Cloning and characterization of genes involved in bioremediation from nocardioform bacteria

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ABSTRACT

The alarming incidence of pollution arising from technological advancement has attracted much concern worldwide. Bioremediation has been considered promising to offer cost-effective, pollution-free and economically feasible means of removing pollutants from the environment. Within this framework, it was desired to clone and characterize genes responsible for two bioremediations, viz cesium accumulation and benzoate degradation. Genomic library of *Rhodococcus erythropolis* CS98 was constructed, using shuttle vector pDA71, in *Escherichia coli* strain MM294-4 and the pool of recombinant plasmids of this library transformed into *Rhodococcus* strain SQ1. The transformants were screened for acquired ability to utilize benzoate as sole carbon source. Various genomic libraries were also screened for tolerance to high concentrations of NaCl. Three different gene inserts of sizes 2.8kb, 3.7kb and 31.8kb were identified from mutants with acquired ability to degrade benzoate. Meanwhile, genomic library of CS98 in strain SQ1 had clones that could confer osmotolerance phenotypes. CS98 therefore, bears genes responsible for benzoate degradation and osmotolerance. Genomic libraries of *Nocardia brasiliensis*, *Mycobacterium avium*, *Rhodococcus australis* A448 and *Rhodococcus rhodochrous* ATCC 12674 all had clones that could confer osmotolerance phenotypes.

DECLARATION

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

Signature of Candidate

3 day of March 1999.

DEDICATION

In memory of my father

Peter Kweku Lemaire

(1924 - 1996)

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May God richly bless you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 *INTRODUCTION*

1.1 Environmental pollution

Environmental protection is of much concern because of the alarming rate of pollution arising from technological advancement. Chemical pollutants of various kinds have been released into environments through various channels including accidents, explosions, weapons testing (Suzuki et al. 1973) and industrial effluents. Effluent from industries includes an incredible range of recalcitrant substances. Fatal diseases have been identified to be associated with these pollutants. An example is the serious health hazards associated with widespread pollution of water and soil environments by the radioisotope of cesium, ^{137}Cs. This has attracted diverse research activities into seeking efficient pollutant-remediation methods.

Technological advancement has been associated with increased use of chemical agents to enhance productivity. This has resulted in the release of large quantities of chemicals, both organic and inorganic into life-supporting environments contaminating them. An example is the use of pesticides, fungicides, fertilizers etc. to enhance agricultural productivity. These and other organic agents increase both the biological and chemical oxygen demand of the environment. The by-product and wastes from many industries become toxic because the chemical constituents are released in proportions above tolerable limits and are not easily accessible to the elemental cycles of nature such as the carbon, nitrogen, phosphorus and sulphur cycles. Because most chemical wastes are readily soluble and disposable in water effluents from industries are always contaminated. Most effluents are discharged into water sources such as rivers, streams, ponds and marine habitats and could contaminate sources of

potable water.

Pollution arising from accidents has contributed to large amounts of pollutants in the environment. An example is the release of large amounts of radioactive nuclides into the environment as a result of the Chernobyl accident (Devell et al, 1986). These included ^{137}Cs, ^{90}Sr, ^{144}Ce, ^{242}Cr and ^{244}Cr that had serious adverse environmental and health implications. The high incidence of cancer that the world of science is battling against now cannot be overemphasised. Similarly, accidents in petrochemical industries have polluted marine and other aquatic environments with benzene and other hazardous hydrocarbons (Prince, 1993; Swannell et al, 1996; Jones, 1998).

Industrial activities including the use of wood for furniture and other commercial products as well as the disposal of many carbon materials through burning also release large amounts of CO_2 and CO into the environment. Aromatic compounds and their derivatives including phenolic quinones, cresol, catechol, lignin, flavonoids, xanthonoes and other groups (Harborne, 1964) have also been released into the environment through industrial activities. Though some of the natural phenolics eg. 2-hydroxyphenyl acetic acid is easy to degrade (Harborne, 1964) varieties of the synthetic ones are very difficult to degrade. What makes many of the hydrocarbons recalcitrant (difficult to degrade) is the presence of chloro-, nitro-, bromo-substituents. The oxygenation steps involved in their degradation are retarded by the presence of these electron-withdrawing substituents (Dorn and Knackmuss, 1978). These recalcitrant compounds are typical of pesticides and petrochemical industrial effluents (Lapinskas, 1989). Polychlorinated biphenyls (PCB's) are synthesised and widely used for making industrial materials because of their high resistance to heat, good insulating properties and high level of

chemical stability, and have now accumulated in the environment (Masai et al, 1995). Aromatic compounds also get into soil and water through the decay of living organisms (Gottschalk, 1979). The majority originates from industrial processes like wood-processing, metal furnishing, petroleum refining, leather tanning making and finishing, ink formulation, manufacturing of automobile parts (Lapinskas, 1989) and the production of pharmaceuticals. These compounds therefore become incorporated into waste-waters, ground and surface waters and can be very detrimental to living organisms.

Heavy metals are toxic to life when they exceed tolerable limits (Hughes and Poole, 1989). When these heavy metals with unknown biological functions compete with or replace metals with known biological functions toxicity results. Metal toxicity to man is an increasingly important environmental concern. Analysis (Nriagu and Pacyna, 1988) shows that the annual toxicity of metals mobilized by human activities exceeds the combined total toxicity of all radioactive and organic wastes generated each year. Perhaps the most difficult environmental hazards are heavy metals since large amounts of energy and money is required to eliminate them from soil and water. Effluent of most metal refinery, mining and processing companies contain high levels of metals.

There is increasing awareness of pollution from industrialization and technological advancement that has forced many industries to be conscious about environmental control. Nevertheless, this cannot hinder advancement and it is therefore appropriate that much attention has rather been directed towards seeking remediation methods to keep pace with the rate of hazardous products release from technological advancement.

1.2 ^{137}Cs and the environment

^{137}Cs is a major product of nuclear power plants (Holm et al, 1983). It has been released into soil and water environments through leakages or effluents from nuclear power plants contaminating the above mentioned life-supporting environments. Particularly, large amounts of ^{137}Cs were released after the nuclear reactor accident at Chernobyl (Devell et al, 1986). After the accident food crops were heavily contaminated with ^{137}Cs (Anspaugh et al, 1988). Contaminated soils were abandoned because they were no longer useful for agricultural purposes.

The consequences of ionizing radiations from ^{137}Cs and other radioisotopes included high incidences of fatal cancer, congenital abnormalities and adverse pregnancies (Goldman, 1987). It is because of these serious health implications that much attention has been focussed on the fate and removal of ^{137}Cs from polluted soil and water environments.

1.3 Bioremediation of pollutants

There have been many research works into developing efficient remediation methods. Though physicochemical methods of remediation such as thermal destruction, chemical oxidation chelating, solvent extraction and detergent extraction have been used quite extensively in the past (Lapinskas, 1989) they have not been economically feasible and also resulted in other environmental problems. Microorganisms play a major role in the breakdown and mineralization of pollutants (Van der Meer et al, 1992).

Bioremediation is the act of adding or improving the availability of materials (eg. nutrients, microorganisms or oxygen) to contaminated environments to cause an acceleration of natural biodegradative processes (Swannell et al, 1996). Bioremediation is considered as the most promising technology to offer cost-effective and pollution free environmental control (Prince, 1993). It certainly provides the potential for contaminant-specific treatment to reduce concentrations of individual or mixed contaminants (Jones, 1998) from the environment.

Bioremediation generally uses microbes (fungi, bacteria, yeast and algae), although higher plants are used in some applications (Bonaventura and Johnson, 1997). It exploits the genetic diversity and metabolic versatility of microorganisms for the transformation of contaminants into fewer harmful end-products. These end-products are then integrated into natural biogeochemical pathways or cycles (Liu and Suflita, 1993) such as the carbon, sulphur, phosphorus and nitrogen cycles. The metabolic versatility of microorganisms could be attributed to their flexible genetic machineries that endow them with diverse adaptability to changing conditions in their natural habitat. For instance, most marine microbes including the *Rhodococcus* species have adapted to using petroleum products for growth because of persistent spillages of such products into the marine environment (Swannell et al, 1996; Jones, 1998). Most often, the addition of other nutrients to the environment enhances their activity for bioremediation. For instance, bioremediation strategies based on the application of fertilizers have been shown to enhance the rates of bioremediation of oils in aerobic intertidal sediments such as sand and cobble (Swannell et al, 1996). Other potential successes of bioremediation include an increasing role in concentrating metals (biomining) and radioactive materials (e.g. ^{137}Cs considered in this work) to avoid toxicity or to recover metals for reuse (Bonaventura and Johnson, 1997).

Bioremediation options encompass diverse biochemical mechanisms that may lead to a target's mineralization, partial transformation, humification or altered redox state (e.g. for metallic elements). Because these various mechanisms produce alternative fates of the targeted pollutants, it is often necessary to use diverse evaluation criteria to qualify a successful bioremediation (Shannon and Unterman, 1993). Thus understanding the ecology, physiology, genetics and evolution of degradation microorganisms are critical for the successful consideration and implementation of bioremediation (Liu and Suflita, 1993). It is also necessary to know the biochemistry of how each element is channelled into the natural biogeochemical cycles.

Bioremediation has already been a beneficial addition or substitute to chemical and physical methods of managing wastes as well as environmental pollutants (Bonaventura and Johnson, 1997). Yet, it would be much more applicable if emerging techniques for the processes (Bonaventura and Johnson, 1997), including molecular biology approach, are employed to rationally improve the versatility of bioremediation. A purposeful enhancement of this natural process can aid in efficient pollutant degradation and waste-site clean-up operations globally.

1.4 Biodegradation

It is a process by which natural or acquired metabolic activities in microorganisms or plants are employed for breaking down the carbon or elemental skeleton of compounds. This process takes place in order to provide the organisms with support or nutrients for growth. Of much importance is the carbon source that has lead to the use of diverse organic compounds, both simple and complex, by microorganisms for nutrients. Of course, the desirable aspects of this

natural process are the transformation or elimination of recalcitrant compounds and the generation of industrially useful products. For instance, *Rhodococcus* species possess an enzymatic pathway that can remove covalently bound sulphur from dibenzothiophene (DBT) for their use without breaking the carbon bonds (Denome et al, 1994). DBT is a model compound for organic sulphur in fossil fuels (Gallagher et al, 1993). It is a representative of a broad range of sulphur heterocyclics found in petroleum products that are recalcitrant to desulfurization via a chemical process called hydrodesulfurization (HDS) (Gray et al, 1996). This biocatalytic principle is being exploited for desulfurization of petroleum fractions and getting rid of DBT. DBT desulfurization has been characterized in a newly isolated *Gordona* strain CYKS1 and its possibility of application for the biocatalytic desulfurization of diesel oils has been demonstrated by Rhee et al (1998). Also, hydrocarbon degrading microorganisms are being used for bioremediation of marine oil spills (Prince, 1993).

Recalcitrant substances are difficult to eliminate but with biodegradation they could be eliminated. The world is now changing from the use of polythene to biodegradable polymers as containers. Even chemical soaps that could be hazardous are gradually being replaced with biodegradable and environmentally friendly ones. An example is the LDC household cleansing agent produced by Golden-Neo-life Diamite (GNLD). Microbes can degrade organic chemicals and the pathway could be either aerobic or anaerobic. It may sometimes require an enhancement effect by nutrient supplements. For instance, oxygen and fertilizers are added to enhance aerobic marine oil spill bioremediation (Prince, 1993).

Whichever the process, the technique would be much more applicable if designed and scaled-up for bioremediation in-situ, in biofilters and in bioreactors. The design of mathematical models

could enhance large-scale applicability of the technology (Murphy et al, 1997). Biodegradation is thus a strong "weapon" of environmental biotechnology. It covers solid-phase and slurry-phase bioremediation for contaminated soils and site-specific bioreactor for contaminated groundwater (Omenn, 1992).

1.5 Bioremediation of organic compounds

Many microorganisms have been identified to utilize many organic compounds as their sole carbon source for growth and increase of their biomass. An exploitation of this natural mechanism provides a useful process in bioremediation of recalcitrant organic compounds. For instance, most molecules in crude oil and refined products are biodegradable and they will eventually leave the environment as they are consumed by microbes (Prince, 1993) in this way. These synthetic organic chemicals are much more resistant to biodegradation. However, recent advances in biotechnology allow the development of strains able to us even the nitro-, or chloro- substituted organic compounds as their sole carbon source (Spain, 1994). The ability to biodegrade recalcitrant organic substrates could be acquired by the microorganism through genetic engineering and manipulations.

As much as current basic research is being focussed on expanding the range of synthetic chemicals amenable to biodegradation, there is much effort at improving and incorporating these natural microbial mechanisms into development of appropriate bioreactors and models of scale-up to make the technology much more applicable in environmental biotechnology (Spain, 1994). In- situ bioremediation has already been demonstrated to be highly effective for petroleum hydrocarbons (alkanes, aromatics, polychlorophenols) and organophosphate

pesticides in soils as well as gasoline by-products (benzene, toluene, xylene) and chlorinated solvents (trichloroethylene) in ground water (Omenn, 1992 & 1993).

In all aromatic hydrocarbons the parent structure is constituted by the benzene ring, so the development of a microorganism system that could attack and degrade the parent structure would be of much importance in bioremediation. This was being sort for in this work as the potentials of the *Rhodococcus erythropolis* strain CS98 to utilize benzoate as the sole carbon source was exploited.

1.6 Benzoic acid and benzoate

The parent benzoic acid in its free state is widely distributed in nature. The gum benzoin, derived from *Styrax benzoin*, may contain as much as 20% benzoic acid in the free state or in states broken up by heat (Opgrande et al, 1992). Many other natural products including acaroid resins, scent glands of beaver, cranberries, prunes and oil of anise seed contains an appreciable amount of benzoic acid (Opgrande et al, 1992).

For scientific purposes and use as preservatives bulk of benzoate is manufactured from benzoic acid by treating it with caustic soda or soda ash. The resultant solution is treated to remove trace impurities and colour bodies and it is dried in steam heated driers (Opgrande et al, 1992). 25% of benzoate usually has a pH of 5-8 (Opgrande et al, 1992). Sodium or potassium benzoate is highly soluble in water and partially soluble in ethyl alcohol, glycerol and methane.

1.7 Uses of benzoate and its impact on environment

The diverse use of benzoate in industrial processes makes it available in life-supporting environments as a contaminant. Benzoate gets into the environment through industrial effluents and disposable materials that contains benzoate. Toxicity limits have been found to be 6mg/kg in humans and up to 2530mg/kg in other mammals (Radian Corporation, 1991). The lowest dose from DNA mutation data is 5mmol/L. Toxicity data reveals skin, eyes and mucous membrane irritation (Radian Corporation, 1991). This shows that above the limits stated benzoate could be an environmental hazard and should be remediated.

Benzoate is widely used as preservative in foods, sauces, fats, ciders, fruit juices etc. because of the effective antimicrobial activity and the low cost of the parent benzoic acid (Opgrande et al, 1992). Other industrial applications that build up benzoate in the environment include its use; as a mordant in calico printing; for curing tobacco; and in pharmaceuticals as antifungal agent, in plasticizers and perfumes (Radian Corporation, 1991). About 1.5% of sodium benzoate is added in automobile and any other radiator cooling systems like compressor, diesel and chiller. Steel blades and other corrosion prone steel products are wrapped in paper containing sodium benzoate to prevent corrosion. Sodium benzoate is added in strippable rubber latex coating for metals. It is also used in added ions water-based paints and added to cutting oils as well as machine oils to prevent corrosion (Radian Corporation, 1991). These several uses result in the pollution of the environment with benzoate. A potentially more economical way to remediate benzoate from industrial wastes and the environment is bioremediation.

1.8 Bioremediation of benzoate

Bioremediation in this perspective would be based on the use of benzoate as a carbon source for microorganisms. Both benzoic acid and benzoate have the parent benzene ring that could be used as carbon source for microorganisms. What could be the difference in their use as carbon source is the acidity of benzoic acid and the salt nature of benzoate which microorganisms would have to tolerate. Aerobic and anaerobic metabolism of benzoate and other aromatic compounds have been identified with some microorganism including *Alcaligenes eutrophus* (Ampe et al, 1997), *Bacillus licheniformis* (Mclnerney et al, 1999), *Pseudomonas* sp. (Parales et al, 1993), and *Acinetobacter* sp. (Collier et al, 1997). The genes responsible for benzoate degradation encode for characteristic proteins that are enzymes in the complex metabolic pathway of benzoate degradation. The major intermediates of both the anaerobic and *ortho* pathways include cis-muconate, β-ketoadipate, succinyl-CoA and acetyl-CoA. These intermediates distribute carbon through the central pathways including the tri-carboxylic acid (TCA) cycle, enabling energy generation and more-rapid feeding of the anabolic pathway enhancing cell growth (Ampe et al, 1997).

Rhodococcus erythropolis strain CS98 could efficiently use benzoate as the sole carbon source for growth. Benzoate is even the best carbon source for CS98 if it is to be used for ^{137}Cs bioremediation (Tomioka et al, 1998). CS98 therefore has the potential for being used in biofilters to bioremediate both benzoate and ^{137}Cs from industrial effluents and water. Unlike the anaerobic metabolic pathway in which the concerted action of many different microbial species is often required for the degradation of benzoate, CS98 could be expected to degrade benzoate all by itself in a pathway which has not been identified.

1.9 Bioremediation of metals

Many metals bind with various degrees of tenacity to the largely anionic outer surface layers of microbial cells. This is sometimes a prelude to their transport into cells and has been exploited biotechnologically to detoxify effluents and concentrate metals of value (Hughes and Poole, 1989). Microorganisms thus remediate metals from environment by either bioadsorption or bioaccumulation. Some of the metals are used as nutrients or support by microorganisms for increasing microbial biomass thereby eliminating them from the environment. Microbes could do this by developing electronic properties, metalloenzymes, metal-containing cofactors and biologically-relevant metal chelating and clustering compounds (Hughes and Poole, 1989). These are usually in response to prevailing or changing environmental conditions that the organisms are to adapt to. Their nutritional requirements and growth characteristics are usually amenable to genetic manipulations in response to external pressures (Hughes and Poole, 1989). The proteins involved in metal binding and accumulation are encoded by genes some of which have been identified in various microorganisms. (Pazirandeh et al, 1998)

Recent research in the area of heavy metal removal from wastewater and sediments has therefore focussed on the development of materials with increased affinity, capacity and selectivity for target metals (Gadd and White, 1993; Totura, 1996). The use of microorganisms to sequester, precipitate or alter the oxidation state of various heavy metals has been extensively studied (Macaskie, 1990; Gadd and White, 1993). Some microorganisms identified to have metal bioremediation properties include *Neurospora crassa* whose genes are expressed in *E. coli* for binding Cd^{2+} and Hg^{2+} (Pazirandeh et al, 1998), *Streptococcus aureus*, *Pseudomonas* species, *Bacillus subtilis* (Beveridge and Murray, 1976), *Saccharomyces*

cerevisiae (Murray and Kidby, 1975), *Aureobasidium pullulans*, *Thiobacillus ferroxidans* and *thioxidans* for bioleaching of minerals (Hughes and Poole, 1989). *Alcaligenes eutrophus* is applicable for nickel accumulation (Hughes and Poole, 1989). *Zoogloea ramigera, Klebsiella aerogenes* and *Pseudomonas* species make use of binding polymers for metal accumulation (Sterritt and Lester, 1980).

What is of much concern in this work is the bioaccumulation of ^{137}Cs from water and soil by *Rhodococcus erythropolis* strain CS98.

1.10 Bioremediation of ^{137}Cs

Bioremediation methods, which involve the exploitation of chemolithotrophic microorganisms for the removal and recovery of ^{137}Cs from water and soil, is most favoured because it could be made pollution-free and inexpensive (Hughes and Poole, 1989).

Many organisms with ^{137}Cs accumulating potentials have been identified such as mosses (Elstner et al, 1987), fungi (Haselwandter et al, 1988), mushrooms and cyanobacteria (Avery et al, 1991). Nocardioform bacteria, especially *Rhodococcus* species, have relatively higher ^{137}Cs accumulation potentials than those organisms mentioned above (Tomioka et al, 1992). Particularly, *Rhodococcus erythropolis* strain CS98 is able to accumulate between 380 to 690μmol/g dry weight in 24hrs with accumulation efficiency of 90% (Tomioka et al, 1992). CS98 could accumulate 8 to 25 times higher proportions of ^{137}Cs compared with the other microorganisms. When CS98 was used in a bioaccumulation system with a semipermeable membrane, it was able to accumulate more than 96% of all ^{137}Cs present in water sources

within 24hrs of incubation in a first-order reaction (Tomioka et al, 1998). This shows an enormous potential of CS98 and other *Rhodococcus* species for ^{137}Cs bioremediation from water and soil. Cloning and characterization of the genes responsible for ^{137}Cs accumulation would allow for the development of much more efficient and economically feasible bioremediation method for ^{137}Cs. Meanwhile, the type of carbon source available to CS98 has much influence on its bioremediation potentials (Tomioka et al, 1998).

1.11 Identified carbon sources for CS98

CS98 is able to utilize quite a diverse group of organic compounds as its sole carbon source. It is able to decompose and makes use of the carbon skeletons of adenine, tyrosine and urea for survival (Tomioka et al, 1992). It is able to utilize more than 0.1%(w/v) of inositol, mannitol, sorbitol, ethanol, sodium adipate, sodium benzoate, sodium citrate, sodium lactate, sodium malate, sodium pyruvate, sodium succinate, glycerol, sucrose, trehalose, and the hormone testosterone (Tomioka et al, 1992). Other carbon sources for CS98 include sodium acetate, ammonium acetate, sodium fumarate, sodium gluconate and saccharose (Tomioka et al, 1998). This shows a great potential of CS98 for the bioremediation of recalcitrant organic compounds.

This work is concerned with the utilization of benzoate by CS98 as its sole carbon source. This would highlight on a great potential of CS98 to be used for the removal of aromatic hydrocarbons from contaminated environment.

1.12 Effect of carbon source utilization on the efficiency of metal bioremediation by CS98

CS98 has different efficiency in metabolizing each of the above-mentioned carbon sources. The highest growth efficiency of CS98 was observed with sodium acetate (96.9mg dry weight/litre) and ammonium acetate (96.7mg dry weight/litre) and these apparently registered the highest ^{137}Cs bioremediation efficiency of 90.1% and 90.0% respectively (Tomioka et al, 1998). By increasing the concentration of the carbon source or the total cell count the efficiency of cesium accumulation also increased (Tomioka et al, 1998). It is therefore reasonable to say that metal bioremediation is energy dependant and therefore requires a high carbon metabolic rate to provide the required calories for driving the process. The growth and survival of CS98 are also crucial in its efficiency in bioremediation.

Other factors that affect growth and survival of microorganisms would definitely affect their performance in bioremediation. One of the factors worth considering in this work is the tolerance of high osmotic concentration (osmotolerance). With the increasing changes in osmotic concentration of soil and water environments resulting from industrial activities and technological advancement, microorganisms for bioremediation should be made to tolerate the harsh physical conditions prevailing in the environment in question. Bioremediation in natural habitats such as estuaries and marine environment would require osmotolerance.

1.13 Osmotolerance in microorganisms

Osmotolerance is the ability to withstand high osmotic concentrations. Osmotic stress could be as result of high concentrations of salts or sugars. The ability of microbial cells to adapt to changes in the osmotic strength of their immediate environment is of crucial importance to their survival (Jakowec et al, 1985) just as their use for bioremediation. The physical conditions of most industrial effluents may vary according to the level of the chemical constituents and the type of materials used for production. So there would be wide variations in pH, temperature and salt concentration etc. These wide variations in physical conditions of effluents would require that bio-treatment plants be always designed to be specific for any particular type of effluent. Due to the high cost that would be involved, it is in the right direction that genes responsible for resistance to such physical conditions be cloned and characterized. The availability of osmotolerance genes carried by broad-host-range plasmids is the first step in the transfer of these genes into other bacterial species (Jakowec et al, 1985). The expression of such genes in bioremediation microorganisms would endow them with the ability to resist any stress caused by those physical conditions. An advantage of osmotolerance in bioremediation organisms is their use for marine and estuary environment bioremediation. By transforming hydrocarbon degraders with recombinant plasmid bearing osmotolerance genes they could be used to bioremediate oil and petroleum products that spill into marine habitats. There is therefore increasing interest in the mechanisms of osmotolerance as well as potential applications in this field (Jakowec et al, 1985).

Bacteria have evolved a variety of adaptive mechanisms, one of which is the intracellular accumul[illegible] oprotective compounds (Le Rudulier et al, 1984). In this mechanism the

cell probably accumulates low-molecular weight nitrogenous compounds such as proline, to balance the osmotic stress of the cytosol with that of the environment (Measures, 1975). In a large variety of microorganisms adaptation to growth in water-stressed environments is associated with, and dependent upon intracellular accumulation of certain solutes such as K^+, L-proline and glycine betaine (Measures, 1975; Le Rudulier et al, 1984). Though mechanisms of osmotolerance are not well understood, they could be homeostatic cellular function trying to neutralize the deleterious effects of osmotic stress.

Genetic studies have led to the identification in enterobacteria of transport systems for each of these solutes and pathways for the synthesis of glycine from choline (Styrvold et al, 1986) all of which are actuated by osmotic stress. One important osmoregulatory locus so identified in both *E. coli* and *Salmonella* is *pro* U which encode an active transport system for L-proline (Csonka, 1982; Gowrishankar 1985) and transport of glycine betaine in *Salmonella typhimuruim* (Cairney et al, 1985). Both L-proline and glycine betaine are able, at submillimolar exogenous concentrations, to promote the growth of organisms of the family *Enterobacteriaceae* in high-osmolarity medium (Cairney et al, 1985). *pro* BA (*pro*-74) mutant alleles also have been cloned which confers proline overproduction and osmotolerance in both *E. coli* and *Klebsiella pneumoniae* (Jakowec et al, 1985). *pro*-74 codes for a mutation resulting in a mutated derivative of γ-glutamyl kinase (the first enzyme in proline biosynthetic pathway encoded by *pro* B) that is about two orders of magnitude less sensitive to feedback inhibition than is the wildtype enzyme (Le Rudulier et al, 1984). The overproduction of proline may therefore be due to either increase in the specific activity of γ-glutamyl kinase compared with the wildtype or lost of feedback sensitivity to proline on the rate limiting step enzyme γ-glutamyl kinase (Smith, 1985).

Osmotolerance is, however, not just species dependent (Blomberg et al, 1988) it could be cell dependent. For *Saccharomyces cerevisiae* osmotolerance was found to vary between cells within an exponentially growing pure culture. The wide variations in osmotolerance mechanisms suggest that cells develop the capacity through adaptive resistance or spontaneous mutations of certain gene loci to cause the overproduction of osmoprotective factors. For instance, increased levels of Sn-glycerol-3-phosphate dehydrogenase (GPDH) (EC1.1.1.8) in the glycerol biosynthetic pathway provides cells with a greater capacity for glycerol synthesis and osmotolerance (Blomberg et al, 1988). The fact that GPDH activity increase under osmotic stress suggests that both GPDH and glycerol may play a major role in osmoregulation in yeast cells (Blomberg et al, 1988).

Other factors may also be involved in the expression of the osmotolerance phenotype. The possibility of a double mutation in *pro* A and *pro* B genes has been suggested by Smith et al (1985). The expression of the osmotolerance phenotype is also related to copy number of the recombinant plasmid bearing the gene. For instance, enhancement of osmotolerance expression is seen only in those strains that have multiple copies of both the *pro* U^+ and *pro* V^+ genes from *E. coli* (Gowrishankar et al, 1986). The plasmid pBR322 failed to confer the osmotolerance phenotype but the broad-host-range plasmid vector pQSR49 derivatives pMJ101 and pMJ1 could confer over-expression of the osmotolerance phenotype (Jakowec et al, 1985). Analysis of copy number revealed that the pQSR49 constructs were present in the cell at a level 6 to 8 fold lower than those of the pBR322 recombinants (Jakowec et al, 1985). A lower copy number therefore favours the over-expression of the osmotolerance phenotype.

The disaccharide trehalose has also been described to play a major role in osmotolerance as mutation of the *tre* A gene locus drastically decreased the osmotolerance ability of the mutant in the liquid culture (Gaballa et al, 1997). The role *sig* B gene (which encode for sigma B factor) from *Listeria monocytogenes* in osmoregulation has also been emphasized by Becker et al (1998). A null mutation in the *sig* B gene led to substantial defects in the ability of *Listeria monocytogenes* to accumulate and use betaine and carnithine as osmoprotectants (Becker et al, 1998). This suggests that sigma B could be a pump or transport channel for betaine and carnithine into the cell.

By principle of adaptive resistance, osmotolerance could be induced under osmotic stress. For instance, 12 osmotic-stress-induced proteins (OSP's) were expressed in cyanobacteria, *Anabaena* sp. Strain L-31, when exposed to 350mM sucrose. The OSP's certainly play a role in osmotolerance in *Anabaena* sp. Strain L-31.

1.14 Nocardioforms

These are gram-positive bacteria which belong to the family *Actinomycetaceae.* They have fungi characteristics as they form branching filaments yet they are rod shaped, nonmotile, aerobic and chemoorganotrophic (Lechevalier and Lechevalier, 1981). They mostly live in the soil as saprophyte and are found predominantly in the rhizosphere where they can interact with plants (Cross et al, 1976). Though nocardioforms have complex classification, some pathogenic forms that live in the soil include *Nocardia asteroides, Nocardia caviae* and *Nocardia brasiliensis. Mycobacteria* are human pathogens and responsible for more deaths worldwide than any other single pathogen (Rook and Hernandez-Pando, 1996). Fresh water and marine

habitats usually contain the *Rhodococcus* and *streptomyces* (Goodfellow and Williams, 1983). Nocardioforms play an important role in the recycling of materials and organic materials in the soil (Prescott et al, 1990).

1.15 *Rhodococcus* species and their implications for Biotechnology

These are nocardioform bacteria, gram positive, aerobic and chemorganotrophic bacteria with G + C content of their DNA ranging from 64% to 69% (Goodfellow and Minnikin, 1977). Members of this genus have a type IV cell wall (Prescott et al, 1990) containing *meso*-diaminopimelic acid, arabinose and galactose (Goodfellow, 1977; Lechevalier, 1986). They have large amounts of mycolic acids and menaquinones (Goodfellow, 1986). Typically, CS98 strain was found to be nonmotile and strictly aerobic (Tomioka et al, 1992). Members of the genus *Rhodococcus* are widely distributed in nature and have been isolated from soil, freshwater, and marine habitats (Lechevalier, 1986). This genus accommodates 14 species and they are characterized by their ability to form hyphae that fragment into rods and cocci.

Rhodococcus species can utilize a variety of substances for growth and survive through a range of temperatures between 10°C and 40°C (Finnerty, 1992). They are extremely versatile in their metabolic capabilities because they possess unusually and widely varied enzymatic systems including monooxygenase and dioxygenase activities (Harayama et al, 1992). This has endowed them with the ability to degrade xenobiotic and naturally occurring recalcitrant compounds (Finnerty, 1992) and therefore thrive on a wide range of organic substrates. Of pharmacological interest is their ability to convert cholesterol into substances which are the precursors of steroid hormones or oral contraceptives (Ferreira et al, 1984). They are also

useful for environmental control because they possess the ability to efficiently degrade oil (Sorkhoh et al, 1990), pesticides (Ferguson and Korte, 1981), acrylamide (Arai et al, 1981), phenol (Haider et al, 1981), lignin-related compounds, surfactants and humic acid (MacDonald et al, 1981). The strain CS98 is able to decompose adenine, tyrosine, and urea. Such desirable functions including their ability to accumulate ^{137}Cs are believed to be controlled by the expression of genes that need to be characterized if their application in bioremediation is to be considered. Their abilities to thrive under certain osmotic conditions and temperature need to be exploited so that their use in environmental bioremediation could be achieved under such desired conditions.

1.16 Growth of CS98 and cesium accumulation

Rhodococcus species grow well in *Luria Bertani* (LB) medium supplemented with 1 to 2% glycine and LB-agar at 28°C and pH 7.0. Many carbon sources have been identified to support growth of these species but ammonium acetate and sodium acetate are the best carbon sources for CS98 (Tomioka et al, 1998). The menaquinone of CS98 is MK-8(H_2) (Tomioka et al, 1992).

In general, microbial uptake of metallic elements has been found to be required for metabolism or adsorption. It might be a requirement for survival in a given medium, hence most of the microorganisms that accumulate Cs require certain concentrations of Cs in order to achieve optimum growth conditions. The metabolic-dependent uptake of metals occurs via monovalent or divalent cation transport systems and is energy-dependent (Avery, 1995). For CS98, both cell yields and the specific growth rate are affected by inadequate levels of potassium (K^+),

rubidium (Rb^+), and (Cs). No growth was observed when there was none of the above cations in the medium (Tomioka et al, 1994). Meanwhile, beyond certain concentrations of Cs the organism begins to lose the Cs accumulated (Avery et al, 1992). This is considered to be due to the response to increased internal osmotic pressure. Hence higher concentrations of Cs inhibit growth. CS98 was found to be saturated when Cs concentration in the growth medium was 0.1mM and further Cs addition did not result in additional uptake (Tomioka et al, 1994). Moreover, isolated CS98 strain accumulate significant levels of Cs in the log phase of growth and released it in the late stationary phase. This feedback inhibition processes and all other mechanisms that affect Cs accumulation against a concentration gradient may be controlled by genes on the DNA of the organism.

With ammonium acetate and sodium acetate as carbon sources for CS98 growth rates of 96.7 and 96.9mg dry weight per litre register 90.0 and 90.1% removal of Cs respectively (Tomioka et al, 1998). The type of carbon source used for growing CS98 therefore determines the efficiency of Cs accumulation.

If the gene responsible for Cs accumulation could be exclusively engineered into a plasmid and the resultant recombinant plasmid cloned in a strain that do not impose such inhibition processes, then the over-expression of the desired gene would enable an efficient bioremediation of ^{137}Cs from soil and water.

1.17 Characterisation of cesium accumulation by K^+ accumulation mechanisms

Cesium accumulation by CS98 strain is competitively inhibited by K^+ in its growth medium (Tomioka et al, 1994). Moreover, *E. coli* with K^+ accumulation systems, which involves protein carriers encoded by *trkD* or *kup* genes, could accumulate high levels of Cs (Bossemeyer et al, 1989). This suggests that both metals are accumulated by similar or related mechanisms. Tomioka et al (1994) suggested that CS98 strain has at least two K^+ uptake systems, one constitutively expressed and concerned with Cs transport across cell membrane. Hence, genes responsible for Cs accumulation could be characterised by K^+ accumulation from the growth medium.

1.18 Mutagenesis

This is a mechanism for generating bacterial strains of desired characteristics. Random mutations in the DNA of bacteria could change the nature of biologically active proteins expressed in the organism, the metabolic potentials of the organism and ultimately alter their growth requirement. For instance, when genes responsible for K^+ accumulation are inactivated a mutant with increased K^+ requirement could be obtained.

N-methyl-N-nitro-N-nitrosoguanidine (NTG) and ethyl methanesulphonate (EMS) are both alkylation agents which react with nucleophilic ring nitrogen or oxygen of DNA and play a major role in the induction of gene mutations (Van de Vliet et al, 1989 and Suziki et al, 1997). N-akylation and O-akylation disrupt the normal hydrogen bonding patterns and induce miscoding upon DNA replication. NTG induces predominantly transitions of G:C to A:T

events (98%) (Van de Vliet et al, 1989). Such transitions are the predominant type of mutations detected in various in-vitro gene mutation assays (Pastink et al, 1989). While NTG methylates EMS ethylates nucleophilic ring atoms. These mutations induced by spontaneous deamination of target cytosines generating inefficiently repaired G:T mismatches or by enzyme-induced C->U deamination which occurs under conditions of reduced levels of S-adenosylmthionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) (Zingg et al, 1996). This indicates that methylation activators or enhancers could increase the efficiency of NTG mutagenesis.

1.19 Vector pDA71

This vector designed by Dabbs et al (1990) bears ampicillin resistance, chloramphenicol resistance, and *EcoR1* suicide genes which are expressed in a host organism under the control of λ promoter. It is able to replicate and allows for positive selection in both *E. coli* and *Rhodococcus erythropolis*. There are useful restriction sites for *Hind* III, *Bgl* II, *Pst* I, and *Sfu*I in the *EcoR1* gene into which various fragments with compatible ends (genes of interest) could be cloned. Self ligation without the insertion of any fragment leads to the expression of the suicide gene which eventually kills the bacterial cell. This results in positive selection of strains with insertions. Though false positive selection could result, it is a factor of concentration and could therefore be reduced by DNA concentration calibration.

1.20 Objectives

The aims of this research were therefore to clone and characte- : the genes responsible for ^{137}Cs accumulation and benzoate degradation from CS98. This involved the construction of a genomic library from DNA of CS98, creation of *Rhodococcus erythropolis* mutant strains with increased potassium requirements, transformation of mutants with recombinant plasmid (pDA71), selection of strains with restored low potassium requirement and characterization of desired genes. Various libraries were also to be screened for the expression of osmotolerance phenotypes.

2.0 *MATERIALS AND METHODS*

2.1 Bacterial species used in this work

Table 1. Nocardioform bacteria and *Escherichia coli* strains used in this work

Strain	Species	Characteristics	Origin
Ri8	*Rhodococcus erythropolis,* ATCC 12674	Highly transformable mutant of ATCC 12674, Rif-R	S. Andersen
SQ1	*Rhodococcus erythropolis*	Highly transformable derivative of ATCC 4277, Rif-R	S. Quan
CS98	*Rhodococcus erythropolis*	Rif-S	N. Tomioka
CS98	*Rhodococcus erythropolis*	Rif-R	This work
MM294-4	*Escherichia coli*	*endA1, hsdR17, gyrA*	E. Dabbs

Key: Rif-R means rifampicin resistant strain and Rif-S means rifampicin sensitive strain.

Table 2. Genomic libraries screened for the expression of osmotolerance phenotypes

Source of DNA	Host strain	Enzyme used	Source
Rhodococcus australis A448	SQ1	*Bgl*II	Neelan Laloo
Rhodococcus rhodochrous ATCC 12674	SQ1	*Sfu*I	Nassem Ahmed
Nocardia otitidiscaviarum IFM 0239	SQ1	*Bgl*II	Selwyn Quan
Nocardia brasiliensis IFM 0236	Ri8	*Bgl*II	Kelly Trenton
Nocardia brasiliensis IFM 0236	Ri8	*Sfu*I	Kelly Trenton
Mycobacterium avium IFM 0414	Ri8	*Pst*I	Melissa Chait

100μl of each library was diluted in 5ml LB and each medium was supplemented with 50μg/ml rifampicin, 20μg/ml chloramphenicol and 200μg/ml cycloheximide. Each medium was put on the wheel at 28°C to grow for 2-3 days.

2.2 Growth and maintenance of bacterial strains

Yeast extract, agar and tryptone for the preparation of growth medium were obtained from Oxoid Ltd., England. *E. coli* strain MM294-4 was used to follow plasmid behaviour in terms of restriction, ligation, and insertion of genes into plasmid. The growth media for this strain was Luria Bertani (LB) liquid medium and LB-Agar (LA) solid medium and incubation temperature was 37°C. For quick growth the LB medium was supplemented with 25μl/ml of glucose. *Rhodococcus* strains CS98, Ri8 and SQ1 were also grown in LB or LA medium at an incubation temperature of 28°C. For transformation purposes LB was supplemented with 1.8% sucrose and 1% to 3% glycine (LBSG). The growth medium was supplemented with 50μg/ml of rifampicin for strains known to be resistant to rifampicin and ≥ 100μg/ml of ampicillin for strains that are resistant to ampicillin, in order to avoid contamination. 200μg/ml of cycloheximide was added each time to avoid fungal contamination. All liquid media were incubated on a wheel at the required temperatures.

2.3 Mini-Plasmid DNA isolation from gram negative bacteria

In principle, bacterial cells were lysed with EDTA and SDS solutions while differential pH from 8.0 by NaOH to 4.8 by CH_3COOK, followed by centrifugation, was applied to precipitate chromosomes with the cell debris. Plasmid DNA in the supernatant was precipitated with isopropanol and then washed with 20X volume of ethanol.

Bacterial cells were grown in 5ml LB supplem nted with 100μg/ml ampicillin, at 37°C with aeration. 0.7ml of culture was harvested by pelleting 1min in microfuge and then resuspended

in 80μl solution 1. 160μl solution 2 was added, mixed gently and then left to stand on bench for 15mins at room temperature. 150μl solution 3 was added, mixed vigorously by shaking and then left on ice for 5mins. It was microfuged in cold for 5mins and the supernatant drawn into sterile eppendorf tubes. The supernatant was warmed. 220μl of isopropanol was added and mixed. It was left to stand on bench for 5mins at room temperature and DNA precipitated by centrifuging for 5mins at room temperature. DNA pellet was then washed with 150μl ethanol, microfuged 1min at room temperature and dried in Speedvac for 20mins. Plasmid DNA was resuspended in 4mM Tris-HCl buffer at pH 8.

2.4 Large-scale Plasmid DNA isolation from gram negative bacteria

Bacterial cells bearing plasmid were grown in 100ml of LB supplemented with 100μl/ml ampicillin, at 37°C with aeration. Culture was harvested by pelleting in centrifuge and then resuspended in 5ml solution 1 in JA20 tube. 10ml solution 2 was added, mixed gently and then left to stand on bench for 15mins. 7.5ml solution 3 was added and shaken vigorously for 15-20secs. The tube containing the mixture was placed on ice/water slurry for 5mins and then centrifuged in pre-chilled rotor at 15000rpm for 10mins. The supernatant was decanted into fresh sterile tubes and warmed at 37°C. 12ml isopropanol was added, mixed well and left to stand on bench for 5mins at room temperature. DNA was precipitated by centrifuging at 15000rpm for 10mins at room temperature. Pellets were washed with 2ml ethanol, re-centrifuged briefly at room temperature and then dried in Speedvac for 20mins. DNA was resuspended in 4ml TE buffer. 4.1g of cesium chloride was added and dissolved by gentle agitation. 400μl of a 1% solution of ethidium bromide was added and the refractive index of the final solution adjusted to 1.387-1.389 using a refractometer. Pasteur's pipette was used

to load final solution into Quickseal tube. The tube was then sealed, balanced and loaded into a vertical rotor. It was run at 45000rpm for 16hrs. The lower band was extracted (using a needle and hypodermic syringe) and transferred into an eppendorf tube. Ethidium bromide was removed by 3-4 extractions with 1/10 volume butanol and CsCl was removed by dilution with 2X volume sterile water. 2X the combined volume of ethanol was added and then microfuged for 20 min at 4°C. It was blotted with a paper towel to remove excess liquid and then dried by Speedvac for 20mins. Plasmid DNA was resuspended in 4mM Tris-HCl buffer at pH 8.

2.5 Genomic DNA isolation from gram negative bacteria

Bacterial culture was grown to stationary phase in 200ml of tryptone yeast glycine broth (TYG) on a shaker. Cells were collected by centrifugation in a Beckman JA-10 rotor at 6000rpm for 10mins. The cells were resuspended in 5ml of solution A with 5mg/ml freshly added lysozyme and then transferred to a centrifuge tube and incubated for 30mins. Cells were then pelleted at 8000rpm for 5mins in Beckman JA-20 rotor and resuspended in 4ml of TE to which a minute quantity of proteinase K had been added. 1/10 volume solution B was added and the tube was incubated for 30mins. The viscous solution was transferred to a 50-Ti tube and spun at 40000rpm for 30mins in a Beckman L5-50 ultracentrifuge. The supernatant was transferred into a clean centrifuge tube and 4.4g CsCl was added. The solution was mixed thoroughly by inverting the tube several times and then centrifuged at 15000rpm for 15mins. The liquid was decanted from under the scum. 400μl of 1% EtBr solution was added and the refractive index of the final solution adjusted between 1.391 and 1.392. The solution was transferred to a Beckman Quick-seal tube and spun in a VTi 65.2 vertical rotor in a Beckman

L5-55 ultracentrifuge at 45 000rpm for 20-22hrs. The genomic DNA (upper) band was extracted using needle and hypodermic syringe and transferred into an eppendorf tube. EtBr was removed by 3-4 extractions with 1/10 volume butanol and CsCl was removed by dilution with 2 volumes of TE. 2.5 volumes of ethanol were added and the DNA precipitated by centrifugation at room temperature for 20mins. Genomic DNA was then resuspended in sterile water.

2.6 Chromosomal DNA isolation from *Rhodococcus* species

Cells were grown in 1-2%glycine at 28°C for 2-3days to weaken the cell walls. Cells were harvested by centrifuging at 3000rpm for 10mins and then resuspended in 400μl in solution 1 containing 5mg/ml freshly added lysozyme. It was then incubated at 37°C for 2hrs with gentle agitation. 1/10 volume of 10% SDS supplemented with proteinase K was added and incubated at 55°C for 2hrs with occasional inversion. 1/3 volume of 5M NaCl and 1 volume of chloroform were added and incubated at room temperature for 60mins with frequent inversion. It was centrifuged at 4500rpm for 15mins and the aqueous phase transferred into a new tube using a blunt-ended pipette tip. DNA was precipitated by adding 1 volume of isopropanol and gently inverting the tube. DNA was transferred into a microfuge tube and rinsed with 70% ethanol. DNA was then dried in a speedvac for 20mins and resuspended in 4mM Tris-HCl buffer.

2.7 Nocardioform mini-plasmid isolation

1ml of bacteria culture was grown in 5ml Bijou bottles on a shaker at 30°C for about 1hr. 300μl of culture was diluted to 1ml with LB and grown on a shaker at 30°C for 2 days. 800μl of solution A containing freshly added lysozyme (5mg/ml) was added to 200-300μl of culture and incubated at 37°C with shaking for 1hr. Cells were pelleted by centrifuging for 1min and resuspended in 280μl TE. 40μl of solution B was added and left on bench at room temperature for 10mins. 40μl of chilled solution C was added, mixed by shaking vigorously and placed on ice/water slurry for 30mins. Precipitates were removed by microfuging at 4°C for 20mins and the supernatant decanted into sterile eppendorf. Phenol-chloroform extraction was done twice on the supernatant. The DNA was precipitated by adding 2.5 volumes ethanol and microfuging at 4°C for 20mins. DNA was then dried in Speedvac for 20mins and resuspended in 20μl of 4mM Tris-HCl.

2.8 Nocardiaform bulk plasmid preparation

Cells were grown in 300ml tryptone yeast glycine broth (TYG) to stationery phase on a rotary shaker at 28°C. Cells were harvested by centrifuging culture at 6000rpm for 10mins in a JA-10 rotor. The supernatant was decanted and pellets were blotted dry on a paper towel. Pellets were resuspended in 5ml solution A and transferred into Sorval tube. The suspension was incubated at 37°C for 30mins and cells harvested by centrifuging at 8000rpm for 5mins in JA-20 rotor. Supernatant was decanted and resuspended in 4ml TE containing freshly added proteinase K. 0.4ml solution B was added and incubated at 37°C for 30mins. The transparent and viscous solution was transferred into Ti tubes using a blue-tip. Solution was centrifuged

at 40000rpm for 30mins and the supernatant transferred into a clean Sorval tube. 4.4g CsCl was added and mixed well by inversions of the tube in order to dissolve. Solution was centrifuged in JA-20 rotor at 15000rpm for 15mins and the supernatant was carefully separated from the scum. 400μl of EtBr (10mg/ml) was added and the refractive index adjusted to between 1.391 and 1.392. The solution was transferred to a Beckman Quick-seal tube and spun in a VTi 65.2 vertical rotor in a Beckman L5-55 ultracentrifuge at 45 000rpm for 20-22hrs. The plasmid DNA (middle) band was extracted using needle and hypodermic syringe and transferred into an eppendorf tube. Ethidium bromide was removed by 3-4 extractions with 1/10 volume butanol and CsCl was removed by dilution with 2 volumes of TE. 2.5 volumes of ethanol were added and the DNA precipitated by centrifugation at room temperature for 20mins. DNA was then resuspended in 4mM Tris-HCl buffer.

2.9 Phenol-Chloroform extractions

This was done on DNA digested with restriction endonuclease in order to deactivated and extract restriction enzymes if DNA were to be transformed into another bacterial strain after the digestion. 80μl of TE-saturated Phenol was added to about 200μl DNA solution and then inverted several times to mix. It was microfuged at 4°C for 5mins to separate the organic and aqueous phases. The upper layer was removed and if necessary a further phenol step was done until there was no visible protein at the interface. The upper aqueous layer was then transferred into 80μl of chloroform and inverted several times to mix well. It was microfuged for 30secs at room temperature. The upper aqueous layer was removed into sterile eppendorf tubes and ions (NaCl) were added to the final concentration of 50mM. DNA was then divided into two tubes. 2X volume of ethanol was added, inverted to mix well and then microfuged

at 4°C for 15-20mins. DNA pellets were dried in the speed vacuum for 20mins and resuspended in 400μl of 4mM Tris buffer.

2.10 DNA manipulations

All restriction enzymes used including *Sfu*I and *Hind*III were obtained from either Boehringer Mannheim, New England BioLabs, Amersham or Promega and were used according to the manufacturer's recommendations. For double digestion an appropriate buffer in which both enzymes showed suitable activity was selected or failing that, the digestions were done sequentially. DNA digestions were done at 37°C. T4 DNA ligase was used for all ligations done at 16°C for 18hrs in a minimal volume (10-20μl).

2.11 DNA fragment size analysis

DNA fragment size analysis to identify restriction, ligation of fragments, and insertions was done by agarose gel electrophoresis with agarose concentrations ranging form 0.4% to 1.2%. Stock solutions of agarose were usually prepared in 0.5X TBE buffer and sterilized by autoclaving. DNA fragments were rendered visible by ethidium bromide intercalation of the DNA fragments and UV detection photographed by Polaroid camera. Size of unknown fragments were determined by running standard molecular weight markers, λII on 0.4% to 0.8% agarose gels and λIII on 1.2% agarose gel along side the unknown fragments. A standard curve of molecular weights verses distance moved on gel from the well was plotted on a semi-log paper and size of unknown fragments were estimated by extrapolations on the curve to the molecular weight's axis.

2.12 Gram-negative bacterial transformations

$CaCl_2$ buffer and heat shock at 42°C was adopted to transform *E. coli* strain MM294-4 with the recombinant plasmid. 200μl of an overnight culture was added to 20ml of fresh LB supplemented with 0.5% glucose and incubated at 37°C with aeration till OD_{590} of 0.2-0.4 was reached. The culture was chilled to 0°C as quickly as possible in ice or ice slurry and cells pelleted in precooled JA20 rotor at 10000rpm for 5mins. Cells were resuspended in a half volume of $CaCl_2$ buffer using a pipette and then left on ice for ≥ 15mins. Cells were pelleted again and resuspended in 1/15 volume of $CaCl_2$ buffer using P1000 Gilson. The suspension was left on ice for 2-24hrs and then aliquot into pre-chilled Eppendorf tubes. Suspension of ligated DNA was added to cells, aerated to mix and left on ice for 10-30mins before for heat-shocked at 42°C for 90secs in a water bath. 1ml of LB was added and then incubated at 37°C for 60-90min to allow phenotypic expression of resistance genes. Cells were poured on selection plates (dried after 48hrs at 37°C) and incubated at 37°C for 14-22hrs.

2.13 Gram-positive bacterial transformations

Cells were grown in LBSG and refrigerated when dense. Aliquots of 1-2ml each in 4 Eppendorf tubes were spined down for 30secs to pellet cells. Cells were then washed with B-buffer and pelleted. 1ml of lysozyme in B-buffer was added to each tube and sucked up and down to resuspend cells. Tubes were placed in 37°C water-bath for 45mins with inversion every 10mins. Cells were pelleted and carefully resuspended in 1ml B-buffer, spined down and supernatant decanted. 600μl of P-buffer was added to each tube content and then aliquots of 200μl were measured into 12 Eppendorf tubes. 20μl of DNA from library was added to each

of 11 tubes leaving the last tube without any DNA. Air was bubbled through to mix and left for 15-20 mins. 220μl of P-PEG to each tube and air was blown through it until the two phases blended into one. Taking 200μl at time cell mixture was dropped onto rifampicin regeneration plates and spread all over plate. Plates were then incubated at 28°C for 8-10hrs after which chloramphenicol underlay was done to each plate and then incubated at 28°C for 5days.

2.14 Mutagenesis

Mutagenesis of *Rhodococcus* strain was done using N-methyl-N-nitro-N-nitrosoguanidine (NTG) as the mutagenic agent and boric acid / borax buffer at pH 8.2. Cells were washed with buffer and then resuspended in 1ml NTG solution. Cells were incubated at 37°C in water-bath for 60-90 mins. Cells were washed twice with buffer and then resuspended in LB containing 0.5%Tween and 50μg/ml rifampicin. They were incubated on a wheel at 28°C for two days, for cells to recover from mutagenesis. Serial dilutions of 10^{-4}-10^{-7} were made and 100μl of each was spread on LA-rifampicin plates. After two days of incubation at 28°C single colonies were transferred unto normal medium and replicated on minimum medium plates in order to check for possible auxotrophs. Ethyl methanesulphonate (EMS) mutagenesis, at a concentration 2%, also followed the same procedure.

Successive mutagenesis, using NTG, was also performed on CS98 and Ri8 strains for 4 rounds. After each round of mutagenesis mutants were plated as described above to check for possible auxotrophs.

Various variable conditions were altered in the above method to check for any effect on the efficiency of mutagenesis. These include the addition of NaCl (based on the finding by Wada et al, 1998 that the effect of NTG in cancer formation in rats was enhanced by NaCl), varying the duration of NTG treatment, making pre-culture in 1-2% LBSG and using pre-culture grown to stationary phase.

Another assay of NTG mutagenesis tested was by growing bacterial strains in 5ml LB supplemented with 50μl of 1M KCl and 100-500μl of NTG solution. Mutants were plated to check for auxotrophs at each stage.

2.15 Osmotolerance

Pre-culture of 6 previously constructed libraries in *Rhodococcus* species were made and 100μl each was spread on plates with increasing NaCl concentration from 100-1000mM. Plates were incubated at 28°C for two days. Growth medium was supplemented with 50μg/ml rifampicin, 100μg/ml ampicillin and 200μg/ml cycloheximide.

2.16 Carbon source testing

Minimum medium plates were made from stock III and 0.1% of the following carbon sources: sodium benzoate, sodium acetate, sodium citrate, sodium deoxycholate and m-cresol. 100μl of pre-cultures of *Rhodococcus* strains SQ1, Ri8 and CS98 were spread onto for each carbon source and then incubated at 28°C for 2-3 days. Carbon sources which supported growth of CS98 but not SQ1 were then used to select their CS98 genomic libraries.

20μl plasmid from CS98 library was transformed into SQ1 strain and twice the clone of CS98 library in MM294-4 was cloned in SQ1. The library in SQ1 was then pooled with 1-2ml LB per plate. The pool was supplemented with 200μg/ml cycloheximide, 50μg/ml rifampicin and 20μg/ml chloramphenicol in order to avoid contamination. Serial dilutions of 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} of the library were made and 100μl of each was spread on selection plates containing 0.1% sodium benzoate.

2.17 Data analysis

1. Simple total count was used to estimate the total number of clones.
2. Arithmetic mean was used to estimate the average insertion size (a).
3. The number of clones (N) required for the construction of the genomic library would then be estimated from the relation;

$$N = \frac{\ln (1 - P)}{\ln (1 - a/b)}$$

where 'b' is the genome size and P is the probability of gene insertion (Brown, 1995).

3.0 *RESULTS*

3.1 Construction of a genomic library of CS98 in *E. coli* strain MM294-4

The genomic library was to be constructed in order to clone genes responsible for cesium accumulation and benzoate degradation. The main idea behind the selection of a particular restriction enzyme was to avoid an enzyme that cuts the DNA too frequently including the gene of interest. Hence an enzyme that cuts the DNA giving fragments of >1-10kb was desired. The enzymes tested included *Sful*, *Pst*I, *Hind*III and *Bam*HI. *Sful*, *Pst*I, and *Hind*III have restriction sites in the *EcoRI* gene of pDA71. Their digestion of the plasmid leaves cohesive ends and allows DNA fragments to insert into the *EcoRI* gene inactivating the suicide gene. This allows for positive selection of colonies bearing the recombinant plasmid. *Bam*HI was also tested because it leaves similar cohesive ends just as *Bgl*II when they digest DNA fragments.

From Fig 1, *Sful* exhibited reasonable digestion of CS98 genome among the restriction enzymes used. *Pst*I and *Bam*HI cut the genome too many. *Sful* was therefore the best enzyme for the construction of the genomic library of CS98. *Hind*III also showed a little restriction of the genome but the average size was too big to allow a representation of as many genes of CS98 in pDA71.

There was the possibility of obtaining false positive clones on the selection plate because nuclease degradation could also prevent a functional *EcoRI* suicide gene from being regenerated after ligation. This situation increases with concentration of the plasmid hence, a

plasmid concentration that would give minimum number of false positive clones was estimated through calibrations. 4μl of plasmid solution was the best concentration to give minimum number of false positive clones per plate.

The plasmid:genomic DNA ratio was also optimized in order to avoid to many background colonies, yet obtaining maximum number of transformants. From the ligation of 1μl, 2μl, 4μl, 9μl and 12μl of genomic DNA to 4μl of plasmid, 9μl of genomic DNA satisfied the conditions desired. Hence, 4μl plasmid DNA:9μl genomic DNA was the best ratio for obtaining a good genomic library of CS98.

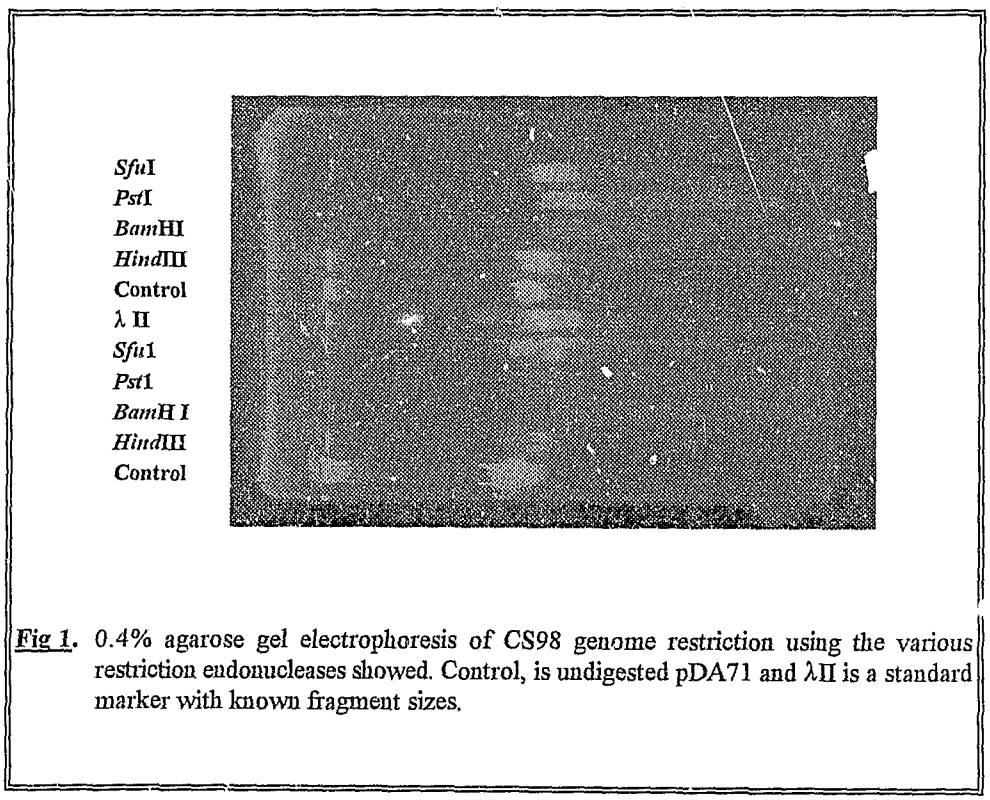


Fig 1. 0.4% agarose gel electrophoresis of CS98 genome restriction using the various restriction endonucleases showed. Control, is undigested pDA71 and λII is a standard marker with known fragment sizes.

Once the optimum ratio of plasmid DNA to genomic DNA was obtained successive ligations were performed. Plasmids were isolated from the single colonies, digested with *Sfu*I and run on 0.8% agarose gel to check for possible gene inserts.

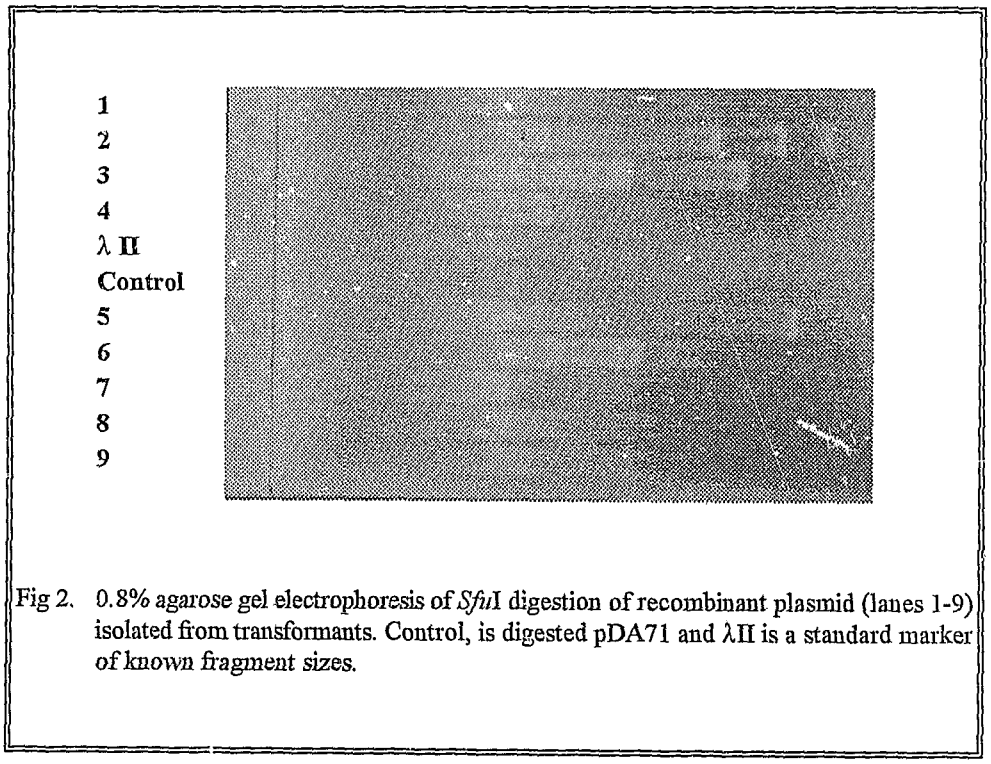


Fig 2. 0.8% agarose gel electrophoresis of *Sfu*I digestion of recombinant plasmid (lanes 1-9) isolated from transformants. Control, is digested pDA71 and λII is a standard marker of known fragment sizes.

From fig. 2, inserts were present in lanes 2, 3, 4, 5, and 6. This shows a 50% chance of obtaining colonies with inserts from each selection plate. Multiple fragments were obtained in lanes 2, 3 and 6 which shows that there was partial digestion of the bulk genomic DNA before the construction of the library.

Table 3. Estimation of fragment sizes of DNA by their distances moved on the gel with reference to that moved by standard λII marker fragments of known sizes.

Sample No.	Distance moved on gel (mm)	Fragment Size (kb)	Total Insert size (kb)
λII marker	31.5, 34, 36.5, 41, 50, 52.5	23.1, 9.4, 6.6, 4.4, 2.3, 2.0	-
1	33	9.4	0
2	33, 37, 60.5, 69, 76, 82	9.4, 5.8, 1.4, 1.0, 0.8, 0.7	9.7
3	33, 44, 48.5, 64	9.4, 3.4, 2.6, 0.8	6.8
4	33, 38	9.4, 5.4	5.4
5	33, 71.5	9.4, 0.9	0.9
6	33, 39.5, 48, 50.5	9.4, 4.8, 2.7, 2.2	9.7
7	33	9.4	0
8	33	9.4	0
9	undigested DNA	-	-
		average insert size =	3.3

Key: kb means kilobase

Fragment sizes from 0.9kb to 9.7kb inserted into the *SfuI* site of pDA71. This was what was desired as described earlier on for the choice of the enzyme. Nevertheless, a complete digestion would have yielded fragment sizes from 0.7kb to 5.8kb as possible inserts into the cloning site of the plasmid.

In order to obtain a good estimation of the average insert size two more transformations of recombinant plasmid into MM294-4 were done and the insert sizes determined as described above.

A *Hind*III digest of one set of colonies yielded inserts cut off the linearized plasmid. This could be seen in fig. 3 below, in lanes 4, 5 and 10. This indicates that there was a *Hind*III restriction site in the inserts of sample 4, 5 and 10.

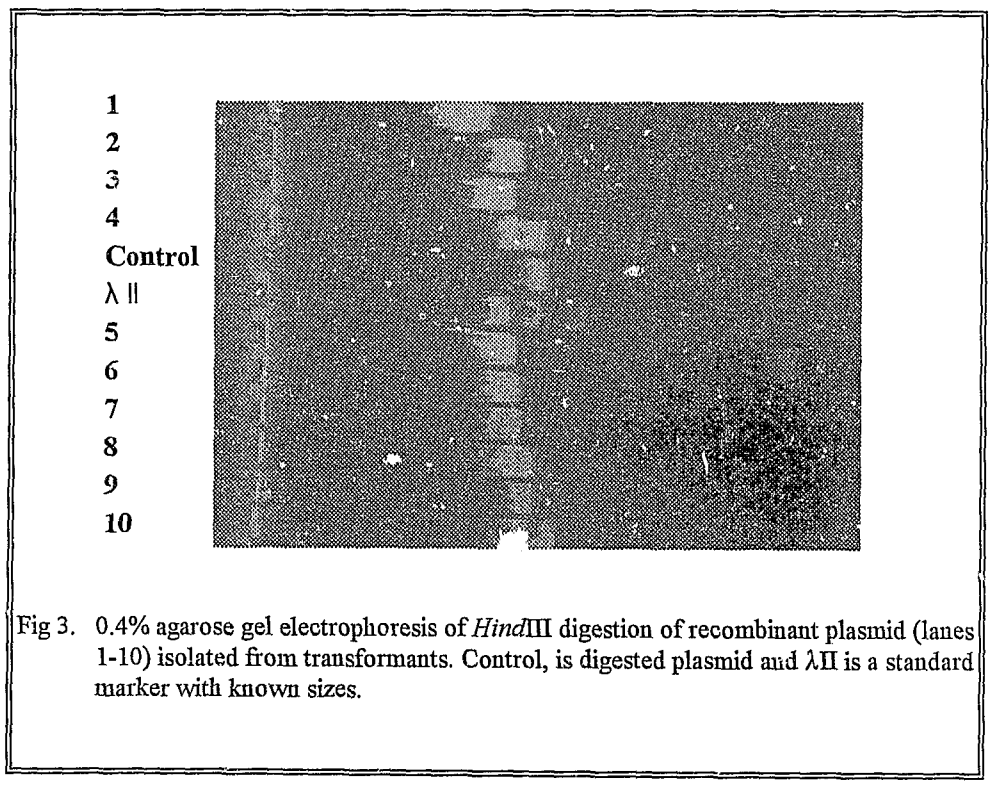


Fig 3. 0.4% agarose gel electrophoresis of *Hind*III digestion of recombinant plasmid (lanes 1-10) isolated from transformants. Control, is digested plasmid and λII is a standard marker with known sizes.

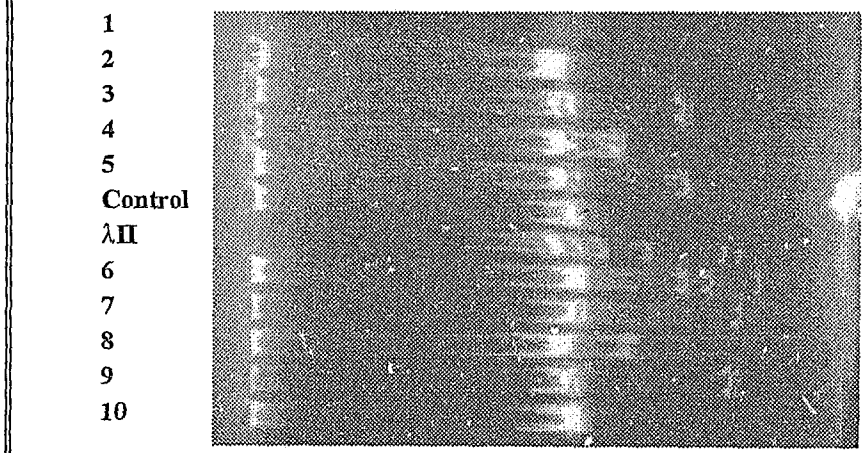


Fig 4. 0.8% agarose gel electrophoresis of *sfu*I digestion of recombinant plasmid (lanes 1-10) isolated from transformants. Control, is digested plasmid and λII is a standard marker with known sizes.

From fig. 4, multiple fragments also occurred in lane 6 just as obtained earlier on in lanes 2, 3 and 6 in fig. 2. This may have also resulted from partial digestion of CS98 genome before its fragments were ligated into the multiple cloning site of pDA71. Hence, larger fragment sizes up to 12.5kb could be inserted at the *sfu*I site on pDA71. The smallest insert size obtained so far from the three determinations was 0.5kb (results not shown). The average insert size for three transformations was calculated to be 3.2kb.

For a 95% probability of cloning any gene of CS98 into pDA71, the total number of transformed colonies (N) required was calculated as follows;

$$N = \frac{\ln (1 - 0.95)}{\ln (1 - 3.2/5000)}$$

where the genome size of CS98 is 5000kb and the average insert size is 3.2kb.

$$N = 4650$$

9120 colonies of transformants were obtained from successive transformations of MM294-4 with the recombinant plasmid. This represents a 99.72% chance of cloning any gene of CS98 at the *sfu*I restriction site on pDA71. An average of 203 colonies was obtained per plate which indicates a good transformation efficiency. 95% probability of cloning a gene into the plasmid would have been enough for the construction of the genomic library, and at this probability 9120 transformants would have given approximately 2X representation of any gene of CS98 in the genomic library pool.

The pooled transformants of CS98 library were tested for resistance to erythromycin, rifampicin, tetracycline and chloramphenicol. There were transformants resistant to erythromycin, rifampicin and chloramphenicol but not tetracycline. When plasmid isolated from colonies of each plate was re-transformed into MM294-4, only the chloramphenicol resistance phenotype was expressed.

3.2 Mutagenesis

It was desired to generate mutants of strains SQ1 or Ri8 or CS98 with increased K^+ requirements to serve as appropriate host for screening genes responsible for cesium accumulation. NTG was the mutagen of choice but EMS was also tested. There was the need to establish the optimum duration for subjecting cells to NTG treatment so that a maximum efficiency of mutagenesis could be obtained with NTG.

Table 4. Mutagenesis on strain SQ1 for establishing the optimum duration for NTG treatment.

Duration of NTG treatment (mins)	15	30	60	90	120	150
Number of colonies	110	110	110	110	110	110
Number of auxotrophs	0	1	2	1	0	0
Recovery period (hrs)	24	24	48	72	98	122
% auxotrophs	0	0.9	1.8	0.9	0	0

From the optimization test in Table 4, 0.9% auxotroph was obtained at 30mins and 90mins of treating cells with NTG solution while 1.8% auxotrophs were obtained for 60mins of treating cells with NTG solution. The best duration for treating cells with NTG solution, at that arbitrary concentration, was therefore 60mins. The recovery period of cells from NTG mutagenesis increased with time.

Table 5. Ethyl methanesulfonate (EMS) mutagenesis on strain SQ1

Duration of NTG treatment (mins)	60	90
Number of colonies	110	110
Number of auxotrophs	0	0
Recovery period(hrs)	24	24

From Table 5, no auxotroph was obtained. EMS, at 2% concentration appeared to have no mutagenic effect on strain SQ1 because cells recovered from mutagenesis at their normal growth rate. A higher concentration may have yielded auxotrophs but much focus was on NTG mutagenesis.

Table 6. NTG mutagenesis on strain SQ1 in the presence of 0.1M NaCl

Duration of NTG treatment (mins)	60	90
Number of colonies	110	110
Number of auxotrophs	0	1
Recovery period(hrs)	24	48
% auxotrophs	0	0.9

The addition of 0.1M NaCl to NTG solution was based on the finding by Wada et al (1998) that the effect of NTG in cancer formation in rats was enhanced by NaCl. Mutagenesis was best at 90mins of treating cells with NTG solution at that concentration. Nevertheless, 0.1M NaCl did not enhance the efficiency of NTG mutagenesis on SQ1.

It could be that SQ1 was much more resistant to NTG treatment so strains Ri8 and CS98 were also tested to see if efficiency of mutagenesis could increase. Simultaneously, the optimum duration for NTG treatment was tested for both strains in case it could be different. Also, a new minimal medium was tested to check how it would affect the total number of auxotrophs obtained. This medium designated minimal medium-Rif was made from Na_2HPO_4/NaH_2PO_4 buffer instead of the usual K_2HPO_4/KH_2PO_4 buffer. Rifampicin was added to the new minimal

medium just to control contamination, but the results obtained (Table 7) generated an interest in the effect of rifampicin in the minimal medium on the number of auxotrophs obtained.

The effect of NaCl on the efficiency of NTG mutagenesis was again tested, but this time with 0.5M NaCl. Strains treated with NTG + NaCl were labelled CS98c and Ri8c. This was an attempt to investigate the possibility of an activator or enhancer to NTG mutagenesis.

Table 7. NTG mutagenesis on strains CS98 and Ri8

	CS98	CS98	CS98c	Ri8	Ri8	Ri8c
Duration of NTG treatment (mins)	60	90	90	60	90	90
Number of colonies	110	110	110	110	110	110
Number of auxotrophs on minimal medium-Rif	0	34	25	0	10	11
% auxotrophs	0	30.9	22.7	0	9.1	10.0
Number of auxotrophs on minimal medium	0	8	-	0	5	-
% auxotrophs	0	7.3	-	0	4.5	-

From Table 7, there appeared to be a major difference between the two media, in terms of number of auxotrophs obtained. A greater proportion of auxotrophs was obtained with minimal medium-Rif (9.1-30.9%) than with minimal medium (4.5-7.3%). The difference could also be due to the presence of rifampicin in the minimal medium-Rif.

CS98 gave more auxotrophs than Ri8. Meanwhile, 0.5M NaCl did not have any enhancement effect on the efficiency of NTG mutagenesis as there was no significant improvement in the proportion of auxotrophs obtained(10.0% and 22.7%) compared with that without NaCl (9.1% and 30.9%). The proportion of auxotrophs was even lower for NTG + NaCl treatment when comparing results from CS98c (22.7%) with that of CS98 (30.9%).

The stability of NTG mutations in the strains was found to be very low because, all the auxotrophs regained their ability to grow on the minimum medium, and even grew to full capacity, when the plates were left in the cold at 4°C for 40 days. Auxotrophs needed to be stable with their newly acquired genetic characteristics so that they could be reliably used as host for screening desired genes.

Further attempt at improving the efficiency of mutagenesis involved the use of LBSG to compromise the integrity of the cell wall; in case the mutagen was not able to penetrate the cell envelope of *Rhodococcus* species. The presence glycine in LBSG was to weaken the cell wall under high osmotic stress provided by 10.3% sucrose. Moreover, cells at stationary phase of growth were also treated with NTG to check if the dormancy period of growth could yield stable mutagenesis. NTG-treated cells from this stage were designated M1 and were used for successive NTG treatments, M2, M3 and M4. This was done to investigate if the process of

NTG mutagenesis could be continued from previous treatments.

Table 8. NTG mutagenesis on strains CS98 and Ri8 for checking the effect of LBSG and stationery phase on the efficiency of mutagenesis.

Sample	Number of colonies on LA medium	Number of auxotrophs on minimal medium	% auxotrophs
CS98 in 1% LBSG	110	0	0
CS98 in 2% LBSG	110	1	0.9
CS98 in LB	110	2	1.8
CS98 at stationary phase	110	0	0
Ri8 in 1% LBSG	110	1	0.9
Ri8 in 2% LBSG	110	1	0.9
Ri8 in LB	110	0	0
Ri8 in stationary phase	110	0	0

From Table 8, 1.8% auxotrophs were obtained for cells grown in LB and 0.9% auxotroph was obtained for cells grown in both 1% and 2% LBSG. The output shows that there was no effect of LBSG and stationary phase on the efficiency of mutagenesis.

From Table 9, successive NTG mutagenesis on both Ri8 and CS98 showed an increase in %auxotrophs from 0% to 4.5% at M2 and then a decrease through 1.8% at M3 to 0% again at M4. However, this was only achieved with cells from stationary phase of growth. Cells treated with NTG at the early exponential growth phase did not yield any auxotroph. Cells for M3 were taken from stationary phase of M2, and for M4 were taken from stationary phase of M3.

It was also of much interest to justify the effect of rifampicin on the % auxotrophs obtained, so another minimal medium was prepared just as minimal medium-Rif but rifampicin (Rif) was not added. This was designated minimal medium-0K since it contained no K^+. When single colonies from CS98 M4 were replica plated on minimal medium-Rif and minimal medium-0K, and the results compared with that on LA plates, 36.4% auxotrophs were obtained for minimal medium-Rif while 10% auxotrophs were obtained for minimal medium-0K.

A calibration of NTG concentration was also done to establish which concentration could yield optimum mutagenesis. From Table 10, 1.8% auxotrophs were obtained at NTG concentrations of 100μg/ml and 1000μg/ml. The higher NTG concentrations did not yield any auxotroph.

Table 9. Successive NTG mutagenesis on strains CS98 and Ri8

Strain	Number of colonies on LA-Rif medium	Number of auxotrophs on minimal medium	% auxotrophs
CS98 M2	110	0	0
Ri8 M2	110	0	0
CS98 Stationary phase M2	110	5	4.5
Ri8 stationary phase M2	110	5	4.5
CS98 M3 (from stationary phase M2)	110	2	1.8
Ri8 M3 (from stationary phase M3)	110	2	1.8
CS98 M4 (from M3)	110	0	0
CS98 M4 (from M3)	110	0	0

Table 10. NTG concentration calibration on strain Ri8

concentration of NTG (μg/ml)	Number of colonies		% auxotrophs
	Normal medium	Minimal medium	
18000	110	0	0
15000	110	0	0
12000	110	0	0
10000	110	0	0
8000	110	0	0
5000	110	0	0
2000	110	0	0
1000	110	1	0.9
500	110	0	0
100	110	1	0.9

The auxotrophs obtained so far were then tested for their K^+ requirement by spot-testing single colonies on minimal medium plates containing increasing concentrations of KCl from 0μM to 1000μM (Tomioka et al, 1994). None of the auxotrophs showed increased K^+ requirement as they all grew well at all concentrations of KCl, even at 0μM KCl.

3.3 Osmotolerance

Since osmotolerance is important in environmental biotechnology, especially in marine environment bioremediation, most of the genomic libraries constructed from *Nocardia brasiliensis*, *Rhodococcus* species and *Mycobacterium avium* in Ri8 and SQ1 were screened for possible expression of osmotolerance phenotypes. Their ability to tolerate concentrations of NaCl from 100mM to 1000mM was investigated.

The results clearly showed the expression of osmotolerance phenotypes in all the genomic libraries screened. As shown in Tables 11a and 11b, the strongest expression of osmotolerance phenotypes (at 1000mM NaCl) was with *Nocardia brasiliensis* in Ri8 and *R. rhodochrous* ATCC 12674 in SQ1. Meanwhile, CS98 in *E. coli* strain MM294-4 could not tolerate > 400mM NaCl. Unlike the growth medium for the library strains, that for the pure strains (SQ1, Ri8, CS98 and *E. coli* MM294-4) used for the control experiment did not contain antibiotics. From the control experiments shown in Tables 12a and 12b, SQ1 exhibited low osmotolerance and could only tolerate up to 400mM NaCl. Strains Ri8 and CS98 exhibited high osmotolerance and could tolerate up to 700mM NaCl. *E. coli* strain MM294-4 exhibited an incredible level of osmotolerance and demonstrated the ability to tolerate > 1000mM NaCl as it showed good growth even at 1000mM NaCl.

Table 11a. NaCl concentration tolerance tests on genomic libraries from *Nocardia brasiliensis* IFM 0236, *Nocardia otitidiscaviarum* IFM 0239, *Rhodococcus australis* A448, *Rhodococcus rhodochrous* ATCC 12674 and *Mycobacterium avium* IFM 0414 constructed in Ri8 and SQ1.

Library	Enzyme for library	Concentration of NaCl (mM)				
		100	200	300	400	500
Nocardia brasiliensis IFM 0236 in Ri8	*Bgl*II	+++	+++	+++	+++	+++
Nocardia brasiliensis IFM 0236 in Ri8	*Sfu*I	+++	+++	+++	+++	+++
Nocardia otitidiscaviarum IFM 0239 in SQ1	*Bgl*II	+++	+++	+++	+++	+++
Mycobacterium avium IFM 0414 in Ri8	*Pst*I	+++	+++	+++	+++	+++
R. australis A448 in SQ1	*Bgl*II	+++	+++	+++	+++	+++
R. rhodochrous ATCC 12674 in SQ1	*Sfu*I	+++	+++	+++	+++	+++
R. erythropolis CS98 in SQ1	*Sfu*I	+++	+++	+++	+++	+++
CS98 in *E. coli* strain MM294-4	*Sfu*I	+++	+++	++	+	-

Key: +++, ++, + and - as used in the results represent strong growth, moderate growth, weak growth and No growth respectively.

Table 11b. NaCl concentration tolerance tests on genomic libraries from *Nocardia brasiliensis* IFM 0236, *Nocardia otitidiscaviarum* IFM 0239, *Rhodococcus australis* A448, *Rhodococcus rhodochrous* ATCC 12674 and *Mycobacterium avium* IFM 0414 constructed in Ri8 and SQ1.

Library	Enzyme for library	Concentration of NaCl (mM)				
		600	700	800	900	1000
Nocardia brasiliensis IFM 0236 in Ri8	*Bgl*II	+++	+++	++	+	-
Nocardia brasiliensis IFM 0236 in Ri8	*Sfu*I	+++	+++	+++	++	+
Nocardia otitidiscaviarum IFM 0239 in SQ1	*Bgl*II	+++	+++	+	-	-
Mycobacterium avium IFM 0414 in Ri8	*Pst*I	+++	+++	++	+	-
R. australis A448 in SQ1	*Bgl*II	+++	+++	++	+	-
R. rhodochrous ATCC 12674 in SQ1	*Sfu*I	+++	+++	+++	++	+
R. erythropolis CS98 in SQ1	*Sfu*I	+++	+++	++	+	-
CS98 in *E. coli* strain MM294-4	*Sfu*I	-	-	-	-	-

Key: +++, ++, + and - as used in the results represent strong growth, moderate growth, weak growth and No growth respectively.

Table 12a. NaCl concentration tolerance test on *E. coli* and *Rhodococcus* strains as control experiment.

Strain	Concentration of NaCl (mM)				
	100	200	300	400	500
SQ1	+	+	+	+	-
Ri8	+++	+++	+++	+++	+++
CS98	+++	+++	+++	+++	+++
E. coli MM294-4	+++	+++	+++	+++	+++

Key: +++, ++, + and - as used in the results represent strong growth, moderate growth, weak growth and No growth respectively.

Table 12b. NaCl concentration tolerance test on *E. coli* and *Rhodococcus* strains as control experiment.

Strain	Concentration of NaCl (mM)				
	600	700	800	900	1000
SQ1	-	-	-	-	-
Ri8	+++	+	-	-	-
CS98	+++	+	-	-	-
E. coli MM294-4	+++	+++	+++	+++	++

Key: +++, ++, + and - as used in the results represent strong growth, moderate growth, weak growth and No growth respectively.

After isolating plasmid from the libraries and then re-transforming them into their respective host cells, all the libraries could grow very well on 1000mM NaCl medium. They appeared to carry cloned DNA conferring an enhanced osmotolerance to > 1M NaCl.

3.4 Carbon source testing and biodegradation of benzoate

The genomic library of CS98 was screened for genes responsible for degradation of various organic compounds. Meanwhile, a suitable host for the recombinant plasmid isolated from the pooled library was required. It was desired to obtain a host related to CS98 so that there would be a high chance of expressing any gene cloned. The three *Rhodococcus* strains Ri8, SQ1 and CS98 were tested for survival on different minimal media containing 0.1% of various carbon sources.

Table 13. Behaviour of *Rhodococcus* species on minimal medium containing various single carbon sources.

Strain	Carbon source				
	Sodium citrate	Sodium benzoate	Sodium deoxy-cholate	Sodium acetate	m-cresol
CS98	+	+	-	+	-
Ri8	+	+	-	+	-
SQ1	+	-	-	+	-

Key: + is for growth and - is for no growth.

The only difference observed from table 13 was the inability of SQ1 to utilize sodium benzoate as sole carbon source while both strains CS98 and Ri8 could do so. Hence, genes responsible benzoate degradation was to be identified from the pool of recombinant plasmid using SQ1 as the host strain. SQ1 was transformed with bulk plasmid isolated from the library in *E. coli*, and the transformants bearing genes for benzoate degradation were screened on minimal medium plates containing 0.1% sodium benzoate.

10^{-7} dilution of the pooled transformants gave 65 colonies on the selection medium which could utilize sodium benzoate as sole carbon source. This gives a high representation of genes responsible for benzoate metabolism in SQ1. CS98 library in MM294-4 could also show colonies on a minimal medium containing 0.1% sodium benzoate as sole carbon source.

Plasmids isolated from single colonies of SQ1 transformants were digested with *Sful* and 0.8% agarose gel electrophoresis was ran to estimate the insert sizes of the genes. Fragment size analysis on *Sful* digest of recombinant plasmids from single colonies showed three different insert sizes; 2.8kb, 3.7kb and 31.8kb (table 14). The 31.8kb fragment certainly resulted from partial digestion of CS98 genome before the construction of the genomic library. It was further digested by *Sful* into 4.0kb, 4.7kb and 23.1kb. One or more of these sub-fragments bear genes responsible for benzoate degradation.

Table 14. Fragment sizes analysis of *Sfu*I digest of plasmid isolated from single colonies found on benzoate plates. Reference was made to distances moved on gel by standard λII marker of known fragment sizes.

Sample	Distance moved on gel (mm)	Fragment size (kb)	insert size (kb)
pDA71(control)	36.0	8.8	-
1	36.0, 47.0	8.8, 3.7	3.7
2	36.0, 52.0	8.8, 2.8	2.8
3	32.0, 36.0, 43.0, 45.5	23.1, 8.8, 4.7, 4.0	31.8

Key: kb means kilobase

4.0 *DISCUSSION*

4.1 Construction of a genomic library of CS98 in MM294-4

Constructing a genomic library of an organism is the first step towards screening and characterizing most of the genes and their products from that organism. Gene expression occurs under the control of promoters so, not all genes in a genome of an organism could be isolated and expressed in another organism. Also, an appropriate restriction endonuclease would be required that would not cut the sequences of the desired gene or its promoter. Moreover, the vector to shuttle the gene between species should have a promoter sequence that would be compatible to the expression of the gene in the host organism. The host organism should not recognize the gene as foreign and destroy it (Brown et al, 1995). Finally, the amount of DNA must be moderate in size in order to be cloned.

It is for these reasons that pDA71 was used as the shuttle vector. It could successively shuttle genes from CS98 between *E. coli* strain MM294-4 and the *Rhodococcus* species used in this work. Moreover, *Sfu*I did not cut this DNA too often and was the best enzyme for the construction of the genomic library (Fig.1). Although the required mutants defective in cesium accumulation were not obtained as host for screening the library for genes responsible for cesium accumulation, CS98 genes conferring benzoate degradation as well as osmotolerance were successfully cloned in *Rhodococcus* strain SQ1.

As shown in Fig. 2 and Fig. 4, there was partial digestion of the DNA used for constructing the library. This process is sometimes desirable (Brown, 1995) as the desired gene could be cut by

complete digestion from the restriction enzyme of choice. Partial digestion usually gives larger sized inserts that would offer a higher chance of expressing most of the genes from the genome. The partial digestion in this case was unintended and may have resulted from inadequate incubation time or temperature variations which did not allow the enzyme enough restriction activity (Brown, 1995). Inserts of sizes up to 12.5kb were therefore obtained.

The chloramphenicol resistance gene in pDA71 was not expected to be expressed in *E. coli* strain MM294-4 yet it occurred. A promoter sequence that allows for it to be expressed in *E. coli* was probably cloned together with the other genes into pDA71 and followed by major plasmid rearrangements. When the isolated recombinant plasmid was re-transformed back into MM294-4 the chloramphenicol resistance gene could be expressed again. This also confirmed that the chloramphenicol resistance gene was plasmid borne.

It could be seen from Fig. 3 that *Hind*III cut off fragments from some inserts. *Hind*III therefore promises to be an enzyme that could be used to construct a genomic library of CS98. It could also be used to subclone any of the genes of CS98.

4.2 Mutagenesis

Though NTG mutagenesis of *E. coli* genes may optimally yield 60-80% auxotrophs *Rhodococcus* sp. usually yield 1-2%, a low frequency which may be due to lack of error-prone repair systems in nocardioforms (Gowan-PhD Thesis, 1994). From Tables 4-10, 0.9-7.3% auxotrophs obtained is comparable to or higher than the 1-2% found for *Rhodococcus* strains (Gowan-PhD Thesis, 1994). The highest percentage of auxotrophs on normal minimal medium

was 1.8 for strain SQ1, 4.5 for strain Ri8 and 7.3 for strain CS98. It therefore appears that CS98 is much more susceptible to NTG mutagenesis than SQ1 and Ri8. Nevertheless, the pattern of NTG mutagenesis exhibited by the three strains was very irregular and appeared to be a matter of chance.

It could be seen from Tables 4-10 that at some occasions no auxotrophs were obtained. The inconsistent trend needed consideration in terms of stabilizing all the random variables that account for that observation. Some random variables thought of included type of mutagen, duration of treating cells with mutagen, type of strain, growth stage of cells and addition of activators. From Table 4, duration of treating cells with mutagen appeared to have effect on efficiency of mutagenesis as 60mins gave the highest percentage of auxotrophs (1.8%). It changed to 90mins in Tables 6 and 7. It created a doubt on the duration factor but concentration was still a random factor as arbitrary concentrations were being used. In Table 5, the type of mutagen was shown to be a factor of mutagenic efficiency. EMS could not produce any auxotroph even within 60-90mins of treating cells with it. Of course, NTG has been found to induce more G:C-----A:T mismatches than EMS (Auerbach, 1976; Lemontt and Generoso, 1982). Probably longer duration or higher concentrations could have improved efficiency of mutagenesis.

From Tables 6 and 7, 0.1M and 0.5M NaCl did not affect the efficiency of mutagenesis in *Rhodococcus* species. NaCl may only enhance NTG mutagenesis in rats as stated by Wada et al (1998) but not in *Rhodococcus* species.

From Table 8, there was no significant difference between the results from using LB and 1-2% LBSG to make pre-culture of cells. This indicates that the cell wall integrity was not a factor of efficiency. The mutagen was able to penetrate the cell envelope into the cell interior. Meanwhile, cells mutagenized from the stationary phase yielded the highest frequency of 4.5%. Meanwhile, 40 days after 4.5-7.3% auxotrophs had been obtained (Table 7) with cells from the early exponential growth phase the auxotrophs had regained their ability to grow on the minimal medium. CS98 M2 and Ri8 M2 still had the auxotrophs on the minimal medium. These suggest that cells mutagenized from the stationary phase produced stable auxotrophs. Probably, DNA repairs are frequent during the exponential growth phase than at the stationary phase so, most DNA errors are corrected before further DNA replication and synthesis occurs.

From Table 9, successive mutagenesis showed an increased efficiency at M2, with 4.5% auxotrophs as compared to none at M1, but decreased through 1.8% auxotrophs at M3 to none again at M4. This indicates that a second phase of mutagenesis enhances the efficiency of mutagenesis. Incomplete mismatches may have been made complete at that stage. At M3 and M4, further NTG treatment may have eliminated auxotrophs, with badly damaged DNA, which probably grow slower than prototrophs. Surviving cells may be those with repaired DNA.

From Table 10, increasing concentration of NTG from saturation point at 18000μg/ml to 100μg/ml also did not show any significant effect on efficiency of mutagenesis. Auxotrophs were obtained at 100μg/ml and 1000μg/ml NTG concentration. Gowan (PhD Thesis, 1994) reported that about 250μg/ml of NTG was enough to yield auxotrophs, and this falls within the range where auxotrophs were obtained in this work. It could be that higher concentrations of NTG only led to severe DNA damage and resulted in a higher cell death rate.

The only consideration left now is the DNA repair mechanism which needs to be controlled in order to achieve higher efficiency of mutagenesis. This is suspected to be the cause of unstable mutagenesis. As reported by Kyriacou et al (1997) *Rhodococcus* species exhibit a surprisingly high level of resistance to NTG treatment which they attributed to highly effective inborn-repair systems. The ability to resist any kind of mutation could be species dependent because I find different species may have different levels of susceptibility to the mutagen. The resistance of *Rhodococcus* species to NTG mutagenesis became evident in this work when strains Ri8, CS98 and SQ1 exhibited normal growth rates in LB containing NTG at concentrations up to 150μg/ml without yielding any auxotroph (results not shown). The repair could be due to the conditions of post-mutagenesis treatment medium (Auerbach, 1976). This is a matter of metabolic effect which determines whether alkylation lesions in DNA be repaired or fixed and, once fixed would lead to expressed mutations (Auerbach, 1976). Severity of the lesions could account for the decision; if too severe cell death may result and if not severe repair may occur. Replication and transcription also play a role in this perspective. Since only normal bases can be replicated, abnormal ones can only produce mutations only by a replication process that results in the incorporation of a wrong-normal base (Auerbach, 1976). The wide metabolic capabilities of *Rhodococcus* species reveal how they would always want to adapt to the harsh conditions of their environment. Hence, they may be so flexible to mutations only when it favours their survival. Ways by which replication and transcription can enter the mutagenesis pathway include the monitoring of mismatches strands by DNA-polymerase at the time of replication (Auerbach, 1976).

Despite the successes in obtaining auxotrophs none of them was due to K^+ as they even survived on minimal medium plates containing 0μM KCl. This means that the probabilities of

obtaining K^+ auxotrophs are very low (N. Tomioka, personal communication). An increase in efficiency of mutagenesis could enhance the chances of obtaining K^+ auxotrophs. Much attention therefore, need to be focussed on how to stabilize random mutations in *Rhodococcus* species.

From Table 7, the 9.1-30.9% auxotrophs obtained could be due the use of Na_2HPO_4/NaH_2PO_4 buffer in minimal medium-0K instead of the usual K_2HPO_4/KH_2PO_4 buffer in the minimal medium. Most of the auxotrophs may require little K^+ for survival but may not necessarily be auxotrophs due to K^+ as expected. Nevertheless, the 36.4% auxotrophs obtained for minimal medium-Rif compared with 10% auxotrophs obtained for minimal medium-0K suggests that the difference was due to Rif in the other medium. The Rif resistance gene is therefore much more prone to NTG mutagenesis.

4.3 Osmotolerance

The ability of microbial cells to resist osmotic stress is said to be inducible (Iyer et al, 1994) and occurs as a means of adaptation to the environment. Hence, not all microorganisms have the capacity to do that. There may be inherent mechanisms in some organisms that are controlled by inducible genes. The expression of those genes may be triggered by chemical elements or electrolytic imbalance which conducts series of signal transductions. These ultimately lead to the production of chemical and biological substances that enable the organism to resist osmotic stress (Iyer et al, 1994; Becker et al, 1998). It could be inferred from the result in general that, osmotolerance phenotypes could be induced by osmotic stress only when an organism has those inherent genetic capabilities to express genes responsible for osmotolerance.

Results from Tables 11a and 11b show that DNA conferring higher osmotolerance phenotypes may have been cloned in the host cells. All the libraries except CS98 in *E. coli* carried clones conferring osmotic tolerance > 900mM NaCl. The presence of antibiotics in the growth medium of the library strains may have even reduced the expression of osmotolerance. From Tables 12a and 12b, the control experiments show the presence of such inducible genes in Ri8 and CS98 as they could tolerate osmotic stress up to 700mM NaCl. The growth medium in this case did not contain antibiotics. However, SQ1 exhibited low tolerance and could not survive > 400mM NaCl. Comparing these results with those of Tables 11a and 11b, the ability of the host cells, Ri8 and SQ1, to resist osmotic stress was enhanced further by genes from the library strains.

E. coli exhibited the ability to grow at > 1M NaCl in its growth medium. This is possible because an osmoregulatory locus was identified in *E. coli* which expresses the *proU* gene (Gowrishankar et al, 1985). The *proU* gene plays a major role in osmotolerance in *E. coli*. The above-mentioned observation suggests that, as an initial host for the genes from the library strains, MM294-4 may have imparted the osmotolerance expression in the recombinant plasmid through recombination and replication. This could have been confirmed only if control experiments were done for the library strains to show that they do not resist osmotic stress. Meanwhile, it could be seen from Tables 11a and 11b that the potential to resist osmotic stress was shut off when CS98 genomic library was constructed in *E. coli*. It could be that certain mechanisms existed in *E. coli* that blocked the expression of the osmotolerance genes in the recombinant plasmid. Those mechanisms did not destroy the genes because they could later be expressed in SQ1. This could be related to the homology-dependent inactivation of transgenes identified with gene transfer in plants (Finnegan and McElroy, 1994; Meyer and Saedler, 1996). It may have resulted from co-suppression arising from the introduction of extra copies of an

endogenous gene in *E. coli*. This may have caused coordinate silencing not only of the introduced gene but also of the endogenous gene (Finnegan and McElroy, 1994). If this is true then the genes responsible for osmotolerance in *E. coli*, *Rhodococcus* species and *Mycobacterium avium* share homology.

It is so interesting that the libraries even gained an enhanced osmotolerance which could allow them to tolerate > 1M NaCl when their recombinant plasmids were isolated and re-transformed into their respective host strains. This is comparable to the osmotolerance of 1.5M NaCl exhibited by *Saccharomyces cerevisiae* (Blomberg et al, 1988). The observation certainly showed that the genes responsible for osmotolerance were plasmid borne. It also suggests that another inducer was involved which triggered further the expression of more genes responsible for osmotolerance. Iyer et al (1994) reported that *Anabaena* species strain L-31 induced the expression of at least 12 osmotic-stress-induced proteins (OSP's) when exposed to 100-350mM sucrose. The above-mentioned findings could therefore be attributed to the presence of 10.3% (300mM) sucrose in LBSG which was used to pre-culture host cells before their transformation with the recombinant plasmid. Spontaneous mutations may have resulted from the osmotic stress induced by sucrose, in order to strengthen the cell wall integrity against possible osmolysis. The presence of glycine in the LBSG may have also contributed to the enhanced osmotolerance because glycine betaine and other amino acids including L-proline were found to play important roles in osmotolerance (Measures, 1975; Jakowec et al, 1985).

4.4 Carbon source testing

The ability of microorganisms to utilize various carbon sources is a property that enhances their survival in the environment. They have developed many metabolic mechanisms for a wide range of organic substances and this is exploited in environmental biotechnology for bioremediation of those substances that may be recalcitrant.

From Table 13, SQ1 was the only strain that could not utilize sodium benzoate as sole carbon source. This means that SQ1 probably lacks the metabolic pathway for channelling the carbon units of the benzene ring into the central metabolic pathway. Ri8 and CS98 on the other hand could utilize benzoate as their sole carbon source.

Benzoate degradation through the *ortho* pathway involves a series of enzymatic reactions yielding major intermediates such as cis,cis-muconate, β-ketoadipate, catechol etc.(Johnson and Stanier, 1971), and ultimately generate succinyl CoA and acetyl CoA which are then metabolized in the central metabolic pathway (Ampe et al, 1997). Certainly, the genes responsible for benzoate degradation encode for enzymes in such metabolic pathways (Collier et al, 1997), especially those at the limiting step. Some also express proteins that play important roles in the transport of benzoate across a cell wall, e.g. the *ben*K gene (Collier et al, 1997).

From Table 14, three different DNA insert sizes, 2.8kb, 3.7kb and 31.8kb were obtained which may represent three different types of genes responsible for benzoate degradation. The 31.8kb fragment could represent a chain or cluster of genes which are involved in benzoate

metabolism. This is because in a 16kb-pair region of the chromosome of *Acinetobacter calcoaceticus* there were 10 genes responsible for benzoate catabolism, identified to be organized in no fewer than 3 transcriptional units (Neidle et al, 1987). This kind of arrangement, termed supraoperonic clustering, has also been identified in *Pseudomonads* (Neidle et al, 1987). If this clustering of genes is true for the 31.8kb fragment then the partial *Sful* digestion of CS98 genome was much appropriate in the screening for genes responsible for benzoate degradation. Complete digestion of CS98 would have digested this fragment destroying the gene cluster. Nevertheless, the genes responsible for benzoate degradation may also be smaller than the sizes obtained and would require sub-cloning.

In any case, there has been a successful cloning of genes responsible for benzoate degradation in pDA71 with SQ1 and *E. coli* strain MM294-4 as hosts. It would be necessary for the recombinant plasmid from such libraries to be re-transformed into host strains in order to confirm that they bear genes responsible for benzoate utilization. It is not surprising at all that *E. coli* strain MM294-4 could express those genes, because *ben*ABCD genes encoding for benzoate 1,2-dioxygenase system have successfully been cloned in *E. coli* (Neidle et al, 1987). This system is composed of NADH-cytochrome C reductase and terminal oxygenase components, and a cis-diol dehydrogenase, all of which are involved in the oxidation of the benzene ring of benzoate (Neidle et al, 1987).

5.0 *CONCLUSION*

Sful could successfully be used to clone CS98 genome into the *EcoRI* site of pDA71, and this library could generate clones bearing genes responsible for benzoate degradation.

Though NTG mutagenesis could not yield auxotrophs with increased K^+ requirement as hosts for screening genes responsible for cesium accumulation, an improved %auxotrophs of 7.3% was obtained with CS98. Moreover, stable auxotrophs (4.5%) could be obtained with strains CS98 and Ri8 when treated with NTG solution at their stationary growth phase.

Genes responsible for osmotolerance had been cloned from microorganisms including *Nocardia brasiliensis* IFM 0236, *Nocardia otitidiscaviarum* IFM 0239, *Rhodococcus australis* A448, *Rhodococcus rhodochrous* ATCC 12674, *Rhodococcus erythropolis* CS98 and *Mycobacterium avium* IFM 0414 into Ri8 and SQ1 as host strains. These genes could successfully be cloned into plasmid vector pDA71 with *Sful*, *BglII* and *PstI*. The osmotolerance phenotype is controlled by inducible genes which are induced only under osmotic stress and could enable microorganisms bearing them to tolerate > 1M NaCl in their growth medium.

CS98 DNA clones of varying sizes 2.8kb, 3.7kb and 31.8kb suggest that more than one enzyme or pathway for degradation of benzoate may exist in this organism. These genes could be expressed in both *Rhodococcus erythropolis* strain SQ1 and *E.coli* strain MM294-4.

APPENDICES

A. Media

LB

1.0% tryptone

0.5% yeast extract

0.5% NaCl

LA

1.0% tryptone

0.5% yeast extract

0.5% NaCl

1.2% agar

LBSG

1.0% tryptone

0.5% yeast extract

0.5% NaCl

3.0% H_2NCH_2COOH

10.3% sucrose

TYG

1.0% tryptone

0.5% yeast extract

2.0% H_2NCH_2COOH

Regeneration plates

30g sucrose

250ml distilled H_2O

3g tryptone

1.5g yeast extract

1.5g glucose

3g $MgCl_2.H_2O$

5.5g agar

After autoclaving, the following were added:

6ml 1M $CaCl_2$ solution

3ml 0.5% KH_2PO_4 solution

70μg/ml cycloheximide

40μg/ml rifampicin

10X Stock 3

91.7g K_2HPO_4

26.8g KH_2PO_4

1.0g $MgSO_4$

1000ml distilled H_2O

10X A-N buffer

91.7g $K_2HPO_4.3H_2O$

26.8g KH_2PO_4

1.0g $MgSO_4.7H_2O$

5.0g $Na_3C_6H_5O_7.H_2O$

1000ml distilled H_2O

10X A-N buffer (Na)

91.7g $Na_2HPO_4.3H_2O$

26.8g NaH_2PO_4

1.0g $MgSO_4.7H_2O$

5.0g $Na_3C_6H_5O_7.H_2O$

1000ml distilled H_2O

Minimal medium plates

Solution A contains

50% (of total volume) distilled H_2O

1.5% (of total volume) agar

Solution B contains

40% (of total volume) distilled H_2O

10% (of total volume) 10X A-N buffer or Stock III

0.1% (of total volume) NH_4Cl

Note: Total volume = solution A + Solution B

Solutions A and B were autoclaved separately and mixed subsequently, before

use.

B. Solutions and buffers

Lysozyme

100% lysozyme prepared to a concentration of 1μg/ml in sterile H_2O

Proteinase K

100% proteinase K prepared to a concentration of 1μg/ml in sterile H_2O

TE

10mM Tris pH 8.0

10mM EDTA

TE saturated phenol

14g C_6H_5OH

10ml 1X TBE

TE-SDS

10mM Tris-HCl

10mM EDTA

made up with 10% SDS and final pH adjusted to 8.0

Solution 1

50mM glucose

25mM Tris-HCl (pH 8.0)

10mM EDTA (pH 8.0)

Solution 2

0.2M NaOH

1% SDS

Solution 3

60ml of 5M CH_3COOK

11.5ml CH_3COOH

28.5ml H_2O

Solution A

50mM sucrose

10mM Tris-HCl

pH 8.0

Solution B

10mM Tris-HCl

10mM EDTA

10% SDS

pH 8.0

Solution C

4.5M CH_3COONa

pH 6.0

Ribonuclease

10mg/ml in sterile H_2O; used at a concentration of 1μg/ml.

Ethidium bromide

10mg/ml in distilled H_2O

Tracking dye

30% $C_3H_8O_3$

0.025% bromophenol blue

A quantity of 2μl was added to each sample

5X TBE

0.89M Tris-HCl

0.089M H_3BO_3

0.002M EDTA

Running buffer

20ml 5X TBE

180ml sterile distilled H_2O

15μl EtBr

$CaCl_2$ transformation buffer

50mM $CaCl_2$

10mM Tris-HCl

(PH 8.0)

30% PEG

3g PEG 6000

10ml distilled H_2O

Basal (b) buffer

10.3g sucrose

25mg K_2SO_4

202mg $MgCl.6H_2O$

87.5ml distilled H_2O

10ml 0.25M TES buffer (pH 7.2)

Protoplast (p) buffer

4.5ml basal buffer

50μl 0.5% KH_2PO_4

125μl of 1M $CaCl_2$

50% PEG Solution

0.5g PEG 4000

1ml protoplast buffer

Boric acid/di-sodium tetraborate buffer

Stock A: 0.2M H_3BO_3

Stock B: 0.05M $Na_2B_4O_7.10H_2O$

50ml A + 7.3ml B diluted to 200ml with sterile H_2O

Adjust pH with NaOH

pH 8.2

NTG solution

NTG pellets were added to Boric acid/di-sodium tetraborate buffer, microwaved and vortexed alternatively until all pellets dissolved to give lime yellow colour

20% Tween

2ml Tween 80

4ml distilled H_2O

5ml C_2H_5OH

C. Antimicrobial and antifungal agents

Ampicillin	(100mg/ml) 1:1 H_2O:C_2H_5OH
Chloramphenicol	(4mg/ml) C_2H_5OH
Cycloheximide	(10mg/ml) H_2O
Erythromycin	(20mg/ml) C_2H_5OH
Nalidixic acid	(10mg/ml) 1:1 H_2O:C_2H_5OH

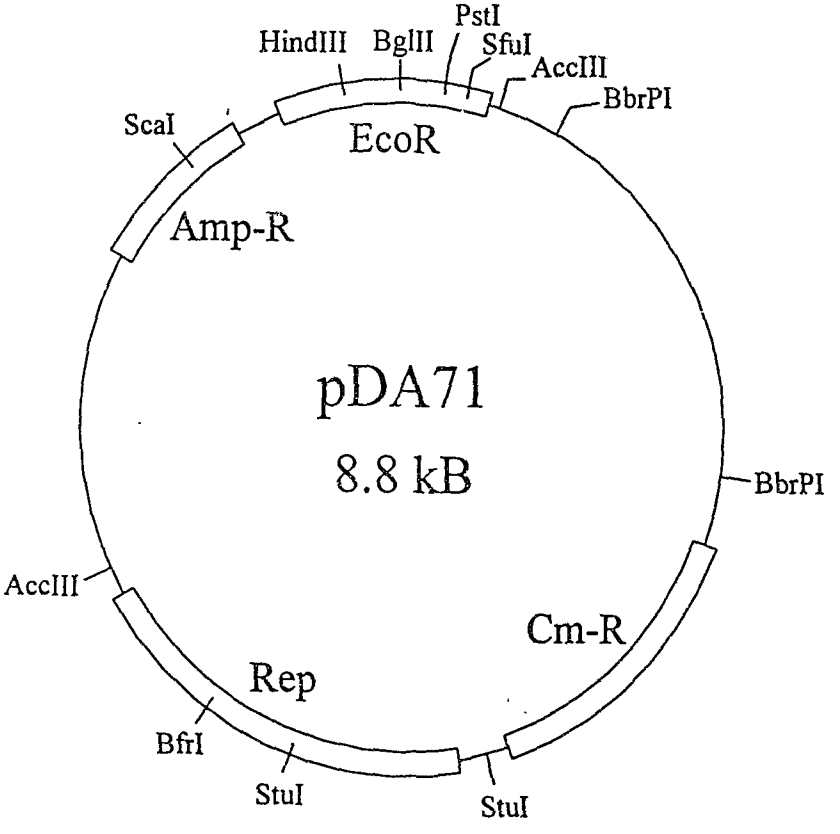
Rifampicin	(10mg/ml) CH_3OH
Tetracycline	(10mg/ml) CH_3OH

D. List of chemicals used

Chemical	Manufacturer
Absolute alcohol	Merck
Agar technical	Oxoid
Agarose HGT	FMC
Alcohol 96%	AAR
Ammonium chloride	Merck
Boric acid	UNIVAR
Butan-1-ol	GPR
Calcium Chloride-2-hydrate	Merck
Chloroform	ACE
Di-sodium tetraborate	AnalaR
Glacial Acetic acid	ACE
Glucose	UNIVAR
Glycine	Merck
Hydrochloric acid	AnalaR
Magnesium chloride	Merck
Magnesium sulphate-7-hydrate	AnalaR
Methanol	AnalaR

N-methyl-N-nitro-N-nitrosoguanidine	Sigma
Polyethylene glycol 4000	Merck
Polyethylene glycol 6000	Merck
Potassium acetate	Merck
Potassium dihydrogen orthophosphate	AnalaR
Potassium dihydrogen phosphate-3-hydrate	Merck
Propan-2-ol	UNIVAR
Sodium chloride	UNILAB
Sodium hydroxide	UNIVAR
Sucrose	UNILAB
Tris	Boehringer Mannheim
Tryptone	Oxoid
Yeast Extract	Oxoid

E. **Restriction map of pDA71, *E. coli* -*Rhodococcus* shuttle vector**



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