Studies in metabolic defects of BCG strains

by

Laura Carranza

Department of Microbiology and Immunology

McGill University

Montreal, Quebec

Canada

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Abstract

Mycobaterium bovis Bacille Calmete-Guerin (BCG) strains are live attenuated vaccines given to prevent tuberculosis. The nature of their attenuation has long been obscure. Recent studies suggest that BCG vaccines are metabolic mutants and antigen mutants, proposed to result in bacteria with decreased persistence and immunogenicity in the host. One specific metabolic defect is that BCG strains are unable to use certain amino acids as nitrogen sources, and paradoxically, that adding L-serine to the culture medium inhibits growth. To explore this further, we studied the growth of BCG strains in different concentrations of serine (both *in vivo* and *in vitro*), screened several candidate genes for mutations and studied the transcription of *sdaA* and *glnA1* genes (encoding for serine deaminase and glutamine synthetase respectively) in the presence or absence of serine. We found that growth inhibition caused by serine could be reversed by over-expressing *sdaA* or *glnA1* via plasmid complementation and by supplementing growth media with glutamine or alpha-ketoglutarate. However, we were unable to detect any genetic mutations that might explain these findings. Consequently, we postulated that BCG strains fail to adequately induce transcription of otherwise genetically-intact *sdaA* and *glnA1* genes in response to serine-induced blockade of glutamine synthetase. To explore the cause of this deficient response, we turned to *Rv3676*, predicted to encode the cyclic AMP receptor protein, for which a number of mutations have been described in BCG strains. We noted that *M. tuberculosis* can utilize glucose, unlike BCG strains, but that BCG strains complemented with wild-type *Rv3676* are able to consume glucose *in vitro*. Additionally, plasmid complementation of BCG strains with *Rv3676* reversed the growth inhibition caused by serine. Together, these results suggest that mutations in the *Rv3676* global regulator gene in BCG strains have resulted in impaired glucose utilization by BCG strains, indirectly impairing sensing of nitrogen status, as measured by serine-associated growth inhibition. The potential implications of this series of metabolic defects for virulence and persistence of the organism in the vaccinated host are discussed. Concretely, we hypothesized that BCG's growth defect in high serine is related to flaws in nitrogen metabolism, or its regulation.

Résumé

Les souches de *Mycobacterium bovis* Bacille Calmete-Guerin (BCG) sont des vaccins vivants atténués qui sont donnés pour prévenir la tuberculose. La nature de leur atténuation a longtemps été obscure. Des études récentes proposent que les vaccins BCG soient des mutants métaboliques et des mutants antigèniques, dont la persistence et l'immunogénicité seraient diminuées chez l'hôte. Un défaut spécifique du métabolisme des souches de BCG reside dans leur incapacité á utiliser certains acides aminés comme sources de carbone, et paradoxalement, l'ajout de L-sérine au bouillon de culture interdit la croissance de ces souches. Pour explorer cette observation, nous avons étudié la croissance de souches BCG en présence de différentes concentrations de serine (dans des conditions *in vivo* et *in vitro*), avons analysé plusieurs gènes en vue d'identifier des candidats porteurs de mutations, et finalement avons étudié la transcription des gènes *sdaA* et *glnA1* (codant pour la sérine déaminase et la glutamine synthétase, respectivement) tant en présence que dans l'absence de sérine. Nos résultats démontrent que l'inhibition de croissance causée par l'ajout de la sérine peut être renversée par une sur-expression de *sdaA* ou *glnA1* (résultats de complémentation par plasmide), ainsi que par l'addition de glutamine ou d'alpha-ketoglutarate dans le milieu de culture. Cependant, des mutations génétiques qui pourraient expliquer ces trouvailles n'ont pas été detectées. Par conséquent, nous avons postulé que les souches de BCG n'induisent pas une transcription adéquate des gènes *sdaA* et *glnA1*, autrement génétiquement intacts, en réponse au blocage de la glutamine synthétase provoqué par la sérine. Pour explorer la cause de cette réponse inadéquate, nous nous sommes tournés vers *Rv3676*, un gène qui coderait pour la synthèse d'une protéine de récepteur AMP-cyclique, et pour lequel un certain nombre de mutations ont été décrits dans les souches de BCG. Nous avons constaté que le *M. tuberculosis* est capable d'utiliser le glucose, contrairement aux souches de BCG, mais que par contre les souches de BCG complémentées avec *Rv3676* (type sauvage) ont la capacité d'utiliser le glucose *in vitro*. De plus, la complémentation par plasmide de souches BCG avec *Rv3676* permet de renverser le défaut de croissance observé en présence de sérine. Ensemble, ces résultats nous suggèrent que les mutations du gène régulateur global *Rv3676* survenues dans les souches BCG aient altérés la capacité de celles-ci á utiliser le glucose, ceci ayant comme effet indirect de diminuer leur potentiel á percevoir le statut en azote tel que mesuré par l'inhibition de croissance associé á la sérine. Les implications potentielles de ces défauts du métabolisme pour la virulence et la persistence de l'organisme dans l'hôte vacciné sont discutées. Concrètement, notre hypothèse soutient que le défaut de croissance du BCG en présence de concentrations élevées de sérine est lié au métabolisme de l'azote, ou bien à sa régulation.

To my beloved son Marco Andrés

who makes it all worth while .....

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Introduction

Bacille Calmete-Guerin (BCG) vaccines are currently provided in much of the world as the only available vaccine against TB, but their efficacy is the subject of ongoing debate (Behr, 2002). BCG is not a single vaccine but a group of live attenuated vaccines that were derived from the strain prepared at the Pasteur institute in 1908 using *Mycobacterium bovis*. The different vaccines obtained thereafter have lost several regions of genomic material over the continuous passages in the different laboratories around the world (Behr, 2002). Through genetic studies of existing BCG strains, it has been possible to catalogue a number of genomic differences between them, indicating that the evolution of BCG vaccines has favoured the elimination of antigenic proteins and regulatory genes (Behr, 2001;Mostowy *et al.*, 2003). The relevance of these mutations in terms of survival and replication of BCG strains in the host and their resulting protective efficacy is the subject of ongoing investigation (Behr *et al.*, 2000;Belley *et al.*, 2004).

An important attribute for bacterial survival in the host is its capacity to adapt to varying levels of available nutrients. In a recent *in vitro* study it was shown that BCG strains differ in their ability to grow in the presence of certain amino acids as the predominant source of nitrogen. Moreover, beyond demonstrating growth differences in the presence of a sole amino acid, it was also shown that higher concentrations of certain amino acids, specifically serine, actually inhibit BCG replication (Chen *et al.*, 2003). Since serine is known to compete for the active site of glutamine synthetase (GS) (Liaw, Pan, and Eisenberg, 1993) and GS is an essential enzyme for *M. tuberculosis* growth (Harth and Horwitz, 2003) with a central role in intracellular nitrogen status sensing and

regulation (Burkovski, 2003), we hypothesized that BCG's growth defect in high serine is related to flaws in nitrogen metabolism, or its regulation.

In order to further determine how serine inhibits the growth of various BCG strains, different approaches were undertaken. We studied the growth of these strains in different concentrations of serine both *in vivo* (cell culture assays) and *in vitro*, screened several candidate genes relevant to nitrogen metabolism for mutations, and studied the expression of *sdaA* and *glnA1* genes (encoding for serine deaminase and glutamine synthetase respectively) in the presence or absence of serine. We also searched for strategies that would reverse the growth inhibition caused by serine by plasmid complementation with *sdaA*, *glnA1* or *Rv3676* genes from *M. tuberculosis* and media supplementation with glutamine and alpha-ketoglutarate. We evaluated the capacity of BCG strains to utilize certain carbon sources like glucose, to explore a potential link between mutations in *Rv3676*, carbohydrate utilization and nitrogen sensing. Finally we discuss our results in the context of nitrogen and carbohydrate metabolism in mycobacteria.

Literature review

I.- Tuberculosis

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis*. *M. tuberculosis* is an intracellular pathogen that resides mainly in macrophages. In order for an infection with mycobacteria to be cleared, mycobacteria need to be sequestered by macrophages (phagocytosed) and later killed by the fusion of the phagosome with the lysosome (containing several anti-bacterial molecules). However, it is well know that *M. tuberculosis*, and other mycobacteria, can arrest phagosomal maturation and prevent phago-lysosomal fusion. Moreover it has been documented that these bacteria can inhibit processing and presentation of antigens to other cells of the immune system. Therefore mycobacteria are able to subvert the immune defences of the host, persist and eventually cause disease (for a review see Hingley-Wilson, Sambandamurthy, and Jacobs, Jr., 2003).

M. tuberculosis is spread mainly through coughing and sneezing. Infected people who develop the disease present primarily respiratory symptoms. It is thought that a person with active TB will infect between 10 and 15 other people per year. To treat these patients and prevent further spread, the World Health Organization (WHO) recommends the DOTS program (Directly Observed Therapy Short-course) as an international strategy to treat active TB. This approach involves direct observation while patients take each dose of anti-TB treatment. The most frequently administered drugs are isoniazid,

rifampicin, pyrazinamide, streptomycin and ethambutol (http://www.who.int/tb/en/). However, because of limitations in this programme, approximately one third of the world's population is asymptomatically infected with *M. tuberculosis*, a state known as "latent infection".

The three major factors that complicate treatment, control and prevention of tuberculosis world wide are the emergence of drug resistant *M. tuberculosis* strains, co-infection with HIV (human immunedeficiency virus), and the lack of an effective vaccine. Isolates of *M. tuberculosis* that are resistant to at least one anti-TB drug have emerged in most countries surveyed by the WHO. Moreover, strains that are resistant to all first-line anti-TB drugs have been reported. Co-infection of *M. tuberculosis* and HIV results in accelerated progression of both diseases. TB is the major cause of death in AIDS (acquired immune deficiency syndrome) patients (13% of deaths worldwide) (http://www.who.int/tb/en/). Prevention of TB would be preferable to treatment, but currently, there is no vaccine that can consistently protect adults from being infected by *M. tuberculosis* or developing pulmonary TB (details below).

II.- BCG and other TB vaccines

Bacille Calmette-Guerin (BCG) strains are the only available vaccines against TB. These vaccines were derived from a strain of *Mycobacterium bovis* at the Pasteur Institute. The preparation of an attenuated vaccine started at the beginning of the 20th century and continued for 13 years. During this time, the strain was grown in glycerine potato bile media, and passaged to fresh media every two to three weeks. Eventually, an attenuated strain appeared that was much less virulent than the progenitor. In 1921

vaccination to prevent TB using BCG started, but it was not until 1961 that BCG-Pasteur was lyophilised. Therefore the *in vitro* conditions that favoured the original attenuation of virulence remained in place further forty years. In 1924 the Pasteur Institute started to distribute aliquots of the vaccine to different laboratories so that the vaccine could be produced locally in different regions of the world. By the time that lyophilisation was available at least 13 different strains had been created. Anecdotal evidence suggested there were differences among these daughter strains, perhaps relating to divergent evolution of these bacteria in the different laboratories. This has since been confirmed by modern molecular analyses. Genetic changes, including genomic deletions (Behr *et al.*, 1999;Mahairas *et al.*, 1996) duplications (Brosch *et al.*, 2000) and single nucleotide polymorphisms (SNPs) (Behr *et al.*, 2000;Spreadbury *et al.*, 2005) have now been documented (for a review see (Behr, 2002). Each BCG strain has a unique pattern of genomic deletions, or "regions of difference" (RD), distinct from the original BCG of 1921. Recent genomic characterization of BCG strains has allowed the reconstruction of BCG history in what has been called the "BCG-phylogeny" (figure 1LR) (Bedwell *et al.*, 2001;Behr, 2002;Behr *et al.*, 1999;Behr and Small, 1999;Behr and Small, 1997;Behr, 2001). Although the consequences of these genetic changes in terms of vaccine performance and bacterial physiology in general are not fully understood, some of them have been related to certain phenotypes. The best documented is the deletion of region of difference 1 (RD1), a genomic element absent from all BCGs and shown to be an important source of attenuation of the vaccine (Mahairas *et al.*, 1996;Lewis *et al.*, 2003). In addition, a large number of regulatory elements have been lost during the evolution of BCG strains through the different genomic deletion events, and by a few point mutations (e.g. genes *Rv3676* and at the starting codon of *sigK*) (Behr *et al.*, 1999;Charlet *et al.*,

2005;Brosch *et al.*, 2000;Lewis *et al.*, 2003;Mostowy *et al.*, 2003;Spreadbury *et al.*, 2005).

The efficacy of BCG vaccines and the state of current TB-vaccine research has been extensively reviewed (for recent reviews see (Andersen and Doherty, 2005;Reed and Lobet, 2005;Orme, 2005;Brennan, 2005;Brennan, Morris, and Sizemore, 2004;Behr, 2002). The overall consensus is that a more effective vaccine is needed. The collective data seems to indicate that BCG effectively protects against TB manifestations in children, but that protection in adults is variable (0-80% efficacy). There are a variety of candidate vaccines (recombinant BCG strains, attenuated *M. tuberculosis* strains, DNA based, and subunit vaccines) presently being tested in animal models and pre-clinical trials. A few of these are currently in or near clinical trials. For example "rBCG30"(Horwitz and Harth, 2003;Horwitz *et al.*, 2000) is a live recombinant BCG that overexpresses antigen-85 (30KD) from *M. tuberculosis*, and is currently in phase I clinical trials.

In general vaccines developed for human use are tested to assure that they are: safe (not harmful when appropriately administrated), pure (the finish product is free from undesired foreign matter) and potent (the efficacy in producing a specific predicted result) (Brennan, Morris, and Sizemore, 2004). Any candidate TB vaccine that will move forward from clinical trial to routine use (in TB endemic countries) will face a number of different challenges; from manufacturing, regulatory, and general infrastructure issues (that may vary from one country to another), to administration strategies. A few examples of the latter factor are: number of doses, target ages of vaccination and pertinence of vaccination (e.g. safety, efficacy) in susceptible groups (like HIV positive and AIDS

patients, active and latent TB patients, and previously BCG vaccinated individuals) (reviewed in (Brennan, Morris, and Sizemore, 2004). However, beyond these practical issues, the success of any possible new TB vaccine is dependent on new research that will increase the current knowledge on how a TB vaccine should confer protection.

Particularly, as some of the leading candidate vaccines are BCG recombinants, understanding what features of the BCG strains need to be improved, will possibly aid in the achievement of the safety, potency and purity desired in the new TB vaccine.

III.- Bacterial metabolism, Serine deaminase and the Citric Acid Cycle

An important attribute of virulent mycobacteria and attenuated BCG vaccine strains is their capacity to survive in the host. Survival is an essential part of the disease process for virulent strains, and is a necessary part of presenting antigens to the host immune system in the otherwise aborted infection process generated by a live, attenuated vaccine. Survival in the host requires a number of strategies that subvert the immune response of the host, but also, more practically, bacterial strategies to acquire and utilize necessary nutrients. For this purpose, the citric acid cycle (CAC) serves as the critical fuel cell of the bacterium (Figure 2LR). Accordingly, mutagenesis screens seeking the list of genes essential to the growth of *M. tuberculosis in vitro* have found that genes annotated as serving the CAC are essential for normal growth (Sassetti, Boyd, and Rubin, 2003).

Serine deaminase (SdaA) is an enzyme that catalyzes the degradation of serine into ammonia and pyruvate. Although the serine deaminase gene (*sdaA*) per se does not seem to be essential in *M. tuberculosis* (Sassetti and Rubin, 2003;Sassetti, Boyd, and Rubin,

2003), the products of serine degradation are important in both nitrogen metabolism (ammonia) and carbohydrate metabolism (pyruvate). Pyruvate from serine degradation along with pyruvate from other carbon sources (e.g. other amino acids and sugars) can be either used for biosynthesis, or to obtain energy.

To enter the CAC, pyruvate is converted into acetyl-CoA (a derivative of coenzyme A). Sequentially acetyl-CoA is combined with a four-carbon precursor called oxaloacetate, to form a six-carbon intermediate (citrate). Six subsequent reactions then release the two added carbons as carbon dioxide and the oxaloacetate is regenerated (hence the cycle can commence again). In these series of reactions eight intermediates are formed: citrate, isocitrate, alpha-ketoglutarate, succinyl-CoA, succinate, fumarate, malate and oxaglutarate (Figure 2LR). Alpha-ketoglutarate, succinyl-CoA and oxaloacetate are also important precursor metabolites for a number of biological reactions including biosynthesis. Each cycle through the CAC produces one molecule of ATP (adenosine triphosphate) (a major form of cellular energy). The cycle also delivers two molecules of NADH (reduced form of nicotinamide adenine dinucleotide), one molecule of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) and one of $FADH_2$ (reduced form of flavine adenine dinucleotide) all of which provide the cell reducing power that can convert ADP (adenosine diphosphate) into ATP.

A deviation of the CAC, called the glyoxylate shunt (figure 2LR), allows bacteria to metabolize acetate or long fatty acids as carbon sources. The first step in the pathway converts isocitrate into glyoxylate and succinate by the action of isocitrate lyase (ICL), and subsequently transforms glyoxylate into malate trough malate synthase (MS). The glyoxylate shunt bypasses the synthesis of alpha-ketoglutarate and succinyl-CoA. Interestingly, the enzymes isocitrate lyase and malate synthase do not exist in humans;

therefore the glyoxylate shunt is frequently acknowledged as a target for antibacterial agents, including treatment of TB. This is a frequent topic in tuberculosis treatment research (for a review see Smith, Sharma, and Sacchettini, 2004).

In general, bacteria favour the glyoxylate shunt when oxygen is limited. In *M. tuberculosis* a series of genes involved in hypoxic metabolism seem to be relevant during the latent phase of infection. In *M. tuberculosis* transcription of the isocitrate lyase gene (*icl* or *Rv0464*), is significantly induced after cultivation in macrophages and mice (Schnappinger *et al.*, 2003). Additionally, ICL is essential for persistence of *M. tuberculosis* in macrophages and mice (McKinney *et al.*, 2000). Recently it was demonstrated that, there are two genes encoding for isocitrate lyase (*icl1* and *icl2*) in *M. tuberculosis*, and they are jointly required for growth *in vivo* and virulence (Munoz-Elias and McKinney, 2005).

Although it was initially thought that *M. tuberculosis* had all enzymes in the CAC (Huynen, Dandekar, and Bork, 1999), it was recently shown that alpha-ketoglutarate dehydrogenase (KDH) is missing (Tian *et al.*, 2005;Tian *et al.*, 2005). KDH converts alpha-ketoglutarate into succinyl-CoA thought the steps of the CAC that are alternative to the glyoxylate shunt (Figure 2LR). Therefore it is though that the absence of KDH is further evidence for the importance of the glyoxylate shunt as an adaptation to low oxygen conditions. It is also hypothesized that alpha-ketoglutarate is transformed into succinyl-CoA via an alternative pathway in *M. tuberculosis*.

IV.- Glutamine synthetase, alpha-ketoglutarate and the nitrogen metabolism

A second important role of Serine deaminase is that serine can block the activity of Glutamine synthetase (GS). GS converts glutamate into glutamine and is a central molecule in nitrogen metabolism (for a review see Burkovski, 2003). Excess serine blocks the active site of GS and inhibits its activity, while serine deaminase activity helps overcome this blockade by degrading serine. (Liaw, Pan, and Eisenberg, 1993).

The system for regulation of nitrogen metabolism has been well described in *Escherichia coli* (Jiang, Peliska, and Ninfa, 1998;Atkinson, Pattaramanon, and Ninfa, 2002;Ninfa and Jiang, 2005;Atkinson *et al.*, 2002) (see Figure 3LR for a schematic summary). When there is high availability of ammonium in the outer media this metabolite diffuses freely through the cell membrane. Then, glutamate dehydrogenase (GDH) uses alpha-ketoglutarate to fix nitrogen in the form of glutamate consuming 1mol of NADPH per mol of ammonium converted. When ammonium is scarce in the bacterial environment, the AmtB transporter protein carries ammonium into the cell. GDH affinity for ammonium is lower than GS, therefore when ammonium concentrations are low (<1mM) nitrogen is stored in the form of glutamine by the action of GS. This reaction consumes 1mol of NADPH and 1mol of ATP per mol of ammonium that is stored. (1 ATP mol extra than in the high nitrogen case).

The shift of nitrogen fixation mechanisms in response to nitrogen availability is regulated at several points. Specifically the action of GS is regulated at the transcriptional and post-translational stages. A two component system (NtrB-NtrC) regulates nitrogen metabolism genes in *E. coli*. Glutamine and alpha-ketoglutarate levels indirectly affect nitrogen metabolism gene expression through interplay with a PII type protein (signal

transduction proteins widely distributed in bacteria). When intracellular levels of alpha-ketoglutarate are low PII interacts with NtrB leading to gene transcription repression by NtrC. Conversely, when concentrations of alpha-ketoglutarate are high, gene transcription of NtrC regulated genes is induced. Glutamine indirectly influences the activity of PII, by determining its uridylylation state. High intracellular glutamine inhibits PII uridylylation, indicating a nitrogen rich status, and is accompanied by base line gene expression of NtrC controlled genes. Uridylylated PII (PII-UMP) occurs when there are low levels of glutamine; it is a flag for poor nitrogen status of the cell and consequently starts a cascade of molecular events that lead to gene transcription. PII also influences the post translational level of control. Unmodified PII induces the ATase enzyme to adenylylate GS. Adenylylated GS is less active than its unmodified form; nevertheless a minimum basal activity is kept to meet the bacterium's requirements of glutamine. In the *E. coli* system both high levels of alpha-ketoglutarate and low concentrations of glutamine are required to fully activate the nitrogen fixation system.

While some lessons from *E. coli* work may pertain to mycobacteria, research on a more-closely related group, the corynebacteria, provides further opportunity to generate hypotheses about glutamine synthesis in mycobacteria. Nitrogen metabolism and regulation in *Corynebacterium glutamicum* has been comprehensively studied by the group of Burvoski and colleagues (Burkovski, 2003;Strosser *et al.*, 2004;Silberbach *et al.*, 2005;Beckers *et al.*, 2005) (see Figure 3LR). Although there are many similarities with *E. coli*, the AmtR global regulatory protein is a repressor and not an activator like NtrC. As a response to low intracellular nitrogen, PII interacts with AmtR releasing it from its target DNA (downstream of most N metabolism genes) and allowing gene transcription (Beckers *et al.*, 2005). As it will be developed later (see Discussion), the effects of the

interaction between alpha-ketoglutarate and PII in *C. glutamicum* have not been described in the literature yet.

The *M. tuberculosis* genome contains homologous sequences to most of the genes that encode nitrogen regulation molecules in the systems described for *E. coli* and *C. glutamicum* (such as *glnB, glnD, glnE, glnA, gltBD, amt, gdh*)(see table 4). Nevertheless, in *M. tuberculosis* there is no candidate sequence for the "global nitrogen regulator" (akin to *AmtR* in *C glutamicum* or NtrB-NtrC in *E coli*). Apart from *glnA1*, a few other genes related to nitrogen metabolism are relevant in *M. tuberculosis*. For example, *glnE* (encoding for ATase enzyme)(Parish and Stoker, 2000), and *gltB* (encoding glutamate synthasa) have shown to be essential *in vitro* (Sassetti, Boyd, and Rubin, 2003). The gene encoding for glutamate synthasa is upregulated in response to bactericidal concentrations of reactive nitrogen intermediates (RNIs) (Tian *et al.*, 2005;Tian *et al.*, 2005).

There are four genes with corroborated GS encoding function in the *M. tuberculosis* genome (*glnA1*, *glnA2*, *glnA3* and *glnA4*). However only *glnA1* is expressed abundantly and is essential for bacterial homeostasis (Harth *et al.*, 2005). Moreover, if *glnA1* is exclusively disrupted, *M. tuberculosis* becomes a glutamine auxotroph (Tullius, Harth, and Horwitz, 2003). The importance of GS in *M. tuberculosis* is extensively documented through the work of Horwitz and colleagues (Harth and Horwitz, 2003;Harth *et al.*, 2000;Harth and Horwitz, 1999;Horwitz and Harth, 2003;Tullius, Harth, and Horwitz, 2003;Harth, Clemens, and Horwitz, 1994). Inhibition of GS activity by L-methionine-SR-sulfoximine (MSO), decreased *M tuberculosis* pathogenicity and virulence in the guinea pig model (Harth and Horwitz, 2003). Additionally, the *glnA1* gene was shown to be essential for the growth of *M. tuberculosis* in human THP-1 macrophages and guinea pigs, attributed to the fact that *M. tuberculosis* in the host

phagosome has limited access to L-glutamine (Tullius, Harth, and Horwitz, 2003). Additionally it is thought that nitrogen regulation by GS may influence phagosomal pH and phagosome-lysosome fusion (Harth, Clemens, and Horwitz, 1994). GS activity is also required in *M. tuberculosis* for the formation of a cell wall component called poly-L-glutamate-glutamine (Harth *et al.*, 2000).

V.- Carbohydrate metabolism gene regulation and CRP

In bacteria most genes are clustered in arrangements called "operons", which constitute functional units of transcription and genetic regulation. The transcription of an operon can be blocked by a "repressor" protein or stimulated by an "activator" protein. A good example is the "lactose operon" (*lac* operon) in the widely studied bacterium *Escherichia coli* (Reznikoff, 1992b).

E. coli is found in lactose-rich environments such as the gut of young mammals, and it requires glucose for much of its cellular processes. The lactose molecule is composed of one glucose and one galactose molecule. When only lactose is available, *E. coli* can convert lactose from environment into the glucose using the enzyme beta-galactosidase. Beta-galactosidase production requires the activation of the *lac* operon, which is repressed in the absence of lactose. The lactose operon includes four genes encoding important proteins implicated in lactose degradation and two DNA regions involved in regulation: *lacI* (repressor), *lacZ* (Beta-galactosidase), *lacY* (permease), *lacA* (transacetylase), and the DNA regions termed "P" (promoter) and "O" (operator) sitting between *lacI* and *lacZ*. In the absence of lactose, *lacI* is continually transcribing the repressor protein. The repressor binds to the "O" region and blocks the access to the

RNA-polymerase (binding to P region upstream). Consequently, the transcription of the genes downstream is impaired. When there is abundant lactose in the environment, a molecule of lactose binds to the repressor protein inducing a conformational change. It then detaches it from the "O" region and allows the RNA-polymerase to transcribe the rest of the operon, enabling the lactose processing mechanism (Reznikoff, 1992a;Kok, 1996).

A second level of control of the lactose operon in *E. coli*, exist and is especially important when there are both glucose and lactose present. In this scenario, glucose is preferred because it is 'easier' to process (less energy required), before activating the lactose operon. Cyclic adenosine monophosphate (cAMP) is a by-product of glucose degradation, therefore when there are high concentrations of unprocessed glucose in the cell cAMP concentrations are low and vice versa. In fact cAMP has been called "the hunger signal" in *E. coli*, because high concentrations indicate low availability of glucose. Concordantly, the regulator that exerts this second level of regulation is sensitive to cAMP levels. This protein is called catabolite activator protein (CAP) or cAMP receptor protein (CRP) and is encoded by the *crp* gene. When glucose is scarce (high cAMP), the complex CRP-cAMP binds to the "P" region and enhances the lactose operon transcription by 50 fold, enabling lactose metabolism (Kok, 1996)

CRP is a global gene regulator that has being extensively studied in *E. coli* (for reviews see (Reznikoff, 1992a;Reznikoff, 1992b;Bruckner and Titgemeyer, 2002). Its regulatory functions are not restricted to lactose metabolism, on the contrary CRP responds to variations in the availability of different carbon sources (e.g. arabinose, tryptophan). When CRP is bound to cAMP, the complex acts as a sequence specific DNA-binding protein that can activate or repress a large number of operons (>100

promoters in *E. coli*). CRP contains two binding sites, one called the "protein binding site" for interaction with RNA-polymerase and a second one called the "DNA binding site" that recognizes a specific sequence at the promoter region of the genes that it controls. There are several hypotheses about the mechanism whereby CRP enhances gene transcription (Busby and Ebright, 1999). In general it is thought that CRP offers an additional site of interaction for RNA-polymerase that further stabilizes the enzyme at the transcription initiation site, and that CRP creates a bend in the DNA strand that facilitates the entrance of the bulky RNA-polymerase into the site.

CRP-like proteins have also been described in other organisms (Bruckner and Titgemeyer, 2002). In *M. tuberculosis* the closest homolog to *crp* is the *Rv3676* gene. When *Rv3676* was deleted from *M. tuberculosis*, growth defects *in vitro* and *in vivo* (bone marrow derived macrophages and mice) where observed. The *Rv3676* mutant had also altered expression of a series of genes including *rpfA* that codes for a "resuscitation activation factor" (predicted to be important in the reactivation of tuberculosis in latent infections) (Rickman *et al.*, 2005).

In BCG strains, the orthologue to *Rv3676* presents point mutations that affect the DNA binding ability of the product protein. This stands in contrast to the paucity of point mutations seen in randomly sequenced genes, and points to a striking rate of polymorphism in this gene. All BCG strains show a non-synonymous mutation (G-to-A at position 532 of the gene sequence) rendering a glutamic acid at position 178 of the protein to change to a lysine. BCG-Japan has a second mutation that changes the valine on position 184 into alanine. BCG-Denmark, BCG-Glaxo and BCG-Pasteur (all obtained from the Pasteur Institute after 1931) have a T-to-C base substitution at position 140 that causes a leucine to change into a proline at position 47. The implications of these

mutations in BCG strains, beyond the ability of the product protein to bind to the hypothetical target sequences, are the subject of ongoing research.

VI. - Literature review summary

BCG vaccine strains are probably impaired in their ability to regulate genes as predicted by different genomic deletions and important point mutations affecting predicted regulatory sequences. Also, BCG strains present an unusual number of mutations in the *Rv3676* gene, possibly involved in regulation of carbon metabolism. BCG strains have documented impaired responses to environmental nitrogen concentrations, and there is a potential link between carbon and nitrogen metabolism converging at the citric acid cycle. In this work we have searched for a relation between these observations, to better understand the metabolic consequences of BCG evolution.

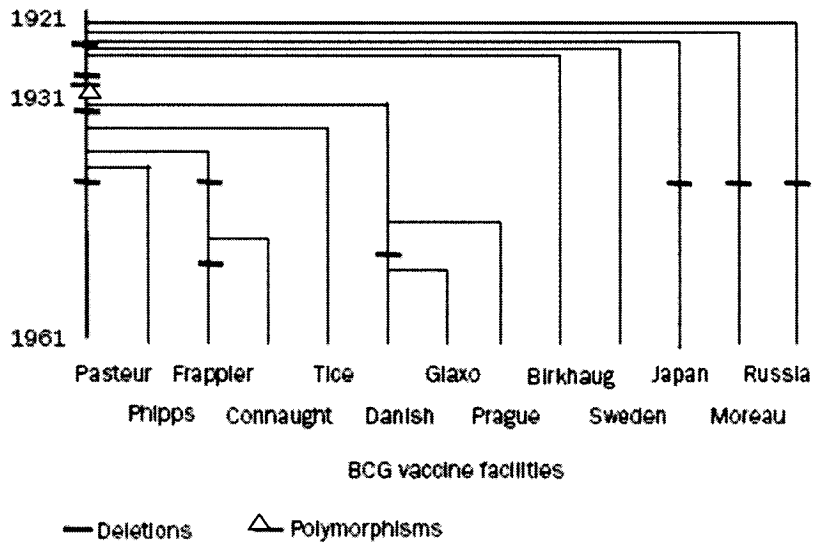


Figure 1LR: Genetic regions deleted from BCG strains propagated around the world. This graph represents the genealogy of BCG vaccine strains based on historical data, showing that all currently available BCG strains differ from that first used in 1921. (From Behr, 2002).

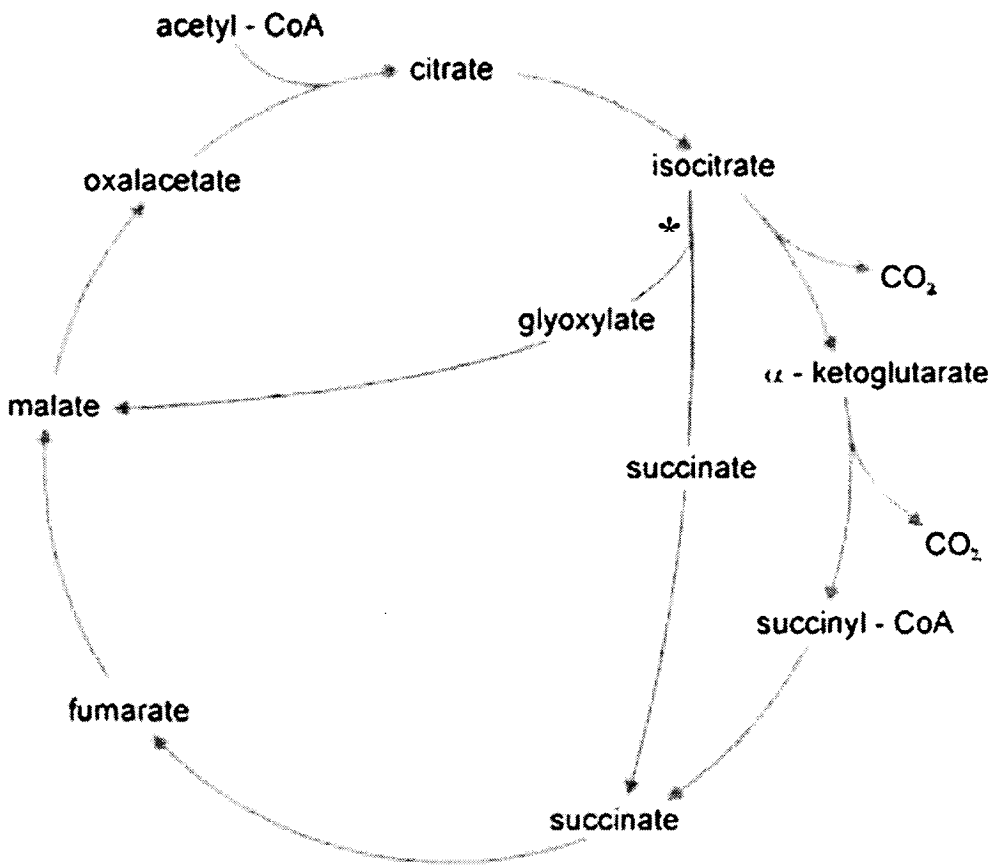
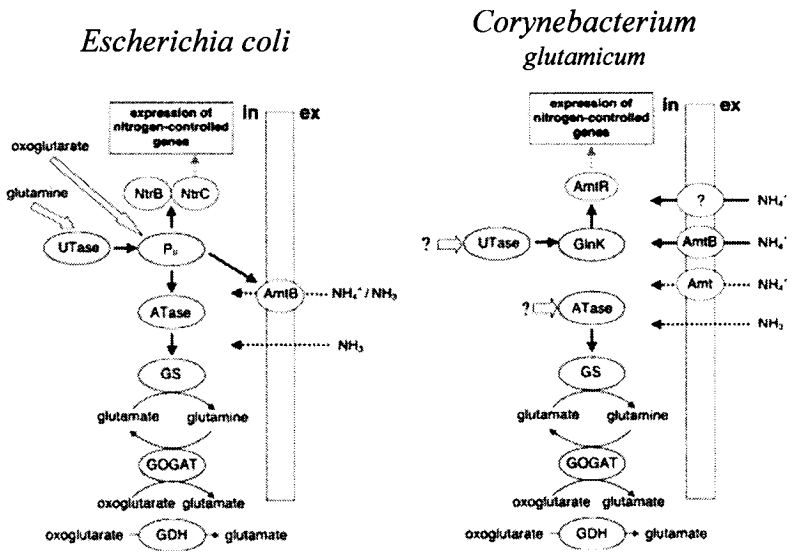


Figure 2LR: Schematic representation of the prokaryote Citric Acid Cycle (CAC). Acetyl-CoA is combined with oxaloacetate, to form citrate. Six subsequent reactions then release two carbon dioxide molecules and the oxaloacetate is regenerated. Eight intermediates are formed in the CAC: citrate, isocitrate, alpha-ketoglutarate, succinyl-CoA, succinate, fumarate, malate and oxaglutarate. The glyoxylate shunt (*) is a deviation of the CAC and bypasses the synthesis of alpha-ketoglutarate and succinyl-CoA (From Ulrichs and Kaufmann, 2002).

Uptake of ammonium and nitrogen regulation



Modified from Burkovski, 2003

Figure 3LR Uptake of ammonium and nitrogen regulation in *E. coli* and *C. glutamicum*. (from Burkovski, 2003).

Methods

I.-Bacterial strains

A) Wild type strains

A collection of thirteen strains of *Mycobacterium bovis* BCG previously curated was studied: BCG-Pasteur, BCG-Phipps, BCG-Frappier, BCG-Connaught, BCG-Tice, BCG-Denmark, BCG-Glaxo, BCG-Prague, BCG-Birkhaug, BCG-Sweden, BCG-Japan, BCG-Moreau and, BCG-Russia. In addition, *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and a clinical isolate of *M. bovis* were used as control strains.

B) Complemented strains

With *sdaA* and *glnA1* genes

Plasmids for complementation of BCG strains with *sdaA* (serine deaminase) or *glnA1*(glutamine synthetase) were previously described (Chen *et al.*, 2003). Briefly, DNA fragments were excised from cosmids of *M. tuberculosis* H37Rv BAC library (BAC339 for *sdaA* and BAC214 for *glnA1*) and ligated to the *Escherichia coli*-mycobacterial shuttle vector pMD31 (extra-chromosomal and kanamycin resistant) BCG-Japan, BCG-Frappier and BCG-Pasteur where individually complemented with each of *M. tuberculosis*-H37Rv *glnA1* and *sdaA* genes using pMD31 plasmid as a vector, resulting in

6 complemented strains: BCG-Pasteur/*glnA1*, BCG-Pasteur/*sdaA*, BCG-Frappier/*glnA1*, BCG-Frappier/*sdaA*, BCG-Japan/*glnA1*, BCG-Japan/*sdaA*.

With *Rv3676* gene

Plasmids for complementation of BCGs with *Rv3676* (putative catabolic repressor protein or CRP) were prepared in house. Briefly, DNA fragments were amplified from *M. tuberculosis* H37Rv DNA (using primers on Table 2.) and ligated to the *Escherichia coli*-mycobacterial shuttle vector pME. BCG-Frappier and BCG-Pasteur were individually complemented with *Rv3676* using pME plasmid as an extra-chromosomal vector containing the kanamycin resistance gene *aph*.

II. -Growth and culture media

A) Routine growth

Frozen 1ml aliquots were thawed and diluted in 20 ml of Middlebrook 7H9 (Difco) supplemented with 0.05% tween, 0.2% glycerol and 10% ADC (bovine serum albumin [fractionV], dextrose, catalase). The suspension was placed in 50 ml tubes (Falcon, Polypropylene, conical tubes), and incubated at 35 °C, while rolling. In the case of transformed bacteria, media was supplemented with 25ug/ml kanamycin. Cultures were grown until an optical density at 600nm (OD_{600}) of approximately 0.5 was achieved.

B) Challenge experiments

Cultures were centrifuged and cell pellets were washed and re-suspended in regular Sauton media (Allen, 1998). This media is one of the classical broths used for culturing maycobacteria, and can be easily prepared in house. It was prepared by adding 0.5gr KH_2PO_4, 0.5 gr MgSO4 hepta-hydrated, 2gr citric acid, 0.05gr ferric ammonium chloride, 60ml glycerol, and 4gr asparagine, to enough distillate water for a final volume of 1L. For growth experiments, modified Sauton media containing 0.27 mM, 2.7 mM or 27mM serine in the place of asparagine was used. All BCG-strains as well as *M. tuberculosis* H37Ra were grown under the above conditions as follows.

For growth experiments, cell suspensions of an OD_{600} of approximately 0.1, were distributed in 8 replicates per strain on 96-well culture plates, followed by incubation for 8 days at 35 °C. Growth was monitored by taking OD_{600} readings on day 0, day 4, and at the end of the experiment. Overall growth increase at the three concentrations of serine was compared to growth of the same strain on regular Sauton media. Due to limited access to level 3 facilities at the time of experiments, this protocol was modified for the study of *M. tuberculosis* H37Rv and *M. bovis* such that only regular Sauton and Sauton 27mM serine were tested and strains were grown in 50ml Falcon tubes instead of on 96 well plates.

To assess the response of BCG-Japan to serine concentrations higher than 27mM, a procedure similar to the described above for other challenge experiments was repeated. This time the media used contained none, 81, 108, 270 or 1000 mM serine. For this particular experiment, growth of BCG-Japan, BCG-Japan/*glnA1*, BCG-Japan/*sdaA* and *M. tuberculosis* H37Ra was studied.

C) Rescue experiments

In order to study the effect of glutamine and alpha-ketoglutarate on the growth of BCG under high serine stress, different concentrations of these reagents were added to Sauton preparations already containing 27mM serine. In these experiments 30mM glutamine as well as 0.1, 0.5, and 5mM alpha-ketoglutarate were tested. The rest of the procedure in terms of repeats, growth conditions, OD_{600} readings etc., is just as described above for the challenge experiments.

D) Growth definitions.

Below are a few definitions used to graph and interpret growth data:

- OD_{600} increase = OD_{600} at the time of measure – OD_{600} at set-up day.
- Percentage of optimal growth = (OD_{600} increase on serine * 100) / OD_{600} increase on regular Sauton media
- Percentage of OD_{600} increased = (OD_{600} increase * 100)/ OD_{600} at set-up day.
- Error: for growth experiments and any other graphs showing error bars, these are calculated as the standard error of the mean (SEM). SEM = standard deviation / square root (number of replicates).

III.-Infection of THP-1 cells and bacteria recovery.

A) THP-1 storage and growth

THP-1 is a human monocytic cell line that can be differentiated to macrophage-like cells. For this work the THP-1 cell line was acquired from ATCC (American Type Culture Collection). For routine growth, the monocytes were maintained in RPMI (Roswell Park Memorial Institute Media) 1640 medium (Wisent) containing, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Wisent) and 20 mM HEPES (Wisent), at 37°C in an atmosphere of 5% CO_2-95% air in vented culture treated flasks (Falcon). The cell density in the culture flasks was kept at approximately 5 x 10^5 cell/ml. Cell density was estimated using a light microscope and cell counting chamber. Cells were passage a maximum of five times, before the culture was discarded. New cultures from frozen aliquots were started by quick thawing, spinning down and resuspending in fresh RPMI complete without DMSO (Dimethyl sulfoxide).

B) THP-1 seeding, differentiation and infection.

The protocol followed was based on that described by the group of Horwitz (Tullius, Harth, and Horwitz, 2003d), with few modifications, as follows. Cells were plated at 2 x 10^5 cells per well in 2-cm^2 24-well tissue treated culture plates (Falcon). Cells were seeded in RPMI complete containing 100 nM PMA (phorbol 12-myristate 13-acetate) (Sigma), allowing 3 days for complete differentiation.

Inocula were prepared by dilution of log-phase broth cultures of BCG grown as described above (routine growth of bacteria). The cfu (colony forming unit) density in broth cultures was estimated using the in house observation that an OD_{600} of 1.0 corresponds approximately to $2x10^8$ cfu/ml. Bacterial strains used were BCG-Pasteur, BCG-Frappier, BCG-Frappier/*glnA1*, BCG-Frappier/*sdaA*, BCG-Japan, BCG-Russia and *M. tuberculosis* H37Ra. The inocula densities were later confirmed by plating serial dilutions (in NaCl 0.85 %) of the inocula in 7H9 agar (Difco) supplemented with 0.2% glycerol and 10% ADC (bovine serum albumin [fractionV], dextrose, catalase, oleic acid), and counting mycobacterial colonies after 2 weeks incubation at 37°C.

The monolayers were infected with bacterial strains at a multiplicity of infection (MOI) of 1 bacterium per THP-1 cell. Six wells per strain were infected. After 2h incubation at 37°C the medium was removed and the cells were washed twice with PBS (Phosphate buffered saline, pH 7.4, Sigma). At this point the 2h sampling was performed as described below.

Out of the six wells infected per strain, duplicate wells received 2ml of either RPMI complete, RPMI 0.2 mM glutamine, or RPMI complete 27mM serine. RPMI complete 0.2mM glutamine was obtained by dilutions with RPMI complete no glutamine (Wisent). This was repeated for each plate (corresponding to different sampling times). Plates were incubated at 37°C in an atmosphere of 5% CO_2-95% air, for 0 to 7 days. The medium was replaced with fresh medium at 3 days for those wells to be harvested after day 3.

C) Bacteria sampling and recovery

After infection, cells were sampled at 2h, and days 1, 4 and 7 to assess bacterial growth. The culture medium was removed and the cells were washed twice with PBS to remove any extracellular bacteria. Cells were lysed by the addition of 1 ml of 0.1% SDS for 2-3 minutes, cells were then removed and resuspended by pipetting. For bacteria viable counts, serial dilutions of the lysates were plated on 7H10 plates as described above for estimation of inoculum density.

IV.- BCG gene sequencing

For sequencing of BCG genes, BCG-Pasteur DNA was always sequenced since this is the BCG strain that has accumulated the most genetic changes (see figure 1LR). In most cases DNA from BCG-Frappier and BCG-Japan was also sequences.

To identify genetic differences that may account for defective serine deaminase or glutamine synthetase, the promoter and gene sequence of *glnA1* gene and the *glyA2-sdaA* operon were determined in our laboratory. The *sdaA* gene had been previously sequenced (Chen *et al.*, 2003), with the exemption of the upstream region and most probably co-transcribed gene *glyA2* (serine methylase 2).

In order to evaluate the sequence integrity and homology of genes involved in nitrogen (N) metabolism, eight different genes were sequenced. The genes selected code for the protein and enzymes with key roles in nitrogen sensing and assimilation: *amtR* (NH_4 transporter), *gltBD* (glutamate synthase subunits B and D or GOGAT), *glnE*

(adenylyltransferase or ATase), *glnK* (pII signal transduction type protein), *glnD* (uridylyltransferase or UTase), *sigF* (sigma factor F, potentially involved in N regulation (DeMaio *et al.*, 1996), *gdh* (glutamate dehydrogenase).

Overlapping sequences spanning the desired regions were amplified by PCR (see Table 1 for primer sequences) and sequenced from both directions to give two folds coverage. DNA sequences were determined by di-deoxy terminal sequencing at the "McGill University and Genome Quebec Innovation Centre" and compared to *Mycobacterium tuberculosis* H37Rv (http://genolist.pasteur.fr/TubercuList/), *Mycobacterium tuberculosis* CDC1551 (http://tigrblast.tigr.org/cmr-blast/), *Mycobacterium bovis*-2122 (http://genolist.pasteur.fr/BoviList/index.html), and BCG-Pasteur (http://www.sanger.ac.uk/Projects/M_bovis/blast_server.shtml) sequences.

V.-Gene transcription assays using quantitative Real-Time PCR

A) Culture preparation

Cultures were grown as described above (bacteria routine growth) to an OD of ~ 0.5 (approximately log phase), separated into two identical aliquots, centrifuged and then washed with saline solution (NaCl 0.85%). For each pair of aliquots, one tube was re-suspended in regular Sauton media and the second in Sauton with 27mM serine in the place of asparagine. The bacteria were incubated in appropriate media for 48h before RNA extraction (Mangan *et al.*, 1997).

B) RNA extraction

Cultures were centrifuged, and the pellet was re-suspended using washing solution (0.5% tween, 0.8% NaCl). This suspension was centrifuged and the new pellet was re-suspended in lysis buffer (Na-acetate 20 mM, SDS 0.5%, EDTA 1mM, pH= 4), and 0.8g of silica beads (Sigma) were added into the vial. Mechanical lysis was achieved in reciprocal shaker (Ribolyzer) using two sequential times for periods of 15 seconds and speeds of 4.5 and 6.5 respectively. The vial was then incubated on ice for 10 min, and immediately centrifudge at 4°C. The top phase was transferred into a new tube and an equal volume of chloroform-isoamylalcohol was added, gently shaken and centrifuged once more. The top phase was placed in a new vial, then equal volumes of isopropanol, and 1/10 volume Na-acetate 3M (Ambion) were added followed by overnight incubation at –70 °C. After, the solution was centrifuged in cold, the supernatant was discarded and the pellet was washed with cold 75% ethanol. RNA was recovered by centrifugation, ethanol was removed and the pellet was air-dried for approximately 15 minutes. Once dry, the pellet was re-suspended in RNAse free water (Ambion). Residual DNA was degraded using the DNAse-I kit (Fermentas) following the supplier's instructions. OD readings to determine purity (at 280nm) and quantity (at 260nm) of the RNA were done in a spectrophotometer (GeneQuant-pro, RNA/DNA calculator). RNA quality was checked on agarose gels. To verify that the residual DNA was effectively cleared, a control PCR using the same primers designed for amplifying the sequences of interest and purified RNA as template was performed. If amplifications were obtained from any other sample than the positive control, the DNAse treatment was repeated.

C) Reverse transcription and Real Time (RT) PCR.

RNA was converted into cDNA using reverse transcriptase (Fermentas) and random hexamer primers, following the supplier's protocol. The resulting number of cDNA copies should be proportional to the original quantities of mRNA templates (Tyagi and Kramer, 1996a). To verify the efficiency of the reverse transcription a control PCR using the same primers designed for amplifying the sequences of interest and cDNA as template was performed, whenever amplification was not obtained, the sample was discarded and a new aliquot of purified RNA was subjected to reverse transcription. cDNA was amplified using a Roche LightCycler with the Roche LightCycler-FastStart DNA kit, in the presence of specifically designed fluorescent molecular beacons for each of *sdaA* and *glnA1*gene sequences. Molecular beacons are probes that fluoresce upon hybridization; they are single-stranded DNA molecules that possess a stem-and-loop structure (Tyagi and Kramer, 1996). The loop portion is designed to bind the target sequence of interest (in our case the corresponding cDNA to the mRNA from the genes of interest *sdaA* and *glnA1*). For primers and beacons sequences see Table 2. The principle behind the method is that as the target sequence is amplified, the molecular beacons bind and in doing so fluorescence is produced. The fluorescence generated is proportional to the amount of target sequence copies amplified, which in turn should be proportional to the amount of cDNA of that particular sequence added to the reaction. Ultimately, the fluorescence produced during RT-PCR can be related to the amount of specific mRNA produced by the bacterial strain.

Results were processed and interpreted using "Light-Cycler Data Analysis" software package version 3.5. The *sigA* gene was used as an internal standard because its

expression was shown not to vary in experimental conditions (Manganelli *et al.*, 1999), We therefore have defined our expression units as "*sigA* equivalents" or "sAe" (sAe = ng of RNA of interest / ng of *sigA* RNA).

VII.-Glucose utilization

Glucose utilization was determined by monitoring glucose levels in 7H9 media inoculated with BCG-Pasteur or *Mycobacterium tuberculosis* H37Ra, and in non-inoculated control tubes. Bacteria where grown as described above. At 0, 1, 4 and 6 days after inoculation 20 µl of media were added onto a test strip # 4, and read on a "blood glucose meter" (Life scan, One Touch, Johnson & Johnson). Additionally, for certain experiments, the glucose utilization was measured as part of a multi-test format called "API' strips, as detailed below.

VIII.-BCG "API" profiles

The "API" identification strips are designed to permit rapid identification of pure, cultured bacteria. They are widely used in clinical and industry settings. We have attempted to use them as a quick tool to check diverse biochemical properties of our strain collection. An API strip system specifically designed for mycobacterium does not exist, so we have chosen the API-coryne, designed for identification of closely related corynebacteria.

Each "API" strip consists of 20 wells each containing a dry metabolite. See table 3 for a full list of biochemical reactions in the API-coryne test strips. When bacteria are added (following the suppliers instructions), and incubated in the strip for sufficient time, a color reaction will indicate if the metabolite has been used or not. Although the recommended incubation time is 24h, up to 4 days are needed for mycobacteria to generate an observable result. Even though API-coryne is not explicitly designed for mycobacteria, results from these tests coincided with those from the glucose utilization essays and with classical biochemical characterization of mycobacteria (e.g. nitrate reduction positive for *M. tuberculosis* strains and negative for *M. bovis* strains).

Name	Sequence
glnA1 pair1 Left-primer	5 '-gttacgatcttgccgaccat -3'
glnA1 pair1 Right-primer	5'- cttgtggcggaccttgtag –3'
glnA1 pair2 Left-primer	5'- caaggccgagaactacctga –3'
glnA1 pair2 Right-primer	5'- accagccgcttgtaggagt-3'
glnA1 pair3 Left-primer	5'- gcactgtcatcagtcgctgt –3'
glnA1 pair3 Right-primer	5'- cgttcttaaacgggctcttc –3'
gltB Left primer	5'-ccggtaacgagaactggatg-3'
gltB Right primer	5'-ctcagggatcatcatcagca-3'
glnE pair1 Left primer	5'-cgctggacgtcatgatcc-3'
glnE pair1 Right primer	5'-gcagtcaactcccgaacact-3'
glnE pair2 Left primer	5'- ctgctggactggatgtcgta -3'
glnE pair2 Right primer	5'- gatcgatgtcgaccatttca -3'
sigF Left primer	5'- atgtagccggcagtgtcttc -3'
sigF Right primer	5'- gctcgccgagatcaagtaag-3'
glyA2 pair1 Left primer	5'-gcctgaccgaaggtgtagag-3'
glyA2 pair1 Right primer	5'-ggtgcatagttctccgaagc-3
glyA2 pair 2 Left primer	5'-acagaccggactgagctgat-3'
glyA2 pair 2 Right primer	5'-tctttggacacctcgtaggc-3'
glyA2 pair 3 Left primer	5'-acaccatcctggggttgtc-3'
glyA2 pair 3 Right primer	5'-ttgtgctgccatcttgaatg-3'
glyA2 pair 4 Left primer	5'-tcgccaagaagatcaattcc-3'
glyA2 pair 4 Right primer	5'-gttccgggtagagcggatag-3'
*gly*A2 pair 5 Left primer	5'-gcttctcccacaacgacttc-3'
glyA2 pair 5 Right primer	5'-aacagatccactcgcatcg-3'

Table 1. Primers used for sequencing and cloning. Genes *glnA1*, *sigF*, *glyA2* and *sdaA*, were completely sequenced. For all other genes sequencing was restricted to the regions where on line databases showed potential differences.

Name	Sequence
sdaA Beacon	5'-gcagc-ccctacgtgtcggcccaag-gctgc–3'
sdaA Left primer to use with beacon	5'-ttcatcgtcacggaacagac–3'
sdaA Right primer to use with beacon	5'-tcgctaattgacacgtcgag–3'
glnA1-Beacon	5'-gcagc-cccccgatcaacctggtctatag-ggctgc-3'
glnA1-Left-primer to use with beacon	5'-cgacggtgaactcctacaag–3'
glnA1-Right-primer to use with beacon	5'-agtcggggcttcggaact –3'

Table 2. Beacons and corresponding primer sequences.

Test name	Reaction
NIT	Nitrate reduction
PYZ	Pyrazinamidase
PyrA	Pyrrolidonyl Arylamidase
PAL	Alkaline Phosphatase
βGUR	Beta-glucuronidase
βGAL	Beta-galactosidase
αGLU	Alpha-gluconidase
βNAG	N-Acetyl-β-Glucosaminidase
ESC	Esculin (β-glucosidase)
URE	Urease
GEL	Gelatine hydrolysis
O	Fermentation control (no sugar)
GLU	Glucose fermentation
RIB	Ribose fermentation
XYL	Xylose fermentation
MAN	Mannitol fermentation
MAL	Maltose fermentation
LAC	Lactose fermentation
SAC	Sucarose fermentation
GLYG	Glycogen fermentation

Table 3 Biochemical reactions in API-coryne test.

Results

I.-Optimal growth

One of the first challenges we encountered involved characterizing the growth expected under optimal conditions for all 13 BCG strains. In order to do so, all BCG strains in our collection where grown on 7H9 and Sauton media, both of which are rich media commonly used in the culture of mycobacteria; we consider these "optimal growth conditions". For any of the tested strains grown in either of the two optimal media, the final OD was equal or greater to the initial OD plus 50% (final OD_{600} = or > initial OD_{600} + ½ [initial OD_{600}]). For the purposes of an operational concept, we have defined "optimal BCG growth" as that achieved in regular Sauton media (figure 1) without added stressor (serine). Strains exhibiting less than 50% optimal growth under stress condition were designated growth inhibited.

II.-Effect of serine on BCG strains growth *in vitro*

As Figure 2 shows, most BCG strains tested follow a classical "dose-response" curve when confronted with increasing concentrations of serine: the growth decreases as the serine concentration increases. Some BCG strains seem to be particularly susceptible to high serine concentrations (e.g. BCG-Frappier, figure 2C). BCG-Japan (figure 2L) and BCG-Russia (figure 2N), and *M. tuberculosis* H37Ra (figure 2O) show little or no growth

inhibition at increasing concentrations of serine. When growth in different concentrations of serine was followed during eight days (figure 3), the effect of serine on bacterial growth increased with time. At eight days of incubation differences were more pronounced in almost all cases (e.g. BCG –Frappier, figure 3C). As it can be seen in Figure 4, for most BCG strains, the final growth achieved after eight days in serine is only fraction of the optimal growth.

The addition of serine did not inhibit growth of *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv and *M. bovis*. In contrast, most BCGs showed some degree of growth inhibition even at the lower concentrations of serine (figures 2 and 3), and demonstrated significant growth impairment at 27mM (figure 4). In particular, growth of BCG-Pasteur and BCG-Frappier was strongly inhibited at this concentration (only 22.2% and 2.1% optimal growth respectively). Certain strains, like BCG-Japan and BCG-Russia, grew nearly as well in media with 27mM serine as in regular Sauton (figure 4). We chose BCG-Pasteur, BCG-Frappier and BCG-Japan for further study, as a follow up of work with lab collaborators. Also, these strains represented the full range of growth responses to high serine, from poor (BCG-Pasteur and BCG-Frappier) to unaffected (BCG-Japan).

BCG-Japan growth did not decrease in the presence 27mM serine, but with serine concentrations of 81 mM and higher, a growth defect was observed that was not manifested by *M. tuberculosis* H37Ra under the same conditions (figure 5).

III.-Growth of BCG strains in THP-1 cells

As shown in figure 6, THP-1 cells were effectively infected with bacteria. The amount of bacteria inside the cells after 3h of contact is proportional to the inocula

concentration. Although most of the bacteria entered the cells, replication was very slow as no significant differences in the amount of bacteria were recovered at the different sampling times. Furthermore, all BCG strains followed the same pattern of low or no replication, regardless of the growth condition (figure 7).

IV.-Effect of serine in gene transcription

BCG strains Pasteur and Frappier showed constant levels of transcription for both genes studied (*sdaA* and *glnA1*), regardless of the growth media. BCG-Japan, *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *M. bovis*, increased the transcription of *sdaA* and *glnA1*, when growing in media with 27mM serine (figure 8). Expression of *sdaA* increased by about one log, while *glnA1* expression increased about 3-5 fold. Transcription of *sdaA* is shown in fig. 8A and that of *glnA1* in fig. 8B. Expression values were also treated as the ratio of gene expression under 27 mM serine to normal culture media. As shown in figure 9, under elevated serine concentrations, each of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *M. bovis* showed statistically and biologically significant induction of both *sdaA* and *glnA1* genes. In contrast, for BCG Frappier and Pasteur, there was no induction of *sdaA* or *glnA1* transcription as a function of elevated serine. BCG Japan manifested an intermediate result, in which transcription of both genes was statistically increased, but the level of induction was lower than observed in *M. tuberculosis* and *M. bovis*.

V.-Effect of gene complementation on growth inhibition caused by serine

Growth of BCG-Pasteur and BCG-Frappier under high concentrations of serine is completely restored by plasmid complementation with either *M. tuberculosis* gene *sdaA* or *glnA1*; and partially recovered (>40% optimal growth) by complementation with *M. tuberculosis Rv3676* (figure 10). BCG-Japan was only complemented with *M. tuberculosis* genes *sdaA* and *glnA1*. As the growth of BCG-Japan was not reduced by 27mM serine, the impact of complementation on growth was modest. While the growth of BCG-Japan was not decreased by 27mM serine, under serine concentrations of 81 mM and higher, a growth decrease was observed that could be overcome by either *sdaA* or *glnA1* complementation, and was not seen for *M. tuberculosis* H37Ra (figure 5).

VI.-Effect of complementation on gene transcription

As expected plasmid complementation with *sdaA* only affected the expression of this gene, increasing its transcription for all 6 *sdaA* complemented strains regardless of the growth condition (figure 11). Concordantly, episomal complementation with *glnA1* only affected the expression of *glnA1*, as demonstrated for all 6 *glnA1* complemented strains, again independent of the serine concentration in the growth media (figure 12).

VII.- Sequencing of genes involved in nitrogen metabolism and serine degradation

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No differences between the BCG gene sequences obtained and those available in databases for *Mycobacterium tuberculosis* were found. All BCG gene sequences were

identical to the corresponding genes in *Mycobacterium tuberculosis*. Although we did no structural or functional studies of the proteins for which this genes code, these results made improbable the hypothesis that the susceptibility to high levels of serine relates to defects in the enzymes encoded by these genes, unless post-translational modifications under the control of other genes were responsible for impaired enzyme activity.

VIII.- Effect of media supplementation on growth inhibition by serine

As expected, direct addition of 30mM glutamine to the growth media containing 27mM serine rescued the growth inhibition rendering a growth nearly equal to that in media without serine (figure 13A). Also, addition of as little as 0.1mM alpha-ketoglutarate to 27mM serine Sauton media enhances growth of BCG-Pasteur in such media (figure 13B). These observations provided a clue into a possible mechanism by which serine inhibits growth of BCG strains, as will be developed further below.

BCG glucose utilization and API profiles

Levels of glucose in 7H9 media remained practically intact over time when BCG-Pasteur was growing, whereas, in *M. tuberculosis* H37Ra cultures the glucose was consumed as time progressed so that by the end of the experiment the concentration of glucose in media had decayed from 17.1mM to 9.6mM over 6 days (figure 14). These results indicate that BCG-Pasteur as opposed to *M. tuberculosis* H37Ra, did not utilize glucose as a preferred carbon source while growing in 7H9. This observation was corroborated by API strips results, in which none of the BCG strains tested was able to

ferment glucose. Only a few biochemical reactions were positive when BCG strains were tested with API strips. Conversely, *M. tuberculosis* H37Ra was able to utilize several of the metabolites offered in the API strips, noteworthy this strain was able to ferment all sugars in the test (table 5). Notably, when BCG-Frappier and BCG-Russia are complemented with *Rv3676* (CRP homolog gene) from *M. tuberculosis*, the ability to ferment glucose was gained by both BCG strains (table 5).

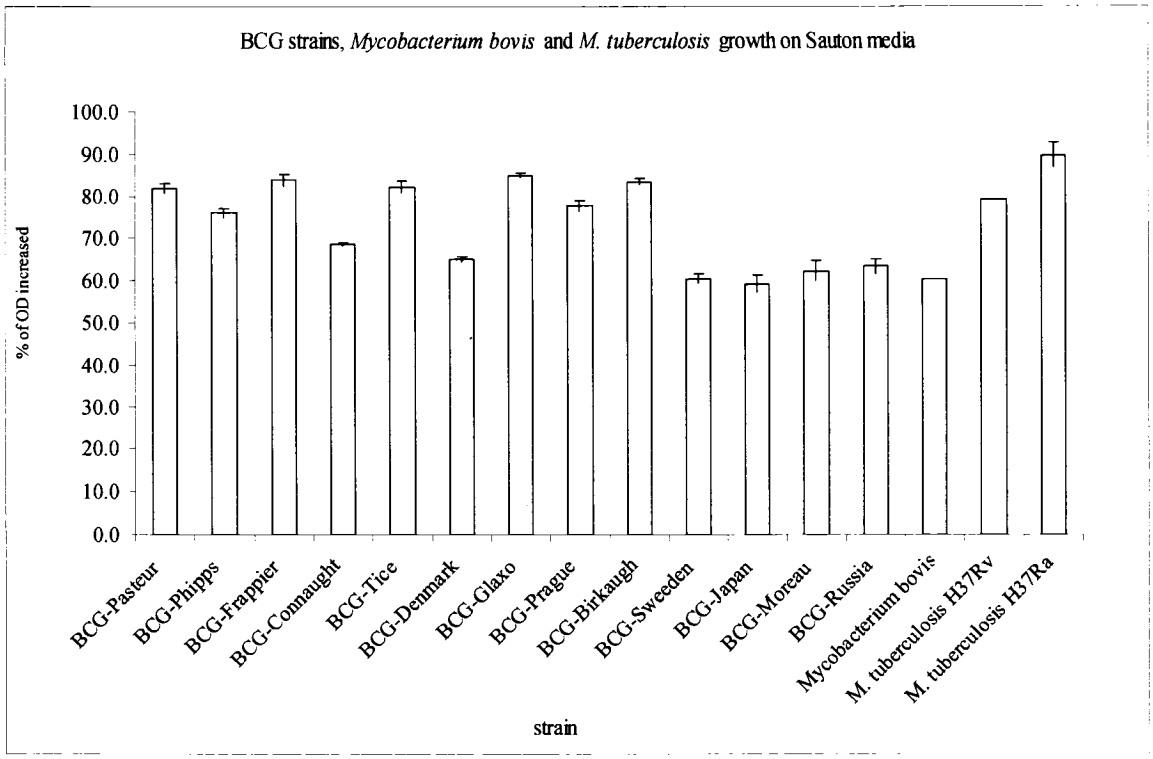
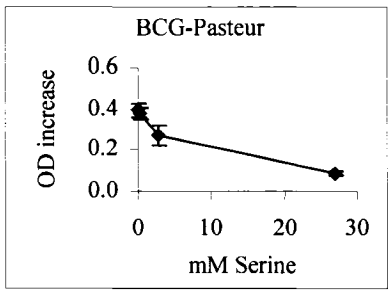


Figure 1. BCG strains, *Mycobacterium bovis* and *M. tuberculosis* growth on Sauton media. Growth is graphed as percentage of OD_{600} increased over 12 days (as defined in the methods). BCG strains are presented along the x- axis in the chronological order they were obtained from the Pasteur Institute (right to left). Note that for all strains, final $OD_{600} \geq$ initial $OD_{600} + \frac{1}{2} OD_{600}$.

A



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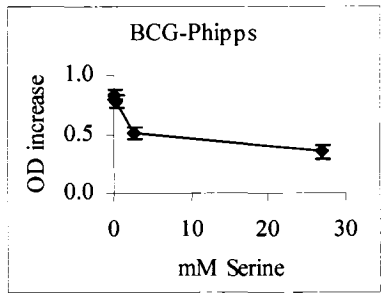

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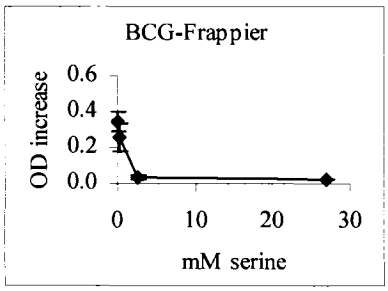

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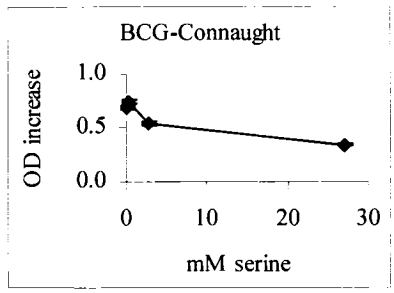

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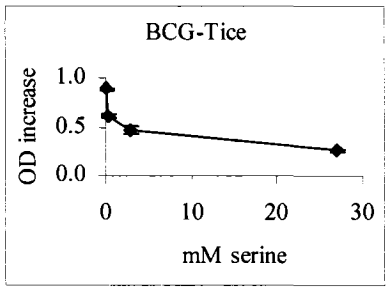

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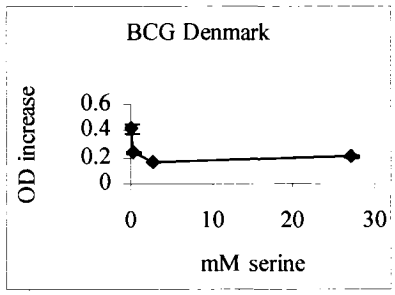

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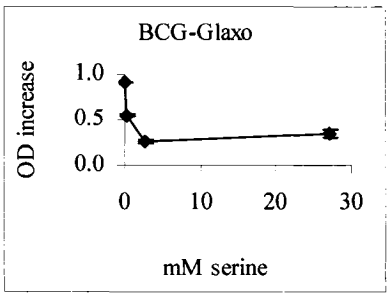

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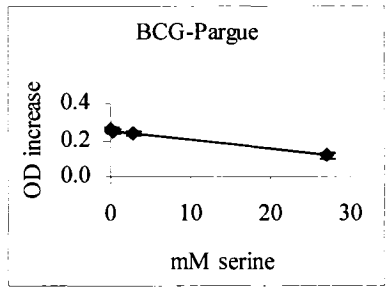


Figures 2A-2H

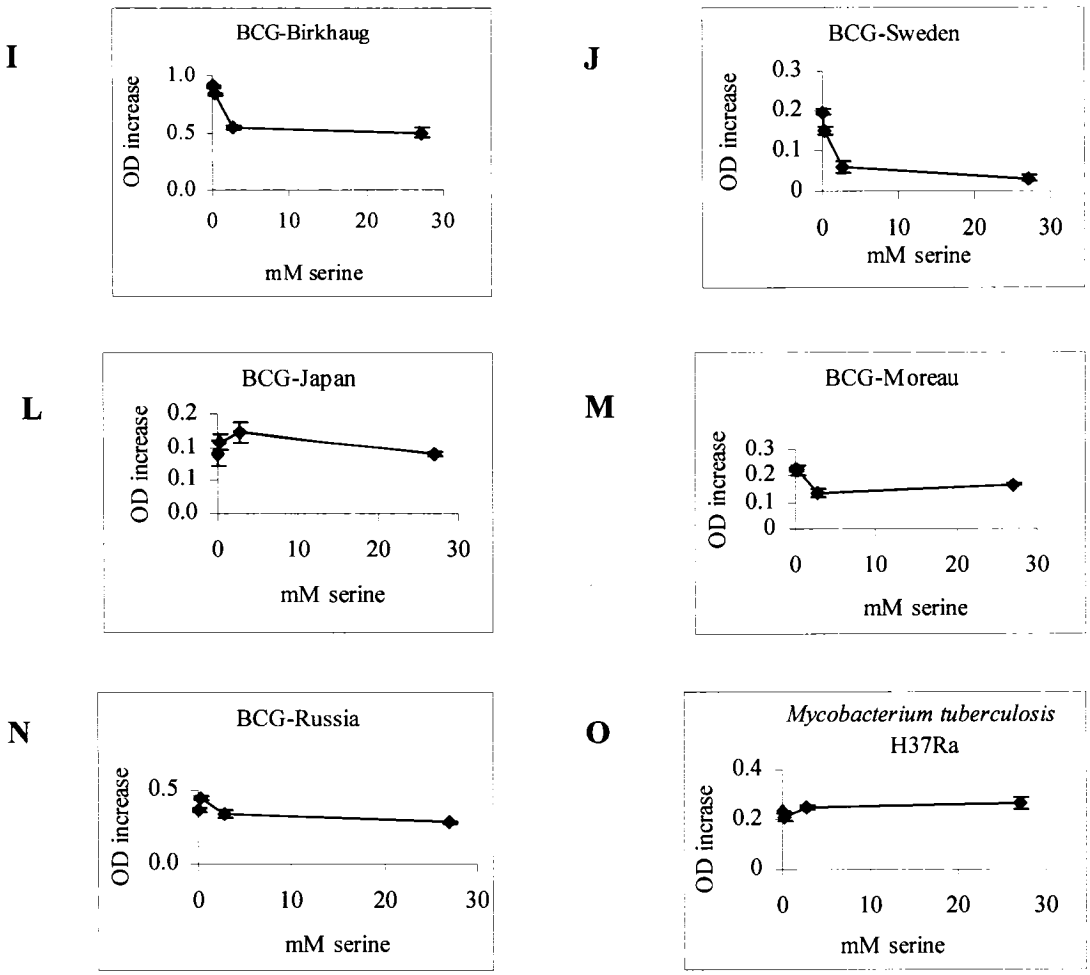
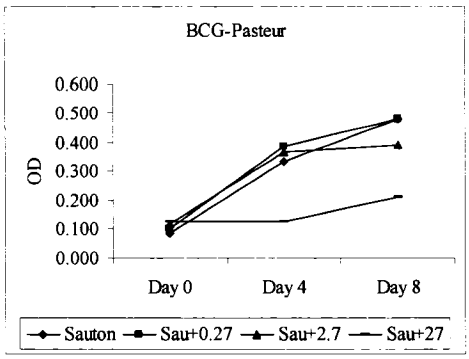


Figure 2I-2O

Figure 2. Dose response curves for all BCG strains and *M tuberculosis* H37Ra. Final growth increase after 8 days measured in OD_{600} is graphed as a function of serine concentration in Sauton media. As opposed to *Mycobacterium tuberculosis* H37Ra, most BCG strains show a classical dose-response curve, where growth is inhibited as serine concentrations increase. Some strains like BCG-Japan and BCG-Russia show a slight growth increase at low concentrations of serine in media. The "y" axis scale has been adapted to maximise the resolution of each graph.

A



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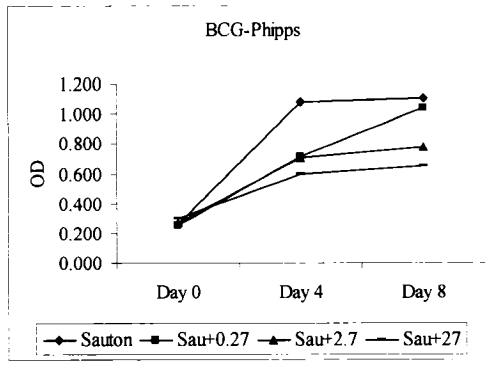

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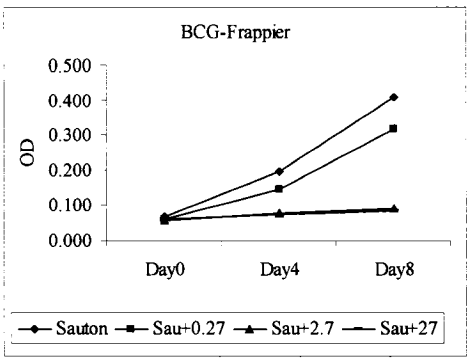

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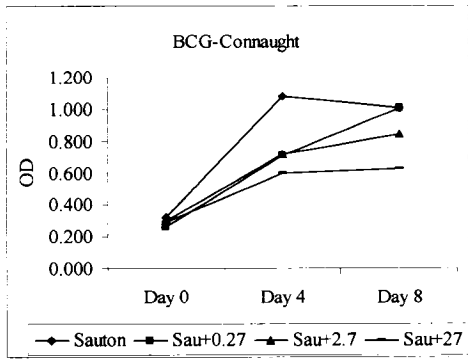

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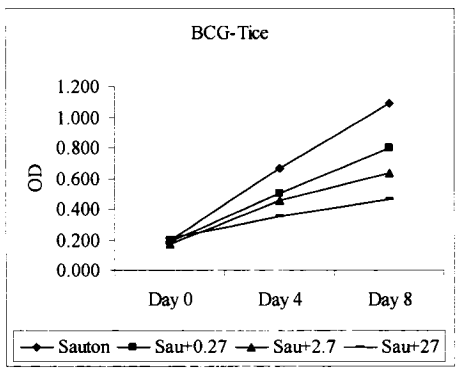

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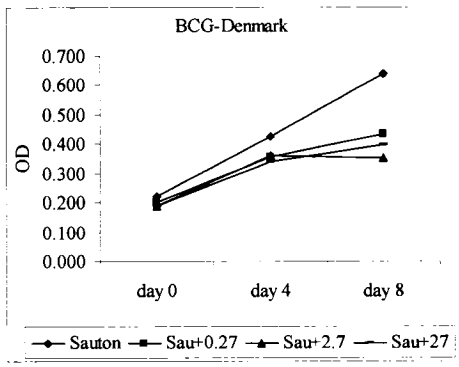
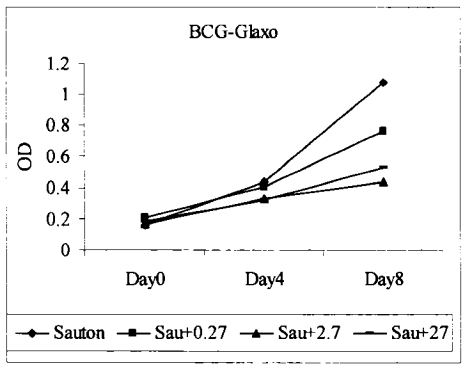


Figures 3-A to 3-F

G



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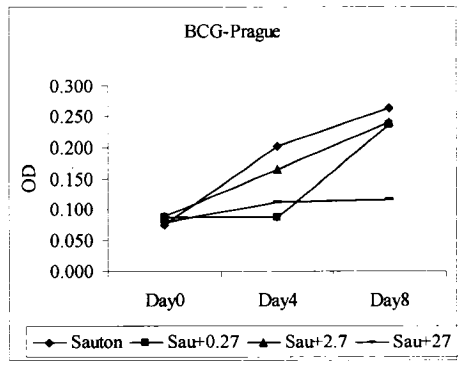

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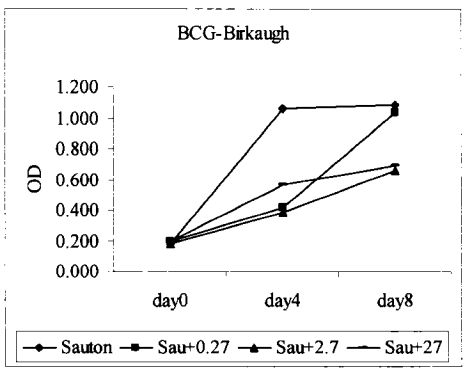

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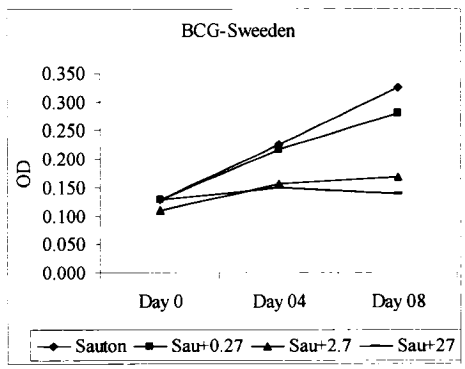

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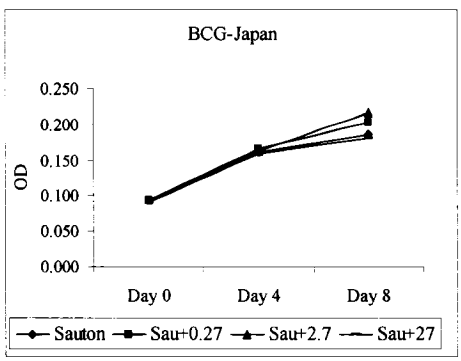

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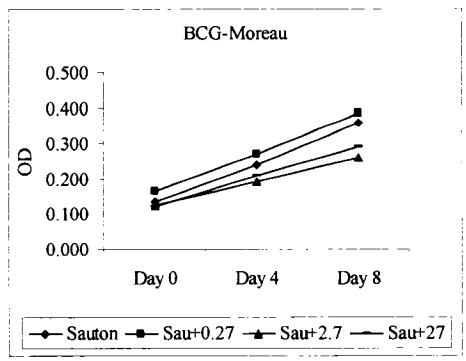
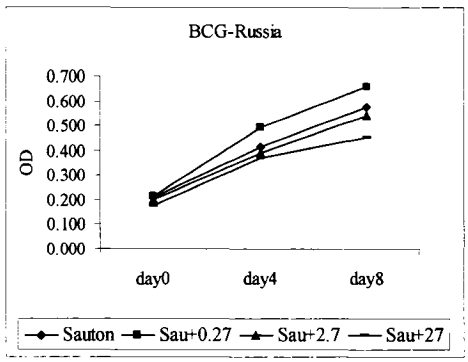


Figures 3-G to 3-L

M



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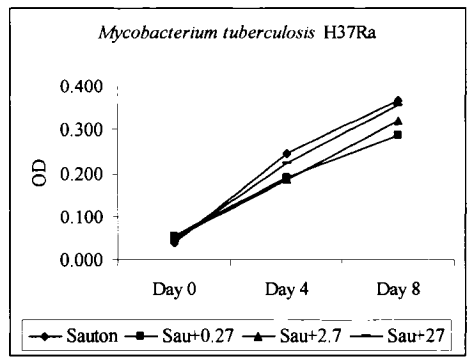


Figures 3-M and 3-N

Figure 3. Eight day growth follow-up of BCG strains and *M. tuberculosis* H37Ra on different concentrations of serine. Growth is graphed as raw OD_{600nm}. Each strain was grown on Sauton media containing either none, 0.27mM, 2.7mM, or 27mM serine. Readings were taken three times in 8 days. The "y" axis scale has been adapted to maximise the resolution of each graph.

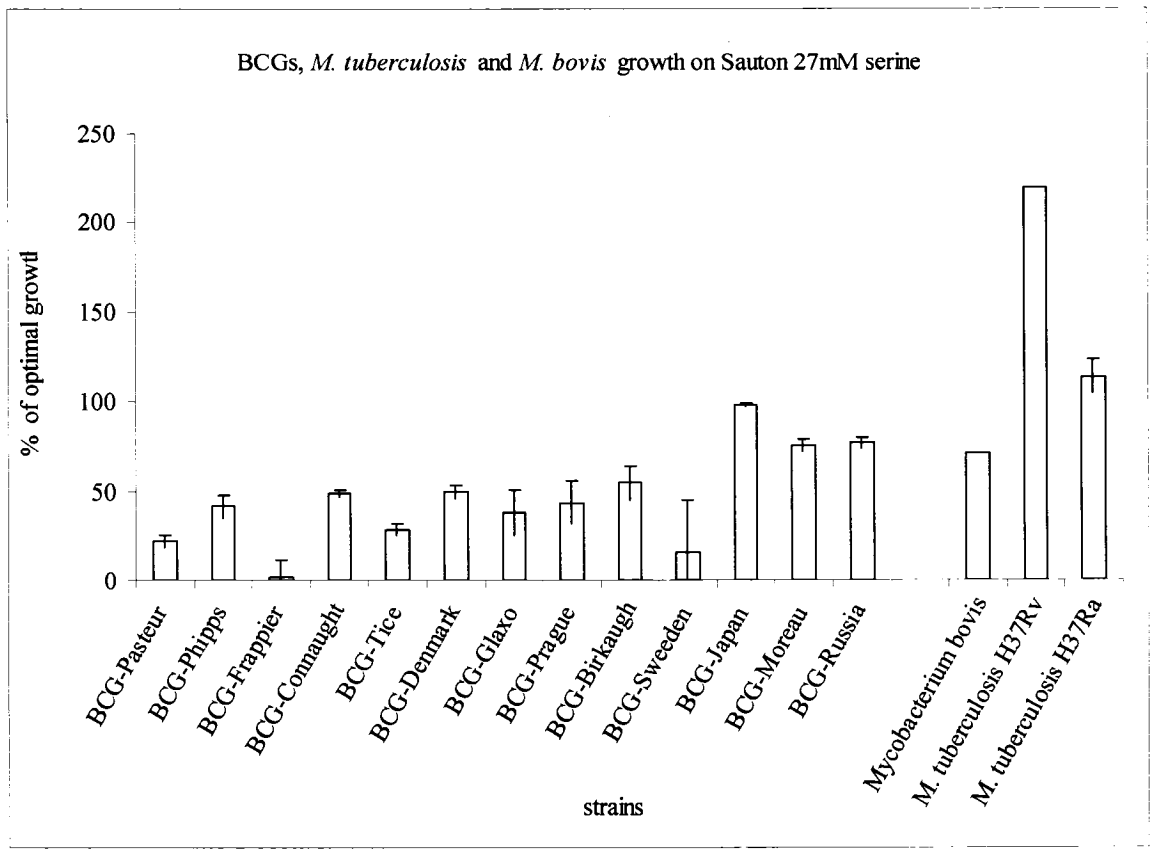


Figure 4. Comparative growths increase for all BCG strains, *M. tuberculosis* (strains H37Rv and H37Ra) and *M. bovis* on Sauton media 27mM serine. Growth is graphed as percentage of optimal growth over 12 days (as defined in the methods). BCG strains are presented along the x- axis in the chronological order they were obtained from the Pasteur Institute (right to left); there is no clear correlation between growth and year of acquisition.

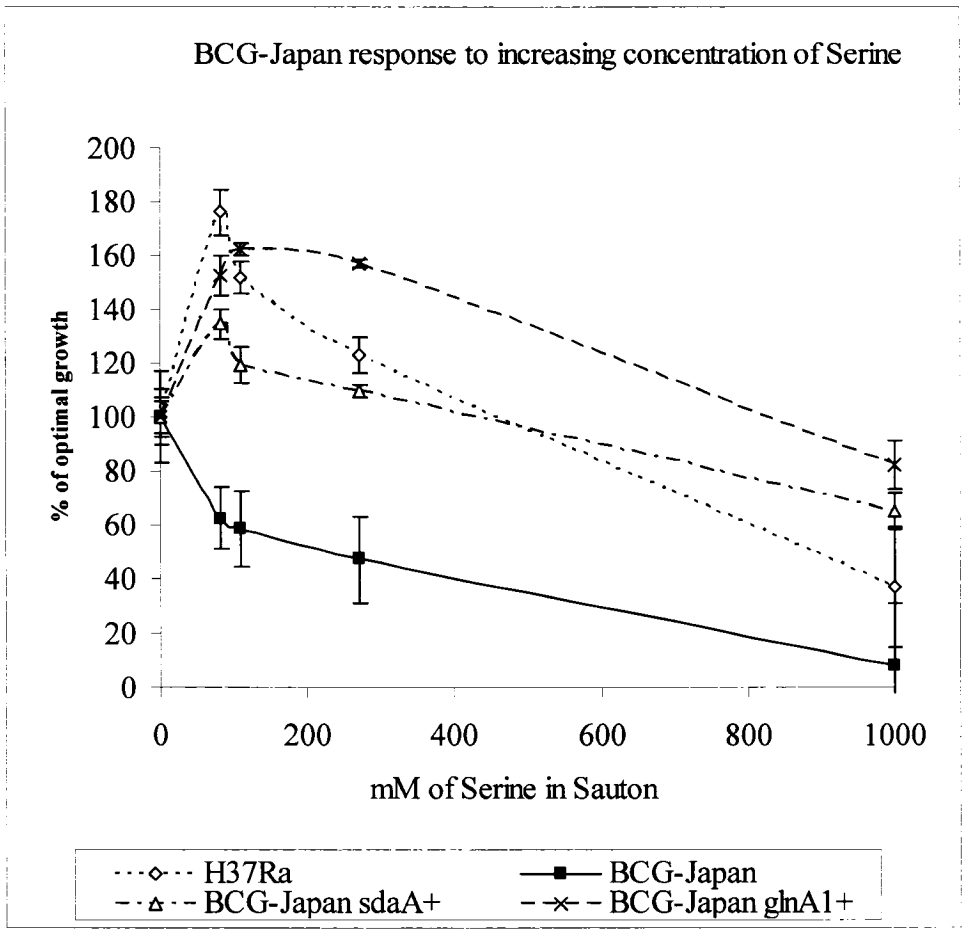


Figure 5. BCG-Japan response to increasing concentration of Serine BCG-Japan, correspondent strains complemented with either *sdaA* or *glnA1*, and *M. tuberculosis* H37Ra growth response to increasing concentrations of serine. Strains where grown on regular Sauton, as well as Sauton 81mM, 108mM, 270mM and 1M serine. Growth is graphed as percentage of optimal growth as defined in the methods. BCG-Japan wild type is the only strain that shows growth inhibition between 81-270mM serine.

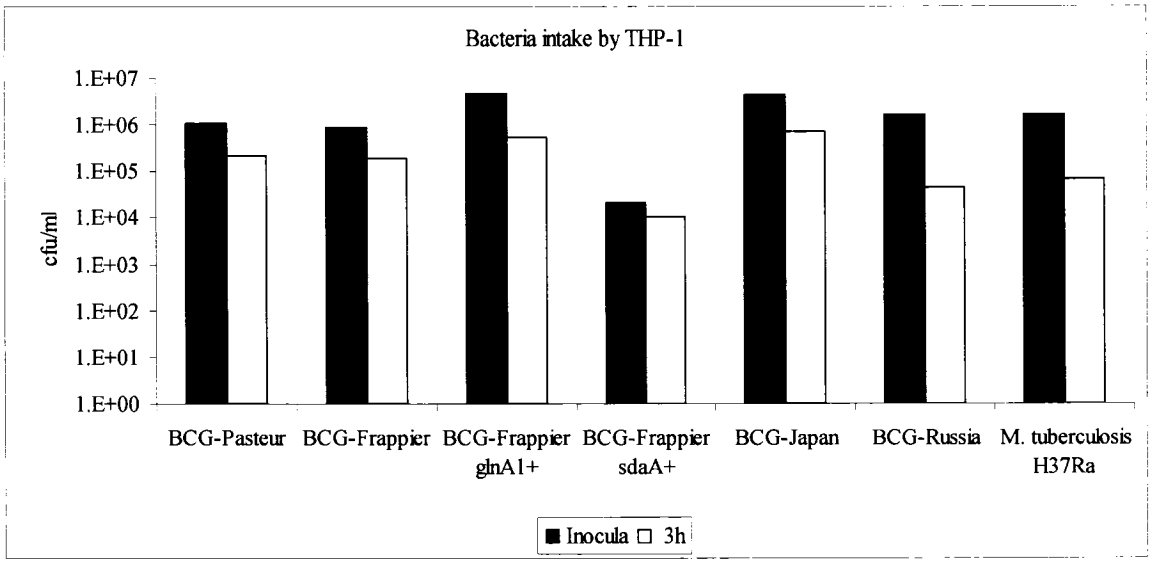
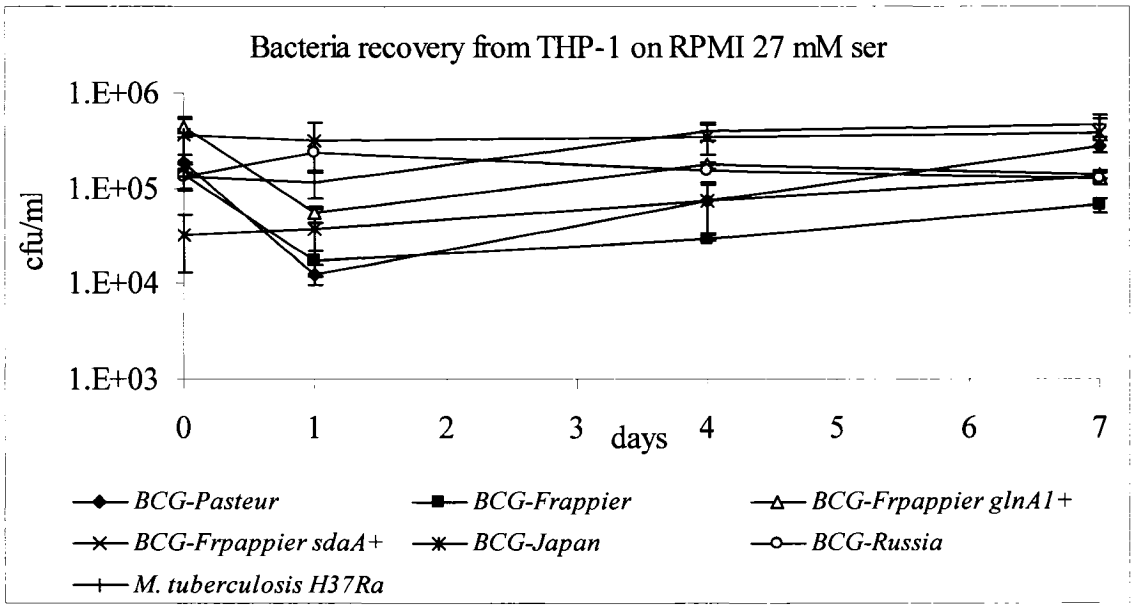


Figure 6. Bacterial uptake by THP-1 cells. Solid bars represent the cfu/ml as estimated by plating serial dilutions of the inocula. The open bars represent the cfu/ml as estimated by plating serial dilutions of the THP-1 cells lysate after 3h of infection. Bacterial suspensions were plated in 7H10 agar, and colonies were counted after 2-3 weeks incubation at 37°C. The results are the average of two independent experiments and Infection of THP-1 cells by BCG strains was effective

A



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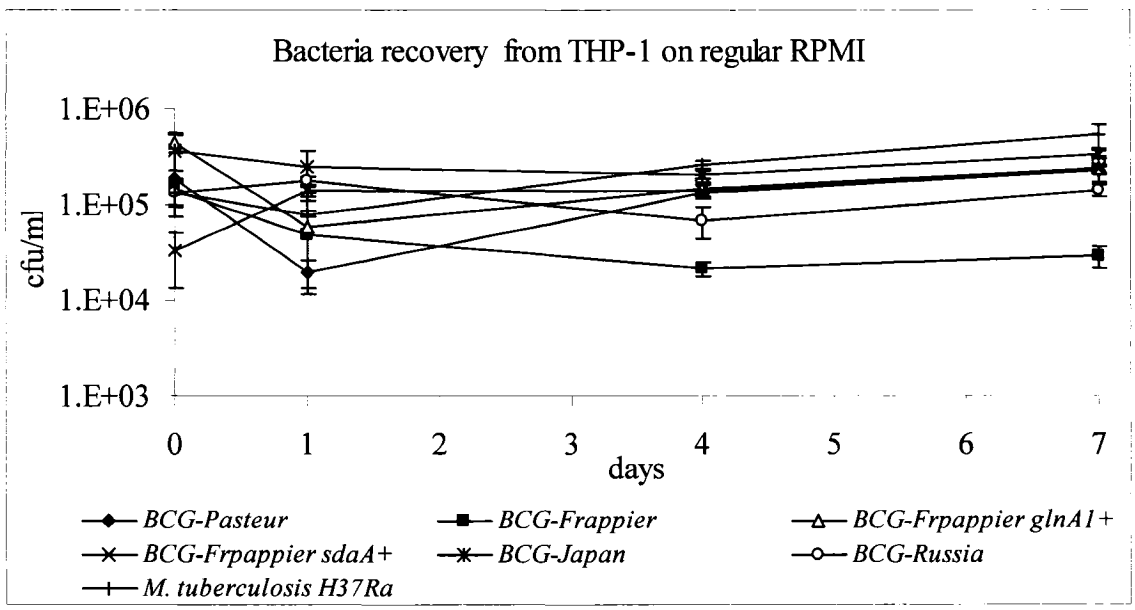


Figure 7A and B

C

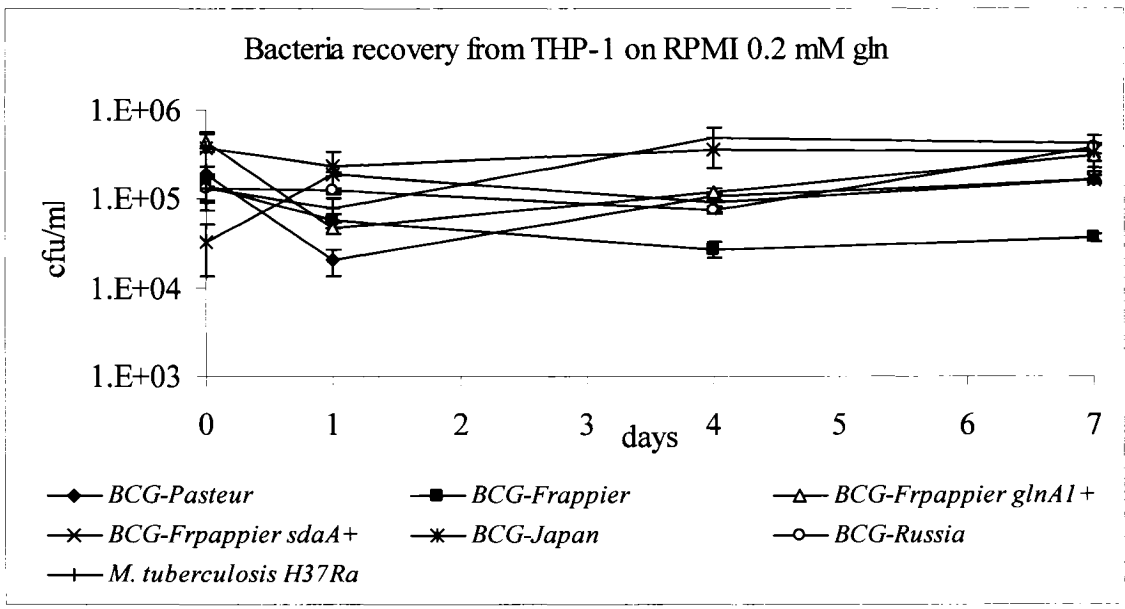
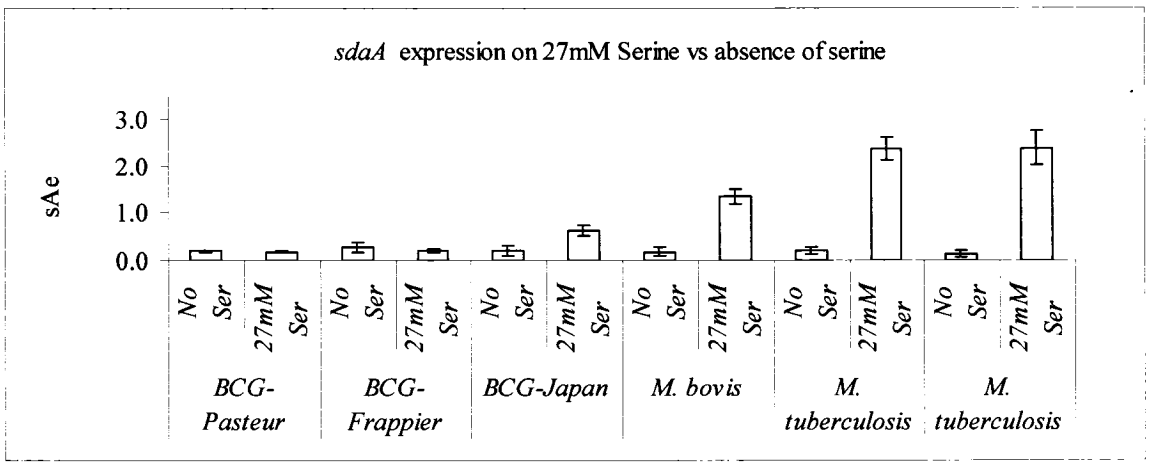


Figure 7C

Figure 7. Bacterial recovery from THP-1 cells infected with BCG strains and *M tuberculosis* H37Ra. The cfu/ml were estimated by plating serial dilutions of the THP-1 cells lysate after 3h (0 days), 1, 4 and 7 of infection. Bacterial suspensions were plated in 7H10 agar, and colonies were counted after 2-3 weeks incubation at 37°C. Results are averages of two independent experiments where duplicates were sampled.

A



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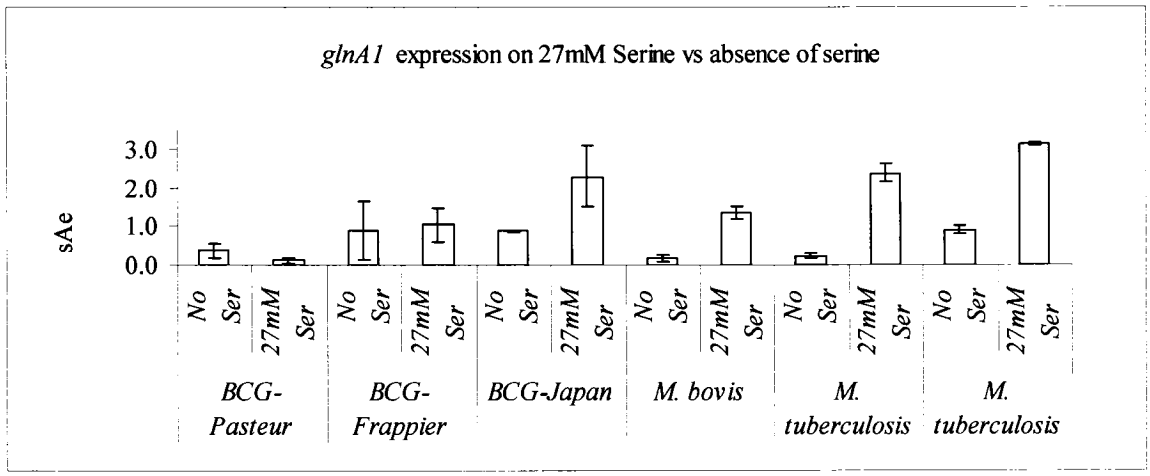


Figure 8 .Glutamine synthetase and serine deaminase gene transcription. Bars show the transcription of *sdaA* (figure 8A) and *glnA1* (figure 8B) genes. The strains tested were BCG-Pasteur, BCG-Frappier, BCG-Japan, *M. tuberculosis* (strains H37Rv and H37Ra) and *M. bovis*. These were grown in either regular Sauton media or Sauton 27mM serine. Results are expressed as *sigA* equivalents (defined in methods).

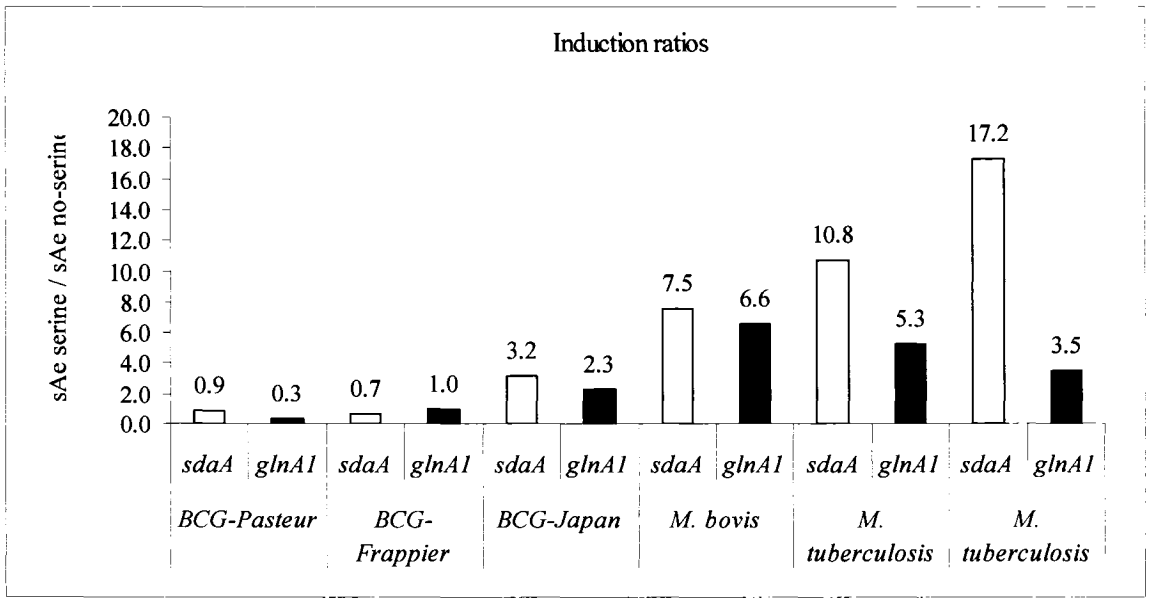


Figure 9. Glutamine synthetase and serine deaminase gene induction. Bars show the ratio of transcription between gene expression on Sauton 27mM serine and expression in regular Sauton media. The strains tested were BCG-Pasteur, BCG-Frappier, BCG-Japan, *M. tuberculosis* (strains H37Rv and H37Ra) and *M. bovis*.

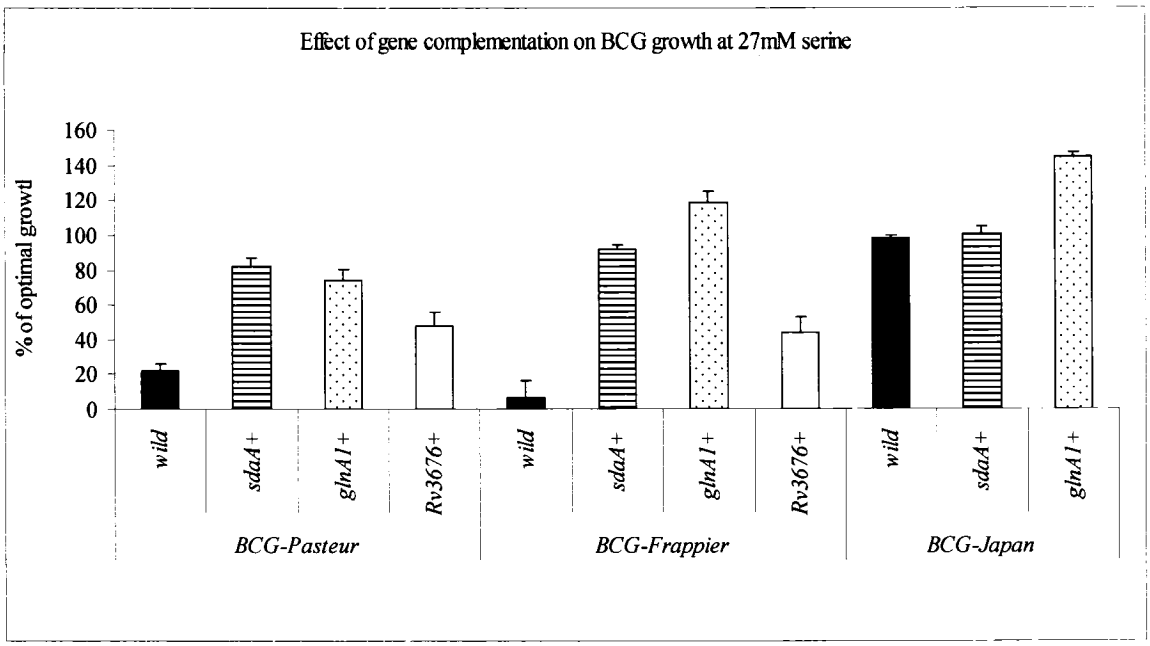
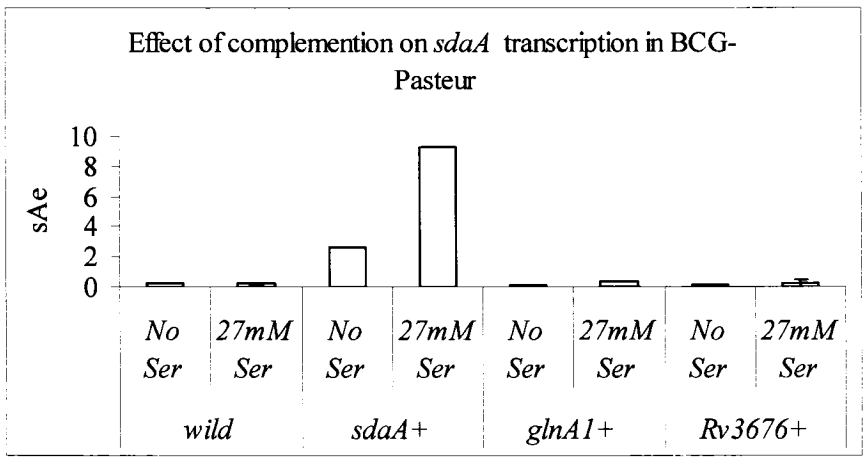
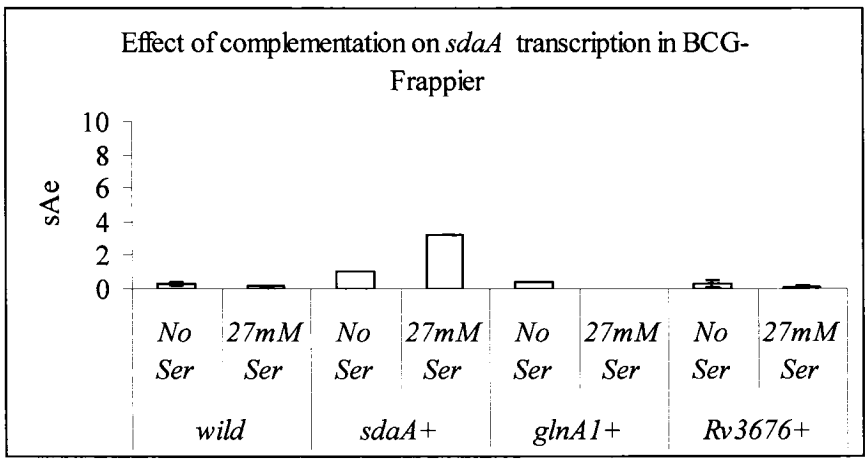


Figure 10. Effect of gene complementation on BCG strains growth at 27mM serine Genes *sdaA*, *glnA1*, or *Rv3676* from *M. tuberculosis*: were used individually, to plasmid complement BCG-Pasteur, BCG-Frappier, and BCG-Japan (not complemented with Rv3676). Growth is graphed as percentage of optimal growth 9as defined in methods) over 8 days. Strains complemented with either *sdaA* or *glnA1* recover growth near or equal to optimal. Complementation with Rv3676 renders about 40% recovery.





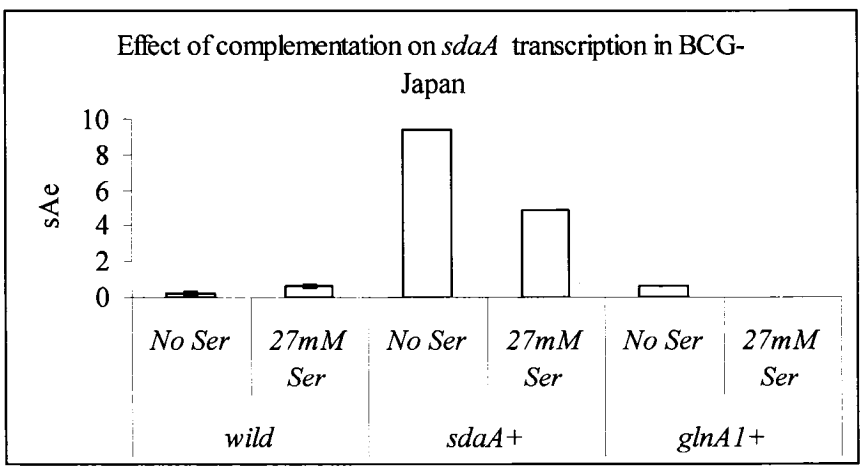
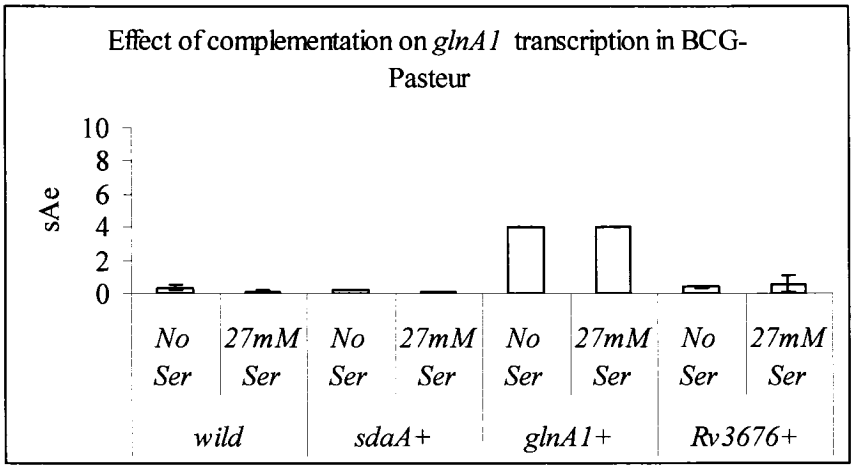
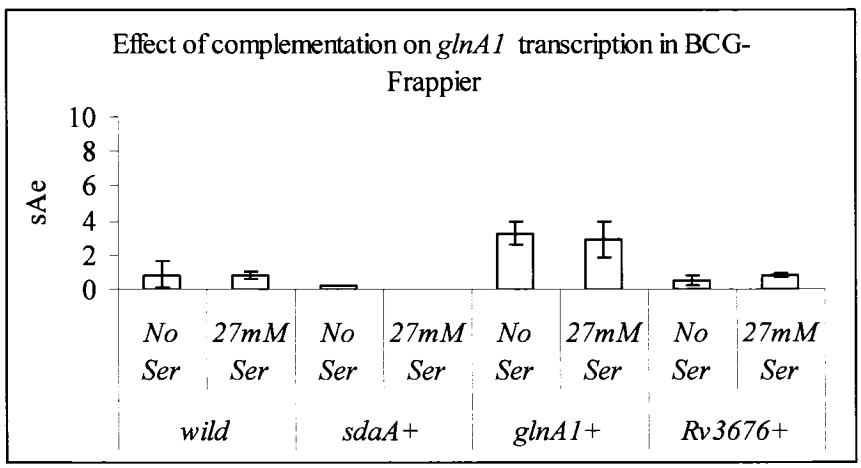


Figure 11. Effect of complementation on *sdaA* gene transcription in BCG strains Bars show the transcription of the *sdaA* gene. The strains tested were BCG-Pasteur, BCG-

Frappier, and BCG-Japan wild type as well as complemented with either *sdaA* or *glnA1*genes from *M. tuberculosis*. These were grown in either regular Sauton media or Sauton 27mM serine. Results are expressed as *sigA* equivalents (defined in methods).





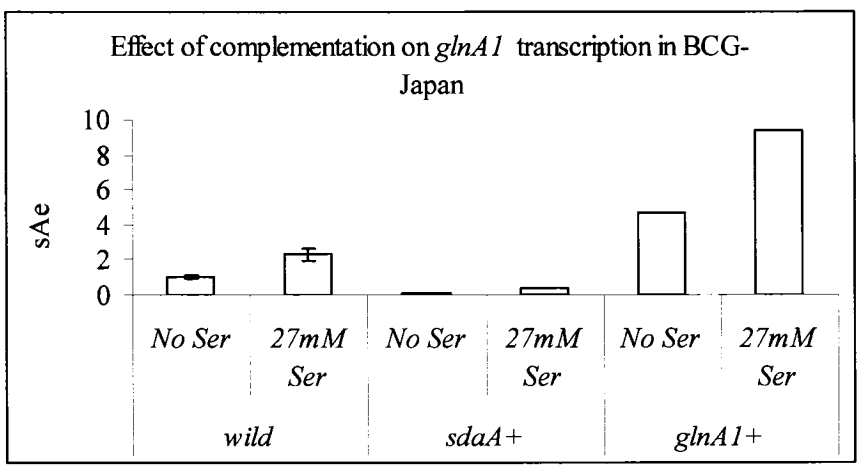
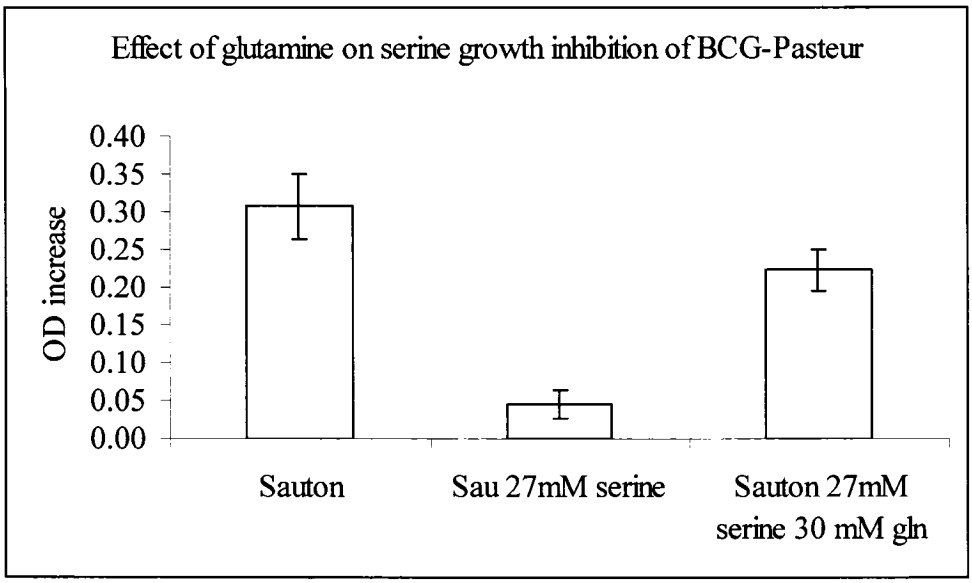


Figure 12. Effect of complementation on *glnA1* gene transcription in BCG strains Bars show the transcription of the *glnA1* gene. The strains tested were BCG-Pasteur, BCG-Frappier, and BCG-Japan wild type as well as complemented with either *sdaA* or *glnA1*genes from *M. tuberculosis*. These were grown in either regular Sauton media or Sauton 27mM serine. Results are expressed as *sigA* equivalents (defined in methods).

Gene	Protein function	Length (base pairs)	BCG sequenced compared to *M.tuberculosis* gene	Comparison by
amt	NH4 transporter	1434	*Rv2920c* : identical	On line databases
glnA1	Glutamine synthetase (GS)	1437	*Rv2220* : identical	Sequencing
gltB	Glutamate synthase (GOGAT) large subunit	4584	*Rv3859c* : identical	Sequencing
gltD	Glutamate synthase (GOGAT) small subunit	1467	*Rv3858c* : identical	On line databases
glnE	Adenylyltransferase (ATase)	2985	*Rv2221c* : identical	Sequencing
glnB	PII-type signal transduction	339	*Rv2919c* : identical	On line databases
glnD	Uridylyltransferase (UTase)	2427	*Rv2918c* : identical	On line databases
sigF	Sigma factor F (possibly related to N metabolism)	786	*Rv3286c* : identical	Sequencing
gdh	Glutamate dehydrogenase	4875	*Rv2476c* : identical	Sequencing
glyA2	Probable serine hydroxymethyl transferase (methylase)	1278	*Rv0070c* : identical	Sequencing
sdaA	Serine deaminase	1386	*Rv0069c* : identical	Sequencing (Chen *et al.*, 2003b)

Table 4. Sequence comparison of genes involved in nitrogen metabolism and serine clearance. Sequences are reported as identical if there are either no differences, or if only synonymous SNPs (single nucleotide polymorphism) were observed.

A



B

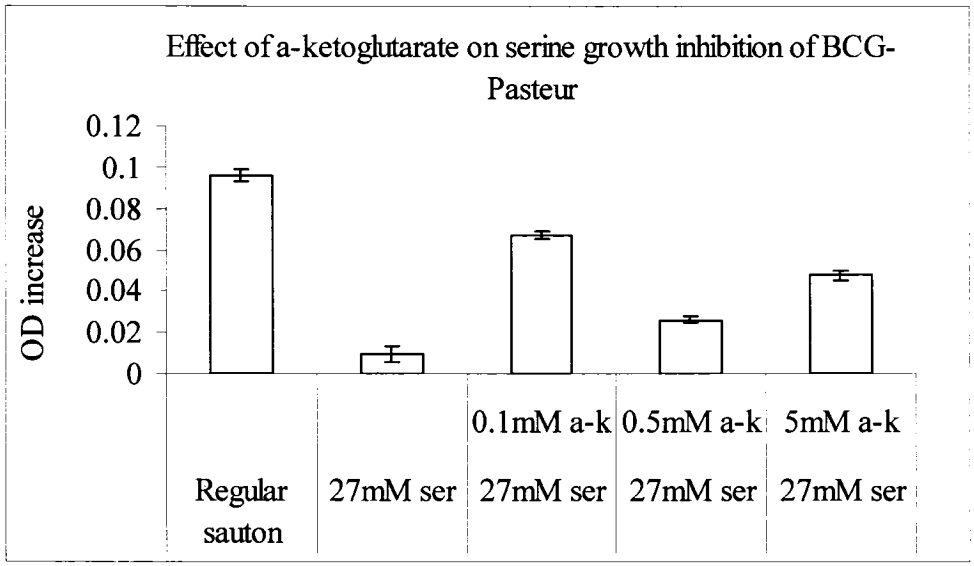


Figure 13. Effect of glutamine and alpha-ketoglutarate on growth inhibition of BCG-Pasteur caused by serine. OD_{600nm} . Each strains was grown on Sauton media containing either none, 27mM serine, 27mM serine and 30mM glutamine, 27mM serine and 0.1mM alpha-ketoglutarate, 27mM serine and 0.5mM alpha-ketoglutarate, or 27mM serine and 5mM alpha-ketoglutarate. . OD_{600nm} increase after 8 days is graphed. The presence of glutamine and alpha-ketoglutarate partially re-stores growth of BCG-Pasteur in Sauton media containing 27mM serine.

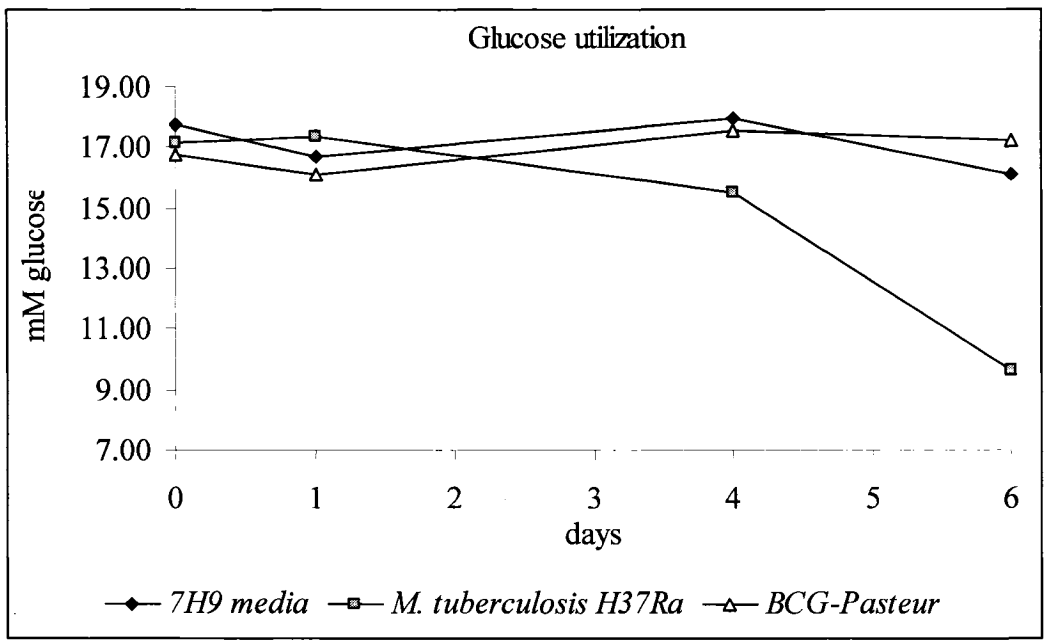


Figure 14. Glucose utilization by *Mycobacterium tuberculosis* and BCG-Pasteur. Graphed points are averages of two replicates. *M. tuberculosis* H37Ra and BCG-Pasteur were grown on 7H9 media. The glucose concentration in the growth media as well as in a sterile control was quantified at 0, 1, 4 and 6 days of incubation (at 37°C). The variation in glucose content of the BCG-Pasteur growth media is equivalent to that of the sterile 7H9 control, whereas the concentration of glucose in the *M. tuberculosis* H37Ra growth media after 6 days is about half of that of the control.

	NIT	PYZ	PYRA	PAL	BGUR	BGAL	AGLU	BNAG	ESC	URE	GEL	O	GLU	RIB	XYL	MAN	MAL	LAC	SAC	GLYG
BCG-Pasteur	-	+/-	-	-	-	-	-	-	+/-	+/-	+	-	-	+	-	-	-	-	-	-
BCG-Phipps	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	-	-	-	-	-	-
BCG-Frappier	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
BCG-Frappier Rv3676+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+/-	-	+/-	-	-	-
BCG-Connaught	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	-	-	-	-	-	-
BCG-Tice	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	-	-	-	-	-	-
BCG-Denmark	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	-	-	-	-	-	-
BCG-Glaxo	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	-	-	-	-	-	-
BCG-Prague	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
BCG-Birkhaug	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	+/-	-	-	-	-	-
BCG-Sweden	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	-	-	-	-	-	-
BCG-Japan	-	+/-	-	-	-	-	-	-	+/-	-	+	-	-	+	-	-	-	-	-	-
BCG-Moreau	-	-	-	-	-	-	-	-	-	-	+	-	-	+/-	-	-	-	-	-	-
BCG-Russia	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+/-	-	-	-	-	-
BCG-Russia Rv3676+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+/-	-	-	-	-	-
M. tuberculosis H37ra	+	+/-	-	-	-	-	-	-	+/-	+/-	+	-	+	+	+	+	+	+	+	-

Table 5. Metabolic profiles for all BCG strains and *M tuberculosis* H37Ra from API strips. Results are the consensus of 2 or 3 independent repeats. This test offers color-qualitative results, for example red is positive yellow is negative, when an intermediate result was obtained (i.e. orange) results were reported as +/-.

Discussion

After defining normal growth of BCG strains in rich media (figure 1), it was clear that most BCG strains presented growth inhibition with added serine. This impairment was accentuated as concentration increased (figure 2) and time progressed (figure 3). Together, these results indicated from the early stages of experimentation, that the phenomena studied (serine stress) could not be simply resolved (normal growth, proper gene induction) by the affected strains regardless of the intensity of the stimuli (serine concentration) or the time elapsed. In fact, the different BCG strains tested varied in their ability to grow on high serine concentrations. For example BCG-Pasteur, BCG-Frappier and BCG-Tice are unable to reach 50% optimal growth in 27mM serine whereas growth of BCG-Japan, BCG-Moreau and BCG-Russia can achieve almost optimal growth in such conditions (Figure 4). Together, the relationship between this growth pattern and the BCG phylogenetic tree (Figure 1LR) is not obvious (Behr and Small, 1999). The lack of concordance between response to serine and the history of BCG was not unexpected. Most BCG strains have individual genetic variations that may generate a unique metabolic profile. Bacteria are well known for genetic mechanisms of compensation and adaptation (e.g. functionally redundant metabolic pathways, and multiple copies of essential genes) (Riehle *et al.*, 2003;Bendt *et al.*, 2004;Kivisaar, 2003;Choi *et al.*, 2003). Therefore, the progressive accumulation of mutations may not necessarily imply an increasing loss of function.

To pursue the study of growth inhibition by serine we chose BCG-Pasteur, BCG-Frappier and BCG-Japan. As growth of the latter strain was not affected by 27mM serine

in media, we wondered if increasing the concentration of serine would reveal a similar growth defect as BCG-Pasteur and BCG-Frappier. As expected, at concentration of 81mM and higher the growth of BCG-Japan was sub-optimal, whereas *M. tuberculosis* H37Ra was able to grow as well or better than in the absence of serine (Figure 5).

As predicted from the literature review, serine deaminase and glutamine synthetase (GS) would be expected to have key roles in the event of excess serine. Serine deaminase (encoded by *sdaA*) breaks serine down into pyruvate and ammonia. GS (encoded by *glnA1*) is blocked by elevated serine therefore impairing glutamine synthesis (Liaw, Pan, and Eisenberg, 1993). Figure 13A shows that 30mM glutamine restores growth in high serine. In the presence of elevated concentrations of serine, an increase in the production of these two enzymes would be expected, as was observed with *M. tuberculosis* H37Rv and H37Ra strains (figure 8). Additionally, complementation with the *sdaA* or *glnA1* from *M. tuberculosis* H37Rv restored growth of BCG strains in 27mM serine (figure 10).

We approached the previous result with two interpretations: either BCG strains produce enzymes that are defective, or they are unable to properly regulate the expression of the corresponding genes. Although no formal biochemical studies of these two enzymes were done, the gene sequence results show no functional differences upon comparison to *M. tuberculosis* H37Rv (table 4, and Chen *et al.*, 2003). Also, complementation of these genes with the corresponding sequence from *M. tuberculosis* restored growth of BCG, arguing against a possible post-translational defect in the vaccine strains. The later suggests that the BCG genes encode functional enzymes. In contrast, our measurements of *sdaA* and *glnA1* gene transcription suggests that regulation is indeed defective (figure 9). The production of serine deaminase and GS mRNA appear invariable in BCG-Pasteur and BCG–Frappier (figure 8). The bacterial strains that were

able to grown on 27mM serine, at least doubled their transcription of both *sdaA* and *glnA1* under when serine concentration was increased to 27mM (figure 9).

The results of *sdaA* and *glnA1* gene transcription suggest that growth inhibition in high serine results from a gene regulation defect. As GS is involved in nitrogen regulation, we looked at genes implicated in nitrogen metabolism that could be mutated in BCG. We found that most sequences were intact in BCG (table 4). Our attention was then brought to the *Rv3676* gene homolog to CRP gene in *E. coli* which codes for cyclic AMP binding protein (Spreadbury *et al.*, 2005), and to *glxR* in *C. glutamicum* that codes for a repressor of the glyoxylate bypass genes (Kim *et al.*, 2004). *Rv3676* is a gene that could potentially be involved in both sugar utilization and nitrogen status sensing in mycobacteria. Several mutations in the *Rv3676* gene of BCG strains have been described previously (Spreadbury *et al.*, 2005). When BCG (Pasteur and Frappier) were complemented with *Rv3676* from *M. tuberculosis* H37Rv about 40% of optimal growth on 27mM serine is recovered. This incomplete phenotype either suggests that *Rv3676* mutations are only partially responsible for the observed effect, or that the levels of expression of *Rv3676* were insufficient to completely restore growth. Therefore, this experiment will require repetition with an over-expression plasmid that produces higher quantities of the *Rv3676* gene product.

To explore the mechanism by which *Rv3676* complementation might overcome growth inhibition by serine, we considered changes in the regulation of the genes implicated. In *E.coli*, CRP has also been related to regulation of *glnA1* (codes for GS) (Tian *et al.*, 2001). Additionally, upregulation of *glnA* and *glnK* (codes for PII) has been shown to depend on carbon and nitrogen sources. Moreover, it has been proposed that the

CRP-cAMP complex may have a role in regulation of all genes under NtrC control (global N metabolism genes regulator in *E. coli*) (Maheswaran and Forchhammer, 2003). To test this possibility, we determined whether complementation with *Rv3676* affected the expression of the other genes studied (*sdaA* and *glnA1*). RT-PCR results showed that complementation with *Rv3676* had no measurable effect on *sdaA* or *glnA1* transcription. In fact only complementation with *sdaA* affects the overall *sdaA* transcription (figure 11). Complementation with *glnA1* increases exclusively *glnA1* mRNA levels (figure 12).

The reason for the lack of expression effect remains to be further understood. One possibility is that extra-chromosomal complementation of *Rv3676* was not sufficient to produce a measurable effect in gene transcription, yet produced a modest effect on *Rv3676* levels enough to produce a moderate improvement in growth. Additionally the effect of *Rv3676* complementation on the transcription of other genes may be more pronounced at earlier time points, not measured in our studies. To continue this line of research, efforts in the laboratory are underway to complement BCG strains with *Rv3676*, under the control of a high transcription promoter and in a chromosome integrating plasmid. Direct measurements of *Rv3676* gene transcription will be done to address the question of how levels of *Rv3676* mRNA relate to growth on high serine.

The mechanism by which *Rv3676* could be implicated in nitrogen sensing remains to be elucidated. Interestingly, alpha-ketoglutarate, which has an important role in directing carbon sources into the different shunts of the CAC, is also involved in N status sensing. In *E. coli*, elevated alpha-ketoglutarate starts a series of molecular interactions that induce NtrC-controlled gene transcription (see literature review). The closest homolog to the *Rv3676* gene in *C. glutamicum* is *glxR*, and its product has shown regulatory activity over the enzymes of the glyoxylate shunt (Kim *et al.*, 2004). When the

expression of these enzymes is upregulated the glyoxylate shunt is favoured and alpha-ketoglutarate production is bypassed. Consistent with this, supplementation of 27mM serine media with 0.1mM alpha-ketoglutarate restored growth of BCG-Pasteur (figure 13B). However the effect varied slightly at higher concentrations of alpha-ketoglutarate. These results suggest that alpha-ketogluatarate levels have a role in overcoming stress caused by excess serine. Most likely, the addition of alpha-ketoglutarate facilitates the induction of nitrogen metabolism genes, such as *glnA1* by restoring the adequate alpha-ketoglutarate/glutamine ratio.

Alpha-ketoglutarate affects gene expression by interaction with a PII type protein which then influences the gene regulator protein in most nitrogen regulation systems (see literature review). The interplay between alpha-ketoglutarate and PII type proteins seems to be a common feature throughout most organisms. The outcome of these interactions varies from one system to another and as a function of alpha-ketoglutarate concentrations (Ninfa and Jiang, 2005). Furthermore, the ways in which PII controls gene expression diverge between organisms. In *E coli*, when alpha-ketoglutarate is low, PII binds to NtrC, and N metabolism gene expression is repressed (reviewed by Burkovski, 2003). Whereas in *C. glutamicum*, binding of the global N gene regulator protein (AmtR) to PII induces N metabolism gene expression (Beckers *et al.*, 2005). The effects of the interaction between alpha-ketoglutarate and PII in *C. glutamicum* have not been described yet. Therefore, we speculate that alpha-ketoglutarate induces expression of N metabolism genes in BCG, under the assumption that regulation of N metabolism genes in mycobacteria and *E. coli* is similar.

Because CRP (*Rv3676* homolog in *E. coli*) is widely accepted as a global regulator of carbohydrate metabolism (see literature review), we also wonder if BCG could be

defective in glucose usage. Both "API strips" (table 5) and glucometer readings (figure 14) show that BCG strains are unable to utilize glucose. These results were consistent with data reported previously (Florczyk *et al.*, 2003). *M. tuberculosis* is able to utilize all sugars offered. When BCG strains (Frappier and Russia) are complemented with *Rv3676* the capacity of fermenting glucose on an "API strip" is restored. The results presented above suggest that BCG strains have a defect in gene regulation associated with mutations in the *Rv3676* global regulator gene that produce both impaired nitrogen status sensing and defective sugar utilization.

Regarding defects in nitrogen status sensing, a study on post-translational regulation of GS in *M. tuberculosis* noted that BCG-Russia's capability to modify levels of adenylylated GS as a function of nitrogen availability in media was impaired (Mehta *et al.*, 2004). Together with our results showing a failure of BCG strains to increase *glnA1* expression, one attractive explanation is that BCG strains suffer from a fundamental defect in nitrogen sensing. There are no analogs of *ntrC* or *amtR* in mycobacteria, but if *M. tuberculosis* has a "global nitrogen-metabolism genes regulator", as seen in *E. coli* and *C. glutamicum*, a defect in this as yet unknown regulator in BCG strains could hypothetically explain the lack of regulation of GS at both the transcriptional and the post-translational levels. As we have looked without success for mutations in BCG genes related to nitrogen metabolism and its regulation, the best candidate to explain our observations is the mutated *Rv3676* (Spreadbury *et al.*, 2005).

Regulatory defects in BCG are not exclusive to nitrogen metabolism. While the attenuation of BCG strains has been linked to the loss of the RD1 region (Lewis *et al.*, 2003;Pym *et al.*, 2003), regulatory defects have also been postulated to contribute to the attenuated phenotype of BCG strains (Mahairas *et al.*, 1996). During the evolution of

BCG strains, changes to putative regulators figure prominently, including the duplication of genes encoding two sigma factors, the deletion of *Rv3405c* in both BCG-Brazil and BCG-Japan, and the deletion of *sigI* in all strains obtained after 1933 (Brosch *et al.*, 2000;Lewis *et al.*, 2003;Mostowy *et al.*, 2003;Behr *et al.*, 1999). Important "single nucleotide polymorphisms" (SNPs) have been identified in regulatory genes of the different BCG strains (Behr *et al.*, 2000;Spreadbury *et al.*, 2005;Charlet *et al.*, 2005). Noteworthy are the mutations in *Rv3676* discussed here, and a SNP in the starting codon of the *sigK* gene that are related to low production of important antigenic proteins (MPB70 and MPB83) (Charlet *et al.*, 2005).

It is possible that this collection of regulatory defects may have some impact on the fitness of BCG strains in the host. As vaccines, BCG strains should be attenuated enough not to cause disease, but must retain some capacity to survive introduction into the host in order to synthesize and present relevant antigenic proteins to the host immune system. Gene regulation is likely important for fine tuning metabolic function that will allow a live attenuated vaccine to successfully survive and replicate in the host. As a first attempt to address the importance of these regulatory defects *in vivo*, we looked at BCG survival in THP-1cells. While no significant difference between BCG strains and *M. tuberculosis* H37Ra were found (figure 7), we suggest that changes in the experimental design may produce alternative results. Personal communications with peers suggest that a higher MOI may help distinguish phenotypes.

The relevance of mutations in regulatory genes incurred by BCG strains is the subject of ongoing investigation in our laboratory, and new experiments assessing the relevance of BCG regulatory defects *in vivo* are an important part of the future directions.

General Conclusions

- Serine concentrations in growth media of 27mM inhibit the growth of some BCG strains (e.g. BCG-Pasteur, BCG-Frappier). The growth of other BCG strains (e.g. BCG-Japan) as well as *M. tuberculosis* strains (H37Rv and H37Ra) and *M. bovis* is not affected by such conditions.
- Poor growth in 27mM serine was related to *sdaA* and *glnA1* transcription induction failure. Optimal growth on 27mM serine, was associated to high *sdaA* and *glnA1* induction rates (>2) and not to a specific quantity (threshold) of mRNA.
- Gene complementation with *sdaA*, *glnA,* or *Rv3676* from *M. tuberculosis* restores BCG strains growth on 27mM serine. Supplementation of serine containing media with alpha-ketoglutarate or glutamine enabled BCG strains to grow optimally.
- None of the nitrogen metabolism related genes sequenced revealed any significant mutations in BCG.
- The BCG strains studied are unable to utilize glucose in the conditions tested (as opposed to *M. tuberculosis* H37Ra). But when BCG strains are complemented with *Rv3676* from *M. tuberculosis*, this impairment is alleviated.
- The observations above suggest that BCG strains have a defect in gene regulation associated with mutations in the *Rv3676* global regulator gene, which produces impaired nitrogen status sensing and regulation as well as a defect in sugar utilization.

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