Starvation/stationary phase survival of *Rhodococcus erythropolis* SQ1: a physiological and genetic analysis

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Abstract

Although the starvation or non-growth state is probably the most common physiological state of bacteria, it has been studied in relatively few organisms. In spite of its importance in pathogenesis, bioremediation and several industrial processes, limited research has been performed on *Rhodococcus* under starvation/stationary phase conditions. The objectives of this study were to analyse the physiological adaptation of *Rhodococcus erythropolis* SQ1 to starvation/stationary phase, and to generate and screen a bank of mutants to identify genetic elements involved in this adaptation.

It was found that *R. erythropolis* SQ1 can survive for at least 43 days in LB and distilled water, and 65 days in chemically defined medium (CDM) containing high (1 % w/v) or low (0.1 % w/v) glucose concentrations. Early stationary phase *R. erythropolis* SQ1 cells grown in 0.1 % glucose also exhibited enhanced resistance to heat and oxidative stress compared with exponential phase cells. A mutant bank of 898 *R. erythropolis* SQ1 mutants was generated and screened; four mutants were of particular interest. The culturability of mutants 4G6 and 10D3 dropped to <0.1 % of the maximum CFU/ml at 27 days incubation, and to <3 % of the maximum CFU/ml for mutants 1B2 and 1H1, when grown in 1 % glucose medium. No drop in culturability was observed when mutants were grown in 0.1 % glucose. Mutant 4G6 had a transposon insertion in *uvrB* (UvrB, part of the DNA excision repair mechanism), while the insertion for mutant 10D3 was immediately downstream of a putative *guaB* gene, which, based on bioinformatic analyses, is followed by another putative IMP dehydrogenase (*guaB*-like) and/or a cholesterol oxidase gene. In mutant 1H1 the transposon inserted 272 nucleotides downstream of a gene encoding a putative phosphoglycerate mutase and upstream of putative thioredoxin and cytochrome *c* biogenesis genes.

In conclusion, *R. erythropolis* SQ1 was shown to present a classic starvation/stationary phase survival response, with the associated increase in resistance to various external stresses. A mutant bank has been generated which can be used in the future to analyse other phenotypes of interest. Several genes linked to starvation/stationary phase survival were identified. These findings show that a wide variety of genes are involved in starvation/stationary phase survival. Indeed, over 100 such genes have been identified in *Escherichia coli* and *Mycobacterium tuberculosis*.

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Introduction

1.1. The genus Rhodococcus

Members of the genus *Rhodococcus* are described in Bergey's manual as aerobic, Gram positive, non-motile, mycolate-containing, partially acid-fast, nocardioform actinomycetes. The shape of cells during growth ranges from cocci to filaments with short projections and elementary branching (Goodfellow, 1989). According to 16S phylogenetic clustering, they are part of the *Corynebacterineae*, which includes Gram-positive actinomycetes with mycolic acid in the cell wall (Stackebrandt *et al.*, 1997).

Over the last seven decades there has been confusion surrounding the taxonomy of the mycolic acid-containing actinomycetes, which currently includes *Rhodococcus*, *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Skermania*, *Millisia*, *Segniliparus*, *Williamsia*, *Tsukamurella* and *Smaragdicoccus* (Adachi *et al.*, 2007; Butler *et al.*, 2005; Goodfellow *et al.*, 1998; Kampfer *et al.*, 1999; Soddell *et al.*, 2006; Stackebrandt *et al.*, 1997). This is largely due to the inability of conventional tests to distinguish amongst different species. In the last ten years, methods have been developed to distinguish *Rhodococcus* from other members of the mycolata group. A study by Bizet *et al.* (1997) showed that pure cultures of *Rhococococcus*, *Gordonia* and *Dietzia* could be discriminated using a carbon source utilisation test based on the "Biotype-100" strips (Biomérieux), in which growth on 99 different carbon sources is analysed with the computer package Taxotron (Taxolab). The system could differentiate between 29 different species, including 10 *Rhococococcus*, 7 *Gordonia* and 1 species of *Dietzia* (Bizet *et al.*, 1997). Primers and specific probes based on the 16S rRNA sequences have also been designed for identification of *R. equi* (Bell *et al.*, 1996), *R. globerulus*, *R. erythropolis*, *R. opacus* and *R. ruber* (Bell *et al.*, 1999), and *R. coprophilus* (Savill *et al.*, 2001). In addition, primers specific to the *vap* virulence

genes of the *R. equi* virulence plasmid have been designed to differentiate between virulent and avirulent equine-derived strains (Takai *et al.*, 1998).

The name "*Rhodococcus*" was first used by Overbeck in 1891, and was subsequently reintroduced by Tsukamura in 1974 (Overbeck, 1891; Tsukamura, 1974). Study of the lipid composition of coryneform bacteria led to further determination of what was called the "rhodochrous" complex (Minnikin *et al.*, 1977). Following a study of 177 strains examined for 92 unit characters (such as colony morphology, growth on sole carbon source, growth temperature), the taxon *Rhodococcus* was definitely established in 1977 (Goodfellow & Alderson, 1977).

The genus is defined primarily on the basis of cell-wall composition. It is restricted to actinomycetes that have: (a) A1γ peptidoglycan consisting of *N*-acetylglucosamine, *N*-glycolylmuramic acid, D- and L-alanine, and D-glutamic acid with meso-DAP as the diamino acid; (b) arabinose and galactose as diagnostic wall sugars (chemotype IV and whole-cell sugar pattern type A); (c) a phospholipid pattern consisting of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides; (d) a fatty acid profile containing major amounts of straight-chain, unsaturated, and tuberculostearic acids (type IV), and mycolic acids with 32–54 carbon atoms; and (e) dehydrogenated menaquinones with eight or nine isoprene units (Briglia *et al.*, 1996; Goodfellow, 1989; Li *et al.*, 2004). The mol% G+C of the DNA ranges from 55.5 to 72 mol% (Goodfellow *et al.*, 1998; Li *et al.*, 2004).

16S rRNA studies subsequently indicated that *Rhodococcus* was indeed a genus of its own and, even though there have been minor changes over the last decade or so, most of the species described as *Rhodococcus* are now well characterised (Goodfellow *et al.*,

1998). More recent work on 29 *R. erythropolis* strains showed very little diversity in the 16S rRNA sequence (Oberreuter *et al.*, 2002). There are now forty-one officially recognised species of *Rhodococcus* (Euzéby, 2007).

1.2. Environmental and industrial applications of Rhodococcus

Rhodococci have been isolated from a large range of habitats such as soil, rocks, ground water, marine sediments, animal dung, the guts of insects and from infected animals, plants and humans (Goodfellow, 1989; Rowbotham & Cross, 1977; Tilford, 1936; Weyland, 1969; Woolcock *et al.*, 1979). They have been isolated from environments ranging from the deep sea (Heald *et al.*, 2001) to the Arctic and Antarctic soil (Bej *et al.*, 2000; Kochkina *et al.*, 2001), and there are even reports of isolation from the air in the Mir space station (Li *et al.*, 2004). Members of the genus *Rhodococcus* have an outstanding range of enzymes that enable them to transform many chemicals, rendering them extremely attractive for many industrial processes, as well as good candidates in environmental biotechnology (Warhurst & Fewson, 1994). This is reflected in the number of patents exploiting *Rhodococcus*; a search for the word "*Rhodococcus*" at http://openaccess.dialog.com/ip/ in all patent sources yielded over 1000 titles in October 2007. This section of the review will focus on the environmental and industrial applications of the genus *Rhodococcus*, with an added emphasis on the species *R. erythropolis*.

1.2.1. Desulphurisation of fossil fuels

The combustion of fossil fuels releases sulphur oxides in the atmosphere, contributing to acid rain and air pollution. To control emissions, environmental agencies worldwide impose strict regulations on the sulphur content of fuels. The United States and European Union environment protection agencies have imposed regulations (Directive

98/70/EC of the European parliament) that allow a maximum sulphur content of 15 ppm since 2006 (Anonymous, 1998). A new objective will be to bring the concentration of sulphur in fuels to 10 ppm by 2009, if new legislation currently proposed by the European Commission is adopted (Anonymous, 2007).

The conventional technique to remove sulphur from petroleum is through a process called hydrodesulphurisation (HDS), which involves reacting oil fractions at high temperature and pressure with an inorganic catalyst and hydrogen, resulting in the production of hydrogen sulphide and a desulphurised product (Gray *et al.*, 1996). This technique is very energy-demanding, and some compounds are recalcitrant to it, for example benzothiophene, dibenzothiophenes (DBT) and DBT with substitutions adjacent to the sulphur group (McFarland, 1999). Several organisms capable of desulfurising DBT have been isolated, in particular *Rhodococcus erythropolis*. *R. erythropolis* IGTS8 (ATCC 53968), patented by the Institute of Gas Technology (Des Plaines, IL, USA), can oxidise DBT to 2-hydroxybiphenyl (HBP), releasing inorganic sulphur that can then be assimilated by the bacteria (Kilbane & Jackowski, 1992). Other strains that can desulphurise fuel have been isolated, such as *R. erythropolis* KA2-5-1 (Hirasawa *et al.*, 2001), *R. erythropolis* H-2 (Ohshiro *et al.*, 1995) and *R. erythropolis* D-1 (Ohshiro & Izumi, 2000). Importantly, the phenyl rings of DBT are left intact following desulphurisation by *R. erythropolis*, and the HBP (2-hydroxybiphenyl) is returned to the fuel, hence its calorific (i.e. fuel) value is not diminished (Kayser *et al.*, 1993).

The oxidation pathway of DBT has been characterised, and the enzymes DszA, DszB, DszC and DszD identified (Denome *et al.*, 1993; Denome *et al.*, 1994). DszA and DszC are flavin-dependent monooxygenases, particular in that they use $FMNH_2$ as a co-

substrate instead of FAD. DszB is the desulfinase that removes the sulphur, and DszD is a FMN:NADPH reductase that supplies DszA and DszC with $FMNH_2$ (Oldfield *et al.*, 1997) (see Figure 1.1).

A number of steps in the pathway are still not well understood, including for example the transfer of these hydrophobic polyaromatic molecules into the cell (Monticello, 2000). The genes involved in the basic desulphurisation steps have been characterised. *dszA*, *dszB* and *dszC* have been located in a single operon on a 150 kb circular plasmid in *R. erythropolis* IGTS8, and on a 100 kb linear plasmid in related strains (Denis-Larose *et al.*, 1997; Oldfield *et al.*, 1997). Their transcription is repressed by the presence of sulphur-containing amino acids, but not by DBT (Li *et al.*, 1996).

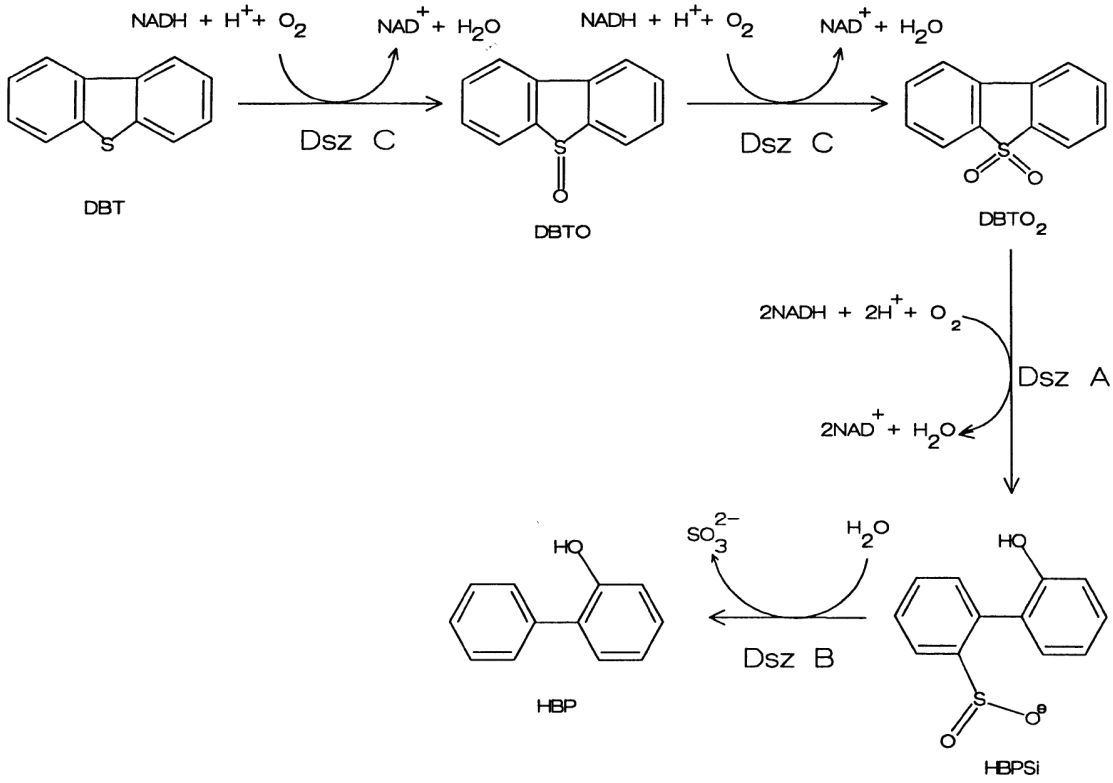


Figure 1.1. Desulphurisation of DBT into HBP by *R. erythropolis* IGTS8. Reproduced from Oldfield *et al.* (1997). DBT, dibenzothiophene; DBTO, dibenzothiophene 5-oxide; $DBTO_2$, dibenzothiophene 5,5-dioxide; HBPSI, 2-(2′H-hydroxyphenyl)benzene sulphinate; HBP, 2-hydroxybiphenyl.

Exploiting the biochemical and genetic information available on the desulphurisation pathway of *Rhodococcus* and indeed other organisms, it was possible to consider manipulating these organisms to produce improved biocatalysts. Work has been done to improve the desulphurisation rate, for example by insertion of multiple copies of the *dsz* operon in *R. erythropolis* I19 (Folsom *et al.*, 1999). An attempt has also been made to increase the desulfinase enzyme activity and broaden its specificity by error-prone PCR of the *dszB* gene (Borole *et al.*, 2003). A clone with higher desulfinase activity was isolated, but analysis of the *dszB* sequence showed the mutation was not within the gene and therefore it is assumed that the modification took place in a regulatory sequence.

There are still many hurdles to overcome before biodesulphurisation of fuels becomes a routine process, including the stability of the biocatalyst. BDS is performed using resting cells, meaning they are non-growing live cells (Guerinik & Al-Mutawah, 2003; Noda *et al.*, 2002; Oldfield *et al.*, 1997). An advantage of using resting cells is that they can be used at higher concentrations than growing cells (Abbad-Andaloussi *et al.*, 2003). Resting cells, harvested at the end of exponential phase for maximum activity, are usually washed and resuspended in potassium phosphate or HEPPS [4-(2-hydroxyethyl)-1-piperazine-propane-sulfonic acid] buffer to which the fuel is added and mixed. Due to the hydrophobicity of *R. erythropolis*, the cell suspension can form a very fine and stable emulsion with the oil, which increases the conversion rate of DBT to HBP (Borole *et al.*, 2002). This, however, does make it difficult to separate the biocatalyst from the reaction medium after the fuel was desulphurised. As a result, an immobilised *R. erythropolis* KA-2-5-1 resting cell system has been developed (Naito *et al.*, 2001). This biocatalyst was found to function well and, when reactivated by incubation overnight in growth medium, could maintain its activity for up to 900 hours.

1.2.2. Production of biosurfactants

Biosurfactants are produced by micro-organisms and represent a broad spectrum of molecules, including normal and hydroxyl fatty acids, glycolipids, cyclic lipopeptides, *N*-acyl amino acids, lipopeptides, mono and diglycerides, and phospholipids. Due to their amphiphatic nature, they have in common their capacity to accumulate at air–water, oil–water and solid–liquid interfaces (for a review, see Christofi & Ivshina, 2002; Lang, 2002). As a result they have solubilisation, emulsification, dispersion, wetting, foaming and detergent effects. The diversity of chemical structures and surface properties may reflect differences in their role in the microorganisms' natural habitat. For example, several biosurfactants increase the bioavailability of hydrophobic substrates (including many xenobiotics), while others have antimicrobial activities (for a review, see Ron & Rosenberg, 2001). Synthetic surfactants have been used in oil washing for secondary oil recovery and to clean oil pipes and oil reservoirs. The impact of biosurfactants on the biodegradation of xenobiotic contaminants in soil and other environments has been investigated and showed that biosurfactants can be used to speed up the remediation of organic and metal-contaminated sites, and their low toxicity makes them suitable for bioremediation by bioaugmentation (for a review, see Christofi & Ivshina, 2002). They are believed to help cell adhesion to hydrophobic phases in two-phase systems, help the entry of hydrophobic compounds into the cell by decreasing the interfacial tension and disperse hydrophobic compounds to increase the surface area for bacteria–molecule contact (Finnerty, 1994). This is illustrated by the increased desulphurisation of fuel by *R. erythropolis* in the presence of surfactants (Feng *et al.*, 2006; Patel *et al.*, 1997). A similar observation was made with *R. erythropolis* EK-1 and surfactants produced by a *Pseudomonas* strain (Karpenko *et al.*, 2006). Similarly, mycolic acids enhanced the degradation of diesel by *Rhodococcus baikonurensis* (Lee *et al.*, 2006).

The predominant biosurfactants produced by members of the genus *Rhodococcus* are glycolipids, with trehalose as the hydrophilic compound and mycolates as the hydrophobic component (Lang & Philp, 1998). The type and quantity of biosurfactant produced by *Rhodococcus* is dependent on growth conditions including growth phase, nutrient limitation and exposure to hydrophobic substrates such as *n*-alkanes. Non-growth-associated production of trehalose lipids has also been observed (Kim *et al.*, 1990). *R. erythropolis* can produce biosurfactants such as trehalose mono- and di-corynomycolates, trehalose-6,6′-diacylates and trehalose-6-acylates and trehalose tetraester, as well as common glycolipids and lipids such as cetyl alcohol, palmitic acid, methyl *n*-pentadecanoate, triglyceride and mycolic acids (Lang & Philp, 1998; Pirog *et al.*, 2004). It was shown that high yields of up to 20 g/l glycolipids could be obtained with resting cells of *R. erythropolis* DSM 43215 (Kim *et al.*, 1990). Production of efficient surfactants (unidentified, monitored by measuring medium surface tension) was also observed in *R. erythropolis* strains DSM 1069 and TA57 (Pizzul *et al.*, 2006). *R. ruber* can also produce a trehalose dicorynomycolate in large quantities (approx. 10 g/l) under nitrogen-limited conditions, as described by Philp *et al.* (2002), that is more hydrophobic than that produced by *R. erythropolis*.

1.2.3. Biosynthesis and biocatalysis

There are many advantages to the use of biocatalysts in chemical synthesis. In particular, complex reactions can be performed in a single step, leading to the use of fewer and cheaper reagents. High yields and high product purity can also be achieved. Milder reaction conditions, typically at near ambient temperature and pressure, can also reduce the cost of the operation (Johannes, 1996; Thomas *et al.*, 2002). Biocatalysis has also been proposed as a way to shift the current main source of chemicals from oil to biomass, thereby reducing CO_2 emissions and hopefully leading to "greener" chemistry (Danner & Braun, 1999). *Rhodococcus* species have been found to catalyse reactions that have applications in the synthesis of fine chemicals (e.g. perfumes, paints), pharmaceuticals and sensors, and more, a selection of examples are presented below.

The most successful use of *Rhodococcus* species in an industrial setting so far has been the production of acrylamide by *Rhodococcus rhodochrous* J1 (Nagasawa & Yamada, 1990). Each year more than 30,000 tons of acrylamide are produced, with a productivity of over 7 kg acrylamide per g of cells (Nagasawa *et al.*, 1993), using non-growing cells fixed inside acrylamide beads [see US patent 7,205,133 by Banba *et al.* (2002)].

Resting cells of *R. erythropolis* DSM 1069 were shown to synthesise cyclopropanol from cyclopropyl methyl ketone (Overbeeke *et al.*, 2003). This reaction has potential for industrial exploitation, as cyclopropanol is difficult to produce by traditional organic chemistry methods and is thus not commercially viable. The possibility to produce cyclopropanol in large quantities for a reasonable cost would open new ways in organic synthesis that could not be investigated before. Another example of potential industrial application came from the observation that *R. erythropolis* DCL14 could grow on limonene as a source of carbon (van der Werf *et al.*, 1999b). This led to the discovery of

enzymes that produce enantiopure epoxides (van der Werf *et al.*, 1999a), essential building blocks in the synthesis of fine chemicals used as fragrances and flavours. *R. erythropolis* IGTS8 and *R. erythropolis* IGTS8 BKO-53, a *R. erythropolis* IGTS8 derivative lacking *dszA* and *dszB* but containing multiple copies of *dszC*, originally developed for biodesulphurisation of fuels, were used to transform simple sulphides into chiral sulfoxides, thus providing interesting building blocks for organic chemistry (Holland *et al.*, 2003a; Holland *et al.*, 2003b). The bioconversion was mediated by resting cells, resuspended in a phosphate buffer containing glucose, ethanol and the appropriate sulphide during a period of 15 hours. In addition to substituted benzyl sulphides, the organism mediated sulphide oxidation of amino acids such as methionine and cysteine.

R. ruber strain DSM 44541 was found to transform *sec*-alcohols into ketones and *vice versa* by an asymmetric hydrogen transfer that uses 2-propanol or acetone as a cofactor (Kosjek *et al.*, 2003). Optically active *R* or *S* alcohols, which are important as intermediates for the synthesis of fine chemicals, are produced depending on the substrate. *R. ruber* has been shown to produce dodecanedioic acid, which is an intermediate in the synthesis of films, fibres, paints and adhesives (Thomas *et al.*, 2002) and to synthesise poly(3-hydroxyalkanoic acid) (PHA), a polymer that can be used to produce biodegradable plastics (Madison & Huisman, 1999).

The exploitation of resting cells of *R. erythropolis* SC 13845 and *R. erythropolis* 16002 as biocatalysts in the production of the HIV-protease inhibitor Atazanavir is being considered. A key step in the process of Atazanavir production includes a diastereoselective reduction which can be mediated by specific *R. erythropolis* isolates with a diastereomeric purity of >98 % and minimum production of undesirable end-

products (Patel *et al.*, 2003). *Rhodococcus* sp. strains B264-1 and I24 were found to convert indene to indandiol, an essential step in the synthesis of another HIV-protease inhibitor, CRIXIVAN (Buckland *et al.*, 1999).

The ability to survive adverse environmental conditions, including high concentrations of toxicants (de Carvalho *et al.*, 2004; Paje *et al.*, 1997), combined with their ability to metabolise a wide range of diverse and unusual compounds (e.g. substituted phenols), offers a rich resource for the development of biosensors capable of detecting these toxic compounds in the environment (de Carvalho & da Fonseca, 2004). Potential sensors currently being developed or evaluated include the use of whole cells of *R. erythropolis* for the detection of 2,4-dinitrophenol (Emelyanova & Reshetilov, 2002). *R. ruber* and *R. erythropolis* also have the capacity to degrade the industrial pollutant acrylonitrile, prompting the development of a biosensor relying on the transformation of acrylonitrile into ammonium acrylate by *R. ruber* (Roach *et al.*, 2003). Another field of interest is the oxidation of cholesterol. *R. erythropolis* can oxidise cholesterol, which leads to various applications (Jadoun & Bar, 1993). Work is being carried out towards the isolation and purification of the cholesterol oxidase of *R. erythropolis*, with the aim of producing a test or a sensor to detect cholesterol (Sojo *et al.*, 1997; Sojo *et al.*, 2002). Disruption of the cholesterol metabolism pathway could also produce mutants that accumulate bioactive steroids, or potential precursors in the synthesis of bioactive steroids (van der Geize *et al.*, 2001).

1.2.4. Biodegradation and bioremediation

Rhodococcus species can metabolise a wide range of xenobiotics, from simple hydrocarbons through to chlorinated and aromatic hydrocarbons, nitroaromatics and chlorinated polycyclic aromatics such as polychlorinated biphenyls (PCBs) (Warhurst &

Fewson, 1994). This makes them potentially useful in bioremediation. A few specific examples are described below.

A wide range of chemicals, in particular aromatics and hydrocarbons, can be degraded by *Rhodococcus*. Natural isolates have been found to degrade polychlorinated biphenyls (PCBs), a common pollutant from industrial oils (Begonja Kolar *et al.*, 2007). *R. erythropolis* UPV-1 can degrade phenol and formaldehyde, and processes are being investigated to use it for decontamination of industrial wastewater (Begoña Prieto *et al.*, 2002; Hidalgo *et al.*, 2002; Prieto *et al.*, 2002). *R. erythropolis* M1 can degrade 2-chlorophenol, phenol and *ρ*-cresol in co-culture with *Pseudomonas fluorescens* (Goswami *et al.*, 2005). *R. erythropolis* HL PM-1 can degrade dinitrophenol (Kitova *et al.*, 2004), and *R. erythropolis* DSM 1069 and TA57 have been found to metabolise polyaromatic hydrocarbons (Pizzul *et al.*, 2006). Additionally, *R. erythropolis* VKM AS-1339D degrades oil and oil products (Yagafarova *et al.*, 2002). Screening of contaminated soil from a chemical plant for benzene degraders resulted in the isolation of a *Rhodococcus* strain of particular interest due to its ability to use liquid benzene as a sole carbon source and at concentrations observed to be toxic to other bacteria. This strain can also co-metabolise toluene and use chlorinated benzenes, making it an ideal candidate for bioremediation (Paje *et al.*, 1997). The persistence of *Rhodococcus* is illustrated by their isolation from soil in Antarctica (Bej *et al.*, 2000). These species were shown to degrade a wide range of alkanes (C6–C20 and branched alkane pristane), as well as being able to grow at temperatures ranging from –2 °C to +15 °C. These characteristics would make these species useful in bioremediation of cold environments where oil is extracted, such as Alaska or Siberia.

R. ruber also can degrade a large number of pollutants. An example is ethyl *tert*-butyl ether, a common pollutant from petrol (Chauvaux *et al.*, 2001). Like *R. erythropolis*, some strains of *R. ruber* can degrade PCBs (Begonja Kolar *et al.*, 2007). Most interestingly, *R. ruber* can metabolise polyethylene, one of the most widespread and most recalcitrant plastic pollutants known (Gilan *et al.*, 2004), which could lead to the bioremediation of landfill.

Strains of *R. erythropolis* have been found to degrade fertilisers (isobutylidenediurea, Jahns & Schepp, 2001), carcinogenic toxins (aflatoxin B1, Alberts *et al.*, 2006), C5–C16 hydrocarbons and motor oils (de Carvalho & da Fonseca, 2005b), diesel (Lin *et al.*, 2005) and to dehalogenate haloalkanes (Armfield *et al.*, 1995). For a review of the metabolic capacities of *R. erythropolis*, see de Carvalho & da Fonseca (2005a).

Rhodococcus strain DN22 has been found to readily degrade RDX (hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine) *in vitro*. RDX is a commonly used explosive, hence a rather frequent pollutant. Since these compounds are toxic and mutagenic, it is important to be able to clean up sites where vast quantities of explosives have been used or stocked, for example by a bioremediation process. Several bacteria are known to biodegrade RDX either aerobically or anaerobically, including *Rhodococcus* strain DN22 (Coleman *et al.*, 1998) and *Enterobacteriaceae* (Kitts *et al.*, 1994), respectively. Initial studies of the RDX biodegradation pathway in *Rhodococcus* strain DN22 indicate that the system is inducible and that the initial steps may be mediated by a cytochrome p-450 system. Coleman *et al*. (2002) suggest that the cytochrome p-450 enzyme is plasmid-encoded. This is of significance since this would enable transfer to other bacteria.

Although *in vitro* studies are important in order to identify the specific organisms involved in biodegradation of a particular toxicant and the metabolic pathways involved, investigation of the performance of individual isolates or microbial consortia in contaminated soil and/or water is absolutely essential. Factors such as the competing indigenous microflora and environmental conditions impact on the survival and catabolic activity of the introduced organism. A number of studies have been initiated, investigating the biodegrading activity of *Rhodococcus* sp. in soil.

The activity of the naphthalene-degrading *Rhodococcus* strain 1BN was investigated in naphthalene-contaminated soil in the presence and absence of an indigenous population of naphthalene-degrading bacteria (Cavalca *et al.*, 2002). *Rhodococcus* strain 1BN was able to survive in the naphthalene-contaminated soil in the presence of the competing indigenous naphthalene-degrading microflora, constituting 13.6 % of the naphthalene-degrading population on the 22^{nd} day of the experiment. Although naphthalene degradation was not significantly different in soil supplemented with *Rhodococcus* strain 1BN compared to uninoculated soil, the presence of 1BN resulted in faster mineralisation of the parent compound to CO_2. Screening of the soil microflora by PCR amplification of *narA* (Gram-positive) and *ndoB*/*nahAC* (Gram-negative) naphthalene-degrading dioxygenase genes revealed that, although after 10 days the *ndoB*-harbouring strains (Gram-negative) were dominant, at 22 days the dominant isolates contained *narA* (Gram-positive). This would suggest that, in substrate-limiting conditions, *Rhodococcus* and other Gram-positive bacteria survive longer than Gram-negative bacteria. Due to their broad metabolic activity, Gram-positive bacteria such as *Rhodococcus* also bring additional degradative properties to the microcosm, for example towards medium- and long-chain alkanes. Similar observations on population changes were made during a study of crude oil biodegradation in soil under laboratory

conditions. Gram-negative bacteria were predominant during the first seven months after which Gram-positive bacteria, including indigenous *Rhodococcus* species, were dominant (Peressutti *et al.*, 2003).

The range of chemicals degraded and metabolised by *Rhodococcus* species is impressive, as outlined above and in several reviews (de Carvalho & da Fonseca, 2005a; Warhurst & Fewson, 1994). With the development of genetic tools for *Rhodococcus* (see e.g. Veselý *et al.*, 2003) and the recent availability of complete genome sequences (McLeod *et al.*, 2006), there is a great scope for the production of genetically engineered "one-cell factories" to use in industry, and for the production of strains that can help clean up the environment from toxicants. What is striking with most of these industrial processes is that they use resting cells (see e.g. Abbad-Andaloussi *et al.*, 2003; Caro *et al.*, 2007; Kim *et al.*, 1990). The cells are grown in the appropriate medium, very often until stationary phase to obtain as much biomass as possible, then harvested by centrifugation. The reactions catalysed then take place in either a phosphate buffer or an aqueous solution of the compound to be metabolised. Even though some of these processes are used commercially, there are no reports on how the physiological state of the cells, in these cases starvation, may affect the production of the product of interest, the half-life of the catalyst or its renewal. Interestingly, in a chemostat biodesulphurisation study, it was found that highest activity was obtained when *Rhodococcus* cells were selectively starved for sulphur, but not for carbon or other nutrients (Kishimoto *et al.*, 2000).

For bioremediation, it is essential that the organisms used can survive in low-energy environments, particularly soil. It has been shown that polyurethane-immobilised *Rhodococcus* could survive for more than six months in soil, and that they increased the

degradation rate of pentachlorophenol (Briglia *et al.*, 1990). Autochthonous populations of *Rhodococcus* were found in soil after a controlled oil spill, even though they could not be detected before the oil spill (Peressutti *et al.*, 2003). This suggests that the population naturally present in soil was growth-restricted, but that it retained the ability to grow and degrade hydrocarbons. *Rhodococcus* species could be found in soil for up to 13 months after the oil spill, also showing that they successfully competed with other species present in the ecosystem (Peressutti *et al.*, 2003). Finally, in a bioremediation study of activated sludge with phenol-degrading *Ralstonia eutropha*, it was found that cells that were starved prior to addition to the sludge survived longer than exponential phase cells (Watanabe *et al.*, 2000). Although this type of protocol has not been investigated with *Rhodococcus*, it could point to an easy and effective way of increasing the efficiency of bioremediation.

1.3. Pathogenicity of Rhodococcus

1.3.1. *Rhodococcus equi* infections: epidemiology and disease

Due to its pathogenic nature, *Rhodococcus equi* is probably one of the best studied *Rhodococcus* species so far. It was isolated for the first time from foals and humans in 1923 and in 1967, respectively. It was first classified as a *Corynebacterium*, then assigned to the *Mycobacterium* genus and finally reclassified as *Rhodococcus* (Mosser & Hondalus, 1996). It has attracted attention because it is the main cause of pneumonia in foals between 1 and 6 months old leading to fatality, and, more recently, due to its emergence as a human opportunistic pathogen.

In foals, the most common clinical manifestation of infection by *R. equi* is a chronic and suppurative pneumonia, with extensive abscessation and lymphadenitis. Intestinal manifestations are present in about 50 % of all foals with pneumonia. The intestinal infection is characterised by ulcerative enterocolitis that can lead to peritonitis, which is usually fatal. It is especially difficult to diagnose since the symptoms are subtle and complex until rather extensive damage has been done to the lung. Joint inflammation, with or without infection can occur, as well as osteomyelitis and subcutaneous lesions (for a review, see Giguère & Prescott, 1997). In adult horses, disease due to *R. equi* is rare and has been associated with immunodeficiency (Freestone *et al.*, 1987). Furthermore, although rare, *R. equi* has been found to cause disease in other animals such as cats, pigs, cattle and dogs (Fairley & Fairley, 1999; Hondalus, 1997).

In humans, *R. equi* is associated with immunocompromisation, including in individuals with AIDS and patients undergoing immuno-suppressive therapy (Prescott, 1991; Takai *et al.*, 1994). The predominant clinical manifestation is pneumonia, but other infections

reported include subcutaneous lesions, osteomyelitis, pericarditis and skin infections. Recurrence is common, and the treatment is long, about six months. The same treatment is applied to horses and humans, and generally consists of a combination of erythromycin and rifampin (Giguère & Prescott, 1997; Perez *et al.*, 2002). The mortality rate is about 20–25 % in immunocompromised patients, but over 50 % for HIV patients (Perez *et al.*, 2002).

Virulence determinants of *Rhodococcus equi*:

In vitro experiments have shown that *R. equi* binds to the macrophage complement receptor CR3 (Hondalus *et al.*, 1993). The bacteria are phagocytosised but the phagosome–lysosome fusion is inhibited (Zink *et al.*, 1987). *R. equi* can therefore replicate in the phagosome, explaining why the treatment of choice includes antibiotics that concentrate in macrophages. Similar behaviour has been observed for other intracellular pathogens such as *Mycobacterium*, *Nocardia asteroides* and *Legionella pneumophila*. Interestingly, avirulent strains have been shown to persist in macrophages but are incapable of multiplying (Hondalus & Mosser, 1994). For a review, see Hondalus (1997).

The main virulence factors of *R. equi* identified to date are plasmid-encoded proteins. All virulent strains isolated from foals contain a plasmid in the 80–90 kb range (Makrai *et al.*, 2002). This plasmid harbours the gene for the 15–17 kDa VapA protein (virulence-associated protein), a cell-surface protein of unknown function (Takai *et al.*, 1991). Sequence analysis of the virulence plasmid revealed a putative pathogenicity island harbouring the *vapA* gene and six additional *vap* genes (*vapC*, *D*, *E*, *F*, *G* and *H*). Deletion and complementation analyses provided clear evidence for VapA as a virulence factor in *R. equi* (Jain *et al.*, 2003). In contrast to foal-derived clinical isolates,

all of which harbour an 80–90 kb VapA-encoding plasmid, the plasmid profile of human isolates is more diverse. Isolates from immunocompromised patients without AIDS were mostly avirulent and plasmid-free, while isolates from AIDS patients harboured a virulence plasmid of 79–100 kb with a degree of relatedness to that harboured by equine isolates (Takai *et al.*, 1994; Takai *et al.*, 1995).

Virulence plasmid gene expression was recently analysed using a DNA microarray containing 66 out of the 69 identified ORFs from the 80 kb virulence plasmid (Ren & Prescott, 2003). A comparison of *in vivo* and *in vitro* growth revealed that, in macrophage-grown *R. equi*, all seven *vap* genes were induced, in addition to 6 ORFs of unknown function located in the putative pathogenicity island (PI). The observation of a putative IdeR binding site upstream of *vapA* is significant since IdeR controls transcription of genes encoding proteins contributing to bacterial survival within macrophages, as well as proteins for iron uptake and sequestration in *Mycobacterium tuberculosis* (Gold *et al.*, 2001). Furthermore, an IdeR homologue has been identified in *R. equi* (Boland & Meijer, 2000). Investigation of the effect of iron depletion on gene expression revealed the up-regulation of *vapA*, *C*, *G* and *H* and down-regulation of *vapD*, suggesting that *vapA* is part of the IdeR regulon in *R. equi* (Ren & Prescott, 2003).

Although *R. equi* infection is rarely fatal, the costs associated with treating the disease are high, especially since infected foals are less likely to race once adult (Ainsworth *et al.*, 1998). This has led The Horserace Betting Levy Board, UK, to fund the sequencing of the complete genome of *R. equi* 103S (see www.sanger.ac.uk/Projects/R_equi/).

1.3.2. *Rhodococcus fascians* infections

The only other *Rhodococcus* known to be pathogenic is *Rhodococcus fascians*. Fasciation of sweet pea was first described in 1927, and the causative agent identified in 1936. Its classification remained problematic until the 1980s, when it was finally named *Rhodococcus fascians*. It is the only known phytopathogen of the genus (Bell *et al.*, 1998; Finnerty, 1992). *R. fascians* initiates the formation of leafy galls that consist of centres of shoot amplification and shoot growth inhibition. It has a very broad host range, across 39 plant families and 86 genera (Goethals *et al.*, 2001).

Both *R. equi* and *R. fascians* are commonly found in soil (Maes *et al.*, 2001; Muscatello *et al.*, 2006). They must therefore survive large variations in temperature, humidity, pH and other physico-chemical characteristics of soil, as well as the intra-macrophage (for *R. equi*) and plant environment (for *R. fascians*). Soil is widely considered to be oligotrophic (Morita, 1993), so these organisms must have some ways of surviving periods of low energy availability.

1.4. Mechanisms of starvation survival in bacteria

1.4.1. Definitions

In this study, we propose to investigate some physiological responses of rhodococci to starvation, and aim to identify molecular elements that participate in the starvation survival of rhodococci. The term "starvation" describes environmental conditions in which bacteria stop growing, or grow at a very slow rate, because of the lack of nutrients. It is also commonly called "stationary phase", although the stationary phase is not necessarily induced by a lack of nutrients.

Starvation survival has been investigated in *Vibrio*, *Escherichia coli*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, *Sinorhizobium* and *Salmonella* to name a few Gram-negative bacteria (Jenkins *et al.*, 1988; Kjelleberg *et al.*, 1993; O'Neal *et al.*, 1994). Among the non-sporulating Gram-positives, *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Listeria*, *Micrococcus* as well as *Lactococcus* have been studied to a limited extent (Besnard *et al.*, 2000; Clements & Foster, 1998; Duwat *et al.*, 2000; Giard *et al.*, 1997; Mukamolova *et al.*, 1998; Trainor *et al.*, 1999). Little work has been done to date on *Rhodococcus* although some work has been done on the close relative *Mycobacterium*, due to its pathogenicity and its ability to remain dormant in the lung (Betts *et al.*, 2002; Kamalakannan *et al.*, 2002; Shleeva *et al.*, 2002).

It has been suggested that bacteria can enter a state where they are "viable but non culturable" (VBNC), meaning that they still have metabolic activities but cannot multiply (see e.g. Besnard *et al.*, 2000). This definition seems somewhat misnamed as some VBNC bacteria have been reported to be "resuscitated" (McDougald *et al.*, 1998), usually by using special growth conditions, adding nutrients, or mediated through an

extracellular protein (Mukamolova *et al.*, 1998). It has been suggested that the term "dormant" describes the state of the cell more accurately, as these cells can return to a "viable and cultivable" state (Kell *et al.*, 1998; Kell & Young, 2000). The following definitions are more precise:

Physiological state	Phenotype
Viable (culturable)	Capable of division; will form a colony on agar plate or proliferate observably in liquid medium
Dormant	In a state of low metabolic activity and unable to divide or to form a colony on an agar plate without a preceding resuscitation phase
Non-viable (non-culturable)	Incapable of division; will not form a colony on an agar plate nor proliferate observably in liquid medium

Table 1.1. Reproduced from Kell & Young (2000).

1.4.2. Physiology of starvation

The physiological responses of non-sporulating bacteria to starvation are extraordinarily similar from species to species, especially in the case of carbon starvation. One of them is the formation of starvation and multiple-stress resistant microcells. The formation of multiple-stress resistant cells has been observed in all bacteria studied so far. Although morphology is not a good marker of starvation, it should be noted that most bacteria change shape, becoming coccoid or round, and dramatically reduce in size, up to 90 % in the case of *Vibrio* (Baker *et al.*, 1983; Kjelleberg *et al.*, 1993). The aspect of the culture itself may change too, from smooth to rough colonies on agar or clumping and biofilm formation in broth. The rugose and biofilm phenotypes are associated with the production of exopolysaccharides in response to nutrient starvation (Wai *et al.*, 1998). The biofilm is believed to trap and adsorb nutrients and protect bacteria from predators, thus providing a more favourable environment for growth (Wai *et al.*, 1998).

A model of starvation has been developed with *Vibrio*, and has subsequently been supported by studies with other organisms. In this model, bacteria adapt to starvation-induced growth arrest by a complex pattern of regulation that turns on the expression of some genes and turns off others (Kjelleberg *et al.*, 1993). This adaptation takes place in three phases during multiple-nutrient starvation *in vitro*. The first phase is described as a stringent control phase marked by decreased rate of macromolecular synthesis, the temporary accumulation of guanosine-tetraphosphate (ppGpp) and an increased rate of protein degradation. In *Vibrio* the stringent phase takes place as soon as the cells face carbon deficiency in the medium and lasts about half an hour (Nyström *et al.*, 1990). During the second phase larger events take place, including changes in membrane fatty acid composition. The reserve material poly-β-hydroxybutyrate (PHB) is degraded and multiple stress resistance to heat, high and low pH and oxidative damage appears (Nyström *et al.*, 1992). This phase extends for up to 6 hours (Kjelleberg *et al.*, 1993). From then on, the bacteria enter the third phase, which appears as a slow shutdown of metabolic functions in which the rates of respiration and of RNA, protein and peptidoglycan synthesis plummet (Holmquist & Kjelleberg, 1993).

It was found for *Vibrio* that only starvation for carbon led to the formation of viable multiple-stress resistant cells, as opposed to cells starved for nitrogen or phosphorus (Holmquist & Kjelleberg, 1993; Nyström *et al.*, 1992). It was shown that carbon starvation could induce the same physiological changes as multiple-nutrient starvation. Comparison of the protein profile, by 2D gel electrophoresis, of cultures starved for carbon, nitrogen, phosphorus and multiple-nutrient starvation proteins showed that few proteins were specific to carbon starvation (Nyström *et al.*, 1992). In these studies, the

carbon source was also the energy source, which would indicate that energy is the most important factor in triggering the starvation survival response.

1.4.3. Molecular components of regulation of the Starvation Survival Response (SSR) in Gram-negative bacteria

1.4.3.1. The stringent response: ppGpp

In response to a non-growing state a phenomenon called the stringent response is initiated resulting in the accumulation of the "alarmone" guanosine 3′, 5′ - bispyrophosphate (ppGpp). This alarmone has been identified in all the organisms studied so far, including *E. coli*, *Salmonella*, and several Gram-positive bacteria (see below) (Gallant *et al.*, 1972; Spector, 1998). ppGpp is usually present at low concentrations in the cell, its level being stabilised by two enzymes, RelA and SpoT (Hernandez & Bremer, 1991; Xiao *et al.*, 1991). RelA is the ribosome-associated ppGpp synthetase I and is activated by uncharged tRNAs as a result of the lack of amino acids. SpoT is a bifunctional enzyme with both weak (p)ppGpp synthetic and hydrolytic properties. The lack of amino acids and starvation for a carbon or energy-source inhibit its hydrolytic activity, leading to a rapid increase in the level of ppGpp in the cell (Murray & Bremer, 1996).

The effects of ppGpp are numerous. High levels of ppGpp block DNA replication at the origin by repressing transcription of *dnaA*, the gene that encodes the initiator protein DnaA (Chiaramello & Zyskind, 1990). Although ppGpp levels were also observed to have an effect on chromosomal replication in *Bacillus subtilis*, replication was initiated but then blocked 100–200 kb downstream of the origin. Replication resumed at the blocked site once the stringent response was lifted. Above all, ppGpp influences the transcription of *rpoS*, encoding the sigma factor RpoS which in turn leads to an increase

in the expression of many starvation inducible genes (for a review, see Hengge-Aronis, 2002a).

1.4.3.2. The stationary phase sigma factor: σ^S

RpoS is the stationary phase sigma factor, also called σ^S or σ^{38}. The vegetative σ factor, σ^{70} (RpoD), is the general "house-keeping factor" that recognises transcription initiation signals on DNA. In exponentially growing cells, low levels of RpoS are present, although there are relatively high levels of *rpoS* mRNA. As the growth rate decreases and the culture enters stationary phase, *rpoS* transcription is increased 5- to 10-fold, and RpoS levels increase dramatically (Lange & Hengge-Aronis, 1994). When the concentration of RpoS increases in the cell, it displaces σ^{70} from the RNA polymerase, thereby diminishing the activity of σ^{70}. Both *in vitro* and *in vivo* work also showed that ppGpp decreased the ability for wild-type σ^{70} to compete with σ^S and bind to the core RNA polymerase (Jishage *et al.*, 2002). RpoS has been identified in *E. coli* and is present in the branch of proteobacteria. It is recognised as the key regulator of the general stress response. Regulation of RpoS occurs at every level: transcriptional, translational and post-translational. For a review, see Hengge-Aronis (2002b).

A number of factors have been implicated in the control of *rpoS* transcription, including ppGpp, polyphosphate and cAMP-CRP. Quorum-sensing systems have also been implicated in the transcriptional regulation of *rpoS* (Lazazzera, 2000). ppGpp and polyphosphates have a part in *rpoS* regulation and RpoS activity at three different points in the pathway; they increase the transcription of *rpoS*, stimulate the activation of inactive *rpoS* mRNA into active mRNA that can be translated, and enhance the binding of σ^S to the RNA polymerase core enzyme (Figure 1.2) (Al-Maghrebi & Benov, 2001; Hengge-Aronis, 2002b). This is supported by the observation of *E. coli* mutants lacking polyphosphate kinase (PPK) or RpoS. *ppk* mutants are unable to accumulate

polyphosphates and demonstrate reduced viability in stationary phase (nutrient deprivation) and are less resistant to heat, oxidative and osmotic stress (Rao & Kornberg, 1996). Interestingly, introduction of extra copies of *rpoS* on a multi-copy plasmid restored the heat resistance of the *ppk* mutants to that of the wild-type strain. Furthermore, *E. coli rpoS*$^{-}$ lacked polyphosphate protection against oxidative DNA damage (Al-Maghrebi & Benov, 2001).

A complex network of proteins and regulatory RNAs is involved in the activation of *rpoS* mRNA (see Figure 1.2), some of which are induced at late log phase by ppGpp and by oxidative stress (HU, histone-like protein; Hfq, host factor for coliphage Qβ and DsrA, small RNA regulator of transcription). UDP-glucose, whose concentration increases in the presence of glucose or galactose, decreases the translation levels of RpoS, although the level at which it acts, its mode of action and whether its cellular levels changes during stress or starvation is unknown. Histone-like protein H-NS is activated by cold and inhibits HU, Hfq and DsrA, as well as the activation of *rpoS* mRNA. Transcription of regulatory RNA OxyS is induced by oxidative stress, including H_2O_2, and it subsequently reduces the transcription of *rpoS* RNA by binding to and inhibiting Hfq. Factors identified to date involved in translational regulation of RpoS include changes in *rpoS* mRNA secondary structure. Theoretical predictions of the *rpoS* mRNA secondary structure have been made, although the *in vivo* structure remains to be determined (Hengge-Aronis, 2002b).

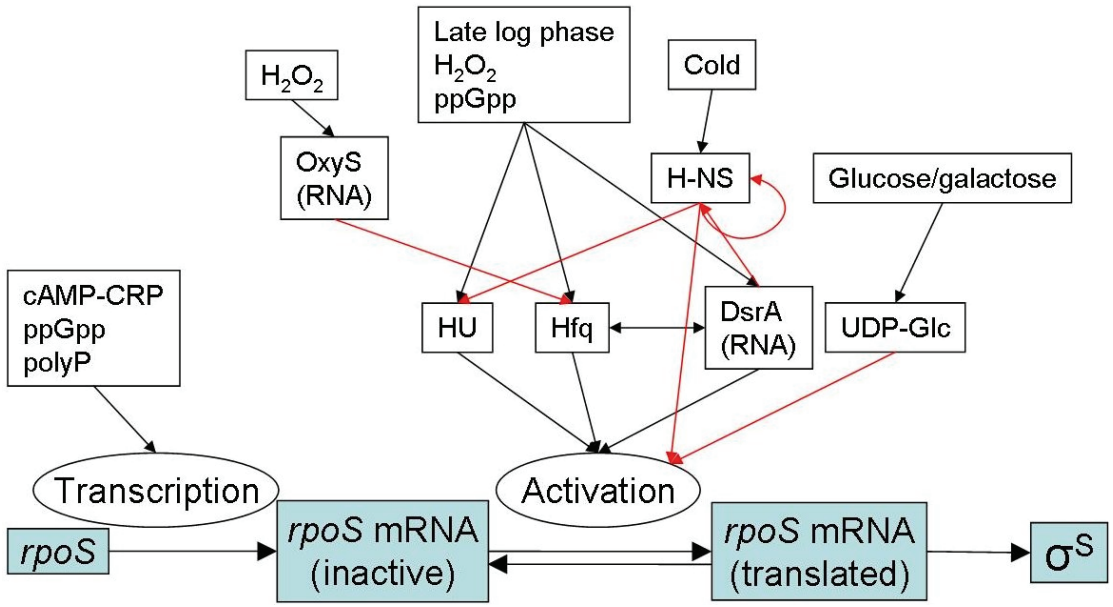


Figure 1.2. Transcriptional and translational regulation of *rpoS*, leading to synthesis of σ^S. Black arrows, induction; red arrows, repression. See text for details. Adapted from Hengge-Aronis (2002b).
cAMP-CRP, cAMP-receptor protein; ppGpp, guanosine 3′, 5′-bispyrophosphate; polyP, polyphosphate; HU, histone-like protein; Hfq, host factor for coliphage Qβ; H-NS, histone-like protein; DsrA, small RNA regulator of transcription; OxyS, regulatory RNA induced by oxidative stress.

A number of small regulatory RNA molecules targeting *rpoS* translation have been identified in *E. coli* and include DsrA, OxyS and RprA (reviewed by Hengge-Aronis, 2002b). DsrA plays a role in low-temperature induction of *rpoS* translation (Sledjeski *et al.*, 1996), while OxyS is induced by oxidative stress (Altuvia *et al.*, 1997). RprA also stimulates RpoS translation, but the physiological conditions by which it responds have yet to be determined.

In addition to transcriptional and translational control, control of RpoS activity is also possible through regulated proteolysis by the ClpXP protease. RssB is a targeting factor which, when phosphorylated, exhibits specificity for RpoS and thereby tags RpoS for proteolysis by the ClpXP protease (see Figure 1.3) (Jenal & Hengge-Aronis, 2003; Weichart *et al.*, 2003). Production of RssB is controlled by σ^S. The integration of all these signals to control σ^S remains to be elucidated.

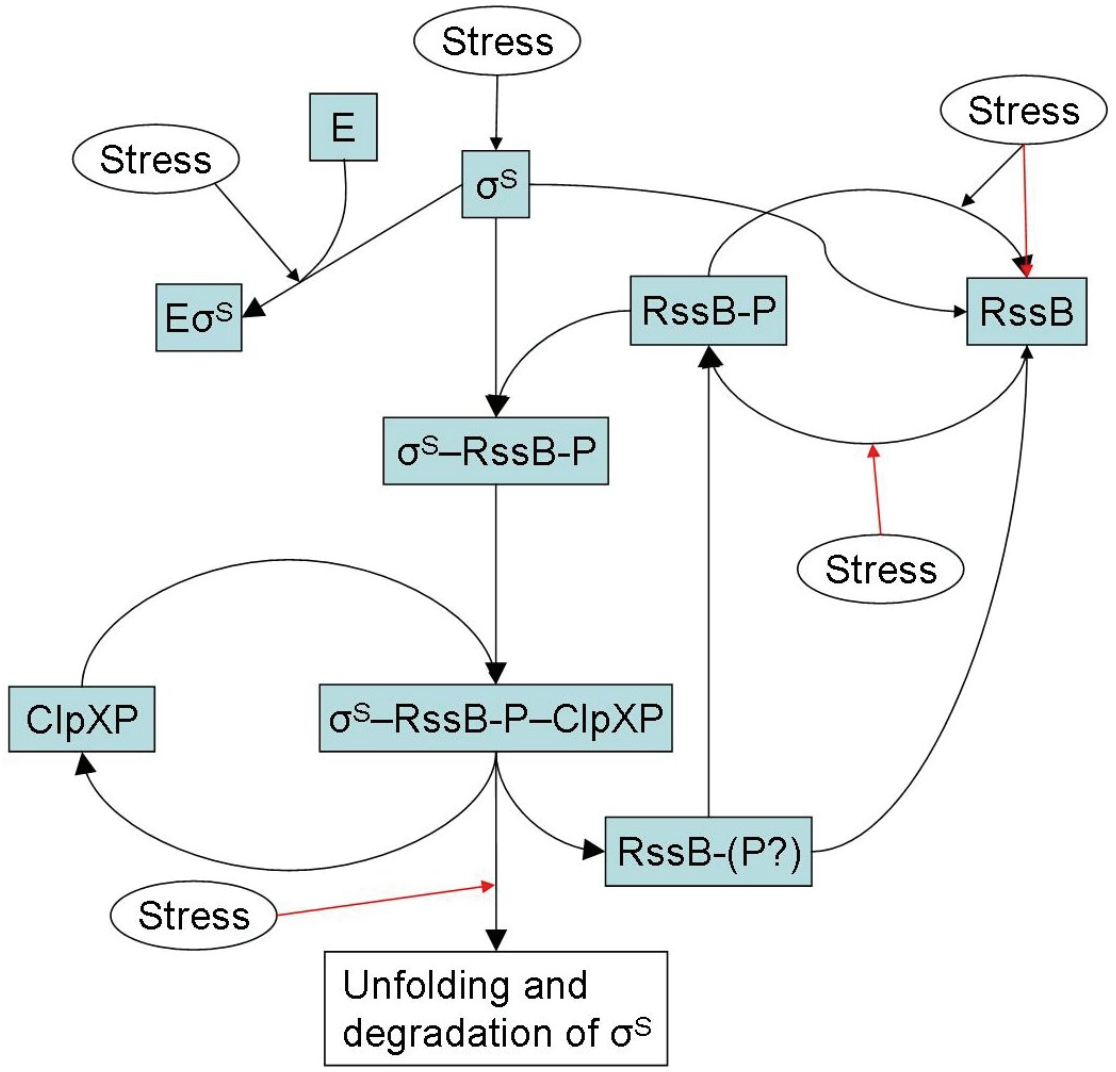


Figure 1.3. Recognition and degradation pathway of σ^S. σ^S displaces σ^{70} from RNA polymerase (E), leading to expression of a different transcriptome. Which stresses intervene at each stage of the pathway has not yet been determined, but the half-life of σ^S is known to increase under sudden carbon starvation, heat shock, osmotic upshift and acidic pH. Black arrows, induction; red arrows, repression. Adapted from Hengge-Aronis (2002b).
E, RNA polymerase; ClpXP, ATP-dependent protease consisting of proteolytic (ClpP) and chaperone (ClpX) subunits; RssB, two-component-type response regulator.

1.4.3.3. Gene expression controlled by σ^S

It is estimated that the expression of more than 70 genes is affected by σ^S, encoding functions leading to altered morphology and metabolism, increased survival during starvation and fluctuating environmental conditions, and also factors leading to increased virulence and cell death (Hengge-Aronis, 1993; Hengge-Aronis, 2002b). Many of the RpoS-dependent genes are not required during the early stationary phase, but provide protection for the cell when *de novo* protein synthesis might not be possible. The *otsA* and *otsB* genes, for example, encode enzymes required for production of the osmoprotectant trehalose. The regulation of these genes is RpoS-dependent and they are induced in response to osmotic shift during the exponential phase (Hengge-Aronis, 1996) and during entry into stationary phase (Hengge-Aronis *et al.*, 1991).

Microarray analysis of the whole genome of *E. coli* K-12 has shown that up to 481 genes are up-regulated by RpoS during transition from exponential to stationary phase (Weber *et al.*, 2005). Of this grand total, 252 were found to be up-regulated during transition to stationary phase, 422 in response to osmotic challenge and 197 in response to acid stress. Interestingly, 140 genes were always up-regulated by RpoS, independently of the challenge. Of those 140 genes, 16 are involved in stress protection (heat, osmotic pressure, oxidative damage etc.), 26 are involved in central energy metabolism (glycolysis, fermentation, pentose phosphate shunt), 11 in regulation, 20 in transmembrane transport, 7 in protein processes and finally 60 were of other or unknown function (Weber *et al.*, 2005). In this study however, the transition was from growth in LB, which is a highly complex medium that can sustain high cell densities, as illustrated by the fact that the authors chose an OD_{600nm} of 4.0 as the transition point between exponential and stationary phase (Weber *et al.*, 2005).

When *E. coli* K-12, grown in a chemostat in chemically defined medium with 0.01 % glucose, was compared with exponential-phase *E. coli* in 0.1 % glucose, 340 genes were found to be up-regulated and 502 down-regulated (increase or decrease by a factor of more than 2) (Franchini & Egli, 2006). Among these, 181 up-regulated genes increased expression by a factor of more than 5, and 81 down-regulated genes decreased expression by a factor of more than 10. Surprisingly few of these were stress resistance-associated genes (6 up-regulated, 5 down-regulated). The other genes are involved in transport (32 up-regulated, 11 down-regulated), carbon and energy metabolism (30 up-regulated, 7 down-regulated), regulation (14 up-regulated, 12 down-regulated), cell structure (7 up-regulated, 9 down-regulated), protein processing (1 up-regulated, 25 down-regulated), amino acid biosynthesis (1 up-regulated, 1 down-regulated), nucleic acid biosynthesis (1 up-regulated, 12 down-regulated), other functions (3 up-regulated, none down-regulated) and unknown or hypothetical functions (56 up-regulated, 15 down-regulated) (Franchini & Egli, 2006). The differences between this study and that by Weber *et al.* (2005) are probably due to the different media employed (i.e. chemically defined *vs* LB) and differences in protocol (chemostat *vs* batch), as well as different cut-off points for inclusion in up- or down-regulated lists (i.e. 2- and 5-fold change *vs* 4-fold change in gene expression).

1.4.4. Molecular components of regulation of the SSR of *Bacillus subtilis*

1.4.4.1. Sigma factor, SigB (σ^B)

In *Bacillus subtilis*, the secondary sigma factor σ^B is a key element in the control of the general stress response (for a review, see Hecker *et al.*, 1996). The σ^B-dependent general stress regulon encompasses over 100 stress genes differentially expressed in response to glucose, phosphate or oxygen starvation, and heat, alcohol, acid and osmotic stress (Voelker *et al.*, 1995). The mechanism by which σ^B is activated has been well studied. In contrast to σ^S, it does not rely on ppGpp but reacts to the ratio of ATP to ADP in the cell. The *sigB* operon comprises eight genes transcribed constitutively from a σ^A (housekeeping factor)-dependent promoter upstream of *rsbR* (Wise & Price, 1995). There is also a second internal promoter recognised by σ^B located immediately upstream of *rsbV*, which leads to increased expression of *rsbV*, *rsbW*, *sigB* and *rsbX* (Duncan *et al.*, 1987). Although all proteins encoded by the *sigB* operon are probably involved in the regulation of σ^B activity, RsbW is the primary regulator.

RsbW can bind either to σ^B, thereby sequestering it and preventing its activity, or to RsbV, resulting in free σ^B, which can then activate transcription of σ^B-dependent genes. The formation of the RsbW–RsbV complex is controlled by RsbW and influenced by RsbU and RsbX (Boylan *et al.*, 1992; Duncan *et al.*, 1987). Scientific evidence to date supports the presence of two independent pathways for activation of σ^B. In the case of starvation for glucose or phosphate, RsbW activity responds to the ATP:ADP ratio. It has been confirmed for *B. subtilis* that glucose and phosphate limitations result in a reduction in the intracellular level of ATP (Voelker *et al.*, 1995). In the presence of low concentrations of ATP, RsbW (a protein kinase) cannot phosphorylate RsbV, as a result of which the RsbW–RsbV complex is then formed. Therefore, the σ^B factor is free to

interact with the RNA polymerase and to drive expression from σ^B-dependent promoters. For reviews, see Hecker *et al.*(1996) and Hecker and Volker (1998).

Activation of *sigB* in response to glucose or phosphate starvation confers multiple-stress resistance to *B. subtilis* through the expression of over 100 genes, involved in heat, oxidative, water and osmotic stress resistance, as well as many with unknown function (Hecker & Völker, 1998; Völker *et al.*, 1999). Interestingly, characterisation of a *sigB* mutant revealed that σ^B, although activated by glucose and phosphate starvation, is not required for starvation survival (Völker *et al.*, 1999). It is therefore believed that σ^B provides additional protection to the bacteria against stresses that could arise during long-term starvation.

1.4.4.2. Stringent response in *Bacillus*

A stringent response mechanism, similar to that in *E. coli*, has been reported in *B. subtilis*. A bifunctional RelA enzyme with the activity of the *E. coli* RelA and SpoT has been isolated from *B. subtilis*. As in *E. coli*, this enzyme provokes an increase in intracellular ppGpp levels under nutrient-limited conditions (Wendrich & Marahiel, 1997). This results in a more than threefold decrease in the expression of translational apparatus genes, including rRNAs, tRNAs, ribosomal proteins (almost all, including *nusA*, *rpoA* and *rpoB*) and translation factors (e.g. *tig*, *infA–C*, *fus*, *tufA* and *tsf*) (Eymann *et al.*, 2002). Genes expected to be expressed in actively growing cells (i.e. involved in nucleotide biosynthesis, synthesis of lipids, energy metabolism, RNA modification, protein modification, cell wall synthesis and DNA replication) are also repressed (Eymann *et al.*, 2002). Relatively few transcription units were up-regulated by the stringent response (approximately 50). These include, in particular, the branched-chain amino acid biosynthetic operon (Ile, Val and Leu; *ilv–leu*) and a γ-aminobutyrate

permease, urease, serine proteases and alkaline protease (*gabP*, *ure*, *vpr* and *epr*, and *aprE*). This possibly reflects the need to relieve the amino acid-induced starvation response (Eymann *et al.*, 2002). Sporulation-associated genes are also up-regulated (e.g. *yvyD*, *ytxGHI*, *spo0A*, and *spoVG*), presumably so that sporulation can occur quickly under worsening conditions. Additionally, a *relA* mutant was found to sporulate less efficiently than the wild-type strain (Eymann *et al.*, 2002). The stringent response in *Bacillus* also results in termination of DNA replication, located approximately 100–200 kb on either side of *oriC*. A contrahelicase, RTP (replication terminator protein), prevents further progression of the replisome by binding constitutively to LSTer and RSTer (left and right stringent termini), approximately 100 kb up- and downstream of the origin of replication on the chromosome. ppGpp does not increase the DNA-binding affinity of RTP. It is interesting to note that, in a mutant lacking RTP, the stringent response retarded the replisome, showing that other mechanisms are involved in stopping replication (Autret *et al.*, 1997).

1.4.5. Starvation survival response of *Staphylococcus aureus*

Staphylococcus aureus is an important human pathogen resulting in diseases ranging from mild skin infection to life-threatening septicaemia. It is of particular concern in hospitals as it is one of the most important causes of nosocomial infection, due to its ability to survive for extended periods of time outside of the host. During this period, *S. aureus* is able to survive long-term starvation and fluctuating environmental conditions. *S. aureus* was proven to survive at least 7 days of carbon starvation in *in vitro* experiments (Clements & Foster, 1998). Further characterisation of the SSR showed responses similar to those already identified in other bacteria, including reductive cell division, and an increase in resistance to acid shock and oxidative stress (Watson *et al.*, 1998b). In order to further investigate this, Watson *et al.* (1998a)

screened a mutant bank for starvation survival deficient mutants (i.e. mutants that were unable to survive starvation-induced stationary phase). Based on sequence analysis, the genes identified encoded a putative superoxide dismutase, a haem A synthase, an RNA polymerase σ subunit, an SOS response component and a phosphoribosyltransferase. Further work confirmed the presence of a haem A synthase (*ctaA*) and a major superoxide dismutase (*sodA*) in *S. aureus* and their role in starvation survival (Clements *et al.*, 1999a; Clements *et al.*, 1999b).

Superoxide dismutases (SOD) detoxify superoxide-anions resulting in production of hydrogen peroxide. In many bacteria, including *E. coli*, *Bacillus* and *Listeria*, increased production of SOD has been observed during stationary phase growth (Dukan & Nyström, 1999; Inaoka *et al.*, 1998; Nyström, 1999; Vasconcelos & Deneer, 1994). This may be part of a mechanism used by the bacterium to protect proteins from oxidative damage, an important activity considering that there is a low protein turnover due to a low level of *de novo* protein synthesis. Analysis of the *S. aureus sod* mutant revealed a low level of survival in stationary phase of growth (Watson *et al.*, 1998a). The *S. aureus sod* mutant demonstrated a survival defect with amino-acid limitation only. During carbon and phosphate limitation it behaved identically to the parent strain (Watson *et al.*, 1998a). The effect of the *sodA* mutation during amino acid starvation is dependent on the degree of aeration. When incubated statically the behaviour of the *sodA* mutant is identical to the parent strain. In contrast, increased aeration results in a dramatic reduction in the viability of the mutants (Clements *et al.*, 1999a). Interestingly, the *sodA* mutant demonstrated increased sensitivity to acid compared to the wild-type strain, although the role of *sodA* in acid resistance remains to be investigated. Using a mouse abscess model to determine the consequence of the *sodA* defect on pathogenicity, no significant differences were observed between the mutant and wild-type strain.

The stringent response in *S. aureus* results in a sharp increase in ppGpp intracellular concentration following nutrient (carbon or amino acid) starvation (Crosse *et al.*, 2000). Immunological analysis revealed that *S. aureus* contains a protein exhibiting cross-reactivity with *E. coli* anti-RelA and anti-SpoT antibodies.

A gene homologous to the *B. subtilis sigB* has been identified in *S. aureus*, although the genetic organisation differs, in that the *S. aureus sigB* locus lacks the upstream *rsbR*, *rsbS* and *rsbT* genes (Kullik & Giachino, 1997; Wu *et al.*, 1996). Analysis of a *S. aureus* mutant revealed that σ^B, although not essential for starvation survival, contributes to increased stress resistance (Chan *et al.*, 1998).

1.4.6. Starvation survival of *Mycobacterium*

Mycobacterium tuberculosis is a pathogen that kills over 2 million people every year, and one third of the world population is believed to be currently infected (WHO, 2002). The incidence of *M. tuberculosis* infection is on the increase in developed and developing countries. One of the main challenges facing control of this pathogen is that *M. tuberculosis* cells can remain dormant in lung lesion tissue and can reactivate years later, leading to tuberculosis. The mechanisms that enable *Mycobacterium* to survive for long periods are poorly understood.

In order to gain an understanding of the molecular basis for dormancy, both *M. tuberculosis* and *M. smegmatis*, a fast-growing relative, have been studied. *M. smegmatis* was shown to be able to survive carbon, nitrogen or phosphorus starvation for 650 days *in vitro* in chemically defined medium (Smeulders *et al.*, 1999). During this time they were shown to undergo reductive cell division and exhibited

resistance to oxidative, osmotic and acid stress. Following starvation, mycobacterial cells tend to clump, probably as a result of changes in surface characteristics (Shleeva *et al.*, 2002; Smeulders *et al.*, 1999).

Non-culturable cells could be induced *in vitro* in *M. tuberculosis* by letting them grow into late stationary phase (4–5 months) in modified Sauton's medium (chemically defined, 6 % glycerol v/v, for details see Shleeva *et al.*, 2002). It is not known whether the cells stop growing because of nutrient exhaustion or cell density. At this stage, the cells lost their culturability (0 CFU for *M. tuberculosis*) on solid agar, but could be recovered by culture in liquid medium. The recovery was much improved by the addition of filtered spent medium to fresh broth or the addition of Rpf from *Micrococcus luteus*. Rpf is the resuscitation promoting factor found to resuscitate dormant cells of *Micrococcus luteus*, and that also stimulates growth of other high G+C organisms, including mycobacteria (Mukamolova *et al.*, 1998). Similar predicted genes were found in *M. tuberculosis*, *M. leprae* and *M. smegmatis*, in addition to several other high G+C such as *Corynebacterium glutamicum* and *Streptomyces*. Rpf from *Micrococcus luteus* was found to resuscitate *M. tuberculosis* and *R. rhodochrous* (Shleeva *et al.*, 2002). Further work on putative *rpf* genes of *M. avium* and *M. tuberculosis* showed the proteins they encode have similar resuscitating activities to Rpf from *Micrococcus luteus* (Zhu *et al.*, 2003).

1.4.6.1. Stringent response in *Mycobacterium*

Homologues of RelA and SpoT were found in a single protein in *M. tuberculosis* and named Rel_{Mtb} (Avarbock *et al.*, 1999). *In vitro* studies demonstrated that Rel_{Mtb} catalyses the synthesis of (p)ppGpp, consistent with the activity of *E. coli* RelA. The purified Rel_{Mtb} also possesses 3′-pyrophosphohydrolase activity, correlating with *E. coli*

SpoT activity. This provides further support for the findings that Gram-positive bacteria possess one protein for both synthesis and degradation of (p)ppGpp. Characterisation of a knockout Rel_{Mtb} mutant revealed that the mutant grew significantly more slowly in synthetic medium than the wild type strain (Primm *et al.*, 2000). Interestingly, no significant differences were observed for the growth rate of the mutant and wild type in human macrophage-like cell lines (Primm *et al.*, 2000).

1.4.6.2. Transcription factors

The first transcription factor from *M. tuberculosis* to be associated with the stationary phase is SigF (DeMaio *et al.*, 1996). It has a close homology to SigF and SigB, sporulation and stress-response sigma factors, respectively, from *B. subtilis*. SigF was shown to be transcribed during stationary phase, nitrogen depletion, cold and alcohol shock, and oxidative stress. Analysis of gene expression in *M. bovis* BCG (Bacille Calmette–Guérin) strain grown in complex medium revealed strong induction of *sigF* expression during stationary phase, nitrogen depletion and cold shock. Weak induction of *sigF* transcription was observed during the late-exponential phase in response to alcohol shock and oxidative stress. Since no measurements were taken during the late-stationary phase, it is not known if *sigF* expression is transitional.

In a study of the transcription of *sigA* and *sigB* in *M. tuberculosis* (Hu & Coates, 1999), the level of *sigA* mRNA remained constant from early exponential phase through to late stationary phase, and was not affected by stress. The level of *sigB* on the other hand was low during exponential phase, increased to its maximum in late-exponential phase and then slowly decreased. Expression of *sigB* was also influenced by environmental stress, in that induction of transcription was observed following oxidative stress and heat shock. These results suggest that SigA may be the housekeeping sigma factor, similar to

σ^{70} of *E. coli*, whereas SigB may be a stationary-phase sigma factor responsive to starvation and environmental stress. SigB and SigF could well work together, each with its own regulon, to ensure survival of dormant cells.

1.4.6.3. Mutant, whole-genome and proteome analyses of *Mycobacterium* – response to starvation

In a study by Keer *et al.* (2000), mutants of *M. smegmatis* were generated by transposon mutagenesis, and screened for starvation survival deficiency, i.e. mutants unable to survive carbon-starvation-induced stationary phase. Five starvation survival deficient mutants were further analysed and the genes disrupted were partly sequenced. Based on bioinformatic studies the predicted genes encoded a putative penicillin-binding protein, a polyketide synthase, a monoamine oxidase, a membrane protein and an enzyme for biosynthesis of biotin.

Disruption mutants of the predicted *bioA* gene were auxotrophic for biotin, thereby confirming the bioinformatic analysis. Interestingly, mutants harbouring a disruption of a gene encoding a putative membrane protein have altered colony morphology when grown on agar. The isolation of a stationary-phase mutant harbouring a disruption of a gene predicted to encode a putative penicillin-binding protein is supported by observations in *E. coli*. Penicillin-binding proteins are needed for cell-wall synthesis. Studies of *E. coli* stationary phase cultures indicate an increase in the level of specific penicillin-binding proteins, in comparison with exponential-phase cultures (Dougherty & Pucci, 1994). The roles of the polyketide synthase and the monoamine oxidase in stationary-phase and starvation survival are unknown.

An interesting study, based on microarray analysis of *M. tuberculosis* starvation, during which the expression of 3649 genes was monitored, has been published (Betts *et al.*,

2002). Monitoring after 4, 24 and 96 hours of multiple-nutrient starvation (cells washed and resuspended in PBS) revealed that several hundred genes were significantly up- or down-regulated. For example, at 96 hours, 279 and 323 genes were up- and down-regulated, respectively (Betts *et al.*, 2002). Up-regulated genes included those involved in antibiotic production and resistance, nucleotide synthesis and metabolism, and sequences related to insertion sequences and phages. In addition to the up-regulation of a number of regulatory genes (e.g. *sigB*, *sigD*, *sigE* and *sigF*), it is interesting to note that the majority of up-regulated genes correspond to proteins with hypothetical or unknown functions (e.g. Rv0116c, encoding a 26.9 kDa protein homologous to a putative secreted protein from *M. leprae*). Down-regulated genes included those involved in the synthesis of amino acids, lipids, prosthetic groups, carriers and cofactors, in addition to genes involved in DNA replication, repair, recombination, restriction and modification, and translation and post-translation modifications. Of the 281 energy metabolism genes, 15 % were significantly down-regulated. In addition, several virulence-associated genes were down-regulated, e.g. the isocitrate lyase required for *in vivo* survival of *M. tuberculosis* and polyketide *pk52* induced during macrophage infection.

Two-dimensional gel electrophoresis analysis was performed in the same study and revealed up-regulation for only three proteins, including an α–crystalline homologue and 24.3 and 25.7 kDa hypothetical proteins, of unknown function. The 16 kDa α–crystalline homologue is believed to enhance long-term protein stability, and it was found that only oxygen limitation could increase its synthesis (Yuan *et al.*, 1996).

Four down-regulated proteins were identified, although two of these proteins were recovered in culture filtrates of *M. tuberculosis*. The down-regulated proteins included

Tig, a chaperone-like protein, and GrpE, which together with DnaJ is a chaperone for DnaK (Pierpaoli *et al.*, 1998). Although few up- or down-regulated proteins were identified in this study, it should be noted that the 2-D gel analysis on the *M. tuberculosis* proteome was performed on 6-week multiple-nutrient-starved cultures. This is in contrast to the microarray experiment which monitored cultures in early stationary phase (Betts *et al.*, 2002). It is well known that there is a sharp increase in protein synthesis just before the cells enter stationary phase, and therefore these changes were presumably captured in the microarray experiment.

Proteome analysis of a carbon-starved *M. smegmatis* culture identified a Dps homologue which is preferentially expressed under starvation conditions (Gupta *et al.*, 2002). The Dps (DNA-binding protein from starved cells) of *E. coli* is expressed during stationary phase and then binds non-specifically to the DNA, thereby protecting it against DNase and oxidative damage in particular (Almirón *et al.*, 1992). The Dps-like protein from *M. smegmatis* (Ms-Dps) was purified and shown to bind non-specifically to DNA. In the presence of Ms-Dps, DNA was protected from oxidative radicals as demonstrated by an *in vitro* DNA damage assay. The Ms-Dps protein also protected the DNA from DNaseI activity (Gupta & Chatterji, 2003). Dps-like proteins have been identified in other Gram-positive organisms including *B. subtilis* and *L. innocua* (Bozzi *et al.*, 1997; Chen & Helmann, 1995).

1.4.7. Starvation survival of *Rhodococcus*

Few studies so far have focussed on the starvation survival of *Rhodococcus*. Most of the information available to date is circumstantial, such as when cells are grown in defined mineral media to stationary phase for accumulation of a biocatalyst (see e.g. Begoña Prieto *et al.*, 2002). Survival of *Rhodococcus* species has also been demonstrated in

bioremediation studies using a mixed culture added to polluted seawater (Shkidchenko *et al.*, 2004) as well as in soil (Briglia *et al.*, 1990; Peressutti *et al.*, 2003). Both of these environments are notoriously low in energy sources and have been described as generally oligotrophic (Morita, 1993). Remarkably, *Rhodococcus chlorophenolicus* was shown to survive for 200 days in soil, all the while degrading polychlorophenol (Briglia *et al.*, 1990). It is also clear from numerous environmental studies that rhodococci are resilient organisms, as they have been isolated from a very wide range of environments, from the deep sea (Heald *et al.*, 2001) to the Antarctic soil (Bej *et al.*, 2000).

In one of the only studies dedicated to the starvation survival of *Rhodococcus*, *R. corallinus* was shown to recover from total carbon or nitrogen starvation for up to 5 months (Sanin, 2003). Following starvation, when placed in normal growth medium (0.4 % glucose, w/v), maximum (non-starved) growth rates were achieved after just 5 days in the case of cells that had been starved for carbon, or 10 days if the cells had been starved for nitrogen. The hydrophobicity of the cells was also measured, but little variation was observed (Sanin, 2003; Sanin *et al.*, 2003).

In another study, a large but transient drop in culturability (5 logs) was observed in *Rhodococcus rhodochrous* by allowing the cells to enter late stationary phase (60 hours growth) in modified Sauton's medium (chemically defined, 6 % glycerol v/v, for details see Shleeva *et al.*, 2002). It is not known whether the cells stopped growing because of nutrient exhaustion or cell density. At this stage, the cells lost their culturability on solid agar, but could be recovered by culture in liquid medium, suggesting that the cells entered a temporary state of non-culturability. Rpf from *Micrococcus luteus* was found to greatly increase "resuscitation" of "non-culturable" *R. rhodochrous* (Shleeva *et al.*, 2002).

1.5. Objectives

As described above, the starvation survival of *Rhodococcus* has not been studied in great detail, in spite of the importance that organisms of this genus hold for industrial processes and bioremediation. Indeed, *R. erythropolis* has been suggested as an important future bioremediation agent (Čejková *et al.*, 2005), and an agent in the biodesulphurisation of fuel (Caro *et al.*, 2007). In addition, with the exception perhaps of *R. equi*, this genus remains relatively unexplored in terms of genetic analyses. Relatively few tools are available for the genetic manipulation of these organisms and complete genome sequences are only now becoming available.

This study consists of two major components. An objective of this research was to study the physiological adaptation of a selected *Rhodococcus* strain, *R. erythropolis* SQ1, to energy and carbon starvation, in particular in terms of (i) its capacity to survive starvation, and (ii) the cross-protection to other environmental insults such as heat and oxidative stress, afforded by the transition to nutrient-induced stationary phase.

In addition, the second objective was to generate a mutant bank of *R. erythropolis* SQ1 and to screen this bank for starvation survival mutants, with subsequent sequence analysis of the mutated genes, in order to gain an insight to the molecular mechanisms involved in the starvation survival response of *Rhodococcus*.

Chapter II
Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The bacterial strains used in this study are described in Table 2.1. *Rhodococcus* spp. and *Escherichia coli* were routinely grown overnight in Luria-Bertani (LB) (Atlas, 1996) or Glucose Yeast Extract (GYE) broth on a rotary shaker (200 rpm) and incubated at 27 and 37 °C, respectively. For growth on solid media, 13 g/l of bacteriological agar (Oxoid) was added to the broth. Media were sterilised by autoclaving at 121 °C and 15 pounds per square inch of pressure for 15 min. When required, media were supplemented with antibiotics at concentrations of 200, 100 and 40 µg/ml of kanamycin, ampicillin and chloramphenicol (all Sigma-Aldrich), respectively, for *Rhodococcus*. Final concentrations of 50 µg/ml of kanamycin or 100 µg/ml of ampicillin were used for *E. coli*. All strains were stocked at −20 and −80 °C in 20 % and 40 % glycerol, respectively.

Species	Strain	Origin/characteristics	Reference
Rhodococcus erythropolis	SQ1	Chloramphenicol- and arsenic-sensitive mutant of type-strain ATCC 4277 with increased transformability.	Quan & Dabbs (1993)
	Ac201*	Isolated from spring water, Perm region, Russia; uses hydrocarbons as sole carbon source; produces biosurfactants when growing on *n*-alkanes.	Ivshina *et al.* (1995)
	8147†	Isolated from soil.	Bisset & Moore (1950)
	8863†	Isolated from chalk soil.	Metcalfe & Brown (1957)
Rhodococcus equi	GV1	Clinical isolate. D. Taylor, University of Glasgow Vet School, UK.	Bell *et al.* (1996)
	VI1	Clinical isolate. Veterinary Investigation Centre, Edinburgh, UK.	
	10027†	Isolated from soil. Can degrade cholesterol.	Tai & Sih (1970); Turfitt (1944)
	10701†	Degradation of cholesterol, US patent 3,056,731.	Isono & Abe (1962)
Rhodococcus ruber	Ac72*	Core sample from the depth 110 m, Byelorussia. Uses propane and *n*-butane as sole carbon source; produces biosurfactants when growing on *n*-alkanes; forms cholesterol oxidase.	Ivshina *et al.* (1995)
	Ac74*	Ordovik age sand soil, Irkutsk region, Eastern Siberia, Russia. Uses propane and *n*-butane as sole carbon source (55); produces biosurfactants when growing on *n*-alkanes.	Ivshina *et al.* (1994)
	Ac82*	Ground water, Masuninskoye oilfield outline zone, Perm region, Russia. Uses propane, *n*-butane, liquid *n*-alkanes and aliphatic alcohols (ethanol, *n*-propanol, *n*-butanol) as sole carbon source.	Ivshina *et al.* (1995)
	Ac87*	Core sample 39 m deep, Uljanovsk region, Russia. Uses propane, *n*-butane, liquid *n*-alkanes and aliphatic alcohols (ethanol, *n*-propanol, *n*-butanol) as sole carbon source.	Ivshina *et al.* (1994)
Rhodococcus fascians	Ac170*	Snow, oil field, Perm oblast, Russia. Can degrade *n*-alkanes and fix cesium.	Ivshina *et al.* (2002); Vomberg & Klinner (2000)
Escherichia coli	DH5α	High-efficiency competent cells, Invitrogen.	Hanahan (1983)

Table 2.1. Species and strains used during this study and their provenance.

*Obtained from the Regional Specialized Collection of Alkanotrophic Microorganisms, Institute of Ecology and Genetics of Microorganisms, Urals Branch, Russian Academy of Sciences, Perm, Russia.
†Obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

2.2. Growth curves of R. erythropolis

Growth curve experiments were performed to establish the growth rate of *R. erythropolis* SQ1 and to determine when cultures entered stationary phase. Optical density (OD_{600nm}) and viable plate count measurements were used to monitor the viability of *Rhodococcus* and entry into stationary phase. Unless otherwise stated, cultures used to inoculate the medium were in the exponential growth phase. To ensure this, *R. erythropolis* SQ1 stored at −20 °C was grown at 27 °C and 200 rpm in the same medium as that used for the growth curve. When this culture reached an OD_{600nm} between 0.5 and 1.0 (early exponential phase), it was inoculated at 1 % (v/v) in the same medium. After overnight growth at 27 °C and 200 rpm, this culture was used to inoculate 100 ml medium (in 500 ml conical flasks) at an OD_{600nm} 0.01. The flasks were then incubated at 27 °C and 200 rpm. Samples were taken periodically to determine the OD_{600nm} and the viable plate count (Figure 2.1).

To measure the viable plate count, 1 ml of culture was sampled for a serial dilution. Dilutions were performed in 9 ml quarter strength (Ringer) saline supplemented with 0.05 % (v/v) Tween. Aliquots (100 µl) of the appropriate dilutions were then plated in triplicate on LB agar. The plates were then incubated for at least two days at 27 °C and the viable plate count calculated as colony forming units per ml (CFU/ml).

Where specified, glucose concentration in the culture medium was determined during the course of growth. A 1 ml sample of culture was taken and centrifuged at 16,000×g for 1 min. The supernatant was then filter-sterilised (0.22 µm, Millipore) before storage at −20 °C. The quantity of glucose in the samples was measured using a colorimetric hexokinase-based method (Glucose HK assay kit; Sigma-Aldrich), following the manufacturer's protocol. The standard curves were done using chemically defined

medium containing 0.1 % and 1 % glucose. When required, samples were diluted with glucose-free chemically defined medium, so that the only variable would be the glucose concentration.

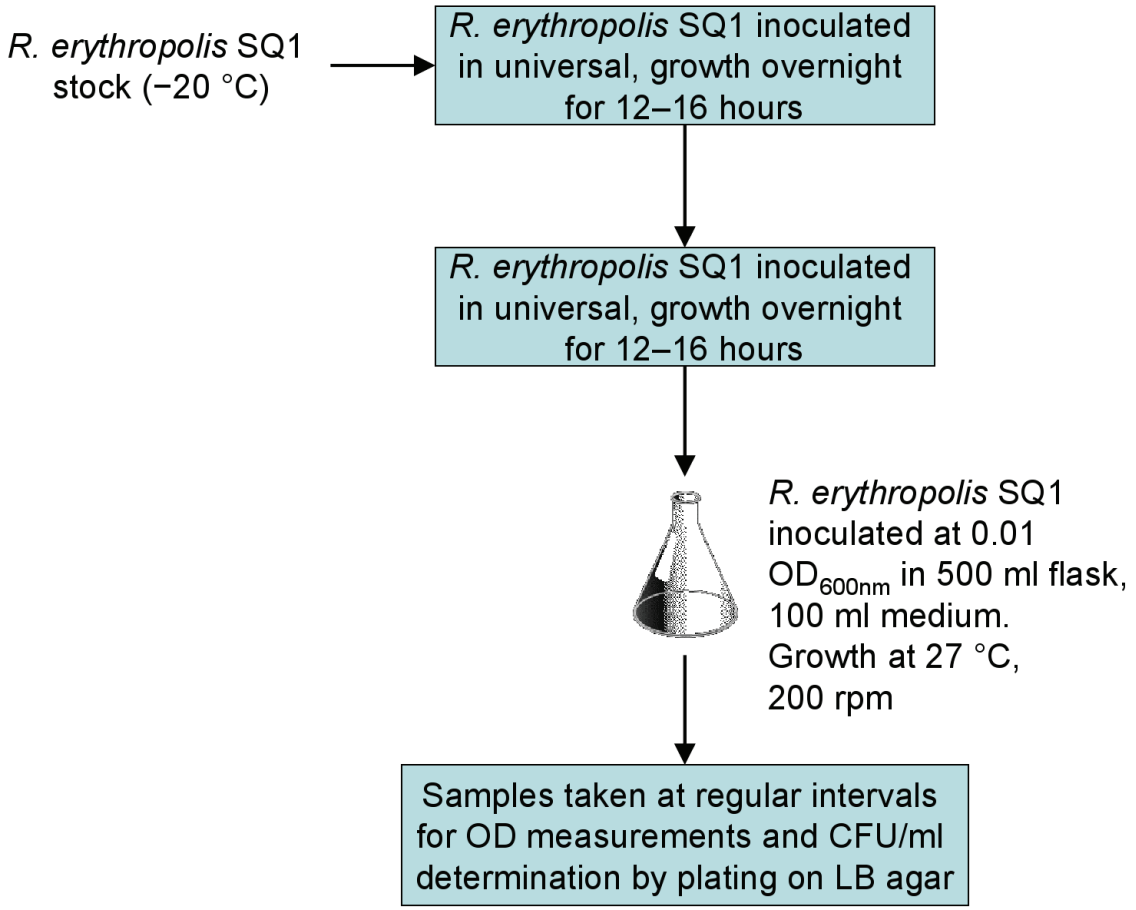


Figure 2.1. Schematic overview of the growth experiments methodology.

2.3. Measurement of impact of heat shock on cell viability

The ability of *R. erythropolis* SQ1 to survive heat shock was evaluated by using a modification of the protocol described by Jenkins *et al.* (1988). *R. erythropolis* SQ1 was grown overnight in 100 ml of chemically defined medium (CDM IIA) containing 0.1 % glucose at 27 °C and 200 rpm to ensure that entry into stationary phase was due to depletion of the carbon source. The inoculum was prepared as described in Section 2.2. The composition of chemically defined medium is provided in Section 2.15. Ten ml samples were taken at mid-exponential and early-stationary phase. The exponential phase samples were taken after approximately 48 h of growth at an OD_{600nm} 0.5. Additional samples were taken after 6 days of incubation (early-stationary phase).

The samples were aliquoted (1 ml) in 1.5 ml eppendorfs pre-warmed at 57 °C. The aliquots were then incubated in a 57 °C waterbath for up to 2 h. At 0, 5, 10 and 15 min one sample was removed from the waterbath and maintained at room temperature for 5 min to cool down. Serial dilutions of the samples were then prepared using quarter strength (Ringer) saline solution supplemented with 0.05 % (v/v) Tween and 100 µl aliquots were plated in triplicate on LB-agar. The plates were then incubated for 48 h at 27 °C and the viable count (CFU/ml) determined. In parallel, an additional late-log-phase sample was diluted prior to heat shock treatment to an OD_{600nm} of approximately 0.5 and a titre of 5.05×10^8 CFU/ml (which was reached in mid-exponential phase) in order to determine whether cell density affected the heat resistance profile. Since no difference was observed, the protocol described above was used.

2.4. Measurement of impact of oxidative damage on cell viability

Exponential and stationary phase cultures were sampled for investigation of the resistance of *R. erythropolis* SQ1 to oxidative damage. Cells were grown as described in Section 2.2. One ml samples were taken and the cells harvested by centrifugation at 16,000×g for 5 min. The supernatant was discarded and the cells resuspended in quarter strength (Ringer) saline. The washed cells were then used to inoculate 2 ml of quarter strength (Ringer) saline to an OD_{600nm} of approximately 0.01 (approx. 1×10^6 CFU/ml). The cell concentration was determined by plating three 100 µl aliquots. Tert-butyl hydroperoxide (tBHP) was then added to 1 ml of cells at concentrations ranging from 1 to 400 mM. To measure cell viability, 100 µl samples were taken at regular intervals for serial dilution. Dilutions were done using 900 µl quarter strength (Ringer) saline supplemented with 0.05 % Tween. Aliquots (100 µl) of the appropriate dilutions were then plated in triplicate on LB-agar. The plates were subsequently incubated for two days at 27 °C and the cell viability calculated as colony forming units per ml (CFU/ml).

2.5. Transformation of cells by electroporation

2.5.1. Preparation of electrocompetent cells of *E. coli* and *Rhodococcus*

Electrocompetent cells of *E. coli* DH5α (Invitrogen) were prepared as described in the manual for the Gene Pulser II (Bio-Rad). LB (100 ml) was inoculated with 1 ml fresh overnight culture and grown at 37°C with vigorous shaking to an OD_{600nm} between 0.5 and 1. The flasks were then chilled on ice for approximately 30 min and the cells were subsequently harvested by centrifugation at 10,000×g for 10 min in a centrifuge cooled to 4 °C (5810R, Eppendorf). The supernatant was discarded and the cells were washed twice in ice-cold 10 % glycerol, and then resuspended in 400 µl of 10 % glycerol. The cells were aliquoted (50 µl) in ice-cold eppendorfs, which were then stored at –80 °C until required for electroporation.

A similar protocol was used for the preparation of electrocompetent *Rhodococcus* but with the following modifications: incubation of the culture at 27 °C to an OD_{600nm} of 1.0 and final resuspension of the cell pellet in 2.5 ml of 10 % glycerol (Tanaka *et al.*, 2002).

The protocol described by Zheng *et al.* (1997) was also used. Briefly, cells for electroporation were prepared by growth of a single colony in 200 ml of LB supplemented with 1.8 % sucrose, 1.5 % glycine and 100 mg ml^{-1} isoniazid, incubated at 27 °C with constant shaking at 200 rpm for 2 days, chilling on ice for 1.5 h, and repeated washing in 10 % glycerol dissolved in pure water.

2.5.2. Electroporation

An aliquot of competent cells was thawed on ice and gently mixed with 0.5 to 2 µg of DNA. The cells were then incubated on ice for approximately 1 min. The electroporator

(Gene Pulser II with Pulse Controller Plus, Bio-Rad) was set at 25 μF, 2.5 kV and 200 Ω for *E. coli* (Bio-Rad protocol), or at 25 μF, 2.5 kV and 400 Ω for *Rhodococcus* (Treadway *et al.*, 1999). The mixture of cells and DNA was transferred to a cold 0.2 cm electroporation cuvette and placed into the electrotransformation chamber. The sample was pulsed once at the above settings, which should produce a pulse of time constant 4.5–5 ms for *E. coli* and 8–12 ms for *Rhodococcus*. The cuvette was removed from the chamber and 950 μl of SOC recovery medium (composition of SOC is provided in Section 2.15) (Sambrook *et al.*, 1987) was immediately added. The cell suspension was transferred to a 17×100 mm sterile propylene culture tube and incubated at 37 °C and 200 rpm for 1 h for *E. coli*. For *Rhodococcus*, the samples were incubated at 27 °C and 200 rpm for 2–4 h. The samples were subsequently plated on LB agar plates containing the appropriate antibiotic.

2.6. Transposon mutagenesis and generation of mutant bank

Transposon mutagenesis exploits the ability of transposons to insert randomly into DNA. The EZ-TN<KAN-2> transposon is an artificial transposon, consisting only of a kanamycin resistance gene from Tn903 flanked by 19 bp inverted repeat mosaic end sequences which serve as binding sites for the transposase (Epicentre Biotechnologies). The DNA is provided as a complex with the EZ-TN transposase enzyme and the resulting transposome can be electroporated into living cells, where the intracellular Mg^{2+} activates the transposase, leading to random insertion of the transposon into the genome (see Figure 2.2), the only requirement being a TA dimer at the point of insertion (Berg *et al.*, 1983). Mutants are obtained by selection for kanamycin resistance.

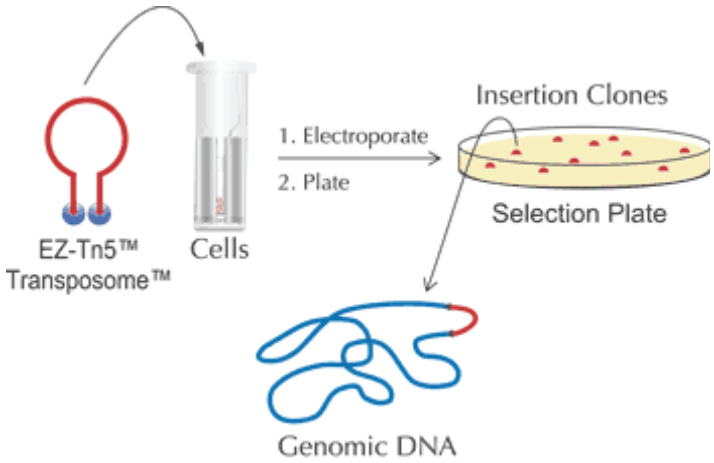


Figure 2.2. Generation of a mutant bank by random transposon insertion using the EZ-Tn*5* Transposome (©2007 Epicentre Biotechnologies).

Transposon mutagenesis was performed on competent cells of *R. erythropolis* SQ1 prepared as described in Section 2.5.1. Instead of plasmid DNA, 1 µl transposome (transposon–transposase complex) EZ-Tn5<KAN-2>Tnp from Epicentre Biotechnologies was used. The electroporated cells were plated on LB agar containing 200 µg/ml of kanamycin and incubated at 27 °C. Each colony growing on the selective

medium was picked using a sterile toothpick and inoculated in 100 µl of LB supplemented with 200 µg/ml of kanamycin in a well of a microtitre plate (stock plates). The plates were incubated overnight at 27 °C in a static incubator and fresh microtitre plates containing 150 µl of LB + kanamycin per well (working plates) were inoculated using purpose-built sterile 96-prong replicators. 100 % glycerol (50 µl) was added to each well of the stock plates and mixed by pipetting, and the plates were stored at −80 °C. In each plate, well A1 was inoculated with wild-type *R. erythropolis* SQ1. Well H12 contained LB only (the non-inoculated negative control).

2.7. Mutant screening strategy

The mutant bank was screened for stationary phase survival mutants following the method described by Uhde *et al.* (1997). Transfers were made from the working plates to plates containing chemically defined medium CDM IIA (see Section 2.15. for details) containing either 1 % or 0.1 % (w/v) glucose broth. The working plates were replicated at weekly intervals on LB agar or CDM 1 % glucose agar to assess the survival of the mutants (see Figure 2.3). The appearance of growth for each mutant was recorded. Further investigation of mutants exhibiting interesting features was continued by going back to the stock plates. Each plate was assigned a number (from 1 to 10) and each well was identified alpha-numerically, so that each mutant has a unique identifying code (e.g. 4C7), allowing easy retrieval of mutants of interest for further analysis.

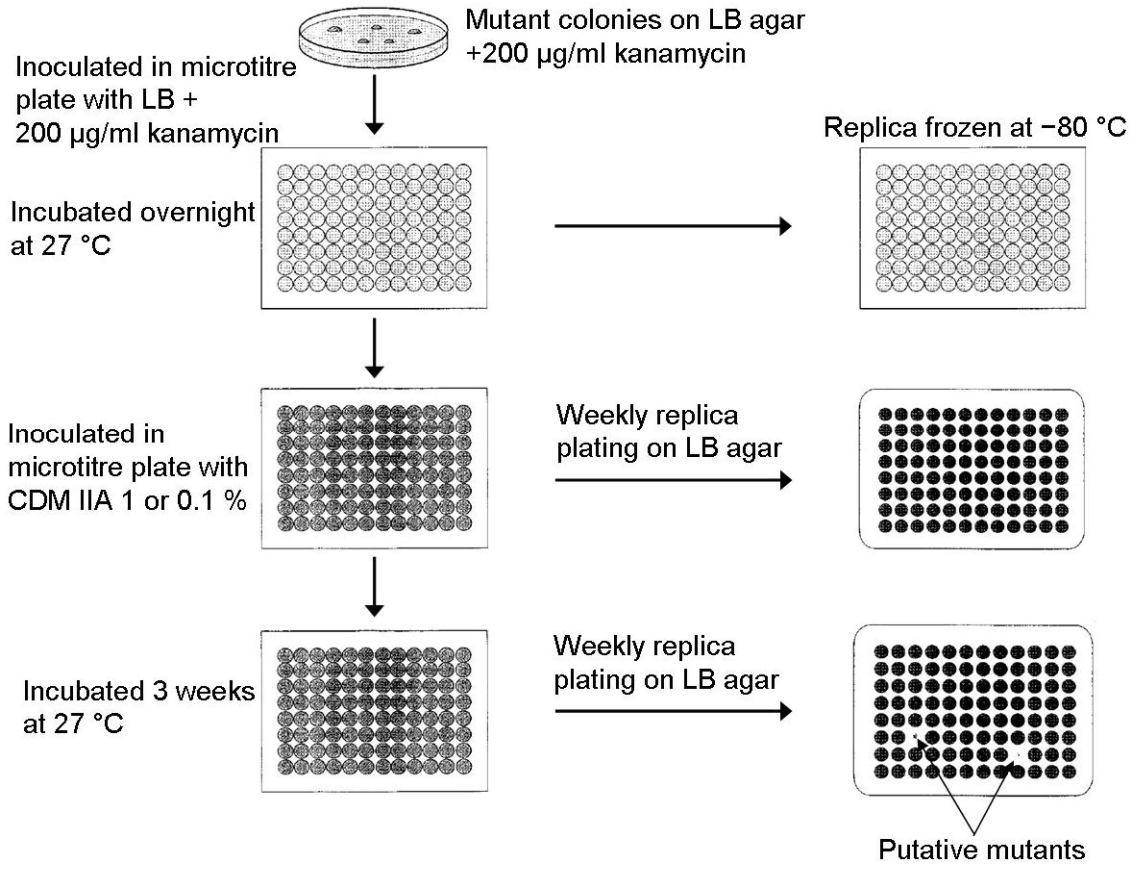


Figure 2.3. Mutant screening strategy (adapted from Uhde *et al.*, 1997). The mutants were not allowed to enter stationary phase until inoculation in CDM II A for screening.

2.8. DNA isolation

2.8.1. Plasmid DNA Isolation (<20,000 bp)

Plasmid DNA was recovered from bacterial cultures using the Wizard SV Miniprep kit (Promega). For extraction of plasmids from *Rhodococcus* species the following adaptation was made: 1–5 ml overnight culture was harvested and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with 20 mg/ml lysozyme (Sigma) and incubated for 2–4 h in a 37 °C waterbath. The procedure was completed using the DNA isolation kit as per the manufacturer's instructions. The plasmids used in this study are listed in Table 2.2.

2.8.2. Genomic DNA Isolation

The FastDNA SPIN Kit for Soil (Qbiogene) was used to extract genomic DNA from *R. erythropolis* SQ1 and mutants thereof. Modifications made to the manufacturer's protocol were as follows. Two to four ml of broth culture (OD_{600nm} ~1.0) were centrifuged at 12,000×g for 10 min. The pellets were resuspended in 978 µl Sodium Phosphate Buffer (SPB) and 122 µl MT buffer (provided in the kit), and subsequently transferred to a Lysing Matrix E Tube. The procedure was continued as described in the manufacturer's handbook, including a lysis step at setting 5.5 for 30 s in the FastPrep Instrument (Qbiogene). In the final step, the genomic DNA was eluted in 100 µl DNase- and pyrogen-free water provided in the kit.

Plasmid	Antibiotic resistance	Description	Reference
pRE7	Km^R	*E. coli – Rhodococcus* shuttle vector (5.9 kb)	Zheng *et al.* (1997)
pDA71	Amp^R, Cm^R	*E. coli – Rhodococcus* shuttle vector (8.8 kb)	Dabbs (1998)
pSRK21	Km^R	*E. coli – Rhodococcus* shuttle vector (5.8 kb)	Veselý *et al.* (2003)
pCR2.1-TOPO	Amp^R, Km^R	*E. coli* cloning vector (3.9 kb)	Invitrogen

Table 2.2. Description of the plasmids used in this study.

Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin.

2.9. Restriction Analysis of DNA

Restriction enzymes used were from Roche Applied Science and used according to the manufacturer's instructions. Routinely, the DNA was combined with the appropriate buffer (10×) and 10 U of enzyme followed by incubation overnight at 37 °C. When performing multiple digests, the buffer was chosen according to the manufacturer's recommendations in order to yield the highest possible activity.

2.10. Agarose Gel Electrophoresis

The results of DNA extraction, DNA restriction analysis and PCR amplification were visualised by agarose gel electrophoresis. Molecular grade agarose (0.8 to 1.0 % w/v, Fluka) was dissolved in Tris-acetate buffer (TAE; 40 mM Tris-base, 20 mM acetate and 2 mM EDTA, pH 8.0) by heating. Ethidium bromide was incorporated in the gel at a final concentration of 0.3 μg/ml. The gel was cast in a mould (Embi Tec) and allowed to solidify prior to being submerged in TAE buffer. The molecular size standard used was the New England Biolabs 2-log DNA ladder. The DNA samples were mixed with loading buffer (0.25 % bromophenol blue, 30 % glycerol in water) prior to gel loading. Samples were electrophoresed through the gel at 150 V in a Run-One electrophoresis cell (Embi Tec) until the bromophenol blue from the loading buffer had migrated half-way through the gel. Gels were visualised and recorded using the Molecular Imager FX Pro and the Quantity One software (Bio-Rad). When necessary, DNA fragments were extracted from gels using the Montage DNA gel extraction kit (Millipore) following the manufacturer's protocol.

2.11. Polymerase Chain Reaction (PCR)

PCR was routinely performed using BioMix (Bioline), unless otherwise specified. BioMix is a complete reaction mix (2×) for PCR that contains 32 mM $(NH_4)_2SO_4$, 125 mM Tris-HCl (pH 8.8), 0.02 % Tween 20, 2 mM dNTPs, 0.05 U/µl *Taq* polymerase and 3 mM $MgCl_2$. A PCR reaction consisted of 25 µl BioMix, 1 µl of each primer (100 pmol/µl), DNA template and the volume was adjusted to 50 µl with dH_2O. The primers used in this study are described in Table 2.3. Water was substituted for the DNA template in the negative controls. The reagents were combined and centrifuged briefly prior to being placed in the Px2 thermal cycler (Thermo Scientific). A typical reaction was carried out as follows: a denaturing period of 5 min at 94 °C, followed by 25 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 3 min of elongation at 72 °C. A final elongation period of 10 min at 72 °C was then followed by cooling down to 4 °C. The PCR products were then analysed by agarose gel electrophoresis as described above.

2.12. Inverse PCR

Inverse PCR (iPCR) is a method designed to recover DNA sequences flanking a region of known sequence (Ochman *et al.*, 1988). In this study, iPCR was performed on mutants of *R. erythropolis* SQ1 harbouring the EZ-Tn<KAN2> transposon (Epicentre Biotechnologies). Genomic DNA was isolated from the mutants and restricted with the appropriate restriction endonucleases, and subsequently religated. The sequence flanking the transposon was then amplified by PCR, as illustrated in Figure 2.4 and described below in further detail.

Primer	Sequence (5′ to 3′)	Details	Tm (°C)
KANR-FP	ATGAGCCATATTCAACGGGAAACGT	Amplify the Tn903 kanamycin resistance gene	56
KANR-RP	TTAGAAAAACTCATCGAGCATCAAA		51
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC	Specific to the 3′ end of Tn903, used to amplify DNA sequence flanking the transposon	56
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG	Specific to the 5′ end of Tn903, used to amplify DNA sequence flanking the transposon	53
M13 uni (−21)	CAGGAAACAGCTATGACC	Sequencing primer	48
M13 rev (−29)	TGTAAAACGACGGCCAGT	Sequencing primer	48

Table 2.3. Primers used in this study.

Tm, melting temperature.

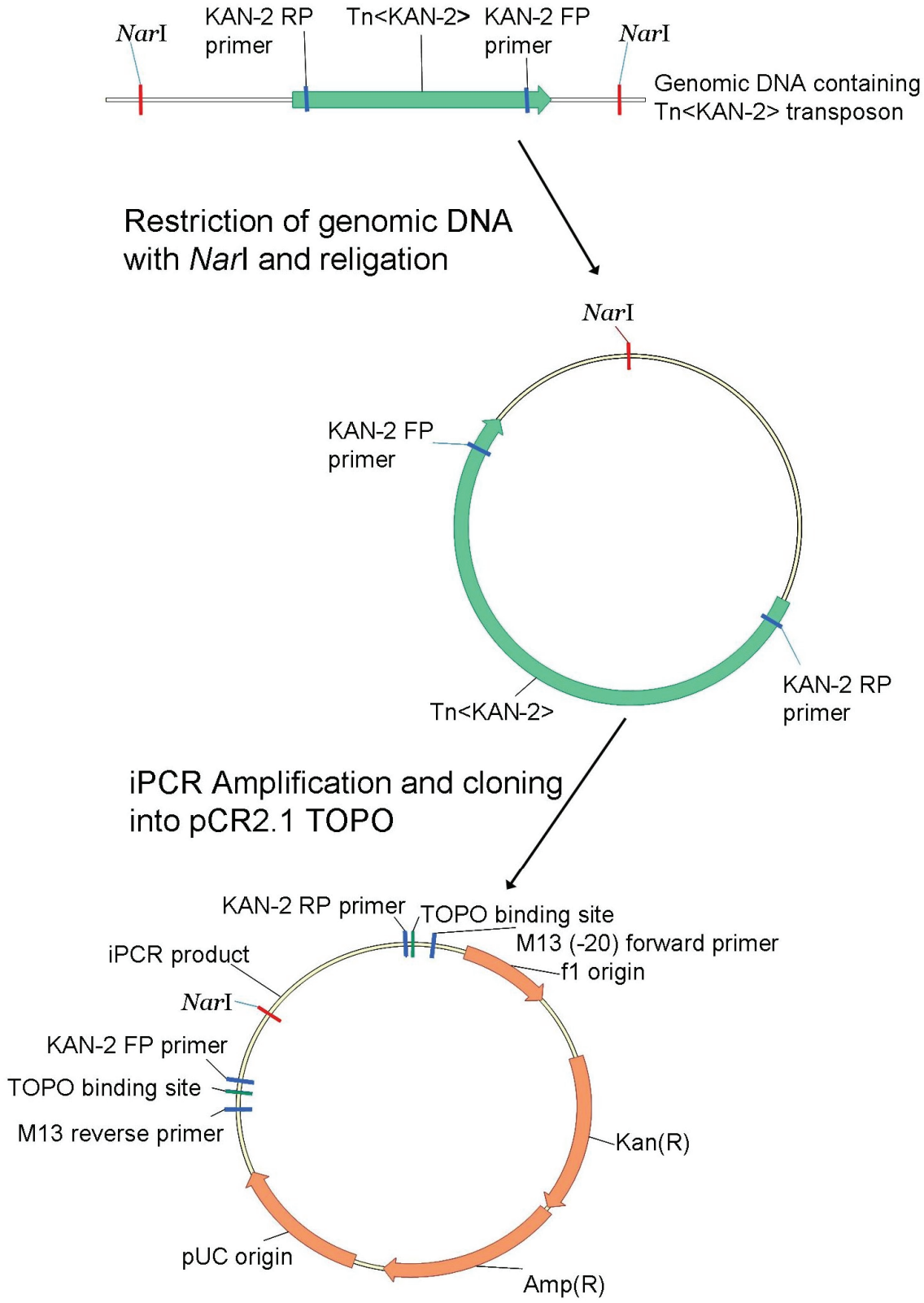


Figure 2.4. Principle of Inverse PCR (iPCR) and cloning. For illustration purposes only the restriction endonuclease *Nar*I is used.

Approximately 2 μg of genomic DNA was digested with a panel of restriction enzymes. The restrictions were performed following the manufacturer's instructions and the efficiency of restriction was checked by agarose gel electrophoresis on 0.8 % agarose gels. The DNA was then separated from the restriction enzymes by phenol/chloroform extraction (Sambrook *et al.*, 1987). Briefly, the DNA was diluted to a volume of 200 μl and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) (Sigma-Aldrich) was added. The contents of the tube were mixed by agitation until an emulsion formed. The mixture was centrifuged at 12,000×g for 15 s at room temperature to separate the organic and aqueous phases. The aqueous phase, containing the DNA, was then transferred to a fresh sterile eppendorf and the DNA precipitated by adding an equal volume of isopropanol (2-propanol). The tube was mixed by agitation and the DNA pelleted by centrifugation at 14,000×g for 10 min. The DNA was resuspended in 17 μl dH_2O, to which 1 μl ligation buffer (10×) and 1 μl of T4 DNA ligase (Roche Applied Science) were added. After incubation at room temperature overnight, the reaction was inactivated by heating in a 80 °C waterbath for 20 min. The ligation mixture was then dialysed against dH_2O on a 0.025 μm MF-Millipore membrane (hydrophilic, Millipore) for 1 h at room temperature prior to iPCR.

The PCR was performed as previously described in Section 2.11 using 1 μl religated DNA as the template. The amplification cycle consisted of a denaturing period of 5 min at 96 °C, followed by 30 cycles of 30 s denaturation at 95 °C, 1 min annealing at 56 °C and 1 min of elongation at 72 °C. A final elongation period of 5 min at 72 °C was then followed by cooling down to 4 °C. The PCR products were then analysed by agarose gel electrophoresis.

2.13. TA-Cloning

The iPCR products were cloned using the TOPO TA Cloning Kit (with pCR2.1-TOPO vector) with One Shot TOP10 chemically competent *E. coli* DH5-α from Invitrogen according to the manufacturer's instructions. The pCR2.1-TOPO vector was used since it has 3′-T overhangs to allow efficient cloning of *Taq* polymerase-generated fragments (*Taq* adds an A to the 5′ end of the region amplified). The T overhangs are also covalently linked to topoisomerase I from the vaccinia virus, mediating the ligation of the PCR-generated fragment into the cloning vector.

2.14. DNA sequencing and bioinformatics analysis

The concentration of purified plasmid DNA was determined by agarose gel electrophoresis. Ethanol-precipitated DNA, provided to MWG Biotech (London) for sequencing, was prepared as follows. The plasmid DNA (1-2 μg) was diluted in a final volume of 100 μl dH_2O, to which 0.1 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of 100 % ethanol were added. The sample was mixed after 5 min at 4 °C and centrifuged at 14,000×g for 5 min at room temperature. The pellet was resuspended in 200 μl 70 % ethanol and centrifugation was repeated. The supernatant was discarded, and the DNA pellet was air-dried at room temperature. DNA sequencing was performed by MWG Biotech using primers M13 uni (−21) and M13 rev (−29) (see Table 2.3).

All sequence manipulations (i.e. cutting and pasting, generation of complement sequence, identification of restriction and primer binding sites) were done using the programme Vector NTI from Invitrogen (http://www.invitrogen.com). The sequence was then analysed using various tools of the Vector NTI package, such as AlignX, ContigExpress, ORF Finder and the Translation tool. The complete DNA sequences obtained were also searched directly against the non-redundant database at NCBI, using

either BLASTN for a nucleotide search or BLASTX to search the database with the sequence translated in all six frames. The resulting putative proteins were searched against the database using BLASTP (Altschul *et al.*, 1997; Schaffer *et al.*, 2001) at www.ncbi.nlm.nih.gov. The search was performed on the non-redundant database (nr) limited to the phylum Bacteria. The most significant results were used to assign a function to the putative protein. Further analysis of the DNA sequence was carried out with a neural network promoter prediction method (Reese & Eeckman, 1995), in an attempt to identify putative promoters. The software NNPP version 2.2 used is available at http://www.fruitfly.org/seq_tools/promoter.html.

2.15. Composition of solutions and reagents

All solutions and media were prepared with deionised water, except where indicated. Bacteriological medium components were obtained from Oxoid. All chemicals, antibiotics and vitamins were purchased from Sigma-Aldrich and were of HPLC grade.

2.15.1. Growth media and supplements

All media were prepared and dispensed prior to autoclaving at 121 ˚C and 15 pounds per square inch of pressure for 15 min.

Luria-Bertani broth (per litre) (Atlas, 1996):

Tryptone	10 g
Sodium chloride (NaCl)	10 g (172 mM)
Yeast extract	5 g

Glucose-yeast extract broth (per litre):

Glucose	10 g (55 mM)
Yeast extract	10 g

CDM I (per litre):

Glucose	2 g (11 mM)
Ammonium sulphate [$(NH_4)_2SO_4$]	1 g (7.5 mM)
Dipotassium phosphate anhydrous (K_2HPO_4)	1 g (5.7 mM)
Salts solution (recipe provided below)	10 ml
Trace elements solution (recipe provided below)	1 ml

CDM IIA 1 % Glucose (per litre):

Glucose	10 g (55 mM)
Ammonium sulphate ($(NH_4)_2SO_4$)	1 g (7.5 mM)
Dipotassium phosphate anhydrous (K_2HPO_4)	2.75 g (15.8 mM)
Potassium phosphate (KH_2PO_4)	2.25 g (16.5 mM)
Salts solution	10 ml
Trace elements solution	1 ml

For CDM IIA 0.1 % Glucose, only 1 g of glucose was used per litre (final concentration 5.5 mM).

Salts solution (per litre):

Magnesium sulphate ($MgSO_4.7H_2O$)	25 g (0.1 M)
Ferrous sulphate ($FeSO_4.7H_2O$)	0.28 g (1 mM)
Manganous sulphate ($MnSO_4.4H_2O$)	1.7 g (7.6 mM)
Sodium chloride (NaCl)	0.6 g (10 mM)
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.1 g (0.4 mM)
Zinc sulphate ($ZnSO_4.7H_2O$)	0.06 g (0.2 mM)
Calcium chloride ($CaCl_2.2H_2O$)	0.01 g (0.07 mM)
Hydrochloric acid, 0.1M	1 litre

Trace elements solution (per litre):

Hydrochloric acid (25 %)	6.5 ml
Ferrous chloride ($FeCl_2.4H_2O$)	1.5 g (7.5 mM)
Borate (H_3BO_3)	60 mg (1 mM)
Manganous chloride ($MnCl_2.4H_2O$)	100 mg (0.5 mM)
Cobalt chloride ($CoCl_2.6H_2O$)	120 mg (0.5 mM)
Zinc chloride ($ZnCl_2$)	70 mg (0.5 mM)
Nickel chloride ($NiCl_2.6H_2O$)	25 mg (0.1 mM)
Copper chloride ($CuCl_2.2H_2O$)	15 mg (0.1 mM)
Sodium molybdate ($Na_2MoO_4.2H_2O$)	25 mg (0.1 mM)

The salts and the trace elements solutions were distributed in 200 ml bottles, autoclaved and stored at 4 °C.

2.15.2. Composition of media supplements

Vitamin solution (per 100 ml) (Atlas, 1996):

Biotin	4 mg (160 μM)
p-aminobenzoic acid	10 mg (260 μM)
Folic acid	4 mg (90 μM)
Calcium pantothenate	10 mg (420 μM)
Nicotinic acid	10 mg (813 μM)
Vitamin B12	0.2 mg (1 μM)
Thiamine hydrochloride	10 mg (296 μM)
Pyridoxine (B6) hydrochloride	20 mg (975 μM)
Thioctic acid	10 mg (485 μM)
Riboflavin	1 mg (26 μM)

The vitamin solution was filter-sterilised to avoid heat degradation, aliquoted in 1.5 ml eppendorfs and stored at −20 °C.

Thiamine solution:

Thiamine hydrochloride	5 mg (0.7 mM)
Distilled water	20 ml

The thiamine solution was filter-sterilised to avoid heat degradation, aliquoted in 1.5 ml eppendorfs and stored at −20 °C.

Amino acid solution (per 100 ml):

L-Gln	292 mg (20 mM)
L-Arg	126.1 mg (7 mM)
L-Ile	52 mg (4 mM)
L-Leu	52 mg (4 mM)
L-Tyr, diNa	52 mg (2.8 mM)
L-Val	46 mg (3.9 mM)
L-His.HCl	42 mg (2.7 mM)
L-Phe	32 mg (1.9 mM)
L-Met	31 mg (2 mM)
L-Glu	15 mg (1 mM)
L-Asp	13.3 mg (0.7 mM)
L-Asn.H_2O	13.2 mg (1 mM)
L-Pro	11.5 mg (1 mM)
L-Ser	10.5 mg (1 mM)
L-Trp	10 mg (0.5 mM)
DL-Ala	8.9 mg (1 mM)
Gly	7.5 mg (1 mM)
Distilled water	100 ml

The amino acid solution was filter-sterilised and stored at 4 °C.

Pyrimidine solution (per 100 ml):

Uracil	20 mg (1.8 mM)
Thymine	20 mg (1.6 mM)
Cytosine	20 mg (1.8 mM)

The pyrimidine solution was gently heated to dissolve all components. It was then filter-sterilised and stored at 4°C.

Antibiotics:

Stock solutions of 100 mg/ml kanamycin and ampicillin were prepared in distilled water, filter-sterilised and stocked at –20 °C. Stock solution of 100 mg/ml chloramphenicol was prepared in 100 % ethanol and stocked at –20 °C.

2.15.3. Reagents

Tris-EDTA (TE) buffer (1×): 10 mM Tris-HCl, 1 mM EDTA pH 8.0.

Tris-acetate-EDTA buffer (TAE) (50×):

Tris-HCl	240 g (2 M)
Glacial acetic acid	57.1 ml
EDTA 0.5 M	100 ml (50 mM)
dH_2O	to 1000 ml

The pH was then adjusted to 8.5. The solution was diluted 50× to prepare agarose gels and used as running buffer.

SOC broth:	Magnesium chloride ($MgCl_2$)	10 mM
	Magnesium sulphate ($MgSO_4$)	10 mM
	Potassium chloride (KCl)	2.5 mM
	Sodium chloride (NaCl)	10 mM
	Tryptone	20 g/l
	Yeast extract	5 g/l
	Glucose	20 mM

SOC broth was dispensed in universals and autoclaved at 121 °C and 15 pounds per inch of pressure for 15 min.

Chapter III

Physiological Response of *Rhodococcus erythropolis* SQ1 to Starvation

Members of the genus *Rhodococcus* are widely distributed and have been isolated from diverse environments, as described in Chapter I. Their ability to survive in low-energy and nutrient environments is significant due to the role they play in bioremediation (e.g. in the case of *Rhodococcus erythropolis* and *Rhodococcus ruber*) and pathogenesis (e.g. in the case of *Rhodococcus equi* and *Rhodococcus fascians*). From an industrial perspective, it has been shown that nitrogen starvation may elicit biosurfactant production in *R. ruber* (Philp *et al.*, 2002) and *R. erythropolis* (Kim *et al.*, 1990). Nitrogen starvation also decreased the hydrophobicity of *Rhodococcus corallinus* (Sanin *et al.*, 2003), with consequences for bioremediation and microbial de-emulsification (Van Hamme *et al.*, 2003). Carbon and/or energy starvation can also be important factors for other chemical/biosynthesis processes. For instance, ultramicrobacteria (UMB) are produced by carbon/energy starvation, and used for microbial-enhanced oil recovery (Van Hamme *et al.*, 2003).

There has been little study to date of the processes by which rhodococci survive starvation, or indeed of the effects of starvation on rhodococci. It has been shown however that *R. corallinus* can grow after incubation for up to 5 months in carbon- or nitrogen-free medium (Sanin, 2003). The objective of the study reported in this chapter was to characterise the physiological response of *Rhodococcus* to starvation (in this study, carbon was chosen as the limiting nutrient) and to investigate whether carbon starvation can induce cross-protection in *Rhodococcus* to a variety of environmental stresses, including heat and oxidative stress.

3.1. Selection of an appropriate Rhodococcus strain for the study of carbon starvation

There are many parameters that influenced the choice of the particular strain of *Rhodococcus* for this study. In order to investigate the physiological response of *Rhodococcus* to starvation (the focus of this chapter), it was essential to be able to accurately limit the nutrient or nutrients for which the bacterium was being starved and to be able to monitor the growth of *Rhodococcus* on this medium. The growth behaviour of *Rhodococcus* on chemically defined medium was therefore considered. The second part of this study (Chapter IV) however is concerned with the generation of a mutant bank and the screening of that mutant bank for starvation or stationary phase survival mutants. Therefore additional criteria were applied to the selection of the appropriate strain. These included sensitivity to antibiotics that may be used as selective pressure to allow for screening of mutants and transformants, and the transformability of *Rhodococcus* with plasmid DNA

3.1.1. Design of a chemically defined medium suitable for studying the effect of carbon starvation on *Rhodococcus*

Thirteen strains representative of four taxa – *R. erythropolis*, *R. ruber*, *R. equi* and *R. fascians* – were selected. These strains are described in Table 2.1. Of the forty-two species of *Rhodococcus* identified to date (Euzéby, 2007), these four species were selected initially for their industrial, environmental and/or medical significance. All thirteen strains selected were able to grow readily on Luria-Bertani (LB) or glucose yeast extract (GYE) media, whether prepared as broth or agar plates. The basic chemically defined medium described by Goodhue *et al.* (1986) was selected as the basis for designing a chemically defined medium appropriate for *Rhodococcus*. It

should be noted that the basic medium described by Goodhue *et al.* (1986) does not include vitamins, although these are widely reported to be necessary for the growth of some rhodococci (Goodfellow & Alderson, 1977; Goodfellow, 1989). Therefore, in the first instance, in this study supplementation with either a complex mix of vitamins (described in Chapter II) or with 0.7 mM thiamine was performed in order to ensure growth of all strains and to avoid complex deficiencies. These modified media were labelled as CDM IA and CDM IB, respectively. Glucose (0.2% w/v) was chosen as the carbon and energy source (Goodhue *et al.*, 1986). Growth was assessed visually after 48 h of incubation at 27 °C and agitation at 200 rpm. The results are shown in Table 3.1.

Strains of *R. ruber* Ac72, Ac74, Ac82 and Ac87 grown in CDM IA and IB broth clumped extensively. A significant amount of clumping was also observed for *R. erythropolis* Ac201. Extensive clumping of bacterial cells is undesirable since this leads to inaccurate cell counts, whether evaluated by determining viable plate counts or optical density. Although dense cultures were obtained for most strains tested in CDM IA and IB, it is possible that the low concentrations of carbon (0.2 % w/v glucose, i.e. 11 mM) and phosphate (6 mM K_2HPO_4) used may be growth-limiting. Furthermore, although there was no evidence of pH fluctuation, the low concentrations of phosphate and the limited buffering capacity provided may lead to large variations in pH during lengthy periods of incubation. Therefore, the glucose concentration was increased from 0.2 to 1 % w/v. The phosphate concentration was also increased to 32.3 mM using 15.8 mM dipotassium hydrogen phosphate (K_2HPO_4) and 16.5 mM potassium dihydrogen phosphate (KH_2PO_4), thereby buffering the medium at pH 6.2–7.4 (Sambrook *et al.*, 1987). Good growth was attained in this medium (CDM IIA; see Table 3.1) for all species and strains tested, except for *R. equi*. Supplementation with

amino acids and pyrimidines (see Chapter II for composition) was required for good growth of *R. equi* (CDM IIB; Table 3.1).

Species	Strain	CDM			
		IA	IB	IIA	IIB
R. erythropolis	SQ1	+++	+++	++++	ND
	Ac201	+++	+++	++++	ND
	NCIMB 8147	+++	+++	++++	ND
	NCIMB 8863	+++	+++	++++	ND
R. equi	GV1	+	+	++	++++
	VI 1	+	−	+	++++
	NCIMB 10027	−	+	+	++++
	NCIMB 10701	+	+	+	++++
R. ruber	Ac72	++	++	+++	ND
	Ac74	++	++	+++	ND
	Ac82	+++	+++	++++	ND
	Ac87	++	++	+++	ND
R. fascians	Ac170	+++	+++	++++	ND

Table 3.1. Growth of *Rhodococcus* species in a range of chemically defined media.

−, no growth; + to ++++, increasing growth; ND, not determined.

CDM IA and CDM IB correspond to a basic chemically defined medium containing 0.2% glucose described by Goodhue *et al.* (1986) supplemented with either 1 % v/v of vitamin mix (described in Chapter II) or 0.7 mM thiamine, respectively.

CDM IIA corresponds to CDM IA with 1 % glucose and is phosphate-buffered.

CDM IIB corresponds to CDM IIA supplemented with amino acids and pyrimidines.

3.1.2. Antibiotic resistance markers for screening of transformants and mutants of *Rhodococcus*

Antibiotic resistance is an essential tool for genetic manipulation of microorganisms. Most resistance markers are encoded by a single gene, thus making them easily transferable from one organism to another. The majority of cloning vectors are designed with specific antibiotic resistance markers, allowing the isolation of the organisms harbouring the vector. Transposons, frequently used for random mutagenesis, generally possess an antibiotic resistance gene. It is therefore essential that the *Rhodococcus* strain selected for this study is sensitive to antibiotics that may be used in a later stage of the project. The cloning vectors used in this study carry resistance genes for ampicillin, kanamycin and chloramphenicol, and the transposon system we proposed to use harbours a kanamycin resistance gene. It was therefore essential to verify the antibiotic sensitivity profile of the available rhodococcal strains.

All the strains considered for this study were plated on GYE agar containing either 100 μg ml^{-1} of ampicillin, 200 μg ml^{-1} of kanamycin or 40 μg ml^{-1} of chloramphenicol. The genes coding for resistance to these antibiotics are used as the selective markers on the cloning vectors used to date in *Rhodococcus* (Dabbs, 1998; Quan & Dabbs, 1993; Veselý *et al.*, 2003). Antibiotic concentrations were chosen according to the literature regarding the use of these vectors in *Rhodococcus* (Quan & Dabbs, 1993; Veselý *et al.*, 2003).

Except for the phytopathogen *R. fascians*, all the strains tested were sensitive to ampicillin, kanamycin and chloramphenicol (Table 3.2). *R. fascians* Ac170 was resistant to all three antibiotics. Based on these observations, the *Rhodococcus–E. coli*

shuttle vectors and the transposon mutagenesis system used later in this study are unsuitable for *R. fascians* Ac170.

Strain	Growth medium			
	GYE	GYE + ampicillin	GYE + kanamycin	GYE + chloramphenicol
R. fascians Ac170	+	+	+	+
R. erythropolis Ac201	+	−	−	−
R. erythropolis NCIMB 8147	+	−	−	−
R. erythropolis NCIMB 8863	+	−	−	−
R. erythropolis SQ1	+	−	−	−
R. ruber Ac72	+	−	−	−
R. ruber Ac74	+	−	−	−
R. ruber Ac82	+	−	−	−
R. ruber Ac87	+	−	−	−

Table 3.2. Growth of selected *Rhodococcus* strains on GYE agar containing antibiotics.

Concentrations of 100 μg ml^{-1} ampicillin, 200 μg ml^{-1} kanamycin and 40 μg ml^{-1} chloramphenicol were used. Cultures were incubated at 27 °C until colonies could be clearly observed, or for up to 4 days.

+, growth; −, no growth.

3.1.3. Transformability of rhodococcal strains

To date, there are only a limited number of publications reporting the genetic manipulation of *Rhodococcus* sp. There are a few reports concerning the introduction of DNA to *Rhodococcus* species using conjugation. Conjugation has been reported for *R. erythropolis* BD2 with plasmid pBD2 (Dabrock *et al.*, 1994), *R. fascians* with plasmid pD188 (Desomer *et al.*, 1988) and *R. erythropolis* SQ1 with plasmids pB264 and pAN12 derivatives (Lessard *et al.*, 2004; Yang *et al.*, 2007b). These conjugative plasmids can however be so large as to be unwieldy for molecular manipulation. pBD2 is approximately 210 kb, while pD188 is 138 kb (Dabrock *et al.*, 1994; Desomer *et al.*, 1988). The transfer efficiency of pB264 (5 kb) is low at 7.3×10^{-7} per recipient (Lessard *et al.*, 2004). A recent study with pAN12 derivatives has yielded promising results with conjugation efficiencies up to 7×10^{-4} tranconjugants per recipient cell (Yang *et al.*, 2007a).

R. erythropolis, *R. ruber*, *R. equi* and *R. fascians* have all been transformed by electroporation (Chauvaux *et al.*, 2001; Desomer *et al.*, 1990; Sekizaki *et al.*, 1998; Treadway *et al.*, 1999). Several shuttle vectors have been designed that allow transfer of genes to and from rhodococci by electroporation, such as pMVS301 that replicates in *E. coli*, *R. equi*, *R. erythropolis* and *R. globerulus* (Singer & Finnerty, 1988); pDA21 that replicates in *E. coli*, *R. equi*, *R. erythropolis* and *R. rhodochrous* (Dabbs, 1998), pRE-7 that replicates in *E. coli*, *R. ruber* and *R. equi* (Chauvaux *et al.*, 2001; Zheng *et al.*, 1997) and pSRK21 that replicates in *E. coli* and *R. erythropolis* (Veselý *et al.*, 2003). In addition, random transposon mutagenesis has been achieved in *R. erythropolis* and *R. equi* using electroporation (Fernandes *et al.*, 2001; Mangan & Meijer, 2001). It was therefore decided to investigate the efficiency of transformation of selected

Rhodococcus strains by electroporation, with the aim of using this technique to generate a mutant bank (Chapter IV).

Three strains, *R. equi* NCIMB 10027, *R. equi* NCIMB 10701 and *R. erythropolis* SQ1, were tested for their transformability (i.e. the ease with which transformants can be obtained in the laboratory). Two protocols were used to produce electrocompetent cells. The main differences between these protocols is that cells are grown in LB alone in one (Denome *et al.*, 1993), whereas in the other LB is supplemented with sucrose, glycine and isoniazid (Zheng *et al.*, 1997). Glycine is often used in the growth medium to prepare competent cells as it disrupts the structure of the peptidoglycan. Isoniazid is an antibiotic that disrupts the synthesis of mycolic acids, a component of the cell wall of actinomycetes. The transformation efficiencies were determined using plasmids pSRK21 and pRE-7.

pSRK21 is a small (5.8 kb) shuttle vector based on *Corynebacterium* vectors and was designed specifically for transfer between *E. coli* and *R. erythropolis* (Veselý *et al.*, 2003). It is based on the fusion of pSR1, a *Corynebacterium glutamicum* plasmid (Archer & Sinskey, 1993), and pK19, an *E. coli* shuttle vector bearing both an *ori* (origin of replication) and a kanamycin resistance gene (Pridmore, 1987). A maximum transformation efficiency of 7×10^4 transformants μg^{-1} DNA was achieved with pSRK21 in *R. erythropolis* CCM2595 (Veselý *et al.*, 2003).

pRE-7 is similar in size (5.9 kb) to pSRK21 and was designed as a shuttle vector between *E. coli* and *R. equi* (Zheng *et al.*, 1997). It was created by combining the *ori* of *R. equi* strain 103 virulence-associated plasmid pOTS, the kanamycin resistance gene of plasmid pACYC177 and the plasmid pBluescript KS(+) (ColE1 *ori*) (Zheng *et al.*,

1997). A maximum transformation efficiency of 2.5×10^7 transformants μg^{-1} DNA was achieved with pRE-7 in *R. equi* 103^- (pOTS-cured strain) .

R. equi NCIMB 10027 and *R. equi* NCIMB 10701 competent cells were initially prepared using the method described by Zheng *et al.* (1997). With plasmid pSRK21, transformation frequencies of 9.5×10^5 and 4.45×10^6 transformants μg^{-1} DNA were obtained for NCIMB 10027 and NCIMB 10701, respectively. *R. equi* NCIMB 10027 was also transformed with plasmid pRE-7, achieving a frequency of 1×10^5 transformants μg^{-1} DNA.

For *R. erythropolis* SQ1, frequencies of up to 1.85×10^5 transformants μg^{-1} pRE-7 DNA were achieved using the protocol of Denome *et al.* (1993). Using the method of Zheng *et al*., (1997), low cell densities were consistently obtained in the growth medium described. Therefore, a modified medium was used in which isoniazid was omitted, leading to frequencies of up to 1.95×10^4 transformants μg^{-1} pSRK21 DNA.

The transformation frequencies obtained were comparable to the ones reported by the researchers cited above, i.e. 10^4-10^5 transformants μg^{-1} DNA for *R. erythropolis* SQ1 *vs* 7×10^4 transformants μg^{-1} DNA for *R. erythropolis* CCM2595 (Veselý *et al.*, 2003). Furthermore, these numbers are close to the transformation efficiency recommended by the manufacturer of the transposon system we proposed to use in subsequent stages of the project for random mutagenesis of *Rhodococcus* (10^5 transformants μg^{-1} DNA; Epicentre).

3.1.4. Selection of the study strain

Representatives of four taxa within the genus *Rhodococcus* were selected in the initial stages of this study on the basis of their industrial, environmental and/or medical significance. *R. erythropolis* and *R. ruber* are of particular interest due to their biodegradation and biocatalysis capacities, and *R. fascians* and *R. equi* are important pathogens (as reported in Chapter I).

The outcome of growth experiments performed on chemically defined media allowed for a preliminary selection of potential study strains to be made. As reported above (Section 3.1.1), supplementation of chemically defined medium with amino acids and pyrimidines was required for growth of *R. equi*. Since amino acids contain carbon, phosphorus, nitrogen and, in some cases, sulphur, these requirements complicate the estimation of the final quantities of each element in the growth medium. In addition, several strains of *R. equi* can use amino acids as their sole source of carbon, nitrogen, phosphorus and energy (Bizet *et al.*, 1997). This renders determination of the limiting growth factor rather complex. Despite shared mechanisms, it is also well documented that starvation survival mechanisms for carbon/energy and amino acids do differ (e.g. the stringent response in the case of starvation for amino acids) (Autret *et al.*, 1997) (see Chapter I). Therefore, if amino acids were to be used in the growth medium, their abundance or depletion could influence the mechanisms that trigger the starvation survival response. On this basis it was therefore decided to disregard *R. equi* as the study organism, due to its complex nutrient requirements.

R. ruber cells are known to be particularly hydrophobic (Philp *et al.*, 2002). This hydrophobicity is responsible for the extensive clumping observed for all *R. ruber* strains in all three growth media tested in Section 3.1.1. This aggregation would lead to

difficulties in accurately assessing bacterial growth and physiological status. The reliability of viable plate counts (CFU/ml) and optical density measurements, for instance, would be questionable.

R. fascians Ac170 grew well on chemically defined medium and no cell aggregation was observed. However, this strain was resistant to all three antibiotics tested in this study (Section 3.1.2). Of particular relevance was the resistance to kanamycin, since the kanamycin resistance marker is present on the cloning vectors and the transposon mutagenesis system at our disposal for this study.

R. erythropolis grew well on all the chemically defined media investigated in this study and, with the exception of *R. erythropolis* Ac201, no cell aggregates were observed (Section 3.1.1). It should be noted that *R. erythropolis* SQ1 (one of the strains tested) is a mutant of ATCC 4277-1 (Quan & Dabbs, 1993), which in turn is an arsenic- and chloramphenicol-sensitive variant of the type strain ATCC 4277 (Dabbs *et al.*, 1990). It is described as having increased transformability compared to the type strain (Quan & Dabbs, 1993), and has also been used in a targeted gene disruption experiment (van der Geize *et al.*, 2001). Indeed *R. erythropolis* SQ1 stands out for its ease of manipulation in laboratory conditions and its high transformability, achieving frequencies of $>10^5$ transformants μg^{-1} plasmid DNA (Section 3.1.3).

On the basis of the analyses above, *R. erythropolis* SQ1 was selected as the study organism for the remainder of this project.

3.2. Response of R. erythropolis SQ1 to starvation

It is generally accepted that the environment is oligotrophic (Morita, 1993), sea water and certain types of soil are especially so (Di Mattia *et al.*, 2002; Kjelleberg *et al.*, 1993). Since most bioavailable energy sources are carbon-based, it is justified to study the response of *Rhodococcus* to carbon starvation. Furthermore, the entrance into stationary phase is of particular interest, since this is when most of the stress-resistance capacities are acquired (see for example Matin, 1991; Smeulders *et al.*, 1999; van Overbeek *et al.*, 1995).

Growth curves are indispensable to the study of the behaviour of bacteria. It was especially important for this project that the growth profile of *Rhodococcus* in non-limiting and in limiting environments was established. To this effect, growth was monitored in LB, a complex medium, and in CDM IIA containing 1 and 0.1 % glucose. Survival of *R. erythropolis* SQ1 in the complete absence of nutrients (distilled water) was also monitored.

3.2.1. Response of *R. erythropolis* SQ1 to growth in complex medium and to multiple-nutrient starvation

The aim of this experiment was to compare the behaviour of *R. erythropolis* SQ1 in a commonly used rich medium (LB) and when subjected to multiple-nutrient starvation. Growth curves were performed by growing *R. erythropolis* SQ1 in complex (LB) and nutrient-free media (distilled water). In both cases exponential-phase cultures (corresponding to a 24 h culture) were used to inoculate flasks containing 100 ml of media to a starting cell density of 0.01 OD_{600nm}. Cultures were then incubated at 27 °C, at 200 rpm. Samples were taken at regular intervals over a period of 43 days. For each

sample, viable plate counts (CFU/ml) and optical density (OD_{600nm}) were determined. *R. erythropolis* SQ1 was shown to survive long-term starvation in both LB and dH_2O (Figure 3.1). High viable plate counts of up to 4×10^9 CFU/ml were observed in LB after 2 days. This figure then decreased to approximately 5×10^7 CFU/ml after 30 days in stationary phase. In contrast, the cultures in distilled water did not exhibit any decrease in viability over the 43 day period. There was a slight increase in cell numbers, from 5×10^6 to 2×10^7 CFU/ml within the first 10 days, presumably due to the metabolism of stored material. The cell numbers then remained stable, dropping only to 1.2×10^7 CFU/ml.

It is interesting to note that, when grown in LB, the viable cell count decreases to approximately the same as that in water. This drop in culturability, of 1–2 logs, is similar to that observed in *E. coli* grown in LB (Siegele *et al.*, 1993) and in *Oenococcus oeni* grown in modified FT 80 medium, containing meat extract, yeast extract, salts and carbohydrates (Cavin *et al.*, 1989; Zapparoli, 2004). Similarly, *Enterococcus faecalis* was found to survive multiple-nutrient starvation in water for up to 80 days (Hartke *et al.*, 1998). *Mycobacterium tuberculosis* and *Mycobacterium kansasii* were shown to survive for up to 2 years in rich nutrient broth or in distilled water (Nyka, 1974). Survival in water was also observed in *E. coli* for up to 20 days (Ozkanca & Flint, 1997), although only when incubated at 4–25 °C. At 30 °C, the culture did not yield colonies on agar after 7 days incubation in water.

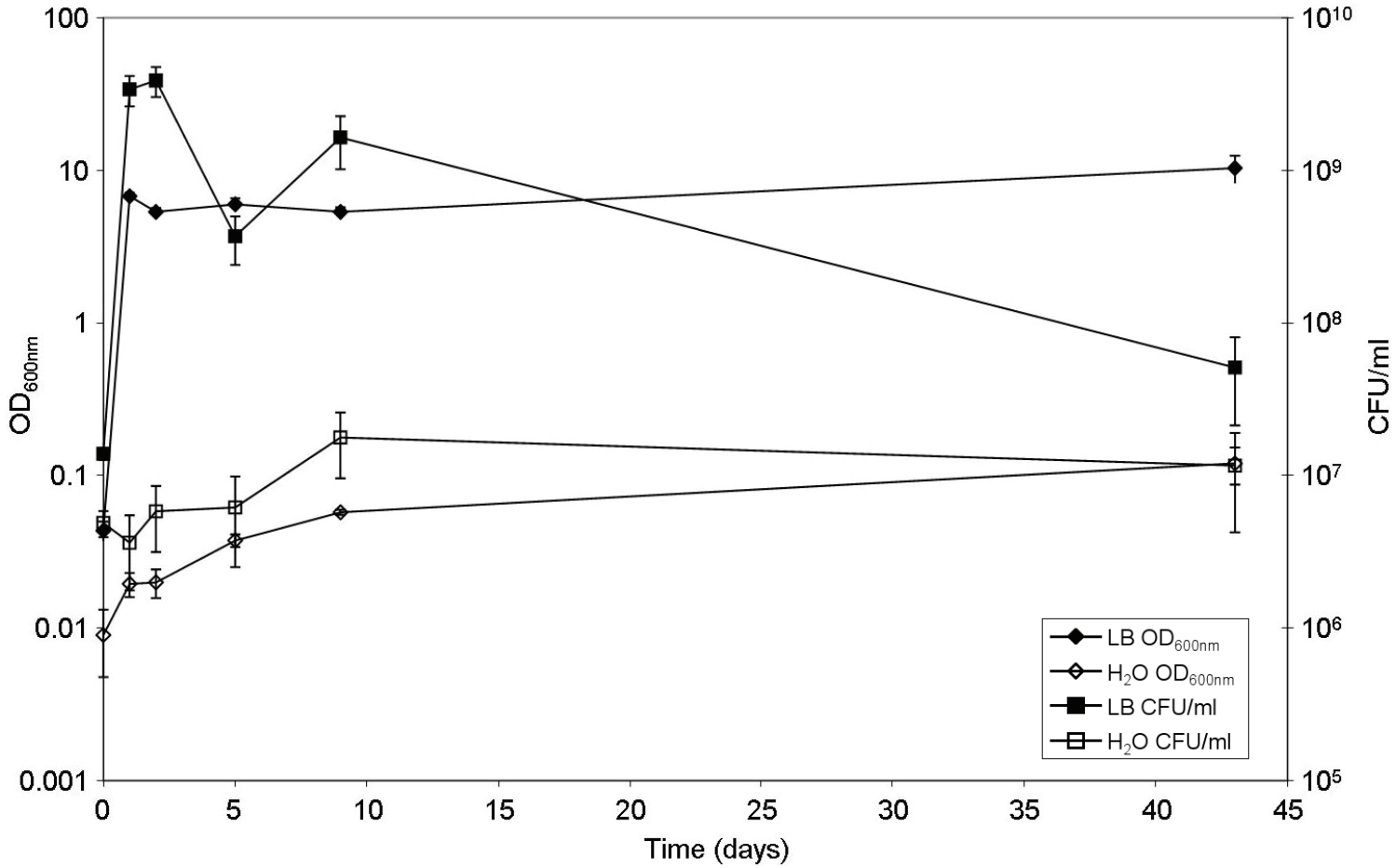


Figure 3.1. Growth curve of *R. erythropolis* SQ1 grown in LB and distilled water. Data are from two separate experiments, plated in triplicate. Closed symbols, LB; open symbols, water. ■ and □, CFU/ml; ♦ and ◇, OD_{600nm}. Error bars represent one standard deviation.

3.2.2. Response of *R. erythropolis* SQ1 to glucose limitation

The response of *R. erythropolis* SQ1 to glucose starvation was initially characterised using the experimental medium CDM IA with two different glucose concentrations – 1 % and 0.1 % w/v. Exponential-phase cultures (corresponding to a 24 hr culture) were used to inoculate flasks containing 100 ml of media to a starting OD_{600nm} of 0.01. Flasks were then incubated at 27^{o}C, at 200 rpm. Samples were taken at regular intervals over a period of 43 days. For each sample, viable plate counts (CFU/ml) and optical density (OD_{600nm}) were determined (Figure 3.2). It should be noted that the flasks were transferred to a static incubator after entry into stationary phase as determined from OD measurements, as described previously in starvation experiments for *Vibrio* and *S. aureus* (Nyström *et al.*, 1990; Watson *et al.*, 1998b). This corresponds to 7 and 12 days for the 1 % and 0.1 % glucose media, respectively.

The culture grown in CDM IA 0.1 % glucose clearly achieves much lower maximum OD_{600nm} (2 *vs* 6.6) and viable plate counts (1.3×10^{9} *vs* 6.1×10^{9} CFU/ml) than that grown in 1 % glucose. This confirms that glucose is the limiting component in the medium. It is worth noting that maximum cell numbers were attained after 7 and 14 days of incubation in CDM IA 1 % and 0.1 % glucose, respectively. The calculated growth rate for the culture grown in 1 % glucose was indeed nearly double that of the culture grown in 0.1 % glucose (i.e. 0.17 hr^{-1} *vs* 0.094 hr^{-1}; Figure 3.3).

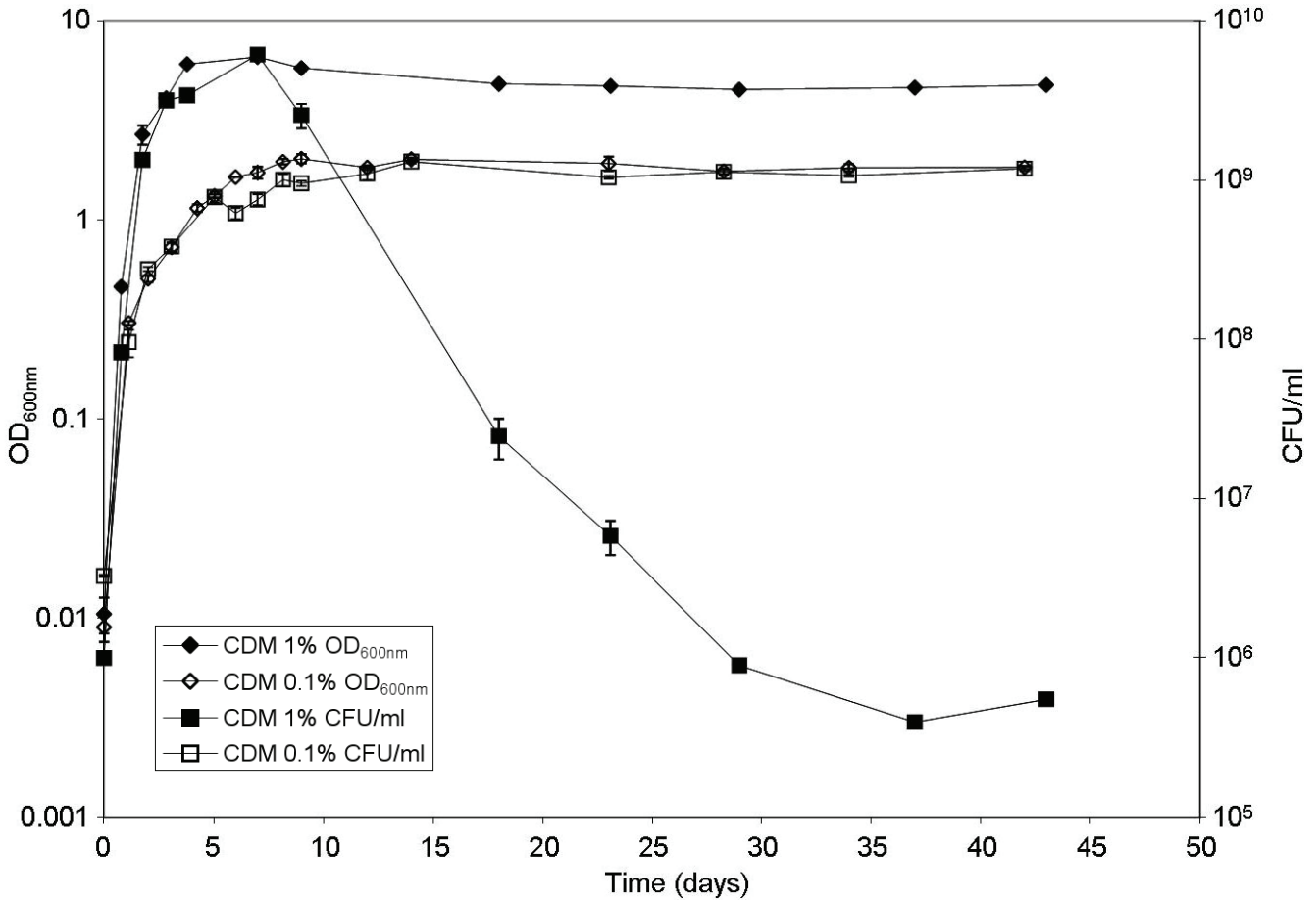


Figure 3.2. Growth curve of *R. erythropolis* SQ1 grown in CDM IA 1 % glucose and 0.1 % glucose with static incubation after entry into stationary phase. Data are from two separate experiments, plated in triplicate. Closed symbols, CDM IA 1 %; open symbols, CDM IA 0.1 %. ■ and □, CFU/ml; ♦ and ◇, OD_{600nm}. Flasks were moved to a static incubator after 7 and 12 days of shaking incubation for 1 % and 0.1 % glucose, respectively. Error bars represent one standard deviation.

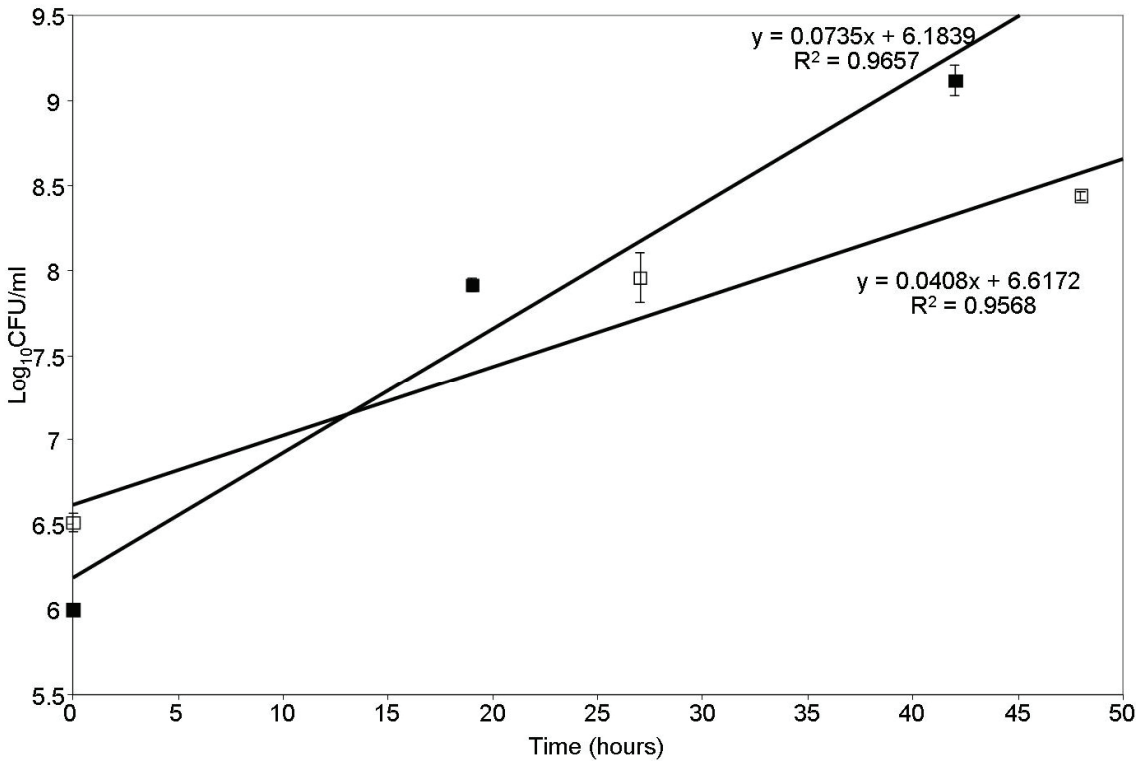


Figure 3.3. Comparison of the growth rates of *R. erythropolis* SQ1 grown in CDM IA 1 % (■) and 0.1 % glucose (□). Data plotted were obtained from two experiments, plated in triplicate. Error bars represent one standard deviation.

Differences in culture behaviour are quite striking after the logarithmic phase of growth – the culture grown in glucose-limited medium reaches a plateau, whereas the culture grown in non-limiting medium (1 % glucose) exhibits a rapid reduction in culturability, as demonstrated by the viable plate counts (Figure 3.2). There is a steady decline in the culturability of the culture, from a maximum of 6.1×10^{9} CFU/ml to less than 10^{6} CFU/ml.

As mentioned in Section 3.1.1. (Design of a chemically defined medium suitable for studying the effect of carbon starvation on *Rhodococcus*), the buffering capacity was a concern with CDM IA. To determine whether the drop in viability observed in Figure 3.2 was due to phosphate starvation and/or variation in pH, this experiment was repeated using the improved phosphate-buffered medium CDM IIA with glucose concentrations of 1 % and 0.1 % w/v. The experiment was conducted as described above with flasks being transferred to static incubation upon entry into the stationary phase, which in the case of the CDM IIA 1 % and 0.1 % glucose corresponded to 10 and 9 days, respectively. There was no significant difference in the behaviour of *R. erythropolis* SQ1 when grown in CDM IA or CDM IIA (Figure 3.4).

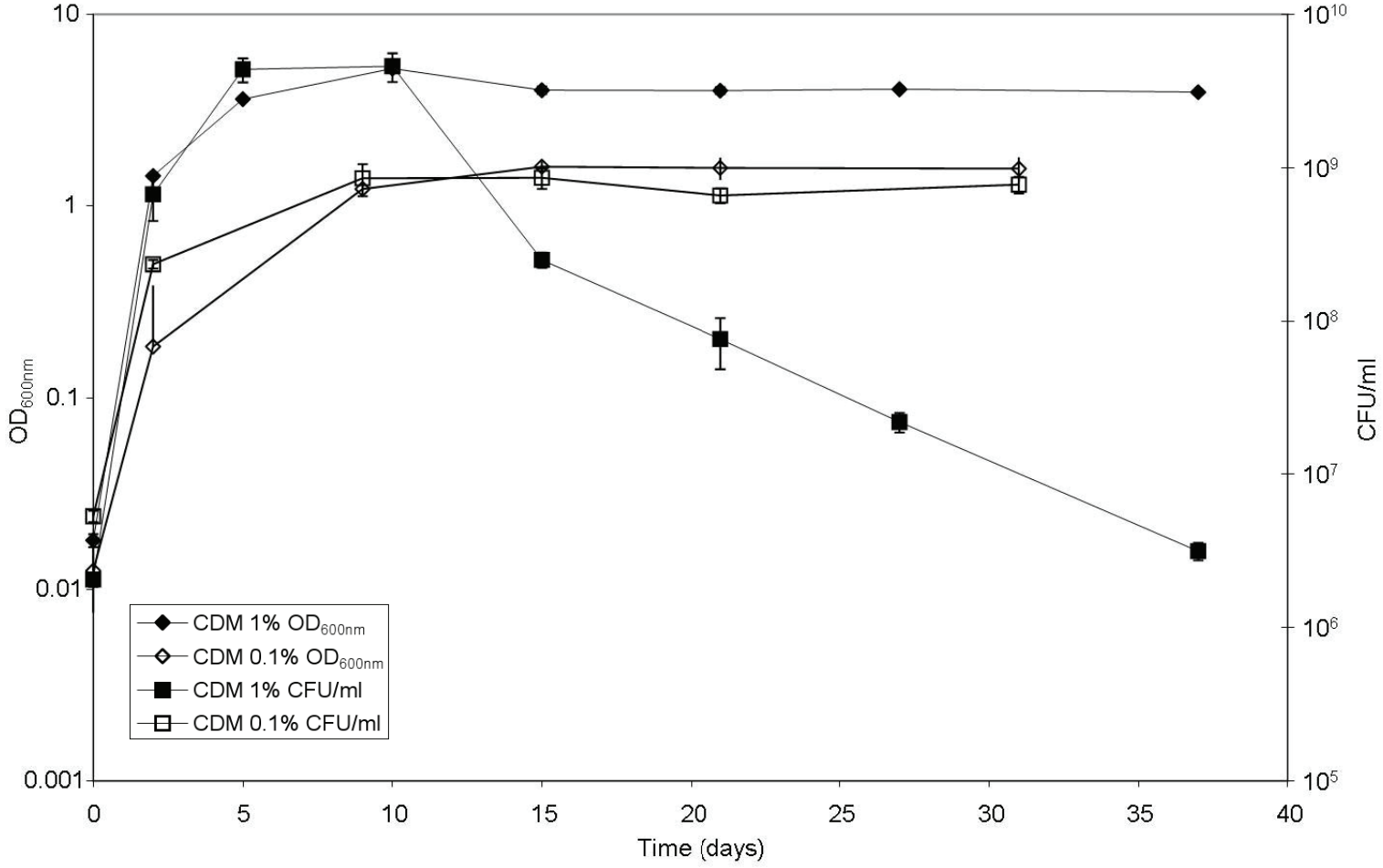


Figure 3.4. Growth curve of *R. erythropolis* SQ1 grown in CDM IIA 1 % glucose and CDM IIA 0.1 % glucose with static incubation. Flasks were moved to a static incubator after 10 and 9 days of shaking incubation for CDM IIA 1 % glucose and 0.1 % glucose, respectively. Data are from two separate experiments, plated in triplicate. Closed symbols, CDM IIA 1 %; open symbols, CDM IIA 0.1 %. ■ and □, CFU/ml; ♦ and ◇, OD_{600nm}. Error bars represent one standard deviation.

It is worth noting that the drop in culturability observed in Figures 3.2 and 3.4 is coincident with the transfer of the culture from shaking to static incubation. Static incubation was the standard method used in the study of starvation survival of *Vibrio* and *S. aureus* (Nyström *et al.*, 1990; Watson *et al.*, 1998b), but interestingly placing flasks of growing cultures in a stationary incubator is the method used to produce dormant *Mycobacterium tuberculosis* (Wayne & Hayes, 1996). In order to determine whether transfer to static incubation impacted on the culturability of *R. erythropolis* SQ1, an experiment was performed in which the growth curves of cultures subjected to continuous agitation were compared with cultures transferred to static incubation following 24 hours (mid-exponential) of shaking incubation (Figure 3.5).

Interestingly, when *R. erythropolis* SQ1 was transferred to static incubation after 24 hours of growth, no dramatic drop in CFU/ml was observed (Figure 3.5a). There is a decrease in viable counts from 1.8×10^9 to 1×10^8 CFU/ml in CDM IIA 1 % glucose, but that figure then remained constant throughout the remainder of the experiment. The growth curves also showed that *R. erythropolis* SQ1 could survive long-term starvation under aerated conditions (Figure 3.5b). Although there is an initial drop in culturability when *R. erythropolis* SQ1 is grown in 1 % glucose, it occurs comparatively late (at 20 days of incubation) and is less pronounced, i.e. less than 1-log reduction in the viable count, compared to a 4-log reduction when agitation is stopped at early stationary phase (Figure 3.2, Figure 3.5b). Furthermore, the viable count remained constant during subsequent incubation.

Since there was a possibility that the drop in culturability associated with transfer to static incubation was due to depletion of oxygen in the media rather than glucose

exhaustion, it was decided that all subsequent growth experiments would be performed under agitation.

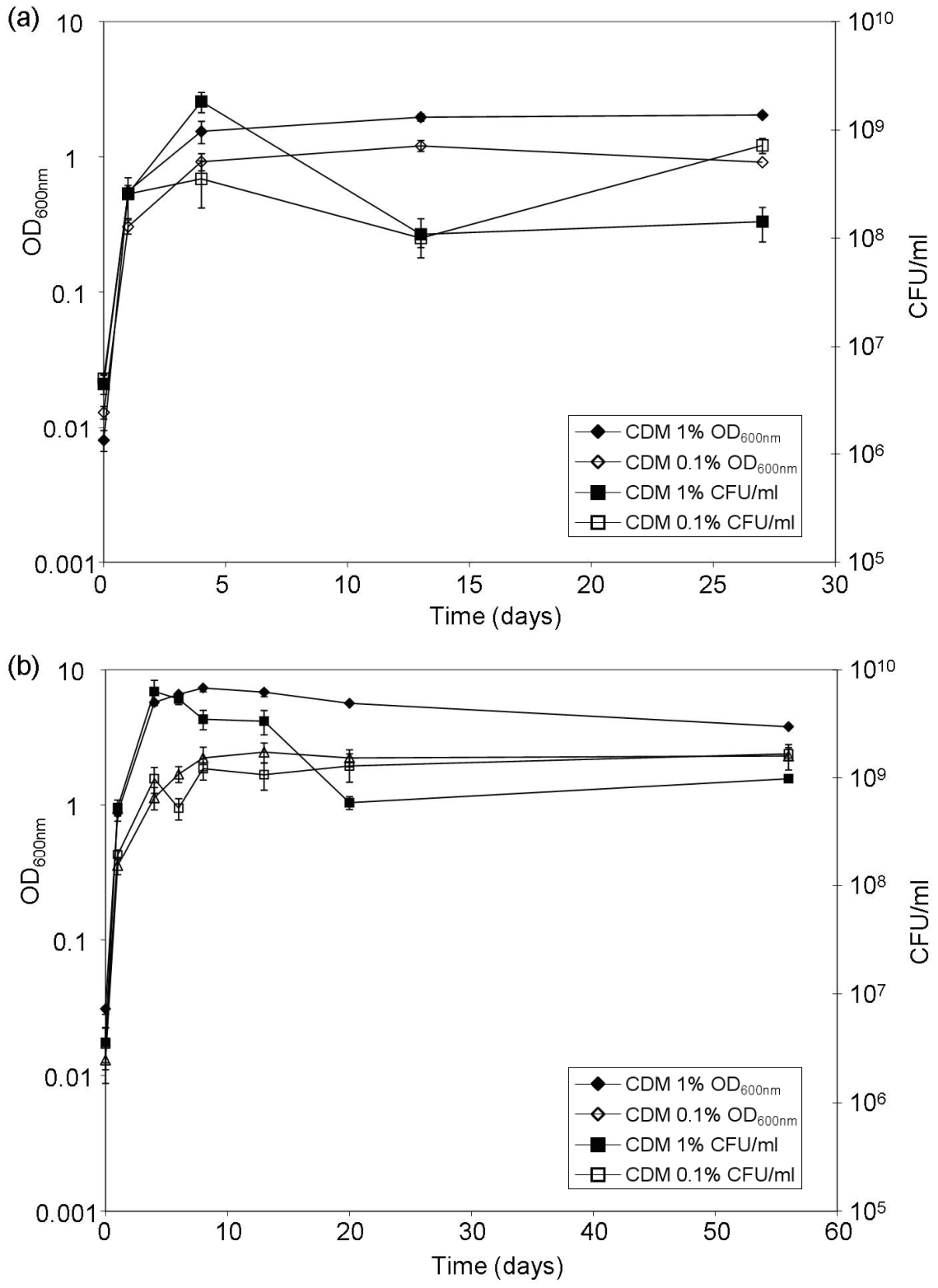


Figure 3.5. Growth curve of *R. erythropolis* SQ1 grown in CDM II 1 % glucose and CDM II 0.1 % glucose with or without static incubation. (a) Flasks were moved to a static incubator after 24 hours of shaking incubation. (b) Flasks were kept in a shaking incubator for the duration of the experiment. Data are from two separate experiments, plated in triplicate. Closed symbols, CDM IIA 1 %; open symbols, CDM IIA 0.1 %. ■ and □, CFU/ml; ♦ and ◇, OD_{600nm}. Error bars represent one standard deviation.

3.2.3. Consumption of glucose in CDM

The glucose concentration in the growth medium was monitored by a colorimetric method in parallel with optical density and viable plate count measurements. Standard curves were performed to allow for accurate measurement of the glucose concentration (Figure 3.6).

Figure 3.7 demonstrates the utilisation of glucose by *R. erythropolis* SQ1 growing in CDM IIA 1 % glucose (Figure 3.7a) and 0.1 % glucose (Figure 3.7b). Note that at 7 days, the glucose is completely exhausted in CDM IIA 0.1 %, whereas there is still in excess of 15 mM remaining in 1 % glucose medium. In both cases the glucose concentration drops sharply, by 50 %, in just 24 hours. It should also be noted that in both the 1 % and 0.1 % glucose media, the glucose concentration eventually drops to levels that are not detectable by the colorimetric method used (<0.1 mM).

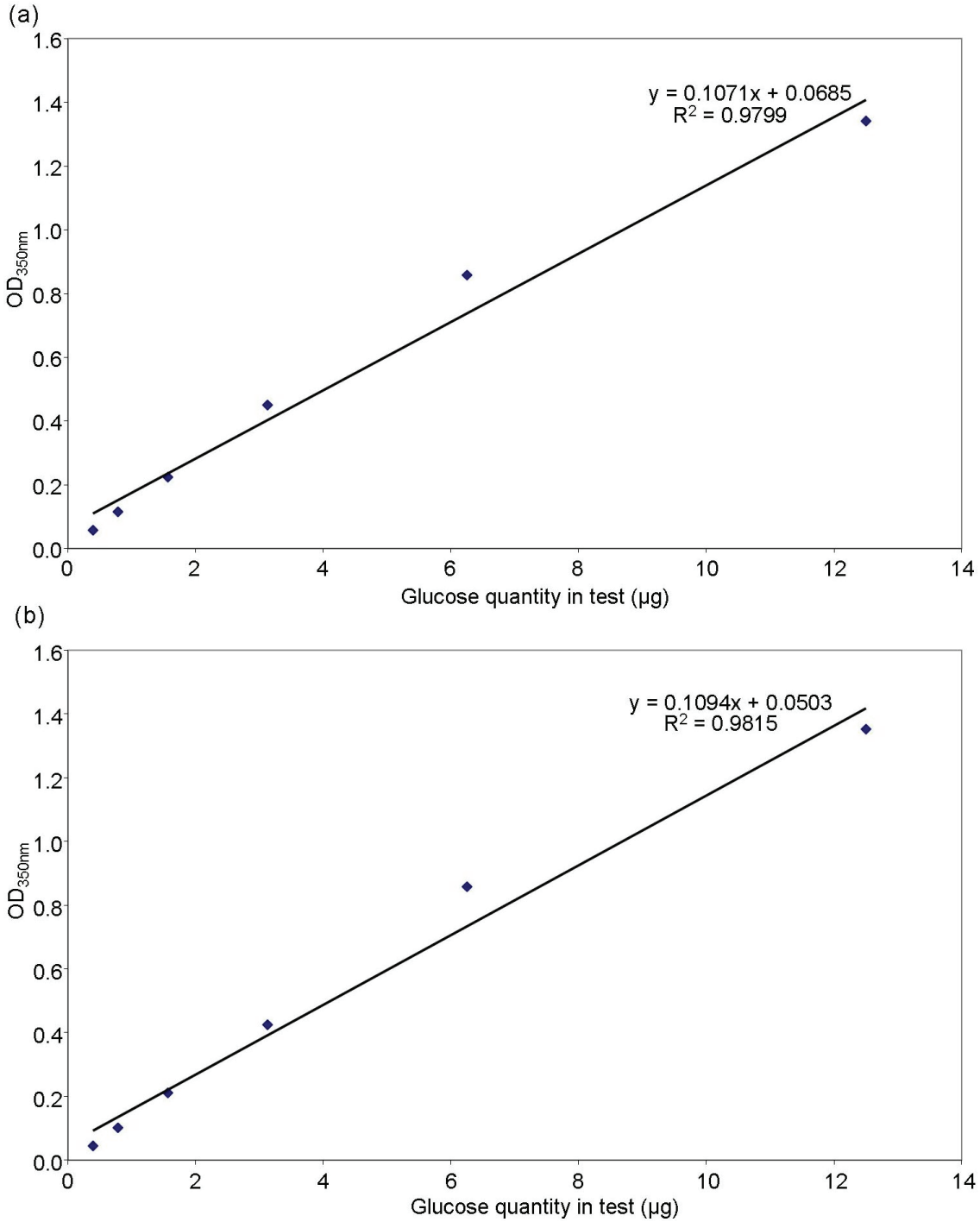


Figure 3.6. Representative standard curves for measurement of glucose in CDM IIA. (a) Standard curve performed with CDM IIA-0.1 % glucose, (b) standard curve performed with CDM IIA-1 % glucose.

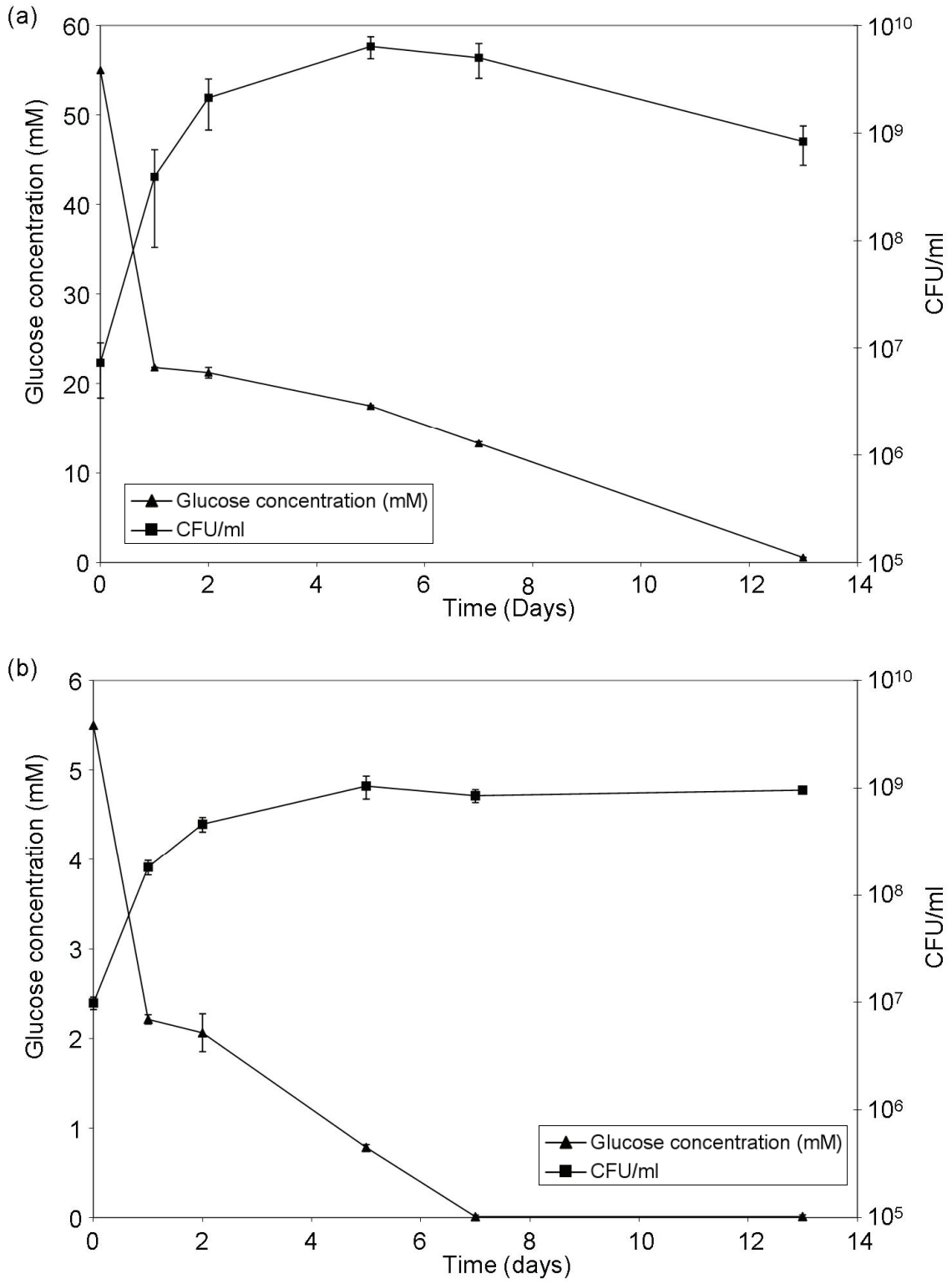


Figure 3.7. Monitoring of glucose concentration during growth of *R. erythropolis* SQ1 in (a) CDM IIA 1% glucose and (b) CDM IIA 0.1% glucose.
Data are from two separate experiments, plated in triplicate. ■, CFU/ml; ▲, glucose concentration in mM. Error bars represent one standard deviation. Note that the glucose concentration ranges up to 55 mM in CDM IIA 1 % glucose, but only 5.5 mM in 0.1 % glucose.

3.3. Starvation of R. erythropolis SQ1: Cross protection induced by carbon starvation

Bacteria in the environment are subjected to permanently changing conditions, especially in terms of nutrient availability and both physical and chemical challenges, including temperature fluctuations and oxidative stress. There have been a number of reports demonstrating a correlation between nutrient starvation and the ability of a bacterium to survive changing environmental conditions. Studies examining the effects of carbon starvation on survival and cross-protection have been performed *in vitro* on *E. coli* (Jenkins *et al.*, 1988). Similar observations have been made in a limited number of bacterial genera including *Vibrio* (Nyström *et al.*, 1992), *Pseudomonas putida* (Givskov *et al.*, 1994), *Salmonella typhimurium* (Seymour *et al.*, 1996), *Enterococcus faecalis* (Giard *et al.*, 1997), *Mycobacterium smegmatis* (Smeulders *et al.*, 1999) and *Listeria monocytogenes* (Ferreira *et al.*, 2001). These studies have shown that bacteria starved for carbon developed cross-protection against a range of stress factors. Such observations may be of particular importance to the survival of non-sporulating bacteria. In this study, the effect of glucose starvation on the ability of *R. erythropolis* SQ1 to survive heat shock and oxidative stress was examined.

3.3.1. The effect of carbon-starvation on the ability of *R. erythropolis* SQ1 to survive heat-shock

CDM IIA 0.1 % glucose was chosen as the growth medium for this experiment in order to ensure that the growth limiting factor was the carbon and energy source. Flasks containing 100 ml of medium were inoculated at a cell density of 0.01 OD_{600nm} with exponential phase cultures (24 hours growth) and incubated at 27^{o}C with 200 rpm agitation. Samples were taken at 48 and 144 hrs (2 and 6 days), corresponding to an exponentially growing and an early stationary phase culture, respectively. In order to investigate the protection afforded by carbon limitation to heat shock, the time points for the shock were chosen according to the glucose concentration of the medium, as illustrated previously in Figure 3.7 as days 2 and 6 – at day 2 when the culture is in exponential phase glucose is still plentiful, while day 6 corresponds to 24 hours before exhaustion of glucose from the medium.

In a preliminary experiment, the exponential and early stationary phase samples taken as described above were aliquoted and incubated in a 57 °C water bath for up to 60 minutes. Aliquots were removed at 5, 10, 15, 20, 30 and 60 minutes and viable plate counts were performed. Percentage survival was determined for each time point as a percentage of the viable count at time of sampling (i.e. a sample not subjected to heat treatment) (Figure 3.8). The early stationary phase culture demonstrated an increased resistance to heat shock in comparison to the exponential phase culture. Since little growth could be observed after 15 minutes heat shock it was decided that, in subsequent experiments, experiments would be performed over 15 minutes with sampling at 5 minute intervals.

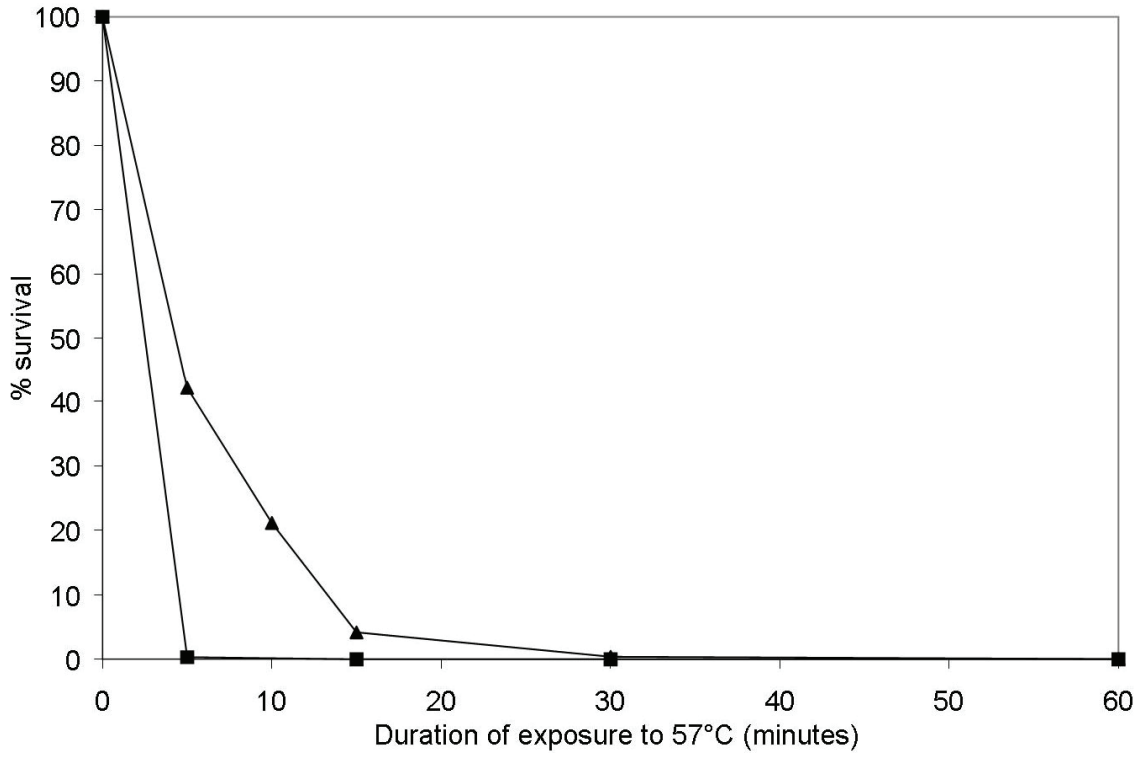


Figure 3.8. Preliminary heat shock experiment in which *R. erythropolis* SQ1 cultures were exposed to heat treatment at 57 ^{o}C.
■, exponential phase culture; ▲, early stationary phase culture. Results shown represent one experiment.

In the experiment described above, the cell density of the two cultures being compared (exponential and early stationary phase) obviously differ. Concerns were raised over the effect of cell density on the survival of *R. erythropolis* SQ1 subjected to heat shock. In order to address this issue, a late exponential phase culture was diluted in CDM IIA lacking glucose to approximately 0.5 OD_{600nm} and submitted to heat shock as described above. The same culture was also submitted to the standard protocol described earlier to provide a control. No significant difference could be observed in the ability to survive heat shock (Figure 3.9), showing that cell density is not a significant factor in the resistance of *R. erythropolis* SQ1 to heat shock. Further experiments were therefore performed without diluting the cells prior to heat shock.

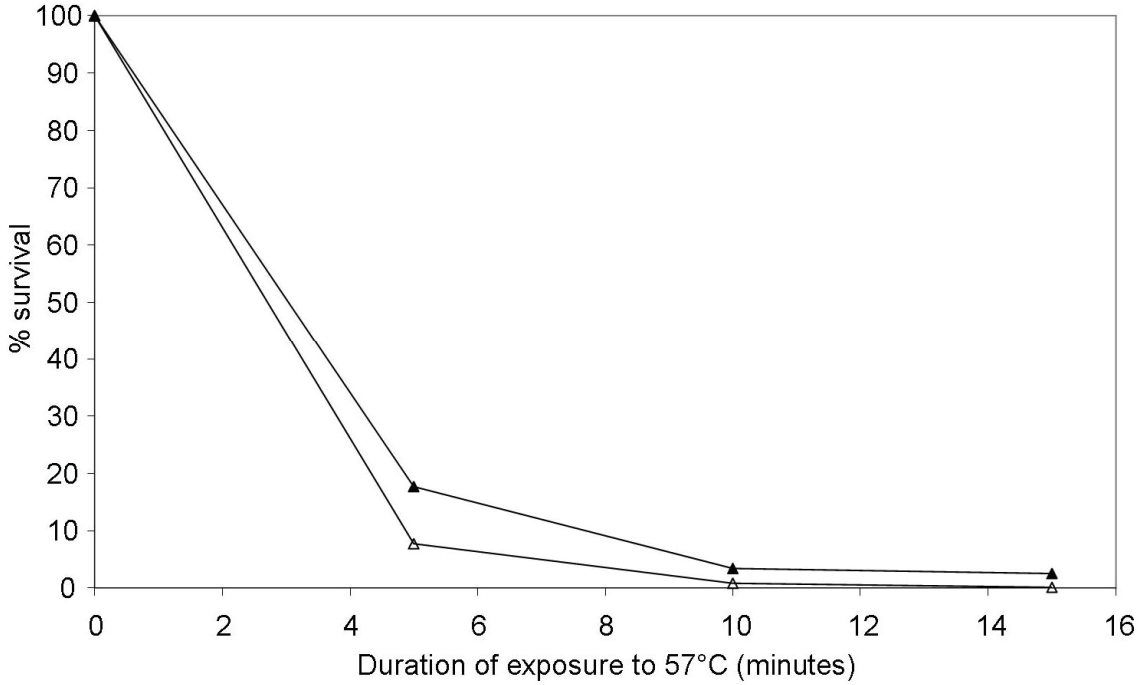


Figure 3.9. Effect of heat shock on diluted and undiluted late exponential phase cultures of *R. erythropolis* SQ1. △, late exponential phase culture (approx. 2.0 OD_{600nm}); ▲, late exponential phase diluted to approximately 0.5 OD_{600nm}.

Chloramphenicol is a bacteriostatic antibiotic that inhibits protein synthesis by blocking the peptidyl transferase activity of the ribosome subunit 50S (Neu & Gootz, 1996). To investigate whether the observed cross-protection induced by carbon starvation could be reduced by inhibiting protein synthesis, the challenged cultures were incubated for 2 h with 40 μg ml^{-1} chloramphenicol prior to heat shock treatment. This chloramphenicol concentration was selected as it is the one at which *R. erythropolis* SQ1 growth is inhibited, as illustrated in Table 3.2, thus ensuring that protein synthesis is inhibited.

In order to investigate the effect of protein synthesis inhibition on heat shock survival, samples were taken of exponential and early stationary phase cultures as described above, aliquoted and either treated with chloramphenicol or kept at 27 °C for 2 hours. All samples were then incubated in a 57 °C water bath for up to 15 minutes. At 5 minute intervals, aliquots were removed and viable plate counts were performed. Percentage survival was determined for each time point as a percentage of the viable count at time of sampling (Figure 3.10).

Figure 3.10 shows very clearly that early stationary phase cells are more resistant to heat shock than exponential phase cells, with 40–50 % cells surviving 5 minutes of shock *vs* 0 %, respectively. Interestingly, treatment of the cultures with 40 μg ml^{-1} chloramphenicol prior to heat shock treatment did not have any substantial effect on the resistance of *R. erythropolis* SQ1 to heat shock, in that their resistance to heat shock was not diminished. It has previously been shown for *E. coli* that when cultures were treated with 100 μg ml^{-1} chloramphenicol for approximately 4 hours after depletion of glucose from the growth medium, stationary phase cells were as sensitive to heat shock as exponentially growing cells (Jenkins *et al.*, 1988). Inhibition of heat shock resistance by chloramphenicol has also been demonstrated for *Enterococcus faecalis* (Giard *et al.*,

1996) and *Clostridium perfringens* (García *et al.*, 2001), although the heat-shock resistance in *Clostridium* was not starvation-induced. It was therefore hypothesised that in *E. coli* and *Enterococcus*, proteins are synthesised upon entry into stationary phase which confer heat resistance capabilities to the cells and that treatment with chloramphenicol blocks the development of heat resistance by blocking new protein synthesis.

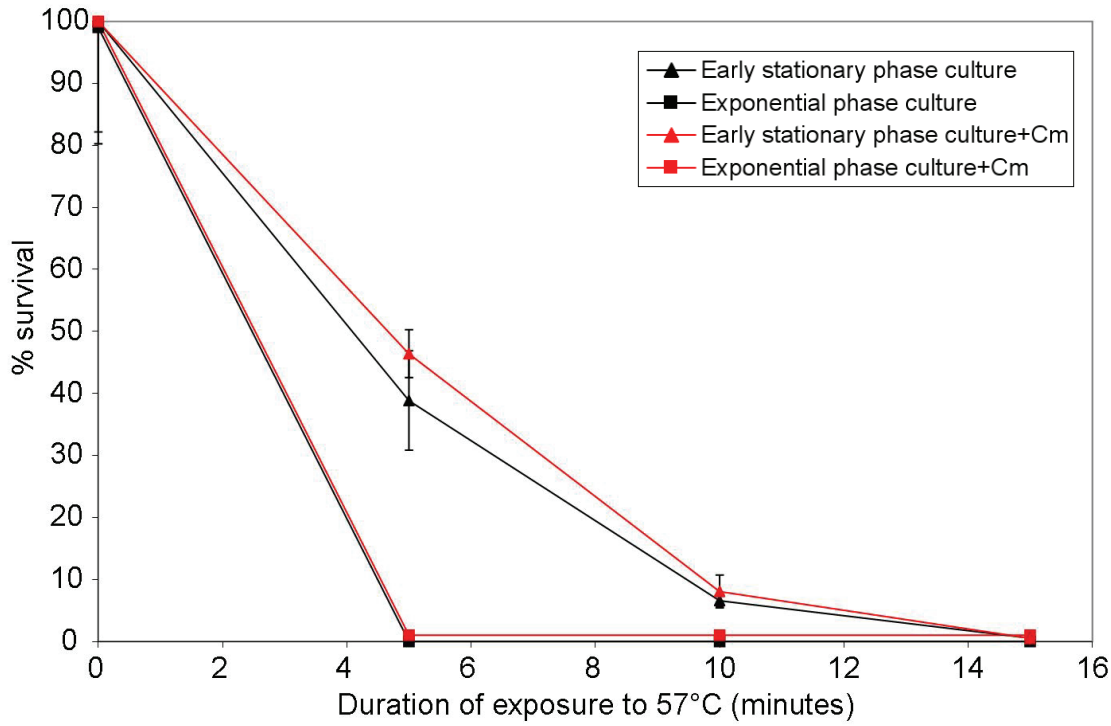


Figure 3.10. Survival of *R. erythropolis* SQ1 subjected to heat shock (57 °C) with and without exposure to 40 µg ml^{-1} chloramphenicol.

■, exponential phase culture; ■, exponential phase culture treated with chloramphenicol; ▲, early stationary phase culture; ▲, early stationary phase culture treated with chloramphenicol. Error bars represent one standard deviation.

3.3.2. Cross-protection against oxidative stress

In studies performed to date examining the interaction between nutrient starvation and the development of resistance to oxidative stress, hydrogen peroxide (H_2O_2) is typically used as the oxidative agent (see for example Giard *et al.*, 1996; Jenkins *et al.*, 1988). H_2O_2 was initially used in this study to induce oxidative stress, but the standard deviations obtained were so large that the results were not significant (data not shown). Additionally, it has been reported that *R. erythropolis* is able to use H_2O_2 as a sole oxygen source (Tarasov *et al.*, 2004). Other oxidative agents that have been used in oxidative damage studies reported in the literature include paraquat, menadione and *tert*-butyl hydroperoxide (tBHP). tBHP was used in oxidative damage studies with *Pseudomonas aeruginosa* (Ochsner *et al.*, 2000) and, in preliminary experiments performed in this study, tBHP was found to give reliable and reproducible results.

A preliminary experiment was performed to estimate the quantity of tBHP necessary to induce measurable cell death. Exponentially growing *R. erythropolis* SQ1 cells were washed in saline and resuspended to approximately 1×10^9 CFU/ml. They were then exposed to an increasing concentration of tBHP in the 1 to 600 mM range. After 5 minutes, the cells were diluted by serial dilution (hence also diluting tBHP) and plated in triplicate on LB agar in order to obtain viable plate counts (Figure 3.11). 400 mM of tBHP gave a drop in culturability of approximately 3 logs, which was deemed adequate for the purpose of further experiments.

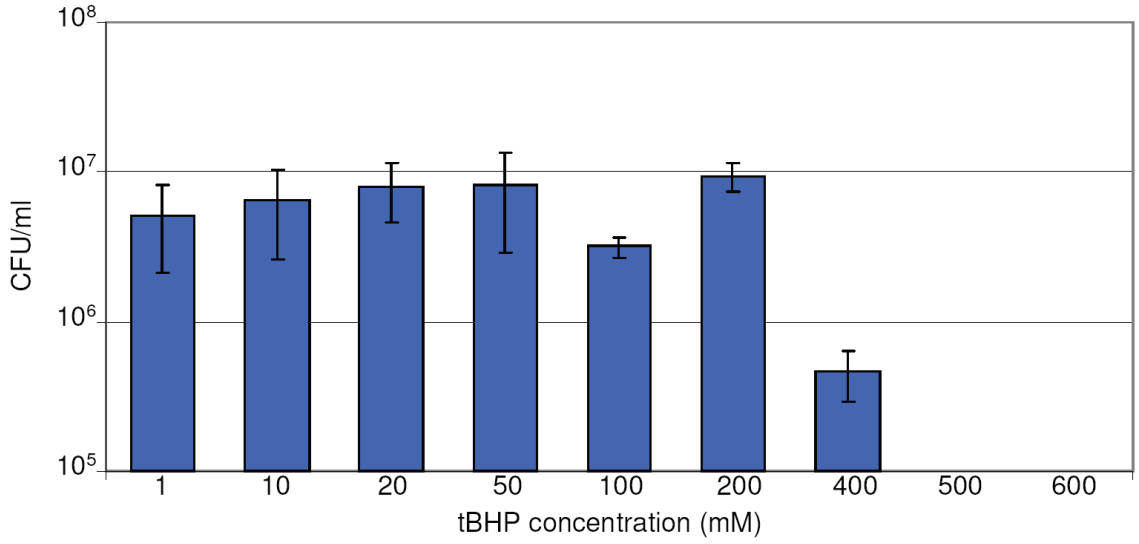


Figure 3.11. Effect of increasing concentrations of tBHP on exponentially growing *R. erythropolis* SQ1 cells.
At each concentration approximately 1×10^9 CFU/ml was treated with tBHP for 5 min and then plated in triplicate. No growth was observed at 500 and 600 mM tBHP. Results presented are that of one experiment.

Having established the concentration of tBHP required to induce oxidative stress, the effect of carbon starvation on oxidative stress resistance was investigated. CDM IIA 0.1 % glucose was chosen as the growth medium for this experiment in order to ensure that the growth limiting factor was the carbon and energy source. Flasks containing 100 ml of medium were inoculated at a cell density of 0.01 OD_{600nm} with exponential phase (24 h) cultures and incubated at 27 °C with 200 rpm agitation. Samples were taken at 48 and 144 hours (2 and 6 days), corresponding to exponentially growing and early stationary phase cultures, respectively. In order to investigate the protection afforded by carbon limitation to oxidative stress, the time points for the shock were chosen according to the glucose concentration of the medium, as illustrated previously in Figure 3.7 as days 2 and 6 – at day 2 when the culture is in exponential phase and glucose is still plentiful, while day 6 corresponds to 24 hours before exhaustion of glucose from the medium.

The exponential and early stationary phase samples taken above were aliquoted, washed and adjusted to 1×10^6 CFU/ml in saline, as described in Chapter II. Aliquots were then subjected to oxidative shock by the addition of tBHP at a final concentration of 400 mM and samples were incubated at 27 °C and under agitation. The percentage survival of the cells was then determined from viable plate counts in which samples were diluted in saline and plated in triplicate on LB agar. Percentage survival was calculated as percentage of the viable count of cells that had been removed from the reaction vessel prior to addition of tBHP.

To investigate whether the extent of cross-protection induced could be reduced by inhibiting protein synthesis, the cultures challenged were also incubated for 2 hours with 40 $\mu g\ ml^{-1}$ chloramphenicol. Immediately after sampling and dilution, two

aliquots for each time point were incubated at 27 °C – one aliquot was treated with chloramphenicol, whereas the other was not. This concentration was selected as it is the one at which *R. erythropolis* SQ1 growth is inhibited, thus ensuring that protein synthesis is inhibited. Oxidative stress was then performed as described above.

At all time points early stationary phase (6 days) *R. erythropolis* SQ1 cultures were found to be more resistant to oxidative stress than exponential phase (2 days) cultures, indicating that carbon starvation induced oxidative stress resistance in *R. erythropolis* SQ1 (Figure 3.12). At 10 minutes of exposure, early stationary phase cultures demonstrated 40 % survival in comparison with only 7 % survival for exponential phase cultures. After 15 minutes of exposure to oxidative stress, no exponential phase cells survived, in contrast to 20 % survival for the early stationary phase culture.

In contrast to the results obtained for the heat shock experiment, resistance to oxidative stress was transitorily decreased by treatment with chloramphenicol (Figure 3.12). This effect could be seen only after 5 minutes of oxidative stress; there was no significant difference in percentage survival between chloramphenicol-treated and untreated cells at 10 and 15 minutes. At 5 minutes, the percentage survival was reduced from 48 (no chloramphenicol treatment) to 26 % (chloramphenicol-treated) for exponential phase cultures, and from 63 (no chloramphenicol treatment) to 45 % chloramphenicol-treated) for stationary phase cultures. The same observation was repeatedly made in four independent experiments.

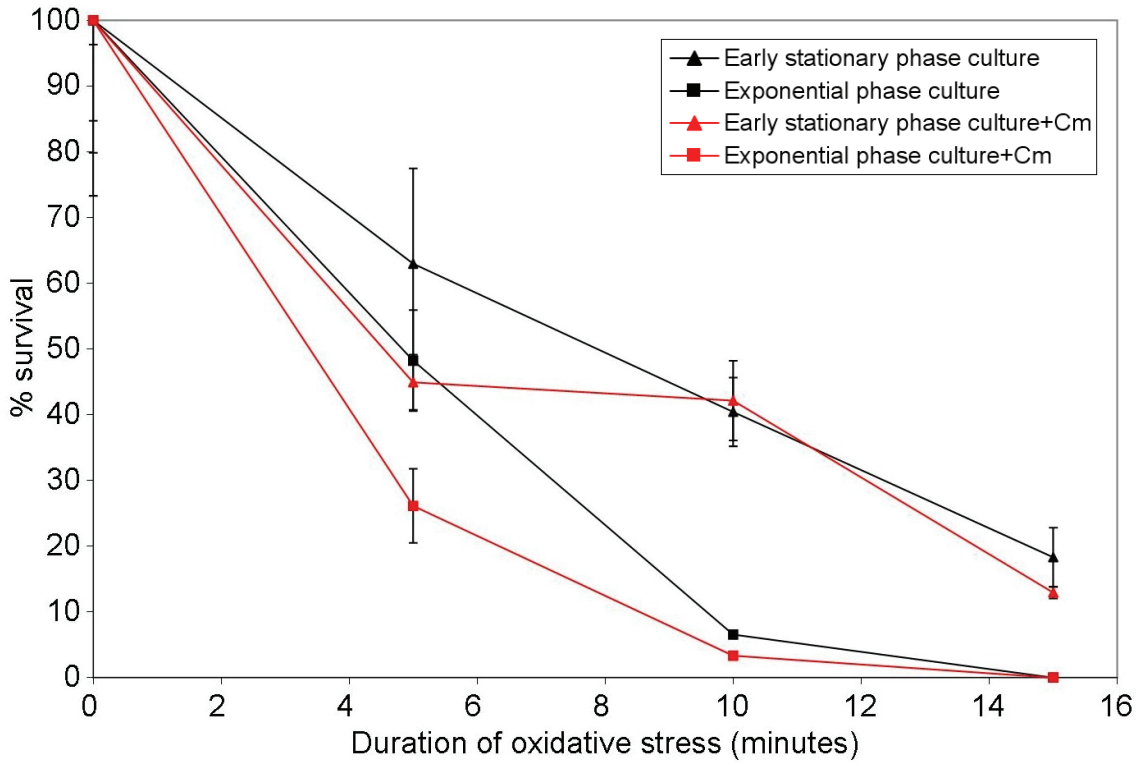


Figure 3.12. Survival of *R. erythropolis* SQ1 subjected to oxidative stress (400 mM tBHP) with and without exposure to 40 µg ml^{-1} chloramphenicol.
■, ; ■, exponential phase *R. erythropolis* SQ1 culture treated with chloramphenicol; ▲, early stationary phase *R. erythropolis* SQ1 culture; ▲, early stationary phase *R. erythropolis* SQ1 culture treated with chloramphenicol.

Chapter IV

Random Transposon Mutagenesis of *R. erythropolis* SQ1 and Genomic Analysis of the Mutants

Mutagenesis, both random and site-directed, is an essential tool in the functional analysis of microbial genomes. Many biochemical pathways and mechanisms have been elucidated by studying mutants. An example is the histidine synthesis pathway of *E. coli* (Haas *et al.*, 1952). In the context of understanding starvation survival, mutagenesis strategies have been used in *Listeria monocytogenes* (Herbert & Foster, 2001), *Staphylococcus aureus* (Watson *et al.*, 1998a) and *Mycobacterium smegmatis* (Keer *et al.*, 2000), amongst others. These studies used random mutagenesis based on transposons. Transposon mutagenesis provides many advantages over other mutagenesis methods. Most transposon systems will allow only one insertion to take place at a time, therefore disabling a single gene or a limited area of the genome (Berg *et al.*, 1983). This is in contrast to physical (UV) or chemical methods (e.g. with methoxylamine or *N*′-methyl-*N*′-nitro-*N*-nitrosoguanidine) that affect the entirety of the genome. The transposon used to disrupt the target DNA also usually contains an antibiotic resistance gene, so that mutants are easily selected. Since the sequence of the transposon is known, it also allows for recovery of the flanking sequences by various PCR methods. One drawback is that the insertion of the transposon is influenced by the target sequence, e.g. *in vitro* the transposon Tn*5* inserts preferentially in G+C-rich areas of the genome (Herron *et al.*, 2004).

Transposon mutagenesis has been reported for a number of *Rhodococcus* species. Mutagenesis using a modified *Himar1* transposon has been reported for *R. equi* (Ashour & Hondalus, 2003). pTNR, based on IS*1415*, was used to mutagenise *R. erythropolis*, *R. fascians*, *R. rhodochrous* and *R. ruber* (Sallam *et al.*, 2006). Transposon mutagenesis based on the use of the Ez:Tn<KAN-2>Tnp transposome from Epicentre has been reported for a number of *Rhodococcus* species. In this commercial system, the transposome consists of the 19 bp inverted repeat sequences of Tn*5* and the Tn*903*

kanamycin resistance gene as a complex with the Tn*5* transposase. The transposome is ready to be electroporated into the bacterial cell, where the intracellular Mg^{2+} activates the transposase. The Ez::Tn*5* transposon is then inserted randomly into the genome. Due to the fact that the Ez::Tn*5* DNA is not on a carrier plasmid, nor is it an intact transposon, introduction of multiple copies in the genome is limited and indeed unlikely (Goryshin & Reznikoff, 1998). To date, the use of the EZ::Tn*5* transposome has been reported for mutagenesis of *Rhodococcus rhodochrous* CW25, *Rhodococcus erythropolis* KA2-5-1 and *Rhodococcus equi* ATCC 33701 (Fernandes *et al.*, 2001; Mangan & Meijer, 2001; Tanaka *et al.*, 2002). This transposome system was therefore chosen in this study to generate a mutant bank of *R. erythropolis* SQ1 for the purpose of isolating carbon starvation or stationary phase survival mutants.

4.1. Generation of a mutant bank of R. erythropolis SQ1

The protocol described by Tanaka *et al.* (2002) was used for mutagenesis of *R. erythropolis* SQ1 with EZ::TN<KAN-2>Tnp and resulted in a mutant bank of 898 mutants. This protocol was selected over others because growth of *R. erythropolis* SQ1 in LB supplemented with glycine, as performed by Fernandes *et al.* (2001) and Mangan & Meijer (2001) was extremely unpredictable and impeded the production of competent cells. Growth could be very quick or plateau around OD_{600nm} 0.5, and it was not possible to predict whether growth would be fast or slow. A transformation efficiency of 4.5×10^4 mutants μg^{-1} of transposome DNA was obtained, which compares favourably with the efficiencies reported in the literature of 3×10^4 mutants μg^{-1} for *R. equi* (Mangan & Meijer, 2001) and 7.5×10^4 mutants μg^{-1} for *R. rhodochrous* (Fernandes *et al.*, 2001). A higher efficiency of 2.3×10^6 mutants μg^{-1} was reported for *R. erythropolis* KA2-5-1 (Tanaka *et al.*, 2002), possibly explained by the lower concentration of kanamycin used to select for mutants (100 $\mu g\ ml^{-1}$ in contrast to 200 $\mu g\ ml^{-1}$ in this study). A higher kanamycin concentration of 200 $\mu g\ ml^{-1}$ was needed for *R. erythropolis* SQ1 in order to avoid isolation of spontaneously resistant mutants. The isolation of spontaneous resistant mutants below 400 $\mu g\ ml^{-1}$ kanamycin was reported for *R. rhodochrous* by Fernandes *et al.* (2001).

In order to ensure that resistance to kanamycin was due to insertion of the Tn*903* kanamycin resistance gene in the genome of *R. erythropolis* SQ1, primers KANR-FP and KANR-RP were designed to amplify the kanamycin resistance gene in its entirety. A PCR amplification product of 816 bp would be expected. The plasmid pRE-7 (Zheng *et al.*, 1997), which contains the Tn*903* kanamycin resistance gene, was used as a positive control. Agarose gel electrophoresis of the PCR products obtained from 20 randomly selected mutant colonies revealed a band of approximately 800 bp

(Figure 4.1). This confirms the insertion of the transposome DNA in the *Rhodococcus* genome. No spontaneously kanamycin-resistant mutants were obtained. In total 898 kanamycin-resistant colonies were obtained.

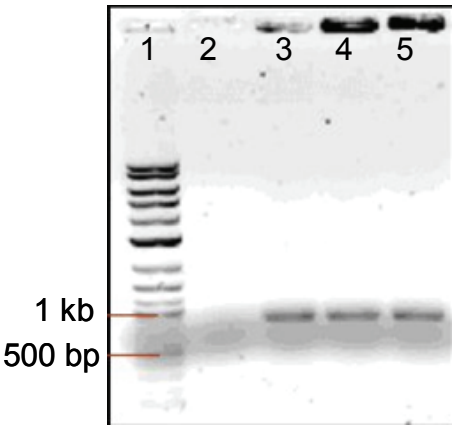


Figure 4.1. Agarose gel (0.8 %) electrophoresis of the PCR products using KANR-FP and KANR-RP, designed to the Tn*903* kanamycin resistance gene.
Lane 1, 2-log ladder (New England Biolabs); lane 2, negative control (no DNA); lane 3, positive control (pRE-7); lane 4, mutant 1; lane 5, mutant 2.

4.2. Mutant screening strategy

The mutant bank generated by transposon insertion was screened for mutants affected in stationary phase survival or recovery. This was done following the method detailed by Uhde *et al.* (1997). Following electroporation, each colony growing on LB agar supplemented with 200 μg $\mathbf{ml^{-1}}$ kanamycin was inoculated in an individual well of a microtitre plate containing 200 μl kanamycin-supplemented LB broth; 898 mutants were obtained in total. After overnight incubation at 27 °C, the mutants were transferred to a fresh microtitre plate using 96-prong replicators, and the original plate was frozen at −80 °C as the master stock. The mutants were again allowed to grow overnight before inoculating microtitre plates containing chemically defined medium CDM IIA 1 and 0.1 % glucose for stationary phase survival and recovery screening. Cultures were never allowed to enter stationary phase except for specific experiments. The mutants, while still in mid-exponential phase, were spotted on LB agar using 96-prong replicators; it was obvious after 48 h growth that important changes had occurred. The extent of growth on agar varied widely from less than 1 mm to approximately 10 mm in diameter. The shape varied slightly, flat growth in particular was observed (Figure 4.2). To facilitate the description and the screening of the mutants, they were classified at this stage in five categories, based on appearance of growth on solid medium (Table 4.1).

Group of mutants	Characterisation of growth
α	Identical or similar to wild-type *R. erythropolis* SQ1, rough and matt, slight variation in size and appearance of edge, approximately 2 mm diameter
β	Extensive growth, rough and matt, thinning towards edge (mycelial-like), serrated edges, approximately 10 mm diameter
γ	Less growth than wild-type, smooth and shiny, very often irregular shape and well-defined edges, 1 to 2 mm diameter
δ	Slow growing, appearance often variable, less than 1 mm diameter
ε	Increased/decreased pigmentation

Table 4.1. Classification of *R. erythropolis* SQ1 mutants on the basis of appearance of growth on agar plates when spotted from microtitre plates.

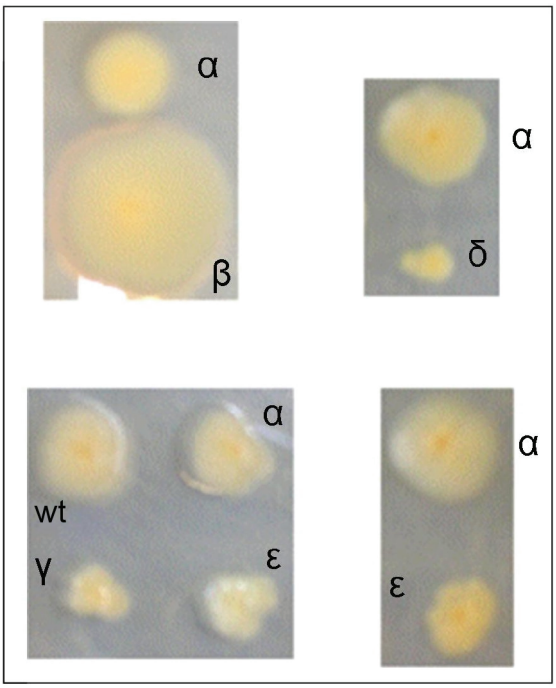


Figure 4.2. Appearance of mutants growing on CDM IIA 1 % glucose agar.
The class of each mutant is indicated on each photo. Growth on LB agar is identical to that on CDM II agar. wt, Wild-type.

The appearance of β mutants on solid medium generally remained constant, in that once the phenotype was observed; it was then reproduced and was not a transitory phase. The growth characteristics observed were also the same whether the mutants were spotted or streaked on agar. The same appearances were observed on CDM II 1 % glucose agar and LB agar. Streaking of mutants on plates also showed that the populations were homogeneous.

To investigate whether the appearance of growth on agar correlated with a change in the appearance of growth in broth, mutants of each category were picked from CDM II 1 % agar plates and inoculated into CDM II 1 % glucose broth. Appearance of growth is noted in Table 4.2. The only obvious differences from the wild-type were observed with mutants of type β, which aggregated in broth compared to the wild-type *R. erythropolis* SQ1. This may possibly be due to a change in the hydrophobicity of the cell membrane. The same appearance was observed with mutants picked from LB agar plates.

Class of mutants	Growth in liquid medium (CDM II 1 % glucose broth) when transferred from CDM II 1 % glucose agar		
	24 h incubation	**48 h incubation**	**>96 h incubation**
α	Small aggregates (less than 0.5 mm)	Predominantly homogenous growth	Predominantly homogenous growth
β	Aggregates (up to 1 mm diameter)	Aggregates (up to 1 mm diameter)	Aggregates (up to 1 mm diameter)
γ	Homogenous growth	Homogenous growth	Homogenous growth
δ	ND	ND	ND
ε	Small aggregates (less than 0.5 mm)	Predominantly homogenous growth	Predominantly homogenous growth

Table 4.2. Appearance of growth of the different classes of mutants in liquid CDM II 1 % glucose. ND, not determined.

In order to screen the mutant bank for stationary phase and carbon starvation survival mutants, the 898 mutants were cultured for up to three weeks in microtitre plates containing CDM II 1 % glucose and CDM II 0.1 % glucose at 27 °C, following an adaptation of the protocol used by Uhde *et al.* (1997) (see Chapter II). The reasoning was that comparison of the growth patterns of *R. erythropolis* SQ1 mutants in CDM II 1 % glucose and 0.1 % glucose would reveal mutants that, for example, could survive stationary phase after growth in low-carbon medium but not high-carbon, and *vice-versa*. Following 1, 8, 14 and 22 days of incubation in the microtitre plates, a replica of each plate was made on LB agar and incubated at 27 °C. After 48 h incubation (for the 1, 8 and 14 day samples) or 96 h incubation (for the 22 day samples) the mutants were classified according to the system described in Table 4.1 and the results recorded. Growth on plates was observed after 4 days for 22 days samples because growth occurred more slowly at that time point, as judged by observation of the wild-type growth in well A1 of each plate. A summary of the results is presented in Table 4.3.

It is interesting to note that in CDM 1 % glucose, the number of class δ mutants remained almost constant, whereas the mutant classes β, ε and γ increased over time. This is particularly so for γ mutants, increasing from 28 at 24 h to 58 at 14 days. Similar observations were made for CDM 0.1% glucose; the number of γ mutants increased from 3 to 54 from day 1 to 14. In addition, the δ mutants increased in number from 8 to 27. Four pigmentation mutants were also observed. Three were white and one was orange, instead of the salmon colour of *R. erythropolis* SQ1.

Nine mutants consistently failed to form colonies on LB agar, in contrast with the mutants above that failed to grow on agar only transitorily. An attempt to culture them

in LB broth failed. They presumably have an insertion in a biosynthetic pathway, the product of which is not provided by LB or CDM.

Growth medium	Mutant class	Incubation time			
		1 day	8 days	14 days	22 days*
CDM 1 % glucose	α	829	804	763	767
	β	0	22	26	29
	γ	28	40	58	53
	δ	19	17	18	20
	ε	0	4	4	10
	No growth	22	11	29	21
CDM 0.1 % glucose	α	866	800	765	781
	β	0	27	27	29
	γ	3	42	54	51
	δ	8	12	27	19
	ε	0	2	2	3
	No growth	21	15	23	17

Table 4.3. Summary of the results of the mutant bank screening experiment.

At each time point indicated, the mutants grown in microtitre plates were duplicated onto LB agar. After 2 days of growth (4 days for 22-day samples), each mutant was scored and the score recorded.

*Total number for this time point is 900, this is due to mutants 4C7 and 9A3 being classified as γ and ε simultaneously, i.e. their growth is similar to γ with in addition an altered pigmentation; 4C7 is orange whereas 9A3 is white.

Sixteen mutants, exhibiting weak or no growth in at least one stage of screening, were selected for further study. Detailed results of the stationary phase/carbon starvation screening for these mutants are shown in Table 4.4. It was expected that stationary phase survival mutants would grow at day 1 and maybe day 8, and then die out (see mutant 3F5). It was also expected that mutants that are deficient in glucose-sensing mechanisms, for example, would die quickly in CDM 0.1 % glucose, since they would not adapt to the low-energy conditions. Auxotrophic mutants were not expected to grow in either 1 or 0.1 % glucose medium.

Mutant	Extent of growth at various time points							
	CDM 1 % glucose				CDM 0.1 % glucose			
	1 d	8 d	14 d	22 d	1 d	8 d	14 d	22 d
1B2	WT	++	WT	++	WT	WT	+	WT
1B3	+	WT	+	+	WT	WT	+	+
1H1	WT	WT	WT	WT	WT	WT	+	WT
3B4	−	+	−	−	WT	WT	−	+
3B10	−	+	−	−	−	+	−	−
3F5	WT	+	−	−	+	+	−	+
4G6	WT	++	++	WT	WT	WT	+	WT
4G11	+	WT	−	+	WT	WT	+	+
5C11	WT	WT	WT	WT	WT	WT	+	+
5F8	−	WT	+	−	+	−	+	−
6B2	WT	WT	WT	+	WT	+	+	+
6E6	−	WT	−	+	WT	WT	−	+
8B7	+	WT	WT	WT	WT	+	+	+
10D3	++	++	++	++	WT	WT	+	WT
10E1	+	WT	+	WT	WT	WT	+	+
10E4	+	+	+	+	+	+	+	+

Table 4.4. Results of screening of selected mutants.
Detailed results for 16 mutants, selected for further study, from the mutant bank stationary phase/carbon starvation screening experiment. Mutants were grown in microtitre plates and plated on LB agar. Growth was observed at 2 days after plating (4 days for 22-day samples). WT, wild-type growth; ++, intermediate growth; +, weak growth; −, no growth was observed.

4.3. Growth curves of selected mutants in chemically defined medium

The growth of wild-type *R. erythropolis* SQ1 was compared with that of mutants. The 16 mutants described in Table 4.4 were further investigated by cultivation in glass flasks, as was described previously for wild-type *R. erythropolis* SQ1. Briefly, an inoculum of the mutant culture was taken from the stock microtitre plate (LB + kanamycin) and inoculated in LB broth (10 ml in a universal). After overnight growth, this culture was used to inoculate 100 ml CDM IIA 1 % and 0.1 % glucose in a 500 ml conical flask to 0.01 OD_{600nm}. Samples were taken at regular intervals. The OD_{600nm} and viable plate counts were measured at regular intervals. Viable plate counts were determined by serial dilution and plating on LB agar, as described previously. Four mutants, 4G6, 10D3, 1B2 and 1H1 were found to behave differently from the wild-type.

As can be seen from Figure 4.3, mutants 4G6 and 10D3 did not survive well during prolonged stationary phase. Mutant 4G6 grown in CDM IIA 1 % glucose reached a maximum cell density of 6.7×10^9 CFU/ml at 3 days, with a subsequent steady decrease in culturability until day 27, when cell counts of 7.6×10^5 CFU/ml were obtained (0.01 % survival compared to maximum) (Figure 4.3a). No drop in culturability was observed for mutant 4G6 grown in CDM IIA 0.1 % glucose, but the cell densities obtained were consistently lower than those of the wild-type *R. erythropolis* SQ1.

Mutant 10D3 grown in CDM IIA 1 % glucose (Figure 4.3b) reached a maximum of 5.5×10^9 CFU/ml at 12 days, showing that it probably grows slower than wild-type (maximum of 6.3×10^9 CFU/ml at 4 days). The viable cell density then decreased to

5.1×10^6 CFU/ml (0.1 % survival) at 27 days. When grown in CDM IIA 0.1 % glucose, mutant 10D3 appeared to behave as wild-type *R. erythropolis* SQ1.

In contrast, only a modest decrease in culturability could be observed for mutants 1B2 and 1H1 in comparison with wild-type *R. erythropolis* SQ1 when grown in CDM IIA 1 % glucose (Figure 4.4). For mutant 1B2, culturability dropped to approximately 1×10^8 CFU/ml at 20 days then remained constant. For mutant 1H1, culturability remained similar to wild-type until 27 days, when it dropped to 1×10^8 CFU/ml. No significant decrease in culturability was observed when the mutants were grown in CDM IIA 0.1 % glucose.

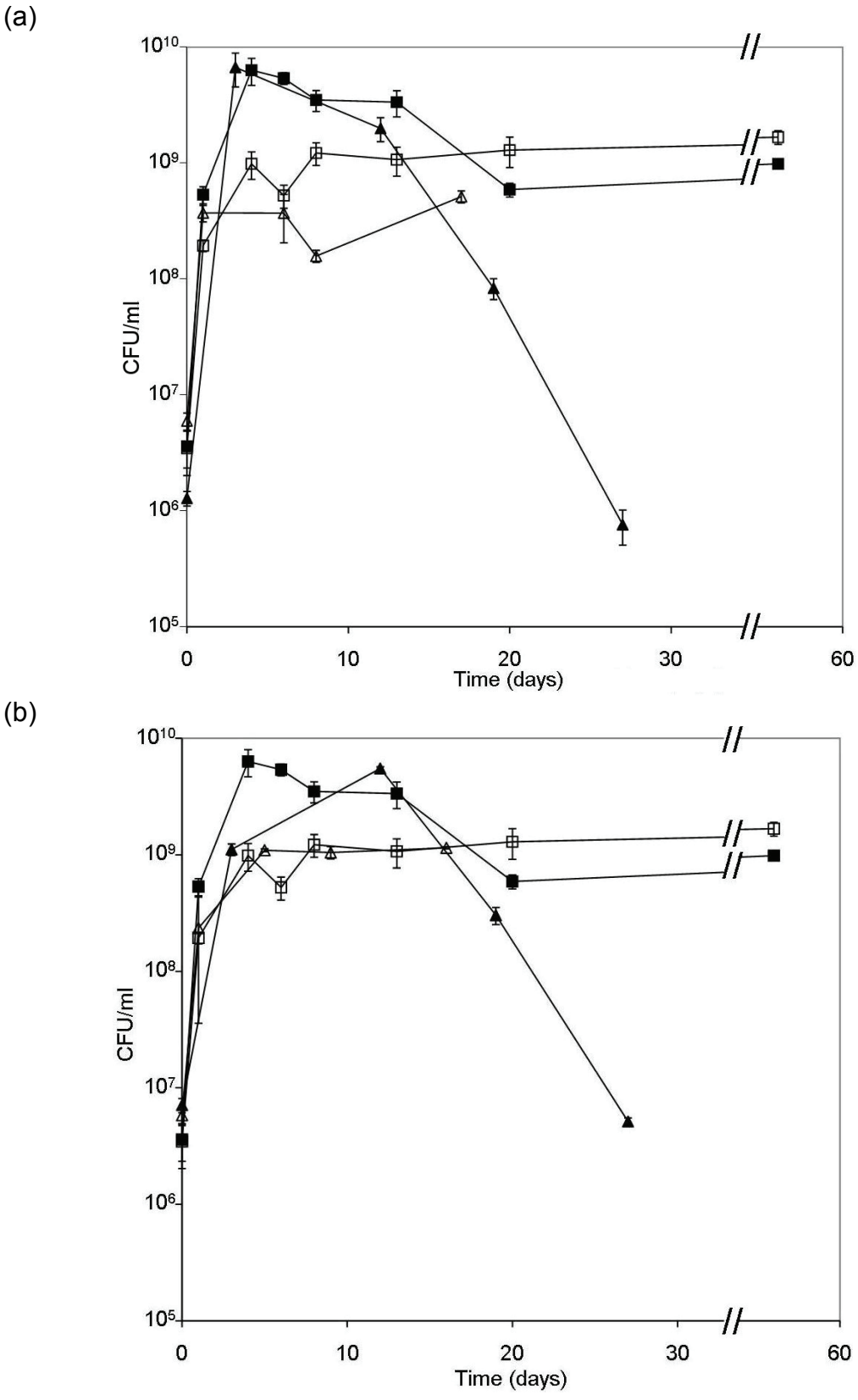
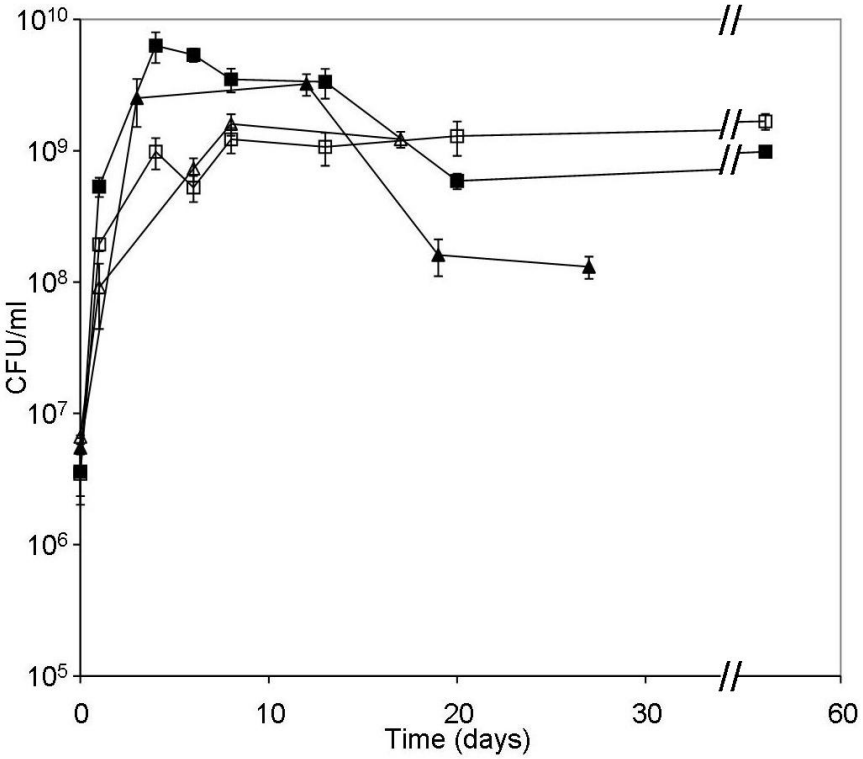


Figure 4.3. Growth curves of mutants 4G6 (a) and 10D3 (b) compared to wild-type *R. erythropolis* SQ1. Data are from two separate experiments, plated in triplicate. Closed symbols, CDM IIA 1% glucose; open symbols, CDM IIA 0.1% glucose. ■ and □, wild-type *R. erythropolis* SQ1; ▲ and △, mutants 4G6 (a) and 10D3(b).

(a)



(b)

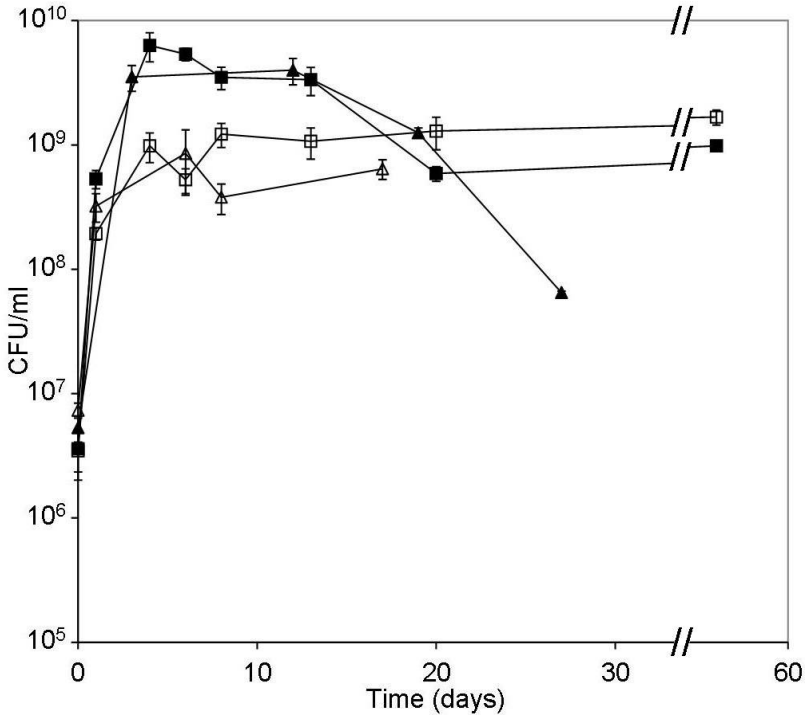


Figure 4.4. Growth curves of mutants 1B2 (a) and 1H1 (b) compared to wild-type *R. erythropolis* SQ1. Data are from two separate experiments, plated in triplicate. Closed symbols, CDM IIA 1% glucose; open symbols, CDM IIA 0.1% glucose. ■ and □, wild-type *R. erythropolis* SQ1; ▲ and △, mutants 1B2 (a) and 1H1(b).

4.4. Identification of genes and functions disrupted by transposon insertion

The strategy used for obtaining the sequence of the DNA flanking the inserted transposon in any particular mutant was based on restriction and religation of the genomic DNA, followed by inverse PCR. Total genomic DNA was purified from the mutants as described in Chapter II and restricted using a panel of restriction endonucleases. The DNA from each restriction reaction was religated. A fraction of the religated DNA should carry the transposon. The DNA flanking the transposon was then amplified by inverse PCR using primers KAN-2_FP-1 and KAN-2_RP-1 (provided in the transposon kit, Epicentre Biotechnologies), that are specific to the 3′ and 5′ ends of the transposon, respectively, and designed to amplify DNA upstream and downstream of the kanamycin resistance gene (Figure 4.5). The amplicons were then cloned into pCR2.1 (Invitrogen) and sequenced using the M13 forward and reverse primers, specific to areas of the vector that are adjacent to the cloned sequence.

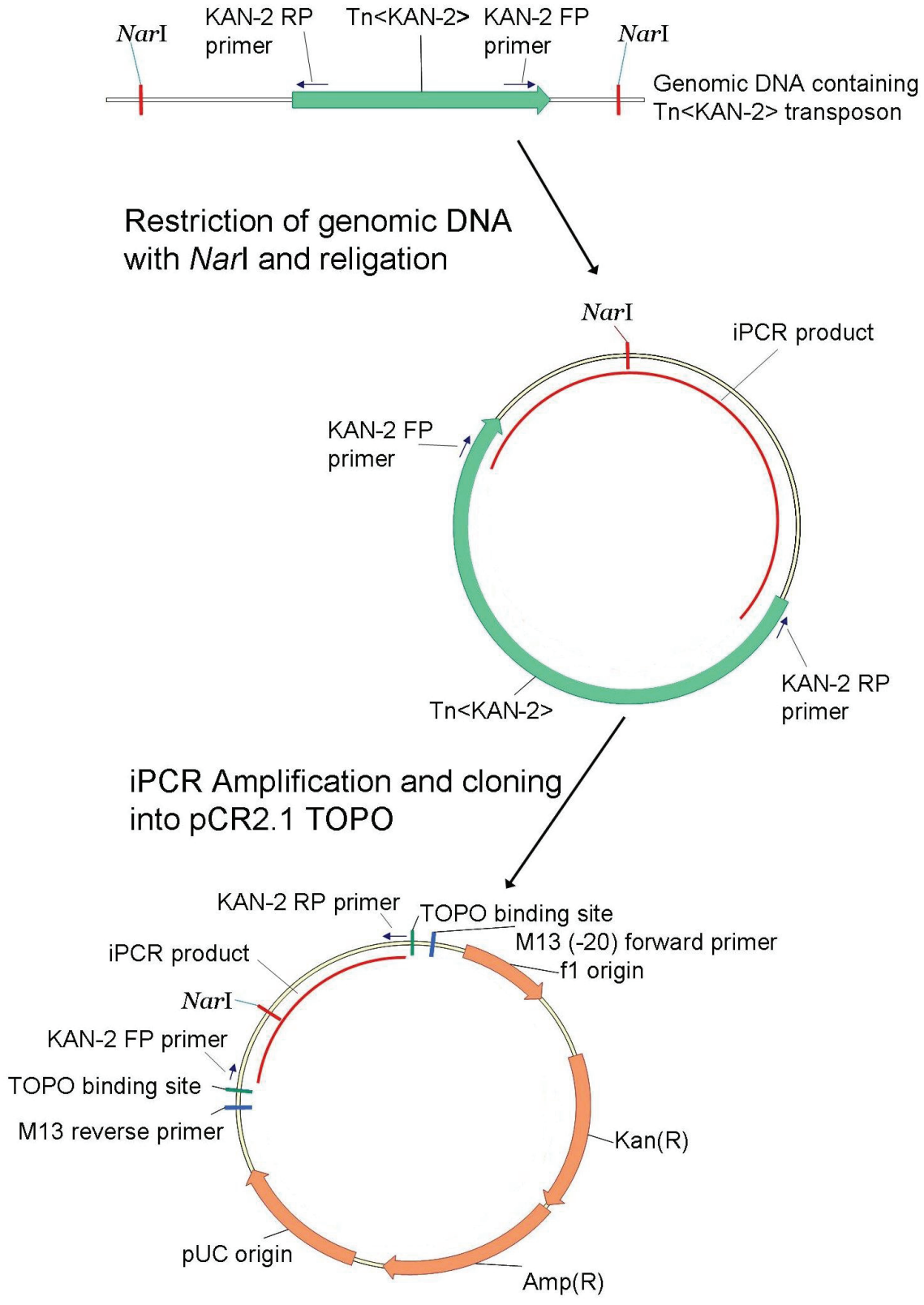


Figure 4.5. Principle of recovering DNA flanking a transposon insertion site by inverse PCR (iPCR). In this example *Nar*I has been arbitrarily selected as the restriction endonuclease for restriction of the genomic DNA in the first step.

Mutants selected by the screening strategy described above were grown in LB broth and their genomic DNA isolated for characterisation of the sequence disrupted by transposon insertion. Since it is not possible to predict which restriction endonuclease will yield a fragment that can be religated and amplified by iPCR, it is important in the first instance to restrict the genomic DNA using a range of restriction enzymes. For instance, using mutant 1H1 as an example, although *Eco*RI cuts the genomic DNA efficiently (Figure 4.6), iPCR products were only obtained for religated *Sal*I-, *Bam*HI- and *Bcl*I-digested DNA. These products were of approximately 200 bp, 1 and 8 kb, respectively (Figure 4.7). Therefore, for all the mutants investigated the first step of the analysis involved treating the genomic DNA with a range of restriction endonucleases, followed by religation and iPCR.

The iPCR amplification products of an appropriate size (0.2–8 kb) were then selected for cloning into the vector pCR2.1 using the TA cloning system (Invitrogen) and subsequent sequencing. The pCR2.1 vector is designed with binding sites for the primers M13 uni (−21) and M13 rev (−29) on either side of the cloning site, which can be used to verify the presence of inserted DNA by PCR and for sequencing the cloned DNA.

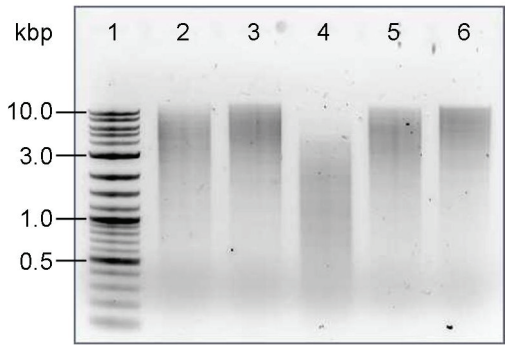


Figure 4.6. Total genomic DNA from mutant 1H1 subjected to restriction endonucleases and analysed by agarose gel electrophoresis. Lane 1, 2-Log DNA ladder (New England Biolabs); Lanes 2-6, DNA restricted by *Bcl*I, *Eco*RI, *Sal*I, *Bam*HI and *Bgl*II, respectively. Sizes of the molecular weight standards are shown on the left.

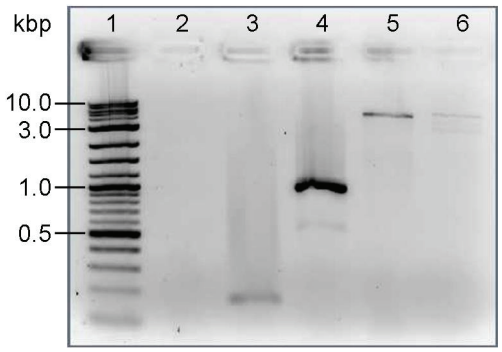


Figure 4.7. Agarose gel electrophoresis of DNA products of inverse PCR on religated DNA from mutant 1H1. Lane 1, 2-log DNA ladder; lanes 2-6, template was DNA restricted by *Eco*RI, *Sal*I, *Bam*HI, *Bcl*I and *Bgl*II, and religated prior to iPCR. Sizes of the molecular weight standards are shown on the left.

For all mutants analysed, the DNA sequence of the cloned insert was determined using M13 uni (−21) and M13 rev (−29) primers. The resulting data was processed and analysed using the bioinformatic suite Vector NTI (Invitrogen) and online resources such as BLASTX and BLASTP at the NCBI (http://www.ncbi.nlm.nih.gov). The first step in analysing the products obtained by inverse PCR was to reconstruct the sequence by firstly identifying the restriction site for the endonuclease used to prepare the DNA template for iPCR and secondly by locating the transposon insertion site. Only the sequences between the restriction sites (if available) and the transposon insertion site were retained. The reverse complement of one of the two sequences was then generated and the two sequences amalgamated by joining at the transposon insertion site, thereby reconstructing the transposon insertion site and enabling analyses of the disrupted sequence.

4.4.1. Nucleotide excision repair mutant 4G6: *uvrB* mutant

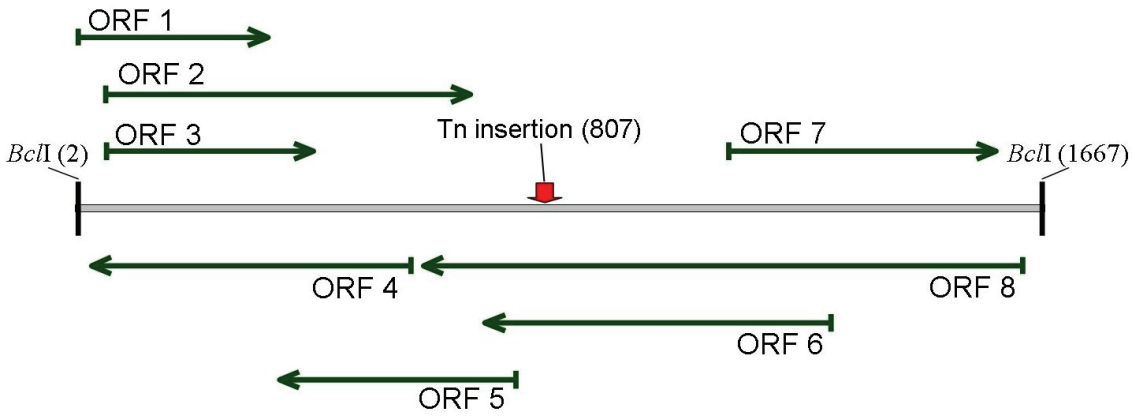
Genomic DNA from mutant 4G6 was isolated, restricted with a range of enzymes and then religated to perform iPCR. A 1.7 kb PCR amplification product was obtained from *Bcl*I-restricted template DNA, which was then cloned and sequenced as described earlier, yielding a sequence of 1671 bp. The sequence was examined for the presence of a 9 bp repeat sequence, a characteristic of Tn*5* transposon insertion (Berg *et al.*, 1983). In mutant 4G6 there is slight variation in the 9 bp sequence upstream and downstream of the transposon. Alignment of the two sequences is thus:

```
GACCGGCCT
 |||  ||||
CACCCGCCT
```

It can be seen that 2 out of 9 bp are different, at positions 1 and 5. Note that two guanines in the upstream sequence are replaced with two cytosines downstream. This did not result in any difference for the open reading frame search; the same ORFs were identified regardless of which of the 9 bp sequence was retained in the reconstructed sequence (Figure 4.8). It should be noted that in all the other mutants analysed (described later in this chapter), the repeat sequences are identical.

Eight putative ORFs were identified in the 1671 bp sequence obtained (Figure 4.8). The ORFs were then analysed by comparison with the non-redundant protein database at NCBI using BLASTX. BLASTX translates the sequences in the six frames then searches the protein database against them; the bacterial genetic code was used for the translation. The most significant result (*E*-values $<1\times10^{-33}$) were for ORF 8 and ORF 4 (Table 4.5). This was corroborated by performing a BLASTP search on all ORF frames. ORF 4 was found to be similar to an hypothetical protein from *Rhodococcus* sp. RHA1 and several *Mycobacterium* species. The protein predicted for ORF 8 was found to possess 73 % amino acid identity to the 350 C-terminal amino acids of UvrB

(excinuclease ABC subunit B) (Figure 4.9). UvrB is typically a protein of 673–721 amino acids (e.g. 673 and 698 amino acids for *M. tuberculosis* and *E. coli*, respectively) (Arikan *et al.*, 1986; Darwin *et al.*, 2003). It seems therefore that the sequence obtained for mutant 4G6 corresponds to the 3′ end of the *uvrB* gene.



Open reading frame	Position	Strand	Size
ORF 1	3–329	Sense	327 bp
ORF 2	50–679	Sense	630 bp
ORF 3	52–408	Sense	357 bp
ORF 4	25–576	Antisense	552 bp
ORF 5	350–757	Antisense	408 bp
ORF 6	705–1301	Antisense	597 bp
ORF 7	1126–1587	Sense	462 bp
ORF 8	598–1632	Antisense	1035 bp

Figure 4.8. Schematic representation of the 1671 bp sequence retrieved for mutant 4G6. The *Bcl*I restriction sites used to retrieve the sequence are shown, as well as the transposon insertion site and putative open reading frames. Size, position and strand of the ORFs identified in the sequence are shown in the table.
ORFs were identified with the ORF Finder function of Vector NTI (Invitrogen, http://www.invitrogen.com).

Organism	Protein	Score
ORF 4		
Rhodococcus sp. RHA1	Hypothetical protein RHA1_ro00975	6×10^{-65}
Saccharopolyspora erythraea NRRL 2338	Hypothetical protein SACE_2169	2×10^{-43}
Streptomyces coelicolor A3(2)	Hypothetical protein SCO6745	1×10^{-34}
Mycobacterium sp. JLS	Hypothetical protein Mjls_2552	4×10^{-33}
Mycobacterium sp. MCS	Hypothetical protein Mmcs_2515	4×10^{-33}
ORF 8		
Rhodococcus sp. RHA1	Excision nuclease ABC subunit B	2×10^{-161}
Mycobacterium gilvum PYR-GCK	Excinuclease ABC, B subunit	8×10^{-144}
Mycobacterium bovis BCG str. Pasteur 1173P2	Excinuclease ABC subunit B *uvrB*	3×10^{-143}
Nocardia farcinica IFM 10152	Excinuclease ABC subunit B	5×10^{-143}
Mycobacterium tuberculosis CDC1551	Excinuclease ABC subunit B	6×10^{-143}

Table 4.5. BLASTX search results for ORF 4 and ORF 8. The genetic code was set to 11 (bacterial) and the search was performed on the non-redundant database.

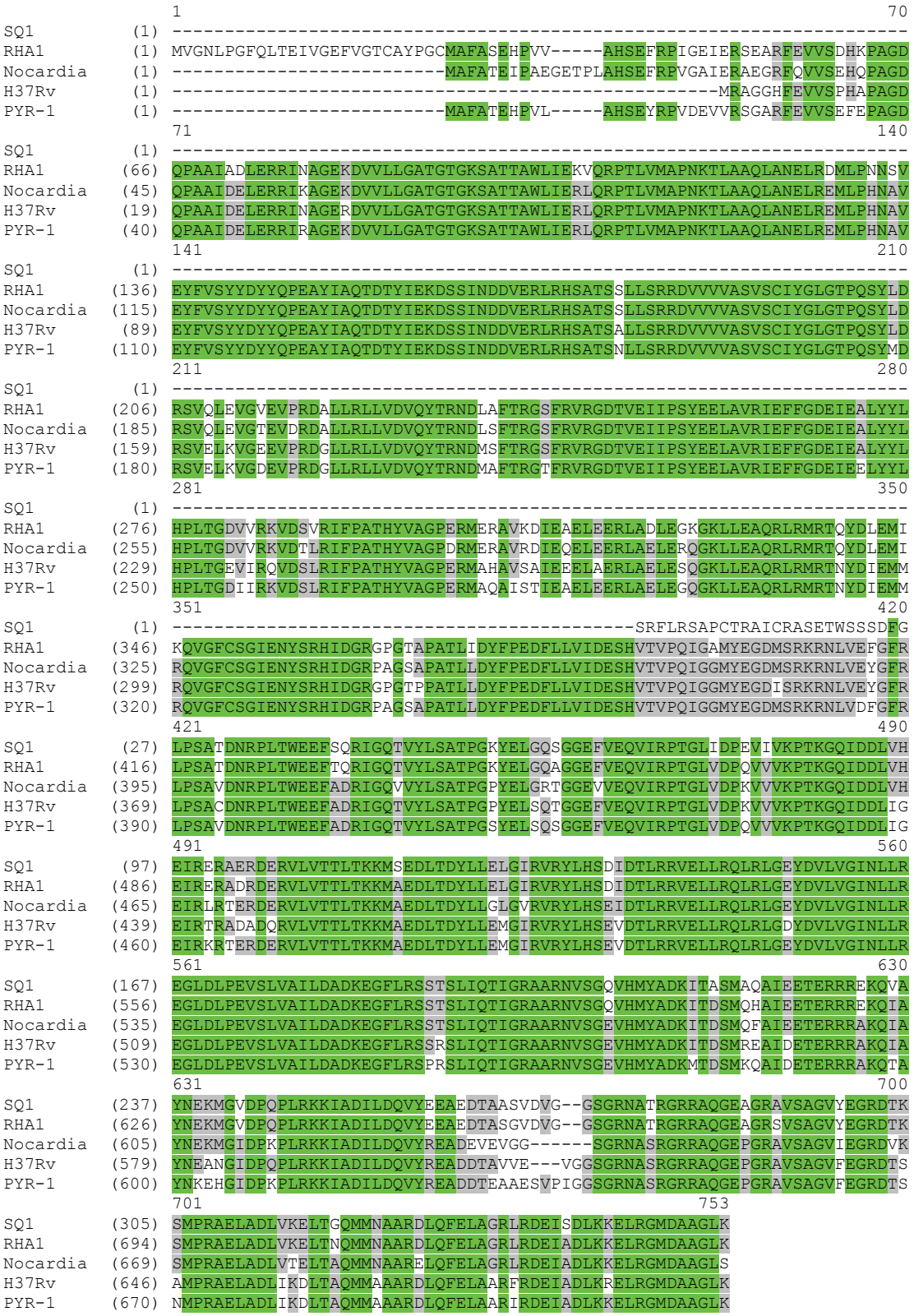


Figure 4.9. Alignment of the putative UvrB sequence from *R. erythropolis* SQ1 with sequences from *Rhodococcus* sp. RHA1, *Nocardia farcinica* IFM 10152, *M. tuberculosis* H37Rv and *M. vanbaalenii* PYR-1. Grey background, >50 % identity; green background, identical residues.

The nucleotide excision repair pathway is ubiquitous in all organisms (Eisen & Hanawalt, 1999), of which UvrB is an important subunit. UvrA and UvrB function, in *E. coli*, as a complex to identify conformational defects caused by DNA damage, by moving randomly along the DNA. The DNA conformation is changed by ATP hydrolysis and binding of UvrB to the DNA, with subsequent release of UvrA from the complex. UvrC will then attach to the DNA–UvrB complex and nick the DNA on either side of the lesion; the DNA–UvrB–UvrC complex is released by a helicase II. The gap is then filled by the DNA polymerase I (Lodish *et al.*, 2000). *M. tuberculosis uvrB* transposon mutants were found to be hypersensitive to nitric oxide and UV light (Darwin *et al.*, 2003). These UvrB mutants were also found to be less virulent in mice than wild-type strains (Darwin & Nathan, 2005). Interestingly, *M. tuberculosis uvrB* mutants were shown to reach the same cell density as wild-type when grown in Middlebrook 7H9 broth supplemented with 0.2 % glycerol, 0.05 % Tween-80, 0.5 % bovine serum albumin, 0.2 % dextrose and 0.085 % sodium chloride (Darwin *et al.*, 2003). Although further work showed that ABC excision repair has a role in the pathogenesis of *M. tuberculosis* (Darwin & Nathan, 2005), and in particular in the resistance to nitric oxide, it is not known whether *uvrB* mutants can survive long-term starvation.

In order to understand the genetic context of this transposon insertion in mutant 4G6, the arrangement of genes coding for the elements of the nucleotide excision repair pathway (*uvrA–D*) was investigated in *Rhodococcus* RHA1, *M. tuberculosis* CDC1551 and H37Rv, *Nocardia farcinica* IFM10152 and *E. coli* K12 using the genome visualisation program BacMap (Stothard *et al.*, 2005), available at http://wishart.biology.ualberta.ca/BacMap. It should be noted that *Rhodococcus* RHA1 (McLeod *et al.*, 2006) and *R. equi* 103 (www.sanger.ac.uk/Projects/R_equi/) represent

the only sequenced *Rhodococcus* genomes to date. It was found that in the genomes the *uvr* genes are not organised in an operon, but rather are distributed across the genome of all these species. A putative ORF was identified downstream of *uvrB* in *R. erythropolis* SQ1 that is very similar to ORF RHA1_ro00975 in *Rhodococcus* RHA1, but both are of unknown function. In other organisms a variety of genes are found downstream of *uvrB*, e.g. the molybdenum cofactor biosynthesis protein in *E. coli* K12 or the puromycin resistance protein in both *M. tuberculosis* CDC1551 and H37Rv.

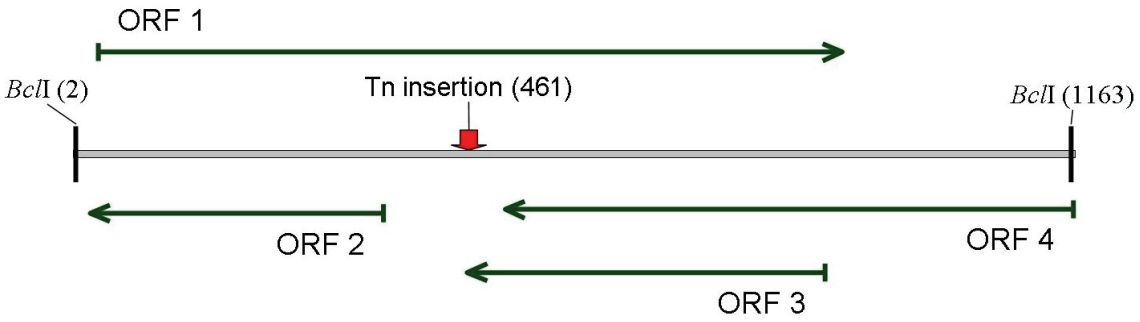
4.4.2. Mutant with insertion downstream of putative IMP dehydrogenase gene *guaB*: mutant 10D3

Mutant 10D3 was found to have a consistently weaker growth than wild type *R. erythropolis* SQ1 on all solid media tested (Section 4.1.2). Growth curves also showed that this mutant did not survive prolonged starvation in CDM IIA 1 % glucose, with the culturability dropping to 0.1 % of the maximum after 15 days in stationary phase. The genomic DNA was isolated and restricted with a panel of enzymes as previously described. iPCR yielded a 1.2 kb fragment from *Bcl*I-restricted genomic DNA, which was subsequently cloned and sequenced. A 1165 bp sequence was reconstructed and analysed for open reading frames (as described above), the results of which are shown in Figure 4.10.

When the nucleotide sequence was analysed against the non-redundant database at the NCBI using BLASTX, a putative inositol-5′-monophosphate dehydrogenase (GuaB) was identified, encoded by ORF 4. Scores lower than 1×10^{-100} were obtained with GuaB from *Rhodococcus* RHA1, *N. farcinica* IFM10152 and several *Mycobacterium* species. This result was confirmed by running the translated product of all the ORFs identified against the non-redundant database using BLASTP. The putative protein sequence obtained by translation of ORF 4 is 221 amino acids long and is highly similar to GuaB from various organisms. For example, amino acid identities of 93, 88 and 85 % were obtained with the GuaB of *Rhodococcus* RHA1, *N. farcinica* IFM10152 and *M. tuberculosis* H37Rv. The alignment of the ORF 4 amino acid sequence with selected IMP dehydrogenases of high similarity shows clearly that the sequence obtained for mutant 10D3 represents the 3′ end of a putative *guaB* gene. Alignment of the available putative *R. erythropolis* SQ1 IMP dehydrogenase sequence with the corresponding

GuaB sequences returns an identity of 77 % for the 200 C-terminal amino acids (Figure 4.11).

The inositol-5′-monophosphate dehydrogenase (EC 1.1.1.205) catalyses the dehydrogenation of inosine 5′-monophosphate into xanthosine 5′-phosphate (Figure 4.12). It is an essential step in the synthesis of guanosine 5′-phosphate (GMP). GMP then feeds directly into the synthesis of guanosine 5′-diphosphate (GDP), and from there into the synthesis of lipoarabinomannan, phosphatidylinositol mannosides and lipomannan (Yamazaki *et al.*, 2006), and of course DNA. It is encoded by the gene *guaB* and is part of the guanine synthesis operon in *E. coli* (Vales *et al.*, 1979).



Open reading frame	Position	Strand	Size
ORF 1	29–895	Sense	867 bp
ORF 2	19–360	Antisense	342 bp
ORF 3	459–875	Antisense	417 bp
ORF 4	503–1165	Antisense	663 bp

Figure 4.10. Schematic representation of the 1165 bp sequence retrieved for mutant 10D3. The *Bcl*I restriction sites used to retrieve the sequence are shown, as well as putative open reading frames predicted by ORF finder and the transposon insertion site. Size, position and strand of the ORFs identified in the sequence are shown in the table.
ORFs were identified with the ORF Finder function of Vector NTI (Invitrogen, http://www.invitrogen.com).

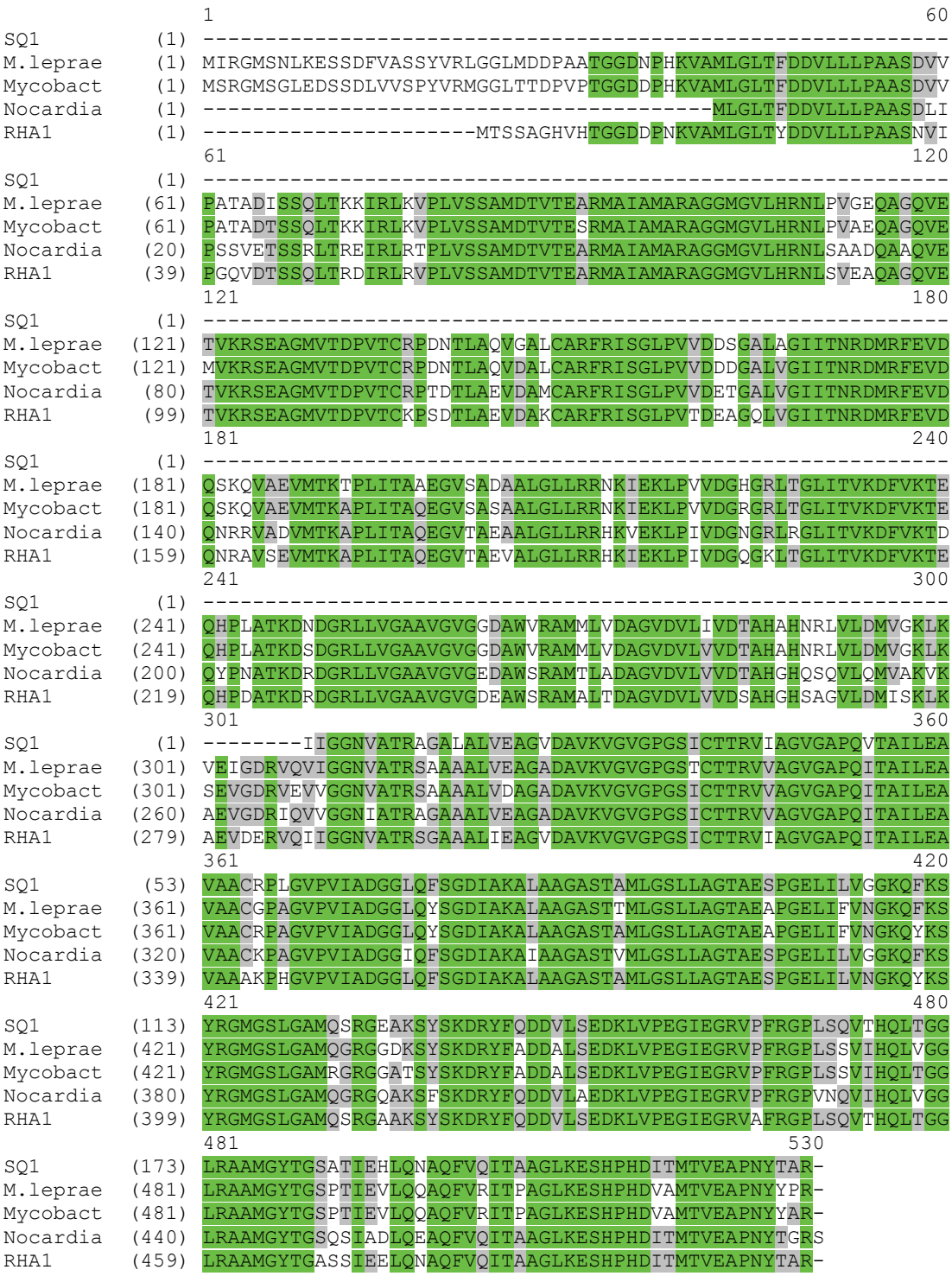


Figure 4.11. Alignment of a putative IMP dehydrogenase sequence from *R. erythropolis* SQ1 with sequences from *Rhodococcus* sp. RHA1 (*guaB1*), *Nocardia farcinica* IFM 10152 (*guaB*), and *Mycobacterium leprae* TN (*guaB2*).
Mycobact is the sequence of a IMP dehydrogenase found in *M. tuberculosis* H37Rv, *M. tuberculosis* CDC 1551 and *M. bovis* AF2122/97 (*guaB2*). Grey background, >50 % identity; green background, identical residues.

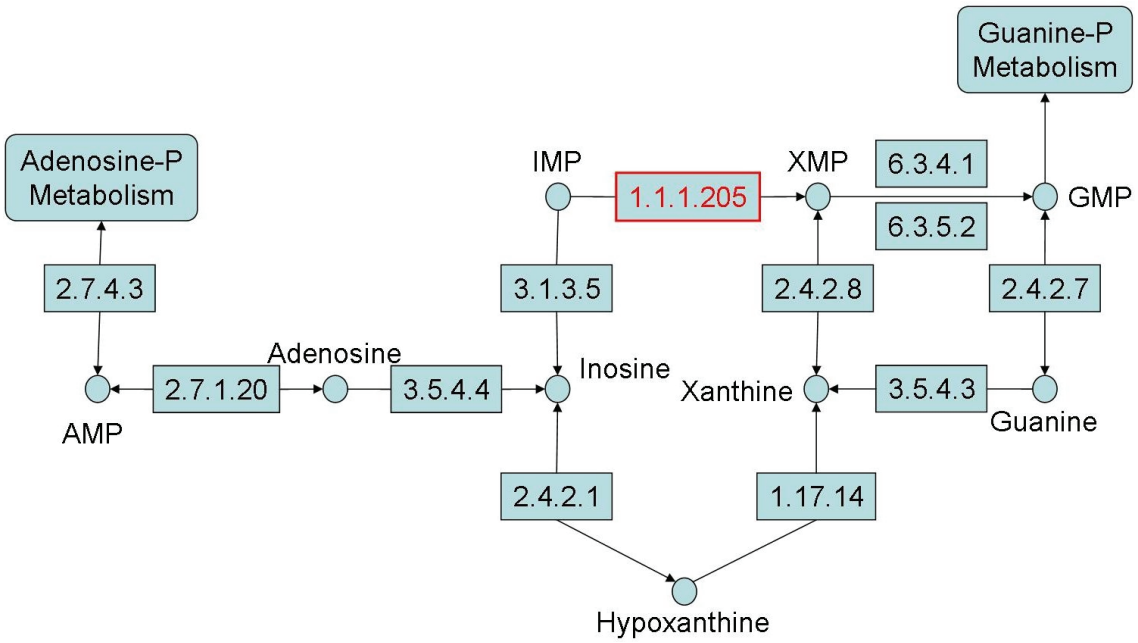


Figure 4.12. Position of the inositol-5′-monophosphate dehydrogenase in the purine metabolism pathway [adapted from the *Rhodococcus* sp. RHA1 pathway from KEGG (Kanehisa *et al.*, 2006)].

It should be noted, however, that in mutant 10D3 the transposon did not insert into the putative *guaB* of *R. erythropolis* SQ1, but 42 bp downstream of it. It is therefore unlikely that the function of the putative *guaB* is disrupted in this mutant. This was confirmed by growing mutant 10D3 in chemically defined medium lacking guanine. The mutant grew like the wild-type, and the addition of guanine to the medium (100 $\mu g\ ml^{-1}$) did not have any noticeable effect on the growth (data not shown). However, it should be noted that the step catalysed by the IMP dehydrogenase can also easily be circumvented via the synthesis of hypoxanthine as shown in Figure 4.12.

Many actinobacteria possess several copies of the *guaB* gene. According to the KEGG gene database *Corynebacterium glutamicum*, *M. tuberculosis*, *M. bovis* and *M. leprae* have three, and *Nocardia farcinica*, *Streptomyces coelicolor* and *Rhodococcus* sp. RHA1 each contain at least two copies. The putative *guaB* of *R. erythropolis* SQ1 was found to be most similar to *guaB1* of *Rhodococcus* RHA1 and *guaB2* of *M. tuberculosis* CDC1551 and H37Rv. In these and other organisms the *guaB* genes appear to be part of an operon, as determined using the genome visualisation program BacMap (Stothard *et al.*, 2005). In *N. farcinica* IFM 10152, *Rhodococcus* sp. RHA1 and *M. tuberculosis* H37Rv, the *guaB* (*guaB1* for RHA1 and *guaB2* for H37Rv) gene is upstream of a second putative IMP dehydrogenase gene (corresponding to the ro_06200 in *Rhodococcus* RHA1, *guaB3* in *M. tuberculosis* H37Rv and nfa8960 in *N. farcinica*). These two genes are then followed by a putative cholesterol oxidase gene in *Rhodococcus* RHA1 and *M. tuberculosis* H37Rv, and by *guaA* (bifunctional GMP synthase/glutamine amidotransferase protein) in *N. farcinica* (see Figure 4.13). A closer examination of the sequence information obtained from mutant 10D3 revealed that, in *R. erythropolis* SQ1, ORF2 (located immediately downstream of the putative *guaB*,

ORF4) possesses high similarity to the translated *guaB1* of *Rhodococcus* RHA1 with a score of 3×10^{-38} when using BLASTX.

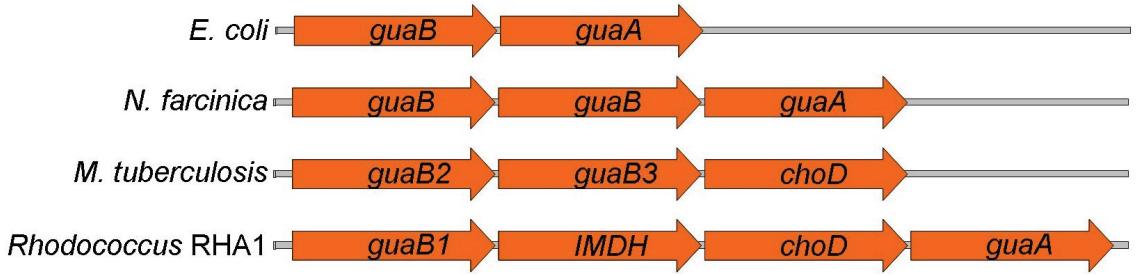


Figure 4.13. Schematic representation of the gua operon of *E. coli* K12, *N. farcinica* IFM 10152, *M. tuberculosis* H37Rv and *Rhodococcus* RHA1.
The putative *guaB* found in *R. erythropolis* SQ1 is most similar to *guaB1*, *guaB2* and the first *guaB* in the *N. farcinica* operon. *guaB* and *IMDH*, IMP dehydrogenase; *choD*, cholesterol oxidase; *guaA*, GMP synthase.

4.4.3. Putative thioredoxin mutant: 1H1

Mutant 1H1 failed to survive long-term starvation in flasks. When grown in flasks in CDM 0.1 % glucose, mutant 1H1 was found to behave like the wild-type (Section 4.3). However, in CDM 1 % glucose, the mutant behaved according to the wild-type until approximately 20 days into stationary phase, then the culturability dropped to 1.6 % of the maximum at 27 days.

Genomic DNA of the mutant was isolated and restricted with a panel of enzymes and then subjected to religation and iPCR. A 1 kb amplicon was obtained when *Bam*HI-restricted DNA was used as the DNA template for iPCR. This fragment was cloned and sequenced. For reasons that remain to be elucidated, sequence information could only be obtained for one side flanking the transposon insertion site. Hence, the sequence obtained was short (844 bp) compared to that obtained for the other mutants (>1200 bp). The details of the open reading frames identified are provided (Figure 4.15).

When the sequence was run against the non-redundant database at NCBI using BLASTX, strong similarity was found between the protein sequence encoded by ORF 1 and the sequence of an hypothetical protein of several actinomycetes, including *Rhodococcus* RHA1 (92 % amino acid identity), *Nocardia farcinica* (82 % identity), *Mycobacterium ulcerans* (69 % identity) and *M. tuberculosis* H37Rv (67 % identity). This protein was identified as a phosphoglycerate mutase in *Saccharopolyspora erythraea*, *M. tuberculosis* CDC1551 and *Streptomyces avermitilis*.

BLASTX identified the area corresponding to nucleotides 644–844 as similar to several putative thioredoxins/redoxins in *Rhodococcus* RHA1 (E-score = 5×10^{-27}), *Nocardia*

farcinica (2×10^{-19}) and several *Mycobacterium* species (minimum 2×10^{-22}, *M. vanbaalenii* PYR-1), as well as a putative cytochrome *c* biogenesis protein in *Nocardia farcinica* IFM 10152 (E-score = 2×10^{-22}). It is not clear whether this sequence is part of an ORF, but it should be noted that the transposon insertion occurs at position 844 (Fig. 4.15) The ORF could potentially start at 575 bp, where an ATT triplet codes for isoleucine and is in-frame for coding of the putative thioredoxin. This would be an unusual start codon, ATT has been predicted to be start codon for four genes in *E. coli* (Makita *et al.*, 2007), but has been demonstrated to be start codon only for *infC* and *pcnB* (Butler *et al.*, 1987; Jasiecki & Wegrzyn, 2006). The only ATG codon in this area of the sequence is at 620 bp, but it is not in-frame for coding of the putative thioredoxin. Alignment and closer analysis of the sequence revealed that, although there is similarity to a segment of the N-terminal end of several thioredoxins, the most N-terminal residues have very low similarity to other thioredoxins (Figure 4.14). The putative thioredoxins identified above are very similar to DsbE from *M. tuberculosis*, which is involved in cytochrome maturation (Goulding *et al.*, 2004). Interestingly, other genes involved in cytochrome production have been identified as important in starvation survival, such as *cta* in *S. aureus* (Clements *et al.*, 1999b), and in resuming growth after stationary phase, such as *cydC* (*surB*) in *E. coli* (Siegele & Kolter, 1993; Siegele *et al.*, 1996).

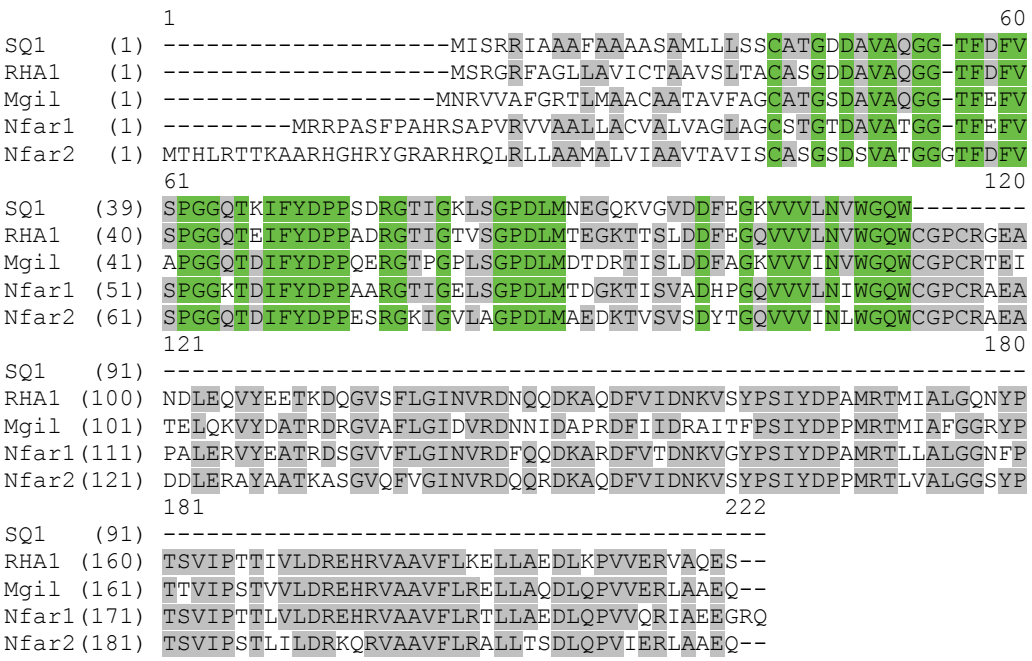


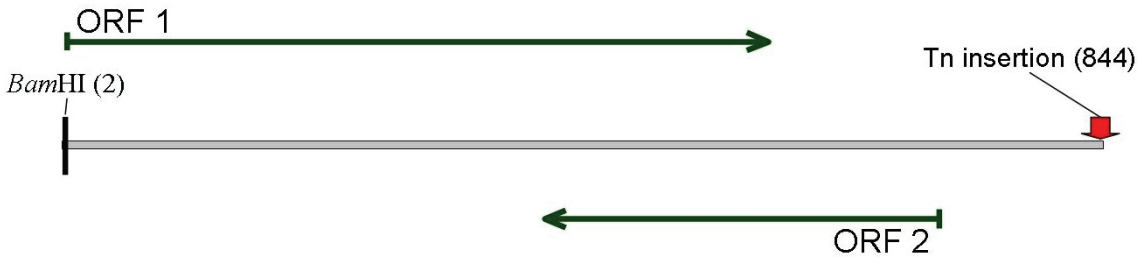
Figure 4.14. Alignment of a putative thioredoxin from *R. erythropolis* SQ1 with sequences from *Rhodococcus* sp. RHA1, *Mycobacterium gilvum* and *Nocardia farcinica* IFM 10152.

SQ1, translation of sequence from 572–844 nt; RHA1, putative thioredoxin; Mgil, alkyl hydroperoxide reductase; Nfar1, putative cytochrome *c* biogenesis protein; Nfar2, putative thioredoxin. All have a similar putative protein disulfide reductases (thioredoxin) function with important roles in cytochrome maturation.

Grey background, >50 % identity; green background, identical residues.

No significant similarity could be found for ORF 2, whether by using BLASTX or BLASTP.

Phosphoglycerate mutase reversibly catalyses the transfer of a phosphate group from C_2 to C_3 of phosphoglycerate (Fothergill-Gilmore & Watson, 2006). It is at the junction of glycolysis and neoglucogenesis, allowing the degradation of glucose into pyruvate and *vice-versa*. Phosphoglycerate mutases form a large family, many members of which have a wide range of substrates (e.g. phosphoglucomutase, involved in both glucose and galactose metabolism; (Berg *et al.*, 2002). The gene coding for the putative phosphoglycerate mutase is upstream of a thioredoxin protein gene and a cytochrome *c* biogenesis protein gene in the organisms with high similarity to ORF 1 of *R. erythropolis* SQ1. These genes are *cssX* and *ccdA* in *Saccharopolyspora erythraea* NRRL 2338, MUL_0625 and *ccsA* in *Mycobacterium ulcerans* Agy99, and Rv0526 and *ccdA* in *M. tuberculosis* H37Rv, respectively. It is not known whether these genes are organised in operons.



Open reading frame	Position	Strand	Size
ORF 1	3–572	Sense	570 bp
ORF 2	391–711	Antisense	321 bp

Figure 4.15. Schematic representation of the 844 bp sequence retrieved for mutant 1H1. The *Bam*HI restriction site used to retrieve the sequence is shown, as well as putative open reading frames predicted by ORF finder and the transposon insertion site. Size, position and strand of the ORFs identified in the sequence are shown in the table. ORFs were identified with the ORF Finder function of Vector NTI (Invitrogen, http://www.invitrogen.com).

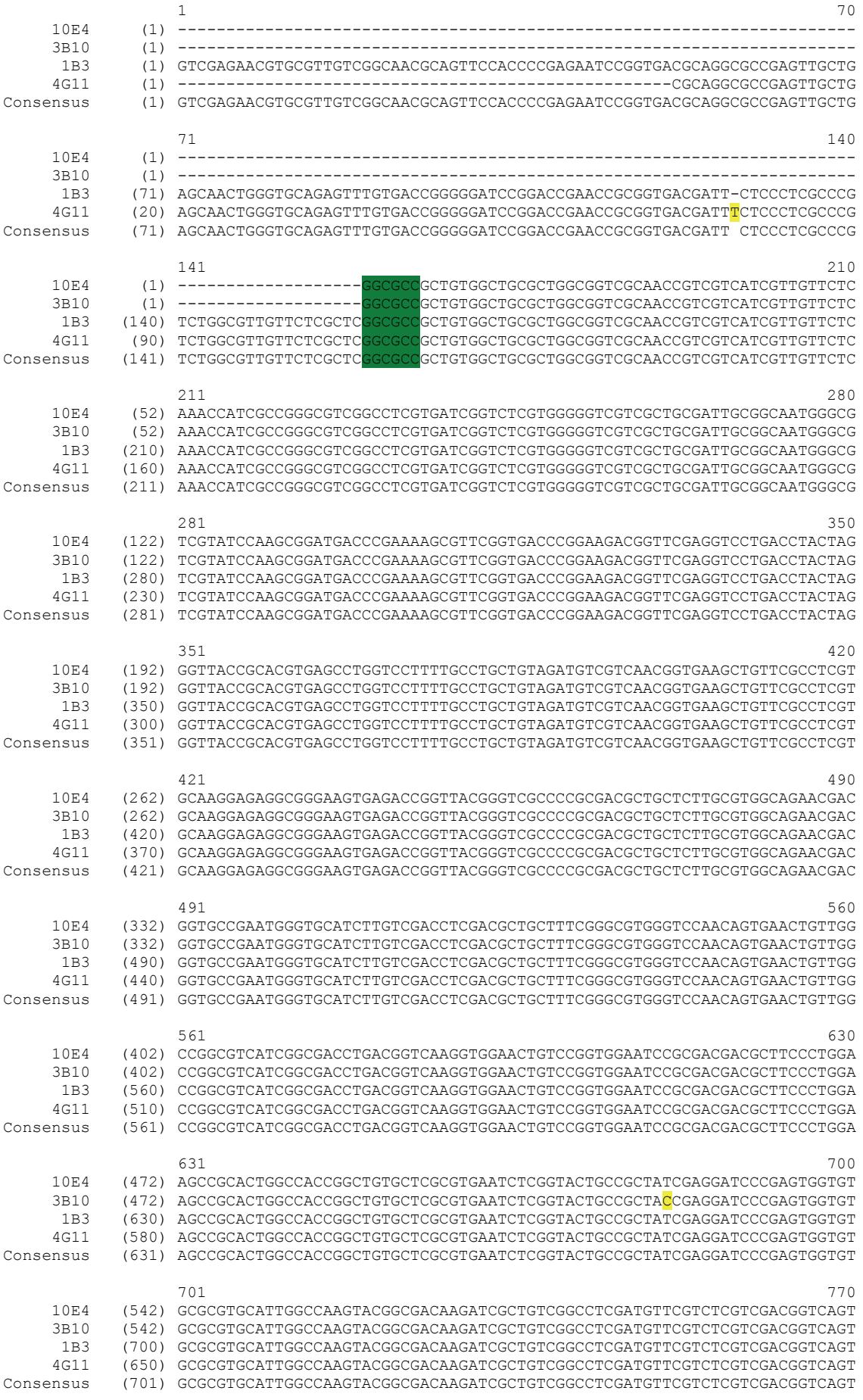
4.4.4. Histidine and Serine/Glycine auxotrophic mutants

In addition to characterising potential starvation and/or stationary phase survival mutants, mutants that displayed different growth behaviour patterns than the *R. erythropolis* SQ1 parent strain were also investigated. These mutants were found to grow well on LB, but poorly on CDM IIA.

4.4.4.1. Histidine auxotrophic *hisA* mutants: 1B3, 3B10, 4G11 and 10E4.

Nucleotide sequence analysis

The cloned inverse PCR products were obtained from *Eco*RI-digested DNA for mutants 1B3 (1317 bp) and 4G11 (1267 bp), and from *Nar*I-digested DNA for mutants 3B10 and 10E4 (both 1039 bp). Alignment of the four sequences obtained for these mutants showed that there was strong similarity between them. Using AlignX (part of Vector NTI) with standard settings (gap opening penalty = 15; gap extension penalty = 6.66 and gap separation penalty range = 8), the nucleotide identity was calculated to be 99 % for all four sequences. The identity variation was introduced by differences in the size of the fragments obtained, due to the use of different restriction enzymes in the initial stage of the analyses, i.e. *Eco*RI for mutants 1B3 and 4G11, and *Nar*I for mutants 3B10 and 10E4 (Figure 4.16).



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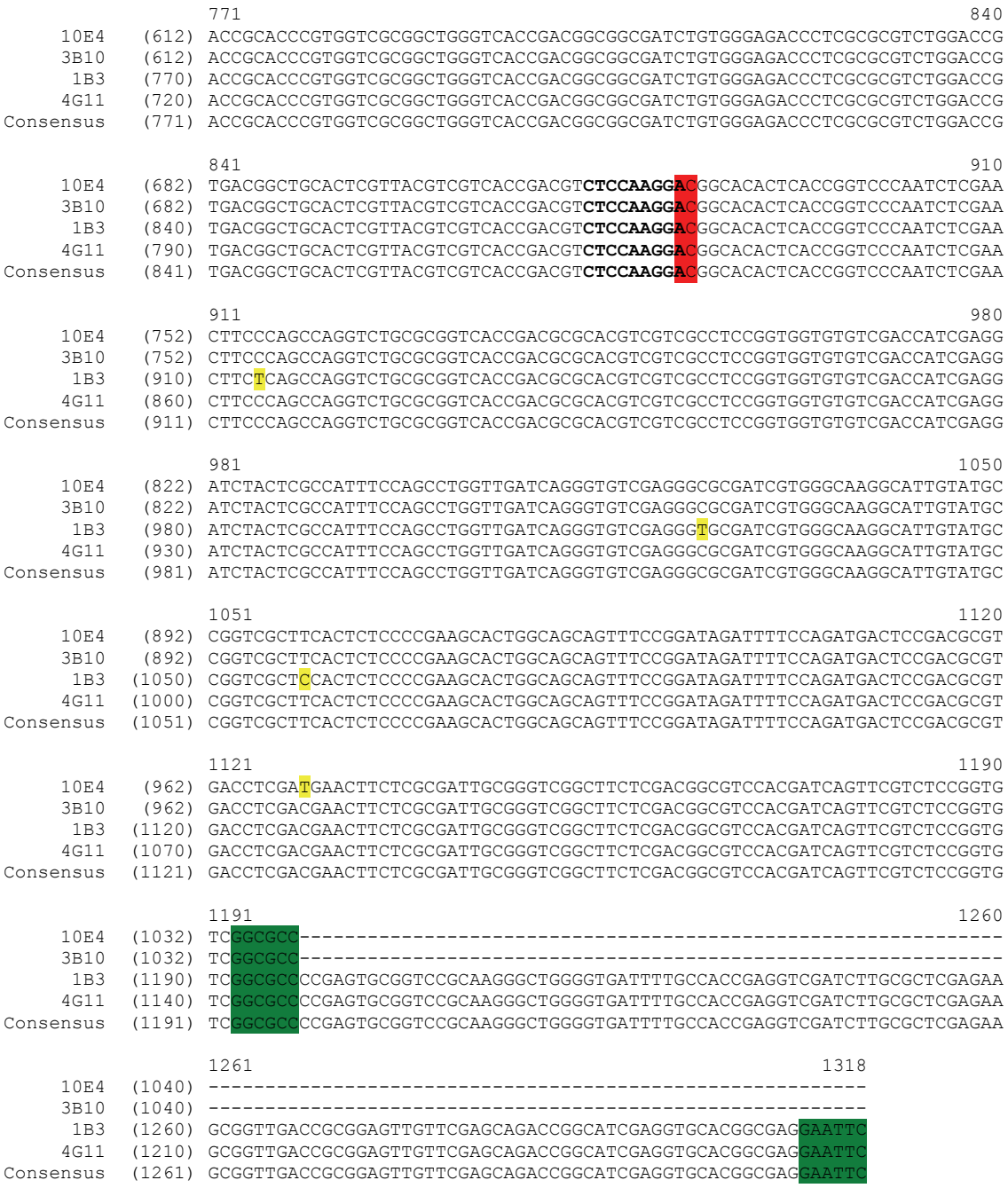


Figure 4.16. Alignment of the sequences obtained from mutants 1B3, 3B10, 4G11 and 10E4.

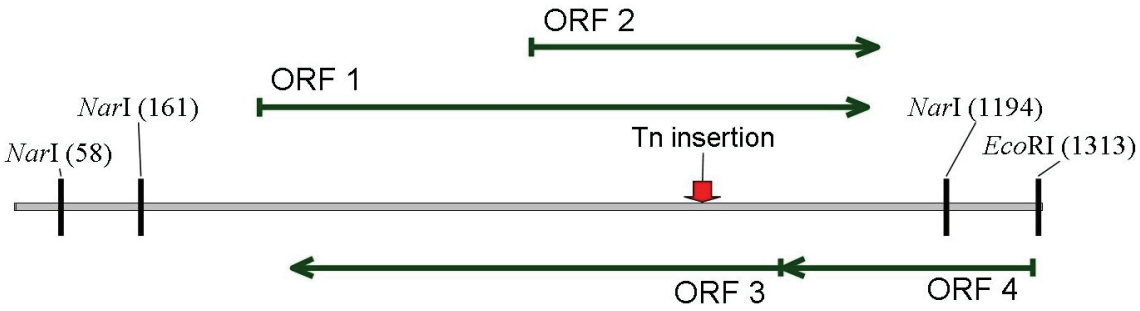
The transposon insertion site is highlighted in red and the 9 nt sequence duplicated upon transposon insertion is in bold. The *Nar*I and *Eco*RI restriction enzyme recognition sites are highlighted in green, and the nucleotides differing between sequences are in yellow (at positions 128, 680, 916, 1024, 1059 and 1129 using the consensus sequence as the reference).

It is clear from the alignment that mutants 1B3, 3B10, 4G11 and 10E4 are clones, or that multiple mutants have been isolated in which the transposon insertion took place at the same site.

Open reading frames (ORF) were identified with the ORF Finder (part of Vector NTI). The default settings were used, with a minimum ORF size of 100 codons. Four ORFs were found – their relative positions and sizes are shown in Figure 4.17, together with the transposon insertion site and the enzyme restriction sites used to recover the sequences by iPCR. The consensus sequence was searched against the NCBI database using BLASTX. All the sequences identified as having high scores were obtained against the protein translated from sequence 383 to approximately 1100 bp in frame +2. This correlates with ORF 1 (Figure 4.16). The region immediately upstream of ORF 1 was analysed for transcription promoter sequences and is shown below:

TGCGGCAATGGGCGTCGTATCCAAGCGGATGACCCGAAAAGCGTTCGGTG
−10 +1

It should be noted that, while it not the most efficient or common start codon, GTG is encountered in many prokaryotes (Kozak, 1983). This is particularly true of high G+C genome organisms, in particular in *Mycobacterium* (Rison *et al.*, 2007). The putative transcription start is highlighted in red, and the −10 area in green. Although there is a poly-dA at −10, it was not possible to find any −35 structure.



Open reading frame	Position	Strand	Size
ORF 1	313–1092	Sense	780 bp
ORF 2	662–1105	Sense	444 bp
ORF 3	355–981	Antisense	627 bp
ORF 4	985–1305	Antisense	321 bp

Figure 4.17. Schematic representation of the genomic area (1317 bp) disrupted in mutants 1B3, 3B10, 4G11 and 10E4.
The restriction enzymes used to retrieve the sequences are shown, as well as the transposon insertion site and putative open reading frames. Size, position and strand of the ORFs identified in the consensus sequence are shown in the table.
ORFs were identified with the ORF Finder function of Vector NTI (Invitrogen, http://www.invitrogen.com).

Translated ORF analysis

All four open reading frames identified in the consensus sequence were translated using the bacterial genetic code, and then run against the non-redundant protein database at NCBI using BLASTP. The 260 amino acid protein obtained by translating ORF 1 was the only one yielding significant scores and *E*-values, confirming what had been determined with BLASTX. All results returned were either HisA or HisA/TrpF proteins; HisA is involved in histidine synthesis and HisA/TrpF in both histidine and tryptophan synthesis. Interestingly, both HisA and the bi-functional HisA/TrpF are the same size - 240 to 260 amino acids. The alignment of the putative HisA from *R. erythropolis* SQ1 with other HisA and HisA/TrpF sequences is shown in Figure 4.17.

There is experimental evidence for the activity of HisA from *Streptomyces coelicolor* A3, and HisA/TrpF of *Mycobacterium tuberculosis* H37Rv (Barona-Gómez & Hodgson, 2003). HisA is a phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide (ProFAR) isomerase (EC 5.3.1.16), catalysing the fourth enzymatic step between phosphoribosyl pyrophosphate (PRPP) and L-histidine (see Figure 4.19a, adapted from KEGG) in the histidine biosynthesis pathway. It mediates transformation of the phosphoribosyl-formimino-AICAR-phosphate (ProFAR) into phosphoribulosyl-formimino-AICAR-phosphate (PRFAR) via an Amadori rearrangement, consisting of the rearrangement of the aminoaldose ProFAR into the aminoketose, PRFAR. This also results in the opening of the ribose ring (illustrated in Figure 4.19b) (Henn-Sax *et al.*, 2002).

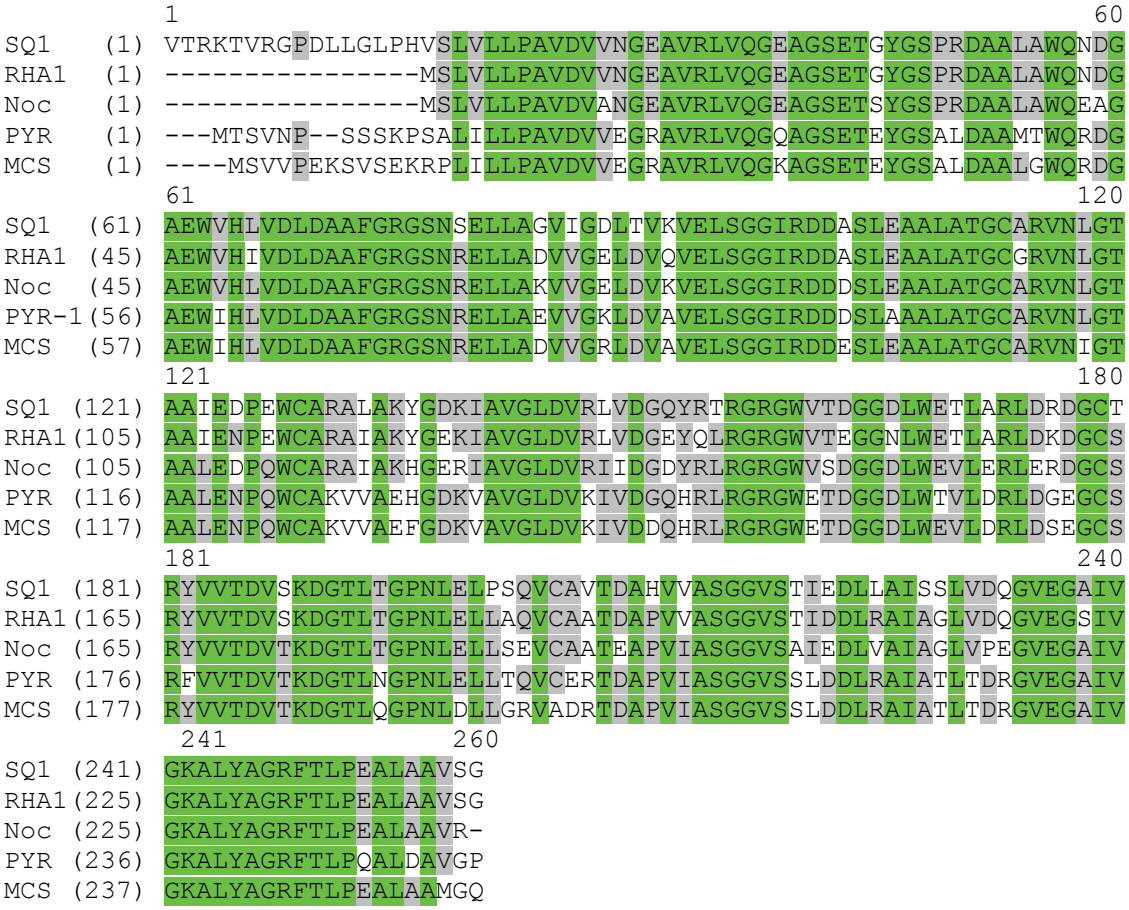
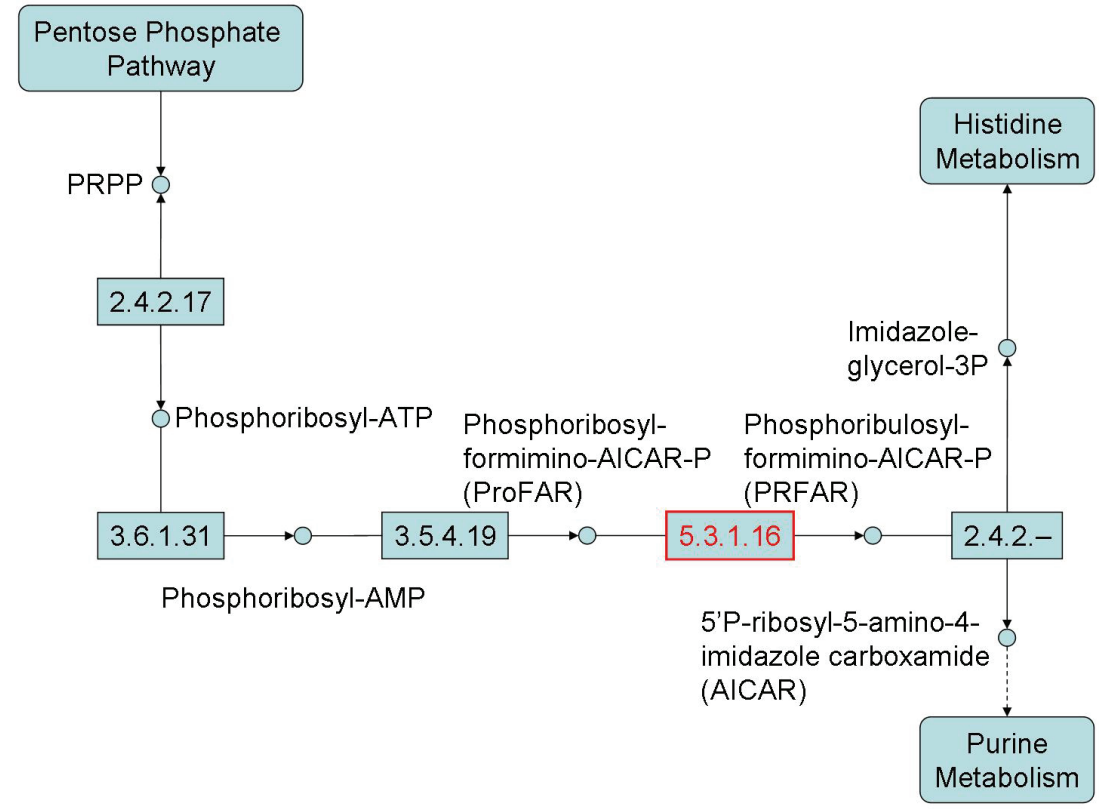


Figure 4.18. Alignment of a putative HisA sequence from *R. erythropolis* SQ1 with HisA sequences from *Rhodococcus* sp. RHA1 and *Nocardia farcinica* IFM 10152 (Noc), and with HisA/TrpF sequences from *Mycobacterium vanbaalenii* PYR-1 (PYR) and *Mycobacterium* sp. MCS.
Grey background, >50 % identity; green background, identical residues.

(a)



(b)

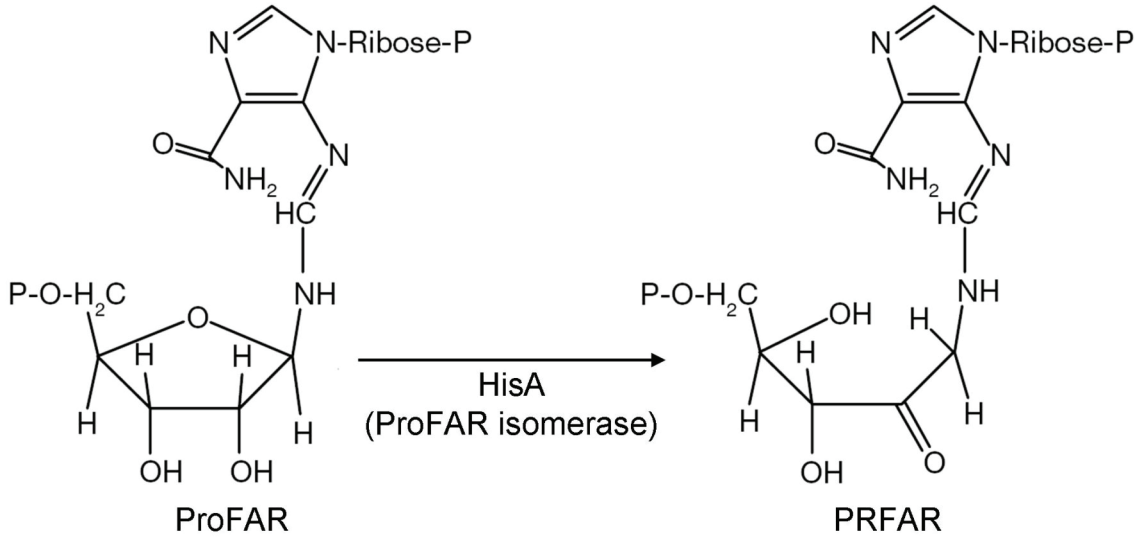


Figure 4.19. Function of HisA in the synthesis of histidine. (a) Position of HisA (highlighted in red) in the histidine anabolic pathway [adapted from KEGG (Kanehisa *et al.*, 2006)]. (b) Amadori rearrangement catalysed by HisA. A similar reaction is catalysed by TrpF. Adapted from Barona-Gómez and Hodgson (2003).

This creates a link between the pentose phosphate pathway, purine metabolism and histidine metabolism. Interestingly, HisA is closely similar to the HisA/TrpF protein from *Mycobacterium* that is involved in both histidine and tryptophan synthesis. Both *M. tuberculosis* and *Streptomyces coelicolor* have a single enzyme to catalyse the Amadori rearrangement described in Figure 4.19b in both the histidine and tryptophan biosynthesis pathways (Barona-Gómez & Hodgson, 2003; Nester & Montoya, 1976).

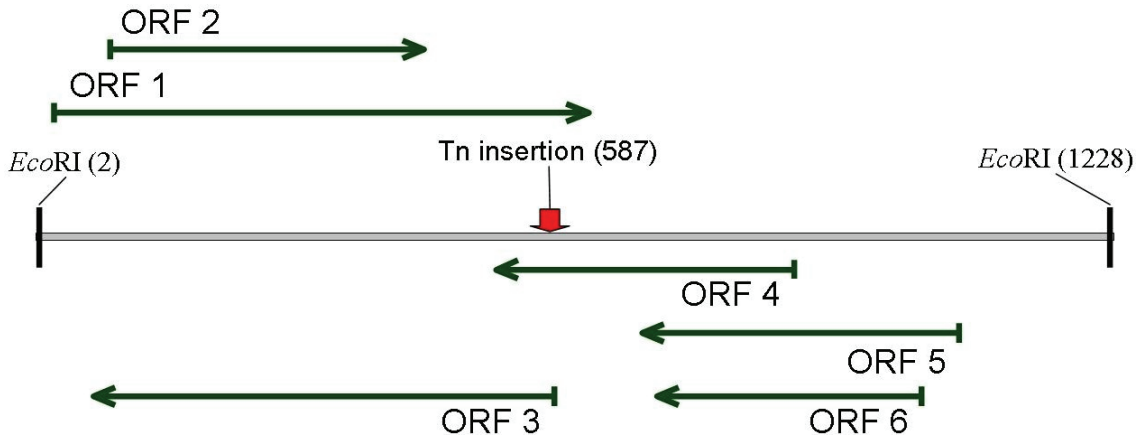
The data above suggests that mutants 1B3, 3B10, 4G11 and 10E4 could be auxotrophic for histidine, and possibly for tryptophan also. To verify this, an overnight LB culture of each mutant was washed twice in saline and inoculated in CDM II 1 % glucose supplemented or not with histidine and/or tryptophan. Mutants 1B3, 3B10, 4G11 and 10E4 were shown to grow on CDM supplemented with histidine, but not on unsupplemented CDM or medium supplemented with tryptophan alone (data not shown). This would suggest that the putative gene disrupted by transposon mutagenesis does indeed code for HisA, and that this protein does not have any function similar to that of TrpF.

4.4.4.2. Histidine auxotrophic *hisB* mutant: 3B4

Similar to the mutants described in the previous section, mutant 3B4 was found to grow poorly on chemically defined media. This was most obvious on CDM IIA 1 % glucose, where growth was observed only at day 8 (section 4.1.2). On CDM IIA 0.1 % glucose, mutant 3B4 grew normally up to 8 days, then failed to produce growth at day 14 and produced only weak growth at day 22. Mutant 3B4 was therefore grown overnight in LB and its genomic DNA was isolated. It was then restricted with *Eco*R1, and inverse PCR was performed. A fragment of 2.4 kb was obtained, which was then cloned and sequenced, and the sequence analysed as described in Section 4.4. Comparison against the non-redundant database at the NCBI with BLASTX identified HisB, a putative imidazole-glycerol-phosphate dehydratase, encoded by an open reading frame of 611 bp. This sequence also corresponds to ORF 1, one of the open reading frames identified using ORF finder (Figure 4.20, Table 4.7).

This observation was confirmed when each ORF was translated using the bacterial genetic code and then analysed against the non-redundant protein database at the NCBI using BLASTP. The only translated ORF to yield significant scores and *E*-values was ORF 1 (lowest *E*-value 2×10^{-96}). The translated protein is 204 amino acids long and is highly similar (65.1 % identity) to the HisB of other *Corynebacterineae.* The putative protein sequence is shown aligned with similar sequences in Figure 4.21. There is experimental evidence for this protein in *N. farcinica* IFM 10152 (accession no. Q5YYP8) (Ishikawa *et al.*, 2004). The C-terminal part of HisB encodes an imidazoleglycerol-phosphate dehydratase function (Alifano *et al.*, 1996), that dehydrates D-erythro-1-(imidazol-4-yl) glycerol 3-phosphate into 3-(imidazol-4-yl)-2-oxopropyl phosphate, the sixth step in the biosynthesis of histidine from phosphoribosyl

pyrophosphate (PRPP) (Figure 4.22). The N-terminal part of HisB has a phosphatase activity that transforms L-histidinol-P into L-histidinol (Alifano *et al.*, 1996).



Open reading frame	Position	Strand	Size
ORF 1	19–630	Sense	612 bp
ORF 2	83–442	Sense	360 bp
ORF 3	64–591	Antisense	528 bp
ORF 4	525–866	Antisense	342 bp
ORF 5	709–1011	Antisense	303 bp
ORF 6	692–1054	Antisense	363 bp

Figure 4.20. Schematic representation of the 1232 bp sequence retrieved from mutant 3B4.
The *Eco*R1 restriction sites used to retrieve the sequence are shown, as well as the open reading frames predicted by ORF finder and the transposon insertion site. Size, position and strand of the ORFs identified in the sequence are shown in the table.
ORFs were identified with the ORF Finder function of Vector NTI (Invitrogen, http://www.invitrogen.com).

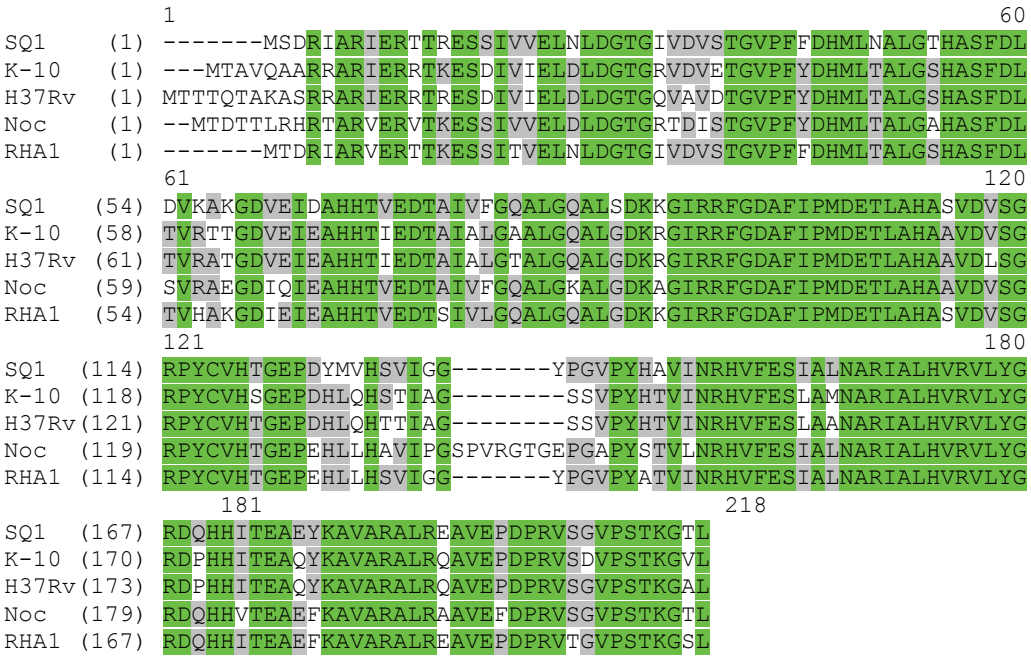


Figure 4.21. Alignment of a putative HisB sequence from *R. erythropolis* SQ1 with HisB sequences from *Rhodococcus* sp. RHA1, *Nocardia farcinica* IFM 10152 (Noc), *Mycobacterium avium* subsp. *paratuberculosis* K-10 and *M. tuberculosis* H37Rv. Grey background, >50 % identity; green background, identical residues.

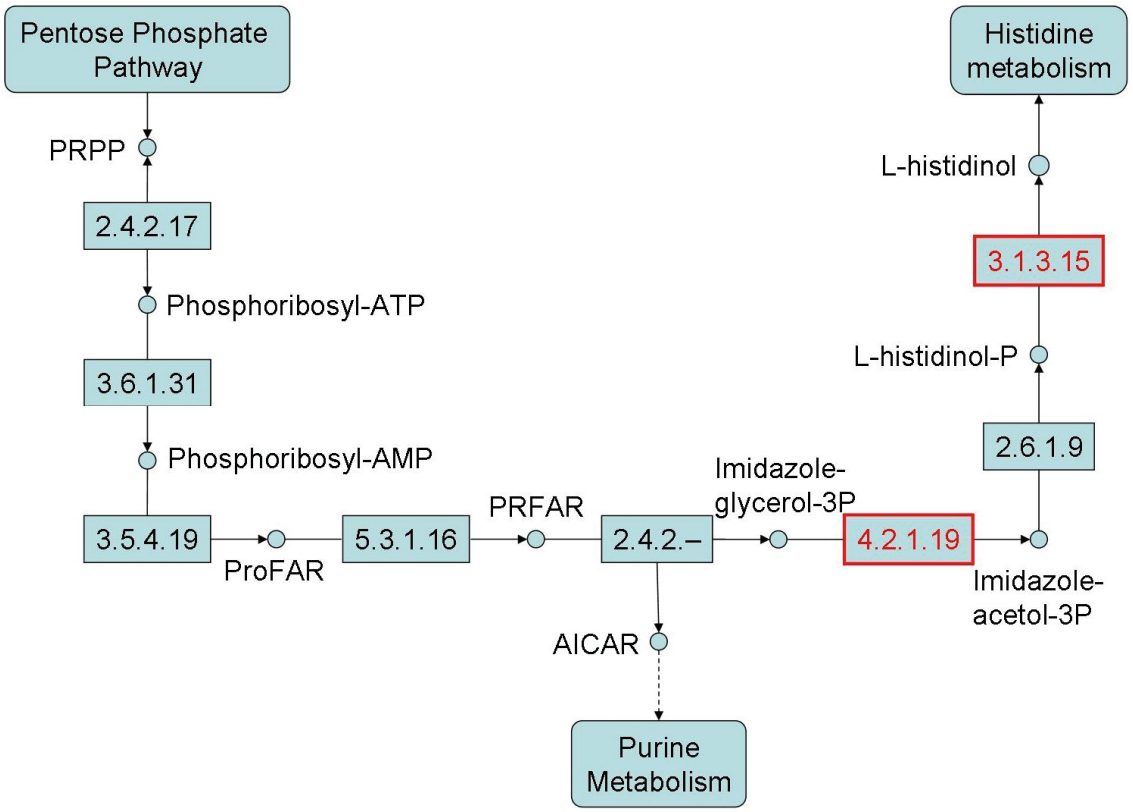


Figure 4.22. Steps catalysed by HisB (highlighted in red) in the histidine anabolic pathway [adapted from KEGG (Kanehisa *et al.*, 2006)].

HisB is encoded by the gene *hisB*, and is part of the *his* operon. Histidine metabolism has been a focus point of genetics for over forty years, contributing to the early hypothesis that prokaryotes possess genes arranged in operons. The histidine operon has since been shown to be present in most bacteria studied, including the actinomycete *Streptomyces coelicolor* (Alifano *et al.*, 1996), *Mycobacterium smegmatis* and *M. tuberculosis* (Parish *et al.*, 1997). The organisation of the *his* operon is remarkably similar across genus and species, as illustrated in Figure 4.23, and has been commented on extensively in the literature (Price *et al.*, 2006). From a genomic perspective, *hisB* is found upstream of *hisH* and *hisA*. It is interesting to note that *Rhodococcus* sp. RHA1, *M. smegmatis* and *M. tuberculosis* carry an inositol-monophosphatase between *hisA* and *hisF* which is involved in cell-wall synthesis (Parish *et al.*, 1997). Although the genes *hisB*, *hisH* and *hisA* are found in the same order in all organisms studied so far (Alifano *et al.*, 1996), interestingly, the *hisBHA* sequence is interrupted in *Nocardia farcinica* IFM 10152, *Streptomyces avermitilis* MA-4680 and *Rhodococcus* sp. RHA1 (Ishikawa *et al.*, 2004; McLeod *et al.*, 2006; Omura *et al.*, 2001). In *Nocardia*, the ORF nfa18490 lies between *hisH* and *hisA*. In *Streptomyces*, the ORF SAV6156 lies between *hisB* and *hisH*. These two ORFs bear no similarity and are of unknown function. *Rhodococcus* sp. RHA1 seems to be quite unique in having two ORFs inserted between *hisB* and *hisH* (ro01020 and ro01027) as well as one between *hisH* and *hisA* (ro01025). In *R. erythropolis* SQ1, the remaining 598 nucleotides of sequence information obtained for mutant 3B4 located downstream of the putative *hisB* gene are highly similar (over 70 % identity) to the corresponding sequence in *Rhodococcus* RHA1. This would suggest that *Rhodococcus* RHA1 and *R. erythropolis* SQ1 share a similar organisation for the *his* operon. Predicted proteins encoded by ORF nfa18490, SAV6156, RHA1_ro01025 and RHA1_ro01028 are of unknown function, while RHA1_ro01027 is a putative major facilitator family transporter (McLeod *et al.*, 2006).

As was observed for the *hisA* mutants, the *hisB* mutant is auxotrophic for histidine in that normal growth was achieved when CDM II was supplemented with histidine (data not shown).

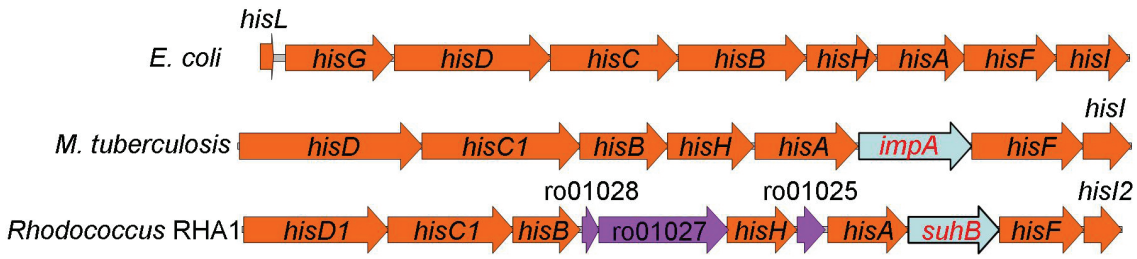


Figure 4.23. Histidine synthesis operons of *E. coli* K-12, *M. tuberculosis* H37Rv and *Rhodococcus* sp. RHA1. The purple arrows show open reading frames encoding proteins of unknown function. *impA* (*M. tuberculosis*) and *suhB* (*Rhodococcus*) both encode an inositol-monophosphatase specific to actinobacteria.

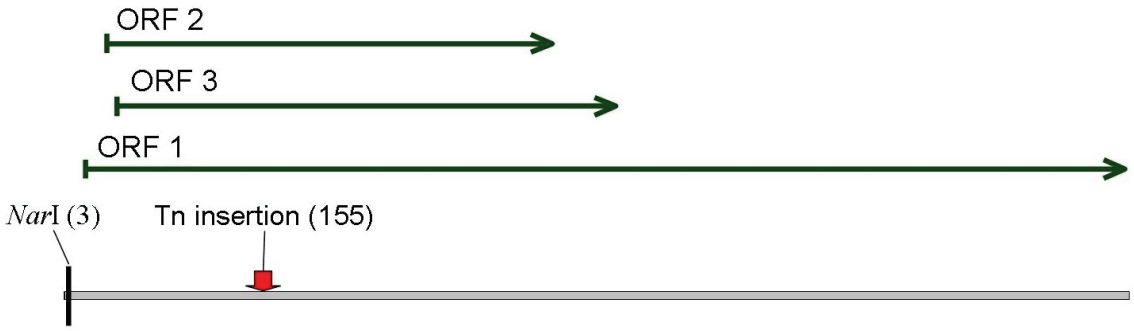
4.4.5. Serine/glycine auxotrophic *serA* mutant: 6E6

Mutant 6E6 failed to grow after 14 days incubation in either CDM 1 % glucose or CDM 0.1 % glucose. In contrast, wild-type *R. erythropolis* SQ1 gave strong growth at each sampling point. However, the wild-type phenotype was observed when mutant 6E6 was grown in LB broth. DNA from mutant 6E6 was isolated and processed in the same way as the mutants previously described and an 831 bp sequence was retrieved (Figure 4.24). Although sequence data was obtained for regions both upstream and downstream of the transposon insertion point, only one restriction site could be located in the sequence obtained. A similar situation arose with mutants 1B3 and 4G11 restricted with *Eco*RI (section 4.4.1). Interestingly, a 10 bp repeat sequence, TCCTCGCTCT, was observed at the transposon insertion point.

The nucleotide sequence obtained was translated and analysed using BLASTX against the non-redundant protein database at NCBI. Strong similarity was found between the sequence encoded by ORF 1 and the sequence of the D-3-phosphoglycerate dehydrogenase (PGDH, encoded by *serA* genes) of several mycobacteria (scores >300 and *E*-values $<2\times10^{-80}$). Each ORF was translated using the bacterial genetic code and submitted to a BLASTP search. ORF 1 was found to be the only one with significant similarity to proteins in the database, specifically to the D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95, SerA).

The 831 bp sequence obtained codes for the C-terminal 271 amino acids only, corresponding to approximately half the size of SerA proteins reported to date (505–531 aa). The sequence from 1 to 831 bp was therefore translated in frame +1 to yield a 276 amino acid sequence that was aligned using Align to the best BLAST hits (Figure 4.25). An identity of 63 % was calculated (using Align) between the amino acid

sequence obtained from *R. erythropolis* SQ1 and the C-terminal 276 amino acids of SerA from *M. tuberculosis* H37Rv.



Open reading frame	Position	Strand	Size
ORF 1	16–828	Sense	813 bp
ORF 2	33–380	Sense	348 bp
ORF 3	41–430	Sense	390 bp

Figure 4.24. Schematic representation of the 831 bp sequence retrieved for mutant 6E6.
The *Nar*I restriction site used to retrieve the sequence is shown, as well as putative open reading frames predicted by ORF finder and the transposon insertion site. Size, position and strand of the ORFs identified in sequence are shown in the table.
ORFs were identified with the ORF Finder function of Vector NTI (Invitrogen, http://www.invitrogen.com).

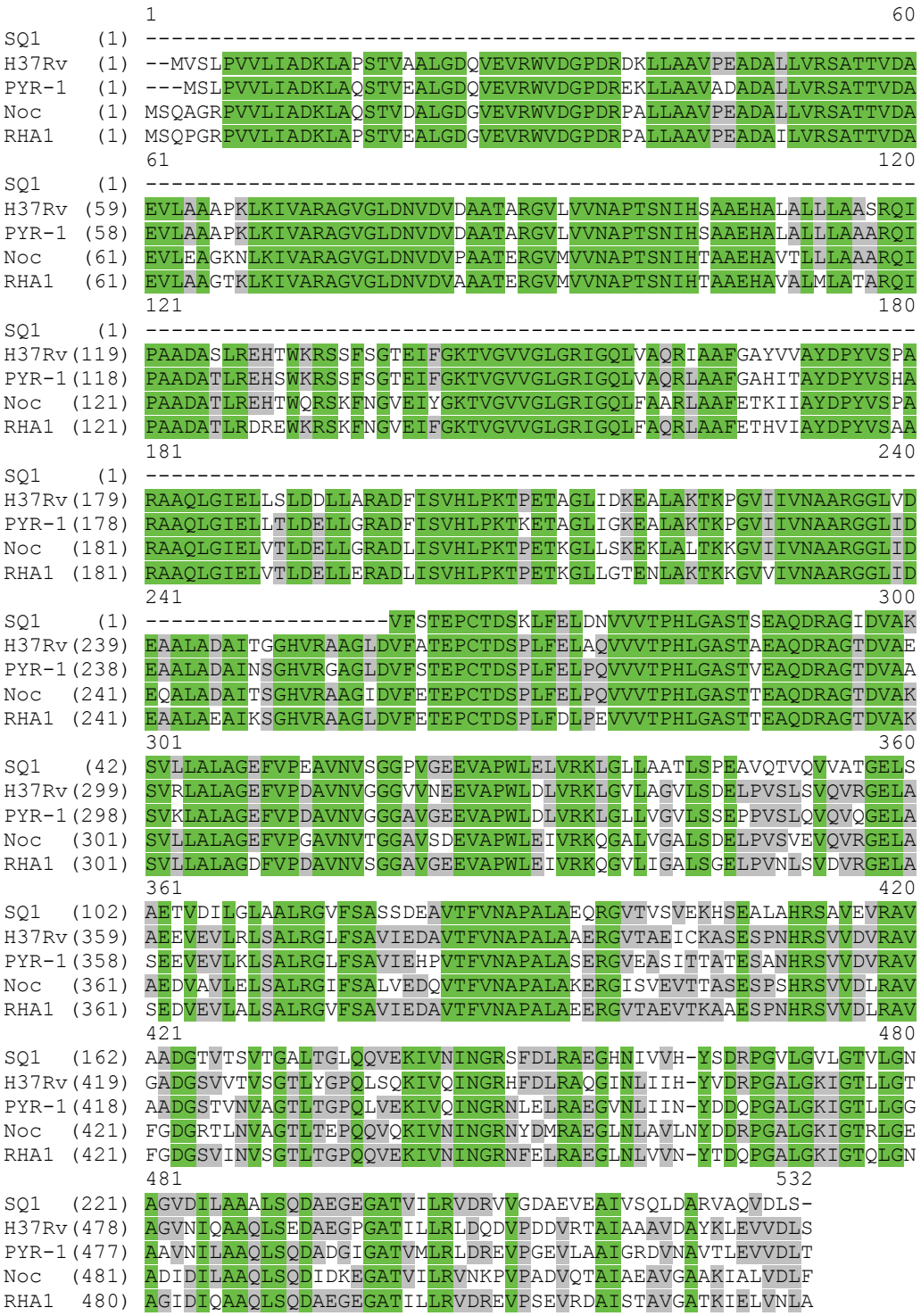


Figure 4.25. Alignment of a putative SerA sequence from *R. erythropolis* SQ1 with sequences from *Rhodococcus* sp. RHA1, *M. tuberculosis* strain H37Rv, *M. vanbaalenii* PYR-1 and *N. farcinica* IFM 10152.
Grey background, >50 % identity; green background, identical residues.

SerA catalyses the conversion of D-3-phosphoglycerate to 3-phospho-hydroxypyruvic acid (or hydroxypyruvic acid phosphate) and *vice versa*. It is one of the first steps in the biosynthesis of serine from glycerate (Figure 4.26) (Ichihara & Greenberg, 1957; Walsh & Sallach, 1966). Since SerA appears essential to the synthesis of serine in *M. tuberculosis* (Sassetti *et al.*, 2003) and could be involved in the biosynthesis of glycine and threonine, a short supplementation study was performed. Growth of mutant 6E6 was assessed in CDM II 1% glucose broth supplemented with glycine, serine, threonine, and combinations of these amino acids (all at concentrations of 100 μg ml^{-1}) (Table 4.6). It is interesting to see that both serine and glycine restored the ability of the mutant to grow in chemically defined medium, presumably because of the possibility to synthesise serine from glycine (EC 2.1.2.1, Figure 4.25). The seemingly complete inhibition of growth by threonine was not completely unexpected, considering its toxic effects on other organisms (Eccleston & Kelly, 1973; Lamb & Bott, 1979).

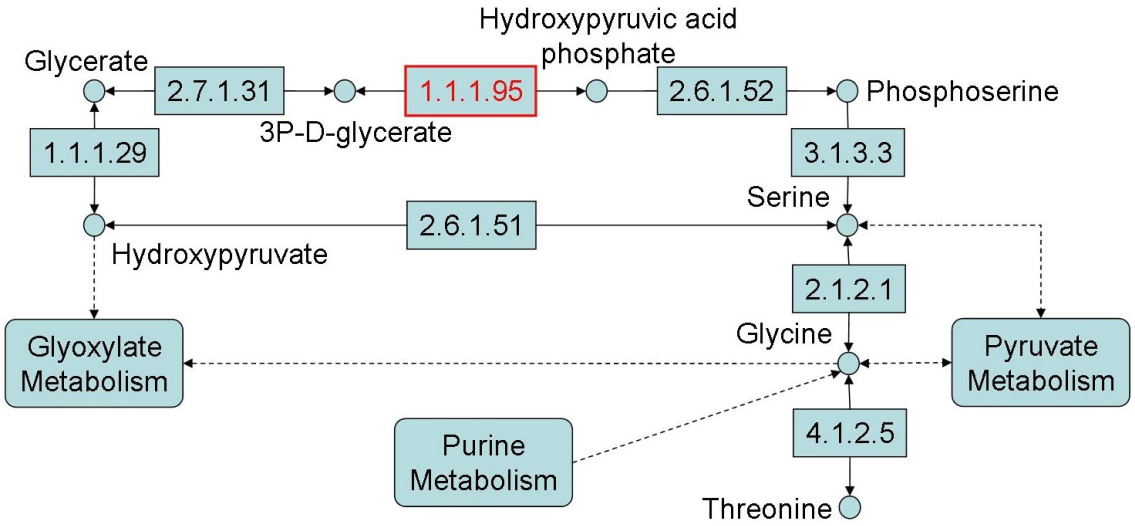


Figure 4.26. Position of phosphoglycerate dehydrogenase in a simplified glycine, serine and threonine metabolism pathway [adapted from the *Rhodococcus* sp. RHA1 pathway from KEGG (Kanehisa *et al.*, 2006)].

Growth medium (CDM II 1% glucose)	Growth density
Non-supplemented	–
+ threonine	–
+ serine	++
+ glycine	+++
+ glycine + serine	+++
+ glycine + threonine	–
+ serine + threonine	–
+ glycine + serine + threonine	–

Table 4.6. Growth of mutant 6E6 in CDM 1% glucose broth supplemented with serine, glycine and threonine (100 µg ml^{-1}).
–, no visible growth; + to +++, increasing growth.

A gene database search (NCBI) revealed that there are at least two genes with the same putative function in *Rhodococcus* sp. RHA1, *serA2* and *serA3*, and a further three putative *serA* genes (RHA1_ro04630, RHA1_ro01308 and RHA1_ro02790). Since mutant 6E6 is auxotrophic for serine/glycine, it seems that only the gene disrupted by transposon insertion is expressed in CDM IIA, in other words the additional gene copies are not fulfilling the activity of the inactivated gene under these growth conditions. The possession of multiple *serA* or *serA*-like genes is common. For instance, two *serA* genes are found in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 (*serA1* and *serA2*); one gene with proven function, in addition to a putative gene are found in *Nocardia farcinica* IFM 10152 (*serA* and nfa13640) and *M. tuberculosis* CDC1551 (*serA*, identical to *serA1* from *M. tuberculosis* and *M. bovis* and MT0753).

Chapter V
Discussion

Although the starvation or non-growth state is probably the most common physiological state of bacteria (Morita, 1993), it has been studied in relatively few organisms. In spite of its importance in pathogenesis, bioremediation and several industrial processes, limited research has been performed on members of the genus *Rhodococcus* under starvation conditions. The objectives of this study were to analyse the physiological adaptation of *Rhodococcus* to starvation/stationary phase, and to generate a bank of mutants to identify genetic elements involved in this adaptation. The study organism, *R. erythropolis* SQ1, was chosen based on ease of growth in chemically defined medium, antibiotic sensitivity profile (of relevance as selective pressure in DNA transformation experiments) and transformation efficiency by electroporation.

In this study, it was shown that, similar to *Vibrio*, *S. aureus* and *M. tuberculosis* (Jenkins *et al.*, 1988; Nyka, 1974; Nyström, 1999; Watson *et al.*, 1998b), *R. erythropolis* SQ1 can survive carbon starvation or nutrient-induced stationary phase for a prolonged period of time. It was found that *R. erythropolis* SQ1 can survive for at least 43 days in LB and distilled water, and at least 65 days in chemically defined medium (CDM) containing high (1 % w/v, 55 mM) or low (0.1 % w/v, 5.5 mM) concentrations of glucose. Interestingly, growth in a low-energy medium (5.5 mM glucose medium) enabled 100 % of the cells in culture (approx. 1.2×10^9 CFU/ml) to form colonies on agar, even after 65 days static incubation (data not shown). Viable cell counts, measured by plating on LB agar, remained more or less constant (variation ≤1 log), regardless of the medium in which the cells were grown. Other organisms were shown to survive long periods of time under similar conditions, e.g. *Listeria monocytogenes*, *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Rhodococcus rhodochrous* (Herbert & Foster, 2001; Shleeva *et al.*, 2002; Watson *et al.*, 1998b). It was verified that, during long-term stationary phase, the glucose was exhausted from

chemically defined medium (<0.1 mM, as measured by a colorimetric method) between 6 and 7 incubation in 0.1 % CDM, and between 7 and 13 days incubation in 1 % CDM, respectively. This shows that *R. erythropolis* SQ1 could survive for long stretches of time in a medium in which there was no source of energy. Additional samples would be necessary to pinpoint the exact time at which glucose was depleted from the medium. The growth rate in exponential phase was found to be much lower when *R. erythropolis* SQ1 was grown in 0.1 % CDM than when grown in 1 % CDM (approx. 0.094 hr^{-1} *vs* 0.17 hr^{-1}, respectively). More frequent sampling would have however been necessary for a more accurate figure to be determined. The observations reported in this study are true of *R. erythropolis* grown with glucose as the sole carbon source. It would be interesting in the future to compare behaviours using alternative carbon and/or energy sources. The modified chemically defined medium described in this work could also be applied to studies investigating other growth-limiting nutrients or elements, such as nitrogen and phosphorous, or trace metals and vitamins. Furthermore, it would be of interest to study the physiological status of immobilised cells, as these have been proposed for use in several industrial processes (see e.g. Begoña Prieto *et al.*, 2002; Kitova *et al.*, 2002; Kitova *et al.*, 2004; Naito *et al.*, 2001; Pirog *et al.*, 2005; Prieto *et al.*, 2002).

Interestingly, the culturability of *R. erythropolis* SQ1 dropped dramatically (by 4 logs) when flasks were transferred from shaking to static incubation, but did not disappear altogether, as cells could still yield colonies on LB agar (approx. 4×10^5 CFU/ml). This behaviour was observed only in high-glucose (1 %) medium. Transfer to static incubation is used to produce dormant cells in *M. tuberculosis* and *M. bovis* growing in Dubos Tween–albumin broth (0.75 % glucose, w/v) (Lim *et al.*, 1999; Wayne & Hayes, 1996). The same effect was observed in *Rhodococcus rhodochrous* grown in modified

Sauton's medium (approx. 6 % glycerol, v/v) (Shleeva *et al.*, 2004; Shleeva *et al.*, 2002). Significantly, *R. erythropolis* SQ1 grown in CDM 0.1 % glucose did not exhibit any drop in culturability upon transfer to static incubation. It can be hypothesised that the lower concentrations of carbon source/energy primed the bacteria for viability in low-oxygen conditions. Previous and current studies on *R. erythropolis* have focussed on high-oxygen conditions for industrial processes (Gomez *et al.*, 2006; Kretschmer & Wagner, 1980a; Kretschmer & Wagner, 1980b; Santos *et al.*, 2006). Further work on the survival of *R. erythropolis* in microaerophilic and/or anaerobic conditions would shed light on its survival in for example the deep sea (Heald *et al.*, 2001).

An association between carbon starvation, or entry into stationary phase, and increased resistance to a range of environmental stresses has been reported for several bacterial genera including *Vibrio*, *Staphylococcus aureus* and mycobacteria (Clements & Foster, 1998; Kjelleberg *et al.*, 1993; Nyka, 1974). The second objective of this study was to determine whether glucose-starved *R. erythropolis* SQ1 possesses enhanced resistance to heat and oxidative stress. The most dramatic increase in resistance was to heat shock: after 5 min exposure to 57 °C, no exponential phase cells survived, whereas nearly 40 % of early stationary phase cells did. The resistance afforded by carbon starvation to oxidative stress was less dramatic, with almost 50 % of exponential phase cells surviving 5 min in the presence of 400 mM tBHP, compared with 63 % of early stationary phase cells. After 10 min of oxidative stress, 40 % of early stationary phase cells survived, *vs* 7 % of exponential phase cells. These results are broadly similar to those obtained in other organisms, e.g. *Staphylococcus aureus* and *Escherichia coli* (Jenkins *et al.*, 1988; Watson *et al.*, 1998b), although like-for-like comparisons are difficult due to the differences in protocol and the fact that different bacteria have different innate sensitivities to stress. Although the tBHP concentration used in this

study was found to be adequate to investigate the cross-protection afforded by entry into stationary phase/starvation, further investigations could be carried out with different concentrations, in particular in the 200–500 mM range. This would help in determining the mode of the killing effect of tBHP on *R. erythropolis* SQ1, as it has been demonstrated previously that hydrogen peroxide has a bimodal killing effect on cells of *E. coli* (Imlay & Linn, 1986).

To investigate whether protein synthesis was involved in the stress resistance mechanisms, chloramphenicol, a bacteriostatic antibiotic that inhibits protein synthesis, was added to the test sample before exposure to the stress. Interestingly, for *R. erythropolis* SQ1, chloramphenicol treatment did not significantly reduce the degree of resistance of exponential or early stationary cultures to heat shock. This is in contrast to previous studies performed on *E. coli* in which it was found to restore exponential-phase sensitivity to stationary phase *E. coli* (Jenkins *et al.*, 1988). It is possible that, in the experiments performed in this study, the timing of exposure to chloramphenicol was not optimal for observation of induction of cross protection and its inhibition by chloramphenicol. The fact that no reduction in heat shock resistance was observed would lead one to conclude that the proteins that protect against heat shock have already been synthesised prior to chloramphenicol treatment. Since exponential phase cells were however sensitive to a heat shock of 57 °C, it is probable that the heat shock proteins were synthesised between the two sampling points, at 2 and 6 days of growth.

For oxidative stress, on the other hand, chloramphenicol treatment decreased exponential phase *R. erythropolis* resistance to tBHP. A transitory effect was observed in chloramphenicol-treated early stationary phase cells, their sensitivity to tBHP at 5 min exposure was the same as untreated exponential phase cells. However, cell

resistance to tBHP at 10 and 15 min was similar to that of untreated early stationary phase cells. It is unclear how resistance to oxidative stress could be restored in the absence of protein synthesis. The point made above about the time of sampling is also valid here. It is well-known that different enzymes are produced by bacteria to protect against peroxides and superoxides, the most well known are catalase and superoxide dismutase, respectively. tBHP is a peroxide, it is quite possible that a superoxide such as veratrate would elicit a different response in *R. erythropolis* SQ1.

Taken together, these results show that *R. erythropolis* SQ1 reacts to carbon depletion in the medium by triggering a starvation survival response, resulting in long-term survival and cross-protection. As long as aeration is maintained in liquid culture, the cells can form colonies on agar plates. The inability to form colonies on agar plates was observed only in high-energy medium (55 mM glucose medium), and only when the cells where transferred to static incubation in early stationary phase.

The behaviour of the cells treated with chloramphenicol can be explained in several ways. Firstly, it is possible that the experimental setup was not appropriate, although the concentration of chloramphenicol used was shown to inhibit *R. erythropolis* SQ1 growth both on agar and in broth, and was shown to act instantly by continuously monitoring the OD_{600nm} of a test culture while chloramphenicol was added at various concentrations. Secondly, it is possible that heat protection proteins (e.g. chaperones) are constitutively expressed in high concentration in *R. erythropolis* SQ1 grown in low-energy medium (5.5 mM glucose); growth in low-energy medium was shown to protect the cells against static incubation, and this may have other cross-protecting effects. This could be investigated by increasing the quantity of glucose in the medium. Under the

conditions used in this study, chloramphenicol did inhibit the development of resistance to oxidative stress, particularly noticeable in the case of exponential phase cells.

The next step in the study was to generate a mutant bank that could be screened for stationary phase survival deficient mutants, in order to then identify the genes involved. Once electroporation settings and recovery conditions had been optimised, the generation of the mutant bank was rather straightforward. The Tn<KAN2> kit (Epicentre) was selected for generation of a mutant bank of *R. erythropolis* SQ1 since it had been used previously to generate mutants of *R. rhodochrous*, *R. equi* and *R. erythropolis* (Fernandes *et al.*, 2001; Mangan & Meijer, 2001; Tanaka *et al.*, 2002) with no particular problems reported. The number of mutants obtained in this study was comparable to that obtained for *R. equi* and *R. rhodochrous* (approx. 600 and 1500, respectively, Fernandes *et al.*, 2001; Mangan & Meijer, 2001), but disappointing compared with that obtained for *R. erythropolis* KA2-5-1 (approx. 46,000, Tanaka *et al.*, 2002).

Following a simple growth experiment in microtitre plates, it was possible to isolate mutants with growth defects. All mutants generated were screened for stationary phase/starvation survival using a method similar to that of Uhde *et al.* (1997). The results for each mutant were recorded on the basis of appearance of growth on LB agar after 1, 8, 14 and 22 days incubation in CDM 1 and 0.1 % glucose broth. Mutants were selected for further study based on the extent and presence of growth in stationary phase. From 898 mutants screened, four mutants, 1B2, 1H1, 4G6 and 10D3, were of particular interest with regards to stationary phase/starvation survival. When grown in 500 ml glass flasks in 100 ml CDM 1 or 0.1 % glucose and compared with the behaviour of the wild-type *R. erythropolis* SQ1 grown under the same conditions the

following observations were made. The culturability of mutants 4G6 and 10D3 dropped to 0.01 and 0.1 % of the maximum CFU/ml at 27 days incubation, respectively, when grown in 1 % glucose medium. A drop in culturability was also observed in 1 % glucose medium for mutants 1B2 and 1H1, to 3 and 2.5 % of the maximum CFU/ml at 27 days incubation, respectively. Interestingly, no significant drop in culturability could be observed for any of these four mutants when grown in CDM 0.1 % glucose.

One aim of this study was to identify genetic elements involved in the stationary phase and starvation survival of *R. erythropolis* SQ1. An essential step was therefore to isolate DNA from the mutants. Many protocols reported in the literature for the isolation of genomic DNA from *Rhodococcus* are based on phenol extractions and proteinase K treatment (see e.g. Treadway *et al.*, 1999; Veselý *et al.*, 2003). Interestingly, protocols include lysozyme, even though it has been reported that several rhodococci are resistant to lysozyme (Mitani *et al.*, 2005; Solovykh *et al.*, 2004). In this study it was found that, although it was possible to lyse *R. erythropolis* SQ1 cells with similar methods, the amount of protein present in the samples of some mutants made it very difficult to isolate clean genomic DNA. Large volumes of reagents and enzyme were required, and it was not possible to design a standard protocol that could be used for all mutants. Therefore a kit (FastDNA SPIN kit for soil; Qbiogene) using a mechanical cell disruption method (glass beads) and a resin-based DNA purification method was used, as advised by Dr Herron, University of Strathclyde, Glasgow, yielding consistently higher quality genomic DNA for further molecular manipulation.

In order to recover the sequences flanking the transposon insertion site in each of these mutants, several approaches were tested. Among these were two methods, arbitrarily primed PCR (AP-PCR) and random amplification of transposon ends PCR (RATE-

PCR), using either degenerate primers or non-stringent binding conditions, respectively (Caetano-Anollés, 1993; Ducey & Dyer, 2002). In the first method, PCR is performed on the template with degenerate primers, a smear of amplified DNA is produced, as verified by agarose electrophoresis, and specific primers are then used to amplify the sequence of interest. This technique has been successfully applied to *Pseudomonas fluorescens* and *Vibrio cholerae* for example (Burrus & Waldor, 2003; O'Toole & Kolter, 1998), but the amplicons are usually short (200–400 bp). Short sequences could be generated with mutant 4G11, but were deemed too short to give a good overview of the genome segment that had been disrupted by transposon insertion.

RATE-PCR has been used to recover transposon-flanking DNA from *Neisseria gonorrhoeae* (Ducey & Dyer, 2002). This method relies on lowering the annealing temperature during PCR, so that non-specific binding occurs. When applied to the analyses of mutants in this study, many bands of varying sizes were produced for each PCR, but not all were amplicons of the transposon-flanking DNA. By the random nature of this technique, the results could not be reproduced, in that bands obtained in repeated PCR reactions in fact consisted of different amplicons. These problems might also be related to the discrepancy in G+C content between the primer and the rhodococcal DNA. The primer is specific to an end of the transposon and therefore has a G+C content of approximately 50 %, *vs* approximately 70 % for *Rhodococcus* DNA. It would therefore seem logical that low-G+C areas of the rhodococcal DNA would be amplified preferentially, even if the transposon inserted elsewhere.

Since neither of the above methods was successful, a third method was tested based on the use of inverse PCR. This yielded much better results (sequences of up to 1 kb were obtained) and allowed DNA amplification products from 9 mutants (out of 16 selected)

to be cloned and sequenced. The limitation of this method was finding for each mutant a restriction enzyme that yields transposon-containing fragments of an appropriate size for religation and subsequent PCR amplification. This meant that many restriction endonucleases had to be systematically tested on each mutant, as there was no easy or rapid way in which to predict which enzyme would yield the appropriate fragments. An added difficulty was the amplification of a low-copy number, high G+C DNA sequence. Although modern *Taq* polymerase kits are very efficient and effective, some DNA regions could still not be amplified and/or sequenced, even using "high-fidelity" or "high G+C" enzymes (e.g. for mutant 1B2, attempts made were made with YB-GCMix, Yorbio; Deep VentR, NEB; GC-rich PCR system, Roche). For such recalcitrant mutants there are additional alternative methods that could still be tested such as ligation-mediated PCR (LMPCR, Prod'hom *et al.*, 1998). For LMPCR, the target DNA is digested with a restriction enzyme that cuts both within and outwith a known sequence, e.g. a transposon. An asymmetric, double-stranded, non-phosphorylated linker is then ligated to the restricted DNA. During the first PCR step, only a primer specific to the known sequence is used, thereby amplifying only the sequence that flanks the known sequence. A standard PCR is then performed, with primers specific to the known sequence and to the linker. Amplicons of mycobacterial sequences of up to 2.8 kb in size have been produced with this method (Prod'hom *et al.*, 1998). However, the issues mentioned above regarding restriction fragment size, PCR amplification and sequencing of complex sequences would still apply, as it has been demonstrated that the failure rate of PCR increases with G+C content and length of the target sequence (Benita *et al.*, 2003).

An alternative method would be to use a transposon like EZ::Tn*5* <R6K*γori*/KAN-2> (Epicentre) (it should be noted that this became available during the later stages of this

project). As can be seen from the name of the transposon, it has been modified to include a plasmid origin of replication, *ori*. It is therefore possible to extract the genomic DNA from mutants of interest, restrict it, perform a self-ligation and electroporate the resulting plasmids into *E. coli*. When applied to a study of *Rhodococcus erythropolis* AN12 (Yang, 2006; Yang *et al.*, 2007a), two such plasmids, of 11 and 16 kb, were recovered. The use of this transposome bypasses the PCR step that was so problematic in the iPCR approach described above.

Starvation/stationary phase mutants were selected on the basis of non-recovery of growth on LB agar after incubation in chemically defined medium containing 1 or 0.1 % glucose (w/v). Sixteen mutants, exhibiting weak or no growth on LB agar in at least one stage of growth in chemically defined medium, were selected for further study. Sequence data was obtained for nine mutants. Sequence analysis of several of the mutants revealed transposon insertion in biosynthetic genes that rendered them auxotrophic for histidine (insertions in *hisA* or *hisB* in the case of mutants 1B3, 3B10, 4G11, 10E4 and 3B4) and serine/glycine (insertion in *serA*, mutant 6E6). Addition of histidine in the growth medium restored wild-type growth in the mutants, thereby confirming the function of the genes disrupted. It is not clear whether mutants 1B3, 3B10, 4G11 and 10E4 are the result of separate transposon insertion events or clones of each other. Since the insertion was in the same locus for all four mutants, the clone explanation is probably the most likely. These auxotrophic mutants probably featured in the screening experiment because of carry-over of organic material from the master plate during replica plating. Since the volumes of medium used were small (200 µl), even very small amounts of carry-over would enable growth. The histidine mutants had inserts in genes that, in other organisms, are part of an operon. Interestingly, it seems that *Rhodococcus* RHA1 contains genes of unknown or unrelated functions in the *his*

operon. More sequence data would be necessary to determine whether these genes are also in an operon in *R. erythropolis* SQ1. This is however very likely, as this operon is ancient and conserved in many species, including actinobacteria (Price *et al.*, 2006). Various studies on the regulation of the *his* operon have revealed that, as expected, the histidine synthesis genes were repressed under stationary phase/starvation conditions in *E. coli* (*hisF*; Franchini & Egli, 2006) and *M. tuberculosis* (*hisA* and *hisI2*; Betts *et al*., 2002). Surprisingly, the entire *his* operon was up-regulated in *B. subtilis* (*hisZGDBHAFI*; Eymann *et al.*, 2002).

The serine/glycine auxotroph mutant (6E6) resulted from transposon insertion in a *serA* gene homologue. The protein encoded by *serA* is a D-3-phosphoglycerate dehydrogenase that catalyses the first step of synthesis of serine from pyruvate. Since this pathway then leads to the synthesis of glycine and threonine, growth of the mutant was therefore tested in chemically defined medium containing serine, glycine, threonine or combinations of these amino acids. The growth of the serine/glycine auxotrophic mutant could be restored by addition of either serine or glycine, singly or together, but was completely inhibited by threonine. It is possible that the aspartokinase of *R. erythropolis* SQ1 is inhibited by threonine, as was described for *Methylococcus capsulatus* (Eccleston & Kelly, 1973), thereby disrupting the synthesis of aspartic acid and aspartate-based amino acids (lysine, methionine, threonine and isoleucine). Eccleston and Kelly (1973) have described how methionine can relieve the inhibition caused by threonine but this was not attempted in this study. In a microarray experiment monitoring gene expression of *E. coli* grown in glucose-limited continuous culture, it was found that the gene *serA* was down-regulated at both 40 and 500 hours incubation (Franchini & Egli, 2006).

One of the mutants that could not survive stationary phase was 4G6, with a transposon insertion in the gene *uvrB*. UvrB, the excinuclease ABC subunit B, is an essential part of the DNA excision repair mechanism; therefore it is not surprising that this mutant would have difficulty in surviving stationary phase. It is known that, as cells stop growing, they accumulate mutations in the DNA (Bridges, 1998; Kivisaar, 2003). The role of *uvrB* in stationary phase mutations is unclear, as it has been shown that *uvrB* mutants accumulate mutations during stationary phase in *E. coli* (Vidal *et al.*, 1998), but the opposite was observed in *Pseudomonas putida* (Tark *et al.*, 2008). Furthermore, a *Mycobacterium tuberculosis uvrB* mutant was found to be extremely sensitive to nitric oxide, and UvrB was essential for resistance to reactive oxygen and reactive nitrogen intermediates. The stationary phase survival of this mutant however was not investigated (Darwin & Nathan, 2005). Interestingly, loss of viability was observed in the *uvrB R. erythropolis* SQ1 mutant grown in CDM 1 % glucose, but not in CDM 0.1 % glucose. Two explanations can be advanced for this discrepancy. Firstly, the high cell concentration obtained in CDM 1 % glucose might mean that high levels of oxidants and waste compounds have accumulated in the growth medium. As cells die and lyse, even more of these compounds would be released, hence increasing their concentration still further. Secondly, it is possible that growth in 0.1 % glucose induced expression of alternative DNA repair mechanisms, or of DNA-protecting proteins such as Dps, as has been shown in *Mycobacterium smegmatis* (Gupta *et al.*, 2002).

Mutant 10D3 was found to have the same growth profile as the UvrB mutant, although the transposon insertion does not seem to have disrupted a gene. The transposon is inserted immediately downstream of a putative *guaB* gene. Similar *guaB* genes are present in other organisms, but are not part of an operon, and are usually followed by another IMP dehydrogenase (*guaB*-like) and/or a cholesterol oxidase. The functions of

these genes are unclear however, so the function disturbed in *R. erythropolis* SQ1 mutant 10D3 is unknown. It is possible that the decrease in viability of this mutant was due not to the disruption of a gene or regulatory sequence, but to overexpression of the kanamycin resistance gene of the transposon. The transposon inserted downstream of the *guaB* gene, a gene essential to the growth of the organism (Gil *et al.*, 2004). It is therefore possible that the KAN2 gene came under regulation of the *guaB* promoter, resulting in overproduction of the kanamycin resistance protein (aminoglycoside 3′-phosphotransferase). Over-expression of foreign proteins has been shown to inhibit bacterial growth and induce an incomplete starvation survival response (Kurland & Dong, 1996), that could affect the long-term stationary phase survival of cells grown in CDM 1 % glucose. However, this seems unlikely, since the genes *guaB* and *guaA* were repressed in *E. coli* grown for 40 h in continuous culture in medium containing 0.01 % glucose (Franchini & Egli, 2006), and *guaB* was repressed in *Bacillus subtilis* in which stationary phase was artificially induced by the addition of norvaline (Eymann *et al.*, 2002). Alternatively, the insertion could have affected the expression of the sequence downstream of the transposon insertion. Interestingly, the loss of culturability was observed only in CDM 1 %, but not in 0.1 % glucose.

Mutant 1H1 was found to lose viability when grown in CDM 1 %. Interestingly, although the flanking sequence from mutant 1H1 could be amplified by iPCR using standard *Taq* polymerase and cloned, and subsequently amplified from the clone, it was not possible to obtain sequence data for one flanking sequence, despite several attempts. The transposon is inserted 272 nt downstream of a gene encoding a putative phosphoglycerate mutase. The mutase identified is probably a fructose-2,6-bisphosphatase involved in five-carbon sugar metabolism. Unfortunately sequence information was not obtained for the sequence downstream of the transposon insertion

site; although in other organisms thioredoxin and cytochrome *c* biogenesis genes have been identified. Closer analysis of the 200 nucleotide sequence immediately upstream of the transposon did reveal significant homology to putative thioredoxins and cytochrome *c* biogenesis proteins of *Rhodococcus* RHA1 and *Nocardia*. It should be noted that in other actinobacteria such as *Saccharopolyspora erythraea*, *Mycobacterium ulcerans* and *M. tuberculosis*, it was found that the phosphoglycerate mutase gene is situated upstream of a putative thioredoxin protein/cytochrome *c* biogenesis protein gene, in the same orientation. It is therefore possible that polar effects of transposon insertion could disturb the expression of these two genes. Interestingly, the phosphoglycerate mutase genes *pgm* and *yibO* were found to be increasingly expressed in norvaline-induced stationary phase in *B. subtilis* (Eymann *et al.*, 2002) and in 500 h 0.01 % glucose continuous culture *E. coli* (Franchini & Egli, 2006). Indeed, many thioredoxin-related genes have been found to be induced by stationary phase; they are shown in Table 5.1. Thioredoxins are involved in many processes in bacteria, in particular DNA synthesis, protein repair, sulphur assimilation, cell division, energy transduction, transcriptional regulation and oxidative stress response (for a review, see Zeller & Klug, 2006). During oxidative stress response, thioredoxins reduce disulfide bonds and scavenge reactive oxygen species. Interestingly, DsbE (a disulfide oxidoreductase) is involved in the maturation of cytochrome *c* in *E. coli* (Fabianek *et al.*, 1998). Cytochrome *c* itself also seems to be involved in maintaining peroxidase and catalase activity in the periplasm (Goodhew *et al.*, 1990).

There are relatively few rigorous studies in which carbon limitation is definitely the stationary phase-triggering factor (see e.g. van Overbeek *et al.*, 1997). In many cases this is because the organism studied requires amino acid supplementation for growth (for example Seymour *et al.*, 1996). The ability of *R. erythropolis* SQ1 to grow in a

rather simple chemically defined medium where all important elements are supplied by inorganic salts is therefore important. The list of vitamins used as supplements in this study could probably be reduced drastically by further modification of the medium. However, it should be noted that, in the medium used in this study the concentration of vitamins used is so low that it is unlikely to support measurable growth. It is nonetheless challenging to screen for mutants deficient in carbon-starvation survival. This is due in no small part to the fact that many of these mutations are probably lethal, given the importance of carbon in every biological process. Different protocols could be used, that simulate "real-world" conditions more closely. For instance, the mutants could have been replica-plated to chemically defined medium agar plates containing decreasing quantities of glucose, or a selection of carbon sources. Investigation of longer term survival would also be interesting, although the growth advantage in stationary phase (GASP) phenotype would certainly eventually arise (Zinser & Kolter, 1999; Zinser & Kolter, 2004).

In all the mutants for which sequence information could be obtained, the transposon insertion was accompanied by the duplication of a short stretch of the target sequence (Figure 5.1). For all the mutants (with one exception), a textbook 9 bp repeated sequence, typical of Tn*5* insertion sites (Goryshin *et al.*, 1998), was observed. Only mutant 6E6 contained a 10 bp repeat, which could be explained by slippage of the transposase–DNA complex during insertion. Interestingly, 10 bp is the kind of duplication seen at the insertion site of IS1166, as observed in *R. erythropolis* AJ270 and AJ300 (O'Mahony *et al.*, 2005). Also, in mutant 4G6 (UvrB mutant) an imperfect duplication was observed, in that only 7 out of 9 bp were identical. It is however plausible that this is a PCR artefact, rather than something directly resulting from the insertion mechanism. Interestingly, symmetry of the duplicated sequence was observed

in a comprehensive study of Tn*5* insertion in *Streptomyces coelicolor*, e.g. GCCCNGGGC (Herron *et al.*, 2004), but no such symmetry was found in *R. rhodochrous* or *R. equi* Tn*5* insertion sites (Fernandes *et al.*, 2001; Mangan & Meijer, 2001). This symmetry therefore seems to be limited to *Streptomyces coelicolor*.

Gene	Function	Organism	Reference
yfcF	GST enzyme with thioredoxin-like domain	*E. coli*	Weber *et al.* (2005)
yncG	GST with C-terminal thioredoxin-like and GST domains	*E. coli*	Weber *et al.* (2005)
ygjK	GST enzyme with thioredoxin-like domain	*E. coli*	Weber *et al.* (2005)
dsbG	Thiosulfide reductase	*E. coli*	Weber *et al.* (2005)
dsbE	Disulfide oxidoreductase	*E. coli*	Franchini & Egli (2006)
yumC	Thioredoxin reductase	*B. subtilis*	Eymann *et al.* (2002)
trxA	Thioredoxin	*B. subtilis*	Hecker & Völker (1998)
trxC	Thioredoxin	*M. tuberculosis*	Betts *et al.* (2002)

Table 5.1. Thioredoxin-related genes up-regulated during starvation/stationary phase. GST, Glutathione *S*-transferase.

Mutants 1B3, 3B10, 4G11 and 10E4:

5'-CACCGACGT **CTCCAAGGA**<TN KAN-2>**CTCCAAGGA** CGGCACACT-3'

Mutant 3B4:

5'- GCGTGAGGC **GGTCGAGCC**<TN KAN-2>**GGTCGAGCC** GGATCCGCG-3'

Mutant 10D3:

5'- ACCCATGCC **GATCTCAAC**<TN KAN-2>**GATCTCAAC** GAGGTCGCG-3'

Mutant 6E6:

5'- AGAGTGTTC **TCCTCGCTCT**<TN KAN-2>**TCCTCGCTCT** GGCAGGCGA-3'

Mutant 4G6:

5'- TGACCGCAC **GACCGGCCT**<TN KAN-2>**CACCCGCCT** CACCCTGCG-3'

Figure 5.1. Transposon insertion sites identified in mutants 1B3, 3B10, 4G6, 10E4, 3B4, 10D3, 6E6 and 4G6. The 9 bp (10 bp for 6E6) repeats either side of the insertion site are shown in bold type.

In conclusion, *R. erythropolis* SQ1 was shown to be a good candidate for a model or test organism for this study, in particular because of its ease of culture and ease of molecular manipulation. It presents a classic starvation/stationary phase survival response, with the associated increase in resistance to various external stresses.

A mutant bank was also generated, that will be available for future screening of other phenotypes, and a method developed to recover transposon flanking sequences. The mutants identified as being deficient in stationary phase/starvation survival had a transposon insertion in genes *uvrB* (nucleotide excision repair), *hisA* and *hisB* (histidine synthesis), *serA* (serine/glycine synthesis) and a putative thioredoxin (cytochrome *c* biogenesis). For one of the mutants (mutant 10D3) the transposon insertion was not within a gene but upstream of *IMDH* (IMP dehydrogenase) and downstream of *guaB* (guanine synthesis).

Similar genes have been identified in stationary/starvation deficient mutants of other organisms. Some of those genes are involved in amino acid metabolism, such as *hprT* (hypoxanthine-guanine phosphoribosyl transferase) in *Staphylococcus aureus* (Watson *et al.*, 1998a), *lysE* (exporter of lysine and arginine) in *Mycobacterium smegmatis* (Smeulders *et al.*, 2004) and *ilvE* (branched-chain amino acid aminotransferase) in *Sinorhizobium meliloti* (Uhde *et al.*, 1997). Others are cytochrome-associated, e.g. *cydC* (cytochrome *d* oxidase) in *E. coli* (Siegele *et al.*, 1996), *cox* (cytochrome *c* oxidase) in *Sinorhizobium meliloti* (Uhde *et al.*, 1997) and *ctaA* (haem A synthase, involved in synthesis of cytochromes aa_3 and caa_3) in *Staphylococcus aureus* (Clements *et al.*, 1999b; Watson *et al.*, 1998a). More work should be performed in order to understand the function of these genes in stationary phase/starvation survival in *Rhodococcus* and also to identify additional implicated genes.

Few complete genome sequences of rhodococci are available to date. However, it should be noted that the availability of the *Rhodococcus* RHA1 genome sequence (McLeod *et al.*, 2006) has been invaluable to the interpretation of the short stretches of DNA sequence retrieved from mutants analysed during the course of this project. The only other genome sequence partially available is that of *R. equi* 103S (http://www.sanger.ac.uk/Projects/R_equi/). *R. erythropolis* RR4 and *R. opacus* B4 are currently being sequenced at the National Institute of Technology and Evaluation in Japan (http://www.bio.nite.go.jp/ngac/e/project-e.html). With additional sequence data, it will be possible to use a site-directed mutagenesis strategy and/or microarrays to progress in this field.

Several sigma factors have already been identified in *Rhodococcus* RHA1 [σ^A, σ^B; McLeod *et al.* (2006)], the sequences of which could be used to identify the corresponding genes in *R. erythropolis* SQ1. A homologue of *relA* can also be found in *Rhodococcus* RHA1, the putative protein sequence of which has high identity (>93 %) with that of *M. tuberculosis* H37Rv, and would therefore be a prime candidate for site-directed mutagenesis. Until sequencing of *R. erythropolis* RR4 and *R. opacus* B4 is completed, it should be possible to identify an equivalent gene in *R. erythropolis* SQ1 by careful design of primers for PCR amplification.

The "resuscitation factor" identified in *M. tuberculosis* could be another target (Mukamolova *et al.*, 1998). Five *rpf* genes have been identified in *M. tuberculosis* so far (Mukamolova *et al.*, 2002), and some mutagenesis work has started in *M. tuberculosis* and *Micrococcus luteus* (Downing *et al.*, 2004; Mukamolova *et al.*, 2006). Some limited

studies have also been performed on *R. rhodochrous* (Shleeva *et al.*, 2002; Voloshin *et al.*, 2005). It should be noted that the procedure to generate 'non-culturable' cells of *R. rhodochrous* is the exact opposite of that observed in this study for *R. erythropolis* SQ1, i.e. shaking *vs* stationary incubation. Comparison of the two species would provide more information on the role of Rpf in rhodococci.

Other genes of interest for site-directed mutagenesis would be *dps*, encoding a DNA-protecting protein (Gupta & Chatterji, 2003), and *ppk*, encoding a polyphosphate kinase (Hirsch & Elliott, 2002).

All the genes highlighted previously for site-directed mutagenesis would also be suitable for microarray study. This would allow the study of the expression of the stationary phase/starvation genes when cells are used in industrial processes, e.g. when in a resting state or even isolated from polymer-bound cells.

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