Mutants of *Mycobacterium smegmatis* impaired in stationary-phase survival

Jacquie Keer,[†] Marjan J. Smeulders, Kathryn M. Gray
and Huw D. Williams

Author for correspondence: Huw D. Williams. Tel: +44 20 75945383. Fax: +44 20 75842056.
e-mail: h.d.williams@ic.ac.uk

Department of Biology,
Imperial College of
Science, Technology and
Medicine, Imperial College
Road, London SW7 2AZ,
UK

A bank of 600 insertional mutants of *Mycobacterium smegmatis* was screened for mutants defective in stationary-phase survival. Of 74 mutants picked by the initial screen, 21 had stationary-phase survival defects and 7 of these were studied in more detail. In general, mutants survived stationary phase significantly less well in rich medium than under carbon-starvation conditions. In all cases the loss of viability in stationary phase was not complete even after prolonged incubation. All mutants showed an initial decrease in viability, during the first 40 d in stationary phase, followed by an increase in viable counts that returned viability close to the levels of the wild-type. Southern hybridization experiments showed that recovery of viability was not a consequence of precise excision or movement of the transposon. Two of the survival mutants differed from the wild-type in their colony morphology, and recovery of their viability in stationary phase was coincident with the return of wild-type colony morphology. It is possible that second-site suppressor mutations accumulate that alleviate the effects of the original mutation. For five of the mutants the DNA flanking the site of transposition was amplified by ligation-mediated PCR and sequenced to identify the disrupted locus. In each case, homologous genes were identified in the *Mycobacterium tuberculosis* genome, three of which have clearly predicted functions in *M. tuberculosis* as a penicillin-binding protein, in biotin biosynthesis and as a polyketide synthase. This is the first identification of genes implicated in the stationary-phase survival of mycobacteria.

Keywords: starvation, dormancy, colony morphology, latency, tuberculosis

INTRODUCTION

Mycobacterium tuberculosis is a major human pathogen, causing 3 million deaths per year. Of individuals infected with tuberculosis, the majority show no clinical signs of disease (Raviglione *et al.*, 1995). In addition, apparently successfully treated patients may still harbour persistent bacteria, and also act as reservoirs of *M. tuberculosis* in the environment. This is due to latent tuberculosis, an asymptomatic infection in which *M. tuberculosis* persists within the human host for years without causing disease. During clinical latency, *M.*

tuberculosis is thought to reside in old pulmonary granulomatous lesions, although it is possible that the bacilli are also located throughout the lungs and other tissues during latent infection (Parrish *et al.*, 1998). About one-third of the world population is estimated to have latent tuberculosis (Parrish *et al.*, 1998) and therefore be at risk of developing reactivated TB at some time in their lives.

A key question is the nature of the physiological state of the bacteria during latent infection. Although the survival state is often referred to as ‘dormancy’, there is no direct evidence to indicate that the bacilli enter a physiological state *in vivo* or *in vitro* analogous to that produced following sporulation of *Bacillus* spp. or the dormant state entered by *Micrococcus luteus* in stationary phase (Barer, 1997; Errington, 1993; Kaprelyants & Kell, 1993; Wayne, 1994). An alternative possibility is

[†] Present address: LGC, Queens Road, Middlesex TW11 0LY, UK.

The GenBank accession numbers for the sequences determined in this work are: AJ277088 (mutant 272A), AJ277089 (mutant 272E), AJ27790 (mutant 317C), AJ277152 (mutant 492A) and AJ276883 (mutant 3910D).

that latent infections involve metabolically active bacilli in a state of non-growth or prolonged stationary phase, during which there is cell turnover and very slow growth but where the net viable population remains constant (Parrish *et al.*, 1998).

Although the stationary-phase survival response of Gram-negative bacteria has been well characterized (Hengge-Aronis, 1993; Kjelleberg, 1993; Kolter *et al.*, 1993; Nystrom, 1999; Spector & Cubitt, 1992), it is only recently that the stationary-phase survival of mycobacteria has started to be studied. Two mycobacterial sigma factors have been implicated in stationary-phase gene regulation: *sigF* (Demaio *et al.*, 1996, 1997; Michele *et al.*, 1999) and *sigB* (Beggs *et al.*, 1996; Doukhan *et al.*, 1995; Hu & Coates, 1999a; Predich *et al.*, 1995). Both sigma factors are up-regulated during O₂-limited stationary phase (Hu & Coates, 1999a), which is an *in vitro* model for mycobacterial persistence developed by Wayne and co-workers (Wayne, 1994; Wayne & Hayes, 1996). In this model, cessation of growth is caused by the depletion of oxygen to the bacteria, by growing them in a sealed container with a limiting amount of oxygen. This model has been used to identify several proteins and genes that are induced during anaerobiosis (Hutter & Dick, 1998; Wayne & Lin, 1982; Wayne & Hayes, 1996): the 16 kDa α -crystallin small heat-shock protein (Hu & Coates, 1999b; Yuan *et al.*, 1996) and the *M. tuberculosis hmp* gene product, which is homologous to *Escherichia coli* flavohaemoglobin (Hu *et al.*, 1999), as well as a histone-like protein in *Mycobacterium smegmatis* (Lee *et al.*, 1998).

We are studying adaptation to stationary phase in the fast-growing, non-pathogenic *Mycobacterium smegmatis*. We have previously reported that upon entry into carbon-limited stationary phase, *M. smegmatis* undergoes physiological changes resulting in increased stress resistance, an increase in mRNA stability, and an overall decrease in protein synthesis. The cells also undergo reductive cell division (Smeulders *et al.*, 1999). During prolonged stationary phase, variants arise that have a growth advantage in stationary phase over exponential-phase-adapted strains. The ability of these variants to take over stationary-phase cultures strongly suggests that a heterogeneous, dynamic population of bacteria is present in stationary-phase cultures of *M. smegmatis* (Smeulders *et al.*, 1999).

Understanding the molecular mechanisms underlying mycobacterial latency would be aided by identifying genes essential for mounting an effective stationary-phase survival response. Although a number of such genes have been identified in enteric bacteria (Groat *et al.*, 1986; Nystrom, 1999; Siegle & Kolter, 1992; Spector & Cubitt, 1992; Tormo *et al.*, 1990) to our knowledge, no mycobacterial mutants have been described with defects in stationary-phase survival. Here, we report the identification of mycobacterial genes important for surviving periods of non-growth. An insertional mutant library of *M. smegmatis* was generated using the transposon delivery vector pCG79

(Guilhot *et al.*, 1994). Mutants were isolated that showed impaired survival during stationary phase, using a strategy similar to that used in the isolation of *sur* mutants of *E. coli* (Tormo *et al.*, 1990). The genes disrupted in five of these mutants have been identified and each has a clear homologue in *M. tuberculosis*, allowing the identification of a number of proteins implicated in mycobacterial stationary-phase survival.

METHODS

Media and growth conditions. *M. smegmatis* mc²155 was grown as described previously either in rich Lab-lemco medium or a carbon-limited minimal medium [Hartmans-de Bont (HdB) medium containing 0.02 % glycerol (Hartmans & De Bont, 1992; Smeulders *et al.*, 1999)]. *M. smegmatis* cultures were grown with shaking at 200 r.p.m. at 37 °C, except for initial selection of pCG79 transformants, when incubation was at 30 °C to permit replication of the temperature-sensitive plasmid, and during the procedure to eliminate the plasmid, when cultures were incubated at 41 °C. *E. coli* cultures were grown in Luria broth at 37 °C. When required, kanamycin was added to a final concentration of 20 µg ml⁻¹.

Isolation of transposon mutants impaired in stationary-phase survival. *M. smegmatis* mutants impaired in stationary-phase survival were isolated from a mutant library constructed by transposon mutagenesis using Tn611, as described by Guilhot *et al.* (1994), with the modification that plasmid loss was induced by raising the temperature to 41 °C. Using the previously recommended temperature of 39 °C resulted in low-frequency loss of the transposon delivery plasmid (data not shown). pCG79 was electroporated into *M. smegmatis* mc²155 and transformants selected on Lab-lemco with kanamycin at 30 °C. Transformants were eluted from plates, diluted to about 5 × 10³ cells ml⁻¹ and grown into mid-exponential phase at 41 °C in Lab-lemco medium without antibiotics, and then plated onto Lab-lemco plus kanamycin. Care was taken to avoid growing the cultures into stationary phase, as this would be expected to select against the survival of mutants with the desired phenotype. Plates were incubated for 2 d at 41 °C, and then pinprick-sized colonies were picked in duplicate into microtitre wells containing carbon-limited minimal medium (HdB medium with 0.02 % glycerol). The microtitre plates were sealed with film to prevent evaporation of the medium during prolonged incubation at 37 °C. One set of plates was frozen at -70 °C immediately and the duplicate set was grown for 30 d at 37 °C, then 5 µl of each culture was plated onto Lab-lemco medium (plus kanamycin) to check stationary-phase survival of the mutant strains. Cultures that failed to grow on plates after 7 d incubation at 37 °C on Lab-lemco medium were revived from the frozen duplicate stock for further investigation.

Viability studies. For stationary-phase survival experiments, wild-type and Tn611-mutant strains of *M. smegmatis* were grown at 37 °C either as 5 ml cultures in 20 ml universal tubes, or as 2 ml cultures in 16 ml test tubes in HdB or Lab-lemco medium containing kanamycin. Cultures were shaken at 200 r.p.m. Viability was assayed by plating samples of cultures, diluted appropriately in PBS + 0.05 % Tween 80, onto Lab-lemco plus kanamycin plates and incubating at 37 °C for 5 d.

Isolation of DNA and recombinant DNA methods. Standard recombinant DNA procedures were used as described by Sambrook *et al.* (1989). Total genomic DNA was prepared from *M. smegmatis* as described by Martin *et al.* (1990). For

Southern hybridization experiments, chromosomal DNA was isolated from Tn611 mutants and digested with *Pst*I; the fragments were separated by agarose gel electrophoresis and probed with the radiolabelled 640 bp *Hind*III-*Pst*I internal fragment of IS6100 (Guilhot *et al.*, 1994).

Identification of mutated genes using ligation-mediated PCR. To identify the genes disrupted in the Tn611 mutants, the DNA sequences adjacent to the site of transposon Tn611 insertion were amplified using the ligation-mediated PCR procedures described by Prod'homme *et al.* (1997, 1998). Briefly, genomic DNA isolated from mutant strains was digested with *Sal*I and then ligated to an asymmetric, non-phosphorylated, double-stranded *Sal*I linker (Prod'homme *et al.* 1997, 1998). PCR was carried out exactly as described previously, using one primer that recognizes part of the transposon sequence and a second primer that recognizes sequence in the *Sal*I linker. PCR products were separated on agarose gels and purified using the Gene Clean kit as described by the manufacturer (Bio101) and cloned into pGEM-T Easy (Promega). The nucleotide sequence data were compiled and analysed using the Lasergene program suite (DNASTAR Inc.). Automated DNA sequencing was done with a Taq DyeDeoxy Terminator Cycle sequencing kit (Perkin Elmer Cetus) with the M13 forward primer.

RESULTS

Isolation of transposon mutants impaired in stationary-phase survival

M. smegmatis stationary-phase survival mutants were sought using a screen originally used to isolate stationary-phase survival mutants of *E. coli* (Tormo *et al.*, 1990). A bank of 600 *M. smegmatis* insertional mutants was generated using the transposon Tn611 (Guilhot *et al.*, 1994), and screened for mutants that failed to survive incubation for 30 d in carbon-starvation-induced stationary phase. Seventy-four mutants were identified by this initial screen, of which 21 showed markedly reduced survival in stationary phase on further testing (Fig. 1), as compared to the

viability of the wild-type after 40 d in stationary phase at 37 °C. Therefore, 3.5% of the mutants screened showed some impairment of stationary-phase survival.

Stationary-phase survival of selected mutants

The stationary-phase survival of a number of mutants was studied in more detail, following growth in rich medium and under carbon-starvation conditions. Tests of mutant viability in stationary phase following growth in Lab-lemco medium were confusing, as often after a single serial subculture with antibiotic selection the impaired survival phenotype was lost. The survival curve of mutant 3910D during the 120 d following entry into stationary phase is shown in Fig. 2(a). This mutant showed a rapid decline in viability to $<10^6$ c.f.u. ml⁻¹ after day 40, only for the viability to then recover to wild-type levels after 120 d. To determine whether the transposon had moved during stationary phase, a Southern hybridization experiment was carried out using a portion of IS6100 to probe *Pst*I digests of chromosomal DNA isolated from 3910D at the start of the experiment and after 120 d in stationary phase (Fig. 2c). Hybridization of *Pst*I digests with the IS6100 probe gave four hybridizing bands (Fig. 2c), three of which (0.9, 1.2 and 2.0 kb) are due to its hybridization to internal fragments of the integrated vector and the fourth, of variable size, results from duplication of IS6100 upon Tn611 transposition (Guilhot *et al.*, 1994). 3910D has a single Tn611 insertion and the hybridization pattern is identical in the DNA isolated from the start of the experiment and after 120 d in stationary phase (Fig. 2). These data show that Tn611 has not moved during prolonged stationary phase and the apparent recovery in the survival of the mutants may be due to suppression of the survival phenotype.

Survival of mutant 412A was also impaired in stationary phase following growth in rich medium (Fig. 2b), and a similar Southern hybridization experiment to that described for 3910D showed that Tn611 did not move during prolonged stationary-phase incubation (Fig. 2c). The 412A culture showed a marked (4 log₁₀) fall in viability up to day 20 followed by a rapid recovery by day 40 to 25% of the initial day 5 viability.

Both 3910D and 412A are colony morphology mutants and when plated for enumeration they had an appearance that was clearly different from the wild-type (Fig. 3). Both had a crinkled appearance but were also easily distinguishable from each other (Fig. 3b, e). Initially following inoculation of 3910D all the colonies had a crinkled appearance (Fig. 3b). However, by day 5 a small proportion ($<5\%$) had wild-type morphology; this typically increased to 10–50% by day 20 (Fig. 3c) and by the end of the experiment virtually all colonies observed on plating were of wild-type appearance (Fig. 3d). A similar transition of colony morphology accompanied the recovery in viability of 412A during prolonged stationary phase (Fig. 3e–g).

The stationary-phase survival of five further mutants

Fig. 1. Viability tests on putative stationary-phase-survival mutants. Mutant strains were grown in Lab-lemco medium plus kanamycin at 37 °C. Viability was assayed by plate counts on entry of the cultures into stationary phase and after 40 d incubation, and the percentage survival over the course of the experiment was calculated.

Fig. 2. (a, b) Long-term viability of wild-type *M. smegmatis* mc²155 and stationary-phase survival mutants 3910D (a) and 412A (b). Strains were grown into stationary phase in Lab-lemco medium plus kanamycin at 37 °C and viability determined by plate counts at intervals during stationary-phase incubation. (a) □, *M. smegmatis* mc²155; ■, 3910D. (b) □, *M. smegmatis* mc²155; ■, 412A. The same control culture is shown in (a) and (b). The labels 1 and 2 indicate the times at which samples of the mutant cultures were taken for the preparation of chromosomal DNA for the Southern hybridization experiment shown in (c). (c) Autoradiograph showing a Southern hybridization of a *Pst*I-*Hind*III fragment containing the repeat element of the transposon with *Pst*I digests of chromosomal DNA isolated from the mutant strains 3910D and 412A. Lanes 1 and 2, DNA from time points 1 and 2, respectively, of the 3910D culture indicated in (a). Lanes 3 and 4, DNA from time points 1 and 2, respectively, of the 412A culture indicated in (b). In each case DNA was isolated from a washed cell sample prepared from the culture. The bands at 2.0, 1.2 and 0.9 kb result from hybridization to fragments internal in the inserted vector, while the fourth, variable, band (*) hybridizes to a junction fragment containing both transposon vector and chromosomal flanking sequences.

Fig. 3. Colony morphology of wild-type (a) and the stationary-phase survival mutants 3910D (b, c, and d) and 412A (e, f and g), grown on Lab-lemco medium. The colonies in (a) are representative of the wild-type under the growth conditions used. 3910D was plated from cultures 5 d (b), 20 d (c) and 120 d (d) into stationary phase in Lab-lemco medium. 412A was plated from cultures 5 d (e), 12 d (f) and 120 d (g) in to stationary phase in Lab-lemco medium. The arrows in (b), (c) and (f) indicate colonies with wild-type-like morphology.

following growth in rich medium is shown in Fig. 4. All the mutants showed similar survival patterns. A significant (3–4 log₁₀) reduction in viability during the

first 40 d in stationary phase was followed by either a levelling off (272A and 272E) or an increase (317C, 228H and 173D) in viability over the subsequent 60–80 d,

Fig. 4. Long-term viability of wild-type *M. smegmatis* mc²155 (□) and stationary-phase survival mutants (■): 173D (a), 272A (b), 272E (c), 228H (d) and 317C (e). Strains were grown into stationary phase in Lab-lemco medium plus kanamycin at 37 °C and viability determined by plate counts at intervals during stationary-phase incubation.

returning viability to levels closer to those of the wild-type at this stage. In addition, if mutants were serially subcultured and allowed to grow into stationary phase, they did not show stationary-phase survival defects on retesting.

Survival following carbon starvation

We also looked at the carbon-starvation survival of the mutants described in Figs 2 and 4, together with a further mutant 492A, which in preliminary experiments showed only modest impairment of survival in rich medium (Fig. 1). Following carbon starvation due to exhaustion of glycerol in the medium, the wild-type *M. smegmatis* strain mc²155 survives extended periods of non-growth with little loss of viability (Smeulders *et al.*, 1999). It reaches viable counts of $\sim 10^9$ c.f.u. ml⁻¹ on entry into stationary phase following reductive cell division (Smeulders *et al.*, 1999) when grown in HdB minimal medium, and over a period of around 80 d

viability drops to around 6×10^8 c.f.u. ml⁻¹. Many of the mutant strains survived carbon starvation markedly less well than the wild-type culture (Fig. 5). Overall, the impairment of survival was less severe under carbon-starvation conditions compared to stationary phase in Lab-lemco medium. In most cases, the rate of loss of viability declined after 40 d in stationary phase, possibly due to the accumulation of second-site suppressor mutations or to the improved ability of the mutants to survive the conditions prevalent late in stationary phase. The clearest impairment of survival was observed for 173D, 3910D, 412A and 228H (Fig. 5). While both 272A and 272E showed significant impairment of their stationary-phase survival in rich medium (Fig. 4), we found that they were unable to grow in unsupplemented minimal medium and that each was likely to be an auxotroph. Auxanography experiments revealed a complex pattern of complementation, but the presence of a vitamin pool (containing pyridoxine, nicotinic acid, biotin, pantothenate and the amino acid alanine) al-

Fig. 5. Long-term viability in carbon-starved stationary phase of *M. smegmatis* mc²155 (□) and: (a) ■, 173D; ●, 492A; ▲, 3910D; (b) ■, 412A; ●, 272E; ▲, 272A; (c) ■, 228H; ●, 317C. Strains were grown into stationary phase in HdB minimal medium containing 0.02% glycerol, conditions under which the cultures enter stationary phase due to glycerol exhaustion (Smeulders *et al.*, 1999), and viability was followed by plating onto Lab-lemco medium plus kanamycin. The same control culture is shown in each panel. The initial c.f.u. ml⁻¹ for each culture upon entry into carbon-starved stationary phase was: mc²155, 2×10^8 ; 173D, 3.5×10^8 ; 492A, 7×10^8 ; 3910D, 6×10^8 ; 412A, 2.3×10^8 ; 272E, 8×10^8 ; 272A, 8×10^8 ; 228H, 4.5×10^8 ; 317C, 5×10^8 .

Table 1. *M. tuberculosis* genes homologous to loci disrupted in five of the *M. smegmatis* mutants

Mutant	<i>M. tuberculosis</i> ORF/locus	% Similarity of mutated gene (identical or similar aa/total aa)	Function of homologous protein	Similarities with genes from other organisms
272A	Rv1568/ <i>bioA</i>	87.5% (21/24)	Probable adenosylmethionine-8-amino-7-oxononanoate aminotransferase	BioA, <i>M. leprae</i> P4548
272E	Rv1180/ <i>pks3</i>	89% (115/129)	Probable polyketide synthase β -ketoacyl synthase domain, similar to the N-terminus of many polyketide synthases	Mycocerosic acid synthase, <i>M. bovis</i>
317C	Rv3682/ <i>ponA</i>	88% (30/34)	Class A penicillin-binding protein	PBP1 (<i>pon1</i>), <i>M. leprae</i>
3910D	Rv0658c	79% (70/89)	Unknown probable membrane protein	Similar to YprB, <i>E. coli</i> (P33774)
492A	Rv3170	77% (35/45)	Probable flavin-containing monoamine oxidase similar to many eukaryotic monoamine oxidases	

The GenBank accession numbers for the sequences obtained from the Tn611-mutated genes are: AJ277088 (272A); AJ277089 (272E); AJ27790 (317C); AJ277152 (492A) and AJ276883 (3910D).

lowed growth on minimal medium. When carbon starvation was tested in minimal medium containing this vitamin pool there was a small (approx. $1 \log_{10}$) reduction in viability of these strains compared to the wild-type over 80 d (Fig. 5b).

Identification of the site of Tn611 insertion in stationary-phase survival mutants

Southern hybridization analysis of the mutants indicated that a single transposition event had occurred through the expected replicative transposition mechanism in all mutants (Fig. 2 and data not shown). To identify the disrupted genes we used a ligation-mediated PCR strategy (Prod'homme *et al.*, 1997, 1998). This method allows the amplification of two DNA fragments, each containing one end of the transposon and the adjacent *M. smegmatis* DNA, which can then be sequenced. This

approach was used to clone the DNA flanking five mutants, 272A, 272E, 317C, 3910D and 492A. One of the flanking sequences was amplified and sequenced from each of these mutants and it was established that the transposon had inserted into five distinct loci. To identify the genes disrupted, the partial *M. smegmatis* sequences were used in a BLAST search of the *M. tuberculosis* H37Rv genome sequence (Cole *et al.*, 1998). All five mutants had insertions into distinct loci, disrupting genes with clear homologues in the *M. tuberculosis* genome (Table 1). Despite numerous attempts, we were unable to amplify the DNA flanking the Tn611 insertion in mutant 412A.

DISCUSSION

This report is, to our knowledge, the first description of the isolation of mycobacterial mutants impaired in stationary-phase survival. We used a mutant screen that

looked directly for a loss of viability following 40 d incubation in stationary phase. This strategy was originally used by Tormo *et al.* (1990) to isolate *sur* mutants of *E. coli* and subsequently, in a modified form, to isolate stationary-phase survival mutants of *Sinorhizobium meliloti* (Uhde *et al.*, 1997). Using this method we screened 600 *M. smegmatis* Tn611 mutants and identified 21 that were putative stationary-phase survival mutants, of which we selected 7 for further study. The alleles disrupted in these mutants almost certainly represent only a fraction of those with roles in the stationary-phase survival of *M. smegmatis*. Indeed, using two-dimensional gel electrophoresis analysis of *M. smegmatis*, we have found more than 400 proteins that are up-regulated in early stationary phase (M. J. Smeulders, J. Keer & H. D. Williams, unpublished results).

All the mutants survived significantly less well in stationary phase following growth in rich medium than under carbon-starved conditions. In Lab-lemco medium an initial decrease in viability, during the first 40 d of stationary phase, was followed by an increase in the viable count to levels approaching those of the wild-type culture after > 100 d in stationary phase (Figs 2 and 4). A similar observation has been described previously for survival mutants of *E. coli* and *S. meliloti* (Tormo *et al.*, 1990; Uhde *et al.*, 1997). In the case of *E. coli*, this was shown to result from the accumulation of second-site suppressor mutations that allowed the mutants to survive stationary phase or resume growth (Tormo *et al.*, 1990). What is the cause of the recovery in mutant viable counts? There are no terminators to isolate Tn611 from surrounding transcription, following its replicative transposition into the chromosome. IS6100 is the only known prokaryotic transposable element that has no means of protecting itself from external transcriptional activation (Smith & Dyson, 1995). So it is possible for the transposon to move to a new locus. However, the experiments with mutants 3910D and 412A clearly showed that the transposon did not move during 120 d of stationary-phase incubation (Fig. 2).

Recovery of viable cell counts may result from the accumulation of secondary mutations that alleviate the original phenotype in some cells of the population. Important in this context is our previous demonstration of the dynamic nature of stationary-phase *M. smegmatis* cultures (Smeulders *et al.*, 1999). Variants emerge in stationary phase that have a growth or survival advantage over exponential-phase-adapted cultures (Smeulders *et al.*, 1999; Zambrano & Kolter, 1993; Zambrano *et al.*, 1996). This recovery of viability could be due exclusively to suppression followed by cryptic growth, but it is not clear whether sufficient nutrients remain or are released by dying cells to allow this to occur. An alternative explanation is that the recovery in colony counts observed does not represent growth but is due to an initial loss and subsequent recovery of plating efficiency. In this case the initial loss of viable counts would not be due to cell death but to a transient loss of the ability of the cells to form colonies which is reversed,

due to changes in either the culture or the intracellular environment, during prolonged stationary phase. It is certainly possible that mutations which are harmful early in stationary phase are not a problem as the environment changes in the ageing culture, allowing cells already present to recover colony-forming ability or a small number of surviving cells to regrow. However, support for the growth of suppressor strains comes from the return to wild-type colony morphology that accompanies the recovery of 3910D and 412A plate counts during stationary phase, and from the finding that after serial subculturing, allowing growth to go into stationary phase, mutants do not have the survival defect on retesting. Recovery in colony counts is not as apparent during carbon starvation, although in most mutants the rate of loss in viability decreases appreciably with prolonged stationary-phase incubation.

Cloning and DNA sequencing identified the Tn611-mutated alleles of five of the mutant strains. An important finding is that each of the genes disrupted has a clear homologue in the *M. tuberculosis* H37Rv genome sequence (Cole *et al.*, 1998). This raises the question of whether these homologous genes are also important for the stationary-phase survival of *M. tuberculosis*. However, experiments have not been performed to rule out the possibility that the impairment of stationary-phase survival in these mutants is a consequence of polar effects on neighbouring genes.

The identification of the Tn611-disrupted gene in the mutant 272A as *bioA* indicates that biotin, and therefore an enzyme(s) containing a biotin prosthetic group, is important for *M. smegmatis* to survive in or recover from stationary phase. This finding is consistent with the auxotrophic nature of 272A and its complementation with a biotin-containing vitamin pool. It is intriguing that the mutant survives poorly in rich medium in stationary phase, although this medium clearly does provide sufficient biotin for growth. It may be that *de novo* biosynthesis of biotin is required in stationary phase, perhaps due to an inability of stationary-phase cells to take up biotin from the medium.

272E was mutated in a gene homologous to polyketide synthase genes of *M. tuberculosis*. *M. tuberculosis* has 15 polyketide synthase genes, whose exact functions are not known (Cole *et al.*, 1998); however, one characterized polyketide synthase is the mycocerosic acid synthase of *M. bovis* BCG, which has a role in the synthesis of cell wall lipids (Azad *et al.*, 1996; Kolattukudy *et al.*, 1997).

Mutant 317C was disrupted in a gene homologous to a *M. tuberculosis* penicillin-binding protein (PBP). PBPs are needed for peptidoglycan cross-linking and cell wall biosynthesis. The apparent role of a PBP in stationary-phase survival of *M. smegmatis* is consistent with the dynamic nature of stationary-phase cultures and the appearance of new variants, which presumably requires growth and cell division (Smeulders *et al.*, 1999). Several genes, including *bolA* and *ftsQAZ* (Ballesteros *et al.*, 1998; Bohannon *et al.*, 1991; Sitnikov *et al.*, 1996), that

are involved in the determination of cell shape and structure in *E. coli* are regulated by the stationary-phase sigma factor RpoS. It has recently been shown that *E. coli* strains lacking both PBP3 and RpoS have a stationary-phase survival defect similar to the defect in *surA rpoS* double mutants. SurA is a foldase that is thought to be important for the proper assembly of the cell-wall-synthesizing apparatus (Lazar *et al.*, 1998), indicating that this apparatus is important in the stationary-phase survival of *E. coli*. In this context, it is interesting that a considerable rise in PBP6 levels has been found in stationary-phase versus exponential cultures of *E. coli* and this, together with a fall in the levels of PBP3 upon entry into stationary phase, is dependent on RpoS (Buchanan & Sowell, 1982; Dougherty & Pucci, 1994; Glauner & Hölte, 1990). A specific role for PBP6 in the stabilization of peptidoglycan during stationary phase has been suggested (Van der Linden *et al.*, 1992).

Given the colony morphology phenotype of 3910D it is interesting that this mutant is disrupted in a putative membrane protein homologous to Rv0658c but its function is not known. The possible function of the protein homologous to flavin-containing monoamine oxidase disrupted in 492A is also unclear at present.

In this study we have identified a number of genes with roles in the survival of *M. smegmatis* under O₂-sufficient conditions in carbon-starved stationary phase, each of which has a homologue in *M. tuberculosis*. Further studies are in progress to examine the role of these genes in O₂-starved stationary phase, an alternative *in vitro* model for mycobacterial persistence (Wayne & Hayes, 1996).

ACKNOWLEDGEMENTS

This work was funded by the Wellcome Trust, via a Wellcome Prize Fellowship to M. J. S. and grants to H. D. W. We are very grateful to Douglas Young for his support at the start of this project.

REFERENCES

- Azad, A. K., Sirakova, T. D., Rogers, L. M. & Kolattukudy, P. E. (1996). Targeted replacement of the mycocerosic acid synthase gene in *Mycobacterium bovis* BCG produces a mutant that lacks mycosides. *Proc Natl Acad Sci USA* **93**, 4787–4792.
- Ballesteros, M., Kusano, S., Ishihama, A. & Vicente, M. (1998). The *ftsQ1p* gearbox promoter of *Escherichia coli* is a major sigma S-dependent promoter in the *ddlB-ftsA* region. *Mol Microbiol* **30**, 419–430.
- Barer, M. R. (1997). Viable but non-culturable and dormant bacteria: time to resolve an oxymoron and a misnomer? *J Med Microbiol* **46**, 629–631.
- Beggs, M. L., Cave, M. D. & Eisenach, K. D. (1996). Isolation and sequence of a *Mycobacterium tuberculosis* sigma factor. *Gene* **174**, 285–287.
- Bohannon, D. E., Connell, N., Keener, J., Tormo, A., Espinosaurgel, M., Zambrano, M. M. & Kolter, R. (1991). Stationary-phase-inducible gearbox promoters – differential

- effects of *katF* mutations and role of sigma-70. *J Bacteriol* **173**, 4482–4492.
- Buchanan, C. E. & Sowell, M. O. (1982). Synthesis of penicillin-binding protein-6 by stationary-phase *Escherichia coli*. *J Bacteriol* **151**, 491–494.
- Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–560.
- Demaio, J., Zhang, Y., Ko, C., Young, D. B. & Bishai, W. R. (1996). A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **93**, 2790–2794.
- Demaio, J., Zhang, Y., Ko, C. & Bishai, W. (1997). *Mycobacterium tuberculosis sigF* is part of a gene cluster with similarities to the *Bacillus subtilis sigF* and *sigB* operons. *Tubercle Lung Dis* **78**, 3–12.
- Dougherty, T. J. & Pucci, M. J. (1994). Penicillin-binding proteins are regulated by RpoS during transitions in growth states of *Escherichia coli*. *Antimicrob Agents Chemother* **38**, 205–210.
- Doukhan, L., Predich, M., Nair, G., Dussurget, O., Mandicmulec, I., Cole, S. T. & Smith, I. (1995). Genomic organization of the mycobacterial sigma-gene-cluster. *Gene* **165**, 67–70.
- Errington, J. (1993). *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol Rev* **57**, 1–33.
- Glauner, B. & Hölte, J. V. (1990). Growth-pattern of the murein sacculus of *Escherichia coli*. *J Biol Chem* **265**, 18988–18996.
- Groat, R. G., Schultz, J. E., Zychlinsky, E., Bockman, A. & Matin, A. (1986). Starvation proteins in *Escherichia coli* – kinetics of synthesis and role in starvation survival. *J Bacteriol* **168**, 486–493.
- Guilhot, C., Otal, I., Vanrompaey, I., Martin, C. & Gicquel, B. (1994). Efficient transposition in mycobacteria – construction of *Mycobacterium smegmatis* insertional mutant libraries. *J Bacteriol* **176**, 535–539.
- Hartmans, S. & De Bont, J. A. M. (1992). The genus *Mycobacterium* – nonmedical. In *The Prokaryotes*, 2nd edn, vol. II, pp. 1215–1237. Edited by A. Balows and others. New York: Springer.
- Hengge-Aronis, R. (1993). The role of *rpoS* in early stationary phase gene regulation in *Escherichia coli* K12. In *Starvation in Bacteria*, pp. 171–200. Edited by S. Kjelleberg. New York: Plenum.
- Hu, Y. M. & Coates, A. R. M. (1999a). Transcription of two sigma 70 homologue genes, SigA and SigB, in stationary-phase *Mycobacterium tuberculosis*. *J Bacteriol* **181**, 469–476.
- Hu, Y. M. & Coates, A. R. M. (1999b). Transcription of the stationary-phase-associated *hspX* gene of *Mycobacterium tuberculosis* is inversely related to synthesis of the 16-kilodalton protein. *J Bacteriol* **181**, 1380–1387.
- Hu, Y. M., Butcher, P. D., Mangan, J. A., Rajandream, M. A. & Coates, A. R. M. (1999). Regulation of *hmp* gene transcription in *Mycobacterium tuberculosis*: effects of oxygen limitation and nitrosative and oxidative stress. *J Bacteriol* **181**, 3486–3493.
- Hutter, B. & Dick, T. (1998). Increased alanine dehydrogenase activity during dormancy in *Mycobacterium smegmatis*. *FEMS Microbiol Lett* **167**, 7–11.
- Kaprelyants, A. S. & Kell, D. B. (1993). Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Appl Environ Microbiol* **59**, 3187–3196.
- Kjelleberg, S. (editor) (1993). *Starvation in Bacteria*. New York: Plenum.

- Kolattukudy, P. E., Fernandes, N. D., Azad, A. K., Fitzmaurice, A. M. & Sirakova, T. D. (1997). Biochemistry and molecular genetics of cell-wall lipid biosynthesis in mycobacteria. *Mol Microbiol* **24**, 263–270.
- Kolter, R., Siegele, D. A. & Tormo, A. (1993). The stationary phase of the bacterial life cycle. *Annu Rev Microbiol* **47**, 855–874.
- Lazar, S. W., Almiron, M., Tormo, A. & Kolter, R. (1998). Role of the *Escherichia coli* SurA protein in stationary-phase survival. *J Bacteriol* **180**, 5704–5711.
- Lee, B. H., Murugasu-Oei, B. & Dick, T. (1998). Upregulation of a histone-like protein in dormant *Mycobacterium smegmatis*. *Mol Gen Genet* **260**, 475–479.
- Martin, C., Timm, J., Rauzier, J., Gomez-Lus, R., Davies, J. & Gicquel, B. (1990). Transposition of an antibiotic resistance element in mycobacteria. *Nature* **345**, 739–743.
- Michele, T. M., Ko, C. & Bishai, W. R. (1999). Exposure to antibiotics induces expression of the *Mycobacterium tuberculosis* *sigF* gene: implications for chemotherapy against mycobacterial persisters. *Antimicrob Agents Chemother* **43**, 218–225.
- Nystrom, T. (1999). Starvation, cessation of growth and bacterial aging. *Curr Opin Microbiol* **2**, 214–219.
- Parrish, N. M., Dick, J. D. & Bishai, W. R. (1998). Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol* **6**, 107–112.
- Predich, M., Doukhan, L., Nair, G. & Smith, I. (1995). Characterization of RNA-polymerase and 2 sigma-factor genes from *Mycobacterium smegmatis*. *Mol Microbiol* **15**, 355–366.
- Prod'hom, G., Guilhot, C., Gutierrez, M. C., Varnerot, A., Gicquel, B. & Vincent, V. (1997). Rapid discrimination of *Mycobacterium tuberculosis* complex strains by ligation-mediated PCR fingerprint analysis. *J Clin Microbiol* **35**, 3331–3334.
- Prod'hom, G., Lagier, B., Pelicic, V., Hance, A. J., Gicquel, B. & Guilhot, C. (1998). A reliable amplification technique for the characterization of genomic DNA sequences flanking insertion sequences. *FEMS Microbiol Lett* **158**, 75–81.
- Raviglione, M. C., Snider, D. & Kochi, A. (1995). Global epidemiology of tuberculosis. *JAMA (J Am Med Assoc)* **273**, 220–226.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Siegele, D. A. & Kolter, R. (1992). Life after log. *J Bacteriol* **174**, 345–348.
- Sitnikov, D. M., Schineller, J. B. & Baldwin, T. O. (1996). Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQZ* involves both *rpoS* and *sdiA*-mediated autoinduction. *Proc Natl Acad Sci USA* **93**, 336–341.
- Smeulders, M. J., Keer, J., Speight, R. A. & Williams, H. D. (1999). Adaptation of *Mycobacterium smegmatis* to stationary phase. *J Bacteriol* **181**, 270–283.
- Smith, B. & Dyson, P. (1995). Inducible transposition in *Streptomyces lividans* of insertion sequence IS6100 from *Mycobacterium fortuitum*. *Mol Microbiol* **18**, 933–941.
- Spector, M. P. & Cubitt, C. L. (1992). Starvation-inducible loci of *Salmonella typhimurium* – regulation and roles in starvation-survival. *Mol Microbiol* **6**, 1467–1476.
- Tormo, A., Almiron, M. & Kolter, R. (1990). *surA*, an *Escherichia coli* gene essential for survival in stationary phase. *J Bacteriol* **172**, 4339–4347.
- Uhde, C., Schmidt, R., Jording, D., Selbitschka, W. & Puhler, A. (1997). Stationary-phase mutants of *Sinorhizobium meliloti* are impaired in stationary-phase survival or in recovery to logarithmic growth. *J Bacteriol* **179**, 6432–6440.
- Van der Linden, M. P. G., de Haan, L., Hoyer, M. A. & Keck, W. (1992). Possible role of *Escherichia coli* penicillin-binding protein-6 in stabilization of stationary-phase peptidoglycan. *J Bacteriol* **174**, 7572–7578.
- Wayne, L. G. (1994). Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur J Clin Microbiol Infect Dis* **13**, 908–914.
- Wayne, L. G. & Hayes, L. G. (1996). An *in vitro* model for sequential study of shutdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun* **64**, 2062–2069.
- Wayne, L. G. & Lin, K.-Y. (1982). Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. *Infect Immun* **37**, 1042–1049.
- Yuan, Y., Crane, D. D. & Barry, C. E., III (1996). Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. *J Bacteriol* **178**, 4484–4492.
- Zambrano, M. M. & Kolter, R. (1996). Gasping for life in stationary phase. *Cell* **86**, 181–184.
- Zambrano, M. M., Siegele, D. A., Almiron, M., Tormo, A. & Kolter, R. (1993). Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259**, 1757–1760.

Received 3 March 2000; revised 16 May 2000; accepted 30 May 2000.