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Rapid identification of *Streptomyces* spp. on potato, the key to integrated management of common scab

PT006

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This report is published by the Horticultural Research and Development Corporation to pass on information as to horticultural research and development undertaken on rapid identification of Streptomyces spp. on potato, the key to integrated management of common scab.

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SYNOPSIS

The aim of this project was to develop a rapid identification technique for pathogenic strains of the potato common scab organism and involved accurately identifying the causal organism before evaluating techniques which would detect levels of the pathogen in the soil environment capable of causing significant disease.

Methods used to identify the pathogen included selective media, scanning electron microscopy, electrophoresis and toxicity of antibiotics. The pathogen was identified tentatively as the actinomycete *Streptomyces scabies* but all methods were unable to differentiate between isolates from scab lesions of varying pathogenicity.

A number of techniques were considered in developing the rapid identification test. DNA probe technology is under development and has potential as a rapid identification tool. However commercialisation of this technology would be expensive and not commercially available for some time. The ELISA test, based on antisera from four pathogenic isolates, was successful in detecting *S. scabies* in infested soil. However, field testing is required to prove accuracy and sensitivity of the test before the method can be used as a predictor for scab. Fluorescent antibody microscopy uses similar technology to that of the ELISA test. This method was not evaluated but it may have potential if the ELISA antiserum proves sensitive and selective. A selective medium for actinomycetes in soil suspension and media for production of microtubers invitro were identified as future options for isolating potential pathogens and establishing pathogenicity more quickly. This has potential in varietal screening for disease resistance.

PROJECT INTRODUCTION

Common scab is a soil borne disease of potatoes causing corky lesions on the surface of the tuber. In recent seasons there has been an increase in the incidence and severity of this disease on the Russet Burbank cultivar which is the preferred french fry processing variety. There is concern that the disease will spread and possibly infect large areas of productive land.

The aim of this project was to develop a rapid identification test for the causal organism of this disease, *Streptomyces scabies*. Such a rapid test would be used to monitor levels of this organism in the potato growing soils of the North West coast. The identification test would also be useful in epidemiological studies to determine which conditions favour or inhibit the growth of the organism. This would enable the research to be carried out to determine how farming methods can be modified to prevent significant losses from this disease.

It was important that the organism causing common scab in Tasmania be accurately identified. To focus on the identity of *Streptomyces* spp. likely to be involved a review of group taxonomy and its current status was undertaken and is reported in the next section.

CHARACTERISTICS OF THE CAUSAL ORGANISM

INTRODUCTION

Common scab disease of potatoes in Tasmania has traditionally been confined to crops grown without irrigation. Dry soil conditions are known to favour the disease. After the widespread adoption of irrigation, incidence of the disease declined until its recent resurgence on the Russet Burbank cultivar.

The occurrence locally of severe scab symptoms on a variety which is recorded in its country of origin (USA) to be tolerant of the disease is baffling. It was considered essential to investigate and characterise the Streptomycete causing scab symptoms in Tasmania to ensure that no other organism or groups of organisms were contributing to disease.

A review of literature revealed that the taxomony of the genus *Streptomyces* and the species *Streptomyces scabies* is far from defined, with the scientific community unable to reconcile current descriptions with historical records and dispute over a type strain for *S. scabies*.

A number of Streptomycete isolates were obtained from scab infected potatoes from different sources. Steps were taken to prove pathogenicity of isolates and to identify those organisms causing disease. Several techniques were used to separate strains or species based on pathogenicity results.

HISTORICAL REVIEW

The common scab disease of potatoes, which is characterised by corky lesions on the tuber surface, is caused by Streptomycetes that historically have been referred to as "*Streptomyces scabies*". However, other *Streptomyces* species can also produce scab. These have been assigned to other species by various researchers or left unassigned. Acid tolerant strains have been described and assigned to a distinct species *Streptomyces acidiscabies*.

Organisms causing common scab were first isolated in 1890 in Connecticut, USA, by Thaxter. The methods to demonstrate bacterial causation of disease had been developed by Robert Koch in 1876 with the disease anthrax. It is likely that Thaxter would have used Koch's methodology to determine which micro-organism was the cause of common scab; firstly, microbes would have been isolated from scab lesions and grown up in pure culture, then these cultures would be used to inoculate potato plants growing in sterile soil. Most potato plants would produce healthy and unblemished tubers, but some isolates gave rise to scabbed tubers once more. Thaxter gave this microbe the name *Oospora scabies* and

described it as : hyphae 0.6 - 1.0μm in diameter, curving irregularly, septate, branching. Aerial hyphae white with grey spores borne in spiral chains and producing a melanin pigment in the media on which it is grown.

The species was renamed *Actinomyces scabies* by Gussow and then *Streptomyces scabies* by Waksman and Henrici in 1948. Waksman redescribed the species, and designated a type strain, ie a strain showing the typical characteristics of the species used for reference. Waksman (1950) outlines a number of studies that led to the conclusion that more than one species is involved in the causation of this disease.

Shirling and Gottlieb (1966) developed a protocol to standardise methods for the International *Streptomyces* Project (ISP). Waksman's type strain represented *S. scabies*. However, Waksman's choice of type strain was in error according to Lambert and Loria (1989). The uncertainty which developed over the taxonomy resulted in the species being considered invalid, listed in Bergey's Manual of Determinative Bacteriology 8th Edition as : `type strain not extant, many taxonomically different strains available`.

In 1989, Lambert and Loria from Cornell University submitted a paper reviving the species as *Streptomyces scabies* and designating Thaxter as the original authority. They state: "In our opinion, the original descriptions of *S. scabies* are correct and have been consistently substantiated by those workers familiar with phytopathogenic *Streptomyces*. Strains belonging to this species form a coherent group and are easily distinguishable from other pathogenic strains by numerous characteristics". Lambert and Loria chose a type strain ATCC 49173. The major characteristics of this species are smooth grey spores borne in spiral chains, melanin production and usage of all ISP sugars. Lambert and Loria consider this group to be the predominant species causing common scab with strains from the north eastern United States, Atlantic, Canada and Hungary being investigated.

Lawrence, Clark and King (1990) have proposed the mechanism by which *Streptomyces scabies* induces the development of scab on the tuber. They have isolated phytotoxins they call thaxtomin A and B which will produce scab in cell-free extracts. Thaxtomin production seems to only occur when the scab organism is in contact with the potato plant, it does not occur in culture media.

METHODS AND RESULTS

The plan for the conduct of this project had three parts:

1. Isolation of the causal organism from infected tubers.
2. Pathogenicity of isolates.
3. Identification and characterisation of isolates.

1. Isolation of the causal organism from infected tubers.

The classical methods of microbiology were developed in the late 19th century. Media were developed solidified with gelatin or agar over which a bacterial culture could be streaked. The different bacterial colonies which appeared could be purified by isolating a colony and repeating the streaking process using sterile techniques. These techniques were used to isolate the common scab organism.

Streptomyces scabies is characterised by the production of a melanin pigment on media containing tyrosine. At first, problems were encountered in finding a medium on which this organism would grow and produce melanin pigment. The tyrosine agar of Waksman (1950) was used without success. It was considered that a non-melanin producing Streptomycete might have produced the scab, but there was no consistent appearance of any one species from diseased tissue.

The glycerol-asparagine-tyrosine (GAT) medium of the International Steptomyces Project (Shirling and Gottlieb, 1966) was found to be successful and colonies producing melanin were isolated.

The isolation method of *S. scabies* was carried out as follows. A thin sliver of tissue was excised from the scab infection site on the potato tuber. The tissue was ground in a mortar with a small amount of sterile water. Five drops of ground tissue was added to 10ml of sterile water or to 10ml of a mixture of lactophenol and water (1:140). The lactophenol helped to reduce the growth of contaminants. A dilution series would be carried out and 2ml of the material at the 10^{-3} or 10^{-4} dilution would be plated onto GAT agar. The agar plates were incubated at 25°C for 2 weeks and the resultant colonies examined.

Thirty-two isolates were obtained from scab infected potatoes from a number of sources. These isolates may represent more than one species, or possibly different strains of the one species varying in their degree of pathogenicity on potato.

2. Pathogenicity of isolates.

The pathogenicity of the actinomycete isolates was determined by inoculating young potato plants and examining the development of scab symptoms on the subsequent tubers.

The thirty-two colonies producing melanin, isolated from scab tissue, were subcultured onto potato dextrose agar (PDA) and incubated for two weeks at 25°C. Colonies were subcultured again, half onto a yeast extract/malt extract slope and half into a flask of yeast extract/sugar broth. The broth culture was used to inoculate soil for the pathogenicity testing and the slope culture kept for later serological testing.

Pathogenicity testing was begun in December with 27 isolates being investigated. Each broth culture was divided into three replicates and mixed with pasteurised potting soil. Rooted stem cuttings and tissue cultured plantlets were planted in pots with the inoculated soil. These were grown in a shadehouse under a regular watering regime. Tubers were harvested after twelve weeks. Eleven isolates showed no pathogenic effect. Ten isolates consistently produced scab on the tubers. Six isolates produced scab on only one of the

replicates. These were referred to as 'weakly' pathogenic. The remaining five isolates became contaminated in broth culture and were unacceptible for inoculation

Table 1. Results of pathogenicity tests on isolates.

Isolate	Scab production (Number of replicates)
9	Scab in 1 replicate
12	Scab in 3 replicates
13	Scab in 2 replicates
15	Scab in 3 replicates
16	Scab in 3 replicates
17	Scab in 3 replicates
18	Scab in 2 replicates
19	Scab in 3 replicates
20	Scab in 3 replicates
24	Scab in 1 replicate
25	Scab in 1 replicate
27	Scab in 3 replicates
28	Scab in 1 replicate
29	Scab in 1 replicate
30	Scab 1 replicate
32	Scab in 3 replicates

Isolates 1 - 5, 7, 10, 14, 23, 26 and 31 induced no scab symptoms on plantlets. Remaining isolates were contaminated and unacceptable for use as inoculant.

Four isolates chosen for the immunological part of the work were 12, 20, 27 and 32.

3. Identification and characterisation of isolates.

A number of methods were investigated to determine; i) the identity of the *Streptomyces* species and ii) the presence or absence of strains, based on morphological or physiological characteristics which would account for differences in pathogenicity of isolates.

A. Colony Morphology

The morphology of the colonies closely agreed with the description of *Streptomyces scabies* of Thaxter and Lambert and Loria, as outlined earlier.

B. Electron Microscopy

Scanning electron microscopy showed the ultra-structure to agree with the literature description of *S. scabies*; smooth grey spores borne in spiral chains. The typical spore chain morphology and spore ornamentation are shown in the photographs. (Plate I)

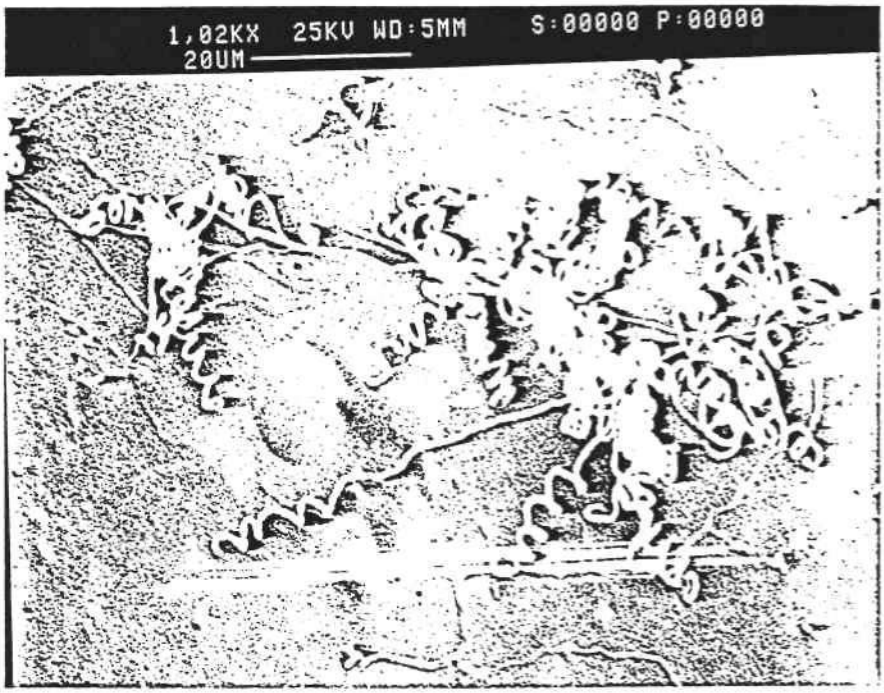
C. Electrophoresis

Electrophoresis is a technique which separates proteins (in this case the extra cellular enzymes) into their components by passing an electric current through a gel coated plate on which the enzymes are spotted. Components of proteins move at different rates through the gel, and their position is determined by their electric charge and molecular size. The result is a gel with stained bands marking the position of the enzymes. This technique has been used to characterise some fungal groups, including Rhizoctonia and Penicillium.

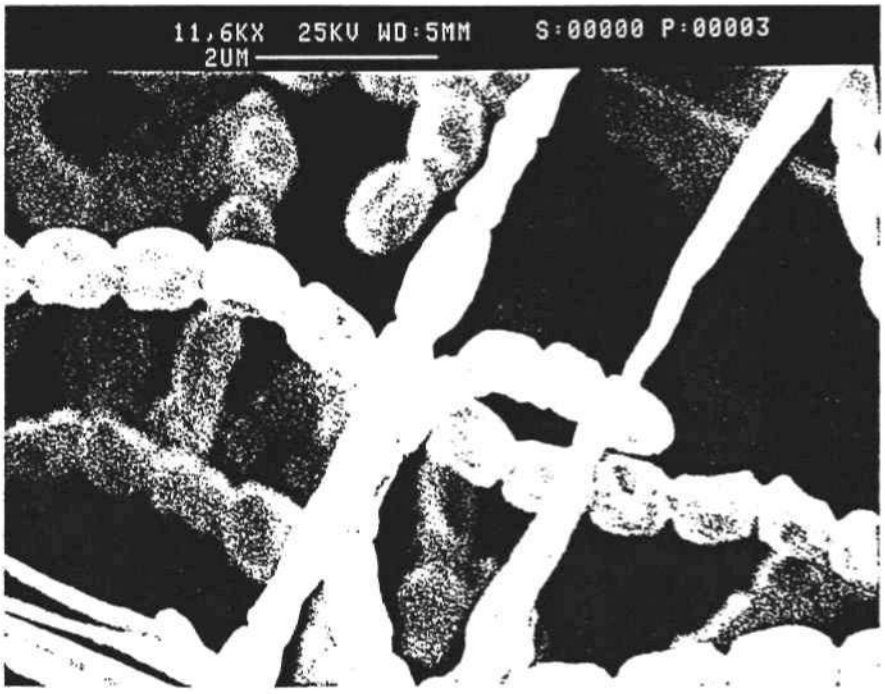
However, the technique could not successfully distinguish differences in pectinase, amylase or tyrosinase enzymes of the isolates. Insufficient quantities of extracellular enzymes may have been produced by the Streptomycete isolates to be detected by this method.

The result of one test for amylase enzymes is shown in the photograph (Plate II). The gel is stained with iodine which reacts with the starch to give a blue colour, the clear areas show where amylase enzymes have broken down the starch.

PLATE 1

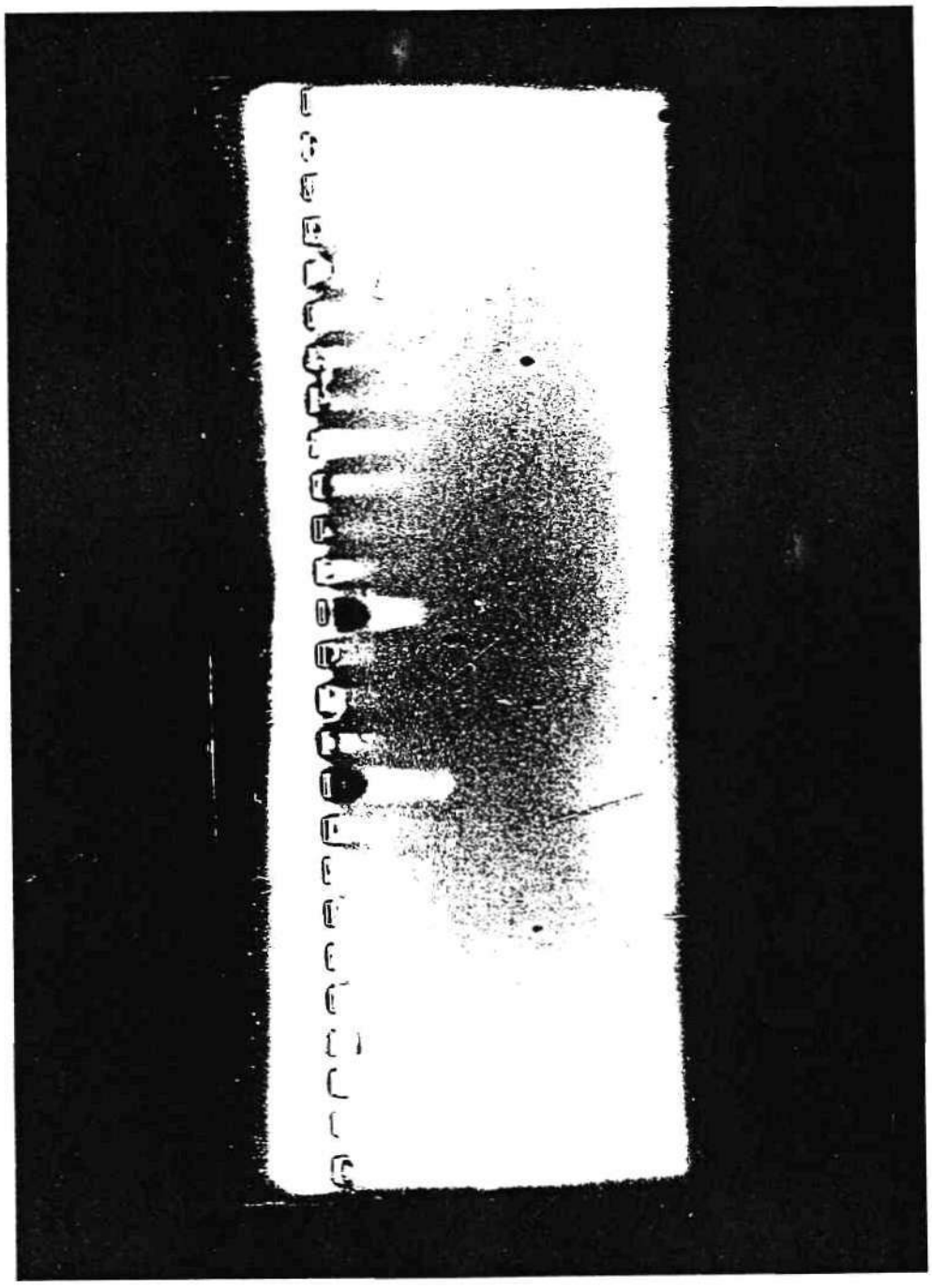


a. Typical spore chain morphology of *Streptomyces scabies*



b. Spore ornamentation of *S. scabies*

PLATE 2



Electrophoretic zymogram for amylase enzymes in isolates of Streptomyces ex potato

D. Toxicity of Antibiotics

The toxicities of antibiotics were determined on modified Bennett agar (per litre; 1g beef extract, 1g yeast extract, 2g tryptone, 10g glycerol, 15g agar).

Antibiotics and ammendments were chosen to specifically differentiate isolates into either *S. scabies* or *S. acidiscabies* species according to Lambert & Loria (1989 (a),(b)). Results are summarised in Table 2.

According to Lambert and Loria, isolates 27 and 17 are closest to *S. acidiscabies*, while sodium chloride reactions of isolates 15 and 29 align then with a non-typical '*S. scabies*'. Most variation between isolates occurs in the strength of growth on streptomycin and the lack of growth on phenol ammended agar. However most isolates appear more closely allied to the 'acidiscabies' group than the 'scabies'. This is consistent with field observations of severe scab symptoms in potato crops in soils of pH 5.3, but not with common culture morphologies which are typically that of 'scabies'.

E. Carbon Utilisation

The utilisation of carbon sources by the isolates will be investigated when the carbon source plates are available. The carbon source plates are a new product and have not been delivered in time to be included in this report. These plates may be of use in distinguishing between the isolates and between *Streptomyces* species.

DISCUSSION AND CONCLUSIONS

Most evidence points to *Streptomyces scabies* being the identity of the scab causing organism in the soils of Northwest Tasmania. However, it is not possible to be certain, and the identity of the scab organism, on present evidence, is yet to be proved.

Table 2. Characterisation of species by use of agar ammendments

Isolate	Penicillin 10 IU/ml	Phenol 0.1 %	Sodium Chloride 7 %	Crystal Violet 0.5μg/ml	Streptomycin 20μg/ml
S scabies *	-	-	-	-	-
S acidiscabies^	+	+	-	+	+
3,10,25,30 19,31,24,14 18,16,20,26	++	-	-	++	+
22, 8, 7	++	-	-	++	++
27	++	+	-	++	+
17	++	+	-	+	+
15	++	-	+	++	+
29	+	+	+	+	-

* Lambert & Loria 1989 (a)

^ Lambert & Loria 1989 (b)

DEVELOPING A RAPID IDENTIFICATION TEST

INTRODUCTION

Four techniques were identified as having a possible role in the detection of *Streptomyces scabies* in soil:

1. DNA probe
2. ELISA (Enzyme-linked Immunosorbent Assay)
3. Flourescent antibody microscopy
4. Combination high and low technology tests

These techniques will be outlined in the light of research undertaken in this project. Their potential role in a rapid identification test for pathogenic *Streptomyces* spp will be discussed and recommendations made at the conclusion.

1. DNA Probe

INTRODUCTION

DNA probe technology is a new and powerful tool for detecting micro-organisms in the environment. Professor E Stackebrandt, of the Microbiology Department, University of Queensland, St Lucia, heads a research team which is at the cutting edge of this technology. Professor Stackebrandt and his team are undertaking a major project looking at the taxonomy of the *Streptomyces* group.

DNA probe technology is based on sequence data from the 16 S sub unit of the organisms ribosomal RNA (rRNA). Sequence data from the 16 S sub unit of the rRNA is collected from as many species of the genus as possible and compared. Much of the RNA sequence

is highly conserved and identical for all species. Stackebrandt found three particular sites where a greater degree of variation was present. This variation differentiated some species of the genus *Streptomyces* from others. Within a species the sequence should not vary.

A DNA probe is developed by manufacturing the DNA sequence with the complementary code to the rRNA sequence of the species of interest. The DNA probe is the match of the rRNA sequence and will bind tightly to the rRNA of that species. It is normally 16 - 20 bases long:

ie *S. scabies* 4 code = GCA UCA GAG AUG GUG CC

A radioactive label is attached to the probe to enable detection of RNA-probe conjugates by exposure of X-ray film.

METHODS

DNA probe methodology is appended in the study tour report of my visit to the University and Professor Stackebrandt's laboratory (Appendix 1).

RESULTS

Twenty isolates were sent to Professor Strackebrandt for identification via one of the DNA probes available for *S. scabies*. The probe 4 was designated the 'scabies' probe because it was developed from a 'type strain' of *S. scabies* housed in a German collection (Strain No. DSM 40078)

Three of the 20 isolates reacted positively to the probe indicating that they were of the same (or related species) as the German type strain. This result points to a number of *Streptomyces* spp. causing common scab and underlines the lack of knowledge of the disease.

DISCUSSION AND CONCLUSIONS

DNA probes have the potential to provide a useful tool in identifying soil microorganisms *in situ*. However the development of a DNA probe requires a research project to be established where the appropriate facilities exist ie. University of Queensland. Later, it would require establishment of a laboratory for processing of samples. Future prospects are outlined in the report at Appendix 1.

There is an opportunity for high quality research on the disease common scab. The facilities are available, and the expertise of a team of researchers is available and the genus *Streptomyces* is their primary research interest. However it rests with the potato industry to pursue with Professor Stackebrandt options for funding this high technology project.

2. The ELISA Technique

INTRODUCTION

ELISA or Enzyme Linked Immuno-Sorbent Assay is a process whereby antibodies to a specific substance or organism are used to identify that same substance in a highly sensitive and specific *invitro* test. In the present project, suspensions of one of four chosen Streptomyces isolates were injected into experimental rabbits. Antibodies were purified and used in the test. Plastic plates were coated with rabbit antibody, the test substance introduced and washed away leaving only compatible molecules tightly bound to the plate via the antibodies. A sandwich was then formed with introduction of more antibodies, this time joined with an enzyme which catalyses a colour change in the presence of an introduced substrate. The intensity of the colour was directly proportional to the number of enzyme molecules bound to the test substance, antibody and plate.

The technique is widely used to detect virus diseases in many hosts, but is only slowly being adapted for detection of fungal and bacterial pathogens.

The ELISA technique was examined in detail, within the context of this project, because of the expertise and facilities available at the New Town Research Laboratories of the Department of Primary Industry, Tasmania, in Hobart.

METHODS

Preparation of antisera

Production of antisera for the development of an ELISA test was begun on 10 April. After consultation with the Animal Ethics committee of the University of Tasmania, four rabbits were purchased. Four pathogenic isolates were chosen (12, 20, 27 and 32) and each rabbit was injected with one of these isolates.

The rabbits were given a code.

Rabbit	Code number	Isolate
White	5	32
Grey	6	20
Dapple	7	27
White	8	12

The rabbits were injected twice more at four week intervals.

A twenty millilitre aliquot of blood was taken from the ear vein of each rabbit on 3 July, before the rabbits were then given away as pets. The serum component of the blood was separated by centrifugation and preserved by mixing with an equal volume of glycerol. The gamma globulin component of the serum was separated by precipitation and purified by dialysis and then by filtration through a cellulose column. The gamma globulins were detected using a spectrophotometer at an Optical Density of 280. They were found to be of

high titre and were diluted until the spectrophotometer gave a reading of 1.4 which corresponded to a concentration of 100µg/ml. The purified gamma globulins were then conjugated with an alkaline phosphatase enzyme. This enzyme provided the means for detection of the gamma globulins later in the ELISA test by catalysing a reaction that produced colour development.

The ELISA technique

The technique has four basic steps:

1. The 96 well ELISA plate is coated with immunoglobins in coating buffer - 100 ul per well

   After each of the first 3 steps the plates are incubated overnight at 4°C or for four hours at 37°C (to ensure binding) and then thorougly washed three times with buffer
2. The plate is coated with the test samples (samples diluted 1:10 W:V in extracting buffer)
3. The conjugate is diluted 1:500 in extracting buffer
4. The plate is coated with the subtrate for colour development

Plates are incubated at room temperature, and optimum colour development occurs between about half to one hour. The concentration of colour is quantified using a spectrophotometer.

The purpose of each step is;

1. to fix the immunoglobins in the wells of the plate,
2. to add the sample to be tested. If the sample has antigenic properties it will bind to the immunoglobins, otherwise it will wash away,
3. to add the conjugated immunoglobins. If the sample is present the immunoglobins will bind to it, and the alkaline phosphatase enzyme will be present,
4. the substrate buffer will produce colour if the enzyme is present.

Therefore, if the sample is identical to the antigen it will bind strongly to the antibodies and there will be strong colour development. If the sample is not related to the antigen it will not bind to the antibodies and be washed away and there will be no colour development. There is usually a small amount of non-specific binding. Details of the ELISA test method and materials are appended (Appendix 2).

RESULTS

Development of the ELISA Test

A number of tests were undertaken to define the specificity of the ELISA test for *Streptomyces* spp. with known pathogenicity to the Russet Burbank potato cultivar.

Test 1 To determine whether the antibodies would bind to the Streptomyces isolates used as antigen.

Different concentrations of antibody and antigen were used to determine the optimum conditions for colour development. Three levels of antibody were used - 0.5, 1.0, 2.0 μg/ml. Four dilutions of antigen were made, 1:10, 1:100, 1:1000 and 1:10,000. Conjugated antibody was added at 1 μl/ml or 2μl/ml. Increased colour development was found at the higher concentrations of antibody and antigen. The differences were not great so the 1 μl/ml was considered on adequate antibody concentration.

The plates were coded 5, 6, 7, 8, and equated to isolates 32, 20, 27 and 12 respectively.
Plate 5 showed the best colour development.
Plate 6 showed the second best.
Plate 8 showed the third best.
Plate 7 showed a poorer level of colour development.

On each plate, all antigens showed colour development. The four antigens were sufficiently alike to induce colour development. In each case, the antigen was initially fixed to the

ELISA plate showed the greatest amount of colour development against its corresponding isolate.

Antigen bound to plate					
	Isolate 32 Plate 5*	Isolate 20 Plate 6	Isolate 27 Plate 7	Isolate 12 Plate 8	Colour Intensity
Order of	32	20	27	12	Good
colour	20	12	12	20	
development	12	32	20	32	
of isolates	27	27	32	27	Poor

* Coded plate 5 or isolate 32 consistently produced the best colour development of the isolates in the ELISA test.

The results of the first experiment indicated the ELISA test would identify the original isolates. It was concluded that the four isolates were related and would react with the rabbit antibodies allowing testing to continue.

Test 2 To determine whether the antibodies could distinguish between the other Streptomyces isolates.

Three replicates of 27 isolates and 5 blanks were tested on the ELISA plate. Results based on averaged absorbance levels (highest absorbance most colour) are presented in Table 2. The table sorts the isolates according to the results of the earlier pathogenicity test.

Table 3. Average absorbance in isolates following ELISA testing, compared with pathogenicity status.

Pathogenic		'weakly' Pathogenic		Non-Pathogenic	
Isolate Number	**Average Absorbance**	**Isolate Number**	**Average Absorbance**	**Isolate Number**	**Average Absorbance**
12	1.12	9	1.28	1	0.60
13	1.26	24	0.88	2	0.72
15	1.10	25	1.34	3	1.76
17	1.13	28	1.28	4	1.26
18	1.19	29	1.28	5	1.49
19	1.20	30	1.14	7	1.37
20	1.18			10	1.29
27	0.39			14	1.10
32	2.14			23	0.58
				26	0.45
				31	0.55

One of the pathogenic isolates showed a low level of colour development (27). Some of the non-pathogenic isolates show higher levels of colour development. All isolates were obtained from potato scab tissue and are unlikely to represent the actinomycete community in soil. This may account for the overlap in absorbance between nonpathogenic and pathogenic isolates viz. all isolates *in vivo* may have the capacity to induce scab symptoms and produce a colour reaction to antibodies that was not fully apparent in pathogenicity trials.

Test 3 To confirm the results of Test 2, and to test potato pulp and skin and soil for reaction with the antibodies.

In this preliminary test soil showed no reaction, two samples of potato skin showed no reaction, and samples of potato pulp showed no reaction. One sample of potato pulp showed a slight reaction.

Retested isolates showed average absorbances similar to those in Test 2. Pathogenic strains tended to have higher absorbances than nonpathogenic but were not statistically different.

Test 4 To determine whether whole cells could be used to elicit the same ELISA response as cells fragmented by sonification.

Better discrimination between isolates may occur when antibodies bind to the cell surface rather than with cell contents. Isolates were tested along with six random bacterial isolates to compare the specificity of antibodies to a range of prokaryotic organisms.

Very little colour developed in the six bacterial isolates tested suggesting little binding of the antibodies took place. Binding was reduced in all whole cell isolates over previous tests using fragmented cells (compare Tables 2 and 3). Variation also occurred within and between all pathogenicity groups.

Table 4. The results for the isolates tested as whole cells for ELISA serum 5 (Isolate 32).

Pathogenic		'weakly' Pathogenic		Non-Pathogenic	
Isolate Number	**Average Absorbance**	**Isolate Number**	**Average Absorbance**	**Isolate Number**	**Average Absorbance**
12	0.45	9	0.28	1	0.17
13	0.54	24	0.55	14	0.29
15	0.31	25	0.40	23	0.20
16	0.26	28	0.29	31	0.16
17	0.25	29	0.30		
18	0.25	30	0.36		
19	0.23				
20	0.48				
27	0.31				
32	0.74				

Test 5 To test further fungal and bacterial isolates for their reaction to the antibodies (specificity). To retest sonicated and whole cells for confirmation of previous results.

The 15 fungal and bacterial samples showed no or a very little colour development. There was no difference in binding between sonicated or whole cell isolates.

Test 6 To determine the sensitivity of the test by using a dilution series and to determine if the presence of soil affects results.

Samples isolates 32 and 12 were sonicated for 60 seconds in extracting buffer at approximately 1g of wet culture in 9ml of buffer. A dilution series was made up to $1:10^8$. A parallel dilution series was made up with 1g of dry soil added to each sample.

The two plates incubated with isolate 12 did not give results. The plates gave a little colour development in all wells without differences between the wells. The reason for this is not known. The plate incubated with isolate 32 gave very good clear results.

The ELISA test was able to detect the antigen at the $1:10^6$ dilution in the pure sample and the sample to which soil had been added. Dry soil used as a control did not give colour development. This result indicated that the presence of soil did not interfere with the detection limits of the ELISA test.

Test 7 In this experiment 27 samples of soil were taken from a site at Forthside Vegetable Research Station where scabby tubers had been grown.

A problem that had arisen in some previous experiments became apparent in this test. The blank wells gave colour development equal to that of the samples and the dry soil used as a control also showed the colour development. Results were therefore inconclusive.

Test 8 To determine the reason for colour development in the blanks and to test some soil *Streptomycete* isolates for antigenic reaction.

Colour development was observed in the blanks containing polyvinyl pyrrolidone (PVP). Future experiments were carried out using PBS-tween rather than extracting buffer. However results in this test may be affected by the problem with the extracting buffer.

The absorbance levels of soil *Streptomycetes* were generally lower that those of the initial isolates.

Table 5. Absorbance of soil Streptomycete isolates and pathogenic isolates from potato.

soil isolates	potato scab isolates	
absorbance	isolate	absorbance
0.38	17	0.84
0.47	12	0.68
0.35	20	0.39
0.29	27	0.39
0.50	32	1.52
0.33	19	0.94
0.37	20	0.90
0.35	22	1.23
0.36		

Test 9 To determine the ability of the ELISA test to detect the scab organism in Forthside soil.

Soil was incubated at room temperature, 25°C and 40°C overnight to determine whether treatments detection be improved.

In this test, and in the absence of PVP, the blanks did not develop colour so that any colour development was due to the detection of the scab organism. Different incubation temperatures did not affect the degree of colour development.

Test 10 To compare colour development from Forthside soil with colour development from a cattle property, 'Aradatha', Sheffield, where potatoes had not been grown.

Forthside soil samples gave greater colour development than soil from the cattle property.

	Forthside	**Aradatha**
No. samples	8	16
Mean absorbance	0.48	0.24
Standard deviation	0.18	0.07

Test 11 In this experiment Forthside soil was again compared with soil from the cattle property 'Aradatha'.

Dark coloured clay soil from beneath eucalypt trees on Aradatha was also compared with red krasnozem soil from a grazing paddock.

	Forthside	Aradatha red soil	Aradatha dark soil
No. samples	8	8	8
Mean absorbance	0.77	0.24	0.34
Standard deviation	0.12	0.10	0.10

Forthside soil showed significantly greater colour development than either Aradatha soil. Soil from beneath trees gave slightly greater colour development than soil from the grazing paddock. This may be due to a more stable Streptomycete community in the root zone of the trees.

Test 12 Nine samples of soil were collected from paddocks in which the common scab status was known.

Forthside soil was used as a positive control while Aradatha soil was used as a negative control.

	Average Absorbance
Aradatha soil	0.32
Forthside soil	2.20

The amount of colour development as measured by the average absorbance should correlate with the amount of common scab in the sample.

Table 6. The average absorbance of each sample and the scab status of the paddock compared with positive and negative controls.

Soil Sample	Average Absorbance	Scab Status
Aradatha	0.32	Negative
Forthside	2.20	Positive
9	2.06	scab
4	1.38	scab
6	0.95	scab
8	0.95	clean
1	0.84	clean
3	0.63	slight scab
5	0.59	scab
2	0.48	clean
7	0.31	clean

Three samples were miscategorized by the test, samples 8, 1 and 5. However the results indicate that the ELISA test has potential as a useful tool in determining the scab status of paddocks, but further investigations of cropping history and wider correlation of the ELISA

test with known levels of disease are needed to eliminate errors and increase the reliability of the test.

Summary of ELISA tests

Tests were undertaken to check specificity and selectivity of antisera against isolates and soil.

The first test sought to check the reaction of antibodies against the original antigens to look for differential response to the 'type' antigen. A gradient of colour intenstiy in the test was recorded against original isolates, suggesting overlap in isolates. 'Type' isolates reacted most strongly with 'type' antibodies.

Test 2 sought to relate pathogenicity of original isolates to intensity of colour development in the ELISA test. Results were not consistent and may indicate a lack of sensitivity between Streptomyces, and may also reflect bias in isolation of original cultures sourced from diseased tubers which gave a positive reaction to the presence of tyrosine.

Test 3 to 6 were undertaken to confirm the specificity of antisera to Streptomyces against background 'noise' in the form of other bacteria, fungi, soil particles and potato tissue. In all cases specificity was proven. No differences in degree of detection was found when sonicated cells were compared with whole cells.

Tests 7 to 8 involved problem resolution while test 9 established that holding temperatures of soil (ie. room, 25°, 40° C) made no difference to colour definition in the ELISA test.

Tests 10 to 12 compared colour development in ELISA plates resulting from comparisons of soil from known scab infested and clean soils. The test was relatively sensitive to populations of scab in soils, particularly at extremes.

3. Fluorescent Antibody Microscopy

Fluorescent antibody microscopy uses the same technology as ELISA relying on antibodies to bind with their compatible organsims. A fluorescent dye is attached to the antibodies which become visible under ultraviolet light. When soil or other specimens are viewed under a microscope marked organisms become visible. In the present project time constrainst have precluded investigation of fluorescent antibody microscopy as a method of detection. The technology may suffer from the same lack of specificity of the anti- *S. scabies* antibodies as in the ELISA test. However the technique should be re-examined when the ELISA test is suitably refined.

4. Combination of high and low technology techniques

If the ELISA test proves to be of limited value and the possibility of a DNA probe test remains in the uncertain realms of high technology, then a rapid identification test for *Streptomyces scabies* is still possible using the classical techniques of isolation on an agar medium and testing of the pathogenicity of the isolates.

Isolation

Streptomyces species are very slow growing and are rapidly overwhelmed in culture by other microorganisms. As a result, dilution plating of soil can result in very few Streptomyces colonies being isolated. In dilution plating experiments conducted in this project there was too much contamination by the other micro-organisms in soil samples and to determine the numbers of Streptomycetes in the soil. However an antibiotic agar has been developed by Keinath & Loria (1989) to inhibit the growth of these contaminants. The Glycerol-asparagine-tyrosine agar is used with the addition of the antibiotics nystatin,

cycloheximide, polymixin B sulphate and penicillin. This medium was not used in this project due to time constraints.

Pathogenicity testing *in vitro*

Colonies isolated from soil via selective media can be transferred into liquid culture, and tested for pathogenicity using microtubers grown *in vitro*.

Such a rapid pathogenicity test was developed by Barker and Lawrence (1963) using tissue cultured microtubers but initial attempts to produce cultured tubers at the Stoney Rise Centre potato propagation unit have been unsuccessful. An alternative growing medium is currently being used for the propagation of *in vitro* tubers but it requires eight to ten weeks to produce tubers and there has not been material available to date, to test *in vitro* rapid pathogenicity techniques.

Once a system for growing tubers *in vitro* is perfected, the SRC glasshouse could provide a constant supply of the microtubers for testing suspect soils. The proportion of *in vitro* tubers showing scab symptoms can be assessed to give an indication of the amount of the common scab organism in any soil.

This system has the potential to be a relatively simple technique for detection of the pathogen, and also to act as a rapid screening test for a scab resistant Russet Burbank clone or for new varieties.

Somaclonal variation is a feature of tissue cultured plantlets and may provide a clone that has scab resistant properties. The rapid pathogenicity test using microtubers has the added advantage of being able to test a large number of somaclones of Russet Burbank more quickly than traditional methods.

PROJECT RECOMMENDATIONS

The ELISA test shows the potential to be a successful rapid identification test for *Streptomyces scabies*. The purpose of a rapid identification test is to provide information on the level of common scab in a paddock so that a farmer could avoid growing potatoes in infected soil. As such, the predictive value of the test is important.

Soils from selected paddocks need to be tested before the planting of potatoes in October and November and the amount of common scab present at harvest compared with ELISA test results. If the ELISA test successfully predicts the outcome, it can then be introduced as a commercial test. This work should begin in the 1991-92 season.

The isolation technique followed by a rapid pathogenicity test should be developed as an alternate to the ELISA test. It would assist in the evaluation of the ELISA test and also provide a rapid screening test for scab resistant clones. The facilities are presently available at the Stoney Rise Centre, Devonport to annually run any rapid pathogenicity test.

The Tasmanian potato industry should consider putting forward a proposal to the HRDC for funds to continue the study of *Streptomyces scabies* using DNA probe technology. The facilities and expertise are presently available at the University of Queensland but it would be in the long-term interests of the potato industry that the taxonomic status and the ecology of *Streptomyces scabies* be better understood.

With refinement of the ELISA test comes the potential use of fluorescent antibody microscopy as a quicker and alternative serological technique.

PROJECT CONCLUSIONS

The development of a rapid identification test for *Streptomyces scabies* has been successfully achieved. The ELISA test has been able to detect the common scab organism in soil but it test needs to be evaluated in conjunction with the classical isolation techniques of dilution plating to determine its accuracy.

Research should also proceed to determine the predictive value of the test. Soils should be tested for the presence of common scab before the planting of potatoes and compared with the amount of scab at harvest.

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APPENDIX 1

DNA PROBE TECHNOLOGY

A report on my visit to Professor E Stackebrandt and his team at the Department of Microbiology of the University of Queensland.

Christopher Gillian

July 1991

INTRODUCTION TO STREPTOMYCES TAXONOMY

The causal organism of common scab of potatoes was first described in 1890 by Thaxter.

In 1961, the species was redescribed and renamed *Streptomyces scabies* by Waksman. However, as research continued, common scab producing organisms were found, and type strains collected, which did not meet the description. *Streptomyces scabies* was dropped as an approved name and the species considered invalid, with many taxonomically different strains available.

In 1989, Lambert & Loria proposed reviving the species *Streptomyces scabies* and cited Thaxter as the original authority.

To quote them: 'In our opinion, the original descriptions of *Streptomyces scabies* are correct and have been consistently substantiated by those workers familiar with phytopathogenic Streptomyces.

Strains belonging to this species form a coherent group and are easily distinguishable from other pathogenic strains by numerous characteristics.

Lambert & Loria consider *Streptomyces scabies* as the predominant scab causing organism.

An acid tolerant species is named *Streptomyces acidiscabies*. Pathogenic isolates not of the scabies type, have been assigned to other species or supply left unassigned.

So the taxonomic identity of the common scab causing organism(s) in Tasmania is an important factor in understanding the disease and in the chances of success of this project.

When Linnaeus (1707-1778) instituted a system of binomial taxonomy, he recognised the existence of microscopic forms of life and placed all in the one group designated 'Chaos'.

There are at present greater than 600 described species within the Genus *Streptomyces*, and Streptomycetes are considered 'the most widespread and important in soil'.

Therefore, a powerful taxonomic tool is needed to differentiate between the various soil Streptomycetes, and DNA probe technology offers that possibility.

I had three reasons to visit the University of Queensland:

1. to determine if DNA probe technology could be useful in the taxonomic identification of my isolates

2. to determine if DNA probe technology could provide a rapid identification test for *Streptomyces scabies*

3. to learn how DNA probe technology works.

I will begin with an explanation of how DNA probe technology works, as practised by Professor Stackebrandt and his research team at the University of Queensland.

DNA PROBE TECHNOLOGY

THEORY

Sequencing of the rRNA is the most powerful technique in the use of DNA technologies in taxonomic studies of procaryotes.

Sequence data from the 16S sub unit of the ribosomal RNA is collected from as many species of the genus as possible and compared.

Much of the RNA sequence is highly conserved and identical for all species.

Stackebrandt found three particular sites where a greater degree of variation was present. This variation differentiated some species of the Genus *Streptomyces* from other species. Within a

species, the sequence would not vary. The sequence data from a 'site of variability' would be obtained for a species.

eg. Streptomyces scabies 4 code = GCA UCA GAG AUG GUG CC.

A probe is developed by manufacturing the DNA sequence with the complimentary code to the rRNA code at the site of variability.

A probe is normally 16-20 bases long.

The DNA probe is the perfect match to the rRNA sequence of that species, and will bind tightly to the rRNA of that species. Some other species may have the same sequence and will also bind.

17 of the Stackebrandt test species bind with 4.

So, many type strains are collected and tested against the probe to determine which species are differentiated by the probe and which are not.

Thus you have a probe which is diagnostic of one or a number of species.

PRACTICAL

Tuesday morning I arrived at University of Queensland and spoke to Prof. Stackebrandt. I was introduced to Ph.D student Cordula Kemmerling and given a preliminary explanation of techniques.

Experiment 1: To determine whether the Universal probe will hybridise with purified RNA.

THE DOT BLOT HYBRIDISATION TECHNIQUE

Method -

1. Place hybond nylon filter in apparatus (apparatus: plastic apparatus with 12 x 10 wells connected to water suction.

2. wash filter distilled water
3. add RNA 1-g in 100-l solution
4. wash filter twice
5. wait 15min to dry
6. recover filter from apparatus, dry in air 30 min.
7. UV light to fix RNA.
8. add hybridising fluid to protect long chain RNA's
9. place in rotating oven
10. add radioactive probe, wait 2 hrs.
11. after 2 hrs remove radioactive probe into bottle
12. add washing solution. ¼ tube wash 20 min.
13. remove filter on to glass plate
    + xray film + xray magnifier
14. place in -70°C freezer overnight
15. develop next morning

Experiment 2: To determine whether the Genus *Streptomyces* probe will hybridise with the RNA of colonies of 20 isolates.

WEDNESDAY COLONY LYSIS

Method:

1. small colony taken from streak plate with tooth pick
2. filter had been placed on agar plate
   place colony on filter - marked corner for orientation
3. into clean petri dish + sucrose buffer
4. 37° incubation room for 1 hr. only
5. remove colony filter to dry in airflow cabinet
6. fix - UV light for 5 min.
7. filter + colony put into tubes with pre hybridisation buffer rotated at 38°C for 2 hrs.

Making up the probe (Genus *Streptomyces* probe)

tube 1	tube 2
3-l buffer	0.6 l 1M MgCl
0.5 g probe	1 l spermadin (protect probe)
	3 l $100M^{-3}$ DTT (protect enzyme)

boil tube 1

combine tubes

onto ice

+ 2-l ATP radioactive

+ 2-l poly nucleotide kiNAse

mix well then 3 hrs. in fridge

THURSDAY

Next separate the radioactive nucleotide from the radioactive ATP Sephadex column

1. wash column with elution buffer
2. add radionucleotide + small vol. buffer
3. collect drops as pass through column
   5 drops in 10 tubes
4. test with geiger counter
   1-2 - very little radioactivity
   3-5 - most radioactivity
   6-10 - less radioactive
5. 3-5 - tubes into freezer

Hybridisation of colony RNA and radioactive probe (following from the work of the previous day)

8. remove pre hybridisation buffer
9. add hybridisation solution + radioactive probe leave overnight in oven

FRIDAY

10. pour off radioactive probe
    add washing solution
11. after washing make up x-ray plates
    into freezer overnight

SATURDAY

12. develop x-ray film

FRIDAY AFTERNOON

Discuss DNA probe use with soil: Werner

Discuss isolation techniques from soil: Therese

Thank Professor Stackebrandt and discuss future possibilities.

RESULTS

Experiment 1:

The x-ray film showed the circular block dots where it had been exposed to the radioactive probe. The space where a black dot did not occur was a blank. Therefore, the universal probe had successfully bound to the purified rRNA as would be expected.

Experiment 2:

The x-ray film showed a faint outline of the two filters tested. There was a black dot corresponding to one colony which showed successful hybridisation of colony RNA to the Genus *Streptomyces* probe.

There are two possibilities: 1. the colonies were not of the genus *Streptomyces* and therefore the probe did not bind OR 2. that the

colony RNA had not bound well to the filter and had not been present for the probe to bind to.

The latter is the more likely explanation as changes had been made to the usual procedure to speed up the process so that an extra day would not be necessary. The incubation in a sugar buffer solution was normally carried out overnight.

THE DNA PROBE AS AN IDENTIFICATION TEST

The second question I wished to address was whether a DNA probe could be used to identify an organism in the soil and whether a practical rapid identification technique could be developed at a reasonable cost.

The answer to the first part is that DNA probes can be used and have been used to identify a specific organism in soil. A practical system that will work for all soils has yet to be developed. There are difficulties due to denaturation of the oligonucleotides by the soil acids.

Research into methods is progressing at a very fast pace, and I can foresee a time when automatic processing of samples would be possible. However, at present the technology is not available.

Saylor and Layton (1990) have written a review article entitled 'Environmental Application of Nucleic Acid Hybridisation', and it is perhaps worth copying their conclusions in full.

CONCLUSIONS AND FUTURE PROSPECTS

The use of nucleic acid gene probes for environmental assays is at a cross-roads. Research has clearly demonstrated the feasibility of developing a wide array of specific probes for organisms and genes of environmental interest. However,

environmental applications of gene probe techniques have been hindered because standard nucleic acid assays are not sensitive enough to detect microorganisms present as only a small percentage in the community.

The prospects for improved sensitivity and detection methods look promising. For example, PCR has greatly increased the sensitivity for medical assays and environmental detection. In addition, several companies have patented and released detection kits for clinical applications. Additional advances in nucleic acid technology may be made with the development of automated equipment for the rapid processing of samples. Already, several types of automated instruments are available for the extraction of DNA from tissue samples, the synthesis of oligonucleotides, the sequencing of DNA, and the amplification of DNA sequence. Therefore, environmental microbiologists may soon be able to apply standard techniques for the detection and enumeration of microorganisms in the environment and for the study of gene transfer and maintenance in natural communities.

The vast diversity of different environments currently under investigation, the types of target and probe nucleic acids employed, and the developmental level of the methods used indicates the interest in utilisation of probe techniques. The diverse forces behind current applications are equally varied, ranging among the desires to provide more quantitative and absolute data for regulatory purposes, to develop faster and more sensitive techniques for environmental monitoring, and to gain more fundamental insight into complex community behaviour and ecological processes.

The diversity also points to the fact that further standardisation and quantification is also needed to accurately interpret the results obtained from the application of the technology. Both qualitative and quantitative internal standards and controls are needed but are often lacking in many applications. This limitation is further magnified by the fact that interpretations of the resulting data are often only as good as the molecular information available on probe and putative target sequences.

THE DNA PROBE AS A TAXONOMIC TOOL

The first question I wished to answer was if the DNA probe would aid in the identification of my isolates.

Prior to my arrival, I had sent 20 isolates of pathogenic and non-pathogenic *Streptomyces* to Professor Stackebrandt. Colonies from these 20 isolates had been fixed to a filter. This filter had been hybridised with probe 4. Probe 4 was designated as the scabies probe. This probe had hybridised with a type strain isolate from the German collection of micro-organisms and designated "*S. scabies*". (Strain number DSM 40078).

Three out of the 20 isolates hybridised with this probe. This indicates that three isolates are of the same or a related species as the German type strain. The other isolates are not related to the German "*S. scabies*".

It points to a number of Streptomyces species causing common scab, and underlines the lack of knowledge of this disease.

FUTURE PROSPECTS FOR WORK WITH DNA PROBES

1. The taxonomic identification of isolates

Professor Stackebrandt is using DNA probe technology to rationalise the taxonomy of the *Streptomyces* group.

He is on the editorial board of the International Journal of Systematic Bacteriology. When I met him, he expressed interest in the taxonomic problems associated with my project and with such a recently accepted species. If contacts are maintained it might be possible that Professor Stackebrandt would do further work with this topic.

2. The DNA probe as an identification test.

The first requirement before a DNA probe can be produced is to gain the sequence data. Then a protocol would need to be developed for an extraction method of the DNA from soil possibly using PCR (Polymerase Chain Reaction). These would need an amount of time and money to fulfil.

It would also require Professor Stackebrandt and his team co-operation in the use of his facilities and expertise to carry out the research.

3. DNA Probe Technology

Once an identification test had been developed, a laboratory would need to be equipped. Another problem arises because of the use of a radio active label on the probe.

Phosphorus is a "hot" radio isotope and there are problems working with these levels of radioactivity. Research into alternate methods of labelling is being carried out.

The DNA probe is a new and powerful technology. I believe it would have the capacity to detect *Streptomyces scabies* in the soil. To use this technology would require a research project to be established where there was appropriate facilities and the establishment of a laboratory for processing of samples.

Two issues arise: the cost of this work and the timing. DNA probe technology is still basically a research tool and it may be better to wait until the technology is more commercially available.

However, it may be the only technique that will give the potato industry the information on *Streptomyces scabies* in soils that it seeks.

APPENDIX 2

ELISA Test Method - *Streptomyces scabies*

Soil sample preparation:

Weigh 1 g soil, add 9 ml PBS/Tween and sonicate for 1 minute.

Volume per well: 100 microlitres (10 ml per plate)

Step 1: Dilute IgG 1/1000 (10 ul for 1 plate), in carbonate coating buffer (pH 9.6) and incubate in plates overnight at 4°C or at 37°C for four hours.
Wash three times in PBS/Tween.

Step 2: Add samples to plate, do not use extracting buffer. Use PBS/Tween in control wells and soil extract from known positive and negative common scab sites as standards.
Incubate at 4°C overnight or at 37°C for four hours.
Wash three times in PBS/Tween.

Step 3: Dilute conjugate 1/500 (20 ul in 10 ml for 1 plate) in PBS/Tween and incubate overnight at 4°C or at 37°C for four hours.
Wash three times in PBS/Tween.

Step 4: Dissolve 2 tablets of PNPP in 10 ml substrate buffer for each plate and incubate at room temperature for 30 minutes to 1 hour. Record colour development.

(**WASHING**: After each stem, wash plate for 10 to 15 seconds in PBS/Tween as described))

ELISA BUFFERS

1. Carbonate Coating Buffer (ph 9.6)

1.59 g Na_2Co_3 (sodium carbonate)
2.93 g $NaHCO_3$ (sodium bicarbonate)
0.2 g NaN_3 (sodium azide)

make up to 1 litre with distilled water, store at 4°C

2. Phosphate Buffered Saline `PBS` (pH 7.4)

8.0 g NaCl (sodium chloride)
0.2 g KH_2PO_4 (potassium phosphate monobasic)
1.44 g $Na_2HPO_4.2H_2O$ (disodium hydrogen phosphate dibasic)
0.2 g KCl (potassium chloride)
0.2 g NaN_3 (sodium azide)

make up to 1 litre with distilled water, store at 4°C

3. PBS 10X Stock (pH 6.7)

80.0 g NaCl
2.0 g KH_2PO_4
14.413 g $Na_2HPO_4.2H_2O$
2.0 g KCl
2.0 g NaN_3

make up to 1 litre with distilled water, store at room temperature. Label "PBS 10x stock". Note that the pH of single strength PBS is 7.4 after 10x stock has been diluted out ten-fold.

4. PBS/Tween

100.0 ml PBS 10x stock
900.0 ml distilled water
0.5 ml Tween 20

5. Substrate Buffer

97.0 ml $[CH_2(OH)CH_2]_2NH$ (diethanolamine) VERY CAUSTIC
0.2 g NaN_3
800.0 ml water

reduce pH with HCl that has been diluted from conc. HCl with purified water to a 1:1 v:v solution (approx 20 ml). Add acid to water slowly and carefully, in the fume cupboard. When pH is 9.8 make up volume to one litre with distilled water using pH meter as it is sometimes necessary to add a few mls HCl/H_2O while making up the volume.

6. **Substrate Solution**

2 x 5 mg tablet PNPP/plate

Dissolve in 10 ml substrate buffer.

NOTE: Sodium azide is highly toxic. It is a directly acting vasodilator with a hypotensive dose of 0.2 to 4.0 ug/kg in humans (0.14 to 28 mg/average sized person)

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ELISA BUFFER METHODS

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