Enzyme-linked Immunosorbent Assay (ELISA) as a Means of Taxonomic Analysis of *Streptomyces* and Related Organisms

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Fourteen *Streptomyces* strains from various numerical taxonomic classes and representatives of three other genera of actinomycetes were studied using an indirect enzyme-linked immunosorbent assay (IND-ELISA) to determine their serological relationships. The IND-ELISA results agreed with those from previous numerical taxonomic analyses and Ouchterlony double-diffusion studies. The IND-ELISA method is quicker, more quantitative and less subjective than Ouchterlony assays and thus should be useful in *Streptomyces* taxonomy. The results indicated that *Frankia* sp. CpII was related to *Streptomyces*.

INTRODUCTION

Three major approaches have been used to study the taxonomy of *Streptomyces*: morphology and pigment production, numerical taxonomy and nucleic acid hybridization. Identification of *Streptomyces* has generally relied on morphology and pigment production. This is subjective in many cases, especially where there is variation in the growth media used and the point in the growth cycle that examination takes place. Numerical taxonomy is more reliable as it uses a wide variety of criteria, but much work is needed to create a comprehensive data base. Nucleic acid hybridization (DNA–RNA or DNA–DNA) requires the use of radiochemicals, the careful preparation of nucleic acid and a high degree of control of hybridization conditions (Mordarski *et al.*, 1981). In a few cases, serology has been used to study the taxonomy of *Streptomyces* and related species with some success (Ridell, 1981; Ridell & Williams, 1983).

Serological techniques allow direct study of the proteins produced by the various *Streptomyces* and therefore should allow quantification of the divergence of these proteins between species. In previous studies using serological techniques, Ouchterlony double-diffusion assays have been used to measure the major protein similarities of *Streptomyces* species (Ridell & Williams, 1983). This approach has three disadvantages. Firstly, only the major soluble antigens can be studied and any minor components are ignored. Secondly, the assay is semiquantitative as only the number of major antigens detected is counted and not their degree of cross-reaction. Finally, the identification of precipitin lines and spurs is subjective and difficult to reproduce in a reaction involving a very complex antigen mixture such as that from total soluble bacterial cell contents. Moreover, the Ouchterlony technique is far less sensitive than indirect enzyme-linked immunosorbent assay (IND-ELISA), which has the added advantage of being usable for membrane bound or insoluble antigens as well as for soluble ones.

IND-ELISA involves the coating of the plastic wells of an ELISA plate with the antigen to be tested, the binding of a known antibody to its specific antigen in the coated well and then detection of the binding by an enzyme-linked second antibody and a specific substrate reaction. The result is a direct quantitative analysis of the amount of antibody bound to the coated antigen. In this study we investigate the potential of IND-ELISA as a taxonomic tool.

Abbreviation: IND-ELISA, Indirect enzyme-linked immunosorbent assay.

METHODS

Test strains. The strains used are described in Table 1. These represent 11 clusters as defined by Williams *et al.* (1983). A strain of *Agrobacterium tumefaciens* was included as a control to measure non-specific antibody binding. It was chosen as a soil bacterium of distant taxonomic relatedness.

Antigen preparation. The strains were inoculated into 100 ml Malt-3 liquid medium (Kirby & Lewis, 1981) and incubated for 3–5 d at 30 °C on a rotary shaker. The cells were harvested by centrifugation at 10000 *g* in a Sorvall GSA rotor for 15 min. The pellet was washed twice with 0.1 M-sodium phosphate, 0.15 M-sodium chloride, pH 7.4 (PBS), then sonicated using an MSE Soniprep 150 fitted with a microprobe. This was done on ice for a total of 2 min in 20 s bursts with 20 s gaps for cooling. The procedure effectively disrupted most of the bacteria. The sonicate was filtered through a 0.4 µm Millex low binding filter. Protein in the sample was measured by the Lowry method and diluted to 1 mg ml⁻¹ in 20% (v/v) glycerol and frozen at -70 °C.

Antisera preparation. Antisera were prepared for *Streptomyces griseus* and *Streptomyces cattleya*. Antigen suspensions (10 mg protein ml⁻¹) were emulsified as a mixture 1:1 (v/v) with Freund's incomplete adjuvant and 1 ml was injected into a rabbit's thigh muscle at 3 week intervals. Blood (20 ml) was taken at weekly intervals from the marginal ear vein starting 3 weeks after the beginning of the course of injections.

IND-ELISA. Polystyrene microtitre plates (Greiner) were coated with twofold dilutions of the antigen samples in PBS, for 2 h at 37 °C, starting with a standardized protein concentration of 1 mg ml⁻¹. In all tests, antigens from *Streptomyces griseus* ISP 5236, *Streptomyces cattleya* C2201, *Frankia* sp. Cp11, *Streptovorticillium griseocarneum* ATCC 12628, *Rhodococcus corallinus* and *Agrobacterium tumefaciens* were included as controls. The plates were washed by soaking for 5 min in three changes of PBS + 0.05% (v/v) Tween 20, and blocked with PBS/Tween + 0.5% (w/v) bovine serum albumin (Sigma) for 2 h at 37 °C. The plates were filled with a 1:100 dilution of the appropriate antiserum in PBS/Tween/BSA for 1 h at 37 °C. After washing, goat anti-rabbit antibody conjugated to alkaline phosphatase (1 mg antibody ml⁻¹; Miles Yeda) was added to the wells at a dilution of 1:1000 and incubated for 1 h at 37 °C. The alkaline phosphatase bound in the microtitre wells was assayed with 1 mg *p*-nitrophenyl phosphate ml⁻¹ (Sigma) in 10% (w/v) diethanolamine, pH 9.8. The *p*-nitrophenol released was measured after 1 h at 405 nm on a Titertek Multiskan microtitre plate reader (Flow Laboratories) relative to the lane containing the dilution series of the control *A. tumefaciens* protein. Absorbance data were first standardized by taking the homologous absorbance reaction value as indicating 100% similarity and the *Agrobacterium* antigen absorbance as indicating 0% similarity. The standardized data from individual plates could then be directly compared as a percentage of the homologous reaction and this was taken as the similarity using this ELISA system.

RESULTS AND DISCUSSION

S. griseus, *S. cattleya*, *Frankia* sp. Cp11, *Stv. griseocarneum* and *R. corallinus* were included in all ELISA plates. In all cases, the duplicate readings for each of these species were within 6% of the mean for each species with a given antiserum. As the protein concentration of all samples was standardized, the reading obtained between plates was consistent throughout all the samples tested. Table 1 shows the similarity of the strains tested by ELISA using the antisera obtained to the reference strains. No cross-reaction was obtained with the *A. tumefaciens* protein, and *R. corallinus* gave a consistently low reaction of <10%. Therefore, the level of response obtained with the ELISA with any given species measures the specific common antigens present in the test strain and the reference strain. A consistent series of decreasing similarities were obtained, which correlated with other classifications of these strains.

Classification of *Streptomyces* by numerical taxonomy places most of the strains tested in different clusters. The $S_{SM}/UPGMA$ value ranges of the test strains compared to the mean IND-ELISA similarities were as follows: the >81% $S_{SM}/UPGMA$ range had a mean IND-ELISA similarity of 79%; the <81% to >77.5% range had a mean similarity of 71%; the <77.5% to >70.1% range had a mean similarity of 43%; while the <70.5% range had a mean similarity of 34%. There was a strong correlation between the two systems, supporting the conclusions of Ridell & Williams (1983), who used Ouchterlony double diffusion assays. However, the IND-ELISA test is rapid and can be easily mechanised, and should prove to be a more reliable and reproducible system for such studies. The results for *S. rimosus* in the IND-ELISA tests agree with the distinctness of the *S. rimosus* cluster as emphasized by Williams *et al.* (1983) and Alderson *et al.* (1985).

Both *Stv. griseocarneum* and *Frankia* sp. Cp11 showed a distinctly higher similarity to *S. griseus* and *S. cattleya* than did *R. corallinus*. This might be expected as *R. corallinus* has almost

Table 1. Bacterial strains and properties

Species	Cluster group	Cluster no.*	Strain no.†	Antibiotic produced	Percentage similarity to:‡	
					<i>S. griseus</i>	<i>S. cattleya</i>
<i>Streptomyces griseus</i>	A	1B	ISP 5236	Streptomycin	100	35 ± 2
<i>S. bikiniensis</i>	A	1B	ISP 5235	Streptomycin	83 ± 5	34 ± 3
<i>S. alboniger</i>	A	1B	ISP 5043	Puromycin	75 ± 5	33 ± 4
<i>S. halstedii</i>	A	1C	ATCC 10897	—	73 ± 3	37 ± 6
<i>S. olivaceus</i>	A	1C	ATCC 3335	β-Lactam	69 ± 4	56 ± 5
<i>S. fulvoviridis</i>	A	3	ATCC 15863	β-Lactam	42 ± 4	39 ± 5
<i>S. venezuelae</i>	A	6	ATCC 10712	Chloramphenicol	57 ± 3	33 ± 3
<i>S. lipmanii</i>	A	7	C1940	β-Lactam	30 ± 5	28 ± 5
<i>S. aureofaciens</i>	A	14	ATCC 10762	β-Lactam	54 ± 5	20 ± 3
<i>S. chartreusis</i>	A	18	ATCC 14922	β-Lactam	38 ± 3	27 ± 4
<i>S. lividans</i>	A	21	ISP 5434	—	45 ± 5	78 ± 4
<i>S. hygroscopicus</i>	A	32	ATCC 31039	β-Lactam	59 ± 4	27 ± 4
<i>S. rimosus</i>	B	42	ATCC 10970	Tetracycline	27 ± 4	45 ± 5
<i>S. cattleya</i>	C	47	C2201	β-Lactam	35 ± 2	100
<i>Streptoverticillium griseocarneum</i>	F	55	ATCC 12628	Hydroxy-streptomycin	41 ± 4	31 ± 3
<i>Frankia</i> sp.	NA	NA	CpII	—	27 ± 2	29 ± 2
<i>Rhodococcus corallinus</i> §	NA	NA	UCT	—	7 ± 2	9 ± 2
<i>Agrobacterium tumefaciens</i>	NA	NA	UCT	—	0	0

NA, Not applicable.

* According to Williams *et al.* (1983), except for *S. hygroscopicus*, which was not the strain they used.

† ISP, International Streptomyces Project; UCT, University of Cape Town stock collection; C1940 and C2201 kindly provided by Glaxo Research.

‡ The similarities to *S. griseus* and *S. cattleya* were measured by IND-ELISA using antiserum to these species and an *A. tumefaciens* control as described in Methods. The results are means of 3 determinations ± SD.

§ *Nocardia corallina* of Kirby & Usdin (1985).

no mycelial development and would thus seem to differ from the other strains tested, which have highly developed mycelia. *Stv. griseocarneum* is classified within the main group of *Streptomyces* by IND-ELISA although with both antisera it showed a similarity approaching that of the least similar *Streptomyces* species. Ridell & Williams (1983) found that the two species of *Streptoverticillium* tested had two precipitin lines in common with a number of *Streptomyces* species. Thus both techniques indicate that the genera *Streptomyces* and *Streptoverticillium* are related but distinct (Williams *et al.*, 1985).

Frankia sp. CpII was included in this study because little is known about the taxonomy of this genus, and it was hoped that IND-ELISA might facilitate the study of the relatedness of *Frankia* spp. directly in their symbiotic nodules without the need to grow the organism in pure culture (Becking, 1981). The IND-ELISA results showed that *Frankia* sp. CpII could be classified within *Streptomyces*. However, the similarity was only 27–29% with the test antisera and below that of *Stv. griseocarneum*. Antiserum raised to *Frankia* sp. CpII showed a 62% similarity with *S. griseus* ISP 5236, 45% similarity with *Micromonospora chalicea* ATCC 12452, 37% similarity with *Stv. griseocarneum* ATCC 12628 and 6% similarity with *R. corallinus*. This suggests that this species is closely related to *Streptomyces* but nevertheless is a member of a distinct genus.

A number of the strains used produce β-lactam-type antibiotics. It is interesting that no correlation was obtained between the β-lactam-producing *Streptomyces* studied using the antiserum to *S. cattleya*, even though this species was taxonomically distinct from most of the strains tested. Thus, the majority of antigens in common between β-lactam-producing *Streptomyces* must relate to their being *Streptomyces*, only a minor proportion being due to proteins related to β-lactam production. Therefore, the direct application of IND-ELISA with antibodies to whole cell extracts as a screening method for novel β-lactam producers is not feasible.

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