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FUSIDIC ACID INACTIVATION IN NOCARDIOFORM BACTERIA, IDENTIFICATION OF THE GENE/S INVOLVED IN THE INACTIVATION MECHANISM

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A B S T R A C T

A number of nocardioform mutant strains were characterised for their response to fusidic acid. It was found that increased resistance to this steroid antibiotic was due to more ready induction of the inactivating enzyme and that increased sensitivity was due to less ready induction, compared to wild-type. A library of DNA from a related nocardioform was built up in Escherichia coli using a putative shuttle vector.

1. INTRODUCTION

Nocardia are a group of ubiquitous, gram-positive, filamentous, soil dwelling bacteria. Some species are the causative agents of tuberculosis, leprosy and nocardiosis (Goodfellow et al., 1977; Goodfellow et al., 1976; Mitchison et al., 1984) and others have the capacity to biodegrade a wide range of compounds, such as acrylamide, some insecticides, phenols and cholesterol (Ferreira et al., 1984; Ferguson et al., 1981). These organisms also have a novel fusidic acid inducible inactivating mechanism (Dabbs, 1987).

Fusidic acid is an antibiotic produced by Fusidium coccineum and a number of other fungal species (Godtfredsen et al., 1982). It is active against gram-positive bacteria including mycobacteria, where its mode of action has been shown to be the inhibition of protein synthesis (Yamaki, 1965). The compound forms a stable complex with EF-G, preventing this protein from recycling, as normally occurs during polypeptide biosynthesis (Tanaka et al., 1968). Chromosomal mutations conferring resistance to fusidic acid have been shown to be due to alterations in the gene for EF-G (Tanaka et al., 1968). Gram-negative bacteria are insensitive to the antibiotic, but since cell-free extracts are blocked in protein synthesis at levels of fusidic acid similar to those effective against gram-positives, it is believed that resistance in gram-negatives is the result of the antibiotic's failure to penetrate the cell.

Fusidic acid is unusual amongst antimicrobial agents, and especially amongst those which inhibit protein synthesis, in having a steroid structure (Vazquez 1979). Though diverse microorganisms are able to perform bioconversion of steroids, nocardioform bacteria are notable for their ability to effect many different types of transformation of steroid molecules. This ability is being exploited to obtain compounds which are difficult to prepare by chemical synthesis but are of pharmacological importance, as intermediates in the synthesis of anti-inflammatory agents or oral contraceptives. The observation that nocardioform bacteria transform steroid compounds ties in with my study to explore the response of these organisms to the steroid fusidic acid.

Fusidic acid sequesters factor EF-G and GDP on the ribosome following a single round of GTP hyrolysis: it thus stabilizes what is normally an unstable ternary complex and leads to inhibition of protein synthesis. The drug does not interact primarily with ribosomes and no mutants with fusidic acid resistant ribosomes as opposed to EF-G have been reported (Reynolds, 1984).

An examination of the available amino acid sequences of GTP-binding proteins has revealed that each contains a polypeptide essentially homologous for all of them. These sequences for elongation factor EF-TU and the human bladder protein, oncogene or proto-oncogene, p 21 exhibit a singular degree of homology 50% (Leberman et al., 1984; Scolnick et al., 1979; Shih et al., 1980; Papageorge et al., 1982).

There is a high degree of homology between the N-terminal sequences of EF-G and ET-Tu suggesting an evolutionary relationship between the two proteins (Jones et al., 1980). Conservation of functional regions in EF-G and EF-Tu suggests that the genes for the two factors are at least in part derived from a common ancestor (Sacedot et al., 1984). Proteins which exhibit this high specific affinity for the guanosine nucleotides GTP and GDP are a diverse group. They include polypeptide elongation factors (EF-G, EF-Tu), tubulin, transducin and receptor protein of eukaryotic adenylate cyclase. It can thus be said that EF-G to some extent is homologous to oncogenes in GTP hydrolysis whose activity is also controlled by steroids, in particular protein p21 and its oncogenic variants.

A 58-year-old man with AIDS improved clinically after the introduction of fusidic acid, 500 mg 3 times a day orally, to his therapeutic regimen. Fusidic acid may have had a direct effect against human immunodeficiency virus (HIV). Fusidic acid has anti-HIV activity in vitro at levels readily attainable in vivo. The drug does not appear to be a reverse transcriptase inhibitor and its mode of action against HIV is not known. Fusidic acid can be given orally and has few side-effects. These results justify fuller evaluation of fusidic acid as therapy against AIDS and HIV infection (Faber et al., 1987).

Fusidic acid resistance / sensitivity can be altered due to the following circumstances:

(a) Differences in the permeability or the uptake of the antibiotic.

(b) Alteration in the target moiety EF-G.

(c) Presence/absence of the inactivating enzyme.

Loss of the inactivating enzyme was the point of interest for this work. The first step therefore was to create mutants that have lost the inactivating enzyme system, i.e. have mutational alteration of the gene/s coding for the enzyme.

The method I will employ for cloning the inactivation gene/s will be cloning by complementation which is by far the easiest way to clone genes. The first objective was to obtain fusidic acid hypersensitive mutants from the wild-type strain 01. These mutants may have mutation of the gene/s involved in the fusidic acid inactivation system, and are thus no longer able to inactivate fusidic acid as the wild-type strain can. Also a number of hypersensitive mutants were needed, as the various complementation groups could thus be examined to give a better idea as to how many gene/s are involved in the inactivation mechanism. The fusidic acid hypersensitive mutants would be obtained by doing mutagenesis experiments with a number of different mutagens, in particular nitrosoguanidine (NTG). Mutagenesis conditions had to be optimised. The fusidic acid hypersensitive mutants would be used as recipients into which chromosomal DNA from a related organism not having mutational alterations of the gene/s involved in coding for the antibiotic inactivation system, would be transformed.

This chromosomal DNA would then complement the hypersensitive mutants resulting in a genotype that could once again inactivate fusidic acid. Nocardioform 14887/1 (_nocardia restricta_, also called _Rhodococcus equi_) chromosomal DNA was used in order to minimise recombination when this was transformed into 01, as no rec⁻ recipient nocardioform strain was available.

It was therefore necessary to build up a genomic library of 14887/1 chromosomal DNA in _Escherichia coli_ strain MM 294-1. This would then be used for transforming the fusidic acid hypersensitive mutants. A putative shuttle vector derived from joining a nocardioform arsenic resistance plasmid pDA22 and a _coli_ plasmid pEcoR251 with ampicillin resistance joined at the BamHl restriction site, was used as the vector for transforming MM 294-1 with the 14887/1 chromosomal DNA. Chromosomal DNA fragments were inserted into the EcoR 1 gene of the pEcoR251 plasmid by using the restriction endonuclease Bgl II, as there is only one Bgl II site within the putative shuttle vector and this is in the EcoR 1 gene. pEcoR251 being a suicide vector it would be easy to screen for transformants, as only those colonies growing on selective plates containing the appropriate concentration of ampicillin to which the putative shuttle vector is resistant are transformants. This is because the inserted chromosomal DNA fragments have caused insertional inactivation of the EcoR 1 gene activity.

The purpose of my work was to work towards the cloning of gene/s encoding the fusidic acid inactivation mechanism in nocarioform bacteria, and this could be achieved by complementation, in which case the putative shuttle vector containing the 14887/1 chromosomal DNA inserts constituting a genomic library of the organism, would be transformed into the fusidic acid hypersensitive mutants, thereby complementing the lack of fusidic acid inactivation in these mutants. It would then be simple to select for those colonies or transformants that have the inactivation gene/s, as only these colonies will grow on selective plates containing the appropriate concentration of fusidic acid.

2. MATERIALS AND METHODS

2.1. (a) NOCARDIOFORM STRAINS USED

ATCC 12674 - Rhodococcus erythropolis

ATCC 14887 - Rhodococcus equi

ATCC 4277 - Rhodococcus erythropolis

14887/1 - Acrylamide resistant mutant derived from 14887.

01 - Mutant derived from ATCC 12674.

01(trp⁻) - A fusidic acid resistant mutant of 01.

01(pro⁻) - A fusidic acid sensitive mutant of 01.

011 - A streptomycin resistant mutant of 01.

012 - A rifampicin resistant mutant of 01.

2.1. (b) ESCHERICHIA coli STRAINS USED

MM 294

MM 294-1 - A rifampicin resistant derivative of MM 294.

C7R - A rifampicin resistant mutant of Arthrobacter oxydans.

AD 14 - An Escherichia coli mutant sensitive to fusidic acid.

ST 47 - An Escherichia coli mutant sensitive to fusidic acid.

2.1. (c) PLASMIDS USED

Putative shuttle vector 1 - A vector comprised of the nocardioform plasmid pDA22 joined to the coli plasmid pEcoR251.

pEcoR251 - A plasmid maintained in any lambda lysogen.

pDA30 - A nocardioform plasmid maintained in 01.

2.2. GROWTH AND MAINTENANCE OF STRAINS USED

All nocardioform strains were grown in T2 medium (see Appendix) at 28°C. Strains were maintained on TYA plates (see Appendix) and stored at a temperature of 4°C. For long term storage strains were placed in 33% glycerol and stored at -80°C.

Escherichia coli strains were grown in LB medium (see Appendix) at 37°C and maintained on LA plates (see Appendix). LA plates were also stored at 4°C, for long term storage 33% glycerol was again used and stored at -80°C.

2.3. DETERMINATION OF RESISTANCE

(Dabbs E.R., Sole G.J., 1987)

The resistance levels of all the strains tested to antibiotics and other compounds were measured by spotting on TYA or LA plates using a replicator. The highest concentration at which growth was confluent was taken to be the resistance level. Aliquots of stock solutions of antibiotics or other compounds were added to plate media after autoclaving and before pouring. All strains used in these assays were firstly streaked to single colonies to ensure accuracy and reproducibility of results. Plates were incubated at 28°C for 72 hours before being assayed.

2.4. ASSAYING FOR FUSIDIC ACID INACTIVATION BY NOCARDIOFORM BACTERIA

The assay plates were 25 ml LA plus 30 µl/ml rifampicin. A 2[illegible] mg/ml stock solution of rifampicin was prepared in methanol. The inhibitory effect of fusidic acid in solution was monitored by measuring the zones of inhibition on a lawn of C7R, the assay organism used. 0.1 ml of a fresh C7R culture was spread on a plate, and 60 µl aliquots of culture or supernatant depending on the assay were pipetted into wells cut into the agar with the broad end of a Pasteur pipette. The culture to be assayed was always prepared from a fresh preculture by making a 1 in 25 dilution into 5 ml of fresh T2 medium. This culture was then allowed to grow for 3 - 4 hours at 28°C before being challenged with fusidic acid of the required concentration. Fusidic acid was obtained from LEO Pharmaceuticals, and 10 mg/ml stock solutions were prepared in 96% ethanol. Plates were incubated at 28° C for 24 hours before being scored.

2.4.1 ANTIBIOTIC INACTIVATION BY CELL-FREE SUPERNATANTS

Cell-free supernatants were obtained by centrifuging 1 ml aliquots of culture in Eppendorf tubes for 1 minute. The supernatant was then transferred to a

new Eppendorf tube and 20 µg/ml rifampicin was added instead of filter sterilizing to avoid any growth in the supernatant due to the carry over of contaminating cells. Fusidic acid to a final concentration of 40 µg/ml was then added. This solution was allowed to incubate at 37°C for 24 hours before the 60 µl samples were loaded into the wells of the assay plates spread with a lawn of C7R.

2.4.2 LIQUID ASSAY OF FUSIDIC ACID INACTIVATION BOTH BY ACTIVELY GROWING CULTURE AND BY CELL-FREE SUPERNATANT

A preculture of the organism/s to be assayed was prepared. 500 ul of preculture was then used to inoculate 10 ml of fresh T2 medium which was allowed to grow at 28°C for 5-6 hours, before optical density (OD) readings were taken and standardized. Once the OD readings of all strains to be assayed were adjusted to the same absorption, cultures were challenged with the required fusidic acid concentration, and this was taken as zero time. The OD's of the cultures were taken at 0,2,4,6 and 8 hours. After each reading 2 ml aliquots of each culture were taken and centrifuged for 1 min in a microfuge. The supernatants were then pipetted into fresh Eppendorfs and heated to 95°C for 5 min to inactivate the fusidic acid inactivating enzyme/s. The supernatants can then be frozen at -80°C. In this assay the supernatants are not assayed on plates as mentioned in 2.4.1,

but were aliquoted into LB containing a 1 in 100 dilution of C7R. 1 ml of supernatant was added to 4 ml of LB with 20 µg/ml rifampicin in autoclaved test tubes. Rifampicin was added in an attempt to prevent contamination by organisms other than the assay organism C7R. 1 ml of various concentrations of fusidic acid made in T2 medium were also aliquoted into 4 ml LB with 20 µg/ml rifampicin and these were used as standards to give an idea of the concentration of fusidic acid left after a given time i.e. the concenteration of fusidic acid which has not been inactivated. All the test tubes were then incubated at 28°C and allowed to grow up on a revolving wheal. Once the cultures became turbid the OD readings were taken.

2.5. OBTAINING HIGH LEVEL RESISTANCE MUTANTS

High level antibiotic resistant mutants were obtained by spreading 1 ml aliquots of the culture of the particular organism onto TYA plates containing 100 µg/ml of the antibiotic or compound to which the resistance was to be made. Plates were then incubated at 28°C until colonies appeared. Each colony was then streaked to single colonies on a fresh TYA plate containing 50 µg/ml of the antibiotic or compound and maintained as such at 4°C.

2.6. NITROSOGUANIDINE (NTG) AND ETHYLMETHANESULPHONATE (EMS) MUTAGENESIS

Strains were grown up in 5 ml T2 overnight. NTG was made up by dissolving 2 mg into 2 ml of buffer. The buffer used was a Tris-HCl buffer for pH 8 and a Tris maleate buffer for pH 4.8. A 2% EMS solution was prepared by dissolving 40 ul of EMS in 2 ml of Tris-HCl buffer pH 7.0. 0,1 ml of the overnight culture was pelleted for 20 seconds and the pellet was washed in the respective buffers. For NTG, the cells were resuspended in 0,9 ml buffer and 0,1 ml NTG to give a final NTG concentration of 100 µg/ml. For EMS mutagenesis, cells were resuspended in 1 ml of the EMS solution. These suspensions were left at 37°C for 2 hours, after which they were again pelleted for 10 seconds. The cells were washed in sterile distilled water and pelleted again for 10 seconds. Cells were resuspended in 100 µl T2 medium. The 100 µl of cells were then aliquoted into an autoclaved flask containing 5 ml T2 medium, 100 ug/ml streptomycin was added to this. A 10 mg/ml streptomycin stock was prepared in 50% sterile distilled water and 50% of 96% ethanol. The culture was allowed to outgrow at 28°C after which it was sonicated for 5 seconds to reduce the clumping of cells. The culture was then diluted to a concentration of 10^{-6}, and 100 µl aliquots of this dilution were spread onto TYA plates. Plates were incubated at 28°C until colonies appeared. These colonies were patched onto TYA plates of varying concentrations of fusidic acid to ensure that mutants were indeed fusidic acid sensitive.

To test the efficiency of mutagenesis colonies were patched onto minimal media (MM) plates (see Appendix) to check for auxotrophs.

2.6.1 MUTAGENESIS OF EXPONENTIALLY GROWING STRAINS

2 ml of exponentially growing cells were taken to which 3 ml of T2 medium was added to give a final volume of 5ml. NTG at pH 4.8 and pH 8 was then added to a final concentration of 100 μg/ml. Growth was continued for 1 hour at 28°C. Cells were pelleted and resuspended in 5 ml T2 medium, in this instance cells were not washed with the respective buffer. The culture was allowed to grow for several hours before 100 μg/ml streptomycin was added. Again the culture was sonicated for 5 seconds to reduce clumping of cells and diluted to 10^{-6}. The 10^{-6} culture was vortexed for 80 seconds before 100 μl aliquots were spread onto TYA plates. Again plates were incubated at 28°C until colonies appeared. Colonies were again patched onto TYA, TYA + fusidic acid, and MM plates.

2.6.2 ENHANCEMENT OF NTG MUTAGENESIS BY CHLORAMPHENICOL (MISHRA, TIVARI, 1985)

A culture was grown overnight in T2 medium. 250 μl of this culture was then spun down and resuspended in 5 ml of TY medium (see Appendix). This was allowed to shake at 28°C for 6 hours until the culture was in exponential phase, and then 100 μg /ml of NTG pH 4.8 was added to one flask

and 100 µg/ml NTG pH 4.8 plus 200 µg/ml chloramphenicol was added to another flask and put at 28°C for 2 hours. TY medium was used rather than T2 medium as calcium seem to enhance killing by chloramphenicol. After the 2 hour incubation 1 ml of each sample was spun down washed with sterile distilled water pelleted again and resuspended in 5 ml T2 to allow for phenotypic expression. After outgrowth the cultures were again sonicated for 5 seconds to reduce clumping and diluted to 10^{-6}. The 10^{-6} dilutions were vortexed for 80 seconds and 100 ul aliquots were spread onto TYA plates. Plates were incubated at 28°C as before. The resulting colonies were patched onto TYA, TYA + 1 µg/ml fusidic acid, and MM plates to screen for fusidic acid sensitive mutants.

2.6.3 ACRIDINE ORANGE AND ETHIDIUM BROMIDE (EtBr) MUTAGENESIS

A culture was grown to stationery phase after which 5 µl of the culture was aliquoted into 5 ml of T2 medium into 3 seperate flasks, to one flask 5 µg/ml acridine orange, to another 10 µg/ml and to the third 15 ug/ml was added and allowed to grow. The same protocol was followed for EtBr. Acridine orange was dissolved in sterile distilled water, filter sterilised and kept in the dark at 4°C. EtBr was prepared in the same way. Once outgrown the cultures were sonicated for 5 seconds and diluted to 10^{-6}. The dilution was vortexed for 80 seconds before 100 µl aliquots were spread onto TYA plates. Plates were incubated at 28°C until colonies appeared. Again colonies were patched onto TYA, TYA + 1 µg/ml fusidic acid and MM plates. All strains used

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for mutagenesis were made either rifampicin or streptomycin resistant and depending on which strain was used rifampicin or streptomycin was added to prevent contamination while cells were being outgrown.

2.6.4 ULTRAVIOLET (UV) MUTAGENESIS

A culture was grown up overnight, the culture was then sonicated for 5 seconds. The sonicated culture was diluted to a final concentration of 10^{-5}. A 200 µl drop of this 10^{-5} dilution was then placed onto the surface of a sterile petri-dish and exposed to UV radiation for varying periods of time. After each time period 10 µl of the droplet was added to a drop of sterile distilled water on a TYA plate and spread. The TYA plates were then incubated at 28°C in the dark for 2 days. A control plate was compared to the other TYA plates onto which were spread the samples of varying exposure to UV light. The colonies on the plate exhibiting the most killing due to UV radiation were patched onto TYA and MM plates as before to screen for mutants.

2.7. BULK PREPARATION OF DNA (BOTH PLASMID AND CHROMOSOMAL) FROM NOCARDIOFORM BACTERIA

The organism was grown in 250 ml TYG (see Appendix) when chromosomal DNA was to be extracted and in 250 ml TYG + 20 mM arsenate when plasmid was to be purified. Arsenate was added to maintain the plasmid which has arsenic resistance. The cells were harvested at 6000 rpm for 20

minutes in a Beckman J2 - 21 rifuge, and resuspended in 10 ml of 10mM Tris pH 8, 10% sucrose. 50 - 100 mg lysozyme was added to this suspension in 5 ml of the same buffer. This was incubated at 37°C for 2 hours. Cells were pelleted at 12 000 rpm for 15 minutes and resuspended in 8 ml TE (see Appendix) to which 0.8 ml TE containing 10% sodium dodecyl sulphate (SDS) was added. This was incubated at 37°C for 2 hours. The sample was then loaded into 50Ti tubes and spun for 30 minutes at 35 000 rpm in a Beckman L5-55 ultracentifuge. The DNA solution was decanted and 8 - 9 g caesium chloride (CsCl) was added and dissolved. The solution was centrifuged at 18 000 rpm for 15 minutes, the DNA solution was decanted out from under the scum which had formed. The refractive index of the DNA solution was measured and ajusted to 1.392 for plasmid DNA and 1.390 for chromosomal DNA respectively. 0.1 ml EtBr was added per ml of solution. The solution was loaded into quickseal ultracentrifuge tubes which were balanced and sealed and centrifuged overnight at 45 000 rpm in a VTi65.2 vertical rotor. The plasmid or chromosomal bands were extracted and pooled, reloaded in an ultracentrifuge tube which was filled with CsCl at a concentration of 1 g/ml in TE. The tube was respun overnight. The plasmid or chromosomal band was again extracted and treated with TE saturated butanol to remove EtBr by inverting the Eppendorf tube several times and then seperating the two phases by a 30 second centrifugation in a microfuge. The EtBr partitions into the upper butanol phase which is removed using a Pasteur pipette. The sample was then dialysed against TE

for 3-4 hours to eliminate CsCl. The DNA concentration was ascertained by measuring the absorbance at 260nm and the sample which was now in TE was frozen at -80°C.

2.8 BULK PLASMID PREPARATION (E.coli)

(Clewell; Helsinki, 1968)

The organism was grown overnight in 200-300 ml LB containing 10 mM magnesium sulphate and 50 µg/ml ampicillin to maintain the plasmid. Cells were pelleted at 6 000 rpm for 10 minutes and resuspended in 10 ml cold TE. Cells were again pelleted at 6 000 rpm for 10 minutes, and resuspended in 2 ml cold 25% sucrose, 50 mM Tris pH 8. 0.25 ml fresh lysozyme solution (10mg/ml) was added and this was gently swirled on ice for 15 minutes. 0.25 ml of 0.5 M EDTA pH 8 was then added and this was swirled on ice for 5 minutes. 2.5 ml of cold detergent solution (see Appendix) was added and swirled on ice for 10 minutes until partial clearing was observed. The material was now highly viscous. This was centrifuged at 18 000 rpm for 45 minutes. The supernatant was decanted and measured and for each ml of supernatant 0.95 g of CsCl was added. 0.1 ml of EtBr was now added (10 mg/ml in sterile water) per ml cleared lysate. The refractive index was measured and adjusted to 1.392. The sample was loaded into ultracentrifuge tubes which were sealed and balanced and spun overnight at 40 000 rpm

in a VTi65.2 vertical rotor. Again EtBr was removed by using water saturated butanol and CsCl by dialysis against TE. The DNA concentration was ascertained and the sample frozen and stored at -80°C.

2.9. PLASMID MINIPREPARATION FOR SCREENING TRANSFORMANTS *(Maniatis et al., 1982)*

Transformants were grown in 4 ml LB + 50 µg/ml ampicillin at 37°C overnight. 1 ml of the culture was harvested by a 1 minute centrifugation in a microfuge. The pellet was resuspended in 100 µl of solution 1 (see Appendix) and stood at room temperature for 5 minutes. 200 µl of solution 2 (see Appendix) was added and mixed gently and stood on ice for 5 minutes. 150 µl of precooled solution 3 (see Appendix) was now added, mixed gently, and stood on ice for 5 minutes. The mixture was centrifuged for 1 minute and the supernatant pipetted into a sterile Eppendof tube. One volume of isopropanol was added to the supernatant and allowed to stand at room temperature for 5 minutes. The DNA was then precipitated by centrifuging for 5 minutes. The pellet was washed with 96% ethanol and spun for 5 - 10 minutes in a microfuge. The ethanol was discarded and the pellet was dried at 60°C for 20 minutes. The dried pellet was resuspended in 12 µl TE + ribonuclease (10 mg/ml) at 42°C before samples were run on a 0.4% agarose gel.

2.10. PLASMID MINIPREPARATION FOR NOCARDIOFORM BACTERIA (Dabbs and Sole; 1987)

5 ml overnight cultures of transformants in TYG + 50 mM arsenate were grown up in a flask at 28°C. 1 ml in each case was pelleted for 1 minute in a microfuge. 800 ul of Trissucrose containing 30 mg/ml lysozyme was used to resuspend the pellets. These suspensions were shaken for 1 hour at 37°C. Cells were pelleted for 30 seconds and resuspended in 280 µl TE. 40 ul TE, 10% SDS was then added and tubes were incubated at 60°C for 10 minutes. 40 µl 4.5 M sodium acetate was mixed in and samples were left on ice for 30 minutes. Suspensions were then microfuged for 20 minutes, the upper layer was pipetted into a new sterile Eppendorf tube. Samples were then treated once with 80 µl phenol and once with 80 µl chloroform: isoamyl alcohol (24:1). 2.5 volumes of cold ethanol was then added and samples microfuged for 20 minutes. Supernatants were discarded, and pellets dried for 20 minutes at 60°C. Dried pellets were resuspended in 20 µl TE containing RNAse. After resuspending for 1 hour at 42°C samples were run on 0.4% agarose gels.

2.11. PREPARATION OF CHROMOSOMAL AND PLASMID DNA FOR LIGATION

The restriction endonuclease Bgl II was used to restrict both chromosomal and plasmid DNA. Chromosomal DNA was partially digested. To ascertain the optimal conditions for chromosomal DNA digestion a serial dilution of the restriction enzyme from 10^{-1} to 10^{-4} for 9 units/µl of the enzyme was made in (1 x) medium restriction buffer.

1 µl of each dilution was then added to chromosomal DNA that had been ethanol precipitated and resuspended in 90 ul sterile distilled water and 10 µl (10 x) medium restriction buffer at 42°C for 1 hour. Samples were restricted at 37°C for 3 hours. Plasmid DNA was digested with 9 units/µl undiluted enzyme for 1½ hours. After restriction a phenol extraction was carried out before ligation was performed to get rid of any remaining restriction endonuclease. An equal volume of phenol was added and the samples spun for 10 minutes. The upper layer was pipetted into new sterile Eppendorf tubes and then equal volume of chloroform-isoamyl alcohol (24:1) was added, this was spun for 5 minutes. Again the top phase was pipetted into new sterile Eppendorf tubes. An ethanol precipitation was now performed. 2.5 volumes ice-cold ethanol and 1/10 volume 1 M NaCl was added to the samples. Samples were spun for 20 minutes and dried for 20 minutes at 60°C. Dried pellets were resuspended in 100 µl ligation buffer (see Appendix) at 42°C for 1 hour. For ligations 50 µl ligation buffer was used to which the restricted chromosomal DNA, plasmid DNA, and T4 DNA ligase was added and allowed to incubate overnight at 14°C. In all cases 1 µl T4 DNA ligase was used, which is equivalent to 1 unit of this enzyme.

2.12. TRANSFORMATION OF NOCARDIOFORM STRAINS

(Bibb et al., 1978)

An overnight culture of CI in TYG was grown up at 28°C.

The optical density of the cells was then taken by diluting 180 µl of cells to 720 µl of TYG and reading the absorbance at 540 nm. 1 ml of the culture was then harvested by centrifuging for 1 minute in a microfuge. The pellet was resuspended in 1 ml of P-buffer (see Appendix) with 5 mg/ml lysozyme and incubated with agitation for 1 hour at 37°C. The cells were again pelleted and resuspended gently in 1 ml P-buffer, again cells were harvested and resuspended in 0.5 ml P-buffer. 75 µl each of cells were then aliquoted into two sterile Eppendorf tubes. To one tube which was the control tube 5 µl TE was added while 5 µl of plasmid pDA30 was added to the other. 80 µl polyethyleneglycol (PEG) made up by adding 1 g PEG to 1 ml P-buffer was then added to each Eppendorf tube. This was mixed gently and left for 4-5 minutes. The samples were then spread onto chilled regeneration plates (see Appendix) and incubated at 28°C. After a 12 hour incubation at 28°C an arsenic underlay was done. 0.5 ml of a 3 M arsenate, 0.5 M arsenite solution was used for the underlay, giving a final molarity of 60mM arsenate, 10mM arsenite. Plates were incubated for approximately five days until colonies appeared.

2.13 TRANSFORMATION OF ESCHERICHIA COLI STRAIN MM294 (Dagert et al., 1979; Norgard et al., 1978; Maniatis et al., 1982)

100 ml of LB was inoculated with 1 ml of an overnight culture of mm 294 which was grown in LB at 37°C. The culture was allowed to grown for approximately 2 hours with vigorous

agitation at 37°C until an optical density of 0.2 was achieved at 540 nm. The culture was then chilled on ice for 10 minutes before centrifugion for 10 minutes at 6 000 rpm in a precooled rotor. Cells were resuspended in 50 ml of ice-cold 50 mM $CaCl_2$ and 10 mM Tris pH 8. This cell suspension was placed in an ice bath for 15 minutes before being centrifuged again at 6 000 rpm for 10 minutes. Cells were resuspended in 6.6 ml of the ice-cold solution mentioned above. 0.2 ml aliquots of this suspension were dispensed in pre-chilled Eppendorf tubes and allowed to stand on ice for 1 hour. Plasmid was then added and the cells heat shocked at 42 C for 1 minute before 1 ml LB at 37°C was added. Samples were incubated at 37°C for 1 hour to allow for phenotipic expression of transformants. Cells were then spun down and the supernatant discarded, cells were spread onto LA + 60 µg/ml ampicillin plates. Plates were incubated overnight at 37°C.

2.14. PREPARATION OF SAMPLES FOR RUNNING ON POLYACRYLAMILE GELS

5 ml of strains in T2 medium was grown up overnight. 500µl of this culture was spun down and the pellet resuspended in 20 ml fresh T2 medium. The culture was grown for 5-6 hours and then challenged with 0, 1, 2, and 4 µg/ml fusidic acid in 20 ml T2 medium. The challenged cultures were left to grow overnight at 28 °C. In the morning the cultures

were spun down at 15 000 rpm for 20 minutes to obtain cell-free supernatants. The volume of each supernatant was measured and for each 1 ml of supernatant 0.66 g ammonium sulphate was added which resulted in 90% saturation. After this 0.1 g aliquots ammonium sulphates were added until the solution was completely saturated, this was done to precipitate the protein/s involved in fusidic acid inactivation. The supernatant was again spun at 15 000 rpm for 20 minutes and the pellet resuspended in 0.5 ml sterile distilled water. Once dissolved the sample was transferred to dialysis tubing and dialysed against 200-300 ml sterile distilled water for 3 - 6 hours. The samples were then frozen at -80°C for 15 minutes before being freeze-dried overnight.

2.15. AGAROSE GEL ELECTROPHORESIS

0.4% agarose gels were used. They were prepared by dissolving 0.4 g agarose in 100 ml TBE (see Appendix). The solution was autoclaved before use. For each gel 25 ml of this solution was used to which 1.5 µl EtBr (10 mg/ml) was added before being poured. The electrophoresis buffer was TBE, 250 ml TBE with 12.5 µl EtBr (10 mg/ml) was used per gel. Gel loading buffer type III (see Appendix) was used in all instances. Gels were run at 80V in a minnie submarine model HE 33, and after completion were immediately observed on a UV transilluminator and photographed.

2.16. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Seperating gels of 12.5% were used throughout and were always poured a day before the gel was to be run. The seperating gel was kept moist and stored in the cold room overnight. The stacking gel was poured the following morning and allowed to polymerise for 2 hours before the gel was run. The s ' sample loading buffer, electrophoresis buffer, stain and destain were used throughout (see Appendix). All gels were run at 120V for 3 - 5 hours. Gels were stained overnight and destained for 4 - 6 hours before being viewed on a illuminator and photographed.

3. RESULTS AND DISCUSSION

3.1. ASSAYING FOR FUSIDIC ACID INACTIVATION IN NOCARDIOFORM BACTERIA

Plate lysate and liquid assays (see 2.4, 2.4.1, 2.4.2) were used for information of fusidic acid inactivation in nocardioform wild-type and mutant strains. The mutant strains used throughout were : 01, derived from ATCC 12674, this 01 mutant has lost the plasmid-borne genes conferring resistance to sodium arsenate and arsenite, cadmium chloride, and chloramphenicol by curing of the plasmid (Dabbs and Sole, 1987). The second mutant 01(trp^-) was derived from 01 by nitrosoguanidine (NTG) mutagenesis. It is an auxotroph requiring tryptophan for growth on minimal medium and has an increased resistance to fusidic acid. Finally, 01(pro^-) which was also derived from 01 by NTG mutagenesis, this auxotrophic mutant requires proline for growth on minimal medium and was more sensitive to fusidic acid than 01.

Growth response to fusidic acid challenge

When actively growing cultures of the three mutants in exponential growth phase were challenged with 10 ug/ml fusidic acid, representative results showed that none of the strains had a clear demonstrative difference in growth response, (see Figure 1) for data see Table 1 in Appendix.

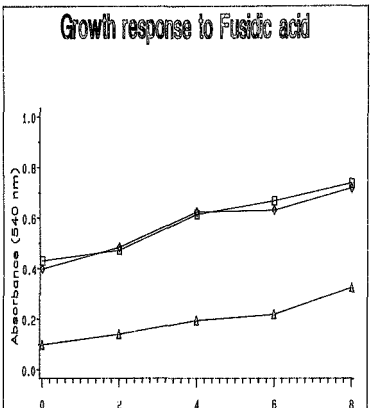


Figure 1:–
Growth response of 01, 01(trp⁻), 01(pro⁻) to 10 μg/ml FUS. (Data from Table 1.)

blue : 01
brown : 01(pro⁻)
red : 01(trp⁻)

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Correlation of antibiotic phenotype and auxotrophy

As 01(trp$^-$) had an increased fusidic acid resistance compared with 01, it was determined whether the increased fusidic acid resistance was due to the mutation resulting in trp$^-$. trp$^-$ to trp$^+$ revertants were selected and tested. The revertants were obtained by spreading an aliquot of an overnight culture of 01(trp$^-$) onto minimal media plates. It was found that all revertants lost the increased resistance to fusidic acid and had the same phenotype on the assay plates as the parental 01 strain. The results therefore indicated that the mutation resulting in trp$^-$ was probably responsible for the increased fusidic acid resistance, and that loss of the mutation results in loss of the fusidic acid resistance conferred by it. pro$^-$ to pro$^+$ revertants could not be obtained suggesting the possibility of a double mutation resulting in the pro$^-$ mutant.

Representative results clearly demonstrated that pro$^-$ and trp$^-$ strains inactivate fusidic acid at approximately the same rate. This suggested that the inactivating enzyme activity was the same or almost the same and that the resistant mutation of trp$^-$ had nothing to do with the antibiotic inactivating system inherent in nocardioform bacteria.

3.1.1. INDUCIBILITY OF THE ANTIBIOTIC INACTIVATING SYSTEM

The fusidic acid sensitive phenotype of 01, 01(trp$^-$), and 01(pro$^-$) was tested with and without the induction of the antibiotic inactivating system. The antibiotic

inactivation mechanism of nocardioform strains is inducible, meaning that the mechanism is only triggered upon induction by fusidic acid. The inactivating enzyme was only present in the supernatant of fusidic acid challenged cultures clearly demonstrating the inducibility phenomenon. The strains were streaked onto TYA plates (uninduced) as well as TYA containing 0,25 µg/ml fusidic acid (induced) plates. This concentration is sub-inhibitory but inducing. Plates were then incubated at 28°C overnight before samples were taken and spotted onto TYA plates with varying concentrations of fusidic acid. These *induced and uninduced spot tests* clearly gave different results (see Table 2).

Numerous assays revealed that the minimum concentration of fusidic acid needed to induce the cell-free inactivation system was 1 µg/ml. Demonstrating that the inducible inactivation enzyme is released into the supernatant. Results also indicated that for 01 and 01(trp^-) the cellfree inactivation system was hyperinducible, whereas 01(pro^-) was hypoinducible compared to the parental strain 01.

TABLE 2 (a) Spot test checking fusidic acid sensitive phenotype of 01, 01(trp$^-$), and 01(pro$^-$) without the induction of the antibiotic inactivating system.

	FUSIDIC ACID CONCENTRATION (µg/ml)						
STRAIN	0	1	2	3	5	10	20
01	+++	+	+				
01(trp$^-$)	+++	+++	+++	++	++	+	
01(pro$^-$)	+++						

(b) Spot test checking fusidic acid sensitive phenotype of 01, 01(trp$^-$), and 01(pro$^-$) with the induction of the antibiotic inactivating system.

	FUSIDIC ACID CONCENTRATION (µg/ml)						
STRAIN	0	1	2	3	5	10	20
01	+++	+++	+++	+++	+++	+++	
01(trp$^-$)	+++	+++	+++	+++	+++	+++	++
01(pro$^-$)	+++	++					

+ + + = maximum growth

+ + = good growth

+ = poor growth

TABLE 2 (a) Spot test checking fusidic acid sensitive phenotype of 01, 01(trp$^-$), and 01(pro$^-$) without the induction of the antibiotic inactivating system.

	FUSIDIC ACID CONCENTRATION (µg/ml)						
STRAIN	0	1	2	3	5	10	20
01	+++	+	+				
01(trp$^-$)	+++	+++	+++	++	++	+	
01(pro$^-$)	+++						

(b) Spot test checking fusidic acid sensitive phenotype of 01, 01(trp$^-$), and 01(pro$^-$) with the induction of the antibiotic inactivating system.

	FUSIDIC ACID CONCENTRATION (µg/ml)						
STRAIN	0	1	2	3	5	10	20
01	+++	+++	+++	+++	+++	+++	
01(trp$^-$)	+++	+++	+++	+++	+++	+++	++
01(pro$^-$)	+++	++					

+ + + = maximum growth

+ + = good growth

+ = poor growth

3.1.2. POLYACRYLAMIDE GEL ELECTROPHORESIS, TO VISUALIZE THE PUTATIVE INACTIVATING ENZYME/S

Assays of cell-free supernatants clearly demonstrated that when cultures of the strains used were induced with fusidic acid the inactivating enzyme was released into the supernatants, and that these supernatants could subsequently inactivate fusidic acid. Cell-free suspernatants of 01, 01(trp^-), and 01(pro^-) were all able to inactivate 40 ug/ml fusidic acid within 20 hours. Protein samples for electrophoresis were prepared as in section 2.14.

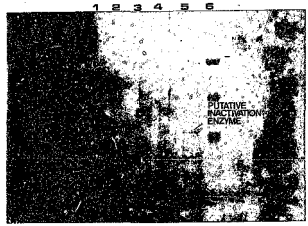


FIGURE 2 Protein samples of cell-free supernatants run on SDS-polyacrylamide gel to visualize putative inactivation enzyme/s

Lane 1 : Protein molecular weight marker
Lane 2 : 01
Lane 3 : 01 + 1 ug/ml fusidic acid
Lane 4 : 01 + 2 ug/ml fusidic acid
Lane 5 : 01 + 4 ug/ml fusidic acid
Lane 6 : protein molecular weigh

3.1.3. LIQUID ASSAYS FOR FUSIDC ACID INACTIVATION

Numerous liquid assays (see 2.4.2) for both actively growing cultures and cell-free supernatants were carried out. This was because when plate assays of cell-free supernatants were done there was only a slight variation in the radius of the zones of inhibition versus large increments in the concentration of fusidic acid used, thereby making it impossible to construct a suitable graph for determining the rate of fusidic acid inactivation. Unfortuantely, although several assay organisms were used, namely, Arthrobacter oxidans, AD14, and ST47, both mutants derived from Escherichia coli, no suitable graphs of inactivation could be constructed. This was primarily because the fusidic acid standards of varying concentrations which were to be used for the calibration curve all grew up, became turbid, at the same or almost the same time, making it impossible to construct any meaningful calibration curve from which data could be extrapolated. With no other option available plate assays of cell-free supernatants were then done. Fusidic acid standards of various concentrations were prepared (see Table 3 (a) in Appendix). It was clear from preliminary experiments that for low concentration fusidic acid standards of between 0-1 ug/ml, assay plates would be incubated in the cold-room for 12 hours before being placed into the 28°C room. This diffusion of the fusidic acid allowed for more accurate measuring of the zones of inhibition. For high concentration fusidic acid standards i.e. greater then 1 ug/ml, assay plates were placed directly into 28°C room. These conditions were optimal for measuring the zones of inhibition.

Representative results showed that the cell-free supernatants of 01(pro$^-$) and 01(trp$^-$) inactivated 5 µg/ml fusidic acid at the same rate, and in addition these strains inactivated the fusidic acid faster than did 01, the wildtype strain (see Figure 3). For data see Table 3 (a) and (b) in Appendix.

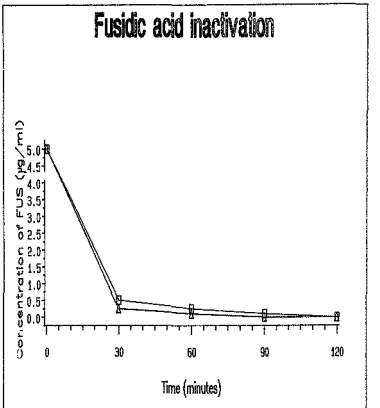


Figure 3:-
Representation of fusidic acid (5µg/ml) inactivation. Data as in Table 3a and 3b.

blue : 01
red : 01(trp$^-$) and 01(pro$^-$)

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3.1.4 ERYTHROMYCIN RESISTANT MUTANTS OF NOCARDIOFORM STRAINS LEAD TO INCREASED FUSIDIC ACID AND RIFAMPICIN RESISTANCE

High level erythromycin resistant mutants of ATCC 12674 as shown from numerous spot tests clearly had increased fusidic acid and rifampicin resistance when compared to the wild-type ATCC 12674 strain. When fusidic acid high level resistant mutants of ATCC 12674 were tested it was found that these had increased erythromycin and rifampicin resistance, and conversely, when rifampicin high level resistant ATCC 12674 mutants were tested they had increased erythromycin and fusidic acid resistance. To determine whether this phenomenon is common in other nocardioform strains, 14887 and 4277 were tested in the same way and indeed the findings were the same, thereby leading to the conclusion that this phenomenon was indeed common in nocardioform bacteria.

To determine whether the above situation also pertains to other gram-positive organisms, Bacillus subtilis strain 1A3 was made high level erythromycin, fusidic acid, and rifampicin resistant and spot tested as before, but representative results were clear, there was no increased fusidic acid or rifampicin resistance.

From these results it appeared that in nocardioforms an amplification of a segment of DNA was taking place. Either it was an amplification of the genes of the target molecules of the various antibiotics, namely, EF-G for fusidic acid, the beta subunit of RNA polymerase for rifampicin, and certain ribosomal proteins for erythromycin (Vazquez, 1979),

or an amplification of the antibiotic inactivating genes involved in each case. Which of the two possibilities it is, is as yet not clear and still being investigated. For the purposes of this work, however, it would be beneficial if amplification of the inactivation genes has taken place as this would increase the number of copies of these genes, and thus enhance the ultimate aim of cloning the gene/s involved in fusidic acid inactivation.

3.2. MUTAGENESIS TO OBTAIN FUSIDIC ACID HYPERSENSITIVE NOCARDIOFORM MUTANTS (Crameri et al., 1986)

In nocardioform bacteria mutagenesis apparently occurs at a much lower rate than in Escherichia coli. The maximum frequency of auxotrophs produced, which was used as a meassure of the efficiency of mutagenesis is approximately 1-2% for nocardioforms. In Escherichia coli the frequency of auxothrophs can be greater than 80% (E.Dabbs Personal Communication). This lack of mutant production could be explained by the lack of an error prone repair system in these organisms, another contributing factor is that nocardioform bacteria tend to clump. In order therefore to optimize mutagenesis the clumping phenomenon was reduced. T2 medium was used instead of TY for the growth of organisms and this reduced clamping as was shown by the fact that the colony forming units at a given optical density were higher. The clumping was further reduced, by approximately ten-fold, when samples were sonicated with a medium tip for 5 seconds after mutagenesis. The number of colonies increased by 50% when samples were vortexed for 80 seconds

before being spread onto TYA plates. Also 10^{-6} dilutions of samples were made before they were vortexed and spread onto plates, this was to ensure that single colonies would result.

To allow for the phenotypic expression of mutations, cultures were outgrown after the mutagenesis and this increased the risk of contamination. To overcome this problem strains used were made resistant to the aminoglycoside streptomycin which was then added to outgrowing cultures. 01 was thus made resistant to streptomycin and was the organism used for all mutagenesis experiments .

TABLE 4 The various mutagenesis experiments performed on 011 (streptomycin resistant 01 mutant)

	011			
TYPE OF mutagenesis	No. of colonies screened	No of auxotrophs	% auxotrophs	Fusidic acid hypersensitive mutants
NTG pH 4.8, growing culture	2200	20	0.9	0
NTG pH 8.0, growing culture	2100	12	0.5	0
EMS pH 7.0, growing culture	1050	11	1.05	0
UV (10 min.)	2000	10	0.5	0
EtBr(15µg/ml), growing culture	2000	12	0.6	0
acridine orange (5µg/ml), growing culture	2000	11	0.55	0

NTG - nitrosoguanidine

EMS - ethylmethanesulphonate

UV - ultraviolet radiation for 10 minutes

EtBr - ethidium bromide

In all mutagenesis experiments colonies that had grown up after a 10^{-6} dilution vortexed for 80 seconds had been spread onto TYA plates, were patched onto TYA, TYA + 1 µg/ml fusidic acid, and MM plates and screened. Colonies that grew up on TYA, but only grew poorly or not at all on MM plates were considered at auxotrophs. Those colonies growing readily on TYA, but not at all or poorly on TYA + 1 µg/ml fusidic acid were considered as possible fusidic acid hypersensitive mutants i.e. candidates for mutants which have lost the fusidic acid inactivating gene/s due to mutational alterations. A number of fusidic acid hypersensitive mutants were wanted in order to examine the different complementation groups, as this is one way to make an assessment as to the number of gene/s involved in the nocardioform inactivation mechanism.

As no fusidic acid hypersensitive mutants were obtained from 011 a rifampicin resistant 01 strain was made and designated as 012. When rifampicin sensitive mutants (isolated by Susan Anderson) were tested for fusidic acid sensitivity these mutants clearly still inactivated fusidic acid, indicating that the rifampicin and fusidic acid inactivating systems were different i.e. different genes are responsible.

TABLE 5 The various mutagenesis experiments performed on 012 (rifampicin resistant 01 mutant)

	012			
TYPE of mutagenesis	No of colonies screened	No of auxotrophs	% auxotrophs	Fusidic acid hypersensitive mutants
NTG pH 4.8, growing culture	2500	35	1.6	7
NTG pH 8.0, growing culture	2500	15	0.6	0
EMS pH 7.0, growing culture	2500	17	0.68	0
UV (10 min.)	1000	11	1.1	0
EtBr(15µg/ml), growing culture	1500	12	0.8	0
acridine orange (5ul/µg/ml), growing culture	1500	11	0.73	0

NTG - nitrosoguanidine

EMS - ethylmethanesulphonate

UV - ultraviolet radiation for 10 minutes

EtBr - ethidium bromide

It is clear from the data in Table 5 that the only condition producing fusidic acid hypersensitive mutants was NTG pH 4.8 mutagenesis of a growing culture of 012. Representative results demonstrated that the mutants were indeed hypersensitive to fusidic acid compared to the wildtype ATCC 12674 strain. The seven mutants were then placed in 33% glycerol and frozen at -80°C. A possible explanation as to why no fusidic acid hypersensitive mutants could be obtained from the streptomycin resistant 011 strain will be given in the next section, 3.2.1.

3.2.1. GENETICS OF FUSIDIC ACID SENSITIVITY

The data presented above suggests that the streptomycin resistance mutation was responsible for not being able to produce fusidic acid hypersensitive mutants. ATCC 12674 has two unstable genetic elements (Dabbs and Sole, 1987), one which is lost spontaneously at a very low rate resulting in 01 and the other which can be cured with mitomycin C treatment. When curing strain 011 with mitomycin C, streptomycin resistance was reduced in two out of seven clones tested. The level of streptomycin resistance was, however, still higher than that of the parental strain, therefore two mutations are involved in the production of streptomycin resistance. One mutation may be chromosomal while the other is probably on the unstable genetic element which is cured by mitomycin C.

Ribosomal protein S12 which is responsible for streptomycin resistance is co-transcribed with the EF-G gene which is the target of fusidic acid suggesting a close functional interaction between the two (personal communication E. Dabbs). The mutation in the S12 gene might result in an increased expression of S12 and thus also EF-G thus preventing the production of fusidic acid hypersensitive mutants.

3.3. OPTIMIZATION OF TRANSFORMATION IN NOCARDIOFORM STRAIN 01

01 was used as the recipient into which the plasmid pDA30 was transformed.

3.3.1. NOCARDIOFORM TRANSFORMATION AND GROWTH PHASE OF 01

Representative results (done in collaboration with Anne Daffey and Susan Anderson) since cultures had to be sampled round the clock, demonstrated that the growth phase of 01 which resulted in the best transformation efficiency was that termed as post-exponential, the absorbance reading of the cells at post-exponential growth phase (540 nm) was 3.89 (see Figure 4). In all transformation experiments 0.1 ug of plasmid pDA30 DNA was used. Each time cultures were sampled to give the results represented in Figure 4, twenty 1 ml aliqots were taken and frozen at -80°C. All future transformation experiments were thus performed using the frozen 01 cells with the optical density of 3.89. For data to Figure 4 see Table 6 in Appendix.

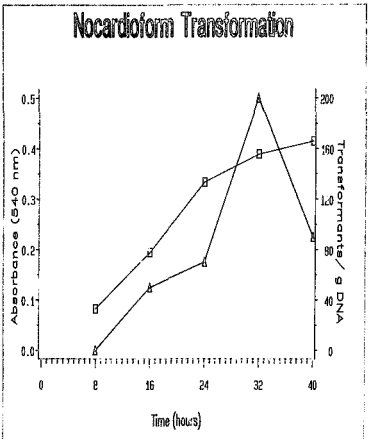


Figure 4:-
Indication of the number of transformants/µg DNA at various growth phases of 01.

blue : plot of absorbances
(all absorbance readings to be multiplied by 10, because of dilution factor)

red : plot of the number of transformants per ug pDA30

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3.3.2. NOCARDIOFORM TRANSFORMATION EFFICIENCY BY VARYING THE AMOUNT OF pDA30 AND CELLS (01)

In these experiments when the amount of plasmid was varied the amount of cells was kept constant and in all cases 50 µl of cells was used. When the amount of cells was varied the amount of plasmid was kept constant and in all cased 5 µl of plasmid DNA was used. 50% polyethyleneglycol (PEG) was used throughout. 5 µl of pDA30 was equivalent to 0.1 ug DNA. It is clear form the data presented in Figure 5 that increasing the concentration of pDA30 DNA resulted in a decrease in the number of transformants per ug of pDA30 DNA, and hence a reduction in the efficiency of transformation. For data to Figure 5 see Table 7 in Appendix.

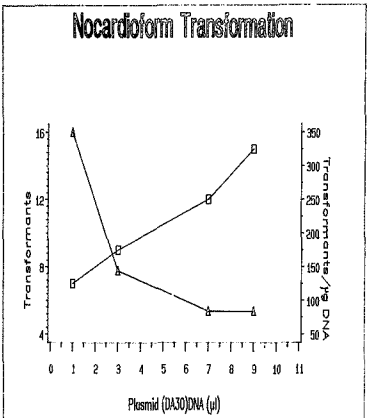


Figure 5:-
Representation of the number of transformants obtained with varying pDA30 DNA volumes.

blue : No. of transformants obtained with 1µl(0.02µg), 3µl(0.06µg), 7µl(0.14µg), 9µl(0.18µg) pDA30

red : No. of transformants per ug pDA30 obtained with the same volumes as in blue above

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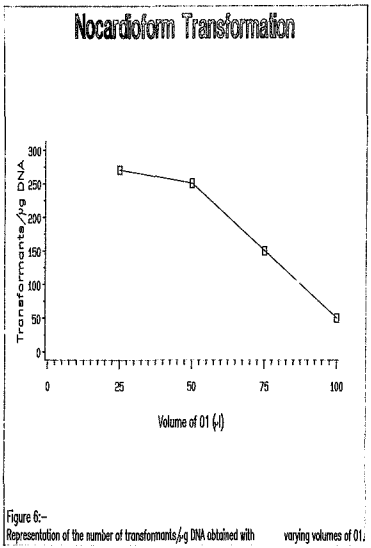


Figure 6:–
Representation of the number of transformants/µg DNA obtained with varying volumes of 01.

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From the data in Figure 6 (for data to Figure 6 see Table 8 in Appendix) it was apparent that increasing the volume of 01 cells resulted in a decrease in the number of transformants per ug DNA. The combined data of Figure 5 and Figure 6 clearly demonstrates that the condition which resulted in the most transformants per ug of pDA30 DNA was that in which 0.02 µg of pDA30 DNA was used, resulting in 350 transformants per µg of plasmid DNA.

To confirm that the recipient 01 strain had indeed been transformed with plasmid pDA30, four random transformants were taken. Plasmid minipreparations were performed on these transformants as well as 01 which would be the control. If transformation was successful then the plasmid pDA30 should be present in all prepared samples except the 01 control (see Figure 7). It is clear from the result in this Figure that in all cases pDA30 has been transformed into 01.

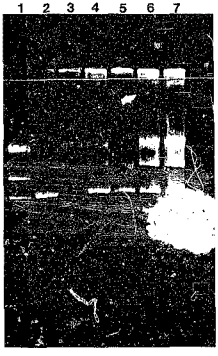


FIGURE 7 A gel of four random transformants, demonstrating that transformation was successful.

Lane 1 - DNA molecular weight marker II

Lane 2 - Plasmid pDA30

Lane 3 - 01 control

Lane 4 - 01 transformed with pDA30

Lane 5 - 01 transformed with pDA30

Lane 6 - 01 transformed with pDA30

Lane 7 - 01 tranformed with pDA30

3.4. CONSTRUCTION OF A GENOMIC LIBRARY OF RHODOCOCCUS EQUI IN THE ESCHERICHIA COLI RECIPIENT MM 294-1

The first step was to ascertain whether the donor strain of Rhodococcus equi 14887/1 did indeed inactivate fusidic acid. This was necessary because if the strain did not inactivate fusidic acid its DNA could not be used for cloning by complementation as it would not have the fusidic acid inactivating gene/s, and could thus not complement the fusidic acid hypersensitive mutants of Rhodococcus erythropolis into which its chromosomal DNA would be eventually transformed. The donor strain was thus grown up and challenged with 20 ug/ml fusidic acid, representative results demonstrated that after 10 hours all the fusidic acid had been completely inactivated. The chromosomal DNA of this strain could thus be used to build a library in MM294-1. The reason 14887/1 was used as the donor was, firstly, because a colleague was using the same strain for her work to build up her library in MM294-1, which meant that our libraries could be shared. Secondly, it was necessary to use chromosomal DNA from various different nocardioform strains to minimise recombination after transformation of this chromosomal DNA back into nocardioform, i.e. into the fusidic acid hypersensitive mutants, as a rec⁻ nocardioform mutant was not available.

The vector used to transform the chromosomal DNA into MM294-1 was a putative shuttle vector made of pDA22 which is a nocardioform plasmid and pEcoR 251 which is a suicide vector. The two plasmids were joined via their single Bam Hl restriction sites.

The putative shuttle vector pDA27 was constructed by Anne Daffey. The suicide vector has ampicillin resistance as well as EcoR 1 end-nuclease activity, a Bgl II restriction site lies within the EcoR 1 gene whose activity is suppressed by a lambda repressor. The chromosomal DNA fragments are inserted into the EcoR 1 gene by using Bgl II resulting in insertional inactivation of the gene, only transformants will thus grow on LA containing ampicillin plates. The nocardioform plasmid pDA22 has arsenate resistance and no Bgl II restriction site.

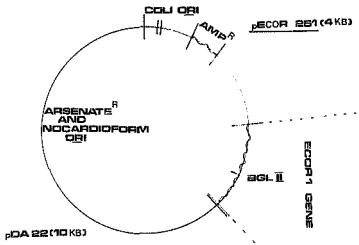


FIGURE 8 Diagrammatic representation of the putative shuttle vector. pDA27

Plasmid pDA27 DNA maintained in E.coli and 14887/1 DNA were then extracted and purified (see Materials and Methods).



FIGURE 9 Gel showing purified pDA27 and 14887/1 chromosomal DNA.

Lane 1 - 14887/1 chromoromal DNA
Lane 2 - 14887/1 chromosomal DNA
Lane 3 - pDA27

pDA27 and chromosomal DNA was digested with restriction endonuclease Bgl II. Before restriction the concentrations of the respective DNA's was ascertained by absorbance readings at 260 nm. pDA27 had a concentration of 254 µg/ml, and 14887/1 chromosomal DNA had a concentration of 295 µg/ml. pDA27 was restricted using 1.2 units of Bgl II for 5 hours. Bgl II was used as there is only one Bgl II site and this is in the EcoR I gene of the pEcoR 251 plasmid, Bgl II was also used to restrict 14887/1 chromosomal DNA so that these fragments would insert into the EcoR gene during ligation and result in insertional inactivation of this gene. After restriction the pDA27 samples were phenol extracted and ethanol precipitated to remove restriction endonuclease, and then resuspended in TE to give a final concentration of 0.3 µg/ul DNA. This was then frozen at -80°C.

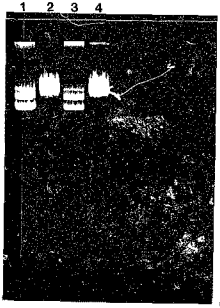


FIGURE 10 Gel showing uncut and restricted pDA27

Lane 1 - uncut pDA27

Lane 2 - cut pDA27

Lane 3 - uncut pDA27

Lane 4 - cut pDA27

To determine the condition which would result in the best partial digestion of 14887/1 chromosomal DNA a serial dilution series of Bgl II (12 units/ul) was done (see Figure 11).

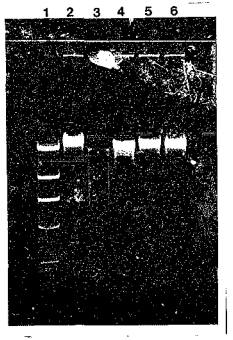


FIGURE 11 Gel showing serial dilution series of Bgl II which was used to restrict the 14887/1 DNA for 3 hours.

Lane 1 - DNA molecular weight marker II

Lane 2 - 14887/1 DNA without Bgl II (control)

Lane 3 - 14887/1 DNA plus 10^{-1} dilution of Bgl II

Lane 4 - 14887/1 DNA plus 10^{-2} dilution of Bgl II

Lane 5 - 14887/1 DNA plus 10^{-3} dilution of Bgl II

Lane 6 - 14887/1 DNA plus 10^{-4} dilution of Bgl II

After observation of the gel it was decided that the 10^{-2} dilution of Bgl II gave the necessary result, 14887/1 DNA was then digested with this dilution of Bgl II. After restriction DNA was again phenol extracted and ethanol precipitated, and resuspended in TE to give a final concentration of 0.3 µg/ul DNA. Again this was frozen at -80°C.

These DNA samples were then ligated and transformed into MM 294-1. The pDA27 concentration was kept constant, 0.3 µg was used throughout. The 14887/1 chromosomal DNA concentration was varied to determine which concentration would give the most transformants, i.e. the best transformation efficiency. The ligated samples were transformed into MM 294-1 which is a rifampicin resistant mutant of MM 294 in order to prevent any contamination in the library. This is because once the library was build up MM 294-1 could be grown up in the presence of rifampicin as well as ampicillin thereby inhibiting any contaminating organisms. A representative result is shown in Table 9.

TABLE 9 A presentation of the number of transformants resistant to ampicillin with varying concentrations of 14887/1 chromosomal DNA.

Transformation sample	Number of ampicillin^{r} transformants
MM 294-1 cells (control)	0
pDA27 0.3 µg	0
0.3 ug pDA27 + 0.3 µg 14887/1 DNA	72
0.3 ug pDA27 + 0.6 µg 14887/1 DNA	429
0.3 ug pDA27 + 1.2 µg 14887/1 DNA	30
0.3 ug pDA27 + 2.4 µg 14887/1 DNA	17

It was clear that 0.3 µg pDA27 and 0.6 µg chromosomal DNA yielded the highest number of transformants. This combination was thus used for all further transformation experiments.

A plasmid minipreparation was prepared on a random sample of six transformants. These samples were run on a 0.4% agarose gel to ascertain whether transformation was successful. What one would expect to see was a gel showing pDA27 with chromosomal DNA inserts of various sizes (see Figure 12). After each transformation experiment the transformants were counted, then the transformants on each

LA + 50 µg/ml ampicillin plate were resuspended in 1 ml LB + 60 µg/ml ampicillin, and transferred to a flask kept at -80°C. Each batch of new transformants was added to the same flask, and the addition was made at 80°C to prevent cells from thawing.

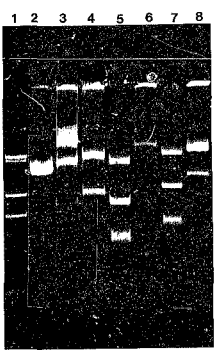


FIGURE 12 Plasmid minipreparation of six random transformants showing chromosomal DNA inserts of various sizes.

Lane 1 - DNA molecular weight marker II
Lane 2 - Cut pDA27
Lane 3 - pDA27 + chromosomal insert
Lane 4 - pDA27 + chromosomal insert
Lane 5 - pDA27 + chromosomal insert
Lane 6 - pDA27 + chromosomal insert
Lane 7 - pDA27 + chromosomal insert
Lane 8 - pDA27 + chromosomal insert

Figure 12 shows a heterogeneous distribution of 14887/1 chromosomal DNA inserts. Another plasmid minipreparation was performed on the same transformants and this was restricted overnight with Eql II. As pDA27 only has one Bgl II site in the EcoR 1 gene of the pEcoR 251 plasmid it was possible to excise the chromosomal DNA inserts that were inserted into the EcoR I gene (see Figure 13).

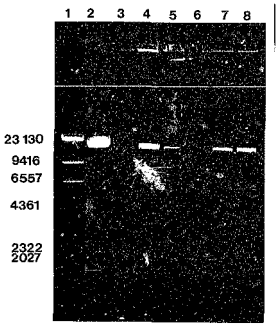


FIGURE 13 Gel showing chromosomal DNA fragments of various sizes after pDA27 had been digested with Bgl II overnight.

Lane 1 - DNA molecular weight marker II
Lane 2 - cut pDA27
Lane 3 - 14887/1 DNA fragments
Lane 4 - 14887/1 DNA fragments
Lane 5 - 14887/1 DNA fragments
Lane 6 - 14887/1 DNA fragments
Lane 7 - 14887/1 DNA fragments
Lane 8 - 14887/1 DNA fragments

The average size of the chromosomal DNA fragments as seen in Figure 13 could then be determined by doing regression analysis. A plot of the log values of the sizes of the DNA molecular weight marker II bands against the distance migrated by each band was done (see Figure 14). For data of regression analysis and Figure 14 see Table 10 (a), 10 (b) in Appendix). The average fragment size would give one an idea as to how large a library is needed to cover the entire 14887/1 genome.

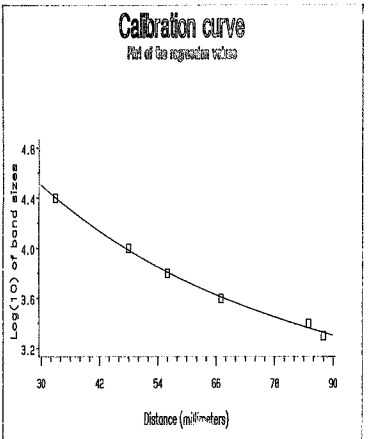


Figure 14:-
Fragment size determination of chromosomal DNA inserts.

10BKTECK 10/02/89 14: 50: 45 081

The average fragment size as determined by regression analysis was 6 569 bp. At present a 14887/1 library of 3 500 transformants has been constructed.

A point has now been reached were fusidic acid hypersensitive mutants are available and a genomic library of 14887/1 with 3 500 transformants has been constructed. The library of the putative shuttle vector containing the 14887/1 chromosomal DNA inserts can be extracted from MM 294-1 and transformed into the fusidic acid hypersensitive mutants in an attempt to screen the fusidic acid inactivation gene/s by complementation.

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A P P E N D I X

T2 medium

1% tryptone

0,5% yeast extract

3mM Sodium Chloride

3mM Calcium Chloride

10mM Magnesium sulphate

TY medium

1% tryptone

0,5% yeast extract

LB (Luria broth)

1% tryptone

0,5% yeast extract

0,5% NaCl

TYG medium

1% tryptone

0,5% yeast extract

1% glycine

TYA

1% tryptone

0,5% yeast extract

1.5% agar

LA

1% tryptone

0,5% yeast extract

0,5% NaCl

1.5% agar

for assay plates ½ the concentration of agar was used.

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0,5% yeast extract

3mM Sodium Chloride

3mM Calcium Chloride

10mM Magnesium sulphate

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0,5% yeast extract

1.5% agar

LA

1% tryptone

0,5% yeast extract

0,5% NaCl

1.5% agar

for assay plates ½ the concentration of agar was used.

Minimal media (MM) plates

10 x Stock of A - N buffer: 458,5g K_2HPO_4 $3H_2O$

134,0g KH_2PO_4

25,0g tri sodium citrate $2H_2O$

5g $MgSO_4$ $7H_2O$

dissolve in 5l H_2O, pH is 7.0 - 7.5

To one 500 ml flask (A) add: 0,3g NH_4Cl

30 ml A - N Stock

120 ml H_2O

To another 500 ml flask (B) add:

1,5 g glucose

4 g agar (noble agar)

150 ml H_2O

autoclave seperately, then add 600 µl of 10% Sodium glutamate to flask (A) and 300 µl Bl (10mg/ml) to flask (B). Add contents of flask (A) to flask (B) mix and pour plates.

TE

10 mM Tris

10 mM EDTA, pH 8.0 with HCl

Cold detergent solution

0,25 ml 20% triton X 100

3,125 ml 0,5 M EDTA pH 8

1,25 ml 1 M Tris pH8

20,375 ml sterile distilled H_2O

Solution 1

50 mM glucose

25 mM Tris pH 8

10 mM EDTA

Soln. 2

0,2 M NaOH

1% SDS

Solution 3

5 M KAc pH 8

P (protoplast) buffer

Sucrose 10,3 g

K_2SO_4 25 mg

$MgCl_2$ $6H_2O$ 0,202 g

87,5 ml distilled H_2O

After autoclaving and before use add:

KH_2PO_4 (0,5%) 1 ml

$CaCl_2$ $2H_2O$ (1M) 2,5 ml

TES buffer (pH 7.2) 10 ml

Regeneration plates

for 500 ml : 0,9 g NaCl

3 g tryptone

1,5 g yeast extract

35 g sucrose

add a little distilled H_2O and heat up in a microwave for a few seconds at a time, until sucrose has dissolved. Then add 4 g agar and make up volume to 280 ml. After autoclaving add 6 ml 1 M $CaCl_2$ and 10 ml TES buffer (0,25 M pH 7.2). Plates can then be poured.

Gel loading buffer for agarose gels

0,25% bromophenol blue

30% glycerol in H_2O

TBE

For 1000 ml of 5 x TBE Stock: 54 g Tris

27,5 g Boric acid

20 ml 0,5 M EDTA

Seperating gel for SDS - PAGE

14 ml 30% acrylamide-bisacrylamide stock

20 ml 0.75 M Tris pH 8.8

0,4 ml 10% SDS

3,6 ml distilled H_2O

2 ml ammonium persulphate (10%), freshly prepared

12 µl TEMED

Stacking gel for SDS - PAGE

3 ml 30% acrylamide-bisacrylamide stock

10 ml 0,25 M Tris pH 6.8

0,2 ml 10% SDS

1 ml ammonium persulphate (10%), freshly prepared

6 µl TEMED

Electrode buffer for SDS - PAGE

14,4 g glycine

33 ml 0,75 M Tris pH 8.8

10 ml 10% SDS

940 ml distilled H_2O

Stain for SDS -PAGE

0,25 g coomasie brilliant blue R250

125 ml methanol

25 ml acetic acid

100 ml distilled H_2O

TBE

For 1000 ml of 5 x TBE Stock: 54 g Tris

27,5 g Boric acid

20 ml 0,5 M EDTA

Seperating gel for SDS - PAGE

14 ml 30% acrylamide-bisacrylamide stock

20 ml 0.75 M Tris pH 8.8

0,4 ml 10% SDS

3,6 ml distilled H_2O

2 ml ammonium persulphate (10%), freshly prepared

12 µl TEMED

Stacking gel for SDS - PAGE

3 ml 30% acrylamide-bisacrylamide stock

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1 ml ammonium persulphate (10%), freshly prepared

6 µl TEMED

Electrode buffer for SDS - PAGE

14,4 g glycine

33 ml 0,75 M Tris pH 8.8

10 ml 10% SDS

940 ml distilled H_2O

Stain for SDS -PAGE

0,25 g coomasie brilliant blue R250

125 ml methanol

25 ml acetic acid

100 ml distilled H_2O

Destain for SDS - PAGE

125 ml methanol

25 ml acetic acid

100 ml distilled H_2O

Sample loading buffer for SDS - PAGE

10 ml 0,25 M Tris pH 6.8

8 ml 10% SDS

4 ml glycerol

2 ml - mercaptoethanol

16 ml distilled H_2O

0,0004 g bromophenol blue

Ligation buffer

200 mM Tris-HCl	1 ml
100 mM $MgCl_2$	1 ml
100 mM DTT	1 ml
0,6 mM ATP	0,6 ml pH 7.6

This was made up to 10 ml with sterile distilled H_2O.

Table 1 - The optical densities at 540 nm of 01, 01(trp⁻) and 01(pro⁻) at various time intervals.

01		01(trp⁻)		01(pro⁻)	
Time after adding fusidic acid (h)	OD(nm)	Time after adding fusidic acid (h)	OD(nm)	Time after adding fusidic acid (h)	OD (nm)
0	0,431	0	0,099	0	0,398
2	0,472	2	0,140	2	0,483
4	0,614	4	0,195	4	0,623
6	0,667	6	0,219	6	0,630
8	0,737	8	0,325	8	0,717

Table 3 - (a) Various fusidic acid standards and respective zones of inhibitation as assayed on LA + 30 µg/ml rifampicin plates matted with a lawn of C7R.

Fusidic acid standards (µg/ml)	Zones of inhibition (radius in cm)
0,1	0,5
0,25	0,8
0,5	0,9
1	1
2	1,1
5	1,3

Table 3 - (b) Fusidic acid (5 ul/ml) inactivation by 01, 01(trp$^-$) and 01(pro$^-$) cell free supernatants after various times as assayed on LA + 30 ug/ml rifampicin plates matted with a lawn of C7R.

01		01(trp$^-$)		01(pro$^-$)	
Time (min)	Zone of inhibition (cm)	Time (min)	Zone of inhibition (cm)	Time (min)	Zone of inhibition (cm)
0	1,3	0	1,3	0	1,3
30	0,9	30	0,8	30	0,8
60	0,8	60	0,5	60	0,5
90	0,5	90	no zone	90	no zone
120	no zone	120	no zone	120	no zone

no zone - implies that all the fusidic acid has been inactivated.

Table 6 - Nocardioform transformation

Time (h)	Absorbance (540 nm)	Number of transformants/ µg DNA
8	0.082	0
16	0.194	50
24	0.333	70
32	0.389	200
40	0.415	90

Table 7 - Nocardioform transformation

pDA30 DNA (µl)	No of transformants	No of transformants/ µg DNA
1 (0,02 µg)	7	350
3 (0,06 µg)	9	144
7 (0,14 µg)	12	84
9 (0,18 µg)	15	84

Table 8 - Nocardioform transformation

Volume of 01 (µl)	No of transformants/ µg DNA
25	270
50	250
75	150
100	50

Table 10 - (a) Calibration curve - molecular markers

Molecular weight (bp)	log of Molecular weights	Distance migrated by Molecular weight bands (mm)	Distance migrated by 14887/1 chromosomal fragments (mm)
23130	4,4	33	50
9416	4,0	48	60
6557	3,8	56	60
4361	3,6	67	69
2322	3,4	85	88
2027	3,3	88	64

Table 10 - (b) Regression analysis to obtain average fragment size

$x - \bar{x}$	$y - \bar{y}$	$(x-\bar{x})(y-\bar{y})$	$(x - x)^2$	$\bar{y}$	(x-x) for each fragment
-30	.4	-12	900	3.75	-15
-15	0	0	225		- 5
- 7	-0.2	1.4	49		- 5
4	-0.4	-1.6	16		4
22	-0.6	-13.2	484		23
25	-0.7	-17.5	625		- 1
		Σ 42.9	Σ 2299		

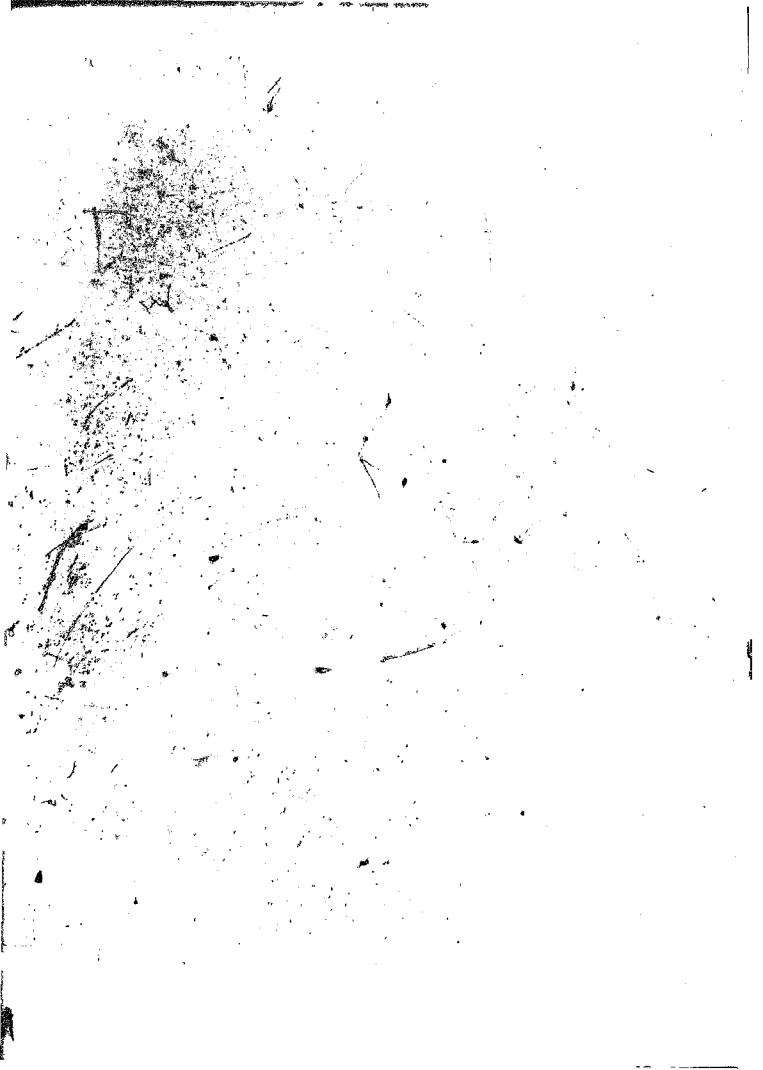
To determine the slope b the following formula was used:

$$b = \frac{(x - \bar{x})\ (y - \bar{y})}{(x - \bar{x})^2}$$

$$b = 0{,}019$$

To determine average 14887/1 chromosomal fragment size the following formula was used:

$$y \text{ (fragment size)} = \bar{y} + b\ (x - \bar{x})$$



Author Golob Harald Karl-heinz
Name of thesis Fusidic Acid Inactivation In Nocardioform Bacteria, Identification Of The Gene/s Involved In The Inactivation Mechanism. 1989

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