospora</i>	1	4

*Adapted from Bérdy.^{4,5)}

The realisation that antibiotics were produced by a taxonomically diverse range of actinomycetes stimulated interest in the development of better procedures for the isolation of uncommon and previously undiscovered strains¹⁾. The improved techniques were designed to eliminate the growth of fungal and unwanted bacterial colonies in order to encourage the development of rare and novel actinomycetes. To date, the introduction of effective selective isolation strategies has been hampered by several factors,³⁾ these include the reliance placed on classical isolation procedures, and poor classification which made it impossible to choose representative actinomycetes for screening programmes and genetic engineering.

The principles applied in the isolation of actinomycetes are well documented.^{6,7,8,9)} This article examines how information in numerical taxonomic data bases can be used to formulate and evaluate selective isolation media designed to provide good quality actinomycetes for industrial screening programmes.

ENTER NUMERICAL TAXONOMY

The theoretical basis of numerical taxonomy is well known¹⁰⁾ and its impact on the classification of actinomycetes has been considered in some detail¹¹⁾. In essence, numerical taxonomy involves the comparison of many phenetic characters of one organism with the same phenetic properties of other organisms. The extent of the similarity between test strains is then estimated using high speed electronic computers. Strains which share many features in common, that is, have a high percentage similarity, will cluster together and a classification can be derived from the groupings obtained. Initially, all characters are given equal weight, but once a numerical taxonomy has been realised, cluster-specific properties can be highlighted and weighted for identification. This approach contrasts with traditional practice in actinomycete systematics as taxa are defined on the basis of overall similarity, not on a small number of subjectively chosen morphological and staining properties. Extensive numerical taxonomic data bases are available for a number of actinomycete genera including *Actinomadura*,¹²⁾ *Mycobacterium*¹³⁾, *Nocardia*¹⁴⁾, *Rhodococcus*¹⁵⁾, *Streptoverticillium*¹⁶⁾ and *Thermomonospora*¹⁷⁾. Similar data bases have been devised for acidophilic¹⁸⁾, neutrotolerant¹⁹⁾ and thermophilic streptomycetes²⁰⁾.

Numerical taxonomy with its emphasis on many strains and tests remains the method of choice for circumscribing poorly classified actinomycetes. It can be important, however, to evaluate numerical taxonomies as over all similarities between strains can be distorted by factors such as test and sampling error²¹⁾. Ideally, following

numerical phenetic surveys, strains representing the range of variation encompassed by clusters should be examined using analytical techniques that cannot readily be applied to large numbers of strains. Chemotaxonomic, molecular and serological techniques have been used for this purpose¹¹⁾.

COMPUTER-ASSISTED IDENTIFICATION

It can be difficult to choose balanced strain sets for industrial screening programmes²²⁾ given the difficulty of separating established actinomycete taxa and recognising new ones. The problem is partly an historical one as the need to identify unknown actinomycetes has rarely been seen as a relevant task for industrial microbiologists. Consequently, current procedures for the identification of actinomycetes owe more to "custom and practice" than to any rational design. This situation is surprising and contrasts markedly with developments in diagnostic medical and veterinary microbiology.

Sound classifications with high information contents are needed for the construction of workable identification schemes. One of the advantages of the numerical taxonomic procedure is that it provides a pool of quantitative data on the test reactions of the strains within each of the numerically defined clusters. This is usually expressed as the percentage of strains within each cluster which show a positive state for each of the characters used to construct the classification (Table 2). When classification is complete, numerical taxonomic data bases can be searched and presumptive diagnostic tests identified. Tests shown to be reproducible can then be weighted and used to provide reliable information for the construction of dichotomous keys, diagnostic tables and computer probability matrices.

Table 2. Percentage distribution of positive characters to streptomycete species-groups defined by numerical classification*

Tests	<i>S. albidoflavus</i>	<i>S. chromofuscus</i>	<i>S. cyaneus</i>	<i>S. diastaticus</i>	<i>S. fulvissimus</i>	<i>S. griseomber</i>	<i>S. griseoviridis</i>	<i>S. lavendulae</i>	<i>S. lydicus</i>	<i>S. rochei</i>
Antimicrobial activity against:										
<i>Aspergillus niger</i>	32	0	10	11	22	0	33	75	100	27
<i>Candida albicans</i>	34	0	3	0	11	0	33	67	27	19
<i>Saccharomyces cerevisiae</i>	32	0	5	0	22	0	5	67	27	15
<i>Bacillus subtilis</i>	28	11	44	21	78	0	100	92	73	35
<i>Escherichia coli</i>	9	0	3	0	0	0	0	83	0	8
<i>Micrococcus luteus</i>	39	11	33	21	89	0	100	83	100	35
<i>Pseudomonas fluorescens</i>	4	0	0	0	0	0	0	50	0	8
<i>Streptomyces marinus</i>	39	22	62	5	100	22	80	100	100	39
Growth on sole carbon source:										
Adonitol	51	22	82	16	89	22	67	11	82	35
D-Melezitose	61	22	72	26	44	56	33	56	82	81
Raffinose	17	220	100	84	89	100	50	11	82	69
Trehalose	99	100	98	100	100	100	100	89	100	100
Growth on sole nitrogen source:										
L-Arginine	85	89	100	95	100	100	100	100	100	89
L-Cysteine	65	67	72	79	44	78	83	33	46	50
L-Histidine	65	78	85	68	100	100	83	100	36	77
L-Serine	83	100	100	95	100	89	83	100	64	85
Production of:										
β -Lactamase	69	25	87	90	40	22	33	92	40	36
β -Lactamase inhibitor	3	38	0	0	20	11	0	0	0	8

*Taken from Williams et al. [19].

Computer-assisted probabilistic identification schemes are more reliable than monothetic sequential keys and diagnostic tables²³⁾. Probability matrices derived from numerical taxonomic data bases contain the minimum number of characters needed to separate numerically defined clusters. Once formed, matrices can be used for the probabilistic identification of representative strains growing on isolation plates. Theoretically sound, workable computer-assisted procedures based on numerical classifications are available for slow-growing mycobacteria²⁴⁾ acidophilic actinomycetes, neutrophilic streptomycetes²⁵⁾ and streptovercillia²⁶⁾.

The theoretical basis of computer-assisted identification and its application to the identification of unknown streptomycetes have already been described^{27,28)}. The current streptomycete probability matrix²⁹⁾ is based on 23 clusters and 50 characters (Table 3) drawn from the 139 unit characters used to construct the sorted streptomycete data base¹⁹⁾. Identification of strains against the probability matrix has been achieved using three co-efficients from the MATIDEN program³⁰⁾.

1. Willcox probability. This is the likelihood of unknown character state values against a particular group divided by the sum of likelihoods against all groups; the closer the score is to 1.0, the better is the fit.

2. The taxonomic distance. This gives the distance of an unknown from the centroid of the group with which it is being compared; a low score indicates relatedness to the group and ideally is less than about 0.15.

3. Standard error of taxonomic distance. This assumes that the groups are in hyperspherical normal clusters. An acceptable score is less than about 2.0 to 3.0 and about half of the members in a cluster will have negative scores, that is, they are closer to the centroid than average.

Table 3. Characters selected for construction of the updated *Streptomyces* identification matrix*

Type of test	Tests and unit characters
Antibiosis	Inhibition of: <i>Aspergillus niger</i> LIV 131; <i>Bacillus subtilis</i> NCIB 3610; <i>Candida albicans</i> CBS 562; <i>Micrococcus luteus</i> NCIB 196; <i>Saccharomyces cerevisiae</i> CBS 1171; <i>Streptomyces murinus</i> ISP 5091
Antibiotic resistance	Neomycin sulphate Oleandomycin Penicillin G Rifampicin
Degradation	Allantoin Arbutin Elastin Pectin Xanthine
Enzyme activity	Hippurate hydrolysis Hydrogen sulphide production Lecithinase activity Lipolysis
Morphological	Aerial spore mass colour - grey or red Spore chain shape - rectus-flexibilis or spiral Substrate pigment colour - red-orange Diffusible pigment produced Diffusible pigment - yellow-brown Melanin production on peptone-yeast-iron and tyrosine agars
Nutritional	Utilisation of sole carbon sources - adonitol, dextran, inositol, mannitol, melezitose, melibiose, raffinose, rhamnose and xylitol Utilization of sole nitrogen sources - DL - α -amino-butyric acid, L-cysteine, L-histidine, L-hydroxyproline, L-phenyl-alanine and L-valine
Physiological	Growth in presence of phenol (0.1%, w/v), potassium tellurite (0.001%, w/v), sodium azide (0.01%, w/v), sodium chloride (7%, w/v) Growth at 45°C.

* Williams et al (29).

The MATIDEN programme and prototype streptomycete probability matrix²⁵) have been used both to identify unknown streptomycetes from soil²⁸) fresh water³¹) and marine habitats³²) and to evaluate the effectiveness of new selective isolation media³³). In these studies the following criteria were adopted for a successful identification:

1. A Willcox probability score greater than 0.850 with low scores for taxonomic distance and its standard error.
2. A first group score significantly better than those against the next best two alternatives.
3. A small number of characters of the unknown isolate listed as being atypical of those of the group in which it is placed.

In Newcastle, over 70% of the unknown streptomycetes examined have been identified to one of the clusters in the streptomycete probability matrix. An example of the print out obtained using the MATIDEN program is shown in Table 4. Examples to illustrate the range of scores obtained for identified and non-identified isoates are shown in Table 5.

Table 4. Example of the output provided by the MATIDEN program where an unknown soil isolate is identified using the Streptomyces identification matrix*

Input The Character Values of The Unknown

Reference Number of The Unknown: B1

Isolate B1 Best Identification: *S. griseoruber*

Scores for Coefficient: 1 (Willcox probability),
2 (Taxonomic distance), 3 (Standard Error of Taxonomic Distance)

	1	2	3
<i>S. griseoruber</i>	0.996	0.367	1.311
<i>S. rochei</i>	0.310×10^{-2}	0.443	2.141
<i>S. diastaticus</i>	0.329×10^{-3}	0.469	2.203

Characters against *S. griseoruber*: None

Additional Characters That Assist in Separating *S. griseoruber* from:

S. rochei: None *S. diastaticus*: None

*See Williams et al.²⁵⁾

Table 5. Examples of identification scores of unknown streptomycetes isolated from soil suspensions plated onto glucose yeast extract agar supplemented with actidione and rifampicin and incubated at 30°C for 3 weeks

Isolate Number	Identification	Identification scores		
		Willcox probability	Taxonomic distance	Standard error of taxonomic distance
1	<i>S. anulatus</i>	0.995	0.458	2.029
5	<i>S. anulatus</i>	0.989	0.461	2.108
6	<i>S. griseoruber</i>	0.999	0.367	1.311
10	<i>S. griseoruber</i>	0.996	0.367	1.311
31	<i>Streptomyces exfoliatus</i>	0.999	0.483	2.466
42	<i>S. diastaticus</i>	0.995	0.478	2.218
57	<i>S. rochei</i>	0.981	0.427	1.714
60	<i>S. rochei</i>	0.937	0.434	1.906
11	Not identified	0.473	0.546	4.083
15	Not identified	0.835	0.523	3.487
65	Not identified	0.767	0.464	3.983

FORMULATION OF SELECTIVE MEDIA

It has repeatedly been shown that differences in nutritional, physiological and antibiotic sensitivity profiles of actinomycetes can be exploited for the isolation of specific fractions of the actinomycete community from natural habitats^{6,7,8,9,34}). It is, therefore, surprising that media formulations have generally been developed on an empirical basis without regard either to the nutritional properties or tolerances of target strains to inhibitory compounds²⁸). Indeed, the logic underlying the composition of media such as colloidal chitin³⁵) and starch casein agars³⁶) is not clear. The selectivity of colloidal chitin agar was based on the view that virtually all streptomycetes had the capacity to hydrolyse this compound³⁷), yet only a quarter of the strains examined in the

numerical phenetic survey on *Streptomyces* were strongly chitinolytic¹⁹⁾. Similarly, starch casein agar supplemented with sodium chloride (4.6%, w/v) to reduce the growth of Gram-negative bacteria³⁸⁾ will not allow the development of streptomycetes unable to tolerate salt at this concentration¹⁹⁾.

Isolation media can be made more selective by the addition of antimicrobial agents that inhibit competing bacteria, including other actinomycetes. A variety of antimicrobial compounds have been used to good effect as they have promoted a reduction in the number of competitors on isolation plates with the consequent growth of resistant strains of actinomycete genera and species normally present in soil in small numbers. Suitable selective agents can be discovered accidentally or by design using numerical taxonomic data. The discovery that Diagnostic Sensitivity Test agar supplemented with tetracyclines was selective for *Nocardia asteroides*³⁹⁾ was based on numerical phenetic^{14, 40)} and antibiotic sensitivity studies⁴¹⁾ that had been carried out previously. Scanning percentage positive frequency tables for suitable selective agents was a logical extension of such work (Table 6). It subsequently became apparent that numerical taxonomic data bases were an ideal resource for the formulation of new selective isolation media as they contained extensive information on the biochemical, nutritional and physiological requirements of numerically circumscribed taxa^{28, 33)}. The formulation of the new media designed by these workers was assisted by the use of the DIACHAR programme⁴²⁾, which selects for the most diagnostic characters for individual clusters within probability matrices.

New media have been designed for the selective isolation of specific fractions of the acidophilic⁵⁰⁾, alkalophilic⁵¹⁾ and neutrophilic streptomycete populations^{28, 33)} of soil. Initial experiments were designed to select against members of the *Streptomyces albidoflavus* group which predominate on colloidal chitin and starch-casein agars. The data matrix (Table 2) showed that a medium based on raffinose and histidine as major carbon and nitrogen sources, respectively, might support reduced numbers of *Streptomyces albidoflavus*, while allowing the growth members of less commonly isolated streptomycete species. Representative isolates growing on control and test isolation media were identified using the computer probability matrix and identification programs described earlier.

Table 6. Antibacterial compounds used in selective media for the isolation of actinomycetes

Selective agent(s)	Actinomycetes selected	Reference
1. Discovered accidentally		
Bruneomycin	Actinomadura spp.	Prehazhenskaya et al. (43)
Nitrofurazone	Streptomyces spp.	Yoshioka (44)
Tellurite	Actinoplanes spp.	Willoughby (45)
Tunicamycin	Micromonospora spp.	Wakisaka et al. (46)
2. Discovered by design		
A. Visual scanning of sorted numerical taxonomic data bases		
Hippurate	Faenia rectivirgula	Mattinson-Rose (47)
Kanamycin	Thermomonospora chromogena	McCarthy and Cross (48)
Rifampicin (25°C)	Streptomyces atroolivaceus and S. diastaticus	Vickers et al. (33)
Rifampicin (30°C)	Actinomadura spp.	Athalye et al. (49)
Rifampicin (50°C)	Saccharomonospora and Thermomonospora spp.	Athalye et al. (49)
B. Selection from data bases using DIACHAR program		
Raffinose and histidine		
	Streptomyces chromofuscus, S. cyaneus and S. rochei	Vickers et al. (33)
Rifampicin	Streptomyces anulatus, S. exfoliatus and S. griseoruber	Goodfellow, unpublished data

EVALUATION OF NEW SELECTIVE MEDIA

It is important to evaluate new media formulations. In the case of the neutrotolerant streptomycetes the employment of objectively designed media, and the subsequent identification of isolates, allowed clear qualitative differences to be detected in streptomycetes derived from the same sand dune sample (Table 7). Comparison of the results with information in the data matrix (Table 2) make it possible to determine whether an observed increase or decrease in numbers of particular streptomycetes on the new media, compared with those on the control plates, was mainly due to selection by media constituents or to the effects of competition.

Table 7. Computer-assisted identification of streptomycetes isolated from a sample of sand-dune soil using three different selective media*

Species	Percentage of total isolation on		
	Starch-casein medium	Raffinose- histidine medium	Starch- casein medium and rifampicin
<i>S. albidoflavus</i>	6.5	0	13.3
<i>S. chromofuscus</i>	2.2	0	0
<i>S. cuaneus</i>	28.3	63.6	0
<i>S. diastaticus</i>	0	2.3	80.0
<i>S. ludicus</i>	37.0	4.5	0
<i>S. rochei</i>	8.7	13.6	6.6
Unidentified isolates	17.4	15.9	0

*Taken from Williams et al.²⁸

It is evident from the data base that *Streptomyces cyaneus* strains can use raffinose and histidine as sole carbon and nitrogen sources, whereas, relatively few strains of *Streptomyces albidoflavus* are able to do so. Competition on selective isolation plates may explain the reduction in the number of strains of other groups, notably *Streptomyces lydicus*, on the raffinose-histidine medium. Conversely, the isolation of large numbers of *Streptomyces diastaticus* strains on the starch casein medium supplemented with rifampicin suggested that competition had previously excluded this group from both control and raffinose-histidine media. The results of this and other studies⁵⁰⁾ underline the value of using objectively formulated media in the continuing search for rare and unusual actinomycetes. Indeed, it seems likely that a broad array of selective media will have to be used if representative samples of actinomycete communities are to be isolated from natural habitats. It is equally clear that the concept of the all-embracing non-selective medium can no longer be sustained.

The importance of evaluating selective isolation procedures was recently demonstrated in Newcastle when representatives of twelve colony types growing on glucose yeast extract agar plates seeded with dilutions of heat pretreated air-dried soil were examined. All seventy representatives of the colony types contained chemical markers consistent with their assignment to the genus *Streptomyces* even though the isolation procedure was designed for the selective isolation of *Actinomadura* strains from the natural environment⁴⁹⁾. Over half of the isolates were found to represent *Streptomyces* species (Table 5) considered to be uncommon in soil. Clearly, selective isolation media can be refined in light of such evaluation exercises.

CONCLUSIONS

The ability to isolate and characterise large numbers of rare, unusual and novel actinomycetes has clear implications for industrial screening programs, especially when it has been shown that particular bioactive compounds are associated with taxa defined on the basis of overall similarity (Table 2). Until recently, the generation of extensive numerical taxonomic data bases and the identification of large numbers of strains were labour intensive and time-consuming exercises. It is, however, now possible to assemble comprehensive data bases using automated procedures and there is some evidence that diagnostic enzymes can be rapidly detected using fluorogenic probes prepared from 4-methylumbelliferone⁵²⁾ and 7-amino-4-methylcoumarin⁵³⁾.

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