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

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

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 & Bell, 1993; Wayne, 1994). An alternative possibility is...
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 (Demiao 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 O$_2$-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 x-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 mc$	extsuperscript{2}$155 was grown as described previously either in rich Lab-lemco medium or a carbon-limited minimal medium [Hartmans–de Bont (HdB) medium containing 0.02% glycerol (Hartmans & De Bont, 1992; Smeulders et al., 1999)]. M. smegmatis cultures were grown with shaking at 200 r.p.m. at 37 °C, except for initial selection of pCG79 transformants, when incubation was at 30 °C to permit replication of the temperature-sensitive plasmid, and during the procedure to eliminate the plasmid, when cultures were incubated at 41 °C. E. coli cultures were grown in Luria broth at 37 °C. When required, kanamycin was added to a final concentration of 20 µg ml$^{-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 mc$	extsuperscript{2}$155 and transformants selected on Lab-lemco with kanamycin at 30 °C. Transformants were eluted from plates, diluted to about 5 × 10$^5$ 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 into duplicate wells containing carbon-limited minimal medium (HdB medium with 0.02% glycerol). The microtiter 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 each of 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 PstI; the
fragments were separated by agarose gel electrophoresis and
probed with the radiolabelled 640 bp HindIII–PstI 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’hom et al. (1997, 1998). Briefly,
genomic DNA isolated from mutant strains was digested with
SalI and then ligated to an asymmetric, non-phosphorylated,
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 SalI 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 se-
quence 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 PstI digests of
chromosomal DNA isolated from 3910D at the start of
the experiment and after 120 d in stationary phase (Fig.
2c). Hybridization of PstI 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 ap-
pearance 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 ac-
panied 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.](image-url)
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 *PstI–HindIII* fragment containing the repeat element of the transposon with *PstI* 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) in to stationary phase in Lab-lemco medium. 412A was plated from cultures 5 d (e), 12 d (f) and 120 d (g) into 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,
Stationary-phase mutants of *M. smegmatis*

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 $6 \times 10^8$ 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-

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**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.
Fig. 5. Long-term viability in carbon-starved stationary phase of *M. smegmatis* mc^2^155 (□) 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-lenco 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: mc^2^155, 2 × 10^8^; 173D, 3 × 10^8^; 492A, 7 × 10^8^; 3910D, 6 × 10^8^; 412A, 2 × 10^8^; 272E, 8 × 10^8^; 272A, 8 × 10^8^; 228H, 4.5 × 10^8^; 317C, 5 × 10^6^.

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

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

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).

allowed 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’hom 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 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; Simikov 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 & Höltinge, 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 Ry0658c 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).

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**Stationary-phase mutants of M. smegmatis**


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