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DEVELOPMENT OF TECHNIQUES FOR CLONING NOCARDIOFORM GENES OF THE ENZYMES INVOLVED IN DETOXIFYING ACRYLAMIDE

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A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in the Department of Genetics, University of the Witwatersrand, Johannesburg.

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ABSTRACT

Acrylamide is a reactive vinyl monomer, widely used in the synthesis of polymers for a variety of industrial applications. The polymers are non-toxic, but the monomer is neurotoxic to both laboratory animals and men.

In this study various Nocardioform strains able to utilize acrylamide as sole carbon and/or nitrogen source were identified. Acrylamide resistant and acrylamide non-utilizing mutants were obtained. Techniques have been developed thus far for the cloning of the acrylamide detoxifiction genes. A genomic DNA library of the acrylamide resistance genes has been constructed and the transformation system in Nocardioform has been optimized.

DEDICATION

To My Parents

DECLARATION

I declare that this is my own, unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university



(Name of Candidate)

28 day of February, 1989.

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1.0 INTRODUCTION

The family *Nocardiaceae* contain aerobic, heterotrophic,Gram positive,acid fast to partially acid fast actinomycetes which produce a primary mycelium that fragments into rod and coccoid-like elements (25). Aerial hyphae are usually formed; strains contain mycolic acids (long-chain 2 alkyl branched- 3 hydroxy acids) and a wall chemotype IV, i.e. strains contain major amounts of meso-diaminopimelic acid (DAP) and arabinose and galactose. The walls of *Nocardiae* and related bacteria like *Mycobacterium*,*Corynebacterium* and *Rhodococcus* species have a similar and complex structure that consists of a peptidoglycan, several classes of free and bound lipid constituents and other polysaccharides or polypeptide compounds. The overall base composition of Nocardioform bacteria is estimated to be between 62 to 77,3 mol percentage guanine and cytocine (G+C). The DNA content of *Nocardiae* has been compared and it is found to be different from strain to strain whereas the DNA content of *Mycobacteria* was more uniform.

Nocardioform actinomycetes are found in the soil and like other bacteria are sensitive to phage. Host range studies have been of value in the classification of *Nocardia*. Phages have been reported to be of value in the tentative identification of fresh isolates and in clarifying the classification of poorly defined Nocardioform taxa.

Although the generation times for *Nocardiae* are not generally known it can be assumed that under optimal growth conditions the generation time for majority of the *Nocardia* could be 3-7 hours; under unfavourable culture conditions, however it can be prolonged to one week or longer.

Regarding their known metabolic activities, *Nocardiae* and Rhodochrous-like organisms seem to be similar to many other aerobic bacteria; this

statement has validity for the utilisation of organic compounds and nitrogen containing derivatives, lipid metabolism and other metabolic steps. A great number of organic substances can be used by Nocardioforms as carbon sources (26). Alkanes are good carbon sources. It seems to be an advantage for *Nocardiae* to use alkanes with relatively longer chains for they can be better metabolised than those with shorter chains. Sodium acetate, propionate and butyrate are good carbon sources for many of the *Nocardia* strains. Inorganic compounds such as NH_4^+, NO_2^- and NO_3^-can be utilised as sole nitrogen source. L-glutamic acid, L-aspartic acid, L-Lysine, L-leucine, L-isoleucine and L-valine support good growth in many *Nocardia* species. *Nocardia* in general have not been found to need specific growth factors, however some strains require thiamine for growth. *Nocardia* can grow on media supplemented with organic substrates such as peptone, amino acids, egg white, egg yolk and milk. Synthetic media are used for special purposes such as in search for growth factors, isolation of auxotrophic mutants, determination of oligocarbophillic properties and in fermentation studies. Given their common metabolic activities *Nocardia* are to some extent a connecting link between *Mycobacterium*, *Corybacterium* and Rhodochrous-like organisms, sometimes showing similar overlapping activities with them.

Nocardioform bacteria are a group notable for their facility of degrading toxic and/or unstable compounds such as phenols, acrylamide, insecticides and herbicides and for interconverting steroids into precursors of pharmacologically important compounds (16). Members of this group also produce a number of antibiotics, such as rifamycins and hygromycin (16). To date progress in the investigation of all these fields have been hampered by the absence of resistance plasmids suitable for development into a cloning vector. Individual species of *Nocardiae* are the aetiological agents of tuberculosis, leprosy, actinomycete mycetoma and an uncommon disease of man and animals called nocardiosis. Nocardiosis is a severe pulmonary infection which frequently disseminates by way of the blood stream and lymphatics to other parts of the body. However it may also

be a primary disease of the central nervous system, kidneys, heart, eyes or other organs. Nocardial mycetomas are characterised as being chronic granulomatous infections that progressively worsen over a period of several months or years. Three species of *Nocadia* are most frequently recognised in human infections. In order to survive within the host and cause disease the *nocardiae* must be able to neutralise or survive the many defence mechanisms of the host. At the same time they must be able to use the body material as a growth medium. The distribution of the pathogenic *nocardiae* has received the most attention and it is generally thought that soil is the primary reservoir for these bacteria since most cutaneous infections in tropical countries occur on the feet or on the back through carrying contaminated sacks. Rhodococci have not been unambiguously implicated as disease agents but are known to form symbiotic associations with a wide variety of blood sucking arthropods (27).

The chemotherapy of infections caused by *Nocardiae* is a matter of increasing concern, both to countries with high living standards where the number of cases of nocardiosis is rising and to countries with low socio - economic levels where more cases of mycetoma are being diagnosed. A number of drugs have been found to be effective in the treatment of nocardiosis and actinomycete mycetoma (27).

Thus at present, little is known of the part Nocardioform bacteria play in the natural habitats although it seems likely that that they will form an integral part of a balanced microbial community and could be involved in the degradation of complex organic substrates. Already improved methods for the recognition of *Nocardia* have shown that they are able to degrade rubber joints in water and sewage pipes (33). Finally it should be recognised that the term "Nocardioform" is merely one of convenience and it should be regarded as a collection of individual genera and species and should be studied as such.

Chemicals and chemistry play vital roles in providing food, clothing, shelter, medication as well as luxury goods for an ever - increasing world population. While the benefits from industrial chemistry remained uncontested for a long time and have been accepted without remorse, the environmental costs have been recognized by the general public only during the past 10 - 15 years. Since chemical production relies on non renewable resources for raw materials, from up to 50% may be left as waste, these costs are high. They have been and sometimes still are paid for by human exposure to a variety of potentially harmful chemicals, by the contamination of water resources and by the endangerment of wildlife.

Environmental pollutants are defined as chemicals of natural or synthetic origin that are released by man's activity into the environment or on man via the environment.

Figure 1 (41). Illustrates the major pathways by which chemicals are released from their containment under human control in the ecosphere.

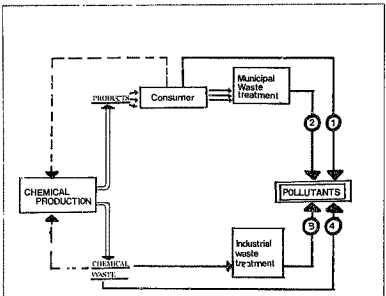


Figure 1. Release of organic chemicals into the enviroment.

1 : Chemicals whose use leads to their entry directly from the consumer to the environment eg. products such as pesticides, aerosol propellants, fertilizers and deliberate release into the environment by illegal dumping.

2 : Chemicals entering the environment in the effluents of municipal sewage treatment systems eg. hard detergents, solvents.

3 : Chemicals resistant to biological degradation in industrial waste treatment systems eg. chlorobenzenes, aminonaphthol sulfonic acids.

4 : Direct discharge from waste disposal sites, losses, spills and accidents leading to the entry of chemicals from production sites into the environment.

Modern agriculture and industry depend on a variety of synthetically produced chemicals including insecticides, fungicides, herbicides and other pesticides. Pesticide usage had increased to 0.75 x 10^9Kg by the end of the decade (54). Problems of chronic exposure to improper waste disposal, environmental damage and other adverse responses to pesticides required that federal laws be promulgated to govern their manufacture, distribution and use. More than 3 million chemical compounds were described in Chemical Abstracts by the year 1980 (24). Some 45 000 substances were traded worldwide and 70 000 are on the US. market. Some 1000 new chemicals are brought on the market *anually and 150 chemicals* are produced in excess of 50 000 tons per annum. The total world production of synthetic organic chemicals is estimated at 300 billion tons per year. Ecotoxicological data are available for less than 1000 compounds.

Today we are faced with certain industrial chemicals that do not readily participate in the global cycles of carbon, nitrogen, phosphorous or sulphur (41). Such compounds cause problems of disposal and may, if they escape containment, lead to adverse effects on the enviroment.
Government regulatory agencies have been controlling the use of pesticides for some time, and the US. Environmental Protection Agency (EPA) has initiated a program to establish procedures for assessing the environmental impact and health hazards of chemicals not specified as pesticides (1). In response to public and government concern and because of intriguing research problems presented, environmental scientists, biologists and chemists have been giving increased attention to identifying and determining the behaviour and fate of organic compounds in natural ecosystems.

For the disposal of chemical wastes the following strategies are generally applied; (24)
1. Improvement and modification of production processes and plants ; reduction of wastes by recycling of acids, salts and gases; reduction of

transformation losses; improved reaction specificities; simplified product isolation.

2. Chemical hydrolysis (esters etc.)

3. Thermal oxidation, eg. high temperature incineration of organic or aqueous process effluents.

4. Microbial oxidations or biodegradation

(a) Degradation in sewage treatment plants

(b) Biodegradation with specialised (salt tolerant) microorganisms in pure or mixed cultures

(c) *Development or isolation of fast growing microorganisms with wide* spectra of biodegradative activities.

Biodegradation offers prospect for an inexpensive but highly efficient method for removing toxic chemicals from contaminated soils (47). The expanding field of biotechnology has helped intensify research programs designed to detoxify pollutants that are in the environment. The approach of biotechnology to the environment's problems of chemical industry consists of developing biological techniques for recycling, detoxification or mineralisation. Among the methods being developed for large scale use is enzyme detoxification. By the 1990's it is very likely that several hydrolase enzymes will be in use on a practical scale in order to aid in the never ending tasks of environmental clean up.

An organic chemical introduced in the terrestrial or aquatic ecosystem may be subjected to nonenzymatic or enzymatic reactions brought about by the inhabitants of the environment. Detoxification by enzymes rather than whole microbial cells is particularly beneficial because enzymes sometimes can tolerate environmental extremes better than whole microbial cells (54). Extremes of pH and temperature as well as high salt and solvent concentrations are often encountered in pesticide production wastewaters. The use of enzymes is also attractive because the transport of pesticides into whole microbial cells can be problematic. Such membrane transport problems could be avoided when soluble enzymes are employed in

disposal processes. Enzymes that have the greatest importance for pesticide detoxification are those that can function without cofactors or coenzymes and can detoxify a pesticide molecule by a hydrolytic or other simple enzymatic reaction.

There is particular interest in developing enzymes for detoxifying a wide variety of waste contaminated waters because such treatment is gentle and save. Once the existing detoxification enzymes have been developed, produced and used on a large scale, then the search for and development of other new enzymes including those that dehalogenate or require cofactors may occur (34).

Some limitations of pesticide detoxification by enzyme technology should be considered here. Some compounds are not detoxified by enzymatic hydrolysis but instead the metabolites have increased toxicity. In such instances, initial enzymatic treatment of a parent compound may cause the molecule to subsequently be degraded more rapidly by whole microbial cells.

To assess the pollution potential of a particular compound one has to consider not only the quantity in which it is released in the environment but also its chemical and toxicological properties. A chemicals structure is of primary importance in determining whether it is accumulated or not, while its concentration and its toxicity determine the environmental impact of accumulation (41). In order to effectively control pollution, potentially hazardous chemicals have to be identified and their concentration limit standards in the environment have to be specified. Obviously toxicity, carcinogenicity and mutagenicity are the most important criteria in evaluating the harmful effects of pollutants.

Since conventional biological treatment procedures are inadequate for the removal of many of the potentially dangerous pollutants, specific technologies for their treatment have to be developed. Microbiology is ex-

pected to make significant contributions to the development of new technologies for industr. waste treatment. To provide microbial strains exhibiting improved bio-degradation capacities is one of the most challenging fields of microbiological research related to pollution control. Strains with degradative capacities for heretofore persistent compounds have to be enriched from nature or generated in the lab by continuous culture techniques.

The technique of enrichment culture is a very simple yet a powerful technique which dates from 1890 - 1900 (14). The compound to be degraded is supplied as the growth limiting and usually sole source of an essential nutrient in the culture medium. Only the organism(s) with the necessary degradative ability will grow significantly under those conditions and these organisms will outgrow the very large number of other organisms also added at the start of the experiment.

Strains with improved degradation rates or with a widened range of degradative ability may also be constructed by *in vivo* or *in vitro* genetic manipulation. Such an approach requires extensive knowledge on the biochemistry of the microbial pathway under investigation. For genetic engineering a key question is expression at the ecological level. You may get the gene expressed in the organism in the laboratory but not in the field. Factors such as temperature, the amount of nutrients and the presence of heavy metals may affect the function of the organism that has been so carefully engineered (46). The real need now is to understand the diverse abilities of natural organisms if we are to have any hope for improving them through recombinant DNA technology.

Microbial ecologist and microbiologists are beginning to unearth a startling array of microorganisms with unexpected abilities to biodegrade some of the toughest and most recalcitrant environmental chemicals (46). Recalcitrant molecules are organic chemicals that endure for long periods in natural ecosystems, owing to the inability of microorganisms to degrade

them rapidly, if at all. A few of these persistent compounds are not hazardous but are aesthetically undesirable - for example many plastics and other synthetic polymers.

Considerable progress has been made in defining the pathways of biodegradation of a variety of synthetic chemicals in laboratory cultures of individual microorganisms. For example there is much information on how individual microbial species cleave simple aromatic molecules in culture and how they bring about the destruction of aliphatic hydrocarbons (1).

The controlled degradation of specific problem compounds using specialized microbial cultures and the improvement of waste treatment plants by the addition of adapted microbial strains are promising applications that need to be developed in the future. The Toxic Substance Control Act and the federal regulations have played a catalytic role in promoting research in biodegradability. In view of this large amount of activity, one can look forward to new technologies and approaches designed to minimise environmental pollution while maintaining the benefits to society of synthetic chemicals. A decrease in pollution caused by product users can be further achieved through consumer education and by banning the use of toxic or potentially toxic chemicals.

The detoxification of acrylamide was the central issue of the work described in this thesis hence its potential toxicity and contribution to environmental pollution will be described.

Acrylamide is a reactive vinyl monomer widely used in the synthesis of polymers for a variety of industrial applications. It is often used in the production of synthetic resins, plastics and as a supporting medium for the electrophoresis of proteins. The polymers are nontoxic, but the monomer is neurotoxic to both laboratory animals and man, producing a classical peripheral neuropathy and having specific influences in the

striatal dopaminergic system (53). Although the compound is highly water soluble its neurotoxicity appears to be related to cumulative dose and dosing schedule. There is an inverse relationship between the dose and the length of the latent period from the start of treatment to the onset of effects.

Acrylamide absorbed through the skin or respiratory tract has been associated with polyneuropathy and CNS lesions (39). Slow recovery occurs in mild cases. Permanent neurologic lesions have been observed in severe intoxications. Several authors have reported other toxic effects of acrylamide including testicular atrophy, effects in bone marrow and spermatogonial cells and carcinogenesis in the mouse. The work of Bull, R.J. *et al* (8) demonstrated for the first time that acrylamide possesses carcinogenic properties. While acrylamide was apparently not capable of producing point mutations in *Salmonella*, it was capable of acting as a tumour initiator in the mouse skin. This data suggests that the carcinogenicity of acrylamide deserves a more thorough evaluation.

In another independent study by Banerjee, S. and Alvins, S. (3) it was demonstrated that acrylamide morphologically transformed mouse fibroblast cells *in vitro*. The acrylamide induced a dose dependent cytotoxic effect on the fibroblasts. Some of the acrylamide polymers are used as coagulant aids in the treatment of drinking water. Polymers used for this purpose are restricted to an acrylamide monomer content of 0.05% because of the neurotoxic properties of acrylamide (8).

Recent interest has focused on development of analytical methods for the determination of acrylamide in sugar samples (15). The interest has been generated because this compound was reported to show some biological activity. Since acrylamide monomer is the raw material for making polyacrylamide, residual monomer may be present in polyacrylamide which is used in the sugar industry as a flocculant. Thus the possible presence of acrylamide monomer in sugar products is of interest. An analytical

technique for determining parts per trillion levels of this monomer in refined sugar has been developed by Cutie, S.S and Kallos, G.J. (1986). Their results indicated that between 30-610 parts per trillion of acrylamide was recovered from refined sugar.

Chemical soil stabilisation has been applied to a wide variety of soils and permitted the building of roadways, dams, banks, blocks and the like. Acrylamide gel is one of the most popular soil stabilisers currently used in the world as it provides tensile strength and some degree of water *proofing even to fine grained clay soils* (2). Soil stabilisation by acrylamide gel involves the incorporation of acrylamide in the form of the monomer into naturally occurring soils and its polymerization *in situ* with the aid of redox catalysts. During the year 1974 to 1975, outbreak of toxicosis characterized by equilibrium disorder attributable by acrylamide monomer were reported in Japan. It was found that the residual acrylamide monomer still present in the polymerised soil stabiliser had contaminated the wells supplying the patients residences with drinking water. For this reason the use of this kind of soil stabiliser has been prohibited by law in Japan since then.

In an attempt to develop a microbiological process for the control of pollution, microorganisms which were capable of degrading and detoxifying acrylamide monomers were searched for by Arai, T. *et al* (2). Several strains of microorganisms degrading and utilising acrylamide monomer were isolated from the sewage of an acrylamide plant. Among these biodegradative microorganisms, an actinomycete strain designated 10021R was found. The organism produced a potential constitutive enzyme, deaminated acrylamide monomer into less toxic acrylic acid and finally converted it to *ammonia* and carbon di oxide. Studies by these researchers on the overall morphology and physiology of the strain suggested that the strain might belong to the Nocardioform genus, *Rhodococcus*

The aim of this study was to identify bacterial strains able to utilise acrylamide, on the assumption that these might convert acrylamide to other less toxic compounds. A simple assay of this ability could be based on the fact that an organism utilised acrylamide as sole nitrogen or carbon source. By selecting mutants that had lost the ability to utilise acrylamide, attempts to clone the acrylamide utilisation genes could be made. A genomic DNA library of an organism possessing acrylamide resistant genes will be constructed. The planned method of cloning will be by complementation of the acrylamide non utilising mutants.

2.0 MATERIALS AND METHODS

2.1 NOCARDIOFORM BACTERIAL STRAINS.

Table 1: *Nocardioe* strains.

Strain	*General Remarks*	Origin
ATCC 12674	requires vitamin B_1 (1µg/ml) and sodium glutamate 2µg/ml) for growth in minimal media.	N. Ferreira
ATCC CW22	derivative of ATCC 12674 cured of the arsenic resistance genes	E. Dabbs N. Ferreira
ATCC 4277		B. Gowan
ATCC 4277-1	streptomycin resistant mutant of the strain 4277 (60µg/ml)	
ATCC 4277-1/1	mutant of ATCC 4277-1 resistant to acrylamide (3mg/ml)	B. Gowan
ATCC 14887		E. Dabbs
ATCC 14887/1	acrylamide resistant mutant of ATCC 14887 (3mg/ml)- utilises it as carbon and nitrogen source	B. Gowan
ATCC 14887/2	acrylamide resistant mutant of ATCC 14887 (3mg/ml)- utilises it as nitrogen source only	B. Gowan
ATCC 14887/1-1	strain ATCC 14887/1 resistant to rifampicin (60µg/ml)	B. Gowan

2.2 ESCHERICHIA COLI BACTERIAL STRAINS.

Table 2: Strains of *E. coli*

Strains	General Remarks	Origin
NM294	restriction⁻ recombination⁺	E. Dabbs
NM294-1	strain NM294 resistant to rifampicin	E. Dabbs

2.3 PLASMIDS

Table 2a : Types of plasmids

Plasmid name	General remarks	Origin
pDA27	shuttle vector obtained by combining the E. coli suicide vector pECOR251 and an arsenic resistant Nocardioform plasmid	A. Daffey
pDA30	arsenic resistence plasmid from Nocardioform	E. Dabbs

2.4 GROWTH OF BACTERIAL STRAINS.

All Nocardioform strains were maintained on TYA plates(see Appendix) or minimal media (mm) plates and stored at 4°C. Utilisation and resistance levels to antibiotics and acrylamide was measured by spotting on plates using a replicator. The highest concentration at which growth was confluent was taken to be the resistance level. The resistance levels were also determined by liquid culture assays. Aliquots of stock solution of antibiotics (generally 10mg/ml) and acrylamide (10%) were added to plate media after autoclaving and before pouring. Nocardioform cultures were grown at 28°C.

All *E. coli* strains were maintained on LA plates (See Appendix) and stored at 4°C. The cells were grown at 37°C.
In addition all strains were also maintained in 33% glycerol and stored at -80°C

2.5 RESPONSE OF NOCARDIOFORM BACTERIA TO ACRYLAMIDE

The strains 42771-1, 4277-1/1, 14887, 14887/1,and 14887/2 were grown in 5ml A-N buffer, 0.1% NH_4Cl and 0.5% glucose. 1ml of each of the precultures was pelleted, washed with sterile distilled water and 10µl inoculated into 5ml of the following liquid media,
A-N buffer +0.1% NH_4Cl + various concentrations of acrylamide which served as sole nitrogen source and A-N buffer + 0.5% glucose + various concentrations of acrylamide which served as sole carbon source. The acrylamide concentrations were 0.06; 0.2; 0.6; 2; 6; and 20mg/ml. Once growth was observed visually for any of the cultures, optical density (O.D) readings were taken at 540 nm on a Spectronic 601 spectophotometer. O.D

readings were taken every 12 hours until the cultures reached maximum growth.

2.6 MUTAGENESIS OF NOCARDIOFORM FOR THE SELECTION OF MUTANTS

2.6.1 N- METHYL - N'- NITRO - N NITROSO -GUANIDINE (NTG) MUTAGENESIS

2.6.1.1 NTG mutagenesis of an exponential phase culture.

Spontaneous streptomycin resistant mutants of the Nocardioform strain 4277 were obtained by spreading 0.1 ml of a preculture onto a TYA plate containing 200µg/ml streptomycin. A single streptomycin resistant colony was grown to the exponential growth phase in 5ml T2 media (see Appendix). 1ml of the cells were pelleted and resuspended in 0.9ml Tris.HCl pH 8.0 buffer and 0.1ml NTG dissolved in the same buffer by gentle heating. The final concentration of the NTG was 100µg/ml. The cells were incubated at 37°C for 2 hours after which they were washed in the Tris.HCl pH 8.0 buffer. Mutagenised cells were outgrown in T2 media containing 100µg/ml streptomycin at 28°C. The cells were sonicated for 5 seconds using a MSE ultrasonic power unit with a medium size tip, to reduce clumping before being diluted to 10^{-6} in sterile distilled water. 0.1ml of the 10^{-6} dilution was spread onto TYA plates to obtain single colonies. Once single colonies were obtained they were patched onto respective media to obtain the various mutants as well as auxotrophs.

A comparison was made using two different buffers;
(1) Tris acid malaate sodium hydroxide buffer pH4.8 (55) (see Appendix)
(2) Tris HCl buffer pH 4.8, pH 8.0 and pH 8.5 (55) (see Appendix)
The Tris.HCl pH 8.0 buffer gave the more satisfactory results with regards to the frequency of auxotrophs.

2.6.1.2 NTG mutagenesis of a growing culture .

The procedure was as described in section 2.6.1.1. except that the cells were grown for 3 hours after which the 0.1ml of NTG was added to the medium. The cells were allowed to grow for a further 2 hours before being washed with the Tris. HCl pH 8.0 buffer and outgrown in T2 media.

2.6.2 METHANESULFONIC ACID ETHYL ESTER(ETHYL METHANESULFONATE- EMS) MUTAGENESIS

The procedure was as described in 2.6.1.1. A 2% EMS solution was made using a Tris.HCl pH 7.0 buffer.

2.6.3 ULTRAVIOLET (U.V) MUTAGENESIS

Nocardioform strain ATCC 12674 was grown in T2 media and sonicated for 5 seconds to reduce clumping. The cells were diluted to 10^{-5} in sterile water and exposed to short wavelength (254nm) U.V. for 2, 3 and 4 minutes

at a distance of 2cm. The control was not exposed to U.V. rays. After the U.V. exposure 10µl of the cells were spread onto TYA plates to obtain single colonies. The plates were incubated in the dark at 28°C to prevent the light dependent repair system from functioning. The single colonies were patched onto selective media to detect the mutants.

2.7 ENRICHMENT PROCEDURE FOR THE SELECTION OF ACRYLAMIDE NON - UTILISING MUTANTS.

The strain 14887/1 was spontaneously made rifampicin resistant by spreading 1ml of a preculture onto a TYA plate containing 60µg/ml rifampicin. The strain was made rifampicin resistant to reduce contamination. The rifampicin derivative was grown to the exponential growth phase in TY media plus 20µg/ml rifampicin. This amount of rifampicin was added at each growth stage. The cells were mutagenised with NTG using the Tris.HCl pH 8.0 buffer for two hours. The mutagenised cells were outgrown in TY media overnight. 1ml of the cells were pelleted, washed twice with sterile distilled water and resuspended equally into 5ml of A-N buffer + 0.1% NH_4Cl 0.5% glucose and 5 ml A-N buffer + 0.1% NH_4Cl + 0.6mg/ml acrylamide (best concentration for growth determined from liquid culture experiments). The cells were allowed to grow for 24 hours before being challenged with 60µg/ml ampicillin for 24 hours. The ampicillin preferentially kills growing cells but not those which are unable to grow because of their inability to utilise acrylamide as sole carbon source. The cells were then pelleted, washed twice with sterile distilled water and grown in A-N buffer + 0.1% NH_4Cl + 0.5% glucose + 0.6mg/ml acrylamide. The acrylamide in this instance inhibited the growth of the cells unable to utilise acrylamide as sole carbon source. The culture was grown to stationary phase before being challenged with 60µg/ml ampicillin for 24 hours. Once again the ampicillin preferentially killed the growing

cells,i.e those that could utilise acrylamide as sole carbon source, and inhibited the growth of the acrylamide non - utilising cells. This process was repeated three times and at the end of the third challenge with ampicillin the cells were diluted from 10^2 to 10^5 and 0.1ml of each of dilution was spread onto TYA + 20µg/ml rifampicin plates. When single colonies were observed they were patched onto selective media plates such as;

(1) A-N buffer + 0.1% NH_4Cl + 0.5% glucose,

(2) A-N buffer + 0.1% NH_4Cl + 0.6mg/ml acrylamide,

(3) A-N buffer + 0.1% NH_4Cl + 0.5% glucose 0.6mg/ml acrylamide and,

(4) TYA

The control cells were diluted from 10^4 to 10^{-3} after the first round of ampicillin challenge and 0.1ml spread onto TYA + 20µg/ml rifampicin plates. The single colonies that grew up were patched onto minimal media plates to select for auxotrophs. Dilutions were also made of the unenriched mutagenized culture and it was treated as the enriched culture. This was done to determine whether the enrichment procedure increased the frequency of auxotrophs or not.

2.8 EXTRACTION OF BACTERIAL DNA

2.8.1 EXTRACTION OF NOCARDIOFORM DNA (LARGE SCALE)

Single colonies of the *Nocardioform* strains were inoculated in 5ml of TYG media (see Appendix) and incubated overnight at 28°C with vigorous shaking. 1ml of the overnight culture was inoculated in 250ml of TYG and the cells were grown at 28°C with vigorous shaking until they reached late log phase. For plasmid containing strains, 50mM arsenate was added to

maintain the plasmid. The stationary phase culture was harvested at 6000 rpm for 20 minutes and the pellet resuspended in 10ml 10mM Tris pH 8.0 10% sucrose with 50mg lysozyme. The cells were incubated at 37°C for 2 hours. The cells were then pelleted for 15 min at 12000 rpm and resuspended in 12ml TE (see Appendix) and 0.8ml TE containing 10% SDS. The cells were incubated at 37°C for 2hours to promote complete lysis to release the DNA before being centrifuged in 50 Ti tubes for 30 mins at 35 000 rpm. The DNA was separated from the proteins and an equal weight of caesium chloride (CsCl) to volume of DNA was added. The CsCl was dissolved and the solution centrifuged for 15mins at 15 000 rpm. The DNA was separated from the scum and the refractive index adjusted to 1.392. 1-1.5ml of ethidium bromide (EtBr) (10mg/ml in H_2O) was added to 12ml of DNA and loaded into quick seal ultracentrifuge tubes. The tubes were sealed , balanced and centrifuged for 16-17 hours at 45 000 rpm at 10°C in a VTi65.2 vertical rotor. DNA bands were observed under U.V. light and removed using a sterile syringe. For the plasmid DNA, the bands from 6 tubes were pooled and rerun on a CsCl gradient. EtBr was removed from the DNA by several extractions with TE saturated butanol. DNA was dialyzed against TE for 4 hours to remove the CsCl.

The concentration of the DNA was ascertained by spectrophotometric analysis on a Varian Cary 210 Spectrophotometer.

2.8.2 THE EXTRACTION OF E.COLI PLASMID DNA (LARGE SCALE)

1ml of a stationary phase preculture of *E .coli* AMN294 containing the putative shuttle vector, pOA27 was inoculated in 250ml of LB (see Appendix) containing 50µg/ml ampicillin and 10mM $MgSO_4$. The cells were grown for 6 hours at 33°C (because the λ had a temperature sensitive repressor). The cells were harvested at 6000 rpm for 10 mins and the pellet resuspended in 2ml cold 25% sucrose. 50mM Tris.HCl pH 8.0 and

0.25ml of a fresh lysozyme solution (10mg/ml) was added. The mixture was gently swirled on ice for 15 mins. 0.25ml of 0.5M EDTA pH 8.0 was added and the mixture swirled gently on ice for 5 mins.

To promote lysis of the cells, 2,5ml of cold detergent (see Appendix) was added and the mixture was gently swirled on ice (for 20-30 mins) until the solution was clear and highly viscous.Cells were centrifuged at 18 000 rpm for 45 mins and the volume of the supernatant was measured. 0.95g of CsCl was added per ml of supernatant. CsCl was dissolved and the solution centrifuged for 10 mins at 10 000 rpm at 4°C. 0.1ml of EtBr was added per ml of cleared lysate. The refractive index measured and adjusted to 1.392. DNA was loaded into quick seal ultracentrifuge tubes, sealed, balanced and centrifuged for 16-17 hours at 45 000 rpm at 10°C. For any plasmid band detected, fractions containing the band from 6 tubes were pooled and rerun on a CsCl gradient. The plasmid DNA was hereafter *treated as described in section 2.8.1.*

2.8.3 CALCULATION OF DNA CONCENTRATION

The absorbance at 260nm is proportional to the concentration with a value of 0.02 units per microgram DNA per millilitre (23).
Hence one absorbance unit at 260nm = 50µg/ml.

2.9 TOTAL DIGESTION OF PLASMID DNA AND PARTIAL DIGESTION OF CHROMOSOMAL DNA WITH RESTRICTION ENZYME BGLII.

DNA was first precipitated by the addition of NaCl to 0.1M and 2.5 volumes of chilled ethanol (-10°C). The solution was mixed well and spun for 20 mins in a microfuge at 4°C. The supernatant was discarded and the pellet dried in a 60°C incubator for 20 mins. The dried pellet was dissolved in sterile distilled water at 37°C for 1 hour.

510µl of chromosomal DNA and 27µl of plasmid DNA was ethanol precipitated and each one dissolved in 45µl of sterile distilled water. 5µl of the 10x stock medium buffer and 1µl of the restriction enzyme BglII (from Boehringer Mannheim) was added to each tube. The digestions were allowed to proceed for 3 hours in the case of the chromosomal DNA and for 1 hour for the plasmid DNA at 37°C.

The Bgl II was denatured with TE saturated phenol. 20µl of phenol was added to the DNA, mixed and spun for 3 mins in a microfuge at 4°C. The upper layer was removed to a clean Eppendorf tube and 20µl of isoamyl alcohol: chloroform (1:24) was added,mixed and spun for 5 mins in a microfuge at 4°C. The upper layer was removed and ethanol precipitated. The dried pellet was resuspended in 300µl of ligation buffer (see Appendix) at 37°C for 1 hour. The digested DNA was stored at -80°C.

2.10 LIGATION OF CHROMOSOMAL DNA TO THE PLASMID DNA

10ul of plasmid DNA and 4µl of chromosomal DNA in ligation buffer were mixed together with 1µl of T4 DNA ligase and incubated overnight at 14°C.

2.11 TRANSFORMATION OF THE STRAIN MM294-1

2.11.1 PREPARATION OF COMPETENT CELLS

A single colony of *E.coli* strain MM294-1 was inoculated into 5ml of LB and incubated overnight at 37°C. 1ml of the preculture was inoculated in 100ml of LB in a side arm flask and the culture grown for approximately 2 hours at 37°C with vigorous shaking. The cells were allowed to grow to a density of approximately 5×10^7 cells/ml. An O.D. at 540nm of 0.2 is = 5×10^7 cells/ml. The culture was chilled on ice for 10 mins and the cell suspension was centrifuged at 6 000 rpm for 10 mins at 4°C . The supernatant was discarded and the cells were resuspended in 50ml of cold transformation solution (see Appendix). The solution was placed on ice for 15 mins before centrifugation at 6 000 rpm for 10 mins at 4°C. The supernatant was discarded and the pellet resuspended in one fifteenth the original culture volume of transformation solution, 0.2ml of the cells were aliquoted into chilled Eppendorf tubes and stored on ice for 1 hour.

2.11.2 TRANSFORMATION OF COMPETENT CELLS

The ligated chromosomal and plasmid DNA (section 2.10) was added to the 0.2ml of competent cells and the samples were heat shocked in a preheated waterbath (42°C) for 1 minute. 1ml of LB was added and the cells incubated at 37°C for 1 hour. Following the incubation the samples were spun for 1 minute in the microcentrifuge at 4°C. Some of the supernatant was decanted and the pellets resuspended in the remaining supernatant. The samples were

spread gently onto LA plates containing 60µg/ml of ampicillin. The plates were incubated overnight at 37°C. The transformants were randomly checked for plasmid inserts and the clones were washed off the LA + 60µg/ml ampicillin plates with LB + 30µ/ml ampicillin and stored at -80°C in a sterile screw top bottle.

2.12 TRANSFORMATION OF PLASMID DNA INTO NOCARDIOFORM BACTERIA

Strain 4277-1 was grown in TYG for 62 hours and 1ml of the cells were pelleted in the microfuge for 1 minute. The pellet was resuspended in 1ml P buffer (see Appendix) containing 5mg of lysozyme. Cells were incubated at 37°C for 1 hour with agitation, after which they were pelleted and washed with P buffer. The pellet was resuspended in half the original volume of cells. 100µl of the cells used for each transformation. 4µl of DNA (final amount 200ng) and 90 µl of a 100% polyethelene glycol (PEG) solution (final concentration 30%) was added. The solution was mixed gently and incubated at room temperature before spreading onto protoplast regenerating media plates (see Appendix). The plates were incubated at 28°C for 12 hours before an underlay of 0.5 ml 3N arsenate, 0.5M arsenite was done. The plates were incubated for a further 7-9 days at 28°C. The regenerated protoplasts were patched onto TYA plates containing 60mM arsenate and 10mM arsenite and they were also screened for plasmids (section 2.13.2).

2.13 DETERMINATION OF PLASMID INSERTS IN TRANSFORMANTS

2.13.1 SMALL SCALE PLASMID ISOLATION FROM E.COLI (MANIATIS ET AL.1982)

Random *E.coli* MH294-1 transformants were grown overnight in 5ml LB containing 50µg/ml ampicillin at 37°C. 1ml of the overnight culture was harvested for 1 minute in the microcentrifuge (4°C) and the pellet resuspended in 100µl solution 1 (see Appendix). After 5 mins at room temperature, 200µl of solution 2 (see Appendix) was added and mixed by gentle inversion. The samples were incubated on ice for 5 mins before 150µl of precooled 3M KAc pH 4.8 was added. The solution was mixed until it was an even suspension. The mixture was placed on ice for 5 mins, centrifuged for 1 min at 4°C and the supernatant removed to a sterile Eppendorf tube.

An equal volume of isopropanol was added to the supernatant and the mixture was allowed to stand at room temperature for 5 mins. DNA was precipitated by centrifuging for 5 mins at 4°C. The pellet was washed with cold 70% ethanol and reprecipitated by centrifuging for 5 mins in a microcentrifuge at 4°C. After drying the precipitate at 60°C for 20 mins it was dissolved in 12µl of TE buffer containing pancreatic ribonuclease (freshly boiled) (1mg/ml stock) for 1 hour at 37°C. The samples were mixed with loading buffer type IV (see Appendix) and analysed by electrophoresis at 80v/cm in 0.4% agarose gels containing 15µg/ml EtBr buffered with Tris - Borate - EDTA (TBE) buffer (see Appendix) which contained 150µg of EtBr/ml.

To determine the size of the inserts, the above procedure was repeated. The precipitate, instead of being dissolved in TE + Rnase it was digested

with BglII. 16µl of the following solution was added to each sample;

180µl H_2O

20µl 10x medium buffer

1µl boiled Rnase (of a 1mg/ml stock)

2µl BglII (24 units)

The DNA was digested overnight at 37°C before electrophoretic analysis on 0.4% agarose gels.

2.13.2 SMALL SCALE PLASMID ISOLATION FROM NOCARDIOFORM.

A modification of the Dabbs, E.R. and Sole, G.J. (1987) method was used. Random transformants were grown overnight at 28°C in 5ml TYG media to which 50mM arsenate was added. 1ml of the culture was pelleted and re-suspended in 800µl of a 5mg/ml lysozyme Tris sucrose, pH 8.0 solution. The sample was incubated for 1 hour at 37°C with shaking. Cells were then pelleted and resuspended in 280µl of TE. 40µl of TE + 10% SDS was added, mixed by gentle inversion and incubated at 60°C for 10 mins. 40µl of 4,5M sodium acetate pH 6.0 was added and the solution was mixed gently. Samples were placed in an ice - water slurry for 30 mins and then pelleted in a microfuge for 20 mins at 4°C. The supernatant was extracted once with 80µl TE saturated phenol and once with 80µl isoamyl alcohol : chloroform (1:24). The DNA was ethanol precipitated and the dried precipitate was dissolved in 20µl of TE buffer containing 1mg/ml pancreatic ribonuclease (freshly boiled). Samples were mixed with loading buffer and analysed by electrophoresis at 80v/cm in 0.4% agarose gels buffered with TBE.

2.13.3 PHOTOGRAPHY

Photographs of agarose gels were taken with a Polaroid Cu - 5 Land Camera. Polaroid type 665 black and white film was used.

3.0 RESULTS

3.1 BACTERIAL GROWTH

3.1.1 GROWTH IN MINIMAL MEDIA

Each bacterial strain responds differently to nutrients in the growth media, therefore as a preliminary to this work the growth response of various Nocardioform strains was checked in minimal media. One A-N buffer had a pH of 7.8 while a second A-N buffer had a pH of 7.0 due to different amounts of K_2HPO_4. The strains 12674, 4277 and 14887 were spot tested using a replicator onto minimal media plates made with both the A-N buffers. The plates were incubated at 28°C for 5 days.

All strains grew better on minimal media containing the pH7.8 buffer. The parental strain 12674 grew poorly in comparison with 4277 and 14887, indicating it has a requirement for sodium glutamate and vitamin B_1 (E.Dabbs - personal communications). This was confirmed by spot testing the same strains onto minimal media containing 5ug/ml sodium glutamate and 1ug/ml Vitamin B_1. Strains 4277 and 14887 grew equally well with or without these additions.

3.1.2 GROWTH IN THE ABSENCE OF A CARBON SOURCE

The same three strains were grown on minimal media(mm) excluding the glucose to determine whether an agarase gene (utilise agar) was present in them or not. The plates were observed for growth after 2 days at 28°C. Results are shown in table 3.

Table 3 : Growth of Nocardioform strains in different media

	Type of media				
Norcardioform strain	TYA	mm	mm^1	mm^2	mm^3
12674	+++	++	+	+++	+++
4277	+++	++	+	++	+++
14887	+++	++	+	+	++

key : mm = A-N buffer + 0,1% NH_4Cl + 0,5% glucose + agar

mm^1 = A-N buffer + 0,1% NH_4Cl + agar

mm^2 = A-N buffer + 0,1% NH_4Cl + sodium glutamate+vitamin B_1 + agar

mm^3 = A-N buffer + 0,1% NH_4Cl + 0,5% glucose+sodium glutamate + vitamin B_1 + agar

+++ = good growth

++ = fair growth

+ = poor growth

All the organisms grow (although poorly) in the absence of glucose, suggesting that the agar was being utilised as a carbon source via an agarase gene. 12674 showed good growth in mm^3, compared to 4277 and 14887. The sodium glutamate and vitamin B_1 serve as additional carbon sources.

It was necessary to obtain an agarase⁻ mutant of 14887 and 4277 so that the utilisation of agar as carbon source did not hinder the use of acrylamide as carbon source. Growth of these strains was tested with 3 types of agar.

3.1.3 TESTING OF GROWTH WITH DIFFERENT AGARS

Strains 4277 and 14887 were spot tested on mm with glucose and without glucose. Three different types of agar were used in each case, they were:
(1) The bacteriological grade, laboratory agar
(2) Purified agar free of inhibitors (obtained from Merck)
(3) Recrystallised Agar (Agar Noble)

The plates were checked for growth after 2 days at 28°C. Both the organisms showed loss growth with the recrystallized agar. This was true for mm with and without glucose. Therefore, in subsequent carbon source determination experiments agar noble was used.

3.1.4 SODIUM CITRATE USED AS CARBON SOURCE

Preliminary studies testing the use of acrylamide by the Nocardioform 4277 as the sole carbon or nitrogen source lead to the discovery that the sodium citrate (Na citrate) in the A-N buffer could be used as a carbon source. Such is not the case for *E.Coli* for which this buffer was devel-

oped. Comparisons of growth with and without the Na citrate in the A-N buffer were made. Visual observations of cell density indicated that exclusion of Na citrate retarded growth and that it could be used as a carbon source. The strain 4277-1 cannot use 0,1mg/ml acrylamide as sole carbon source (see table 4).

Table 4 : Growth of Norcardioform 4277 with and without sodium citrate.

		Growth
1.	buffer 1 + NH_4Cl + glucose	Yes
2.	buffer 2 + NH_4Cl + glucose	Yes - but less than with buffer 1
3.	buffer 1 + NH_4Cl	Yes
4.	buffer 2 + NH_4Cl	No
5.	buffer 1 + NH_4Cl + 0,1mg/ml Acryl	No
6.	buffer 2 + NH_4Cl + 0,1mg/ml Acryl	No

Key : buffer 1 = A-N buffer with citrate
buffer 2 = A-N buffer without citrate

A comparison of 3 and 5 suggests that citrate was used as a carbon source.

To confirm that citrate was used as a carbon source the organisms were also spot tested onto minimal media containing the 2 different buffers. Results from this experiment agreed with the liquid culture experiment. The spot test further confirmed that the strains have an agarase gene because some growth was observed on media without glucose and sodium citrate. The only carbon source present was the agar noble.

For all further experimentation, minimal media without the Na citrate was used. Once the growth media for the Norcardioforms was tailored, mutants had to be obtained.

3.2 MUTAGENESIS

In this study obtaining acrylamide non utilisation and acrylamide resistant mutants of the Norcardioform bacteria was essential, since the acrylamide detoxification genes were to be cloned by complementation of the non-utilisation mutants.

NTG mutagenesis of *E.coli* may yield 60-80% auxotrophs (E. Dabbs - personal communications) whereas the frequency of auxotroph production after NTG mutagenesis in Nocardioform was between 1-2%. Attempts were made to improve upon this with several different mutagens.

3.2.1 CHEMICAL MUTAGENESIS

3.2.1.1 Mutagenesis of bacterial cells with NTG

NTG is a mutagen that acts by alkylation. Bacteria carrying an NTG-induced mutation are usually found to be multiple mutant in the sense that there are often additional mutations in the gene and in other genes. The mutations are clustered in genes that are adjacent in the genetic map. The explanation for this phenomenon is that NTG exerts its most powerful affect in the replication fork.

Choice of mutagenesis buffer: The *E.coli* strain MM294 was mutagenised with NTG for 2 hours using a Tris HCl buffer pH8.0 and a Tris-acid maleate buffer pH 4.8. Mutagenized cells were diluted and spread onto LA plates

and incubated O/N at 37°C. The efficiency of the mutagenesis can be measured by the % killing. Plates were checked for % killing by colony counts of the control and mutagenized cells.

NTG in the pH 8.0 buffer system killed 14% of the cells whilst in the pH 4.8 buffer system it killed 91% of the cells. These results suggest that a buffer of pH 4,8 is effective in mutagenizing Gram negative *E.coli* cells. The Nocardioform strain 4277-1 was mutagenized as was *E.coli* HH294 and the % killing compared for the two different buffer systems. There was a 42% killing with the pH 4.8 buffer and 65% with the pH 8,0 buffer. Thus a buffer of pH 8.0 is more effective in mutagenizing Gram positive cells.These results also imply that the structure and the permeability of the cell wall plays an important role in the uptake of the buffer by the cell.

Tris.HCl buffers of pH 8.5 and pH 4.8 were also used to mutagenise 4277-1 to see if there was a substantial difference in the killing. No significant difference was detected, therefore all further mutagenesis were conducted using the Tris.HCl pH 8.0 buffer.

Frequency of auxotroph production by mutagenesis: A stationary phase culture of strain CW22 ("cured" of its arsenic resistant plasmid) was mutagenized with NTG for 2 hours using a Tris. HCl pH 8,0 buffer. The cells were diluted and 264 colonies were screened on TYA and minimal media plates for auxotrophs. Five auxotrophs were detected, hence the frequency of auxotroph production by NTG was 2,2%.

Nocardioform bacterial cells aggregate to form clumps therefore attempts were made to reduce clumping. Four factors are known to reduce aggregation of the cells, each of which will be discussed in the following sections.

Growth media: When the cells are grown in TY media supplemented with divalent cations such as calcium, magnesium and sodium, there is a 10-50

fold increase in colony forming units per optical density (E. Dabbs personal communication).

All growth during mutagenesis was done in this media (T2 media).

Vortexing: A NTG mutagenized culture of the Nocardioform strain CW22 was vortexed for 0, 20, 40, 60 and 80 seconds before spreading 0.1ml of a 10^{-4} dilution onto TYA plates. The colonies were counted after 2 days and are tabulated in table 5.

Table 5 : Effect of vortexing on reducing aggregation of the Nocardioform cells.

Time (seconds)	No. of colonies
0	222
20	215
40	296
60	246
80	306

The results indicate that there is a direct relationship between vortexing and reduction in aggregation of the cells. Vortexing increases the number of colony forming units by approximately 40%.

Sonication: A NTG mutagenized culture of 4277-1 was sonicated for 5 and 10 seconds using a MSE ultrasonic power unit with a medium size tip. The effect of vortexing together with sonication was also investigated. The sonicated culture was diluted 10^{-3} fold, vortexed for 80 secs and 0.1ml spread onto TYA + streptomycin plates.

Table 6 : The effects of vortexing and sonication on number of colony forming units.

	vortex time	
	0 sec	60 sec
0 sec sonification	91	87
5 sec sonification	194	182
10 sec [illegible]cation	134	62

From the colony counts, the conclusion is that 5 second sonication with the medium sized tip doubles the number of colony forming units. Vortexing for 60 seconds in combination with sonication has no significant effect on reducing clumping.

All further mutagenised cultures were sonicated for 5 seconds with the exclusion of the 60 second vortexing.

Growth of cells in Sodium Taurocholate: The strain 4277-1 was tested for growth in the presence of sodium taurocholate, which is a detergent.

The cells were initially grown in 0,1% sodium taurocholate but growth was greatly inhibited hence a lower concentration of 0,02% was used. After 2 days at 28°C, the culture was diluted to 10^{-6}, vortexed for 60 seconds and 0,1 ml plated on TYA plates containing streptomycin. Colony counts after 2 days indicated that growth of cells in 0,02% sodium taurocholate does reduce aggregation. A total of 386 colonies was obtained which is highly comparable to the number obtained after 60 seconds of vortexing.

All four processes clearly increases the number of colony forming units by 50 to 100%. Sonication appears to be most satisfactory since it doubles the colony forming units by 100%. For this reason sonication of the mutagenised culture grown in T2 media was the method of choice. The use of sodium taurocholate and vortexing were not further pursued.

3.2.1.2 Mutagenesis of growing bacterial cells with NTG

The frequency of auxotrophs obtained from mutagenesis of a stationary phase culture was 2,2%. In an attempt to increase the frequency of auxotrophs, it was decided to mutagenise a growing culture of strain 4477-1.

Cells were grown for 3 hours before the NTG was added. The culture was grown for a further 2 hours before the cells were washed and outgrown in T2 media. Thereafter the cells were treated as for the stationary phase culture.

Upon screening 99 colonies, 2 auxotrophs were identified. This process slightly increased auxotroph production (2,5%), therefore cultures in the mid-log phase of growth were used for all future mutagenesis.

3.2.2 MUTAGENESIS OF BACTERIAL CELLS WITH EMS

EMS is an alkylating agent that reacts primarily with guanine and to some extent with adenine resulting in transversions and transitions.

A 2% EMS solution in a Tris.HCl buffer of pH 7,0 was used to mutagenize exponentially growing cells of 4277-1 in T2 media. The cells were mutagenized for 2 hours at 37°C. The mutagenesis procedure was identical to that of the NTG mutagenesis.

198 mutagenized colonies were screened and 5 auxotrophs were identified, giving a frequency of 2,5%. The experiment was repeated several times and each time the yield of auxotrophs was between 1.8-4,5%.

3.2.3 MUTAGENESIS OF BACTERIAL CELLS WITH ULTRAVIOLET IRRADIATION

Ultraviolet light causes damage to the DNA by producing thymine dimers. When a RecA bacterium is UV irradiated, no mutants result; from this it is apparent UV-induced mutagenesis requires a DNA repair mechanism (23). It is generally thought that the system responsible for mutagenesis is the error-prone SOS repair system.

An exponentially growing culture of strain 12674 was sonicated for 5 seconds. The cells were diluted to 10^{-1} in T2 media and 200µl exposed to UV irradiation for the times 0, 1, 2, 3 and 4 minutes at a distance of 2 cm. 10µl of the UV exposed cells were spread onto TYA plates and incubated at 28°C in the dark (to prevent photo-reactivation repair). Colony counts after 3 days are tabulated in table 7.

Table 7 : Effect of UV on mutagenesis

Time of UV irradiation (mins)	Number of colonies
0	85
1	75
2	105
3	132
4	80

After 4 minutes of UV irradiation only 6% of the cells were killed. This suggested that the T2 media used to dilute the cells was absorbing the UV radiation, therefore the experiment was reposted with sterile distilled water as the dilutent, The % killing of cells when diluted in T2 and sterile distilled water was compared (table 8).

Table 8 : Comparison of % killing by U.V.

	UV irradiation time		
	0 min	10 min	% killing
Dilution in sterile dH_2O	804	8	99,0
Dilution in T2	1236	508	59,0

These results indicated that components of the T2 media were absorbing the UV radiation.

When 271 colonies were screened , 6 auxotrophs were observed, giving a frequency of 2,2%.

3.2.4 CHARACTERISATION OF AUXOTROPHS

It is useful to have a pool of different auxotrophs for genetic analysis. From all the mutagenesis (NTG, EMS and UV irradiation) a total of 37 auxotrophs were obtained. These were tested on minimal media supplemented with 19 different amino acids.

The plates were scored after 4 days at 28°C. An arganine, isoleucine valine (ilv) , serine, proline, leucine and 3 histidine requiring auxotrophs were identified.

3.2.5 SCREENING OF ACRYLAMIDE NON-UTILISING MUTANTS

336 colonies of the strains 4277-1 and 12674 which were exposed to different mutagenesis conditions (NTG, EMS and UV irradiation) and previously screened for auxotrophs were further screened for acrylamide non-utilizing mutants, on minimal media containing 0,1 mg/ml acrylamide as sole nitrogen source.

The screening gave no positive results hence an alternate method for selecting this mutant had to be adopted - the process of enrichment

3.2.5.1 The enrichment procedure

It is often the case that induced mutagenesis does not increase the mutant fraction to more than one mutant per 10^6 cells, so that 500 replica plates will need to be screened to find a single mutant (23). In that case, mutant enrichment procedures are used that favour the growth of the mutant over the wild type. One such procedure is the ampicillin selection technique. This method is based upon the fact that ampicillin kills growing bacteria but not cells that are quiescent.

In this study it was necessary to obtain an acrylamide non-utilising mutant, so acrylamide was the growth limiting nutrient. Strain 4277-1 was grown in minimal media with 0.6mg/ml acrylamide as sole nitrogen source. Three rounds of selection for the mutant were done with 60 µg/ml ampicillin and enrichment in minimal media containing glucose. When 224 colonies were screened on minimal media plates containing 0.6mg/ml acrylamide no mutants were detected.

The experiment was repeated using the Nocardioform strain 14887/1 which can utilise acrylamide both as carbon and nitrogen source. Acrylamide was supplied as sole carbon source in the growth media. 60µg/ml ampicillin was added which preferentially killed the growing cells i.e. those able to utilise acrylamide as sole carbon source were amplified in minimal media containing 0,5% glucose and 60mg/ml acrylamide. This sequential process was repeated thrice. Two types of mutants are likely to be obtained, via acrylamide non-utilising and those that are inhibited by acrylamide. By adding the acrylamide in the amplification step, the frequency of the mutants inhibited by acrylamide is decreased, and thus increasing the probability of obtaining an acrylamide non-utilising mutant. Upon screening 224 colonies after one round of enrichment, 3 apparent acrylamide non-utilising mutants were obtained. Only one possible

acrylamide non- utilising mutant was obtained when 224 colonies were screened after the third round of enrichment.

This result indicates that various conditions in the enrichment procedure (eg. ampicillin concentration) will have to be optimized to increase the frequency of mutants. However, when the frequency of auxotroph production was compared between the enrichment process and the NTG mutagenesis there was a ten fold increase in the production of auxotrophs by the enrichment process. This clearly suggests that the former method is of greater value in the selection of mutants.

3.3 SELECTION OF NORCARDIOFORM MUTANTS RESISTANT TO ACRYLAMIDE

Two factors are involved in the ability of the bacterium to use acrylamide, via the ability to metabolise acrylamide per se modified by the inhibitory effects of this substance. Therefore apart from the genes involved in acrylamide metabolism or utilisation, one would also like to identify genes responsible for resistance to acrylamide.

Norcardioform strains 12674, CW22, 4277-1 and 14887 were spot tested on media containing different concentration of acrylamide to ascertain the level of resistance.

The growth of strains 12674 and CW22 is greatly inhibited at an acrylamide concentration of 1mg/ml, whilst at a 2mg/ml concentration growth of all organisms is inhibited. The strain 4277-1 and 14887 grow fairly well with acrylamide concentrations of 0,5 mg/ml , 1 mg/ml and 1,5 mg/ml , hence all subsequent experiments were focused on these two strains.

Preliminary experiments also showed that the strain 14887 can utilise 1mg/ml acrylamide as sole nitrogen and carbon source but the strain 4277-1 can only use it as sole nitrogen source.

To make the bacterium acrylamide resistant, both 4277-1 and 14887 that could use acrylamide as sole nitrogen source were grown on plates containing 3mg/ml or 5mg/ml acrylamide. Once 2-3 colonies were observed, they were restreaked onto minimal agar media containing 3mg/ml or 5mg/ml acrylamide respectively, to purify the mutant.

Acrylamide resistant mutants of 4277-1 and 14887 were successfully obtained from the minimal media plates containing 3 mg/ml and 5 mg/ml acrylamide. The 14887 acrylamide resistant mutants grow relatively fast (7 days) compared to the 4277-1 resistant [illegible] (14 days). To ensure that the 14887 mutant was not a contam[illegible] analysed with a bacteriophage specific for that strain.

3.3.1 ANALYSIS OF ACRYLAMIDE RESISTANT MUTANTS

Nocardioform sensitivity to phage is of value in the tentative identification of strains. A lysate of a bacteriophage specific for the strain 14887, obtained from K.Downing was useful in identifying the 14887 acrylamide resistant mutant.

An acrylamide resistant colony of 14887 was dispersed in TY media supplemented with 10mm $CaCl_2$ (TYC). 2ml of top agar was added and poured onto a chilled TYC plate. The plate was dried at 42°C for 30 mins after the top agar had hardened. 20µl of the phage lysate was poured in the centre of the plate. The plate was incubated overnight at 28°C. The bacteriophage lysed the bacterial cells since a clear plaque was observed

upon examination of the plate. This clearly indicated that the acrylamide mutant was not a contaminant. The mutants were tested for their potential to utilise acrylamide as sole carbon and/or nitrogen source and for their level of resistance to this compound.

3.3.2 ABILITY OF NOCARDIOFORM BACTERIA TO USE ACRYLAMIDE AS SOLE CARBON OR NITROGEN SOURCE AND THEIR LEVEL OF RESISTANCE.

The acrylamide resistant mutant together with their respective parental strains were spot tested on minimal media containing varying amounts of acrylamide. The aim of the test was to ascertain the level of resistance of each strain to acrylamide and their ability to utilise it as sole carbon or nitrogen source.

The results are tabulated in Table 9 .

Table 9 : Response of Nocardioform bacteria to acrylamido.

		Nocardioform strain					
type of media	AM conc	14887	4277	4277-1	4277-1 (grown on 3mg/ml AM)	14887 (grown on 5mg/ml AM	14887 (grown on 3mg/mlAM)
TYA	0	+++	+++	+++	+++	+++	+++
mm	0	+++	+++	+++	+++	+++	+++
mm	2,5mg/ml	+	+	+	+	+++	+
mm	5mg/ml	+	+	+	+	+++	+
mm	7,5mg/ml	-	+	+	+	+	+
mm	10mg/ml	-	+	+	+	+	+
mm^1	0	+	+	+	+	+	+
mm^1	2,5mg/ml	+	+	+	+	+++ ($)	- (θ)
mm^2	0	++	++	++	++	++	++
mm^2	2,5mg/ml	+	+	+	+++ (*)	+++ ($)	+ (θ)

Key : mm = A-N buffer + 0.1% NH_4Cl + 0.5% glucose.

mm^1 = A-N buffer + 0.1% NH_4Cl.

mm^2 = A-N buffer + 0.5% glucose.

AM = acrylamido

+++ = good growth

++ = *fair growth*

\+ = poor growth

The results indicate that the 4277 acrylamido resistant strain can use acrylamido as sole nitrogen source (*).The acrylamido resistant strain

14887 selected in 5mg/ml acrylamide utilised acrylamide as both sole carbon and nitrogen source ($), whilst the resistant mutant selected in 3mg/ml acrylamide can use the compound poorly as sole nitrogen source but cannot use it as sole carbon source (#).

The acrylamide resistant strains were designated as 4277-1/1; 14887/1; and 14887/2

3.3.3 ACRYLAMIDE DOSE RESPONSE GROWTH CURVES

Cultures of strain 14887/1, 14887/2, 4277-1/1 and the wild type strains 14887 and 4277-1 were grown in 5 ml minimal media. 1ml of each of the precultures were pelleted, washed and 10 µl resuspended in minimal media with 0; 0,06; 0,2; 0,6; 2; 6 and 20 mg/ml acrylamide. The acrylamide was either the carbon or nitrogen source.

Growth of cells was monitored by optical density measurement at 540nm on a Spectronic 601 spectophometer.

The results are represented graphically on a 3 cycle logarithmic scale.

3.3.3.1 Response of the strains 4277-1 and 4277-1/1 to acrylamide

Acrylamide as sole nitrogen source: The 4277-1/1 and the wild type strain was grown in 0; 0,06; 0,2; 0,6; 2; 6 and 20 mg/ml acrylamide as sole nitrogen source in minimal media. Growth was detected by optical density measurements at 540nm after 72 hours.

The results are represented graphically in figure 2.

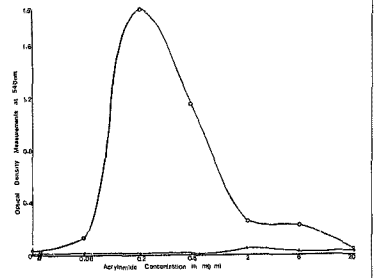


Figure 2. The response of the acrylamide resistant and wild type strains of 4277-1 to varying concentrations of acrylamide as sole nitrogen source.

—o— 4277-1

—o— 4277-1/1

The results clearly indicate that the acrylamide resistant strain is able to utilise acrylamide as sole nitrogen source far better than the wild type strain. The cells grow maximally with 0.6 mg/ml acrylamide

Acrylamide as sole carbon source: The strains 4277-1 and 4277-1/1 were grown in the presence of 0; 0,06; 0,2; 0,6; 2; 6 and 20 mg/ml acrylamide as sole carbon source. Upon spectrophometric measurements at 540nm no detectable growth was observed even after prolonged incubation (163 hours) of the cultures. This suggests that these strains cannot use acrylamide as sole carbon source. This result agree with that obtained from the spot test (section 3.3.2).

3.3.3.2 Response of the strains 14887, 14887/1 and 14887/2 to acrylamide

Acrylamide as sole nitrogen source: The *14887 acrylamide resistant and* wild type strains were grown in 0; 0,06; 0,2; 0,6; 2; 6 and 20 mg/ml acrylamide as sole nitrogen source. Optical density measurements at 540 nm were taken after 72 hours. The results are represented graphically on a 5 cycle log scale in Figure 3.

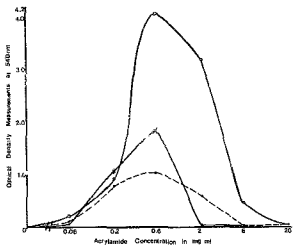


Figure 3. The response of the acrylamide resistant and wild type strains of 14887 to varying concentrations of acrylamide as sole nitrogen source

– –•– – 14887

—o— 14887/1

—•— 14887/2

The result[illegible]cate that both the acrylamide resistant strains are more capable of utilising acrylamide as sole nitrogen source than the wild type

strain. The 14887/1 strain grew at a faster rate than 14887/2. Maximal growth occurred with 0,6 mg/ml acrylamide.

Acrylamide as sole carbon source: The 14887 acrylamide resistant and wild type strains were grown in 0; 0,06; 0,2; 0,6; 2; 6 and 20 mg/ml acrylamide as sole carbon source in minimal media. Growth was detected by optical density measurement at 540nm.

The results are represented graphically on a 3 cycle log scale in Figure 4.

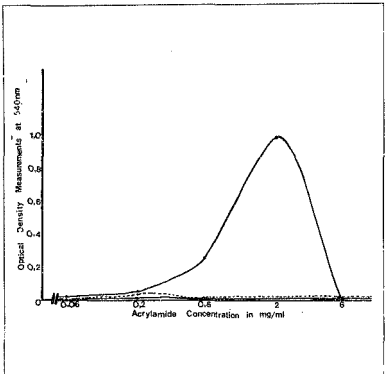


Figure 4. The response of the acrylamide resistant and wild type strains of 14887 to varying concentrati of acrylamide as sole carbon source

– – –14887

——14887/1

——14887/2

The results agree with those obtained from the spot tests (section 3.3.2).The strain 14887/2 apparently cannot use acrylamide as sole carbon

source. The strain 14887/1 grows optimally in the presence of 2 mg/ml acrylamide.

3.4 CONSTRUCTION OF THE NOCARDIOFORM GENOMIC DNA LIBRARY IN E.COLI MM294-1

The principle strategy for cloning the genes involved in acrylamide metabolism/ detoxification/ utilisation is by complementing mutants that have lost the ability to utilise acrylamide. Hence construction of a genomic library for the donor DNA (from strain 14887/1) was the first essential requirement for the cloning process.

3.4.1 THE PUTATIVE SHUTTLE VECTOR

The putative shuttle vector pDA27 (constructed by A. Daffey) is a combination of the *E. coli* plasmid pECOR251 (suicide vector) and a Nocardioform arsenic resistant plasmid, pDA22. The vector is maintained in the *E. coli* strain MM294. The strain is recombination$^+$ therefore the shuttle vector is likely to integrate into the host chromosomal DNA by a set of enzymes and proteins that constitute the Rec$^+$ system. Hence a Rec$^-$ strain coupled with a λ repressor to maintain the plasmid pECOR251 is necessary. Thus far such a recipient cell has not been constructed but further research *should soon meet this requirement.*

Fig 5 shows some of the features of the shuttle vector.

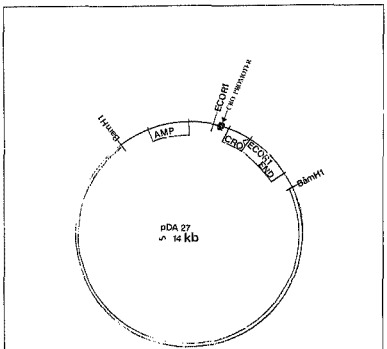


Figure 5. Schematic representation of the shuttle vector, pDA27.

——— suicide vector, pECOR251

═══ Nocardioform arsenic resistance plasmid

AMP = Ampicillin

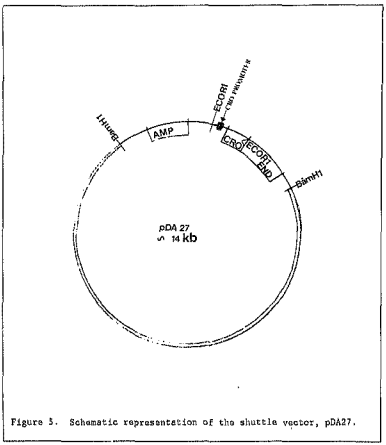


Figure 5. Schematic representation of the shuttle vector, pDA27.

——— suicide vector, pECOR251

═══ Nocardioform arsenic resistance plasmid

AMP = Ampicillin

The first useful feature of the shuttle vector is that it carries the resistance genes for ampicillin. This resistance can be used as a selectable marker for cells containing the plasmid.

The second feature of this vector is that the ECOR1 endonuclease site contain the unique restriction site BglII which can be very useful in cloning. Only transformants that have a DNA segment cloned into it will grow. Therefore it is a useful selection system.

The third advantage of the shuttle vector is that the ECO R1 END. gene is under the control of a λ repressor. The λ repressor binds to the promoter of the cro gene, thereby repressing the transcription of the ECO R1 END. genes. Alternatively the ECO R1 END. genes could be inactivated by the insertion of DNA into the unique BglII site.

3.4.2 CHOICE OF RESTRICTION ENZYME TO DIGEST CHROMOSOMAL DNA

Nocardioform chromosomal DNA from the strain 14687/1 and the shuttle vector from the *E. coli* strain Lam294 were extracted and both digested with BglII. The chromosomal DNA could be digested with either BglII, BamHI, BclI or Sau3A since all these restriction enzymes produce the same sticky ends (see table 10).

Table 10 : Restriction sequences of restriction enzymes.(42)

Restriction enzyme	Recognition sequence
BamHI	G↓GATCC
BclI	T↓GATCA
BglII	A↓GATCT
Sau3A	↓GATC

Although these enzymes produce the same sticky ends they have different flanking regions, thereby producing different hybrid sites upon ligation of the chromosomal and plasmid DNA. Size determination of the plasmid insert will be difficult since the hybrid site will not be recognized and digested. Hence for simplicity, BglII was used to digest both the chromosomal and plasmid DNA.

3.4.3 DETERMINATION OF THE AMOUNT OF BGLII REQUIRED TO PARTIALLY DIGEST THE CHROMOSOMAL DNA.

Partial digestion results in cleavage of only a limited number of the restriction sites. As well as the standard fragments produced by total digestion, additional fragment sizes will also be obtained. These are molecules that comprise two adjacent restriction fragments, separated by a site that has not been cleaved (6). Hence partial digestion ensures that the DNA is not restricted into very small fragments.
The chromosomal DNA from the strain 14887/1 was digested with diluted BglII for 3 hours at 37°C. 4 dilutions were made; 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. The digested DNA was analysed by agarose gel electrophoresis.

Restriction enzyme	Recognition sequence
BamHI	G↓GATCC
ScIZ	T↓GATCA
BglII	A↓GATCT
Sau3A	↓GATC

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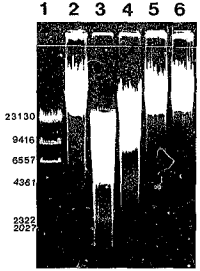


Figure 6. 0.4% agarose gel of diluted BglII digested DNA

Lane 1 : λ II molecular weight marker

2 : uncut chromosomal DNA

3 : chromosomal DNA digested with 10^{-1} diluted BglII—1.6 units/µg DNA

4 : chromosomal DNA digested with 10^{-2} diluted BglII—0.16 "

5 : chromosomal DNA digested with 10^{-3} diluted BglII—0.018 "

6 : chromosomal DNA digested with 10^{-4} diluted BglII—0.0018 "

The figure shows that the 10^{-3} dilution of BglII partially digests 1µg of chromosomal DNA. Therefore to digest 100µg of DNA (amount used for constructing library) 1µl of undiluted BglII was used.

3.4.4 CALCULATION OF DNA CONCENTRATION

3.4.4.1 Concentration of Nocardioform chromosomal DNA

O.D. reading at 260nm = 0.0195
DNA was diluted 1 in 200
Therefore the final concentration was 0.0195 x 200 x 50 = 195µg/ml

3.4.4.2 Concentration of the shuttle vector

O.D. reading at 260nm = 0.0371
DNA was diluted 1 in 200
Therefore final concentration was 0.0371 x 200 x 50 = 371µg/ml

3.4.5 MAXIMIZING TRANSFORMATION FREQUENCY

The concentration of chromosomal DNA and plasmid DNA that yielded maximum number of transformants had to be determined. This was done by ligating a constant concentration (0.1µg/ml) of the digested shuttle vector to four different concentrations (0.1µg/ml, 0.2µg/ml, 0.4µg/ml and 0.8µg/ml) of the digested chromosomal DNA.
For each ligation 10µl of the digested DNA and 0, 1, 2, 3, and 4µl respectively of digested chromosomal DNA was used. The volume in each case was made up to 20µl with ligation buffer and 1µl of T4 ligase was added. The DNA was ligated overnight at 14°C and transformed in the *E.coli* strain MM294-1.

200µl aliquots of transformed and control samples were plated on LA plates containing 60µg/ml ampicillin and incubated overnight at 37°C

Table 11: Number of transformants obtained with varying amounts of DNA.

conc.of undigested DNA		conc. of digested DNA		
plasmid (µg/ml)	chromoso-mal (µg/ml	plasmid (µg/ml)	chromoso-mal (µg/ml	# of trans-formants
0	0	0,1	0,1	15
0	0	0,1	0,2	16
0	0	0,1	0,4	53
0	0	0,1	0,8	56
0	0	0	0	3
0,1	0	0	0	2
0	0	0,1	0	2

The above data suggested that 0.1µg/ml of plasmid DNA ligated to 0.4µg of chromosomal DNA was the best condition with regards to yields of transformants. There was no significant difference between 0.4µg/ml and 0.8µg/ml of chromosomal DNA. Hence, for further construction of the library 0,4µg/ml of chromosomal DNA was used.

3.4.6 PLASMID SCREEN

Cultures of several transformants were grown and screened for the plasmid. The samples were analysed by electrophoresis.

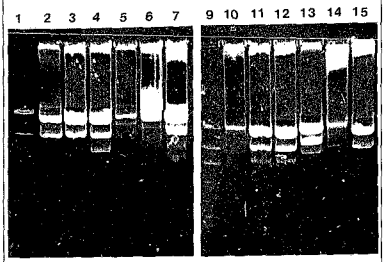


Figure 7. 0.4% agarose gel of plasmid DNA isolated from *E.coli* strain MM294-1

Lanes 1 and 9 : λ DNA II molecular weight marker

Lanes 2 and 10 : *purified shuttle vector*

Lanes 3- 7 and Lanes 11-15 : plasmid DNA isolated from transformants

Figure 7 shows that most of the transformants have the plasmid DNA insert when compared with the purified plasmid DNA. The plasmid DNA isolated from the transformants migrate slower than the *purified plasmid* DNA (vector) because of its larger size. The control in lane 2 is not very clear. It should appear as one distinct band (as in and 10).

The 3 bands could represent covalently closed circular, open circular and linear DNA. In addition the bands could also be due to concatomers.

3.4.7 SIZE DETERMINATION OF CHROMOSOMAL DNA

The approximate size of the inserts had to be determined in order to calculate the number of clones needed for the genomic library of Nocardioform. The plasmid was isolated from several transformants and digested overnight with BglII at 37°C. The samples were analysed by electrophoresis.

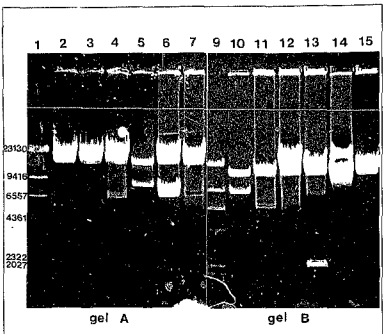


Figure 8. Restriction digest of plasmid DNA from transformants

Lanes 1 and 9 : λ DNA II molecular weight marker

Lanes 2-7 and Lanes 10 -15 : BglII digested plasmid DNA

Figure 8 shows that the chromosomal DNA inserts are large. The average size of the DNA was calculated from a curve based on the relative mobility of λ DNA *marker II* (Fig 9). The *fragment* lengths of the λII molecular weight marker are given alongside the figure

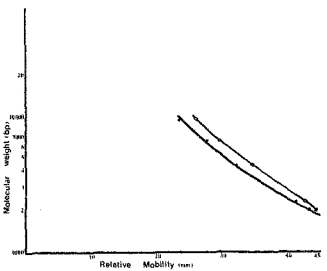


Figure 9. Relationship between molecular weight and electrophoretic mobility of restriction fragments of λ DNA II marker.

—●— - mobility of λ DNA marker of gel A

—○— - mobility of λ DNA marker of gel B

Table 12 : Approximate size of BglII digested chromosomal DNA inserts.

gel no	digested sample	size in Kbp
A	2	7,4
	3	7,4
	4	6,8
	5	9,0
	6	7,0
	7	7,4
B	10	9,4
	11	7,1
	12	7,5
	13	9,0 & 2,25
	14	no plasmid
	15	6,6

These sizes were calculated by measuring the DNA fragment lengths (in mm) in Fig 8 and the corresponding size in base pairs (bp) was obtained from the graph (Fig 9).
From the above data the approximate size of the insert is 7.5 Kbp (from gel A) And 7.0 Kbp (from gel B). Hence the average size is 7.25 Kbp.

The size of the chromosome of most of the Nocardioform bacteria is not known, however the size of DNA from *Mycobacterium tuberculosis* is 6.3×10^3 Kbp (S. Anderson - personal communication).
For *E. coli* at a probability of 95% that any particular gene will be present in the library, a 17Kbp fragment of a 4×10^3 bp genome size requires 700 clones. Hence, on the assumption that the Nocardioform DNA size is compatible with that of the *Mycobacterium* species, a average fragment size of 7.25Kbp will require 2247 clones to

ensure that at a 95% probability any particular gene will be present in the library. Presently the Nocardioform strain 14867/1 genomic DNA library contains 2655 clones.

The transformants were washed off the plates with LB containing 30µg/ml ampicillin. The clones are frozen at -80°C.

3.5 NOCARDIOFORM TRANSFORMATION

The Nocardioform transformation conditions had to be optimized to allow for the efficient transformation of the library into the recipient strain 4277-1. The strain 4277-1 was chosen as the recipient cell since it cannot use acrylamide as a sole carbon source. The DNA from the strain 14887 acrylamide served as donor DNA since it can utilize acrylamide as both carbon and nitrogen source.

3.5.1 TRANSFORMATION OF PLASMID pDA30 INTO NOCARDIOFORM 4227-1

A plasmid transformation system for the Nocardioform recipient strain 4277-1 had to be developed which allowed for the cloning of the DNA at an optimum frequency.

The system involved the uptake of the Nocardioform plasmid pDA30 (confers arsenic resistance) by protoplasts of the Nocardioform strain 4277-1 in the presence of PEG and the visual detection of transformants, after regeneration of the protoplasts.

pDA30 is normally maintained in the Nocardioform strain 12674. When DNA from this strain is transformed into the strain 4277-1 much of it may be destroyed by the restriction system of the strain 4277-1. To overcome this host restriction, the plasmid pDA30 from the 12674 was transformed into the strain 4277-1. *Transformants* were checked on TYA media containing 60mM arsenate and 10mM arsenite.

Cultures of these were grown in TYG media containing 50 mM arsenite (to maintain the plasmid) and screened for plasmids. The samples were analyzed by electrophoresis. (Fig 10)



Figure 10. 0.4% agarose gel of plasmid DNA isolated from Nocardioform strain 4277-1.

Lane 1 : DNA isolated from the strain 4277-1

Lanes 2-6 : plasmid DNA isolated from transformants.

Figure 10 shows the presence of the plasmid in all the transformants when compared with the control. The plasmid band in lane-2 has the greatest intensity, hence the plasmid pDA30 was bulk extracted from this transformant.

The concentration of the DNA was calculated as follows:

O.D. reading at 260nm = 0.005

DNA was diluted 1 in 200

Therefore the final concentration was 0,005 x 200 x 50 = 50µg/ml

All subsequent experiments were carried out using this plasmid from the 4277-1 background.

3.5.2 OPTIMIZATION OF NOCARDIOFORM TRANSFORMATION.

3.5.2.1 *Optimum concentration* of arsenate and arsenite for maintainance of plasmid

Nocardioform transformants from preliminary experiments were tested for the level of resistence to arsenate and arsenite. They were spot tested together with 4277-1 on TYA plates with 100mM, 200mM, and 300mM arsenate and 10mM, 20mM, 30mM and 40mM arsenite.

The results suggested the best combination to be 60mM arsenate and 10mM arsenite. These results conform to the ones obtained for the strain CW22 (S.Dabbs-personal communication).

For all future experiments 60mM arsenate and 10mM arsenite were used to select the plasmid.

3.5.2.2 *Frequency of transformation* with growth phase of cells

The optical density of the Nocardioform 4277-1 culture was measured 14 hours after initiation of growth and thereafter every 12 hours for a pe-

riod of 62 hours at 540nm on the Spectronic 601 spectophotometer. At each stage ten 1ml samples were frozen at -80°C in Eppendorf tubes. At the end of the 62 hours, 75µl of culture from each time interval was transformed with 100ng of the plasmid pDA30. The PEG concentration was 25%, lysozyme incubation was for 1 hour and the arsenate - arsenite underlay was after 12 hours of incubation of plates.

The effect of cell growth on the frequency of transformation of Nocardioform 4277-1 by pDA30 is shown in figure 11.

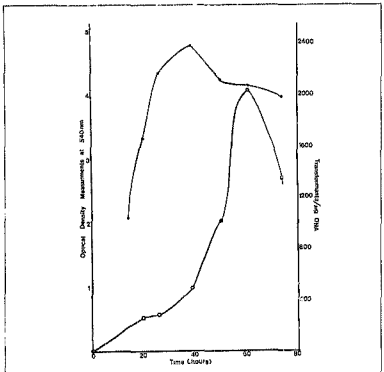


Figure 11. The effect of cell growth on the frequency of transformation of Nocardioform 4277-1 with 100ng of pDA30. The regenerated protoplasts were assayed for the presence of pDA30 as described in materials and methods.

—▲— *Measurements of optical density at various time intervals.*

—○— Measurements of transformants / ug of DNA at various time intervals.

The results clearly indicate that the Nocardioform 4277-1 is most competant for DNA uptake in the post stationary phase. A 62 hour old culture produced the maximum number number of transformants, hence for all subsequent experiments these cells were used (frozen at -80°C). The experiment was carried out twice to demonstrate replicability.

3.5.2.3 Effect of lysozyme incubation time on the frequency of transformation

The walls of the *Nocardia* and related bacteria have a similar and complex structure that consists of a peptidoglycan, several classes of free and bound lipid constituents and other polysaccharides or polypeptide compound (1). To enhance DNA uptake by the bacterial cells, the cell wall has to be removed. Weakening of the cell wall is brought by lysozyme, which digests the polymeric compounds that give the cell wall its rigidity (28). Once the cell wall is destroyed by the lysozyme, the cell membrane is attacked, which may result in the disruption of the cell.

To ascertain maximum yeild of protoplasts, the length of the lysozyme treatment had to be monitored. The plasmid pDA30 was transformed into cells grown from a 62 hour culture, with varying lysozyme incubation time. Cells were transformed after 0min, 15min, 60min, and 120min of lysozyme treatment. All other variables such as PEG concentration, arsenate/arsenite underlay time, concentration of DNA and concentration of cells was as described in section 3.5.2.2

The effect of varying lysozyme incubation time on the frequency of transformation of the Nocardioform strain 4277-1 is shown in figure 12.

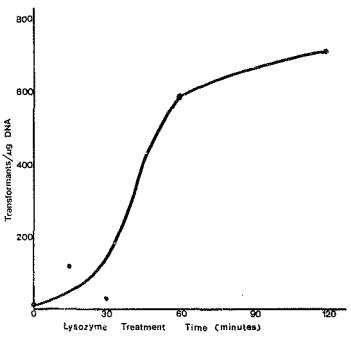


Figure 12. The effect of the varying lysozyme incubation period on the frequency of trasformation of Nocardioform 4277-1 with 100ng of pDA30. The regenerated protoplasts were assayed for the presence of pDA30 as described in the materials and methods section.

The results indicate that the maximum number of transformants were obtained when the cells were incubated with lysozyme for 2 hours.

3.5.2.4 *The effect of PEG concentration on the frequency of* transformation

Nocardioform cells from a 62 hours old culture were transformed with 100ng of plasmid pDA30. The PEG concentration was varied from 0 % to 30 % . All other variables were as described in section 3.5.2.2

The effect of the PEG concentration on the frequency of transformation is shown in figure 13.

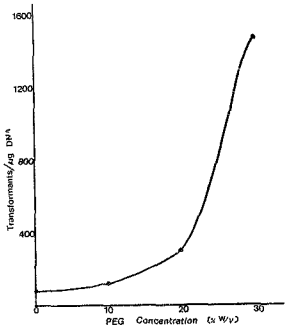


Figure 13. Effect of PEG concentration on the transformation frequency of Nocardioform 4277-1 with pDA30 DNA. pDA30 DNA was added to the protoplasts to a final concentration of 100ng/2µl at various PEG concentrations. The regenerated protoplasts were assayed for the presence of pDA30 as described in the materials and methods.

The results suggest that a concentration of 30 % PEG (w/v) seems to be optimal.

The mechanism by which PEG induces transformations is not known. It may interact with the cell membrane to make it more permeable to DNA. Alternatively, because nucleic acid molecules have been shown to adopt compact forms in solutions containing high concentrations of PEG, it is possible that the stimulation results from a conformational change in the DNA molecule facilitating penetration into the protoplasts (4).

3.5.2.5 The effect of arsenate - arsenite underlay time on the frequency of transformation

Nocardioform cells grown for 62 hours were once again transformed with 100ng pDA30 DNA. The protoplasts were obtained after 1 hour of treatment with lysozyme, and the PEG concentration was 25%. the time of arsenate - arsenite underlay was varied from 0 hours to 20 hours.
The results shown in Table 13.

Table 13 : Effect of underlay time on transformation frequency of Nocardioform 4277-1 with 100ng of pDA30 DNA.

Transformed cells were underlayed with 0.5ml 3N arsenate and 0.5M arsenite every 4 hours for 20 hours. The regenerated protoplasts were assayed for the presence of pDA30 as described in materials and methods.

Underlay Time(hours)	Transformants/µg DNA
0	0.6×10^2
4	8.7×10^2
8	2.2×10^2
12	1.93×10^3
16	1.26×10^3
20	1.68×10^3

Although the results showed great variability, 12 hours after transformation seems to be an ideal time for an arsenate -arsenite underlay.

Effects of DNA amount on the frequency of transformation

The transformation procedure was repeated using Nocardioform cells grown for 62 hours. The cells were incubated in lysozyme for 2 hours (results of lysozyme experiment was known at this stage) and the arsenate - arsenite underlay was done after 12 hours. Varying amounts of pDA30 DNA was transformed into a constant volume of Nocardioform 4227-1 protoplasts.

The results are represented graphically in Fig 14

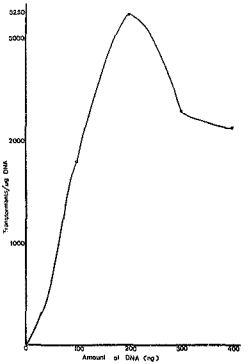


Figure 14. Effects of DNA amount on transformation. Nocardioform protoplasts were treated with various concentrations of pDA30 DNA in the presence of 25% PEG. Regenerated protoplasts were assayed for the presence of pDA30 as described in materials and methods.

The results clearly indicate that 200ng of pDA30 DNA yields the maximum number of transformants.

3.5.2.7 Effect of protoplast amount on the frequency of transformation

The transformation procedure *was repeated as in* sectin 3.5.2.6. except in this case the protoplast volume was varied. The protoplasts were transformed with 100ng of DNA.

The results are represented graphically in Fig 15

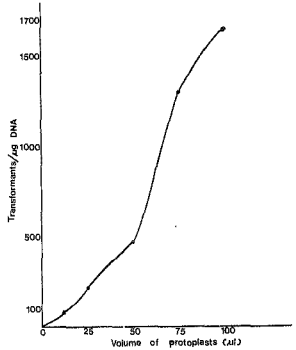


Figure 15. Effect of amount of protoplasts on the frequency of transformation of Nocardioform 4277-1. 100ng of pDA30 DNA was transformed into varying amounts of protoplasts in the presence of 25% PEG. Regenerated protoplasts were assayed for the presence of the plasmid as described in materials and methods.

The results suggests that 100µl of protoplasts are optimally transformed with 100ng of DNA.

3.5.3 SUMMARY : OPTIMAL CONDITIONS FOR THE NOCARDIOFORM TRANSFORMATION

The results discussed in the preceding section described the optimal conditions of the Nocardioform transformation system for achieving maximum number of transformants.

The optimal conditions are summarized in Table 14.

Table 14 : Optimal conditions for Nocardioform transformation.

Factor	Optimal Condition
1. arsenate-arsenite concentration	60mM arsenate and 10mM arsenite
2. growth phase of cells	post stationary phase after 62 hours at an O.D.= 4.14
3. length of lysozyme incubation	2 hours
4. PEG concentration	30%
5. time of arsenate arsenite underlay after transformation to regenerate protoplasts	12 hours
6. amount of plasmid DNA	200ng
7. amount of cells	100µl

3.5.4 NOCARDIOFORM PLASMID SCREEN

It was necessary to ensure that the regenerated protoplasts were "real transformants" and not spontaneous arsenate - arsenite mutants.The simplest method for clarifying this was to screen the regenerated protoplasts for plasmid.

Regenerated protoplasts were first assayed on TYA plates containing 60mM arsenate and 10mM arsenite. Random positive transformants and spontaneous arsenate - arsenite mutants from the control plates were screened for the presence of the plasmid, pDA30.

Cultures were grown in TYG media supplemented with 50mM arsenate. The strain 4277-1 was grown in TYG as the control. The plasmid was extracted as described in materials and methods. Samples were analysed by gel electrophoresis (Fig 16).

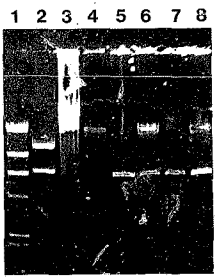


Figure 16. 0.4% Agarose gel of plasmid DNA isolated from the Nocardioform strain 4277-1

Lane 1 : λ DNA II molecular weight marker

2 : purified plasmid, pDA30

3 : DNA from 4277-1

4: DNA from spontaneous arsonate - arsenite mutants

5 - 8 : plasmid DNA isolated from transformants

Fig. 16 shows that all the transformants have the plasmid pDA30 cloned into it. This ensured that the transformation system was functioning efficiently.

4.0 DISCUSSION

The objective of this work was to study Nocardioform strains that were capable of utilising or degrading or metabolising acrylamide. Therefore the first requirement was to tailor the minimal media such that acrylamide could be supplied as sole carbon and/ or nitrogen source.Experiments showed that sodium citrate, a component of minimal media,was capable of being used as a carbon source by the bacteria. For this reason it was omitted from the media in all subsequent experiments.

Two factors are involved in the ability of the bacterium to use acrylamide, viz, the ability to metabolize acrylamide per sé tempered by the *inhibitory effect of acrylamide on the bacterium*. Resistance levels of several strains was tested. Growth of strains 12674 and CW22 was greatly inhibited at an acrylamide concentration of 1mg/ml. Strains 4277-1 and 14887 grew fairly well with acrylamide concentrations of up to 1,5 mg/ml, hence all further experiments were conducted using the latter two strains. At an acrylamide concentration of 2mg/ml growth of all organisms was inhibited.

The strains 4227-1 and 14887 were made acrylamide resistant by selecting them on minimal media plates containing 3mg/ml or 5mg/ml acrylamide. The acrylamide was supplied as sole nitrogen source since both strains were capable of utilising it.

Once the acrylamide resistant mutants of 4277-1 and 14887 were obtained, they were tested for their ability to utilise acrylamide as sole carbon or nitrogen source on minimal media plates. The results indicated that 4277-1 acrylamide resistant strain (4277-1/1) was capable of utilising acrylamide as sole nitrogen source only. In the case of the strain 14887, two types of mutants were obtained, viz, 14887/1 which was able to use

acrylamide as both sole carbon and nitrogen source and mutant 14887/2 which utilised acrylamide as sole nitrogen source only.

Liquid culture growth experiments were also conducted to investigate the dose response of these mutants to acrylamide. *These results confirmed that* 4227-1/1 was incapable of utilising acrylamide as sole carbon source, whilst the 14887/1 mutant could. This experiment also showed that maximal growth of these mutants was in the presence of 0.6 mg/ml acrylamide, when supplied as sole nitrogen source.With acrylamide as sole carbon source, the mutant 14887/1 grew to the highest density at a concentration of 2mg/ml.

Graphically these results could be interpreted as shown in figures 17(A) and 17(B).

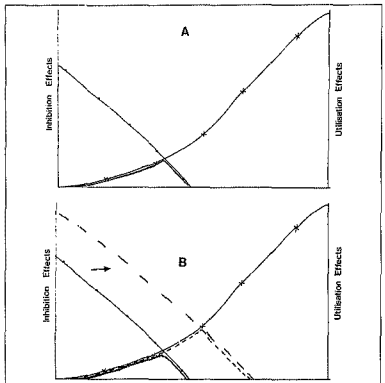


Figure 17. Bacterial dose response curves to acrylamide.

—✕— utilisation curve

—●— inhibition curve

——— growth curve

– – – shifted inhibition curve

– – – growth curve after shift in inhibition curve

The arrow indicates a shift in the inhibition curve.

The growth response curve of the acrylamide mutant is due to an envelope resulting from a combination of both the utilisation and inhibition effects. A reduction in the inhibitory effect leads to increased growth of the mutant.(fig 17B).

The ultimate aim of this study was to clone the genes responsible for acrylamide utilisation or detoxification or metabolism. The principle strategy for cloning was to be by complementing acrylamide non-utilising mutants.

To obtain these mutants, NTG, EMS and U.V. mutagenesis was carried out on both *stationary* phase and logarithmic phase cells. However, all of these attempts failed to yield the required mutant. The frequency of auxotroph production from the mutagenesis was between 1.8 - 2.5%. One factor, namely, reduction in the aggregation of cells was optimized, but clearly much more future work is required in order to have the mutagenisation system *functioning efficiently.As an alternate route* the process of ampicillin enrichment was pursued. This process yielded four apparent acrylamide non-utilising mutants.

Secondly the cloning process required the construction of a genomic library of the donor DNA, 14587/1. This was constructed in the *E. coli* strain HM294 and thus far contains 2655 clones.

The final requirement was to optimize the transformation system in the recipiant strain, 4277-1. Results from these experiments indicated that post stationary phase 4277-1 cells (62 hours of growth) with an O.D. at 540nm of approximately 4.14 were best for the uptake of the Nocardioform plasmid, pNA30. The maximum amount of plasmid DNA and protoplasts needed for optimal transformation was 200ng and 100µl respectively. The optimal PEG concentration was 30%. The time of lysozyme incubation was 2 hours and the time of 60mM arsenate - 10mM arsenite underley was 12 hours after transformation of the protoplasts with plasmid.

5.0 APPENDIX MEDIA AND SOLUTIONS

5.1 MEDIA

5.1.1 MINIMAL MEDIA

(A) 100ml A-N buffer (10 x stock)
1g NH_4Cl
400ml water

(B) 500ml water
15g agar
5g glucose
(A) and (B) autoclaved seperately, mixed and plates poured.
For liquid minimal media the agar is omitted.

5.1.1.1 *A-N buffer* stock solution (10x) - modified for Nocardioforms

91,7g K_2HPO_4 $3H_2O$
26,8g KH_2PO_4
1g $MgSO_4$ H_2O
1l H_2O
pH 7,-
Kept sterile by the addition of 10ml chloroform

5.1.2 TRYPTONE YEAST (TY) MEDIA

1% Tryptone
0,5% Yeast

5.1.3 TRYPTONE YEAST AGAR PLATES (TYA)

1% Tryptone
0,5% Yeast Extract
1,5% Agar

5.1.4 LURIA BROTH

1% Tryptone 0,5% Yeast Extract
0,5% NaCl

5.1.5 LURIA AGAR

1% Tryptone
0.5% Yeast Extract
0,5% NaCl
1,5% Agar

5.1.6 T2 MEDIA

1% Tryptone
0,5% Yeast Extract
3mM NaCl
3mM $CaCl_2$
10mM $MgSO_4$

5.1.7 TRYPTONE, YEAST, GLYCINE (TYG) MEDIA

1% Tryptone
0,5% Yeast Extract
1% Glycine

5.1.8 PROTOPLAST REGENERATING MEDIA PLATES *

0.9g NaCl
3g Tryptone
1,5g Yeast Extract
35g Sucrose
4g Agar
Make volume up to 280ml with water.
After autoclaving add,
6ml $CaCl_2$ $2H_2O$
10ml TES(NTris (hydroxymethyl) methyl - 2 aminoethanesulfonic acid) buffer (0.25M, pH 7,2)

5.2 SOLUTIONS

5.2.1 AMPICILLIN (10MG/ML STOCK)

10mg Ampicillin
5ml Ethanol
5ml Sterile distilled water

5.2.2 10% SODIUM GLUTAMATE

100mg Sodium Glutamate
5ml Ethanol
5ml Sterile distilled water

5.2.3 10% ACRYLAMIDE

1g Acrylamide
10ml Distilled water
sterilized by filtration

5.2.4 RIFAMPICIN (10MG/ML STOCK)

10mg *Rifampicin*
1ml Methanol

5.2.5 *MUTAGENESIS BUFFERS*

5.2.5.1 Tris(Tris(Hydroxymethyl)aminomethane) acid maleate- sodium hydroxide buffer

0,2M Tris acid maleate buffered with 0,2M NaOH to pH 4,8

5.2.5.2 Tris.HCl buffer

0,02M Tris buffered with 1M HCl to pH 4,8 or pH 8.0 or pH 8,5

5.2.6 SOLUTIONS FOR LARGE SCALE DNA EXTRACTION

5.2.6.1 TE buffer

10mM Tris
1mM EDTA (ethylene diamine tetra acetic acid disodium salt)
pH8.0

5.2.6.2 Lysozyme

10mg Lysozyme
1ml 0,25M Tris.HCl pH8,0

5.2.6.3 Ethidium Bromide(EtBr)

10mg EtBr
1ml Sterile distilled water

5.2.6.4 Cold Detergent

20% Triton X -100
0,5M EDTA pH 8,0
1M Tris pH 8,0
Make up to volume with distilled water.

5.2.7 PREPARATION OF DIALYSIS TUBING

Small pieces of dialysis tubing were boiled for ten minutes in a large volume of 20% sodium bicarbonate and 1mM EDTA. The tubing was rinsed thoroughly in distilled water and boiled for 10 minutes in 0,001M EDTA. After cooling it was stored at 4°C ensuring that the tubing was always submerged.

5.2.8 LIGATION BUFFER

10mM Tris.HCl
10mM $MgCl_2$
10mM dithioerythritol
0,6mM ATP pH 7,6

5.2.9 TRANSFORMATION SOLUTION

50mM $CaCl_2$ $2H_2O$
10mM Tris.HCl pH8.0

5.2.10 SOLUTIONS FOR SMALL SCALE PLASMID ISOLATION

5.2.10.1 *Solution 1*

50mM glucose
25mM Tris. HCl pH 8,0
10mM EDTA

5.2.10.2 Solution 2

0,2N NaOH
10% SDS (sodium dodecyl sulphate)

5.2.10.3 Tris Borate Buffer (TBE)

To make a 5x stock solution;
0.089M tris base
0,089M Boric acid
0,002M EDTA pH8,0
Make up to 1l with distilled water.

5.2.10.4 Gel loading buffer Type IV

0,25%Bromophenol blue
40% (w/v) sucrose in distilled water
Stored at 4°C

5.2.11 SOLUTIONS FOR NOCARDIOFORM TRANSFORMATION

5.2.11.1 P(Protoplast) Buffer

16,3g Sucrose
25mg K_2SO_4 0,202g $MgCl_2$ $6H_2O$
87,5ml Distilled water
Dispense in 10ml aliquots and autoclave. Before use add to each flask in order ;
1ml KH_2PO_4

2.5ml $CaCl_2 \cdot 2H_2O$

10ml TES buffer (0,25M , adjusted to pH 7.2).

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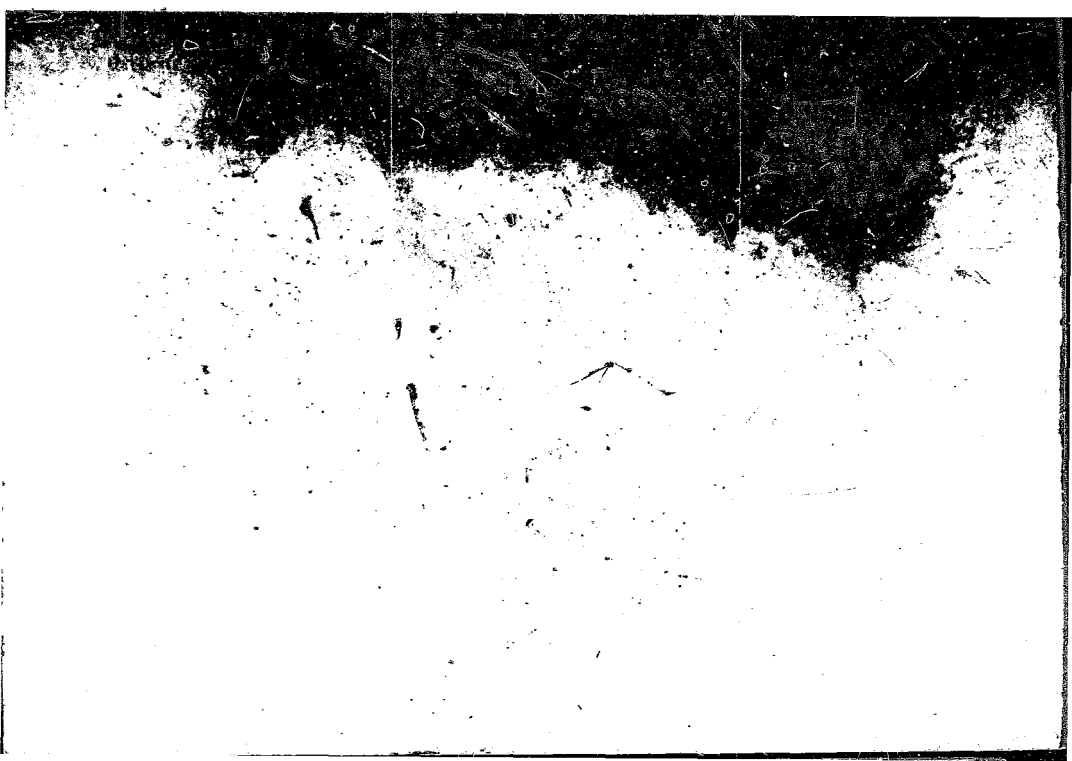
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