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# The Role of the Glyoxylate Cycle in the Pathogenesis of Mycobacterium tuberculosis

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# The Role of the Glyoxylate Cycle in the Pathogenesis of *Mycobacterium tuberculosis*

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# ABSTRACT

According to the World Health Organization a third of the world's population is infected with *Mycobacterium tuberculosis*. The unparalleled success of *M. tuberculosis* as a pathogen reflects the bacterium's extraordinary ability to persist in its host in spite of eliciting a robust immune response. Currently available treatment is inadequate and drug resistance is rapidly spreading. New antibiotics are desperately needed. The substrates and metabolic pathways utilized by pathogens during infection are largely unknown and represent an under-exploited area of investigation. Uniquely, evolution of the genus *Mycobacterium* has involved extensive duplication of fatty acid metabolism genes, including two homologs encoding prokaryotic- and eukaryotic-like isoforms of the glyoxylate cycle enzyme isocitrate lyase (ICL). The glyoxylate cycle is employed by cells when fatty acids are the main carbon source available. Here, we show that these enzymes are jointly required by *M. tuberculosis* for growth on fatty acids and for virulence in experimental infections. Although deletion of *icl1* or *icl2* had little impact on replication of *M. tuberculosis* in macrophages and mice, deletion of both genes abrogated intracellular growth, and resulted in rapid bacterial clearance from the lungs. A dual-specificity ICL inhibitor similarly blocked replication of *M. tuberculosis* on fatty acids *in vitro* and in macrophages. The absence of ICL orthologs in mammals, and recent findings implicating the glyoxylate pathway in the virulence of other bacterial and fungal pathogens makes this metabolic pathway an attractive novel target for drug development.

**A Roxana Elías, mi madre, por su infinita abnegación y amor…**

**"When men lack a sense of awe, there will be disaster."**

**Lao Tsu**

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# PART I

# CHAPTER 1

## Bacterial Persistence: Strategies for Survival

### INTRODUCTION

Most bacteria cause acute infections that cannot withstand the onslaught of the host's adaptive immune response. Less commonly, some bacteria such as *Salmonella typhi* and *Mycobacterium tuberculosis* cause infections that persist, often for the lifetime of the host. Persistence is responsible for the unusual epidemic characteristics of diseases like tuberculosis. A relevant metric is the "critical community size" (CCS) required for endemicity. The theoretical and observed CCS for measles virus is a staggering 250,000 individuals; in smaller populations, measles self-extinguishes after exhausting the pool of naive susceptible hosts (Keeling & Grenfell 1997; Keeling, 1997). Human populations on this scale have existed for only a few thousand years, suggesting that measles is a newcomer to the human niche. This view is supported by field studies of epidemiologist Francis Black, who found that imported measles did not persist in small, isolated human populations — e.g., in the South American Amazon basin

(Black, 1975). In contrast, tuberculosis was endemic in many of these smaller communities. Modeling studies put the CCS for tuberculosis at less than 500 individuals (McGrath, 1988), perhaps explaining its antiquity as a human disease (Daniel *et al.* 1994; Haas & Haas 1996). Persistence is also reflected in the contrasting epidemic dynamics of diseases caused by persistent and non-persistent pathogens — e.g., measles epidemics wax and wane over wks or months (Fig. 1.1A) (Keeling, 1997), whereas tuberculosis epidemics span centuries (Fig. 1.1B) (Grigg, 1958; Blower *et al.*, 1995).

*M. tuberculosis* is not unique in its ability to cause persistent infections. Indeed, the roster of persistent pathogens continues to lengthen as technological advances allow the identification of previously unknown species. An important example is the recognition that gastritis, gastric and duodenal ulcers, and gastric neoplasms and lymphomas are often linked to colonization of the human stomach by *Helicobacter pylori* (Dubois *et al.*, 2000). Via mechanisms that are only partly understood, this Gram-negative spiral bacterium has evolved to exploit a niche that was assumed to be devoid of microbial life. This is a remarkably successful strategy — by current estimates, the stomachs of half the global human population harbor *H. pylori*, often for the lifetime of the host (Blaser & Atherton, 2004). As the list of persistent pathogens grows, so does our appreciation of the diversity and sophistication of the mechanisms that allow these microbial invaders to survive "in the face of opposition or adversity." A comprehensive survey of persistent

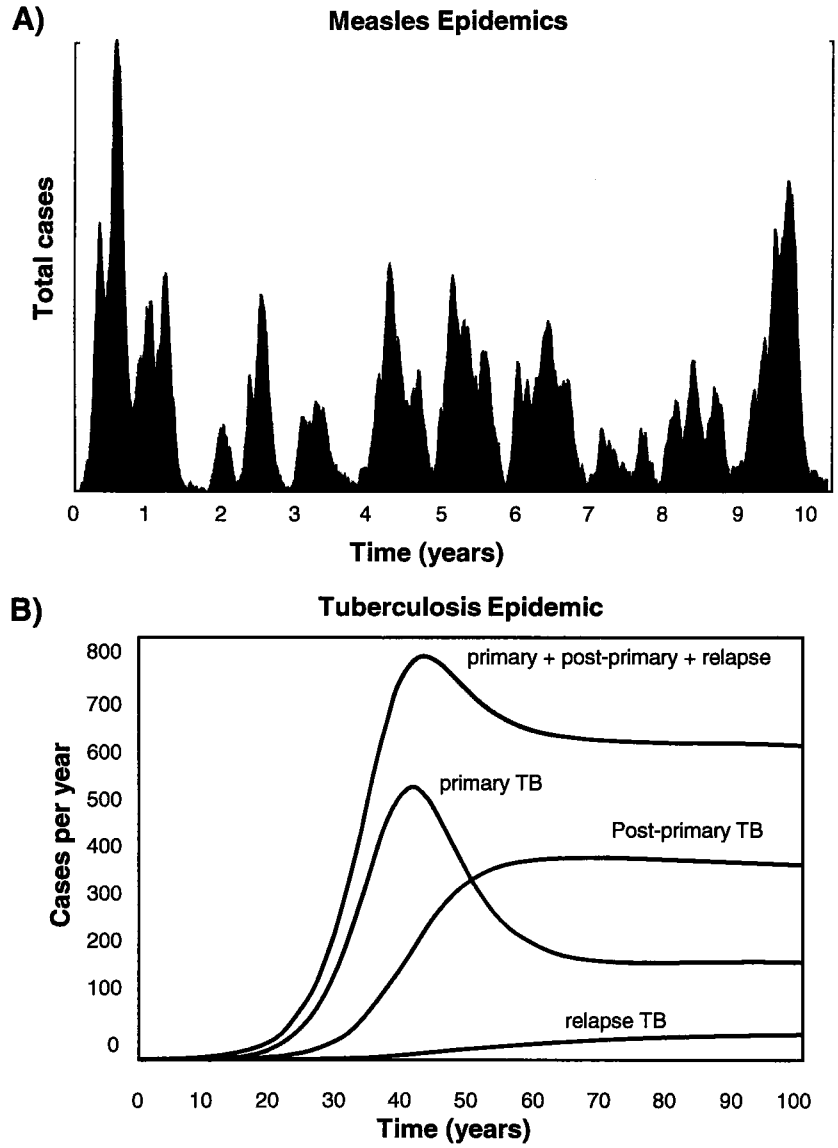


**Fig. 1.1 Contrasting epidemic dynamics of measles and tuberculosis.** (A) Simulation of a measles epidemic initiated by entering one infectious case at time zero into a susceptible population of 500,000. The simulation illustrates successive waves at ~6-12 month intervals (Keeling, 1997). (B) Simulation of a tuberculosis epidemic initiated by entering one infectious case at time zero into a population of 200,000. It illustrates the relative contribution of 3 types of disease to the overall incidence: progressive primary, post-primary, and relapse tuberculosis. A fourth type, exogenous reinfection (not included), presumably would increase the peak level and duration of the epidemic. Redrawn from Blower et al. (1995). The contrasting epidemic dynamics of measles and tuberculosis are also reflected in the "critical community size" (CCS) required for endemicity, which is roughly 250,000 for measles (Keeling, 1997) and 500 or less for tuberculosis (McGrath, 1988).

bacterial pathogens is beyond the scope of this chapter, which will focus on a few organisms as paradigms of persistence strategies.

## 1.1. *Treponema pallidum* — Why run if you can hide?

Several spirochetes of the genus *Treponema* cause persistent infections in humans, including *Treponema pallidum*, the etiologic agent of syphilis. Spirochetes are a phylogenetically distinct group of prokaryotes with an outer membrane and a periplasmic space, similar to the Gram-negatives; however, the outer membrane of treponemes has a higher lipid:protein ratio and lacks the pro-inflammatory constituent lipopolysaccharide (LPS) (Radolf *et al.*, 1989; Radolf, 1995). The periplasmic space contains the flagella of these highly motile, slender, and elongated "corkscrew" bacteria. Syphilis was first recognized in the late fifteenth century after the first European explorers returned from the Americas, where the disease was probably endemic (Quétel, 1990). When syphilis first erupted in Europe, it was highly virulent and usually fatal. As the spirochete spread throughout Europe it was rapidly attenuated, and a milder, more chronic form of disease became dominant. Why? It may be that the attenuated strains were favored because the horrific manifestations of the early, virulent form of syphilis were a deterrent to sexual transmission. It is also tempting to speculate that attenuated treponemes enjoyed a selective advantage because prolonged survival of the infected host ensured greater opportunity for transmission. Field evidence

in favor of this idea derives from the epidemic of myxomatosis that was introduced into the feral rabbit population of Australia in 1950/51 (Fenner & Fantini, 1999). Following an initial period of high host mortality, attenuated strains of myxoma virus emerged and became dominant. Rabbits infected with attenuated viruses were protected against superinfection with the virulent parental virus, and, because they lived longer and were more active, dissemination of the attenuated strains was favored.

The incidence of syphilis has declined steeply since the introduction of penicillin in 1943. However, the disease continues to pose a significant health problem worldwide. The steady decline in the incidence of syphilis from 1991 to the present in the United States should not necessarily be interpreted as a victory for public health because syphilis epidemics display naturally periodic dynamics with approximately ten-year cycles. Recently, these cycles, which previously had been attributed to changes in "environmental" factors such as sexual behavior, have been shown to be due to intrinsic host immunity-pathogen interactions (Grassly *et al.,* 2005). The immunity that follows syphilis, though imperfect, allows for exhaustion of susceptible individuals after an epidemic, leading to a decline in incidence (Morgan *et al.,* 2003). However, this period is typically followed by a surge in the number of cases as a new supply of susceptible individuals becomes available. The periodic dynamics of syphilis epidemics underscores the need for unceasing vigilance in case detection and treatment (Garnett & Brunham, 1999).

Syphilis is a slowly progressing, multistage disease (Singh & Romanowski, 1999). Infection is transmitted horizontally by sexual contact and vertically by transplacental migration of treponemes. During sexual intercourse, spirochetes present in the genital tract of the donor invade the recipient through small abrasions in the mucous membranes or skin of the genitalia. Replication of the organism at the site of entry triggers massive leukocyte infiltration, blood vessel damage, and tissue necrosis, resulting in the appearance of a primary lesion or "chancre." In most patients, the chancre heals spontaneously after a few weeks and disease progression is arrested. In the less fortunate minority, dissemination from the primary lesion occurs a few weeks after initial infection, leading to generalized infection of almost every tissue of the body, with a notable tropism for the vascular system. The onset of secondary syphilis is marked by the appearance of nodular lesions or papules at these metastatic sites of infection. In most cases of primary and secondary syphilis, infection is resolved spontaneously by the adaptive immune response usually in less than 6 months post-exposure (Garnett *et al.,* 1997). However, small populations of treponemes may persist in a few isolated lesions and eventually give rise to tertiary syphilis, a chronic disease that usually endures for the lifetime of the host. Treatment of primary or secondary syphilis with penicillin is highly effective, but once disease progresses to the tertiary or latent stages it becomes refractory (Ghinsberg & Nitzan, 1992). Tertiary syphilis is characterized by the persistence of mature granulomatous lesions ("gummas") containing small numbers of spirochetes. Gummas may occur in

almost any organ system, causing gradual physiological deterioration. Persistent infection of the CNS can lead to destruction of neural tissue, loss of motor activity, and paresthesia, as in the jerking movements and paroxysmal pains that are characteristic of *tabes dorsalis* (sclerosis of the posterior columns of the spinal cord). Gummas occurring in other regions of the CNS may give rise to the most dreaded manifestations of syphilis — insanity and generalized paralysis. It is not clear how treponemes damage tissues. No bacterial toxins have been identified, and it is possible that the pathology is due to chronic inflammatory host responses to persistent spirochetes.

In the pre-chemotherapy era, longitudinal studies of untreated syphilis revealed that the great majority of patients do not progress beyond the primary stage (Gjestlaand, 1955), and reinoculation studies established that syphilitic patients were partially protected against reinfection (Fitzgerald, 1981). Both humoral and cellular adaptive immune responses play a role in resistance to *T. pallidum* (Rich *et al.,* 1933). Passive immunization of rabbits with immune serum before infection with *T. pallidum* delays the appearance of syphilitic lesions and attenuates their severity (Bishop & Miller, 1976a), and resistance to re-infection is correlated with the appearance of anti-treponemal antibodies (Bishop & Miller, 1976b). Antibodies from immune rabbit serum promote complement-mediated killing of treponemes (Blanco *et al.,* 1984) and block the interaction of *T. pallidum* with extracellular matrix components (fibronectin, laminin, and collagen), which could interfere with the invasion and dissemination of spirochetes (Fitzgerald *et*

*al.,* 1984). Syphilitic patients typically develop IgM and IgG responses to a slew of treponemal antigens (Baker-Zander *et al.,* 1985).

A role for cellular immunity is suggested by the dramatic infiltration of lesions by mononuclear leukocytes, which occurs as early as one week post-infection in the rabbit model (Lukehart *et al.,* 1980a; Sell *et al.,* 1980). The cellular infiltration peaks by two weeks post-infection and is composed predominantly of mononuclear phagocytes and T cells, with *Treponema*-specific T cells emerging early in the course of infection (Lukehart *et al.,* 1980b; Baker-Zander *et al.,* 1988; Arroll *et al.,* 1999). Lesions of syphilitic patients contain activated $CD8^+$ cytolytic T lymphocytes (van Voorhis *et al.,* 1996a) and show evidence of a dominant type-1 $CD4^+$ T cell cytokine response — i.e., IFN-γ, IL-12, and IL-2, but not IL-4 (van Voorhis *et al.,* 1996b). Activation of macrophages by type 1 cytokines is thought to be important for treponemal clearance and lesion resolution (Sell *et al.,* 1980; Lukehart *et al.,* 1980a; Lukehart, 1982). Degraded treponemes are found inside the phagolysosomal compartments of infiltrating macrophages in regressing syphilitic lesions (Ovcinnikov & Delektorskij, 1972), and rabbit peritoneal macrophages are capable of phagocytizing and killing treponemes in vitro (Lukehart & Miller, 1978; Baker-Zander & Lukehart, 1992), particularly in the presence of opsonizing antibodies (Baker-Zander *et al.,* 1993). However, both opsonized and non-opsonized treponemes are unusually resistant to phagocytosis by macrophages, which presumably enhances bacterial survival in inflammatory lesions (Alder *et al.,* 1990; Lukehart *et al.,* 1992).

In the rabbit model of syphilis, long-lasting immunity to *T. pallidum* does not develop until about three months post-infection, and fails to develop altogether if the course of infection is interrupted by antibiotic therapy (Fitzgerald, 1981). Although adaptive immunity curtails further disease progression, sterilization does not occur, and "immune" animals continue to harbor viable treponemes indefinitely. There is a clear parallel to human disease, in which tertiary syphilis may occur years or decades after initial infection. Two key questions arise: why does acquired immunity develop so slowly in syphilis, and why is the pathogen not eliminated? Poor immunogenicity and immune evasion by treponemes may provide partial answers.

Persistent treponemes harvested from rabbits at later stages of infection, after >99% of the bacteria have been cleared, are less susceptible to phagocytosis by macrophages (Lukehart *et al.,* 1992), suggesting that persistent infection may be due to a subpopulation of treponemal persistent bacteria that evade opsonization and phagocytosis. It has been suggested that this type of bacteria might adopt a "stealth" strategy *in vivo*, altering their surface composition in order to reduce their antigenicity (Radolf, 1994). Indeed, ultrastructural studies reveal that the outer membrane of *T. pallidum* is largely devoid of membrane-associated proteins (Radolf *et al.,* 1989), and intact treponemes are poorly reactive with immune rabbit serum unless the outer membrane is removed or damaged (Cox *et al.,* 1992). A number of cell wall-associated *T. pallidum* lipoprotein antigens have

been identified, but these are apparently localized to the inner (plasma) membrane rather than the outer membrane (Radolf, 1994; Radolf, 1995).

*T. pallidum* is a highly invasive pathogen (Haake & Lovett, 1990), so the paucity of surface-exposed proteins begs the question, what are the ligands that allow the organism to attach and invade after entering the host? These presumably surface-exposed bacterial components are likely targets of the adaptive immune response. Two proteins termed Tromp1 and Tromp2 ("*Treponema pallidum* rare outer membrane proteins") were identified by freeze-fracture electron microscopy (Blanco *et al.,* 1997). Although localization of Tromps to the outer membrane is disputed (Akins *et al.,* 1997), anti-Tromp antibodies are treponemicidal and correlate with protective immunity in rabbits (Lewinski *et al.,* 1999). The sequence of the *T. pallidum* genome reveals another group of potentially surface-exposed molecules comprising a novel twelve-member gene family termed *tpr* ("*Treponema pallidum* repeat") (Fraser *et al.,* 1998). The Tpr proteins contain central domains of variable length and sequence flanked by conserved N- and C-terminal domains, including conserved hypothetical transmembrane domains in some family members. One of these, TprK, is surface-exposed and targeted by the protective immune response in rabbits (Centurion-Lara *et al.,* 1999). Antiserum from rabbits that are immunized with TprK is opsonizing for phagocytosis and TprK-immunized animals are partially protected against challenge with virulent treponemes. The role of the Tpr proteins in treponemal pathogenesis is not known, but these studies suggest that they could be attractive subunit candidates

for a vaccine. Strain differences in *tprK* copy number and sequence have been identified, suggesting that variation of TprK might contribute to immune evasion (Centurion-Lara *et al.,* 2000). Possibly, the advent of a subpopulation of antigenic variants during infection could account for the failure of adaptive immunity to eliminate small numbers of persistent bacteria, resulting in the prolonged periods of latency that can be observed during syphilis.

## 1.2. *Borrelia* spp. — Antigenic variation: a change of face wins the race

Many microbial pathogens control gene expression by programmed rearrangements of specific DNA sequences (Borst & Greaves, 1987). These "contingency loci" (Moxon *et al.,* 1994) affect processes at the host/pathogen interface, including colonization, invasion, adaptation to different tissue environments, and evasion of immune defenses (Deitsch *et al.,* 1997). Programmed rearrangement of genes encoding surface antigens ("antigenic variation") is essential for the evasion of humoral immunity by extracellular pathogens such as the *Borrelia* spp. that cause relapsing fever and Lyme disease.

**1.2.1. Relapsing fever.** Relapsing fever (RF) is characterized by recurrent febrile episodes, which correspond to intervals of high bacteremia (Fig. 1.2A) (Barbour & Hayes, 1986). The causative agents are arthropod-transmitted spirochetes of the genus *Borrelia*. The RF borrelias persist at titers of up to ~$10^8$ bacteria per ml of

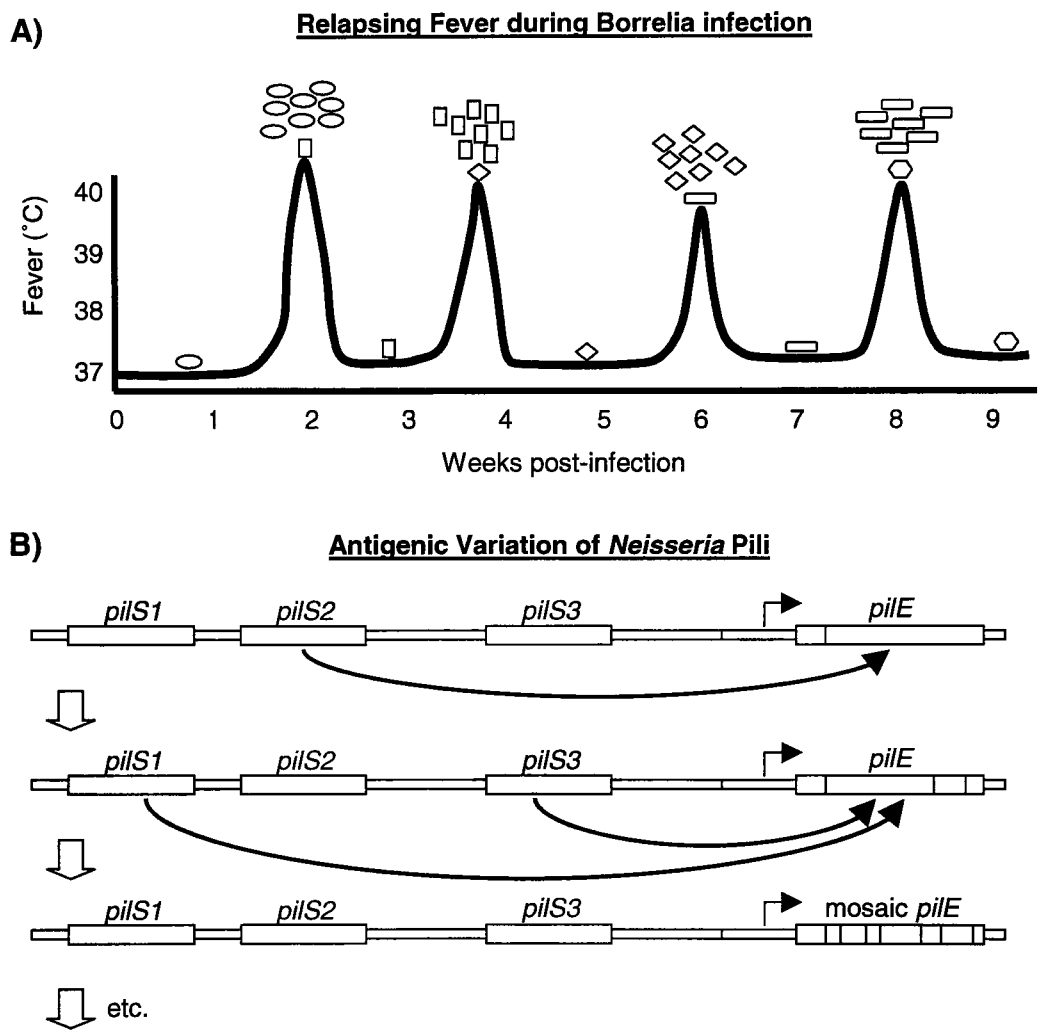


**Fig. 1.2. Antigenic variation in Relapsing Fever and Gonorrhoeae.** (**A**) Relapsing Fever. Spikes of fever are accompanied by waves of spirochetemia. In each successive wave, a new antigenic variant dominates (indicated by different shapes). As the bacteria are cleared by the specific humoral response, the fever subsides. However, expansion of a newly emergent variant brings about another febrile episode within a few days. (**B**) Antigenic variation of pili in *Neisseria gonorrhoeae*. Pilin is encoded by one or two *pilE* expression loci. Variant pilin sequences are stored in silent *pilS* cassettes, which lack the 5' coding and promoter sequences required for expression. Nonreciprocal recombination between *pilS* and *pilE* sequences gives rise to expression of variant pilin subunits. Modular recombination can generate mosaic *pilE* loci containing sequences derived from several distinct *pilS* loci.

blood, which ensures transmission of the pathogen to the insect vector during a blood meal. However, extracellular bacteria residing in the blood are exposed to the onslaught of the humoral immune response; survival in this perilous environment requires the pathogen to keep one step ahead of the host, which the RF borrelias accomplish by means of antigenic variation (Barbour, 1990). Referring to the spirochete-specific antibodies generated during an episode of RF, Meleney (1928) wrote: "These substances [antibodies] are specific for the strain of spirochetes which was present during the preceding attack, but have no influence on the spirochetes of the succeeding relapse. The spirochetes of the relapse give rise, in turn, to immune substances which are specific for them but not for the spirochetes of the first attack." A key question was whether a single bacterium could give rise to the observed waves of variants, or whether relapses were due to simultaneous infection with a mixture of variants that were expanded and eliminated in succession. This issue was resolved by infection of rats with a single bacterium (*B. turicatae*) and recovery of antigenically distinct relapse populations (Fig. 1.2A) (Schuhardt & Wilkerson, 1951). The population dynamics of borrelial antigenic variation were clarified by the application of serotype-specific immune sera to identify relapse isolates of *B. hermsii*, which arose at frequencies of ~ $10^{-3}$ to $10^{-4}$ per generation (Stoenner *et al.,* 1982). Only a small proportion of the spirochete population converted to other serotypes; this switch was abrupt and coincided with the clearance of the dominant (parental) population. Given the high rate at which serotype switching occurred, it was (and still is) puzzling that

only one or a very small number of serotypes were found in each relapse. Inhibition of antibody production had no effect on the emergence of serotype variants, demonstrating that humoral immunity was not driving the generation of new spirochete serotypes, but merely selecting against pre-existing types. The serotype-specific antibodies responsible for spirochete clearance recognize a family of surface-exposed and serotype-specific lipoproteins, the "variable major proteins" (VMPs) (Barbour *et al.*, 1982).

The high-frequency variation of VMPs was later shown to result from programmed rearrangements of the *vmp* genes (Meier *et al.*, 1985). The DNA sequences encoding a serotype-specific VMP variant are present in all serotypes, including those that express a different VMP variant (Plasterk *et al.*, 1985). However, serotypes that express a particular VMP contain not one but two DNA loci encoding that variant, i.e., duplication of a given *vmp* is associated with its expression. Duplication occurs via a non-reciprocal recombination event between a silent *vmp* "cassette" and a unique, unlinked "expression site". Recombination between these non-homologous loci is targeted by homologous regions flanking the expressed and silent *vmp* genes. The repertoire of *vmp* genes is carried on multi-copy, linear plasmids, with silent and expressed loci located on different plasmids (Saint Girons & Barbour, 1991; Kitten & Barbour, 1992; Wilske *et al.*, 1992). This arrangement allows recombination to occur at high frequency without jeopardizing chromosomal integrity. Interestingly, the *vmp* expression site is linked to the telomere of the linear plasmid that carries it (Kitten & Barbour,

1990). Although the significance of telomere linkage is unclear, it is shared by the system responsible for high-frequency variation of the immuno-dominant "variant surface glycoprotein" (VSG) antigen in *Trypanosoma brucei* (Rudenko *et al.,* 1998).

Two additional mechanisms contribute to antigenic variation in *B. hermsii.* The first involves activation of a pseudogene downstream of an expressed *vmp* by intra-plasmid (rather than inter-plasmid) recombination (Restrepo *et al.,* 1994). The second involves the selective hyper-mutation of expressed *vmp*s by an unknown mechanism; polymorphisms in expressed *vmp* genes accumulate with time and are absent in silent *vmp* genes (Restrepo & Barbour, 1994).

None of the *vmp* genes is expressed in the insect vector, suggesting that the *vmp* system has evolved to promote bacterial survival exclusively in the vertebrate host (Schwan & Hinnebusch, 1998). What is the role of the VMP system in pathogenesis? Is it simply to offer a constantly changing face to the vertebrate immune system? Or do the VMPs contribute directly to infection and dissemination within the host? Emerging evidence suggests that VMP variation may influence the organ tropism and dissemination properties of individual spirochetes (Cadavid *et al.,* 1994; Cadavid *et al.,* 1997; Pennington *et al.,* 1999). It is tempting to speculate that these "knock-on" effects of VMP variation could promote the adaptation of *Borrelia* spp. to previously unavailable niches, possibly including new host species.

**1.2.2. Lyme disease.** Lyme disease (LD) is a chronic syndrome caused by the tick-borne spirochete *Borrelia burgdorferi*. The ecological cycle of the pathogen is complex, involving several small and intermediate-sized vertebrate reservoirs, which transmit bacteria to the insect vector during blood meals (Gern & Humair, 1998). Humans are thought to be a "dead end" host. LD can be divided into three clinical stages (Steere, 1989; Evans, 1999). The first stage is marked by a transient skin lesion at the site of inoculation (erythema migrans). In the second stage, lasting up to six months, dissemination of the spirochetes gives rise to secondary skin lesions and other intermittent and variable pathologies, such as muscular and skeletal pain, meningitis, and carditis. These signs and symptoms often resolve spontaneously, leading to a subclinical or latent phase of variable duration. The third stage of disease, occurring months to years after initial infection, is characterized by chronic arthritis, cardiomyopathy, and neurological abnormalities. Borrelial persistence has been documented in clinical studies of LD patients (Fikrig *et al.,* 1994; Kuiper *et al.,* 1994) and in animal models (Barthold *et al.,* 1993; Pachner *et al.,* 1995; Roberts *et al.,* 1995; Gylfe *et al.,* 2000). The mechanism(s) that allow *B. burgdorferi* to cause persistent infection - whether chronic or latent - remain poorly understood.

Although LD patients typically produce antibodies against a broad range of *B. burgdorferi* antigens, antibody production may be significantly delayed (Steere, 1989). Studies in animal models of infection also suggest that *B. burgdorferi* may in some cases induce a non-protective immune response. In different strains of

mice, susceptibility seems to correlate with production of the "type 1" cytokine interferon-γ (IFN-γ), whereas resistance correlates with production of the "type 2" cytokine interleukin-4 (IL-4) (Sigal, 1997). This association has also been noted in Lyme disease patients (Oksi *et al.,* 1996), and may reflect the importance of antibody-mediated mechanisms of protection against borreliosis (Hu & Klempner, 1997), since type 2 cytokines are associated with dominant humoral immune responses.

There is accumulating evidence that *B. burgdorferi* evades the humoral response by antigenic variation of surface-exposed lipoproteins encoded by *vls* genes (for "*vmp*-like sequence") (Zhang *et al.,* 1997). The parallel to the *vmp* system of the RF borrelias is striking: the *vls* coding sequences are carried on a linear plasmid, and serotype switching occurs by non-reciprocal recombination between silent *vls* cassettes and an expressed copy (*vlsE*) that is telomere-linked (Zhang & Norris, 1998a, 1998b). Because recombination between *vlsE* and segments of the silent *vls* cassettes is combinatorial, the number of unique VlsE variants that could arise by shuffling of cassette segments is potentially enormous. Antigenic variation of VlsE in mice is associated with loss of seroreactivity, and the rate of variation is higher in immune-competent mice than in immune-deficient SCID mice (Zhang & Norris, 1998b). These observations suggest that VlsE variation may be an important mechanism of immune evasion. However, the rate of VlsE variation in SCID mice, although relatively low, is still much higher than observed during in vitro passage, suggesting that VlsE variation may play an

adaptive role in vivo in addition to its probable role in immune evasion. This idea is supported by the observation that spirochetes lacking the *vslE* plasmid are less infective to both immune-competent and SCID mice (Norris *et al.,* 1995). Interestingly, the fact that while most of the bacterium's chromosome is ~25% G+C in content while the *vlsE* region is about ~50% G+C, suggests that this 10 kb region might not be autoctonous to *B. burgdorferi* species (Casjens *et al.,* 2000).

The genome of *B. burgdorferi* potentially encodes as many as 150 different lipoproteins (Fraser *et al.,* 1997). Lipoproteins of the *o*uter *s*urface *p*roteins family (OspA through OspG) are immuno-dominant and elicit potent inflammatory responses (Sigal, 1997). *B. burgdorferi* does not display high-frequency variation of Osp proteins during infection, suggesting that Osps probably do not undergo antigenic variation (Barthold, 1993; Persing *et al.,* 1994; Stevenson *et al.,* 1994). However, selection of low-frequency, spontaneous, escape mutants has been observed *in vivo*, and could account for the considerable polymorphism of Osps within natural populations of spirochetes (Fikrig *et al.,* 1993; Marconi *et al.,* 1993; Livey *et al.,* 1995). Strain variation in Osp serotypes could play an important role in enhancing survival during infection of a host who had previously been exposed to a different antigenic variant.

In addition to high-frequency variation and population-level heterogeneity of borrelial antigens, antigenic "modulation" may also play a role in persistence. Expression of surface lipoproteins, e.g. OspA and OspC, fluctuates as the bacteria travel from vector to host and back again (Philipp, 1998). Spirochetes express

high levels of OspA in the gut of the flat tick, but precipitously down-regulate its expression as they begin their migration to the salivary glands of the feeding tick (de Silva *et al.,* 1996). While levels of OspA remain low in the course of infection, expression of OspC is increased and sustained. However, this expression is not uniform: Bacteria not expressing *ospC* are selected *in vivo* just as anti-OspC antibodies titers emerge ~17 days post-infection. Interestingly, these bacteria can regain expression of *ospC* in culture or when transferred to naïve mice. Thus, differential expression of *ospC* might represent an important mechanism of immune evasion in Lyme disease (Liang *et al.,* 2002). Several other genes of *B. burgdorferi* that are expressed in the host but not in the tick vector or *in vitro* have been identified (Suk *et al.,* 1995; Das *et al.,* 1997; de Silva & Fikrig, 1997), some of which are induced specifically during the persistent phases of infection (Fikrig *et al.,* 1999), but their role in the host-pathogen interaction remains to be elucidated.

Some late manifestations of LD may not be related to persistence of viable spirochetes. Some LD patients develop refractory arthritis. It is not clear whether arthritis is due to local persistent infection or to autoimmune disease initiated by infection but maintained in the absence of viable bacteria (Klempner & Huber, 1999; Evans, 1999; Hemmer *et al.,* 1999). The controversy stems from the fact that many patients with Lyme arthritis do not respond to antibiotic therapy and may not have detectable borrelial DNA in their synovial fluid.

The persistence strategies of the RF and LD borrelias are markedly similar, but several important differences should also be noted. Both pathogens cause septicemia, but the spirochete blood load in RF is much higher than in LD. The waves of spirochaetemia and fever that are the signature of RF are not seen in LD. Antigenic variation and immune selection of variants occur at much lower rates in LD than in RF. These differences likely reflect the divergent ecologies of the causative spirochetes, their vectors, and their intermediate hosts.

### 1.3. *Neisseria gonorrhoeae* — Traveling incognito to escape recognition

Gonorrhea, caused by the Gram-negative diplococcus *Neisseria gonorrhoeae*, is one of the most prevalent sexually transmitted diseases of humans — every year, 78 million infections occur worldwide (WHO, 1995). Gonorrhea is usually a self-limiting, uncomplicated acute infection of the urethra or the cervix with localized inflammation, which usually resolves within a weeks of exposure (Garnett *et al.*, 1999). However, in some instances, acute infection is followed by more serious sequellae, such as epididymitis in men, and pelvic inflammatory disease (PID), salpingitis, and endometritis in women (Paavonen, 1998). Damage to the upper reproductive tract in women can lead to infertility and ectopic pregnancy. Mucosal colonization and invasion results in disseminated gonococcal infection (DGI) in ~ 0.5-3.0% of cases, leading to arthritis, dermatitis, tenosynovitis, and (rarely) carditis or meningitis (Kerle *et al.*, 1992). Gonoccocal arthritis is the most

common type of joint infection in sexually active adults (Cucurull & Espinoza, 1998).

"Gonorrhea in the male, as well as in the female, persists for life in certain sections of the organs of generation, notwithstanding its apparent cure in a great many instances" (cited in Oriel, 1991). Thus, the persistent nature of gonorrhea was recognized by the noted veneorologist Emil Noeggerath in 1872, seven years before Albert Neisser identified the gonococcus as the etiologic agent. Persistent infection can be symptomatic or subclinical; the high frequency of asymptomatic carriers is a major obstacle to prevention and treatment. In clinical studies, approximately half of infected males who were examined because their sexual partners were known to have gonorrhea were aymptomatic (Handsfield *et al.,* 1974; Crawford *et al.,* 1977), and asymptomatic infection rates may be even higher in women (McCormack *et al.,* 1977).

The persistence of *N. gonorrhoeae* in a population is largely possible because infection, despite eliciting massive inflammation, uniformly fails to generate an immune response capable of conferring immunity to reinfection (Gray-Owen *et al.,* 2001). Infection initiates with entry of *N. gonorrhoeae* into the lower urogentital tract. Rapid attachment to the mucosal epithelium of the genital tract is mediated by surface appendages called pili (Nassif & So, 1995). Loose attachment is followed by a more intimate association involving the surface-exposed opacity (Opa) proteins (Dehio *et al.,* 1998). Certain Opa proteins also promote the transmigration of gonococci across the epithelial layer to the

submucosa. Colonization of the (sub)mucosa is followed by extensive bacterial multiplication and shedding of bacteria into the genital secretions, triggering the massive localized inflammation that is responsible for the clinical signs and symptoms of gonorrhea. The inflammatory response begins with a massive infiltration of phagocytes, predominantly polymorphonuclear cells (PMNs), in response to pro-inflammatory cytokines and chemokines such as interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor-α (TNF-α (Naumann *et al.,* 1997). These are produced locally in response to factors released or triggered by the bacteria, including bacterial cell-wall peptidoglycan and lipooligosaccharide (LOS), and complement component C5a.

PMNs phagocytize and kill gonococci by the oxidative burst, and other mechanisms such as defensins, cathepsin G, and other cationic antimicrobial proteins (Shafer & Rest, 1989). Gonococci may, however, be able to counter these defenses. Neisserial cell-wall porins inhibit phagocytosis and degranulation by PMNs (Bjerknes *et al.,* 1995) and interfere with both the classical and alternative pathways of complement activation (Ram *et al.,* 1999; Vogel & Frosch, 1999). The critical role of complement in host defense against *Neisseria spp.* is evidenced by the hyper-susceptibility of individuals with heritable complement deficiencies to infections by these species (Figueroa & Densen, 1991). Virtually all patients develop specific, opsonizing antibodies to the major surface proteins of the gonococcus (Jarvis, 1995), but these do not necessarily prevent re-infection (Brooks & Lammel, 1989). In part, re-infection may reflect the ability of the

gonococcus to subvert humoral immunity. Like several other mucosal pathogens, *N. gonorrhoeae* produces an IgA1-specific serine protease that cleaves the hinge region of IgA antibodies (Kilian *et al.,* 1988; Kilian *et al.,* 1996). The IgA1 protease may also promote the intracellular survival of gonococci by cleaving the lysosome-associated membrane protein 1 (LAMP-1) (Lin *et al.,* 1997), which could be important for transcytosis of gonococci across the genital epithelium (Hopper *et al.,* 2000).

Antigenic variation also plays an important role in survival of *N. gonorrhoeae* in the face of adaptive immunity. Variation of several cell-surface components of the gonococcus — notably, the pili, outer membrane Opa proteins, and LOS — is controlled by distinct and complex mechanisms. In addition to immune evasion, the organism's ability to invade and colonize different anatomical niches may also be determined by changes in surface components. Furthermore, recent evidence suggests that some of these surface proteins might be involved in active suppression of T-cell responses, which could be the reason why infection by *N. gonorrhoeae* is not followed by immunity that protects from reinfection with bacterial strains of the same serotype (Hobbs *et al.,* 1999).

**Pili.** Pioneering experiments in human volunteers established the essential role of pili in infection (Kellogg *et al.,* 1963). Pili promote the initial colonization of the urethral tract by mediating adherence to the genital epithelium (Swanson, 1973) via the CD46/MCP receptor (Kallstrom *et al.,* 1997, 1998). Pili are immuno-dominant antigens and antibodies directed against them block gonococcal

adherence to epithelial cells (Rothbard *et al.,* 1985). However, high-frequency variation of the pilin subunits that comprise pili occurs throughout the course of human infection, including early stages before the emergence of the adaptive humoral immune response (Hagblom *et al.,* 1985; Seifert *et al.,* 1994). These observations suggest that pilin variation might play two roles in the gonococcal life cycle: persistence in an infected individual by evasion of the adaptive immune response, and persistence at the community level by permitting transmission to individuals who had previously encountered the parental serotype. The mechanism of pilin variation in *Neisseria* is remarkably similar to the mechanisms used by the *Borrelia* to vary their surface antigens (Segal *et al.,* 1985; Seifert, 1996). The gonococcal genome encodes a large number of sequence-variable pilin genes, but only the pilin gene present at an expression locus (*pilE*) is actually transcribed and translated into protein (Fig. 1.2B). The silent pilin loci (*pilS*) are pseudogenes that lack the 5'-terminal coding sequences and the promoter sequences required for transcription. A pilin switch occurs when sequences stored at a *pilS* locus are transferred to a *pilE* locus by non-reciprocal recombination. Thus, the *pilE* gene that is expressed at any given time is a mosaic of sequences derived from various *pilS* cassettes, which can be endogenous or exogenous in origin, since *Neisseria* are naturally transformable (Gibbs *et al.,* 1989). Pili can also undergo on/off/on... phase variation by imprecise recombination between *pilS* and *pilE* generating aberrant forms of pilin that are not assembled and exported (Seifert, 1996). Phase variation of pili can also occur by on/off/on... switching of

PilC, a minor constituent of pili that is essential for pilus assembly and maturation. Phase variation of *pilC* occurs by "slipped-strand replication" mediated by runs of G residues near the 5' end of the gene. In addition to immune evasion, phase variation of pili might play a role in traversal of the genital epithelium, because pili interfere with invasion of epithelial cells and transcytosis of epithelial barriers (Ilver *et al.,* 1998). If non-piliated gonococci are responsible for mucosal invasion, then reversion to the piliated state must occur rapidly, because gonococci present in the blood of DGI patients are uniformly piliated.

**Opacity (Opa) proteins.** The surface-exposed Opa proteins are encoded by a family of related but unlinked genes; a single isolate of *N. gonorrhoeae* may encode as many as twelve *opa* genes and may express zero, one, or multiple Opa proteins (Dehio *et al.*, 1998; Nassif *et al.*, 1999). Gonococci isolated from human patients or volunteers are predominantly Opa-positive, even when initial infection was with Opa-negative bacteria, and simultaneous expression of multiple Opa proteins is apparently favored (Swanson *et al.,* 1988; Jerse *et al.,* 1994). The Opa repertoire expressed by a given bacterium affects invasiveness as well as tissue tropism (Kupsch *et al.,* 1993; Dehio *et al.,* 1998; Nassif *et al.,* 1999). Opa proteins phase vary in vitro at a frequency of $10^{-3}$ to $10^{-4}$ (Stern *et al.,* 1984, 1986) and the mechanisms involved in this variation include "strand slippage" during DNA replication, and intergenic recombination of *opa* genes, from both intra- and extra-genomic sources. This promiscuous reshuffling of coding sequences is

presumably responsible for the enormous repertoire of *opa* alleles observed in natural populations of gonococci (Meyer *et al.,* 1990).

Opa proteins might also play a role in the suppression of T-cell responses during gonoccocal infection. Binding of bacteria expressing OPA to CEACAM1, a surface molecule present on lymphocytes, blocks $CD4^+$ T cell activation and proliferation i*n vitro* (Boulton & Gray-Owen, 2002). This likely occurs via signaling through ITIM motifs found in the cytoplasmic tails of CEACAM1 molecules, which can recruit SHP1 and SHP2 phosphatases that can antagonize the kinase-dependent signaling required for lymphocyte activation (Ravetch & Lanier, 2000).

**Lipo-oligosaccharide (LOS).** The outer-membrane lipooligosaccharide (LOS) of *Neisseria* spp. differs from the typical lipopolysaccharide (LPS) of Gram-negative bacteria in that it lacks the repetitive O-antigen moiety. In addition to its structural role in the cell wall, LOS is thought to facilitate mucosal invasion and survival of gonococci (Dehio *et al.,* 2000). The resemblance of the carbohydrate moieties of LOS to host glycosphingolipids may promote immune evasion by "molecular mimicry" (Mandrell & Apicella, 1993; Moran *et al.,* 1996). Extensive variation of LOS oligosaccharide moieties can result in expression of several structurally distinct LOS molecules by a single bacterium (van Putten & Robertson, 1995; Minor & Gotschlich, 2000). LOS variation is determined by phase-variable expression of the LOS glycosyltransferases that sequentially add sugars to the LOS variable chain (Gotschlich, 1994; Minor & Gotschlich, 2000).

Individual *lgt* genes contain poly-G tracts that expand and contract by slipped-strand replication, resulting in on/off/on... expression of the corresponding LOS glycosyltransferase (Danaher *et al.,* 1995; Yang & Gotschlich, 1996; Minor & Gotschlich, 2000). Certain LOS variants are sialylated by a bacterial sialyl transferase using host CMP-NANA (van Putten & Robertson, 1995). This modification promotes survival by blocking the bactericidal activity of normal human serum and specific antibodies against cell-surface antigens such as porins or LOS itself (Parsons *et al.,* 1989; Smith *et al.,* 1995; Ram *et al.,* 1999). On the other hand, LOS sialylation interferes with invasion of epithelial cells and transcytosis across mucosal barriers, suggesting that modulation of LOS sialylation could be advantageous at different stages of infection (van Putten, 1993). This idea is supported by studies in human volunteers, where sialylation levels were low during early stages of infection (facilitating mucosal invasion) but high after colonization and traversal of the mucosal barrier (conferring serum resistance) (Schneider *et al.,* 1991). This temporal modulation of surface components in the course of infection is one of the more striking examples of the sophistication with which the *Neisseria* have learned to blindside the defense mechanisms of the host.

## 1.4. *Salmonella typhi* — Chronic carriers, a galling problem

Typhoid fever is major threat to human health anywhere treatment of sewage and drinking water is inadequate. The etiologic agent is the Gram-negative bacillus *Salmonella typhi*. Over 16 million people suffer from the disease annually, and about 0.6 million die from it (Pang *et al.,* 1998). Antibiotics are available but resistance is increasing — some regions in India report rates of multi-drug resistance in excess of 50% (Rowe *et al.,* 1997). A vaccine based on the capsular polysaccharide antigen Vi is effective but its application has not been sufficiently widespread (Hessel *et al.,* 1999). *S. typhi* is an exclusively human pathogen so transmission depends on contact with an infectious case, either an acutely infected individual or a chronic carrier. Because infection occurs via the oral route, transmission is often mediated via contaminated foodstuffs, as in the infamous case of Mary Mallon ("Typhoid Mary") (Scherer & Miller, 2001).

Ingested bacteria invade the intestinal wall by transcytosis of enterocytes or M cells in Peyer's patches. Bacterial replication in the submucosa is followed by entry into the bloodstream and dissemination throughout the body. Replication within macrophages in the spleen and liver leads to release of bacteria into the bloodstream. Signs and symptoms of typhoid include enterocolitis, diarrhea, anorexia, fever, convulsions, and delirium. Much of the pathology of typhoid fever is attributed to the massive release of cytokines triggered by bacterial LPS. Invasion of the gallbladder leads to shedding of bacteria into the intestine. High

fevers, accompanied by constant severe abdominal pain, persist for about four weeks, followed by slow convalescence with continued shedding of bacteria in the feces (Hornick *et al.,* 1970). Both humoral and cell-mediated immunity have been implicated in the protective immune response (Jones & Falkow, 1996; Jones, 1997). Most individuals recover fully and clear the infection; however, about 1% to 5% of those infected become asymptomatic chronic carriers and continue to shed bacteria in their stool and urine for the remainder of their lives. Although chronic carriers have none of the signs and symptoms of acute typhoid, they are at increased risk of developing cancer (Dutta *et al.,* 2000; Shukla *et al.,* 2000).

*S. typhi* has adopted a novel strategy to evade destruction by the host immune response: by invading and adapting to an immune-privileged anatomical niche (the gallbladder), the organism puts itself out of harm's way. Little is known about its survival in the bilious environment of the gallbladder. Curiously, persistence of *S. typhi* in the gallbladder is more likely if gallstones are present — indeed, *S. typhi* can often be cultured from gallstones. The gallbladder also seems to provide a safe haven from the onslaught of chemotherapy. Antibiotics, although generally effective in treating acute infection, often fail at eliminating infection from chronic carriers: About 25-50% of carriers are never cured despite long-term chemotherapy, and relapses are common, particularly in those with biliary or gallbladder calculi (Hornick, 1985; Gilman, 1989). In clinical studies of chronic carriers, subsets of patients had negative stool cultures for up to two years after chemotherapy yet eventually relapsed (Kaye *et al.,* 1967; Johnson *et al.,*

1973). This largely undetected source of contagion poses a formidable obstacle to public health efforts to eliminate typhoid. Therefore, it is rather disheartening that the carrier state has received scant attention from the scientific community.

A major obstacle to the study of typhoid fever and the carrier state has been the lack of a suitable animal model. The related species *S. typhimurium*, a significant source of food-borne gastro-enteritis in humans, produces a disease in mice that resembles typhoid in humans. Extrapolations from studies in the mouse model to human typhoid must be made with caution, since it is not yet clear whether the mechanisms of persistence are conserved between two bacterial species. When mice are infected with *S. typhimurium* orally, survival *en route* from the stomach to the intestine is dependent on the "acid tolerance response" (ATR) controlled by the alternative sigma factor RpoS (Foster., 1995; Slauch *et al.,* 1997). The intestinal mucosa is breached by invasion of bacteria into enterocytes or M cells lining the Peyer's patches. Bacterial "invasion factors" are injected into the cytoplasm of the target cell by a type III secretion system encoded by the "*Salmonella* pathogenicity island 1" (SPI1) (Ochman *et al.,* 2000). Injected factors include SopE (Hardt *et al.,* 1998) and SptP (Fu & Galan, 1999), which function as a GDP/GTP exchange factor (GEF) and a GTPase-activating factor (GAP), respectively, for GTPases Cdc42 and Rac-1. The sequential activation/deactivation of Cdc42/Rac-1 directs the transient actin cytoskeleton reorganization and membrane ruffling that accompany invasion. Once bacteria breach the mucosa they invade and replicate within macrophages in the underlying

submucosa. Alternatively, bacteria may be phagocytized on the lumenal face of the intestinal wall and ferried across to the submucosa, thus bypassing the SPI1-mediated invasion pathway altogether (Vazquez-Torres *et al.,* 1999).

Once the intestinal wall is breached, the infection is rapidly generalized throughout the reticuloendothelial system, especially in the liver and spleen. The pathogenesis of *Salmonella* hinges on the ability to survive and replicate within phagocytes of the monocyte-macrophage lineage. *Salmonella* inhabits a "loose" vacuole that acidifies to the normal phagolysosomal pH of 4.5-5.0 (Rathman *et al.,* 1996), but whether this compartment is a true phago-lysosome or a modified vacuole is controversial (Rathman *et al.,* 1997; Sinai & Joiner, 1997). Functions required for survival and replication within macrophages are encoded by a second "*Salmonella* pathogenicity island", SPI2, which also encodes a second type III secretion system, Spi/Ssa (Ochman *et al.,* 1996), whose expression is activated intracellularly (Valdivia & Falkow, 1997). The SPI2 type III secretion system translocates the bacterial protein SpiC from the *Salmonella* vacuole into the cytoplasm of the host cell, where it interferes with phagosome-lysosome fusion (Uchiya *et al.,* 1999). SPI2 also encodes the PhoP/PhoQ two-component regulatory system that controls expression of Pho-activated genes (*pag*) and Pho-repressed genes (*prg*), which are critical for intracellular survival (Groisman & Ochman, 1997). SPI2-encoded factors are responsible for evasion of the oxidative burst via exclusion of the NADPH-dependent phagocyte oxidase complex from the membrane of the *Salmonella* vacuole (Vazquez-Torres *et al.,* 2000). The

foregoing discussion highlights the important contributions of the susceptible mouse model of typhoid to our current understanding of the molecular basis of invasion, intracellular survival, and dissemination of *Salmonella* spp. This model, however, has shed little light on the asymptomatic carrier state, a key aspect of human typhoid that merits more attention.

In the past, some attempts were made to model certain aspects of persistent *Salmonella* infections in susceptible mice. For instance, certain strains of *S. typhimurium* are capable of causing a persistent infection in the intestine, spleen, liver, and occasionally even the gallbladder of these mice that can last for up to two months, thus allowing the study of some aspects of the immune response to a more chronic infection (Sukupolvi *et al.,* 1997). More recently, a significant step toward the development of a model of prolonged persistent infection was taken by simply shifting the study of *Salmonella* pathogenesis from the historically-preferred *Salmonella*-sensitive mouse strains to resistant ones. Monack *et al.* (2004) demonstrated that infection of mice carrying the wild-type *Nramp1* allele (resistant) infected orally with wild-type *S. typhimurium* bacteria resulted in a chronic infection: bacteria lingered in the organs of these mice for upto a year, mostly in macrophages of the mesenteric lymph nodes. Interestingly, IFN-γ-dependent immunity was found to be required for maintaining the bacteria in check. Though, no persistence-specific bacterial genes have yet been found, two genes, *mig-14* and *virK*, are apparently important in both the acute and persistent phases of the infection using this newly developed *Salmonella* persistence model

(Brodsky *et al.*, 2005). These factors promote bacterial survival in macrophages through inhibition of the bactericidal effects of the anti-microbial peptide (CRAMP), which is highly expressed in IFN-γ-activated macrophages.

## 1.5. Biofilms — Staying alive by staying put

Pathogens persist in their hosts via phenotypic adaptation to a multitude of different tissue environments. Studies of these processes in bacteria have focused on the behavior of individual cells, but accumulating evidence suggests that this is an overly reductionist view (Costerton *et al.*, 1999). Many bacterial species fluctuate between planktonic growth and sessile growth within biofilms, which typically complex and highly structured microbial communities (Costerton *et al.*, 1995), frequently composed of several species engaged in symbiotic relationships. Bacteria living in biofilm communities appear to be strikingly different in their physiology and metabolism from those growing planktonically (Costerton *et al.*, 1995; Costerton *et al.*, 1999).

Biofilms begin to form when bacteria growing in nutrient-rich environments develop adhesive behavior, leading to attachment to a surface and the formation of a monolayer of cells, followed by aggregation into characteristic micro-colonies (Fig. 1.3) (Costerton *et al.*, 1995; Pratt & Kolter, 1999). These micro-colonies secrete highly hydrated exo-polysaccharides (EPS), which compose up to 80-90% of a mature biofilm's mass (Lawrence *et al.*, 1991) and

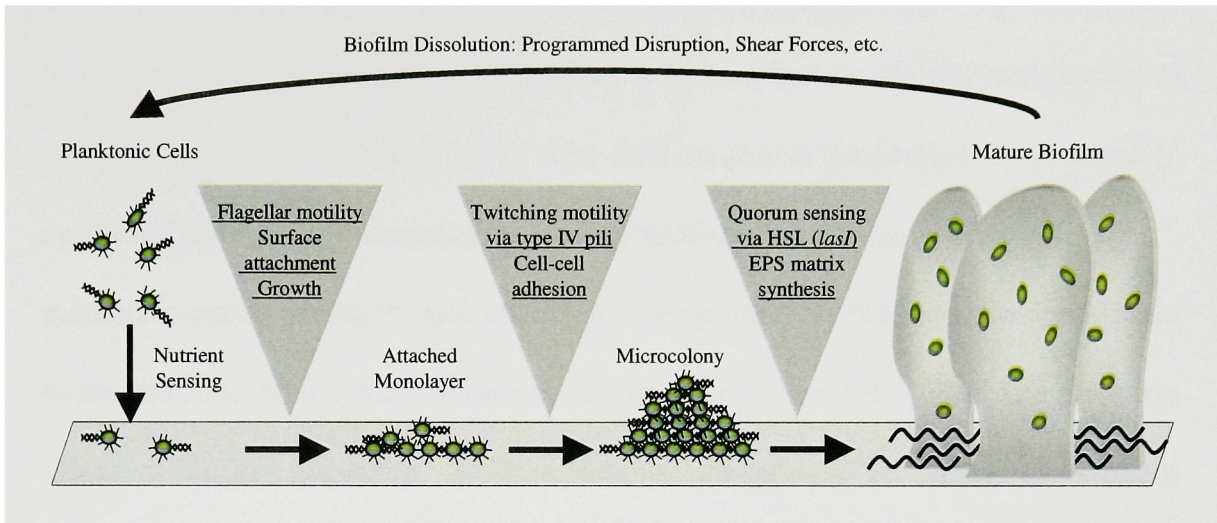


**Fig. 1.3. Biofilm life cycle.** Planktonic bacteria swim to a surface via flagellar motility and attach (mediated by flagella and nutrient sensing mechanisms). Replication on the surface leads to the formation of a monolayer of cells, which aggregate by twitching motility (mediated by type IV pili) and adhere to each other, forming surface-attached microcolonies. These microcolonies become embedded within a thick layer of secreted exopolysaccharides (EPS). As cell density increases, quorum sensing via homoserine lactones (HSLs) synthesized by LasI triggers the developmental changes required for formation of the correct architecture and thickness of the fully mature biofilm. The mature biofilm is penetrated by fluid-filled channels that deliver nutrients and remove waste. Bacteria within the biofilm are less susceptible to antibodies, phagocytes, and antimicrobials than planktonic cells. The steps leading to dissociation of biofilms and reversion to the planktonic form are poorly understood, but may involve environmental sensing and programmed disruption, or simple physical forces such as shear stress from fluid flow. Adapted from Costerton et al. (1999).

form an architecturally complex EPS matrix which is essential for biofilm formation and structure (Watnick & Kolter, 1999; Yildiz & Schoolnik, 1999), as well as resistance to environmental insults, antimicrobials, and predation by free-living protozoa or, in the case of biofilm infection, by host phagocytes (Jensen *et al.* 1990; Costerton *et al.,* 1995). The EPS matrix is penetrated by a complex system of fluid-filled channels that mediate nutrient distribution, elimination of metabolic waste products, and chemical communication between micro-colonies in the biofilm. Nutrient availability is an important determining factor in the early stages of biofilm formation. In *Pseudomonas aeruginosa*, the carbon metabolism sensor Crc controls the expression of functions required for biofilm genesis, such as the type IV pili that mediate twitching motility (Wall & Kaiser, 1999; O'Toole *et al.,* 2000). Both flagellar motility and twitching motility are required for the early stages of biofilm formation by *P. aeruginosa* and *Vibrio cholera* (O'Toole & Kolter, 1998; Watnick & Kolter, 1999). Flagella are required for initial bacteria-surface contacts, while type IV pili promote the aggregation of surface-adherent bacteria into micro-colonies.

The mechanisms that control the later stages of biofilm development remain largely unknown, but quorum sensing via secreted homoserine lactones (HSLs) seems to play a critical role. In *P. aeruginosa*, the *lasI* locus responsible for HSL synthesis is dispensable for the formation of a nascent and structurally simple biofilm, but essential for its further maturation (Davies *et al.,* 1998). Apparently, bacteria within the developing biofilm must talk to each other in order

to coordinate the events that generate the complex architecture of the mature biofilm. Production of HSLs has been demonstrated in naturally-occurring biofilms from aquatic environments (McLean *et al.,* 1997) and from urethral catheters removed from patients (Stickler *et al.,* 1998). The latter observation suggests the possibility that inhibitors of HSL production or function might be useful for preventing biofilm formation on indwelling medical devices.

Little is known about the mechanisms that mediate the dissolution of biofilms and reversion to planktonic growth. Recent work in *V. cholera* suggests that quorum sensing is also involved in this process. During *V. cholera* infection, bacteria in an apparently superinfectious state in biofilm-like structures (Merrel *et al.,* 2002) are ingested orally. After passage through the gut (the biofilm state is needed to withstand the stomach's acidity), they must be able to exit the biofilm in order colonize the intestine. Quorum sensing is apparently essential for the formation of a biofilm of just the right thickness so that rapid dissociation can take place once the bacteria get to the intestine because mutants of the quorum sensing regulator *hapR*, which overexpress the *vps* genes of EPS biosynthesis and make thick biofilms, are poor colonizers, despite having increased acid resistance (Zhu & Mekalanos, 2003).

One of the most relevant aspects of bacterial biofilms vis-à-vis infections is the increased resistant of biofilm bacteria to killing by antimicrobials (Costerton *et al.,* 1999). Inadequate drug penetration may contribute to the problem, as well as the altered physiology of bacteria within biofilms. The heterogeneous architecture

of biofilms creates chemical micro-environments that support slow- and non-growing bacterial populations (Costerton *et al.,* 1995), which are known to be less susceptible to antimicrobials (Eng *et al.,* 1991; Evans *et al.,* 1991). Indeed, the drug susceptibility and growth rate of biofilm bacteria are inversely related (Gilbert *et al.,* 1990).

Among medically relevant biofilm formers, *P. aeruginosa* is the best understood. This Gram-negative opportunistic pathogen afflicts immune-compromised individuals, including burn victims, neutropenic patients on cancer chemotherapy, and particularly cystic fibrosis (CF) patients (Govan & Deretic, 1996). Inherited defects in the chloride ion channel CFTR (cystic fibrosis transmembrane regulator) cause CF patients to suffer from buildup of dehydrated mucus in the respiratory tree, defective mucociliary function, and other defects in lung defenses. CF patients are unusually prone to develop chronic pulmonary infections by opportunistic pathogens, most of which respond to antibiotic therapy. However, virtually all CF patients are eventually colonized by *P. aeruginosa*, which resist eradication.

Flagella are required for the early stages of *Pseudomonas* biofilm formation (see above) and for virulence in mouse models of burn wound sepsis (Drake & Montie, 1988) or pulmonary infection (Feldman *et al.,* 1998). However, flagella can also promote non-opsonic phagocytosis of bacteria by neutrophils and macrophages (Mahenthiralingam & Speert, 1995). This conundrum might be resolved by temporal modulation of flagella expression in the course of infection.

In a prospective clinical study, bacteria isolated from CF patients at early stages of disease were flagella-positive, motile, and sensitive to phagocytosis, whereas bacteria isolated from persistently infected patients were flagella-negative, non-motile, and resistant to phagocytosis (Mahenthiralingam *et al.,* 1994). Flagella phase variation could be accelerated by the emergence of hyper-mutable subpopulations of *P. aeruginosa* in the CF lung (Oliver *et al.,* 2000).

Hyper-mutation might also promote the replacement of non-mucoid strains, which are responsible for initial colonization, by mucoid variants, which dominate later stages of infection. Mucoid variants arise by spontaneous mutations in genes controlling synthesis of alginate, an EPS matrix component, and their appearance is correlated with a poor prognosis for the patient. The alginate matrix shields the bacterial micro-colonies from both arms of the host immune response -humoral and cellular. Some variants that spontaneously arise during infection have been shown to have an enhanced ability to form biofilms as well as resistance to multiple antibiotics (Drenkard & Ausubel, 2002). These variants, which can readily be selected upon antibiotic exposure in vitro, might be selected *in vivo* by antibiotic exposure itself or as a result of exposure to host-mediated stresses.

Most CF patients develop high levels of specific antibodies to *P. aeruginosa,* but these are ineffective against bacteria buried in the alginate matrix (Govan & Deretic, 1996; Costerton *et al.,* 1999). Indeed, deposition of immune complexes on the surface of *Pseudomonas* biofilms is believed to contribute to lung inflammation and pathology in CF. Lung damage may also result from the

chronic influx of neutrophils, which are unable to clear the infection because the alginate matrix interferes with phagocytosis and killing. Bacterial communication (quorum sensing) within the biofilm is apparently required for optimal induction of protective mechanisms, including the superoxide dismutases (Mn-SOD and Fe-SOD) and catalase (KatA) that detoxify phagocyte-generated ROI (Hassett *et al.,* 1999). Interestingly, the biofilm also supports the appearance of variants that

The study of microbial biofilms and their role in persistent infections is still at an early stage. The environmental signals that promote biofilm formation and dissolution, and the signals that the bacteria use to communicate with each other, are just beginning to be deciphered. In addition to species-specific signals, a common language of secreted pheromones is apparently used for inter-species communication (Bassler, 1999). Elucidation of the signalling pathways that control biofilm dynamics could point the way to novel interventions targeting not individual bacterial cells, but their collective behavior as biofilms.

# CHAPTER 2

## Tuberculosis and the tubercle bacillus

### INTRODUCTION

*Mycobacterium tuberculosis* is arguably the single most successful pathogen humankind has ever known. As Koch (1882) put it in The Etiology of Tuberculosis: "If the number of victims is a measure of the significance of a disease, then all disease, even the most dreaded infectious diseases, such as plague or cholera, must rank far behind tuberculosis." Today, 120 years later, *President* Mandella tells us: "We cannot cure AIDS without curing TB."

The unparalleled penetrance of TB in the human population reflects the extraordinary ability of *M. tuberculosis* to cause a lifelong persistent infection, a peculiarity which most likely is the result of a long dynamic interaction with its host, one that may have begun before modern civilization (Morse *et al.,* 1964; Salo *et al.,* 1994). Though microbial infections including those caused by highly pathogenic bacteria tend to run an acute course, with infectious cycles of a few weeks, mycobacterial infections such as leprosy and TB are unusually protracted due to these pathogens' proclivity to persist in their hosts (Fig. 2.1) (Gomez & McKinney, 2004; Britton & Lockwood, 2004). Persistence by *M. tuberculosis* can

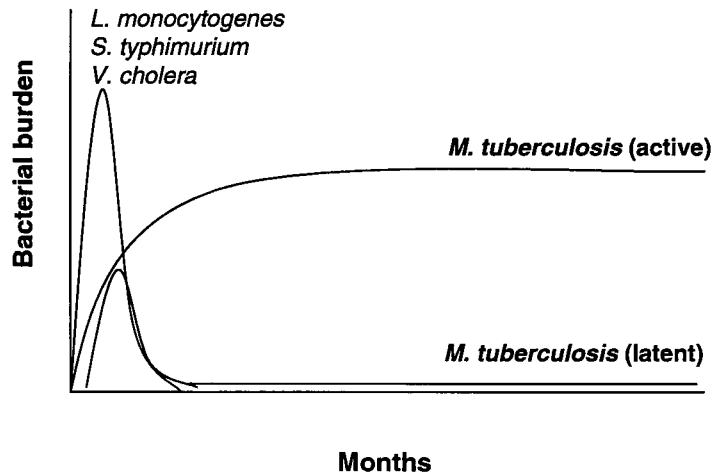


**Fig. 2.1. Acute vs. chronic active and latent bacterial infections.** Acute infections are rapidly cleared by a sterilizing immune response that often protects from reinfection. Chronic infections last much longer, can be controlled, but usually are not eradicated by immunity, which seldom protects from reinfection. *M. tuberculosis* causes both chronic active and latent infections.

occur in the form of chronic active disease and latent infection; in either form, the biology of *M. tuberculosis* persistence is poorly understood (Manabe & Bishai, 2000; Gomez & McKinney 2004; Boshoff & Barry, 2005). Because available antibiotics are inefficient in eradicating persistent bacteria, treatment must be extended, and often lasts between 6 and 9 months. In spite of the inherent difficulties in implementing these sorts of regimens, antibiotics have effectively led to a scale back of TB in most of the industrialized world, however, they have had a much smaller impact in the rest of the world, where lack of resources make implementing such cumbersome drug treatments considerably more difficult (Dye *et al.,* 1999). Most people live in the "developing" world," and it is in this other 'forever–developing' world -where poverty is extreme, public health deplorable, and malnutrition and overpopulation widespread - where TB relentlessly and inexorably continues to exact its deadly toll (Farmer *et al.*, 1991).

Though it is clear that combating the current TB epidemic would be greatly facilitated by the development of new and more efficient drugs and vaccines, and that in order to generate these, a better understanding of the biology of *M. tuberculosis* during infection would be greatly beneficial (McKinney, 2000; Andersen, 2001; Flynn, 2004; Mitchison, 2004), the major obstacle in dealing with TB and other major infectious diseases such as malaria and HIV is the uwillingness of the industrialized world to make the investments that would be needed to facilitate drug discovery (Nathan, 2004) and the affordable and efficient distribution of already available drugs (Farmer, 1996; Farmer & Campos, 2004).

## 2.1. Global burden of tuberculosis

**2.1.1 Epidemiology.** Despite improvement in the prognosis of TB in affluent nations, the disease continues to be the leading cause of death among bacterial infections worldwide (Dye *et al.,* 1999; Dye *et al.,* 2002). Furthermore, it has been estimated that close to 2 billion people harbor latent tubercle bacilli, constituting a reservoir that will result in about 100 million cases of reactivation TB (Raviglione *et al.,* 1995). According to WHO estimates, of circa 50 million deaths occurring per year, approximately 2 million are from TB, and over 95% of those deaths occur in the developing world (WHO, 2004).

TB epidemics are extremely slow, typically expanding over centuries (Blower, *et al.*, 1995) (Fig. 1.1B). Though the incidence of TB worldwide was in a downtrend since the 1950's, this changed considerably in recent times: from 1985 to 1992 the incidence of TB rose by 20% in the United States (Bloom & Murray, 1992), and a considerable percentage of the cases were drug resistant (Moore *et al.*, 1996). Among the most important reasons for such reversal were the AIDS pandemic, reduction in public health resources in inner cities, and continued immigration from areas of high TB prevalence (Zuber *et al.,* 1997).

**2.1.2. TB and AIDS.** AIDS in HIV-infected individuals, e.g., low CD4+ T cells, is one of the most important contributors to the development of TB in co-infected individuals in many parts of the world including the United States (Chaisson *et al.,*

1987; Modilevsky *et al.,* 1989). The low $CD4^+$ T cell counts translate into suboptimal macrophage activation and an inability to mount a protective response. HIV-coinfection is the greatest single risk factor for progression from *M. tuberculosis* infection to disease. The risk of developing disease after infection with *M. tuberculosis* in an HIV-coinfected person is about 5-15% per year, compared to a lifetime risk of developing active TB after infection of about 10% in immuno-competent individuals. Furthermore, the inability to mount an effective adaptive immune response leads to a more progressive course of TB in HIV-infected individuals (Sepkowitz, 1995; Corbett *et al.,* 2003; Aaron, 2004). Miliary TB, a highly disseminated form of TB that can affect multiple organs (Mert *et al.*, 2001), is also more frequent in AIDS patients (1990). Furthermore, some evidence suggests that the interaction might be reciprocal, since active TB might stimulate viral replication, thereby accelerating the onset of AIDS (Whalen *et al.,* 1995), perhaps through TNF-α, which induces viral transcription, and/or activation of the $CD4^+$ T cells that support viral replication (Fauci, 1996; Bafica *et al.,* 2003). Current estimates indicate that there are about 11 million individuals coinfected with HIV and *M. tuberculosis* worldwide. A recent study found that of an estimated 1.8 million deaths from TB, 12% were attributable to HIV, and similarly, TB was responsible for 11% of all deaths due to AIDS in adults (Corbett *et al.,* 2003).

The emergence of drug resistant strains of *M. tuberculosis* is another important factor modifying the current TB epidemic (Farmer *et al.,* 1998). In

general, a strain is considered multiple drug resistant (MDR) if it is resistant to at least isoniazid and rifampicin, the two most effective drugs for TB. Rapidly spreading MDR *M. tuberculosis* strains, that are resistant to all four first line TB drugs, including isoniazid, rifampin, pyrazinamide and embuthal, have already emerged (Bifani *et al.,* 2002; Post *et al.,* 2004). According to WHO estimates, circa 5% of all active TB cases worldwide are caused by MDR strains. In some parts of the world, MDR strains represent a much higher percentage of the TB cases; in Estonia, for instance, MDR TB represents close to 15% of all cases, while in Russia, ~25% of all TB cases are MDR (Farmer, 2001).

## 2.2. The tubercle bacillus

*Mycobacterium tuberculosis* belongs to the class Actinobacteria, order Actinomycetales, suborder Corynebacterineae, family Mycobacteriaceae. The suborder Corynebacterineae encompasses other high G+C Gram-positive bacteria including members of the Nocardia, Rhodococcus and Corynebacterium families. *M. tuberculosis* bacilli are non-motile rod-shape bacteria with general dimensions ranging from 1 and 4 μm in length and 0.3 to 0.6 μm in diameter. Some forms of bacilli that are smaller and round have been observed in histopathological studies (Reviewed in Lurie, 1939a). Though *M. tuberculosis* requires oxygen for growth, it can grow at low oxygen tensions and can survive complete oxygen deprivation (Wayne & Hayes, 1996).

**2.2.1. Mycobacterial growth rates.** Mycobacteria are notorious for being very slow growers. *M. smegmatis*, a fast growing innocuous mycobacterial saprophyte used as a surrogate to study *M. tuberculosis*, has a division time of about 3 hr in axenic culture and takes 3-4 days to form a colony on agar. *M. tuberculosis* has a doubling time of about 20 hours in culture and formation of a colony on agar requires 18-21 days (Wayne, 1994). Pathogenic mycobacteria have adapted to slow growth at many levels. For example, *M. tuberculosis* takes about 10 hours to replicate its genome, while *E. coli* can do it in 0.5 hours; and while *E. coli* has seven rRNA operons, *M. tuberculosis* has only one (Cole *et al.,* 1998).

**2.2.2. Mycobacterial cell wall.** One of the most striking features of mycobacterial cells is their tremendously complex lipid-rich envelope, which weigths half of the cell's dry weight. This property causes mycobacterial cells to aggregate into clumps in culture, making their experimental manipulation difficult. Limited studies suggest that mycobacterial cells might be relatively less permeable to hydrophilic substrates due to the presence of mycolic acids and mycosides; this impermeability could prevent transport of hydrophilic substrates into the cell. This lipid "shield," which causes *M. tuberculosis* to retain Carbol fuchsin dye despite acid treatment ("acid fastness"), plays an important protective function from physical and chemical stress, and accumulating evidence indicates that its components mediate complex interactions with the host immune response (Manca *et al.,* 2004; Reed *et al.,* 2004; Karakousis *et al.,* 2004). These properties might

also limit the ability of certain drugs to efficiently enter the mycobacterial cell (Barry & Mdluli, 1996; Kolattukudy *et al.,* 1997, Brennan, 2003).

The peptidoglycan of mycobacterial cell walls has alternating N-acetylglucosamine and muramic acid residues. The muramic acid residues are N-acylated with glycolic acid rather than acetic acid. Arabinogalactan, a branched-chain polysaccharide made up of a galactose and an arabinose chain, is attached to the peptidoglycan. Some of the arabinose units are linked to penta-arabinose units, which are esterified with mycolic acids. Mycolic acids, long-chain 1-alkyl 2-hydroxy fatty acids, are essential for formation of the mycobacterial cell wall (Brennan, 2003).

**2.2.3. Mycobacterial genomes.** The first *M. tuberculosis* genome sequenced was that of the virulent laboratory strain H37Rv (Cole *et al.*, 1998). A recent clinical isolate, termed CDC1551, was sequenced shortly after (Fleischman, 2001), and a third clinical isolate (strain 210) is near completion. The sequencing of the genomes of several other mycobacteria has been completed or is near completion (Garnier *et al.,* 2003; NCBI). This wealth of information has uncovered unique aspects about mycobacterial biology. The constant G+C content of ~65% throughout mycobacterial genomes suggests the absence of horizontally acquired pathogenicity islands, which are widespread in other pathogenic bacteria. Mycobacterial genomes have several novel multigene families and an unusually high number of duplicated 'housekeeping' genes, as well as repetitive insertion

sequences (Brosch *et al.,* 2001). Interestingly, though the proportion of the genome that has arisen through gene duplication is not particularly high (~51%), the duplicated genes show low divergence, which might contribute to functional overlap or outright redundancy.

The most salient characteristic of the *M. tuberculosis* and other mycobacterial genomes so far sequenced is the presence of a vast number of genes dedicated to lipid metabolism, including catabolic and anabolic functions (Cole *et al.,* 1998; Fleischman, 2001; Garnier *et al.,* 2003). Over 8% of the *M. tuberculosis* genome is made up of genes encoding proteins putatively involved in some aspect of lipid metabolism and considering that near 40% of the dry weight of the mycobacterial cell wall (Anderson, 1940) is made up of lipids, it is reasonable to conclude that lipid metabolism plays a major role in the biology of mycobacteria.

## 2.3. Clinical tuberculosis

*M. tuberculosis* contagion occurs when a susceptible person inhales bacilli-containing infectious droplets that have been expelled from the lungs of a tuberculous individual by sneezing, coughing or simply talking. Of those infected, only a minority (~10%) will develop disease; about half will do so within a few months to a couple of years immediately following infection (primary TB), while the other half will do so only after an indeterminately long period of latency (post-primary TB) (Comstock, 1982).

**2.3.1. Primary TB.** After reaching the alveoli, phagocytosis of the bacilli by resident macrophages (Ernst, 1998; North & Jung 2004) is followed by unrestricted bacterial exponential intracellular growth. Uptake of bacteria by dendritic cells (DCs) and migration of infected DCs to the draining lymph nodes for antigen presentation likely contributes to the lympho-hematogenous dissemination of bacteria to extrapulmonary organs and uninfected parts of the lung, leading to the formation of secondary lesions (Lurie, 1964; Dannenberg & Rook, 1994a). Exponential bacillary growth is thought to continue in primary and secondary lesions until a specific T-cell response is generated around each lesion, curtailing bacillary growth and likely killing some of the bacteria within macrophages as these become mycobacterio-static or -cidal after activation (Flynn & Chan, 2001) (Fig. 2.2).

The caseous centers of newly formed lesions are surrounded by activated macrophages. Presumably bacterial antigens being released by the bacteria (Beatty & Russell, 2002) or captured from them are constantly being processed and presented to surrounding T cells in the context of IL-12 and other cytokines that induce a Th1 type of immune response. Constant presence of antigen likely contributes to expansion of the T cell populations and continued activation of macrophages, which avidly ingest and inhibit/kill bacilli that accumulated during the acute phase of the infection. In a lesion where the bacteria are effectively controlled by CMI, the caseous center becomes surrounded by a capsule, forming a protective granuloma. Overtime, the number of viable bacilli in these

granulomas likely diminishes. Old caseous foci of this sort contain little to no bacilli, and some of the old lesions can ossify and be reabsorbed (Sweany *et al.*, 1943; Opie & Aronson, 1927). The interactions just described result in control of the infection in most infected individuals.

For reasons poorly understood, some individuals fail to generate the protective type of immune response just described. In these cases, bacteria continue to replicate and the granulomas become increasingly necrotic. It is believed that part of the tissue damage that occurs is the result of immunopathology, i.e., an excessive host-mediated inflammatory response to bacillary antigens (Dannenberg & Rook, 1994a). When the centers of granulomas undergo caseating necrosis, the sheaths of monocytes and T cells which normally suppress the intracellular growth of the bacilli can also become necrotic; as the bacteria continue to replicate in nonactivated macrophages at the periphery of the lesion, progressive destruction of lung tissue ensues, ultimately leading to the death of the host. Massive inflammation and tissue damage can also be caused directly by some components of the bacterial cell wall as well as by secreted lipids and proteins; lipoarabinomannan (LAM), for instance, triggers TNF-$\alpha$ secretion by macrophages, while some secreted lipids lead to secretion of proinflammatory IL-6 (Karokousis *et al.*, 2003).

Although *M. tuberculosis* is not an obligate intracellular pathogen, it is unclear whether it is capable of extracellular growth in the acellular necrotic centers of caseous lesions. In contrast, solid caseum that has undergone

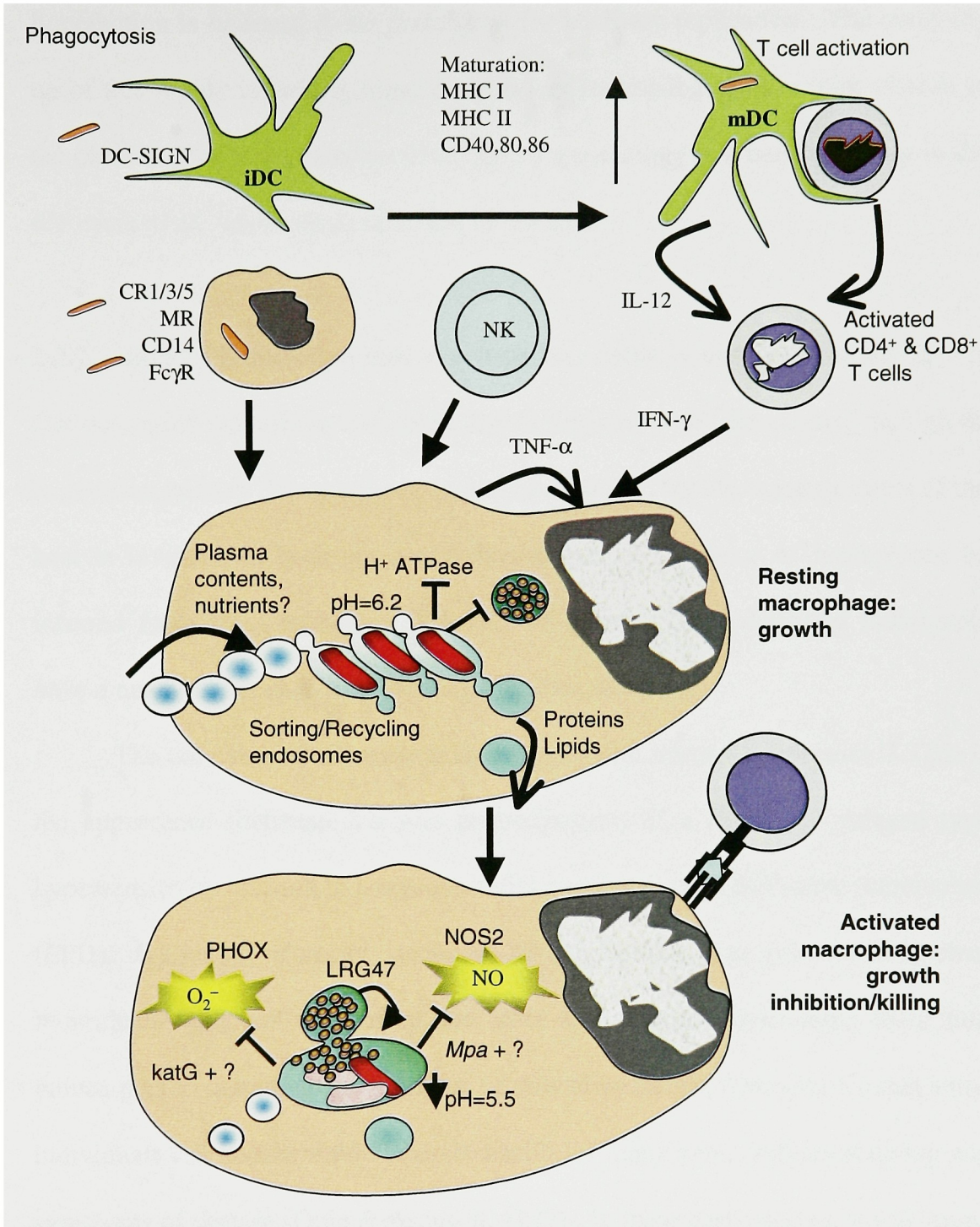


**Fig. 2.2. Cellular immune response to *M. tuberculosis* infection.** Depicted are some aspects of the innate and adaptive cellular immune responses to infection by *M. tuberculosis* as inferred from studies using *ex-vivo* and *in-vivo* models of TB. The bacteria are assumed to reside mostly within vacuoles in macrophages. Not shown are CTL- and gd T cell-mediated proposed mechanisms of protection.

liquefaction is believed to be permissive for bacillary replication. The coughing up of this highly infectious material is in fact responsible for the transmission of the bacilli. These observations illustrate how pathology is a necessary step in the life cycle of *M. tuberculosis* infection in humans.

**2.3.2. Latent TB infection and reactivation.** Little is known about the factors that determine whether an individual that is exposed to *M. tuberculosis* will go on to develop primary TB or become latently infected. That the immune status of the host is involved in both the establishment and maintenance of latency can be inferred from the observation that HIV-*M. tuberculosis* coinfected individuals have a much higher risk of developing TB after infection.

The only sign of infection in individuals with latent TB infection (LTBI) is the appearance (between 2-6 wks post-exposure) of a detectable delayed type hypersensitivity reaction to a mycobacterial purified protein derivative (tuberculin) (PPD). By taking necropsy specimens of lung lesions from immune-competent individuals who had died of causes other than TB and inoculating them into guinea pigs to determine the presence of infectious bacilli, it was shown that some individuals can harbor viable tubercle bacilli for many years without showing any symptoms of disease (Opie & Aronson, 1927). In these early studies, it was found that while specimens from encapsulated or calcified lesions rarely were infectious, those isolated from caseous lesions in the lung's apex generally were. Unexpectedly, apparently healthy tissues were also often infectious. Several other

studies from the pre-antibiotic era have confirmed that lung lesions from asymptomatic individuals can harbor viable bacilli to different degrees (Feldman & Baggentoss, 1938; 1939; Sweany *et al.,* 1943; Manabe & Bishai, 2000; Gomez & McKinney, 2004). Recent evidence of potential LTBI in normal human lung tissue was provided by the finding that about 30-50% of samples from individuals from endemic regions (Mexico and Ethiopia) could harbor *M. tuberculosis* DNA (Hernandez-Pando *et al.,* 2000). Molecular evidence of endogenous LTBI reactivation LTBI comes from a recent case study in Denmark concerning an individual who had become infected 30 years prior with the same strain shown to be responsible for the reactivation and development of active TB (Lillebaek *et al.,* 2002).

Perhaps the most compelling evidence for the prevalence of LTBI has come from the observed increased rates of TB in AIDS patients, as well as the high reactivation TB associated with the intake of TNF-α inhibitors prescribed for rheumatoid arthritis. These observations indicate that many individuals with LTBI accomplish life-long suppression of the bacillus, but never actually achieve sterilization.

Reactivation TB usually presents clinically as a slowly progressive, chronic condition; an individual with chronic sub-acute TB may infect scores of contacts without realizing that (s)he has the disease (Hoge *et al.*, 1994; Valway *et al.*, 1998). Patients with cavitary TB are particularly infectious because they shed enormous numbers of tubercle bacilli in their sputa. Cavitation is caused by the

liquefaction of necrotic tuberculous tissue and its expulsion via the airways; liquefaction is linked to a strong DTH response and is an important example of immuno-pathology in TB (Dannenberg & Rook, 1994a). The molecular basis of cavity formation has not been determined, nor is it clear whether the immune mechanisms responsible for pathogenesis and protection are the same.

## 2.4. Experimental studies of tuberculosis

**The Mouse model of chronic tuberculosis.** Animals infected with *M. tuberculosis* — mice, guinea pigs, or rabbits — typically develop a chronic, progressive form of the disease which varies in severity depending on the species, the guinea pig being the most susceptible, the rabbit the most resistant, and the mouse somewhere in between.

The mouse has become the animal of choice in the study of TB. In fact, Koch himself employed mice in his pioneering experiments over 100 years ago. A chronic infection in mice can be established by inoculation of bacilli by intravenous injection (Hart & Rees, 1950; McCune & Tompsett, 1956) or by aerosol exposure (Orme & Collins, 1994). The bacilli multiply logarithmically during the first 2-4 wks post-infection and then stop increasing in numbers in the lung and start to slowly decline in numbers in the liver and spleen. Depending on the titer of the initial inoculum, the chronic infection can last from months to over a year (Rhoades *et al.,* 1997). The model is useful in that it allows host-*M.*

*tuberculosis* interactions to be studied in the context of a prolonged persistent infection. The maintenance of a relative high bacterial load, accompanied by accumulating pathology, might pertain to human active TB, while the apparent lack of bacterial replication might be relevant to both active TB and LTBI. Among the limitations of the mouse model are differences in the composition and organization of the granulomatous structures compared to those formed in human lungs, and in the location of the bacilli in the infected lungs – almost exclusively intracellular in mice, considerably more extra-cellular in humans.

Spontaneous *M. tuberculosis* latency in animals only occurs in nonhuman primates (Capuano *et al.*, 2003; Flynn *et al.*, 2003). A drug-induced model of latency known as the "Cornell model", in which *M. tuberculosis*-infected mice are sterilized by drug treatment, and then allowed to reactivate spontaneously or by immunesuppression, was developed in the 1950's (McCune & Tompsett 1956; McCune *et al.* 1956, 1966a, 1966b). This model allows host-pathogen interactions to be studied in the context of an infection where very low numbers of bacteria are present and has been used to study host factors involved in establishing and maintaining this type of "latency" (Scanga *et al.*, 1999; Botha *et al.*, 2002; Botha & Ryffel, 2003). However, the relevance of the model to true latency awaits validation.

## 2.5. Host-pathogen interactions in TB

Bacilli reaching the lung alveoli are phagocytosed by resident macrophages (Lurie & Zappasodi, 1942) via mannose, complement (Schlesinger *et al.,* 1990; Schlesinger, 1993)), and Fc receptors (in differentiated macrophages) (Malik *et al.,* 2000). Uptake through Fc receptors increases phagolysosomal fusion (Armstrong & Hart, 1975) and triggers the oxidative burst (Kobzik *et al.,* 1990), leading to killing of bacteria in human macrophages (Malik *et al.,* 2000; Malik *et al.,* 2001). Regardless of the receptor involved, uptake apparently requires recruitment of cholesterol to the phagosome (Gatfield & Pieters, 2000). Uptake by dendritic cells occurs via DC-SIGN (Tailleux *et al.*, 2003).

**2.5.1. Cellular interactions leading to a protective immune response.** A protective immune reponse to *M. tuberculosis* is mediated mainly by T-cells (CMI) (Suter et al., 1961; MacKaness, 1968; Murray, 1999; Flynn & Chan, 2001; North & Jung, 2004) (Fig. 2.2). As early as one wk post-infection, bacteria-specific $CD4^+$ and $CD8^+$ T cells migrate to the lung (Serbina & Flynn, 1999), peaking in numbers at around 4 wks post infection (Feng *et al.,* 1999). Studies in immune-deficient mice have established that IFN-γ (Cooper *et al.,* 1993; Dalton *et al.,* 1993; Flynn *et al.,* 1993; Kamijo *et al.,* 1993), TNF-α (Flynn *et al.,* 1995; Adams *et al.,* 1995), and IL-12 (Flesch *et al.,* 1995; Cooper *et al.,* 1997) are the main ytokines involved in a protective immune response. The relevance of

IFN-γ and IL-12 in antimycobacterial immunity is supported by human genetic data (Altare *et al.*, 1998); the recent association between administration of TNF-α inhibitors used to treat rheumatoid arthritis and the reactivation of LTBI has underscored the protective role of this cytokine in control of *M. tuberculosis* in humans (Nuñez-Martinez *et al.*, 2001).

The initiation of the immune response occurs via presentation of *M. tuberculosis* antigens, presumably by dendritic cells (DCs) (Guermonprez *et al.*, 2002), on MHC class I and II molecules to naïve T cells, which in the context of IL-12 and IL-23 (Flesch et al., 1995; Fulton *et al.*, 1996; Cooper *et al.*, 2002; Verreck *et al.*, 2004) produced by infected macrophages and DCs themselves, are instructed to differentiate into IFN-γ-secreting cells (T helper type I, Th1) cells (Trinchieri, 2003) (Fig. 2.2). Both $CD4^+$ and $CD8^+$ T cells contribute to the activation of infected macrophages via IFN-γ secretion (Tascon *et a*l., 1998), while $CD8^+$ T cells might additionally contribute to protection by causing apoptosis of infected macrophages through the Fas-FasL pathway, as well as via secretion of granulysin and other microbicidal molecules (Stenger *et al.*, 1998; Serbina *et al.*, 2000). However, the relative contribution of $CD8^+$ T cells to the overall protective CMI might not be as significant as that by $CD4^+$ T cells since *M. tuberculosis*-infected mice deficient in the former live almost as long as infected wild-type mice, while those deficient in the latter are significantly more susceptible (Sousa *et al.*, 2000; Mogues *et al.*, 2001).

Natural killer (NK) cells might contribute to the early control of *M. tuberculosis* replication by generating IFN-γ and granulysin during the first few days of the infection (innate phase), however since their depletion does not affect bacterial loads, it is likely that other innate sources of IFN-γ exist (Junqueira-Kipnis *et al.,* 2003). In addition to NK cells, the rapid early activation of 'bystander' $CD8^+$ T cells has been implicated as an early source of IFN-γ during bacterial infections (Lertmemongkolchai *et al.,* 2001). The lack of early $CD8^+$ T cell-derived IFN-γ could explain why in the lungs of $CD8^+$ T cell-deficient mice *M. tuberculosis* bacilli replicate to 1 $\log_{10}$ higher than in wild-type mice, before their numbers stabilize (Mogues *et al.,* 2001). Later on in the infection NK cells might also help control the infection by producing granulysin or by inducing apoptosis in infected monocytes, via a Fas-FasL independent mechanism (Brill *et al.,* 2001).

Mice lacking $CD4^+$ T cells are highly susceptible to *M. tuberculosis* infection (Caruso *et al.,* 1999), and exhibit reduced macrophage activation, which is associated with lower IFN-γ production (Barnes *et al.,* 1993; Orme *et al.,* 1993). Even though $CD8^+$ T cell production of IFN-γ can compensate and restore the concentrations of IFN-γ back to normal levels in $CD4^+$ T-defecient mice, the latter still succumb to the infection earlier than wild-type mice, suggesting a role for $CD4^+$ T cell-derived IFN-γ early in the infection, or perhaps an IFN-γ-independent mechanism that is important early in the infection (Caruso *et al.,* 1999). An IFN-γ-independent $CD4^+$ T cell-mediated mechanism dependent on NOS2 has recently

been shown *in vitro* and suggested to be important *in vivo* by the demonstration that IFN-γ-deficient mice can be effectively protected by BCG vaccination in a $CD4^+$ T cell-dependent manner (Cowley & Elkins, 2003).

CMI to *M. tuberculosis* promotes the formation of granulomas – infected macrophages surrounded by antigen-specific lymphocytes, multinucleated giant cells, and a ring of fibrous deposition (Dannenberg & Rook, 1994b). Granulomas typically encompass the entire infection foci and effectively isolate the bacilli from the rest of the healthy lung. Granuloma formation is largely dependent on TNF-α and GM-CSF (Kindler *et al.*, 1989; Shibata *et al.*, 2001), which are produced by infected macrophages, T cells (Saunders *et al.*, 2003), and other hemaetopoetic cells, and drive tissue-resident stromal cells to produce macrophage-inflammatory protein-1β (MIP-1β), MIP-1α (Czermak *et al.*, 1999) and other chemokines (Algood *et al.*, 2003) needed to recruit neutrophils, lymphocytes, and monocytes to the site of infection. TNF-$\alpha^{-/-}$ mice infected with *M.* tuberculosis fail to form organized granulomas, are incapable of controlling *M. tuberculosis* replication, and succumb to *M. tuberculosis* within 20-30 days post infection (Flynn *et al.*, 1995; Bean *et al.*, 1999). The levels of TNF-α must be tightly regulated since overproduction can lead to excessive pathology (Bekker *et al.*, 2000). Likewise, GM-CSF-deficient mice are highly susceptible to *M. tuberculosis* infection (mean-time to death, 35 days) (Gonzalez-Juarrero *et al.*, 2005).

**2.5.2. Survival of *M. tuberculosis* in the macrophage.** Pathogenic mycobacteria reside and readily replicate within tight vacuoles of non-immune-activated macrophages (Armstrong & Hart, 1971; Rusell, 2001). Pioneering studies by D'Arcy Hart and co-workers showed that tubercle bacilli prevent the fusion of lysosomes to *M. tuberculosis*-containing vacuoles, which prevents their normal acidification and facilitates bacterial survival and replication (Armstrong & Hart, 1971, 1975). Vacuoles containing live virulent *M. avium* have a relatively high pH (6.3-6.5), which correlates with a lower density of vesicular $H^+$ ATPase pumps on their membranes (Sturgill-Koszycki *et al.,* 1994). Mycobacterial vacuoles reside within the recycling/sorting endosomal compartment — markers of early endosomes (Rab5) are retained, markers of late endosomes (Rab7) and lysosomes (LAMP1-1 and LAMP-2) are largely excluded; communication with the cytoplasmic membrane appears to be maintained as suggested by the cycling of transferrin in and out of the vacuoles (Clemens & Horwitz, 1996; Sturgill-Koszycki *et al.,* 1996). Diversion of the mycobacterial phagosome from the normal maturation pathway correlates with retention of the actin-binding coronin "TACO" on the phagosomal membrane (Ferrari *et al.,* 1999).

Active evasion of phagolysosomal fusion by *M. tuberculosis* involves bacterial "effectors" such mannose-capped lipoarabinomannan (ManLAM) which interferes with phagolysosomal fusion by blocking the acquisition of the lysosomal cargo and syntaxin 6 from the trans-Golgi network (Fratti *et al.*, 2003). LAM alone can specifically block the cytosolic $Ca^{2+}$ increase necessary for a

signaling cascade involving phosphatidylinositol (PI)3 kinase hVPS34, which is needed for production of PI 3 phosphate (PI3P) on phagosomes (Vergne *et al.*, 2003).

The importance of type-1 cytokines in human immunity to *M. tuberculosis* infection has been confirmed by studies of naturally occurring polymorphisms in the genes encoding type-1 cytokines and their receptors in humans (Atare *et al.*, 1998; Ottenhoff *et al.*, 2002). The principle role of type-1 cytokines in anti-TB immunity is to activate the anti-microbial functions of macrophages. IFN-γ activation and infection of murine macrophages with *M. tuberculosis* results in the differential expression of some 40% of the macrophage genes, an indication of just how dynamic the host-*M. tuberculosis* interaction is (Ehrt *et al.*, 2001). Many of these differentially-expressed genes likely encode functions involved in the curtailment of growth and killing of the intracellular bacilli.

**2.5.3. Effector mechanisms of the immune-activated macrophage.** Schaible and colleagues (1998) found that upon activation of *M. avium*-infected macrophages with IFN-γ and LPS, the mycobacterial vacuoles fuse to each other, become more acidic (pH 5.3), and as a result of fusion with lysosomes accumulate hydrolases. These and other changes in the intracellular milieu lead to stasis and killing of the bacilli.

One of the facilitators of phagolysosomal fusion and acidification downstream of IFN-γ that has been shown to be essential for protection in TB is

LRG47 (Feng *et al.*, 2004; MacMicking *et al.*, 2003), a member of a family of IFN-γ-dependent GTPases that mediate fusion events in endosomal networks (Taylor *et al.*, 1996). That these GTPases play different functions in response to infection by different pathogens is suggested by the findings that $IGTP^{-/-}$ and $IRG\text{-}47^{-/-}$ mice, which are susceptible to infection by viral, bacterial, and eukaryotic pathogens (Taylor *et al.*, 2000; Collazo *et al.*, 2001), are resistant to infection by *M. tuberculosis*. In contrast, $LRG47^{-/-}$ mice succumb to infection by *M. tuberculosis* with kinetics similar to those of $NOS2^{-/-}$ mice, the other major pathway of antimycobacterial immunity (Chan *et al.*, 1992; Chan *et al.*, 1995; MacMicking *et al.*, 1997).

The immune system relies on chemically reactive micromolecules as well as enzymes to neutralize and eliminate invading pathogens and their products. Some of the most efficient of these reactive molecules in killing and controlling *M. tuberculosis* include the reactive nitrogen intermediates derived from nitric oxide produced by nitric oxide synthase 2 (Nathan, 1992). *M. tuberculosis* is killed by murine peritoneal macrophages and cell lines activated with IFN-γ and either LPS or TNF-α via RNI production, and IFN-γ and TNF-α synergistically increase NO synthesis by macrophages (Flesch & Kaufmann, 1991; Chan *et al.*, 1992; MacMicking *et al.*, 1997a; Nathan & Shiloh, 2000). The demonstration that $NOS2^{-/-}$ mice are highly susceptible to *M.* tuberculosis infection provides clear functional genetic evidence that NOS2 is essential for the protective immune response against *M. tuberculosis* (MacMicking *et al.*, 1997b; Scanga *et al.*, 2001).

Even though macrophage activation and resulting RNI production result in bacteriostasis, *M. tuberculosis* is nonetheless able to resist eradication in mouse lungs and in activated macrophages in tissue culture, despite the fact that *in vitro* reagent NO is a potent mycobactericidal (Nathan & Ehrt, 2003). Having evolved to infect the mammalian lung, *M. tuberculosis* appearently has ways of countering the nossive effects of NO and related RNI. One such mechanism involves the product of the *mpa* gene, which forms hexamers that have ATPase activity, and is believed to be associated with a mycobacterial proteosome. Δ*mpa M. tuberculosis* bacteria are attenuated in wild-type mice and show some revertion to virulence in $NOS2^{-/-}$ (Darwin *et al.,* 2003). It remains to be shown whether Δ*mpa M. tuberculosis* bacteria have defects in protein degradation via this presumptive proteasome, and whether this proposed function is involved in protecting the bacteria from RNI. An additional strategy adopted by *M. tuberculosis* to deal with RNI appears to be simple avoidance of NOS2. Recent, microscopy studies indicate that *M. tuberculosis* might be able to prevent NOS2 from being recruited to bacteria-containing phagosomes (Miller *et al.*, 2004).

The role of NO in protection against *M. tuberculosis* in humans is somewhat controversial (Nathan & Ehrt, 2003). While some investigators have reported NOS2 protein induction, NO, which is measured by determining the concentration of its breakdown products nitrate ($NO_3$) and nitrite ($NO_2$), cannot be consistently detected in *M. tuberculosis*-infected cultures of human monocytes/macrophages (MacMicking *et al.,* 1997; Nathan & Ehrt, 2003).

Furthermore, no mutations in *nos2* in humans have yet been associated with susceptibility to *M. tuberculosis*. However, patients with active TB apparently exhale high levels of NO, which correlate with high expression of NOS2 and spontaneous production of nitrite by alveolar macrophages (Wang *et al.*, 1998).

Another reactive micromolecule that is a potent antimicrobial effector of the immune response is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (PHOX)-mediated production of reactive oxygen intermediates (ROIs) - reduction products of $O_2$ such as superoxide, hydrogen peroxide, and hydroxyl radical (Nathan & Shiloh, 2000). ROI generation is an effective antimicrobial defense against many bacterial pathogens including *Salmonella* (Vazquez-Torres & Fang, 2001) and *Burkholderia cepacia* (Segal & Holland, 2003). However, the protective role of ROI in TB appears to be less prominent than during *Salmonellae* infections. CGD patients which lack phagocyte oxidase have not been reported to be more susceptible to *M. tuberculosis* infection (Nathan & Ehrt, 2003). Furthermore, $gp91^{(phox-/-)}$ mutant mice, which lack a functional phagocyte oxidase are not more susceptible to *M. tuberculosis* than wildtype mice (Hisert *et al.*, 2004; Ng *et al.*, 2004), while mice lacking the gp47 subunit of PHOX are less able to control *M. tuberculosis* replication only transiently (Cooper *et al.*, 2000). Furthermore, *M. tuberculosis* appears to be able to effectively counteract the damaging effects of ROIs. ROI detoxification mediated by the KatG catalase is essential for bacterial persistence in mice following induction of adaptive immunity (Li *et al.*, 1998; Pym *et al.*, 2002), and an *M. tuberculosis*

mutant of *katG* is attenuated in wild-type mice, but reverts to wildtype levels of virulence in mice lacking PHOX (Ng *et al.,* 2004). The product of *katG* is also a peroxynitritetase, thus it may protect *M. tuberculosis* from botrh RNI and ROI (Wengenack *et al.*, 1999). At least one of the superoxide dismutases of *M. tuberculosis* contributes to intracellular survival (Piddington *et al.*, 2001).

**2.5.4. Immunity during *M. tuberculosis* chronic infection.** Several of the components that are important for generating a protective immune response to *M. tuberculosis* infection are also important for maintaining the equilibrium between host and pathogen during the chronic phase of the infection. As expected, Th1 maintainance is important for controlling *M. tuberculosis* during the chronic phase of the infection and sustaining this type of response requires IL-12 (Feng *et al.,* 2005). Neutralization of TNF-α by antibodies or by infection with an adenovirus expressing a soluble TNF-α receptor resulted in marked increases in bacterial loads and exacerbation of disease in the mouse model of chronic TB and in a variation of the Cornell model of drug-induced latency (Adams *et al.,* 1995, Scanga *et al.,* 1999; Mohan *et al.,* 2001). Also using the Cornell model of TB, IFN-γ has been shown to be involved in preventing reactivation (Scanga *et al.,* 1999). Similarly, chemical inhibition of NOS2 in chronically infected mice resulted in rapid increases in lung bacterial loads (Flynn *et al.,* 1998). NOS2-inhibiton also reactivated *M. tuberculosis* in variations of the Cornell model of latency (Scanga *et al.,* 1999; Botha *et al.*, 2002).

## 2.6. *M. tuberculosis* mechanisms of immune evasion

Some of the bacterial mechanisms *M. tuberculosis* uses to directly protect itself from ROI/RNI effectors of the immune response have already been discussed. But like many persistent pathogens, *M. tuberculosis* has evolved mechanisms to evade and subvert host immune responses at many levels (Flynn & Chan, 2003). In addition to directly blocking phagosome maturation (See above), *M. tuberculosis* interferes with IFN-γ signalling via STAT1 (Ting *et al.*, 1999). STAT1 deficiency in humans has been associated with increased susceptibility to mycobacterial infection. Down-regulation of antigen-presenting (MHC class II and CD1) and costimulatory (B7) molecules on infected macrophages may affect activation of IFN-γ-secreting T cells (Wojciechowski *et al.*, 1999; Flynn & Chan, 2003). Macrophage activation, antigen presentation, and T cell activation may also be inhibited by cytokines secreted by macrophages as a result of the infection such as IL-10 (Murray, 1999), IL-6 (van Hyningen *et al.* 1997; Nagabhushanam *et al.*, 2003), and transforming growth factor-β (TGF-β) (Vanham *et al.*, 1997). In addition to blocking antimicrobial signaling pathways, many pathogens prolong their survival by triggering apoptosis of leukocytes (Weinrauch & Zychlinsky, 1999). However, in *M. tuberculosis* infection apoptosis of the infected macrophage has been implicated as a mechanism of host protection which could deprive the bacterium of its intracellular niche of replication (Oddo *et al.*, 1998; Gil *et al.*, 2004). Indeed accumulating evidence suggests that pathogenic

mycobacteria may interfere with apoptosis of infected macrophages in order to perpetuate their intracellular "safe haven" (Fratazzi *et al.,* 1999; Kornfeld *et al.,* 1999; Keane *et al.,* 2000).

## 2.7. Extracellular persistence of *M. tuberculosis in vivo*

In the human lung *M. tuberculosis* is thought to reside both intracellularly and extracellularly, depending on the type of lesion (Grossett, 2003; Kaplan *et al.,* 2003; Boshoff & Barry 2005). Pioneering work by Lurie (1964) and continued by Dannenberg (Dannenberg & Rook, 1994b) in the rabbit model showed the existence of extra-cellular *M. tuberculosis* in caseous necrotic lesions. Since immunity to TB was thought to be mainly mediated by activated phagocytes which kill intracellular bacteria (Mackaness, 1968; North, 1974), it was suspected that the extra-cellular milieu represent a safe heaven for bacterial replication and perhaps persistence. In order to study this population of bacteria directly, Lurie devised a very ingenious method of cultivating bacteria extracellularly *in vivo*: Parlodion-film encased silk bags that were impervious to host cells, but not to plasma, were inoculated with bacilli, and then implanted subcutaneously in rabbits. These "extracellular" bacteria replicated unhindered during the first two wks of infection, but then leveled off at circa 2 wks post infection. When immunized animals were used, bacteria stopped growing earlier and were killed more efficiently than in naïve counterparts, strongly implicating acquired

immunity in controlling the growth of extracellular bacteria. Since control of growth correlated with a more rapid mobilization of mononuclear cells toward the encased focus of infection, it was proposed that factors secreted by activated macrophages rendered the fluids entering the infection foci inimical to bacterial replication (Lurie, 1939a,b).

Recently, Karakousis and collagues (2004) adapted Lurie's strategy to the widely used model of murine TB: *M. tuberculosis* was encapsulated in cell-impermeable hollow fibers, and these were implanted subcutaneously into mice for *in vivo* cultivation. As expected, an initial period of bacterial replication was followed by curtailment of growth, followed by a considerable decline in numbers, and, interestingly, an eventual stabilization of the counts. These stationary-state CFU counts, similarly to those in the equilibrium that characterizes the chronic phase of infection in the mouse model of chronic TB (Rees & Hart, 1961; Muñoz-Elías *et al.,* 2005) represented a somewhat static scenario: they found that about half of the encapsulated bacilli were alive by differential staining; a highly dynamic equilibrium would have resulted in a large accumulation of dead bacteria and a decreasing proportion of live ones. Furthermore, indirect analysis - comparison of ATP utilization and CFU at corresponding time points - indicated that soon after implantation, bacteria decreased their metabolism. The rapid cessation of multiplication and rather swift reduction in metabolic activity suggest that innate defenses or perhaps immune-independent mechanisms prompt these adaptations. This was also suggested by Lurie's studies using vaccinated animals,

which implicated an unknown plasma factor in bacterial growth inhibition. This factor could be nitric oxide, which is known to block *M. tuberculosis* replication *in vitro* by inhibiting bacterial respiration (Voskuil *et al.*, 2003). Careful analysis of the hollow-fiber's contents in this extracellular model of persistence *in vivo*, including concentrations of NO, oxygen, essential elements, and nutrients might be of value.

## 2.8. Chemotherapy: drug tolerance and resistance

**2.8.1. Chemotherapy and drug tolerance.** Current antibiotic treatment regimens for active pulmonary TB are costly and burdensome, typically consisting of an early phase of daily treatment with four drugs (isoniazid, rifampin, pyrazinamide, and ethambutol or streptomycin) for two months, and a prolonged four-month-long phase of isoniazid and rifampin treatment. However, even when adequate antibiotic therapy is completed, persistent bacilli may not be eradicated completely (Kopanoff, 1988), and relapses are relatively frequent, especially among $HIV^+$ individuals (Perriens *et al.*, 1995). Most drugs currently used to treat TB target functions that are required for bacterial growth and division; see Table 2.1 for a list of available drugs and their proposed targets.

**2.8.2. Drug Resistance.** The long duration and complexity of currently available TB drug regimens contribute to high levels of non-compliance and the emergence

of widespread drug resistance and multiple-drug resistant (MDR) strains of *M. tuberculosis*. The global prevalence of MDR-TB has recently been estimated at 4.3% (Heymann *et al.,* 1999); alarmingly, almost 80% of MDR-TB strains worldwide are resistant to 3 of 4 first-line drugs. In some eastern European and former USSR nations, MDR-TB has reached epidemic proportions (Farmer *et al.*, 1998; TB alliance, 2005 report; tballiance.org).

**2.8.3. Drug tolerance *in vivo*.** It is not clear why treatment of TB is so difficult and slow. The same antimicrobials that take months to cure a patient or to sterilize the tissues of an experimentally infected animal, will sterilize a culture of *M. tuberculosis in vitro* within days (Fig. 2.3B&C) (McDermott, 1958; McKinney, 2000; Mitchision, 2004). Drug penetration might be a problem, though apparently at least for isoniazid adequate penetration does occur (Barclay *et al.,* 1953). Neither increasing drug dosages (McDermott, 1958), nor giving patients more drugs (Mitchison, 1980), led to increases in rates of killing *in vivo*.

**Drug tolerant bacterial populations.** One influential model of persistence in the face of chemotherapy posits that the phenomenon results from physiological heterogeneity of mycobacteria in the tissues, which might include slow- and non-dividing bacteria which resist killing by antimicrobials (Mitchison 1979, 1980). Supporting evidence for this model was provided by a remarkable clinical study in which tuberculous lung lesions were cultured from patients who had received prolonged multi-drug chemotherapy (Fig. 2.3A) (Vandiviere *et al.*, 1956). Lesions

**Table 2.1. Currently available drugs to treat TB in humans**

| **Drug** | **Cellular process targeted** | **Molecular target (*gene*)** |
|---|---|---|
| **Isoniazid** | Cell wall mycolic acid synthesis | Enoyl-ACP reductase (*inhA*)<br>β-Ketoacyl ACP synthase (*kasA*) |
| Ethionamide | Cell wall mycolic acid synthesis | Enoyl-ACP reductase (*inhA*) |
| **Pyrazinamide** | Cell wall fatty acid biosynthesis | FAS-I fatty acid synthase (*fas*) |
| **Ethambutol** | Cell wall arabinogalactan synthesis | Arabinosyltransferase (*embAB*) |
| Cycloserine | Cell wall peptidoglycan synthesis | D-alanyl, D-alanine ligase (*ddlA*)<br>Alanine racemase (*alr*) |
| **Rifampin** | RNA synthesis | RNA polymerase β subunit (*rpoB*) |
| Streptomycin | Polypeptide synthesis | 16S rRNA (*rrs*)<br>S12 ribosomal protein (*rpsL*) |
| Fluoroquinolones | DNA synthesis | DNA gyrase (*gyrAB*) |
| PAS (*p*-amino-salicylic acid) | Folic acid synthesis | Dihydropteroate synthase (*folP*) |

Names in bold lettering indicate 1st line drugs. *InhA* (Banerjee *et al.*, 1994); *kasA* (Mdluli *et al.*, 1998); *fas* (Zimhony *et al.*, 2000); *embAB* (Belanger *et al.*, 1996); *ddlA* (David *et al.*, 1969); *alr* (Caceres *et al.*, 1997); *rpoB* (Telenti *et al.*, 1993); *rrs* and *rpsL* (Finken *et al.*, 1993); *gyrAB* (Drlica *et al.*, 1996); *folP* (Nopponpunth *et al.*, 1999). Table adapted from McKinney (2000).

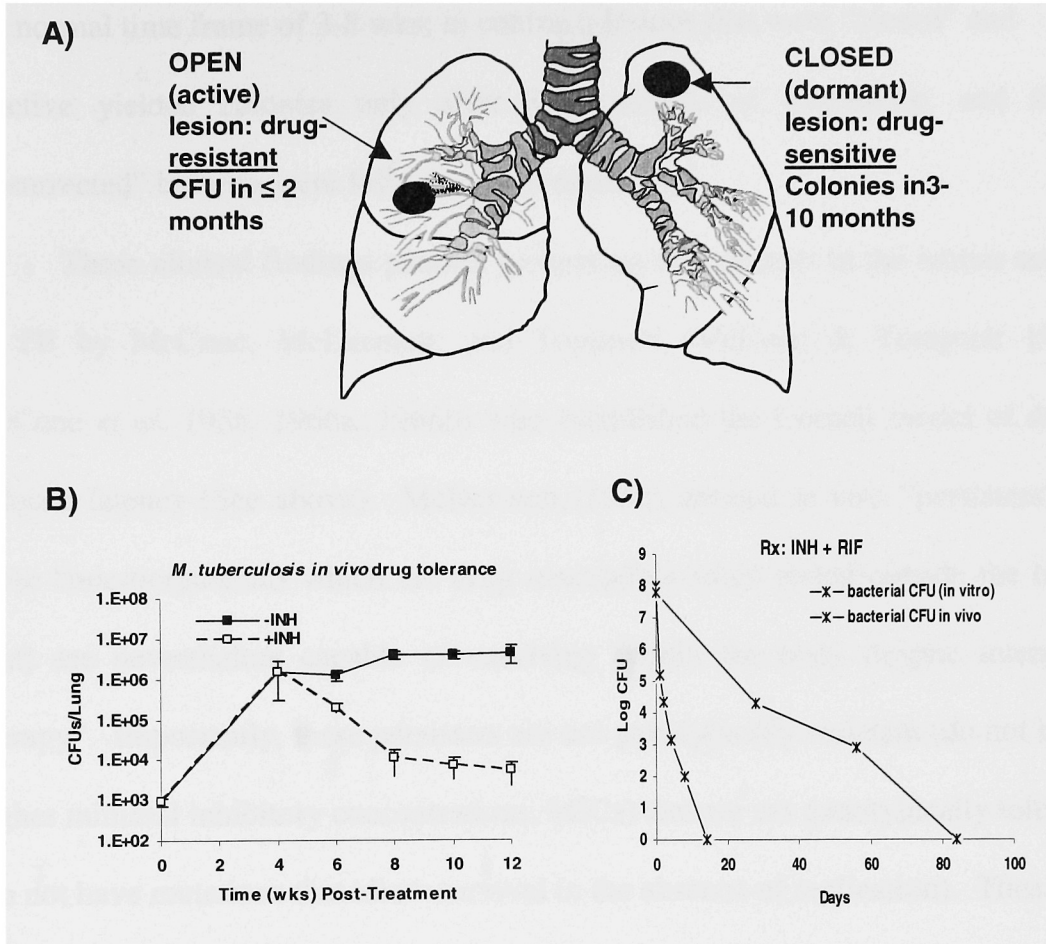


**Fig. 2.3. *In vivo* drug tolerance**. (**A**) Resected lung lesions from patients on chemotherapy were cultured. "Open" and active cavitary lesions, with patent connections to the airways, yielded drug-resistant tubercle bacilli in the normal timeframe (less than 2 months' incubation). "Closed" and dormant encapsulated lesions, with no apparent connections to an airway, yielded drug-sensitive bacteria only after extended incubation (3 to 10 months). Adapted from Vandiviere et al. (1956) by Munoz-Elias & McKinney (2002). (**B**) *In vivo* drug tolerance in mice. Mice infected with *M. tuberculosis* (Erdman) were put on INH monotherapy from wk 4-12 post-infection (empty) or not (solid). (**C**) *In vitro* vs *in vivo* susceptibility of *M. tuberculosis* isoniazid (INH) and rifampin (RIF); (Fig. C: courtesy of J.D. McKinney).

that were "open" and active yielded colonies of drug-resistant tubercle bacilli in the normal time frame of 3-8 wks; in contrast, lesions that were "closed" and inactive yielded colonies only after 3-10 months of incubation, and these "resurrected" bacteria were fully drug susceptible.

These clinical findings parallel pioneering experiments in the mouse model of TB by McCune, McDermott, and Tompsett (McCune & Tompsett 1956; McCune *et al.* 1956, 1966a, 1966b) who established the Cornell model of drug-induced latency (See above). McDermott (1958) defined *in vivo* "persisters" as those "microorganisms which are drug susceptible when tested outside the body [but] are nevertheless capable of surviving within the body despite intensive therapy". Importantly, these persisters are not genotypically resistant (do not have higher minimal inhibitory concentrations, MICs) and are not genotypically tolerant (do not have mutations that allow survival in the absence of replication). Thus, the nature of these persisters largely remains an enigma.

**The nature of 'persisters.'** This type of bacterial persistence was first recognized by Bigger (1944), who observed that penicillin treatment of a growing culture of *Staphylococcus aureus* consistently failed to kill a fraction of the population ($10^{-6}$). Remarkably, these bacteria, which he called "persisters," could survive exposure to multiple antibiotics and stresses and were therefore postulated to be in a modified state in which they were neither able to replicate, nor permanently incapacitated since they retained the ability to grow like wild-type when cultured in the absence of antibiotics.

One important question regarding the nature of "persisters" is whether they generate spontaneously or are induced by exposure to antibiotics. Recently, Balaban *et al.,* (2004) took advantage of two previously described *E. coli* strains which formed high numbers of persisters, hipA7 (Moyed & Bertrand, 1983) and hipQ (Wolfson *et al.,* 1990), and microfluidic devices which allowed the analysis of the history of individual cells after subjecting the population to antibiotic-mediated killing, to demonstrate the existence of at least two types of persisters, both of which were shown to be present in the population prior exposure to antibiotic. Type I persisters are a preexisting subpopulation of non-growing cells generated at stationary phase. The size of the population of type I persisters is directly proportional to the number of stationary phase cells inoculated into a culture. Type II persisters are a subpopulation of cells that multiply more slowly than the majority of the cells in the culture, a state that appears to be inherited for several generations. Since type II persisters are constantly generated during exponential growth, the number of type II persisters present in a growing culture increases in step with the population, and is not influenced by the size of the inoculum.

Since these persisters survive treatment with antibiotics but their generation is independent of exposure to them, they are probably generated during natural infections and might play a role in persistent infections including TB. How might these persisters arise during *M. tuberculosis* infection? Most bacteria in the sputum of TB patients are susceptible to killing by antibiotics, as suggested by

rapid conversion to sputum negativity soon after onset of chemotherapy; thus it is unlikely that many of these bacteria are persisters. Since *M. tuberculosis* infection involves the inhalation of infectious droplet nuclei containing between 1-10 bacilli, natural transmission of persisters is unlikely. Persisters could be generated as the infection progresses. Type II persisters could be generated during the initial period of replication before the onset of CMI and probably in smaller numbers during periods of active replication throughout the chronic phase of the infection. Type I persisters could be generated during periods of no replication, which might be similar to stationary-phase. If bacterial replication resumes sporadically during latency, only to be curtailed by the protective immune response, type I persisters, by virtue of their lag in coming out of stationary phase, might be protected from such killing and enriched for. Both types of persisters would be intrinsically tolerant to the cidal action of antibiotics, but type II persisters would be expected to be more sensitive to killing by antibiotics than type I persisters because replication and cell division require macromolecular synthetic processes that are targeted by currently available drugs.

But why is *M. tuberculosis* so recalcitrant to eradication by the host and by antibiotics compared to other bacteria that also have the ability to form persisters? One reason might be its intrinsic resistance to chemical and physical stress, which could act as a barrier to the efficient penetration by drugs. Additionally, *M. tuberculosis* might have evolved a high rate of generation of persisters. The existence of high persistence mutants in other bacteria indicates that the ability to

form persisters is under genetic control and therefore subject to evolution. Bacterial species might have evolved specific rates for generation of persisters, which fit their particular life styles, e.g., it is possible that bacterial species that spend a lot of time in biofilms have a high basal rate of persister generation, and that the resistance to killing displayed by bacteria in a biofilm might be due to this high fraction of persisters. *M. tuberculosis*, having evolved to cause chronic and latent infections in humans, would likely benefit from having an intrinsically high persister generation rate. In LTBI, acquired immunity, which effectively controls and kills most of the infecting bacteria, might fail in eradicating all the bacteria because surviving cells might be persisters that are phenotypically resistant to host antibacterial chemical compounds (ROIs, RNIs, acid, hydrolases, etc) as well as to commonly used antibiotics. Interestingly, recent modeling suggests that high persister generation might be a physiological adaptation well suited to life in environments where antibiotics (stresses) are intermittently encountered, while low rates of persister generation might be better suited for life in an environment where exposure to anbiotics (stresses) is rare (Kussell *et al.*, 2005). Generation of persisters may be important for pathogens like *M. tuberculosis* which depend on long-term survival in the dynamic environment of the immune-aware host for transmission. Whether environmental signals might influence the manifestation of the persister phenotype is unknown; there could be significant interplay between the myriad of mechanisms involved in stress survival by bacteria and the mechanism(s) of persister generation.

# CHAPTER 3

## Bacterial Metabolism *in vitro*

### INTRODUCTION

We have learned much about the metabolism of bacteria since the observation of these animacules under Leeuwenhoek's crafty lenses, and their early cultivation in Pasteur's expertly sterile hands (de Kruif, 1926). Most of this knowledge has been gained from the study of a handful of species, mostly the enterics *Escherichia coli* and *Salmonella enterica* serovar *typhimurium*, and has resulted in our vast current understanding of physiology and metabolism. However, since most of these studies were and continue to be conducted in the laboratory, generally under artificially optimized conditions, much less is known about the physiology and metabolism of bacteria living in their natural environments. This void in our understanding extends to those few species that are arguably the most relevant ones, those bacteria that as part of their intended or accidental life cycles inhabit and cause malaise in humans, and occasionally their death. This chapter is a general discussion of bacterial metabolic pathways with an emphasis on carbon metabolism, largely drawing from the work on enterics; however, an effort has

been made to highlight what is known to occur or might occur in *M. tuberculosis* and other mycobacterial species.

### 3.1. General metabolic considerations

In order to sustain themselves, chemotrophic bacteria need a number of elements in varying quantities. Some elements such as carbon (C), oxygen (O), hydrogen (H) and nitrogen (N), which are abundant constituents of cells, as well as calcium (Ca), chloride (Cl), iron (Fe), phosphorous (P), potassium (K), magnesium (Mg), and sulfur (S), which are needed for various cell functions, are required in relatively high concentrations (>100 μM). A number of other elements such as manganese and zinc are needed in trace amounts. All of these elements are generally found in substances referred to as nutrients, which are also energy sources since their catabolism can lead to ATP generation, the energy currency of the cell. Some of this ATP can be produced directly from metabolic intermediates containing high-energy phosphoryl bonds by the transferring of this group to ADP (substrate-level phosphorylation). However, most ATP molecules are generated through respiratory chains in which the flow of electrons from negative redox-potential donors to more positive redox-potential acceptors is linked to the transfer of the phosphoryl groups to ADP molecules. ATP is employed to activate metabolic intermediates for further chemical reactions necessary for cell maintainance, growth, and genetic perpetuation. While the electron donor can be

either an organic or an inorganic compound, the electron acceptor is in most cases oxygen, though other electron acceptors such as carbon dioxide, nitrate, and sulfate are sometimes used.

In addition to the elements so far listed, different types and amounts of a variety of compounds must sometimes be obtained from the environment, depending on whether the bacteria can synthesize them themselves. These include prosthetic groups of various enzymes such as nicotinic acid (precursor of coenzymes $NAD^+$ and $NADP^+$), lipoic acid, riboflavin B2 (precursor of flavin mononucleotide, FMN), flavin adenine dinucleotide (FAD) and thiamine (precursor of thiamine pyrophosphate), as well as other vitamins and some amino acids.

### 3.2. Substrate uptake

Passively diffused substrates (oxygen, $CO_2$) get into cells without interaction with cell membrane components until equilibrium is established; their transport does not involve energy expenditure. Similarly, facilitated diffusion requires no energy, but does involve a membrane carrier. Active transport, in contrast, is substrate specific and requires energy. One type of active transport involves the electron transport chain or ATP hydrolysis at the membrane and its coupling with the carrier's ability to bind and transport the substrate into the cell. Another type involves substrate-specific binding proteins, which usually hydrolyse ATP. Group

translocation by chemical modification of the transported substrate via formation of a phosphate ester is also common and in this case the donor is a phosphorylated substrate specific enzyme that traps the substrate in the membrane and frees it into the cytoplasm in the phosphorylated form. The transport of sugars in *E. coli* occurs through this mechanism via the phosphoenolpyruvate:glucose phosphotransferase system (PTS), where the phosphate donor is phosphoenol pyruvate (PEP). This pathway is found mostly in facultative and obligate anaerobic bacteria and is apparently absent in mycobacteria (Romano, 1970).

### 3.3. Carbon catabolism and cellular biosynthesis

Bacteria can either use organic (carbon heterotrophs) or inorganic compounds (carbon autotrophs) to generate energy and cell constituents. Mycobacteria are heterotrophs and can use a variety of carbon substrates as sources of carbon and energy. In addition to chemotrophy, mycobacteria are also capable of chemolithotrophy since they all contain a CO dehydrogenase and can grow on CO (Park *et al.,* 2003). Bacteria can grow on a number of carbohydrates including glucose, fructose, lactose, and pentoses, on several organic acids including pyruvate, malate, and succinate, as well as on a number of amino acids, and fatty acids of different lengths, including acetate and propionate. Carbon utilization studies *in vitro* have shown that *M. tuberculosis* and other mycobacteria can

oxidize a number of carbon substrates including carbohydrates, lipids, and amino acids (Wheeler, 1984).

Carbon substrate catabolism provides the bacterial cell with energy in the form of reducing equivalents to generate ATP, and with essential biosynthetic precursors. Glucose-6-P, fructose-6-P, 3-P-glycerate, PEP, pyruvate, acetyl-CoA, oxaloacetate and alpha-ketoglurate are all precursors of monomers such as sugar phosphates, amino acids, vitamins, fatty acids, and nucleotides, which are then assembled into macromolecular cellular components such as proteins, polysaccharides, lipids, and nucleic acids that make up the cell. A partial list is provided in table 3.1.

**Table 3.1. Important precursors of biosynthesis and their products**

| **Precursor** | **Product** |
|---|---|
| Fructose-6-P | mannose-6-P, hexoseamines (N-acetylglucosamine of LPS and peptidoglycans) |
| Dihydroxyacetone phosphate | Phospholipids |
| 3-phophoglycerate | Glycine, Serine, Cysteine |
| PEP | Phenylalanine, Tryptophan, Tyrosine |
| Pyruvate | Alanine, Valine, Leucine |
| Acetyl-CoA | Fatty acids |
| Propionyl-CoA | Fatty acids |
| 2-ketogluteraldehyde | Arginine, Glutamate, Glutamine, Proline |
| Succinyl-CoA | Porphyrins |
| Oxaloacetate | Asparagine, Aspartate, Isoleucine, Lysine, Methionine, Threonine |

### 3.4. Pathways of carbohydrate catabolism

Sugars such as glucose and other hexoses are used by many bacteria as sources of carbon and energy. Glucose is not only a highly versatile biosynthetic precursor, but thanks to its relatively high potential energy, it is also an excellent fuel for ATP generation. A typical bacterial cell uses half of the glucose available to it for biosynthetic purposes and the other half for ATP generation (Fraenkel, 1996). In several bacteria, the transport of glucose into the cell is linked to its phosphorylation via the PTS, which is unique to bacteria (Anderson and Wood, 1969). Searches of the *M. tuberculosis* genome with the glucose-specific (or fructose-specific) phosphotransferase enzyme IIA or other components of the PTS systems yielded no homologues. Some bacteria, including *E. coli* and *Salmonella* have a hexokinase (*glk*) and while *glk* mutants grow normally on glucose medium, PTS-deficient bacteria grow slowly on glucose relying on this hexokinase. *M. tuberculosis* does not have a clear *glk* homologue (possibly Rv0650), but it does have a clear polyphosphate glucokinase homolog, and transposon mutants of *ppgK*, encoding this putative sugar transporter, grow slowly on glycerol/dextrose-containing plates (Sassetti *et al.*, 2003).

The three main pathways of sugar intermediary metabolism are the Embden-Meyerhof pathway (glycolysis), the pentose phosphate pathway, and the Entner-Doudoroff pathway (Romano & Conway, 1996; Peekhaus and Conway, 1998).

**3.4.1. Entner-Doudoroff pathway.** Some bacteria metabolize sugars through 6-phosphogluconate via the Entner-Doudoroff pathway (EDP) (Fig. 3.1) (Entner & Doudoroff, 1952). Though the EDP is widely distributed and apparently one of the most ancient in evolution, its significance in carbon catabolism is only beginning to be understood (Romano & Conway, 1996). Studies carried out over the past few decades, most of which analyzed *E. coli* grown in bactch cultures and high glucose concentrations, indicated that the EDP played a minor role in carbohydrate metabolism. In contrast, recent functional genetic analyses suggest that the EDP is heavily employed and is essential for the survival of *E. coli* in the mammalian intestine (Chang *et al.,* 2004). Furthermore, recent analysis of bacterial species not routinely studied in the laboratory is uncovering a much more central role for the EDP in bacterial sugar catabolism than previously realized (Fuhrer *et al.,* 2005). Genes encoding enzymes of the EDP, namely glucose oxidase (*gcd*), 6-P-gluconate dehydrase (*edd*), and 2-keto-3-deoxy-6-P-gluconate aldolase *(eda*) are not found in *M. tuberculosis* or *M. leprae* genomes as determined by BLASTP searches with the *E. coli* sequences. Likewise homologs of genes involved in the transport of gluconate (gluconate permeases: *gntP* and *gntT*) or its activation (gluconate kinase: *gntK*) are also missing.

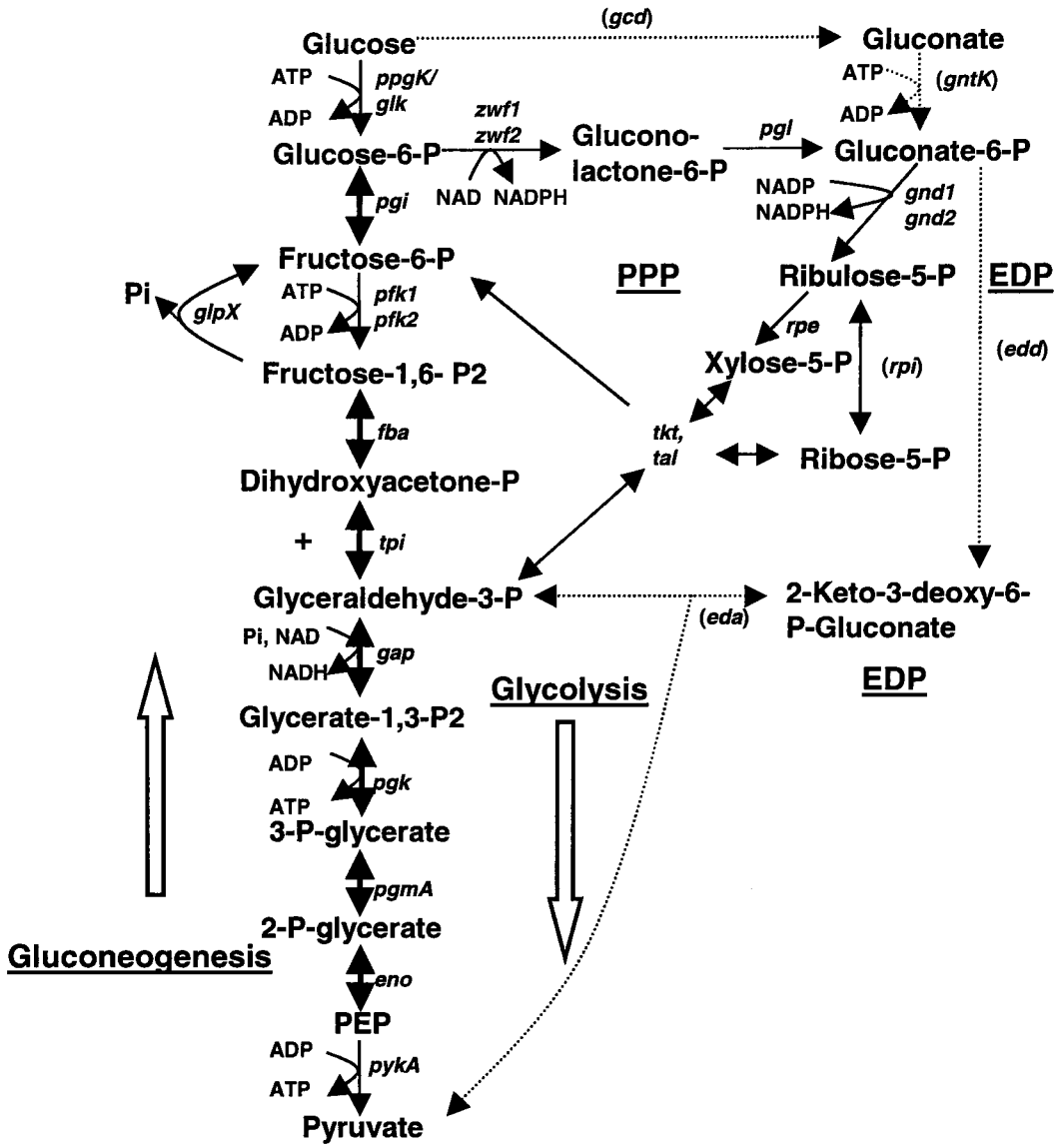


**Fig. 3.1. Carbohydrate metabolism pathways.** The best-characterized pathways of carbohydrate catabolism are glycolysis, the pentose phosphate pathway (PPP), and the Entner Doudoroff pathway (EDP). Glucose anabolism occurs via gluconeogenesis. Enzymes common to glycolysis and gluconeogenesis are indicated by thick double-headed arrows. Irreversible reactions: phosphofructokinase and pyruvate kinase (glycolysis), and fructose-1,6-biphosphatase and PEP synthase (gluconeogenesis). *pts*, PEP:glucose phosphotransferase system; *glk*, glucose kinase; *pgi*, phosphoglucose isomerase; *pfk*, phosphofructokinase; *fbp*, fructose-1,6-bisphosphatase; *fba*, fructose-1,6-bisphosphate aldolase; *tpi*, triosephosphate isomerase; *gap*, glyceraldehyde 3-phosphatedehydrogenase; *pgk* phosphoglycerate kinase; *pgm*, phosphoglyceratemutase; *eno*, enolase; *pyk*, pyruvate kinase; *pps*, PEP synthase; *ppdk*, pyruvate phosphate dikinase; *gcd*, glucose dehydrogenase; *gntK*, gluconate kinase; *zwf*, glucose-6-phosphate dehydrogenase; *pgl*, 6-phosphogluconolactonase; *edd*, 6-phosphogluconate dehydratase; *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase; *gnd*, 6-phosphogluconate dehydrogenase; *rpe*, ribulose-5-phosphate epimerase; *tkt*, transketolase; *tal*, transaldolase. Genes absent in mycobacteria are indicated by parenthesis/dashed arrows (Modified from Fraenkel, 1996).

**3.4.2. The pentose phosphate pathway.** The pentose phosphate pathway (PPP) (Fig. 3.1) allows the oxidation of one molecule of glucose to 3 of $CO_2$ and 1 glyceraldehyde-3-P with the concomitant generation of NADPH; NADPH is required for reactions in which NADH cannot act as co-enzyme. The PPP also generates pentose phosphates, which are precursors of nucleotide biosynthesis, and erythrose-4-P, which together with phosphoenol pyruvate (PEP), are precursors of aromatic amino acids (Romano & Conway, 1996). In *B. abortus* the PPP alone can be used for sugar metabolism. *M. tuberculosis* has homologs of the genes of the PPP, including glucose-6-P dehydrogenase (*zwf1* and *zwf2*), phosphogluconolactonase (*pgl*), 6-phosphogluconate dehydrogenases (*gnd1* and *gnd2*), transaldolase (*tal*) and transketolase (*tkt*). *E. coli* oxidize up to a third of the glucose available throuhg the PPP, and the rest through glycolysis. However, other species use the PPP mostly for biosynthetic purposes. Importantly, the PPP pathway can function as a cycle and allow aerobic growth on carbohydrates in the absence of a functional TCA cycle (Fig. 3.1). Glucose-6-phosphate dehydrogenase activity (GND) was long ago demonstrated in extracts from fast and slow growing mycobacteria (Sharma *et al.,* 1985), and *M. tuberculosis* transposon mutants in several PPP genes (*gnd2*, *tkt*, *tal*) are impaired for growth on standard mycobacterial medium (Sassetti *et al.,* 2003).

**3.4.3. Embden-Meyerhof-Parnas pathway (Glycolysis).** Glycolysis (Fig. 3.1) is widely used for the degradation of glucose-6-P to pyruvate (Fraenkel, 1996).

Lacking a PTS, *M. tuberculosis* likely activates glucose by phosphorylating it via polyphosphate glucokinase (PPGK). In *E. coli*, the inter-conversion of glucose-6-P and fructose-6-P is catalyzed by P-glucose isomerase (*pgi*). Δ*pgi E. coli* grow slowly on glucose, and do so relying on the PPP since deleting either *zwf* or *gnd* in a *pgi*-null background abrogates growth (Fraenkel & Levisohn, 1967). Similarly, PGI-deficient *M. smegmatis* grow on glucose but at a lower rate and after a protracted lag phase (Tuckman *et al.*, 1997). *M. tuberculosis pgi* mutants are also impaired for growth on glucose-containing media (Sassetti *et al.*, 2003).

Fructose-6-P is converted to Fructose-1,6-P2 by phosphofructokinase (*pfk*). *E. coli* has two PFK isoenzymes, neither of which is essential for growth on glucose, however, *pfkA* appears to encode the main PFK in this species since *pfkA* mutants are more markedly impaired for growth on glucose than mutants in *pfkB* (Doelle, 1975; Vinopal & Fraenkel, 1975; Vinopal *et al.*, 1975). Similarly, *M. tuberculosis* has two putative PFKs, which appear to overlap functionally (Sassetti *et al.*, 2003). Interestingly, *E. coli* Δ*pfkA*/Δ*pfkB* mutants cannot grow on glucose, but constitutivity of the glyoxylate cycle (GC), an anaplerotic pathway normally employed during growth on even-chain fatty acids (C2 substrates), as a result of a mutation in the GC regulator *iclR*, can compensate for the partial loss of PFK (Δ*pfkA*) (Vinopal & Fraenkel, 1974), which suggests that when there is reduced flow of carbon into into the lower part of glycolysis, the GC might supply sufficient PEP via PEP carboxykinase (PCK) (Kornberg & Smith, 1970).

Fructose-1,6-P2 aldolase (*fba*) splits fructose-1,6-P2 into dehydroxyacetone-P and glyceraldehyde-3-P, and interconversion of these two products is catalyzed by triose-P-isomerase (*tpi*) (Bai *et al.*, 1982). As expected, *M. tuberculosis tpi* mutants do not grow well on standard 7H10 plates where glucose and glycerol are the main carbon sources (Sassetti *et al.,* 2003). When *E. coli* use glycerol as a carbon source, its conversion to dihydroxyacetone-P must be accompanied by its isomerisation to glyceraldehyde-3-P to avoid the production of methylglyoxal, which can be toxic to cells (Cooper & Anderson, 1970). This might not be an issue during glycerol catabolism in mycobacteria because methylglyoxal synthase (*mgsA*) seems to be absent in these species.

Glyceraldehyde-3-P is converted to glycerate-1,3-P2 by glyceraldehyde-3-P dehydrogenase (*gap*); glycerate-1,3-P2 can be interconverted to 3-P-glycerate by P-glycerate kinase (*pgk*), which can be interconverted to 2-P-glycerate by P-glycerate mutase (*gpm*), of which several homologues exist in *M. tuberculosis*. 2-P-glycerate is dehydrated to PEP by enolase (*eno*). Though functional TPI, GAP, PGK and ENO enzymes are required for growth on either glycerol or succinate alone, if both substrates are provided, these steps are dispensable (Anderson & Wood, 1969; Fraenkel, 1996). Up until this point in glycolysis, the only irreversible reaction dicussed is that catalyzed by PFK.

### 3.5. Conversion of PEP to pyruvate and the fate of pyruvate

Conversion of PEP to pyruvate by pyruvate kinase (PYK) is coupled to ATP generation (Clark, 1989). *E. coli* lacking PYK (PYKI and PYK2) grows slowly on glucose, relying on the PPP for sugar catabolism and energy, the PTS system for pyruvate generation, and PEP carboxylase (PPC) for replenishment of oxaloacetate (Ponce *et al.,* 1998; Peng & Shimizu, 2003). PYK-deficient *E. coli* can only grow on sugars that are transported by the PTS because pyruvate is formed when the phosphoryl group of PEP is donated (Anderson & wood, 1969). *B. subtilis* PYK-deficient mutants grow slowly on glucose (Fry *et al.,* 2000) despite lacking a PPC, likely because their PTS provides the necessary pyruvate.

Since *M. tuberculosis* lacks both a PTS and PPC, a PYK-deficient mutant should not grow on sugars. While *M. tuberculosis* has a functional PYK enzyme, the related species *M. bovis* lacks this activity (Garnier *et al.,* 2003) and cannot grow on glycerol or dextrose (Keating *et al.,* 2005). *M. bovis* grows well on several C2 and C3 (odd chain fatty acids) substrates *in vitro* (unpublished, E.J.M.).

Most commonly, pyruvate is oxidized through decarboxylation to acetyl-CoA via the pyruvate dehydrogenase (PDH) complex, which is composed of pyruvate dehydrogenase (E1), a dihydrolipoamide acetyl (succinyl-) transferase (*sucB*, E2), which liberates the acetyl-CoASH molecule, and dihydrolipoamide dehydrogenase LPD (E3), which regenerates the dihydrolipoic acceptors

covalently attached to lysine residues on the E2 acetyl (succinyl-) transferase component (Argyrou & Blanchard, 2001). The PDH complex is present in almost all aerobic heterotrophs so far studied (Neveling *et al.*, 1998). The LPD subunit is the same as that in the E3 of 2-ketoglutarate dehydrogenase (α-KGDH). *M. tuberculosis* LPD and the SUCB components of PDH/α-KGDH apparently participate in the detoxification of peroxynitrite *in vitro* (Byrk, *et al.*, 2002).

### 3.6. The tricarboxylic acid cycle (TCA cycle)

The TCA cycle is the predominant cycle of intermediary metabolism in animal tissues, where it oxidizes acetyl-CoA to $CO_2$ and provides several important biosynthetic precursors. However, the TCA cycle is generally more widespread in eukaryotes than it is in prokaryotes; bacterial genomes often lack one or more genes of the pathway, most commonly an α-ketoglutarate dehydrogenase component (Cordwell, 1999).

In the TCA cycle, oxaloacetate (oxaloacetate) (a C4-dicarboxylic acid) condenses with acetyl-CoA (high potential-energy C2 compound) yielding citrate (C6 compound) (Fig. 3.2) (Cronan & LaPorte, 1996). The cycle then oxidizes this C6 compound back to the C4 releasing two $CO_2$ molecules and gaining ATP, GTP, $NADH^+$, and $FADH_2$ molecules in the process. Acetyl-CoA, which can originate from sugars (glycolysis and PDHC), fatty acids (β-oxidation), or through direct synthesis from acetate (acetyl-CoA synthase) enters the TCA cycle through its

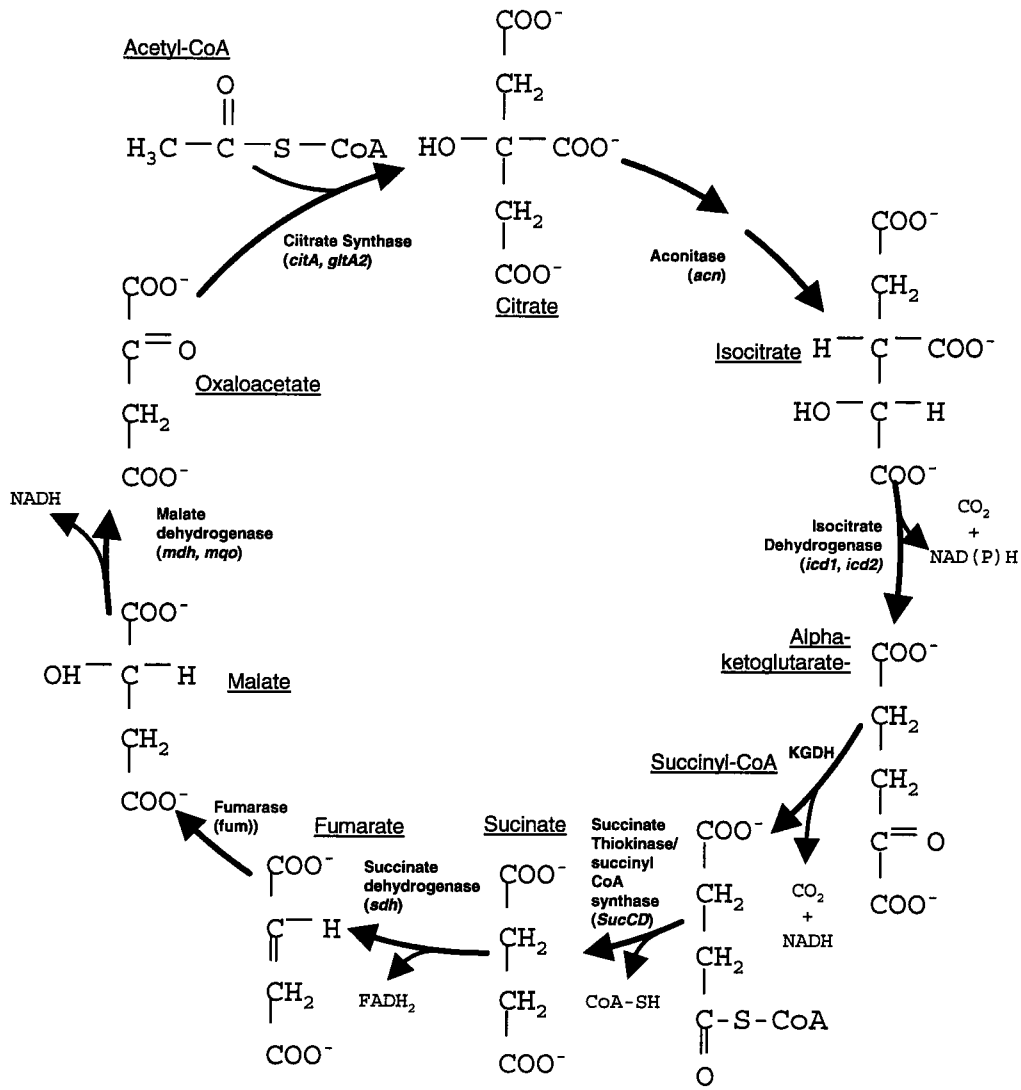


**Fig. 3.2. Tricaboxylic Acid Cycle.** The TCA cycle allows the oxidation of acetyl-CoA to $CO_2$ with concomittant gain of reducing equivalents. Quantity and type of carbon substrate, as well as oxygen levels, affect expression of TCA cycle enzymes; induction of the TCA cycle is most pronounced when bacteria grow on C2 substrates under aerobic conditions. Gene names are those used for *M. tuberculosis* (H37Rv annotation) (TubercuList: http://genolist.pasteur.fr/TubercuList/).

condensation with oxaloacetate by action of citrate synthase. *M. tuberculosis* has three genes that could encode this activity (*gltA1*, *gltA2*, *citA*). The isomerization of citrate to isocitrate is catalyzed by a two step reaction carried out by aconitase (ACN, *acs*), which first dehydrates citrate to the enzyme-bound form cis-aconitate, and subsequently adds a water molecule to this intermediate to produce the free isocitrate. Isocitrate is then oxidized and decarboxylated to α-ketoglutarate by isocitrate dehydrogenase. *M. tuberculosis* has two isocitrate dehydrogenases (IDHs) (*icd1*, *icd2*) (Sharma *et al.,* 1985). Similarly, *Ralstonia* species encode two IDHs and these are functionally redundant (Wang *et al.,* 2003a).

The oxidation of α-ketoglutarate to succinyl-CoA is catalyzed by the α-ketoglutarate dehydrogenase complex (α-KGDH), which is analogous to the PDH complex which oxidizes pyruvate to acetyl-CoA. In *M. tuberculosis*, PDH and α-KGDH complexes apparently have in common not only the LPD (E3) component, but also the lipoate dehydrogenase (E2) component, since the only lipoylated protein in the *M. tuberculosis* proteome is SUCB (E2) (Bryk *et al.,* 2002). The conversion of succinyl-CoA to succinate is catalyzed by succinate thiokinase (*sucCD*) and is accompanied by the generation of an ATP molecule by substrate level phosphorylation by taking advantage of the high energy thioester group in succinyl-CoA. In *E. coli*, α-KGDH dehydrogenase genes (*sucAB*) and the succinate thiokinase genes (*sucCD*) are in an operon (*sucABCD)*; in *M. tuberculosis*, *sucCD* form an operon, but *sucA* and *sucB* do not.

Succinate dehydrogenase (SDH, *sdhA-D*) then oxidizes succinate to fumarate, which is subsequently hydrated to malate by fumarase (FUM, *fum*). In *E. coli,* an NAD-dependent malate dehydrogenase (MDH) is primarily responsible for the oxidation of malate to oxaloacetate. Malate:quinone-oxidoreductase (MQO), an FAD-dependent membrane-associated protein, which can also catalyse the oxidation of malate to oxaloacetate while the electrons are donated to quinones of the electron transport chain instead of to $NAD^+$ (Kather *et al.,* 2000), is only required if MDH is missing (Molenaar *et al.*, 2000; van der Rest *et al.,* 2000). In contrast, MQO is the principal enzyme catalyzing the oxidation of malate to oxaloacetate in *C. glutamicum*, and Δ*mqo* mutants of this species cannot grow on C2 substrates. In fact, purified *C. gltuamicum* MDH readily catalyzes the reduction of oxaloacetate to malate (Molenaar *et al.,* 2000). Similarly, in *P. aeruginosa* MQO rather than MDH oxidizes malate to oxaloacetate and is needed for growth on acetate; the function of MDH in this species is not known (Kretzschmar *et al.,* 2002). These observations suggest that in at least some bacteria, MDH is involved in the reductive branch of the TCA cycle (See Fig. 3.3), while MQO functions in TCA cycle and/or the GC during growth on C2 substrates (See Fig. 3.2, 3.6). Mycobacteria have both types of malate dehydrogenase. Addition of cAMP to low-oxygen cultures of *M. bovis* (BCG) upregulates expression of *mdh*, which could mean that mycobacteria increase flow of carbon through the TCA and glyoxylate cycles under these conditions (Gazdik & McDonough, 2005), or, alternatively, that the TCA cycle functions reductively

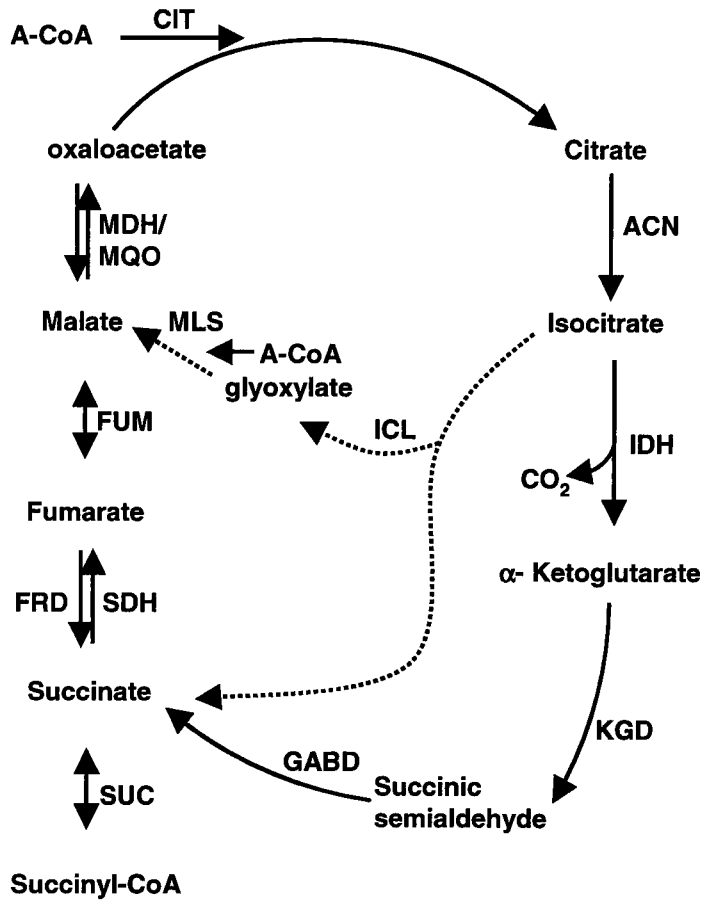


**Fig. 3.3. Branched and 'modified' TCA Cycle.** The branched (biosynthetic) mode of operation of the citric acid cycle is employed by bacteria growing under aerobic conditions in excess glucose or anaerobically on any carbon substrate, and by bacteria that lack a-ketoglutarate dehydrogenase, the pathway plays a strictly biosynthetic role. ATP is obtained from glycolysis and from acetyl-CoA catabolism via the PTA/ACK pathway (not shown). The glyoxylate cycle is needed for anaplerosis. In *M. tuberculosis* the TCA cycle might operate in the branched mode via KGD and GABD replacing a-ketoglutarate dehydrogenase. Abbreviations: CIT, citrate cynthase; ACN,aconitase; IDH, isocitrate dehydrogenase; SUC, succinyl-CoA synthetase; KGD, a-ketoglutarate decarboxylase; SSADH, succinic semialdehyde dehydrogenase; SDH, succinate dehydrogenase; FRD, fumarate reductase; FUM, fumarase; MDH, malate dehydrogenase; MQO, malate:quinone oxidoreductase; ICL, isocitrate lyase;MLS, malate synthase.

under these conditions, depending on whether mycobacterial MDH is more similar in function to the MDH of *E.coli* or *C. glutamicum*, respectively.

All the TCA cycle enzyme activities have been demonstrated in protein extracts from animal tissues and many bacteria (Cronan & LaPorte, 1996). Mycobacterial genomes have homologs of every step of the cycle. Many of the activities including those of ACN, IDH, SDH, FUM and MDH have been demonstrated in several fast and slow growing mycobacteria including *M. vaccae, M. phlei, M. avium,* and *M. leprae* (Sharma *et al.,* 1985; Katoch *et al.,* 1987). Activities of the TCA cycle enzymes have also been demonstrated in *M. tuberculosis* (Murthy *et al.,* 1973), except for α-KGDH activity, which was extremely low; however, since the TCA cycle is an inducible pathway, several growth conditions should be tried before rulling out the presence of a particular activity (Iuchi & Lin, 1988; 1991; Guest & Russell, 1992; Hayashi *et al.,* 2002; Lorenz *et al.,* 2004). Recently, it has been reported that *M. tuberculosis* indeed lacks α-KGDH activity, and that instead, an α-ketoglutarate decarboxylase converts α-ketoglutarate to succinic semialdehyde, which is then converted to succinate (Tian *et al.*, 2005) (Fig. 3.3).

The main factors affecting expression of TCA cycle enzymes are oxygen, and the type and concentration of carbon source available to the cell (Gray *et al.,* 1966; Spencer & Guest, 1987, Lynch & Lin, 1996; Park *et al.,* 1997). TCA cycle enzymes are expressed in low levels in anaerobiosis and in glucose-excess aerobic growth, and in high levels during aerobic growth on C2 compounds such as fatty

acids or acetate (Amarasingham & Davis, 1965; Ornston & Ornston, 1969; Chin *et al.,* 1989; Holms, 1996; Lynch & Lin, 1996; Peng & Shimizu, 2003). Gene expression and carbon flux analyses in *C. glutamicum* suggest a similar control of the TCA cycle (Weindisch *et al.,* 2000; Hayashi *et al.,* 2002). Conditions affecting the expression of key enzymes of the TCA cycle also affect the way the cycle operates (Gray *et al.,* 1966). As mentioned previously, during anaerobic growth on glucose, *E. coli* obtains energy from glycolysis and utilizes the TCA cycle in a bifurcated mode (Fig. 3.3) in which the oxidative branch [citrate synthase, aconitase, isocitrate dehydrogenase] produces 2-ketoglutarate, while the reductive branch [malate dehydrogenase, fumarase, fumarate reductase (replaces succinate dehydrogenase), and succinate thiokinase (a.k.a succinyl-CoA synthase)] produces succinyl-CoA (Amarasingham & Davis, 1965). Interestingly, succinyl CoA synthase activity is high in leprosy bacilli grown *in vivo* (Wheeler, 1984).

In the absence of a suitable electron acceptor, *E. coli* converts acetyl-CoA to a mixture of acetic acid and ethanol via fermentation (Cozzone, 1998). Whether fermentative or acetate producing pathways are employed by mycobacteria is not known; however, genes putatively encoding fermentative enzymes such as formate lyase (*pfl*) or lactate dehydrogenase (*lld*), as well as acetate producing enzymes (*ack*, *pta*) are present in mycobacterial genomes.

Although much has been learnt about sugar metabolism from model organisms such as *E. coli*, *Salmonellae*, and *B. subtilis* over the last century, it is becoming apparent that some of the tenets of central metabolic pathways do not

apply to bacterial strains/species that are not laboratory-adapted. For instance, recent carbon $^{13}C$ tracer flux analysis experiments with several non-laboratory-adapted phylogenetically distinct bacteria indicate that unlike *E. coli* or *B. subtilis* laboratory strains, many bacteria catabolize sugars via the EDP rather than glycolysis, and rely on the PPP almost exclusively to fulfill biosynthetic needs. Importantly, all aerobes analyzed apparently employed a full respirative TCA cycle for oxidation of acetyl-CoA without any significant overflow of carbon in the form of acetate when grown in glucose under aerobic conditions (Fuhrer *et al.*, 2005). Although these observations are only of a quantitative nature, they suggest that the significance and function of a given metabolic route in one species in a particular setting is likely to be much less of a generalizable characteristic than previously thought, with different bacteria using different routes of carbon utilization to different degrees depending on the conditions of growth.

### 3.7. Glycerol catabolism

Glycerol can be used as a carbon source by many bacteria, and in particular the cultivation of mycobacteria *in vitro* is greatly enhanced by addition of this carbohydrate (Katoch *et al.*, 1987; Wheeler, 1984). In *E. coli* glycerol is first phosphorylated to glycerol-3-P by a glycerol kinase (Hayashi & Lin, 1967). Glycerol-3-P is then oxidized to dihydroxyacetone phosphate by glycerol-P dehydrogenase (Schryvers *et al.*, 1978). *M. tuberculosis* has a glycerol kinase

(*glpK*, Rv3696c) and two putative glycerol-P dehydrogenases (*glpD1*, Rv2249c; *glpD2*, Rv3302c). Glycerol-3-P is an important precursor of phospholipids, and in the absence of an exogenous source of glycerol, cells make glycerol-3-P from dihydroxyacetone phosphate employing a glycerol-3-P synthase. The proteins encoded by the *glp* regulon can also be used by the cell for recycling the glycerol moiety of phospholipids and triglycerides.

### 3.8. Fatty acid catabolism

Cells need to metabolize fatty acids to fulfill several physiologic needs. Fatty acids provide the building blocks for phospholipids, glycolipids and lipoproteins, and their derivatives act as hormones and messengers in many cell types. Furthermore, fatty acids are excellent fuels. It has been estimated that 40% of the weight of a mycobacterial cell is made up of lipids (Anderson, 1940; Wheeler, 1988) and about 8% of the *M. tuberculosis* genome is made up of genes putatively involved in lipid metabolism, including over 100 putative catabolic functions (Cole *et al.*, 1998; Cole, 2002).

Though a permease that can transport acetate into *E. coli* cells has recently been identified (Gimenez *et al.*, 2003), little is known about the uptake of other short- and medium-chain fatty acids (SCFAs, MCFAs), most of which might get into the cell by diffusion. The transport of long-chain fatty acids (LCFAs) into *E. coli* cells is mediated by a carrier mechanism which requires *fadD* and *fadL*, which

encode a fatty acyl-CoA synthase (Kamede and Nunn, 1981) and an outer membrane protein (Black *et al.,* 1985), respectively. The exact function of FADL is not known, but it increases binding and uptake of MCFAs and LCFAs. Mycobacteria lack an outer membrane and have no homologs of *fadL* genes; nothing is known about how fatty acids cross the thick lipid-rich wall and plasma membrane of a mycobacterial cell.

**3.8.1. The cycle of beta-oxidation.** Bacteria, like eukaryotes, degrade fatty acids primarily via a cyclic oxidation pathway known as β-oxidation (Black & DiRusso, 1994). Some eukaryotes including plants, fungi, and mammals have a mitochondrial as well as a peroxisomal pathway of β-oxidation, the components of which differ depending on the type of fatty acid they oxidize (Kunau *et al.,* 1985). Through β-oxidation, even-numbered fatty acids (C2 substrates) are oxidized stepwise into acetyl-CoA subunits, while odd-chain fatty acids (C3 substrates) are similarly catabolized into acetyl-CoA units and a terminal propionyl-CoA unit. Acetyl-CoA and propionyl-CoA then entered central metabolism where they are further oxidized or serve as precursors for lipid synthesis (Kolattukudy *et al.,* 1997; Campbell & Cronan, 2001). Under aerobic conditions, *E. coli* can grow on LCFAs (>12 carbon-long acyl chains) as sole source of carbon, but growth on MCFAs (C6-C12) can take place only after β-oxidation enzymes are induced as a result of the transport and activation of LCFAs inside the cell (Overath *et al.,* 1969).

Each turn of the β-oxidation cycle is accompanied by the reduction of one $FAD^+$ (acyl-CoA dehydrogenase reaction) and one $NAD^+$ (3-hydroxyacyl-CoA dehydrogenase reaction), and the shortening of the acyl chain by two carbons, which are released as an acetyl-CoA (CoA-dependent thiolytic cleavage) (Fig. 3.4). The shortened fatty-acyl-CoA molecule reenters the degradation cycle.

The principal enzymatic steps are as follows:

**1.** Activation of the free fatty acid to an acyl-CoA thioester by acyl-CoA synthase (Fatty acid:CoA ligase; EC 6.2.1.3). This step requires two high-energy phosphate equivalents per molecule of free fatty acid activated. Once a fatty acid is thus activated, no further energy expenditure is required; thus, the longer a fatty acid the more energy it can yield. While *E. coli* has a single acyl-CoA synthase with broad specificity for medium and long chain fatty acids, FADD (Overath *et al.,* 1969; Klein *et al.,* 1971), *M. tuberculosis* has 36 putative *fadDs.* At least some of these FADDs, particularly those adjacent to lipid biosynthetic genes such as the polyketide sythases (PKS), function as long-chain fatty acyl-AMP ligases which activate fatty acids as acyl-adenylates which might then be transferred to the PKS for further processing (Trivedi *et al.,* 2004).

**2.** Next, the CoA ester is oxidized to an enoyl-CoA by a fatty acyl-CoA dehydrogenase (FADE). Again, while *E. coli* has a single *fadE*, *M. tuberculosis*

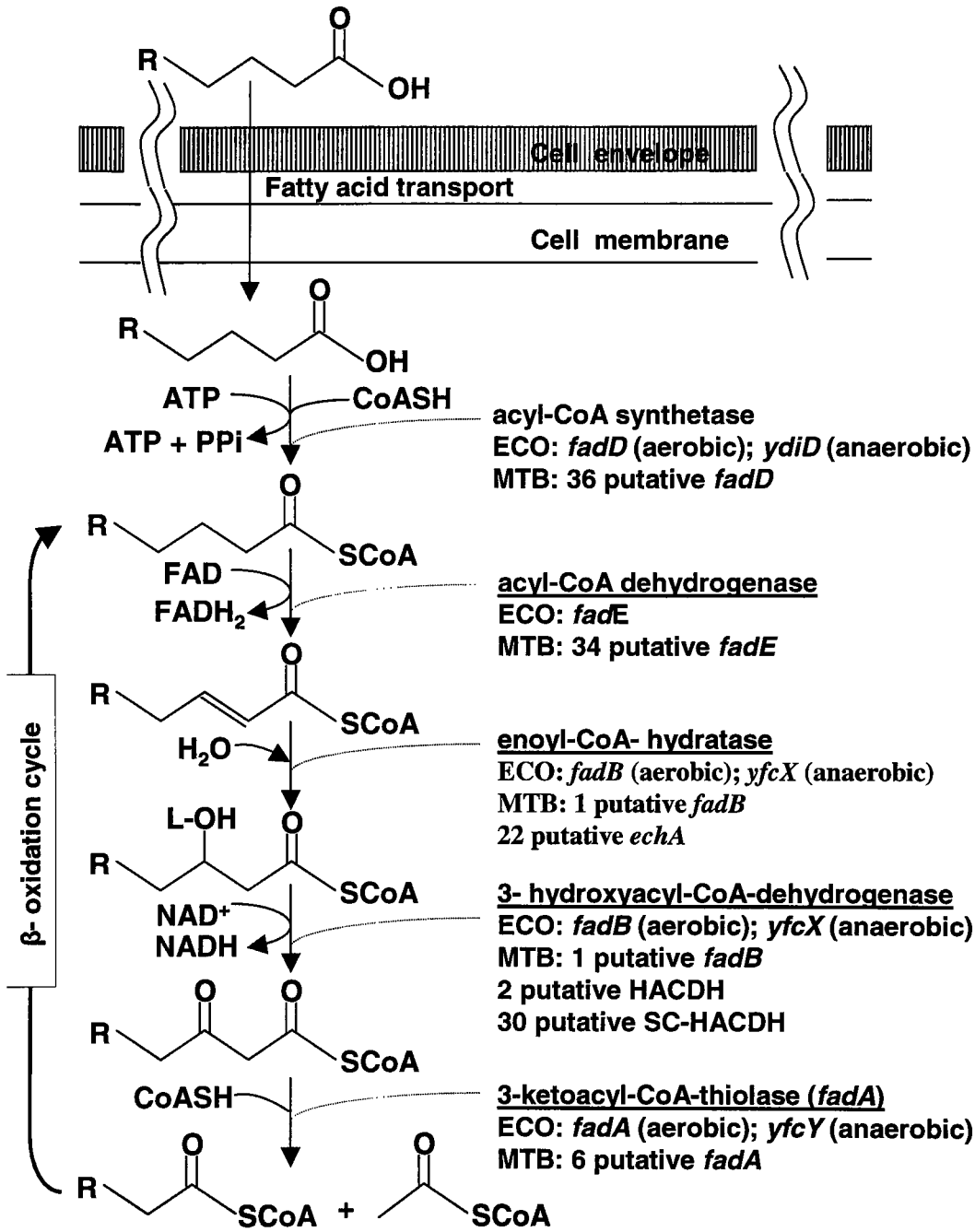


**Fig. 3.4. b-oxidation pathway of fatty acid degradation in *Mycobacterium tuberculosis*.** Based on homology searches of the *M. tuberculosis* genome (H37Rv; http://genolist.pasteur.fr/TubercuList/) using bacterial, fungal and mammalian b-oxidation genes. Gene names are those used for *M. tuberculosis*. *E. coli* (ECO) and *M. tuberculosis* (MTB) enzymes are listed. Gene names are italicized. Abbreviations: HACDH, 3-hydroxyacyl-CoAdehydrogenase; SC-HADH, short-chain 3-hydroxyacyl-CoA-dehydrogenase. (Modified from Clark and Cronan, 1996).

has 34 *fadE* homologs, resembling eukaryotic cells in which many acyl-CoA dehydrogenases with different specificities are present (Kunau *et al.,* 1995).

**3-5.** The remaining steps are catalyzed by enoyl-CoA hydratases (ECHs), which hydrate the enoyl-CoA to 3-hydroxy(OH)-acyl-CoAs, 3-hydroxyacyl-CoA dehydrogenases (FADBs), which oxidize the 3-OH-acyl-CoA to 3-keto-acyl-CoA, and 3-ketoacyl-CoA thiolases (FADAs), which cleave away the terminal acetyl-CoA, freeing the already activated fatty acyl-CoA, which enters another oxidative round. The *M. tuberculosis* genome contains 22 *echA* genes putatively encoding enoyl-CoA hydratases/isomerases. It also has two short 3-OH-butyryl-CoA dehydrogenases (*fadB2* and *fadB3*), as well as a putative multifunctional long FADB (*fadB*), which has ECH and hydroxyacyl-CoA dehydrogenase (HADH) domains. FADB2 and FADB3 belong to a large family of short chain hydroxyl-acyl dehydrogenases (SC-HADH) with members in the mitochondrial β-oxidation systems of eukaryotes, belonging to the superfamily of short-chain alcohol dehydrogenases (Krozowski, 1994).

*M. tuberculosis* has many genes potentially encoding SC-HADHs (Cole *et al.,* 1998), including around 30 or so SC-HADHs with homology to fungal peroxisomal β-oxidation SC-HADHs. *A. nidulans* mutants lacking a peroxisomal SC-HADH (Δ*foxA*) are impaired for growth on long chain fatty acids, but grow normally on C6 fatty acids relying on a mitochondrial short chain β-oxidation system (Maggio-Hall & Keller, 2004), and interestingly, the dehydrogenases of these mitochondrial pathways are homologous to the *E. coli* and *M. tuberculosis*

canonical *fadB* products. Thus, considerable functional overlap exists between FADBs and non-canonical short chain dehydrogenases in fungi; whether this is the case in *M. tuberculosis* remains to be determined.

**3.8.2. Catabolism of short chain fatty acids as carbon substrates.** In some bacteria, including *E. coli*, growth on SCFAs (C4-C6) requires, in addition to some β-oxidation enzymes, the induction of an additional set of activities encoded by the *atoA, D, B* and *C* genes (Pauli & Overath, 1972). *AtoA* and *atoD* encode the subunits of an acetoacetyl-CoA transferase involved in the activation of acetoacetate and short-chain fatty acids into acyl-CoAs; *atoB* encodes a ketohydroxylacyl thiolase. In addition to repression by FadR (a regulator of LCFA utilization), these genes are subject to transcriptional control by a positive regulator encoded by *atoC* (Overath *et al.,* 1969). Wild-type *E. coli* cannot grow on SCFAs unless FadR is released by activated LCFAs and the ATO system is induced by SCFAs.

In contrast to enterics, mycobacteria readily grow on SCFAs without requiring prior exposure to LCFAs. In fact, both *M. smegmatis* (Muñoz-Elías *et al.,* in preparation) and *M. tuberculosis* (Muñoz-Elías & McKinney, 2005) grow comparably well in SCFAs and glucose. *M. tuberculosis scoA* and *scoB* genes have homology to *atoD* and *atoA* genes, respectively, and *fadA* genes have some homology to *atoB*. Thus, it is possible that *scoA/B* gene products are involved in activating SCFAs to acyl-CoAs. Alternatively, one or more of the FADD acyl-

CoA synthases could perform this function. After processing by a FADE and an ECH, the 3-OH-acyl-CoA could then be oxidized by a FADB or one of the other SC-HADHs, into the keto form, which might then be cleaved by a FADA thiolase. Given that the *scoB/A* genes are in an operon with *accA1/accD1* genes, which putatively encode acetyl-/propionyl-CoA carboxylases, it is also possible that the degradation of some SCFAs might be linked to the metabolism of propionyl-CoA via the methylmalonyl-CoA pathway.

**3.8.3. Regulation of fatty acid utilization.** In *E. coli* the genes encoding the functions involved in the transport and β-oxidation of MCFAs and LCFAs form the *fad* regulon, which is under the transcriptional control of the negative regulator FadR, which in the absence of LCFAs, represses oxidation and induces fatty acid biosynthesis instead (Cronan & Subrahmanyan, 1998; Black *et al.*, 2000). In *E. coli*, the *fadBA* operon has a FadR binding site in its promoter, and binding of acyl-CoAs (>11 carbons in length) to FadR, releases it from the *fadBA* promoter and allows the synthesis of β-oxidation enzymes. Utilization of fatty acids requires the anaplerotic GC, the expression of which is indirectly controlled by FadR through IclR (see below) (Ornston & Ornston, 1969). A homolog of *fadR* is absent in *M. tuberculosis* and essentially nothing is known about the regulation of fatty acid catabolism in mycobacteria.

**3.8.4. Utilization of acetate and propionate.**

**Acetate.** Acetate might diffuse freely or be transported into the *E. coli* cell by a permease (Gimenez *et al.,* 2003), and after activation to acetyl-CoA by an inducible acetyl-CoA synthase (ACS) (Brown *et al.,* 1977), it either enters biosynthesis or is oxidized to $CO_2$ via the TCA cycle (Fig. 3.5A). In *Salmonella,* the histone deacetylase SIR2 is required for ACS activity, and for growth on acetate and propionate (Starai *et al.,* 2003). Growth on acetate, similarly to growth on other C2 substrates, requires the GC for anaplerosis (Kornberg & Krebs, 1957; Kornberg & Sadler, 1960).

*E. coli* and *C. glutamicum* growing in high concentrations of glucose employ the PTA/ACK pathway, which is composed of a phosphotransacetylase (PTA) that reversibly converts acetyl-CoA and Pi to acetyl-P and CoASH, and acetate kinase (ACK), which reversibly converts acetyl-P and ADP to acetate and ATP (Rose *et al.,* 1954) (Fig. 3.5A) to produce acetate from acetyl-CoA (Kakuda *et al.,* 1994). However, the pathway can function in the opposite direction, especially during growth in high concentrations of acetate, and allows Δ*acs* mutants to grow, though poorly, on low concentrations of acetate (Kumari *et al.,* 1995) (Fig. 3.5B); Δ*acs*/Δ*pta*/Δ*ack* mutants cannot grow on acetate (Kumari *et al.,* 1995). In addition to eliminating excess acetyl-CoA in the form of acetate, the PTA/ACK pathway also provides acetyl-P, which has been proposed as a signaling molecule based on *in vitro* studies using two-component systems and acetyl-P as phosphate donor, though the significance of these observations is not clear since signaling in wild-type strains with normal two-component systems has

**Acetate metabolism:**

**A)**

**acetate + ATP + CoA-SH → acetyl-CoA + AMP + PPi (acetyl-CoA synthase, *acs*)**

**B)**

**acetyl-CoA + Pi ←→ acetyl-P + CoASH (acetate kinase, *ack*)**

**acetyl-P + ADP ←→ acetate + ATP (phosphotransacetylase, *pta*)**

**C)**

**Propionate metabolism:**

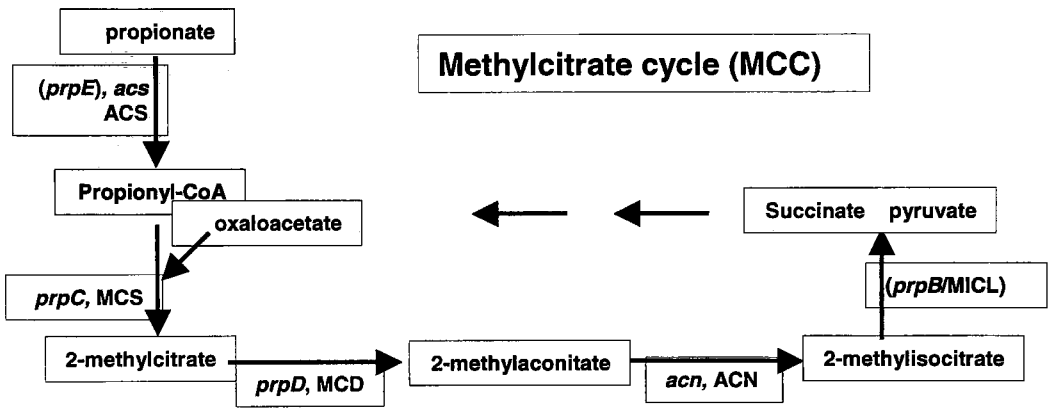


**Fig. 3.5. Pathways of acetate and propionate metabolism.** (**A-B**) Acetate metabolism. (**A**) Acetate is assimilated via acetyl-CoA synthase. ACS can also use propionate as a substrate. (**B**) PTA/ACK is used for acetate production and also provides the acetyl-P signaling molecule. Enzyme names are indicated next to corresponding reaction. (**C**) Odd-chain carbon substrate metabolism via the methyl citrate cycle (MCC). The MCC pathway essentially converts propionyl-CoA into pyruvate. Enzymes involved: acetyl/propionyl-CoA synthase (ACS, *acs*), 2-methylcitrate synthase (*prpC*, MCS), 2-methylcitrate dehydratase (*prpD*, MCD), and 2-methyl-isocitrate lyase (*prpB*, MICL). Aconitase (ACN, *acn*) is in common with the TCA cycle, as are the enzymes converting succinate to oxaloacetate (not shown). Gene/enzyme names are based on the *M. tuberculosis* genome (Tuberculist.org), although not always annotated as such; when missing, the *E. coli* name is used (in parentheses). (**C**) Adapted from Tabuchi & Uchiyama (1975).

not been found to be affected by loss of either *ackA* or *pta* (Cleary & Stock, 1994; Chamnongpol & Groisman, 2000). More recently, acetyl-P has been implicated in glucose-starvation (Chang *et al.,* 1999), osmoregulation (Bang *et al.,* 2002), biofilm development (Wolfe *et al.,* 2003), as well as in nitrogen and phosphate assimilation (Reviewed in Wolfe, 2005).

Mycobacteria have homologs encoding functions of both acetate metabolism pathways. It appears that similarly to *E. coli,* in mycobacteria ACS is the major pathway involved in acetate utilization as a carbon source, since Δ*acs* mutants of *M. smegmatis* cannot grow on acetate (L. Merkov, personal communication). The function of the PTA/ACK pathway and acetyl-P intermediate in mycobacteria is unknown.

**Propionate.** In mammalian cells, the metabolism of propionate-, branched-chain amino acids-, and odd-chain fatty acids-derived propionyl-CoA takes place via propionyl-CoA carboxylase (PCCA), a mitochondrial enzyme that carboxylates propionyl-CoA to d-methylmalonyl-CoA, which is then rearranged to succinyl-CoA. Mutations in PPCA are the cause of propionic acidemia, the most frequent autosomal recessive disorder of organic acid metabolism in humans (Wolf *et al.,* 1981). This pathway is also used by several bacterial species (Halarnkar & Blomquist, 1989) and has been proposed to function in *E. coli* (Evans *et al.,* 1993).

However, mounting evidence indicates that several bacteria including *E. coli* (Textor *et al.,* 1997), *S. typhimurium* (Horswill & Escalante-Semerena, 1997;

1999), *C. glutamicum* (Claes *et al.,* 2002), and *Ralstonia eutropha* (Bramer & Steinbuchel, 2001), catabolize propionate via a pathway known as the methylcitrate cycle (MCC). The role of the MCC in C3 substrate catabolism was discovered three decades ago in *Candida lipolytica* (Tabuchi & Serizawa, 1975; Tabuchi & Uchiyama, 1975), and has more recently been demonstrated in two other fungi, *S. cervisiae* (Pronk et at., 1994) and *A. nidulans* (Brock *et al.,* 2001). In the MCC, a molecule of propionyl-CoA is condensed with oxaloacetate by methylcitrate synthase (MCS, *prpC*) to form methylcitrate, which is then isomerized by the combined action of a dehydratase (MCD, *prpD*) and aconitase (ACN, *acs*) into methylisocitrate, which is cleaved into succinate and pyruvate by methylisocitrate lyase (MICL, *prpB*) (Tabuchi & Uchiyama, 1975) (Fig. 3.5C). The *M. tuberculosis* genome has homologs of *prpC* (MCS) and *prpD* (MCD), in addition to the other TCA cycle enzymes in common with the MCC; however, a *prpB* (MICL) is apparently missing.

The first step in propionate utilization is its activation to propionyl-CoA. This can occur via a specific propionyl-CoA synthase (PRPE) as in *Salmonellae* (Horswill & Escalante-Semerena, 1999), or by ACS which, in addition to acetate, can use propionate as a substrate. However, not all ACS can use propionate; for example *S. cereviase* has 2 ACS, and while one can use both acetate and propionate as a substrate, the other can only use acetate (van den Berg *et al.,* 1996). Mycobacteria have only one ACS (*acs*), whose specificity is unknown. Alternatively, propionate activation can occur via the PTA/ACK pathway, which

usually operates in the reverse orientation (acetate production). The first step can be catalyzed by a specific propionate kinase - *tdcD* gene in *E. coli* and *Salmonellae* - which produces propionyl-P. The closest homolog to either of these kinases in *M. tuberculosis* is *ackA*. ACK from *E. coli* can function as a propionate kinase but is less efficient than the canonical propionate kinase. In the second step in the activation of propionate, propionyl-P is converted to propionyl-CoA by PTA.

In *E. coli* and *Salmonellae*, the *prp* genes are subject to catabolite repression by glucose (Lee *et al.*, 2005). In *M. tuberculosis*, transcription of *prpDC* is upregulated after phagocytosis by macrophages and *in vivo* (Schnappinger *et al.*, 2003).

### 3.9. PEP generation and gluconeogenesis

Regardless of whether carbohydrates are available as carbon sources or not, bacteria need to generate several glycolysis intermediates that serve as biosynthetic precursors. In the absence of sugars as carbon sources, cells accomplish this via gluconeogenesis, which is essentially glycolysis backwards since most glycolytic enzymes readily catalyze the reverse reactions (Fig. 3.1). Only the conversion of fructose-1,6-P2 to fructose-6-P requires a non-glycolytic enzyme and this is because the PFK-catalyzed reaction is not readily reversible. This strictrictly gluconeogenic enzyme is called fructose-1,6-biphosphatase (*fbp*),

and mutants lacking this activity fail to grow on acetate, dicarboxylic acids and other gluconeogenic substrates (Fraenkel & Horecker, 1965). In *M. tuberculosis*, this reaction is carried out by a class II fructose 1,6-bisphosphatase encoded by *glpX* (Movahedzadeh *et al.*, 2004). The transcriptional activator/repressor Cra (*cra*) is apparently responsible for controlling the direction of carbon flow in *E. coli* and *Salmonellae* and mutants of *cra* cannot grow on glucogeogenic substrates (Saier & Ramseier, 1996). No such regulator has been identified in mycobacteria.

The starting point of gluconeogenesis varies depending on the carbon source available for growth but it is usually pyruvate or PEP. The most common precursor of PEP is oxaloacetate, but PEP can also be generated by reactions around pyruvate (Fig. 3.6). During growth of *E. coli* on C2 substrates or dicarboxylic acids, PEP carboxykinase (PCK) decarboxylates oxaloacetate to PEP. PCK-deficient *E. coli* can grow on glucose or pyruvate, but not on succinate, fumarate, or malate (Anderson & Wood, 1969). PEP can also be generated from pyruvate by PEP synthase (PPS), and *E. coli* lacking PPS cannot grow on pyruvate, lactate, or alanine (Cooper & Kornberg, 1965; Oh *et al.*, 2002), indicating that *E. coli* cannot use the pyruvate-oxaloacetate-PEP route (via PCA and PCK) for PEP generation under these conditions. Growth of *E. coli* on glucose or PEP requires upregulation of the anaplerotic PEP carboxylase (PPC), and downregulation of PCK and PPS; PCK is induced by acetyl-CoA, while PPS is induced by pyruvate (Cooper & Kornbeg, 1967). *M. tuberculosis* and *M. bovis* do not have a PPS homolog, but have a putative pyruvate phosphate dikinase

(PPDK), which could generate PEP via phosphorylation of pyruvate as in *E. coli*, thus possibly replacing PPS (Cooper & Kornberg, 1965).

An alternative route for generating PEP from C2 substrates is via malic enzyme (MEZ), which decarboxylates malate to pyruvate (Fig. 3.6) (Hansen & Juni, 1975). During growth of *E. coli* on dicarboxylic acids, MEZ produces pyruvate, which is oxidized to obtain necessary acetyl-CoA (Pertierra & Cooper, 1977). In *B. subtilis*, the pyruvate produced by MEZ apparently also serves as as a substrate for pyruvate carboxylase (PCA). In this species, MEZ and PCA form a "pyruvate shunt" whose function might be to provide pyruvate during growth on gluconeogenic substrates and then recycle back the pyruvate left to acetyl-CoA (Diesterhaft & Freese, 1973). A recent quantitative carbon flux study found that several non-model bacteria substantially (up to ~30% of carbon flow) relied on the pyruvate shunt to obtain oxaloacetate during growth on glucose (Fuhrer *et al.*, 2005). *M. tuberculosis* has both *pca* and *mez* genes, but while *pca* transposon mutants are impaired for growth on glucose-containing plates, *mez* mutants are not, suggesting that *M. tuberculosis* might not need to generate pyruvate from malate during growth on glucose (Sassetti *et al.*, 2003).

In *E. coli*, both PCK and MEZ/PPS(PPDK) pathways can be used to generate PEP from gluconeogenic substrates. While single Δ*pckA* or Δ*pps* mutants grow on acetate, a double mutant cannot. Likewise, deletion of both genes encoding malic enzymes (*sfcA* and *maeB*) does not abrogate growth on acetate, unless *pckA* is also missing (Oh *et al.*, 2002). In *C. jejuni*, the absence of

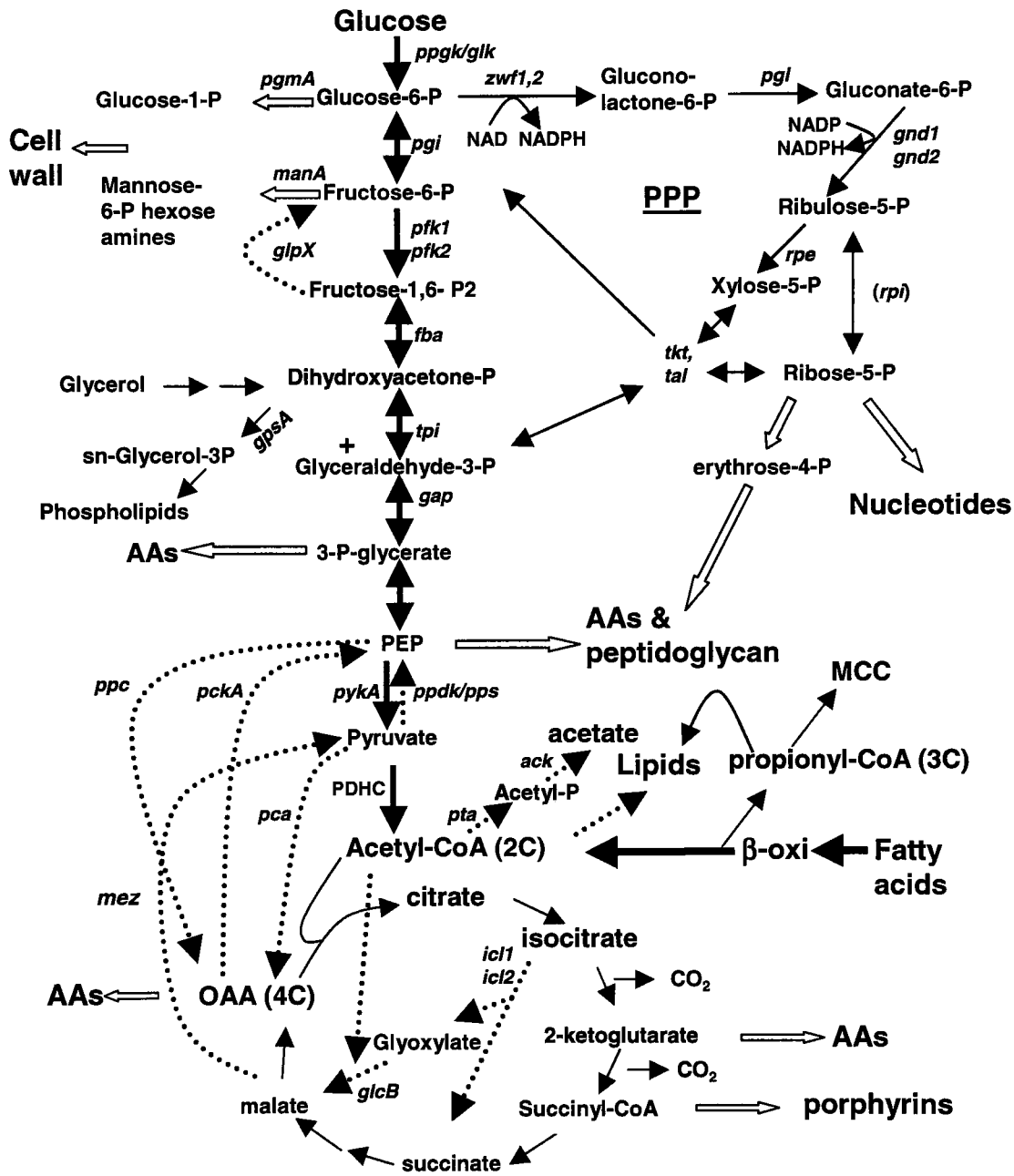


**Fig. 3.6. Cellular biosynthesis and anaplerosis.** Anaplerotic reactions are essential for maintaining the balance between metabolic pathways. Catabolism of carbohydrates through glycolysis and of fatty acids through $\beta$-oxidation yield acetyl-CoA, which feeds into the TCA cycle and/or glyoxylate cycle (GC). Anaplerosis on carbohydrates is via PPC or PCA, growth on C2 substrates is via the GC: isocitrate lyase (*icl1 & 2)* and malate synthase (*glcB*). Gluconeogenesis starts with PEP carboxykinase (*pckA*). A *ppc* homolog is absent in *M. tuberculosis.*

PPS/PPDK makes PCK essential for gluconeogenesis (Velayudhan & Kelly, 2002). In *C. glutamicum*, the purified MEZ favors the decarboxylation reaction (toward gluconeogenesis), but deletion of *malE* (MEZ) has no effect on growth on C2 substrates, suggesting that the absence of *mez* might be compensated by PCK as in *E. coli* (Gourdon *et al.*, 2000). During growth on glucose, *C. glutamicum* generates oxaloacetate from PEP by PPC, making PCA or the MEZ-PCA pyruvate shunt dispensable. Though the function of *mez* has not been investigated in mycobacteria, *M. smegmatis* seems to rely mainly on PCK to generate PEP during growth on C2 or gluconeogenic substrates because a Δ*pck* mutant could not grow on acetate, pyruvate, succinate or malate, but grew normally on dextrose or glycerol (A. Upton, personal communication). This indicates that MEZ and PPDK cannot compensate for the loss of PCK in gluconeogenesis. Furthermore, a PCK-deficient mutant of *M. bovis* grows normally on standard media where glycerol and dextrose are the main carbon substrates, suggesting that PCK is not needed for anaplerosis during growth on carbohydrates (Liu *et al.*, 2003b); the mutant was not tested for growth in C2 substrates.

### 3.10. Anaplerosis: the replenishment of intermediary metabolites

In addition to its role in energy generation, i.e., ATP production by substrate level phosphorylation and reducing power in the form of reduced pyridine and flavine nucleotides (NADH, FADH2) which are oxidized through the respiratory chain to

generate more ATP, the TCA cycle also provides essential biosynthetic precursors and does so in the form of its intermediates such as oxaloacetate and α-ketoglutarate. If these C4-dicarboxylic acids of the TCA cycle were not replenished, the cycle would quickly run out of intermediates and come to a stop. Cells avoid such a fate by constantly replenishing such intermediates from the outside directly. Reactions of this sort, which allow net carbon gain, are termed anaplerotic reactions (Kornberg, 1966). The anaplerotic route an organism employs depends on the type and concentration of carbon substrates, as well as on the electron acceptor(s) available to it (See Fig. 3.6). Some of these anaplerotic routes will be discussed in the next sections.

**3.10.1. Anaplerosis during growth on sugars.** When *E. coli* grow on sugars in batch culture (standard sugar concentrations ~ 0.1-0.5% w/v), oxaloacetate is replenished by carboxylation of PEP by PEP-carboxylase (PPC). PPC-deficient *Salmonellae* grow poorly on glucose unless TCA cycle intermediates are provided (Theodore & Englesberg, 1964). Interestingly, the slow growth on glucose of *E. coli* Δ*ppc* mutants (~20% slower than wild-type bacteria) is facilitated by upregulation of the GC, since when PPC-deficient mutants are grown in relatively high glucose (0.4-0.5% w/v), they increase carbon flow through the GC in order to obtain sufficient oxaloacetate (Peng *et al.,* 2004). Thus the anaplerotic function of PPC on dextrose can be compensated by the GC. During growth on carbohydrates, other bacteria, including *B. subtilis* and *C. glutamicum*, replenish

TCA cycle intermediates via carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase (PCA) (Diesterhaft & Freese, 1973; Peters-Wendisch *et al.,* 1998;). Inspection of recently published genome sequences from enterics indicates that *pca* is missing in this group of bacteria. *M. tuberculosis* and *M. bovis* lack a *ppc* homolog and Δ*pca* transposon mutants are impaired for growth on standard 7H10 plates (glucose and glycerol) (Sassetti *et al.,* 2003), suggesting that *M. tuberculosis* employs PCA for anaplerosis on carbohydrates and that compensation by the GC may not occur. Other mycobacteria, including *M. smegmatis,* do have homologs of PPC, suggesting that the loss of *ppc* might have been a recent event in the *Mycobacterium* genus.

**3.10.2. The Glyoxylate Cycle: anaplerosis during growth on fatty acids and other substrates metabolized via acetyl-CoA.** As discussed above, many bacterial species can utilize short, medium and long fatty acids including acetate and propionate, as sources of carbon and energy. Acetyl-CoA produced by β-oxidation of fatty acids (Fig. 3.4), is further oxidized to $CO_2$ via the TCA cycle (Fig. 3.2). When fatty acids are the only carbon source available, replenishment of TCA cycle intermediates cannot be accomplished via PPC or PCA. Under these conditions, an anaplerotic pathway called the GC becomes indispensable (Kornberg & Krebs, 1957; Kornberg, 1966; Ornston & Ornston, 1969) (Fig. 3.6). In the first step of the GC, isocitrate lyase (ICL) cleaves isocitrate into glyoxylate and succinate, and in the second step, malate synthase (MLS) condenses the

glyoxylate with another molecule of acetyl-CoA to form malate (Vanni *et al.*, 1991). The succinate is oxidized to fumarate by succinate dehydrogenase (SDH), or converted to succinyl-CoA by succinyl-CoA synthase (SUCCD). ICL and MLS-catalyzed reactions together with the TCA cycle enzymes SDH, fumarase (FUM), malate dehydrogenase (MDH), citrate synthase, and aconitase (ACN) constitute the cycle (Fig. 3.6). In addition to mediating the incorporation of two new molecules of acetyl-CoA per turn of the cycle, the GC also bypasses the decarboxylation reactions of the TCA cycle (IDH and αKGDH steps, Fig. 3.3), which further contributes to carbon conservation.

In *E. coli*, the genes encoding ICL (*aceA*) and MLS (*aceB*) are in an operon with *aceK* (*aceBAK*), which encodes a regulator of IDH (see below). In *M. tuberculosis* ICL and MLS are encoded by *aceA* (a.k.a. *icl1*) and *glcB*, respectively, and they are not together in the chromosome (Cole *et al.*, 1998). A gene encoding another putative ICL (*icl2*) is present in the genome of all mycobacteria. Interestingly, in at least one strain of *M. tuberculosis icl2* might be a pseudogene (Cole *et al.*, 1998), while in *M. leprae icl1* is a pseudogene (Cole *et al.*, 2001). Cell-free extracts from *in vitro*-grown *M. tuberculosis, M. smegmatis* and armadillo-grown *M. leprae* have both ICL and MLS synthase activities (Kannan *et al.*, 1985; Bharadwaj *et al.*, 1987). The data on *M. leprae* indicates that the product of *icl2* is expressed *in vivo* and that it indeed encodes an ICL.

**The control of carbon flow through the GC.** Isocitrate can serve as substrate for either IDH (TCA cycle) or ICL (GC), and thus constitutes a branch

point in central metabolism, where two enzymes are competing for the same substrate. Given the kinetic constants of the *E. coli* enzymes (IDH's Km = 8 μM; ICL's Km = 600 μM), it is not surprising that during growth in excess glucose most of the isocitrate is preferentially oxidized by IDH. Carbon flux into the GC is facilitated in two ways: the GC genes are induced and IDH is deactivated (Walsh & Koshland, 1984). In *E. coli*, *aceK* of the *aceBAK* operon encodes an IDH kinase/phosphatase which can phosphorylate IDH and thereby drastically deactivate the enzyme, thus shifting the flow of carbon toward ICL. Addition of glucose to a culture of bacteria growing on acetate, despite increasing growth, rapidly lowers carbon flow through the TCA cycle (by 5 fold). Simultaneously, the IDH kinase/phosphatase dephosphorylates IDH, which rapidly increases its activity and almost completely stops carbon flux through the GC (Walsh & Koshland, 1985). It is not clear how glucose mediates these effects (Corzone *et al.*, 1998).

Expression of the GC in enterics is under the indirect transcriptional control of FadR (of fatty acid catabolism) and the direct control of IclR, a repressor which binds to its own promoter and that of the *aceBAK* operon, thereby repressing expression of the GC (Maloy & Nunn, 1982; Gui *et al.*, 1996a). This binding is inhibited by PEP (Cortay *et al.*, 1991). Transcription of *iclR* is positively regulated by binding to its promoter by FadR (Gui *et al.*, 1996b). No homologs of either FadR or IclR have been identified in *M. tuberculosis*. In *C. glutamicum*, a transcriptional repressor called RamB that binds to the promoter of the *aceA* (ICL)

and *aceB* (MSL) genes and partially prevents their expression has been described recently. However, mutation of *ramB* does not significantly impair growth on acetate or glucose, suggesting that other regulators exist (Gerstmeir *et al.,* 2004). Interestingly, *M. tuberculosis* apparently has two putative transcriptional regulators that have homology to RamB. One of these, Rv0465c, is upstream of *icl1*, and the promoter of *icl1* has a motif that is a close match to the well conserved motif recognized by RamB in *C. glutamicum* promoters (Gerstmeir *et al.,* 2004). The other putative regulator, Rv1129c, is located upstream of homologs of *prpC* and *prpD,* which are putatively involved in metabolism of C3 substrates (see above & Chapter 10). A second repressor of the GC has been found in *C. glutamicum* and it is called GlxR. GlxR bears homology to the cAMP receptor–binding protein (CRP) regulator from *E. coli.* In *E. coli,* cAMP is the mediator of catabolite repression: when glucose is low, intracellular cAMP increases, binds CRP and cAMP-CRP activates transcription of several genes involved in the utilization of alternative sources of carbon (Lin, 1996). To the contrary, in *C. glutamicum,* growth on glucose leads to high cAMP intracellular concentrations, and cAMP-GlxR complexes exert transcriptional repression of genes encoding enzymes involved in utilization of alternative carbon substrates including GC genes. During growth on acetate, cAMP levels are low, and since the repressor cannot bind cAMP, it remains inactive (Kim *et al.,* 2004). ORF Rv3676 of *M. tuberculosis* is over 75% homologous to *glxR*.

### 3.11. Alternative routes of C2/glyoxylate metabolism

The glyoxylate produced by the GC is most commonly condensed with acetyl-CoA by malate synthase to generate malate. However, other routes of glyoxylate metabolism exist; some of these are discussed below.

**3.11.1. Glycerate Pathway.** In the glycerate pathway, glyoxylate carboligase catalyzes the condensation of two molecules of glyoxylate into one molecule of tartronate semialdehyde (Gupta & Vennesland, 1964), which is then phosphorylated to 3-phosphoglycerate by tartronate semialdehyde reductase and glycerate kinase; 3-P-glycerate is then catabolized via glycolysis to pyruvate. *M. smegmatis* can grow on glyoxylate, though poorly (unpublished observations). Since this pathway results in the net conversion of two molecules of glyoxylate to one of PEP/pyruvate, which could then be converted to oxaloacetate by PCA, this routing of the carbon could bypass the need for malate synthase during growth on C2 substrates. Though the genes of this pathway appear to be present in several mycobacteria, they are apparently absent in *M. tuberculosis*. In *E. coli* glyoxylate can alternatively be reduced to glycolate by a constitutive glyoxylate reductase (*ycdW*) (Ornston & Ornston, 1969; Nuñez *et al.*, 2001).

In *E. coli*, glycolate can be metabolized by the enzymes encoded by the *glc* operon, which includes glycolate oxidase (GOX) and a glyoxylate-induced malate synthase (MLSG, *glcB*). In this bacterium, either acetate or glyoxylate can induce

the glc and *ace* operons and the *glcB*- and *aceB*-encoded malate synthases can compensate for each other (Pellicer *et al.*, 1999). *M. tuberculosis* has a putative GOX and its only malate synthase is closer in homology to the *glcB*-encoded MLS than to the *aceB* encoded enzyme (acetate-induced).

**3.11.2. The Dicarboxylic Acid Cycle.** Using a citrate synthase *E. coli* mutant, Kornberg & Sadler (1960) demonstrated that oxidation of glyoxylate could take place independently of a complete functional TCA cycle as long as glutamate was provided. Cells given labeled acetate rapidly labeled oxaloacetate, PEP, and pyruvate in the presence of unlabeled glyoxylate indicating that acetyl-CoA was preferentially metabolized via the GC to oxaloacetate. Oxaloacetate was then decarboxylated (presumably by PCK) to PEP, which was subsequently oxidized (via PYK) to pyruvate, which could then be dehydrogenated to acetyl-CoA to keep the cycle running. Interestingly, it has been recently shown that in contrast to *E. coli* grown in batch culture in excess glucose (~0.1-0.5 w/v), starving bacteria (<0.002% glucose) grown in a chemostat, preferentially metabolize glucose via the dicarboxylic acid cycle rather than the TCA cycle (Fischer & Sauer, 2003).

## 3.12. Catabolism of amino acids

Amino acids are often catabolized by converting them to intermediates of central intermediates of metabolism. In general, amino acids are first converted to the

corresponding amino acid via oxidative deamination reactions catalyzed by amino acid specific dehydrogenases (e.g., CH3-CH(NH2)-COOH (alanine) → CH3-CO-COOH (pyruvate). The catabolism of certain amino acids, e.g., threonine, methionine, valine, and isoleucine results in the generation of propionyl-CoA (Hesslinger *et al.,* 1998).

# CHAPTER 4

## Bacterial Metabolism during Infection

### INTRODUCTION

In order to successfully infect, grow, and survive in their hosts, pathogens must be able to find appropriate and sufficient sources of carbon and energy (Rhen *et al.,* 2000). With few exceptions, little is known about the nutrient sources and metabolic pathways utilized by pathogens during infection (Smith, 2000). Even though the importance of microbial metabolism *in vivo* might appear self-evident, it is an area of research that has received little attention in the past, largely because it has been assumed that we know more than we actually do know about microbial metabolism and also because interfering with central metabolic pathways was deemed unfeasible due to structural conservation of the enzymes involved. However, evidence is emerging that pathogens acquire nutrients from the host utilizing factors (transporters, enzymes, cofactors, etc.) that are sufficiently distinct, and in some cases altogether absent, from those in their mammalian hosts, to warrant targeting such factors for chemotherapeutic intervention (Walsh, 2003; Fidock *et al.,* 2004; Nathan, 2004). In fact, some of these microbial pathways could be viewed as important "virulence" determinants in at least two senses.

Firstly, they are almost certainly needed by pathogens to adapt to the specific "metabolic niches" they inhabit, and thereby provide the pathogen with the constituents and energy required to be able to counteract, evade or deactivate, host antimicrobial effectors. Secondly, emerging evidence indicates that at least some metabolic enzymes, possibly bifunctional, might be directly involved in chemical reactions that deactivate host antimicrobial effector molecules (Bryk *et al.,* 2002; Lundberg *et al.*, 1999).

Defining the way parallel and opposing metabolic pathways operate is a difficult task, especially because, as discussed in the previous chapter, the functions of these pathways are highly dependent on environmental conditions, e.g. growth parameters and nutrients available. The challenge becomes considerably more formidable when the goal is to determine which metabolic pathways are relevant in the highly dynamic environment of an infection. The following paragraphs review our current, rather scant, understanding of metabolic pathways of carbon utilization employed by bacterial pathogens during infection.

### 4.1. *Escherichia coli*

Even though our knowledge of *E. coli* metabolism during axenic growth in the laboratory is extensive, little is known about nutrient acquisition and metabolism of *E. coli* living in the intestines as a commensal or as a pathogen. The intestinal epithelium is covered by mucus, a complex of glycoproteins and glycolipids that

includes acetylglucosamine, *N*-acetylneuraminic acid, *N*-acetylgalactosamine, galactose, glucuronate, galacturonate, mannose, fucose, sialic acids, and ribose. Accumulating evidence indicates that carbohydrate metabolism is important for survival of *E. coli* in the mammalian large intestine as first suggested by the finding that *E. coli* mutants that cannot grow on mucus fail to colonize the intestine of mice (Peekhaus & Conway, 1998). In support of this view, Δ*eda* mutants of the Entner-Doudoroff pathway (EDP) are defective in intestinal colonization in the streptomycin-treated mouse model, and a gluconate transporter (GntP) mutant was also impaired for intestinal growth (Sweeny *et al.,* 1996b). The utilization of glucuronate is suggested by the severe (100 fold < wildtype) colonization defect of Δ*uxuA*, which lacks a dehydratase involved in the catabolism of this compound (Sweeny *et al.,* 1996b).

Recently, gene expression studies of *E. coli* grown in glucose versus mucus revealed that during growth in mucus bacteria upregulate, as expected, genes involved in the catabolism of amino sugars, pentoses, and amino acids (Chang *et al.,* 2004). Less expectedly, *E. coli* also upregulates genes involved in phospholipid degradation as well as the gluconeogenic genes *pckA* and *ppsA* (*pps*). Genes involved in the synthesis of purines, pyrimidines and several amino acids are in contrast repressed suggesting *E. coli* scavenges these from mucin (Chang *et al.,* 2004). These investigators also evaluated several metabolic mutants for their ability to colonize the mouse intestine, and their findings largely confirmed the importance of the EDP for *in vivo* growth and survival. However, these studies

also revealed that mutants differentially deficient in their ability to utilize carbohydrates had different *in vivo* phenotypes. For instance, mutants of the EDP, which could not use gluconate, failed to grow and to persist in the host. However, those incapable of using fucose, mannose, or ribose were impaired specifically during persistence. Mutants of the pentose phosphate pathway (Δ*gnd*), TCA cycle (Δ*sdhB*) and gluconeogenesis (Δ*pps*, Δ*pckA*) did not show an *in vivo* phenotype. Interestingly, in a separate subsequent study, Δ*pps* or Δ*pckA* mutants were found to be impaired for persistence in the context of a co-infection with wild-type bacteria (Miranda *et al.*, 2004).

Together these data support the notion that during its sojourn in the intestine, *E. coli* bacteria largely use carbohydrates for growth and survival. However, to do so they apparently must be able to use a wide variety of substrates. This could be because these different substrates become variably available throughout the infection and in particular might become more limiting during the persistent phase of the infection and/or in the context of a co-infection.

### 4.2. *Helicobacter pylori*

A large fraction of the world's population is believed to be persistently infected by *H. pylori* (Blaser & Atherton, 2004). Nothing is known about the type of carbon sources used by *H. pylori* in the stomach of its host. However, the *H. pylori*

genome sequence indicates that it has a modified TCA cycle in which the conversion of α-ketoglutarate to succinyl-CoA is carried out via a keto-glutarate ferredoxin oxidoreductase instead of via the canonical α-ketoglutarate dehydrogenase (Tomb *et al.,* 1997). Interestingly, the α-ketoglutarate ferredoxin oxidoreductase appears to be essential, making this enzyme of central metabolism an attractive target for drug discovery (Hugues *et al.,* 1998). The *korA-D* genes of *M. tuberculosis* are homologous to those that encode the aforementioned enzyme complex in *H. pylori. H. pylori* lacks the genes encoding the GC, making it unlikely that these bacteria use fatty acids or gluconeogenic substrates are carbon sources in the stomach.

### 4.3. *Listeria monocytogenes*

Virulence of *L. monocytogenes* requires entrance into intestinal epithelial cells, escape from the phagosome into the cytoplasm, replication therein, and actin-based motility which eventually facilitates infection of neighboring cells (Lecuit & Cossart, 2002). Some of these events are regulated by the transcriptional activator PrfA (Kreft *et al.,* 2002). Based on the observation that *in vitro* conditions that activated the expression of *prfA*, and of PrfA-dependent virulence genes, also endowed *Listeria* with the ability to utilize glucose-1-P as a carbon substrate, and the fact that glucose-1-P is a breakdown product of glycogen which might be readily available in the cytosol of host cells, it was hypothesized that hexose

phosphates could be used by *Listeria* as carbon substrates during infection (Ripio *et al.,* 1997). Indeed, while wild-type *Listeria* microinjected directly into a mammalian cell's cytosol could readily grow and replicate, a mutant in *htp*, encoding a hexose transporter, could not (Goetz *et al.,* 2001). Furthermore, this Δ*htp* mutant could not utilize several glucose-1-P-related sugars including glucose-6-P, fructose-6-P, and manose-6-P *in vitro* (Chico-Calero *et al.,* 2002), and was attenuated during infection in mice, e.g, mutant bacteria grew more slowly and killed infected mice later than wild-type. This adaptation by *Listeria* to the intracytoplasmic niche it inhabits might be shared by other pathogens. An Hpt homolog is also found in *Shigella*, which like *Listeria* escapes from the phagosomal vacuole and grows in the cytoplasm of host cells.

### 4.4. *Salmonellae*

Our understanding of *Salmonella* carbon metabolism *in vivo* is rather rudimentary. The *Salmonellae* vacuole is likely limiting in essential elements such as $Mg^{++}$ and $Ca^{++}$, since the mtgC gene encoding a $Mg^{++}$ transporter is required for virulence (Buchmeier *et al.,* 2000), and the two-component system PhoP/PhoQ, which regulates transcription of genes whose function is required for intracellular survival is activated by low concentrations of $Mg^{++}$ (Miller *et al.,* 1989). Amino acids and purines also appear to be limiting since auxotrophs are attenuated for intracellular survival (Hoiseth & Stocker, 1981; Fields *et al.,* 1986).

Based on a comprehensive examination of *Salmonellae* gene expression isolated from infected macrophages, it has been suggested that these bacteria use gluconate, galactone and similar sugars as carbon sources during infection (Eriksson *et al.,* 2003). This was based on the finding that although expression of genes of the EDP pathway was unchanged, some genes encoding transporters of gluconate were upregulated. The fact that both glycolysis and the pentose phosphate phosphate pathway were drastically downregulated was interpreted as suggesting specificity in the types of sugars metabolized by the bacteria. These observations, though informative, are not sufficient to establish the relevance of sugar metabolism during infection of *Salmonellae*. Furthermore, some puzzling observations in the same study -that several genes encoding enzymes of amino acid biosynthesis were downregulated, some of which are known to be essential for *Salmonellae* survival in the macrophage vacuole (Hoiseth & Stocker, 1981; Fields *et al.,* 1986) - need to be clarified.

Alternatively, it has been suggested that *Salmonella* may depend on C2 substrates during infection. An *S. typhimurium* (SR-11) mutant that could not grow on C2 substrates *in vitro* was found to be attenuated in mice (Utley *et al.,* 1998), and was later shown to be a mutant of the catabolite activator/repressor *cra* (Allen *et al.,* 2000). Cra positively controls genes involved in the utilization of gluconeogenic substrates including the GC and gluconeogenesis (Saier & Ramseier, 1996). Suggestively, a mutant of malic enzyme (Δ*mez*), which converts malate to pyruvate, was also found to be attenuated *in vivo* (Valentine *et al.,*

1998). Recently, an *S. typhimurium* isocitrate lyase (Δ*aceA*) mutant of the GC was found to be attenuated "late" in the infection (day 10) in the susceptible mouse model, despite growing similarly to wild-type early on in the infection, or during a 24 hr macrophage infection (Fang *et al.,* 2005). The mutant also showed impaired survival during the chronic phase of infection when tested in a recently described *Salmonellae* persistence model in resistant mice (Monack *et al.,* 2004), however, for this experiment, the Δ*aceA* was combined with an *aroA* mutation, complicating the interpretation of the results. Though clearly the issue merits further investigation, considerable evidence supports the idea that C2 substrates might play an important role in the metabolism of *Salmonellae in vivo*, especially in the persistence phase of the infection.

### 4.5. *Mycobacterium tuberculosis*

*M. tuberculosis* is an intracellular pathogen that chiefly resides within vacuoles in macrophages (Armstromg & Hart, 1971; 1975; Clemens *et al.,* 2002). Although some aspects of the mycobacterial vacuole such as its pH in resting and activated macrophages or its interaction with other components of the endosomal network such as lysosomes have been partially characterized (Clemens & Horwitz, 1995; Clemens, 1996; Russell & Sturgill-Koszycki, 1996; Russell, 2001), other aspects such as its chemical composition in terms of essential elements and nutrients that might be available to the bacterium within it, remain poorly understood.

Some lines of evidence indicate the tubercle bacillus resides in a nutrient-limited environment *in vivo*. Amino acid and purine auxotrophs isolated in both *M. tuberculosis* and *M. bovis* are impaired for growth in macrophages and/or animal models of infection (Hondalus *et al.,* 2000; Smith *et al.,* 2001; Jackson *et al.,* 2003). The fact that Δ*mbtB* mutants, which lack the high affinity siderophore mycobactin and cannot grow in medium containing low iron *in vitro*, are also incapable of replication in human (THP1) macrophages (De Voss *et al.,* 2000) suggests that *M. tuberculosis* might also experience some iron-limitation during infection. Siminarly, magnesium also appears to be limiting within the mycobacterial vacuole (Rodriguez & Smith, 2003, Buchmeier *et al.,* 2000).

Some evidence gathered over the years regarding carbon utilization by *M. tuberculosis* suggested a slowing down of metabolism and the prevalence of C2 (fatty acid) catabolism *in vivo*. *M. tuberculosis* and *M. bovis* bacteria isolated from ~20 day-infected mouse lungs respired at much lower levels than *in vitro*-grown bacteria, which suggested reduced metabolism. Curiously, it was also observed that fatty acids but not carbohydrates significantly stimulated the ability of these bacteria to reduce a tetrazolium dye; *in vitro*-grown bacteria readily oxidized both (Segal & Bloch, 1956; Kanai & Kondo, 1974). Kondo *et al.,* (1970) also found that mycobacteria harvested from infected lungs appeared to be closely associated with lipids, and showed that *M. tuberculosis* could digest liposomes in suspension, releasing fatty acids (Kondo & Kanai, 1976; Kondo *et al.,* 1985). Mycobacteria apparently have extracellular lipases that might have this function

(Casal & Linares, 1984; Kannan *et al.,* 1987) including four partially redundant phospholipases C (Raynaud *et al.,* 2002), which jointly are required for virulence of *M. tuberculosis*. Furthermore, some 20 genes annotated as putative lipases (*lipC-lipW* genes) are also present in the genome.

**The glyoxylate cycle and persistence of *M. tuberculosis*.** As reviewed in Chapter 3, many bacteria can utilize fatty acids as sources of carbon and energy, and to do so bacteria specifically require the $\beta$-oxidation cycle and the GC (GC). Early studies seeking to determine the metabolic capabilities of mycobacteria revealed that both fast and slow growing mycobacteria including *M. tuberculosis* and *M. leprae* encoded activites of the TCA cycle and the GC (Murthy *et al.,* 1973; Seshadri *et al.,* 1972; 1976; Kannan *et al.,* 1985; Katoch *et al.,* 1987; Bharadwaj *et al.,* 1987). Analysis of *M. leprae, M. microti* and *M. avium* grown *in vivo* also revealed the presence of GC enzymes isocitrate lyase and malate synthase (Bharadwaj *et al.,* 1987; Wheeler & Ratledge, 1988). These findings together with the demonstration that activity of ICL increased in late stationary-phase *M. tuberculosis* cultures (Murthy *et al.,* 1973), led some of these early investigators to suggest that GC-mediated metabolism might represent a strategy employed by pathogenic mycobacteria such as *M. leprae* and *M. tuberculosis* to persist in the host in such ill-defined states as dormancy (Segal & Bloch, 1956; Bharadwaj *et al.,* 1987; Gupta & Katoch, 1997). This "alternate" (to carbohydrates) mode of metabolism based on the GC, it was speculated, could be linked to phenotypic drug tolerance and the common failure of antitubercular

drugs to kill recalcitrant 'persisters' (Murthy *et al.,* 1973; Kannan *et al.,* 1985; Gupta & Katoch, 1997).

Functional genetic evidence supporting the hypothesis that fatty acids and specifically the GC might indeed be relevant to the metabolism of the tubercle bacillus during infection was first provided by McKinney and colleagues (2000). An *M. tuberculosis* mutant lacking ICL1 (Δ*icl1*) was impaired for survival specifically during the persistence phase of the infection in the mouse model of chronic tuberculosis, despite growing indistinguishably from the parental strain during the acute phase (Fig. 4.1A). The mutant also elicited less pathology than wild-type bacteria (Fig. 4.1B&C).

The extensive duplication of genes encoding fatty acid β-oxidation enzymes in *M. tuberculosis* (Cole *et al.,* 1998) presents a major obstacle to the genetic analysis of the role of fatty acid catabolism in TB pathogenesis due to potential functional redundancy between homologs. In contrast, the GC in *M. tuberculosis* apparently comprises a single gene encoding malate synthase and two genes encoding isocitrate lyases (*icl1* and *icl2*) (Cole *et al.,* 1998). Thus, we decided to focus our efforts on investigating the role of this latter pathway in the pathogenesis of *M. tuberculosis.*

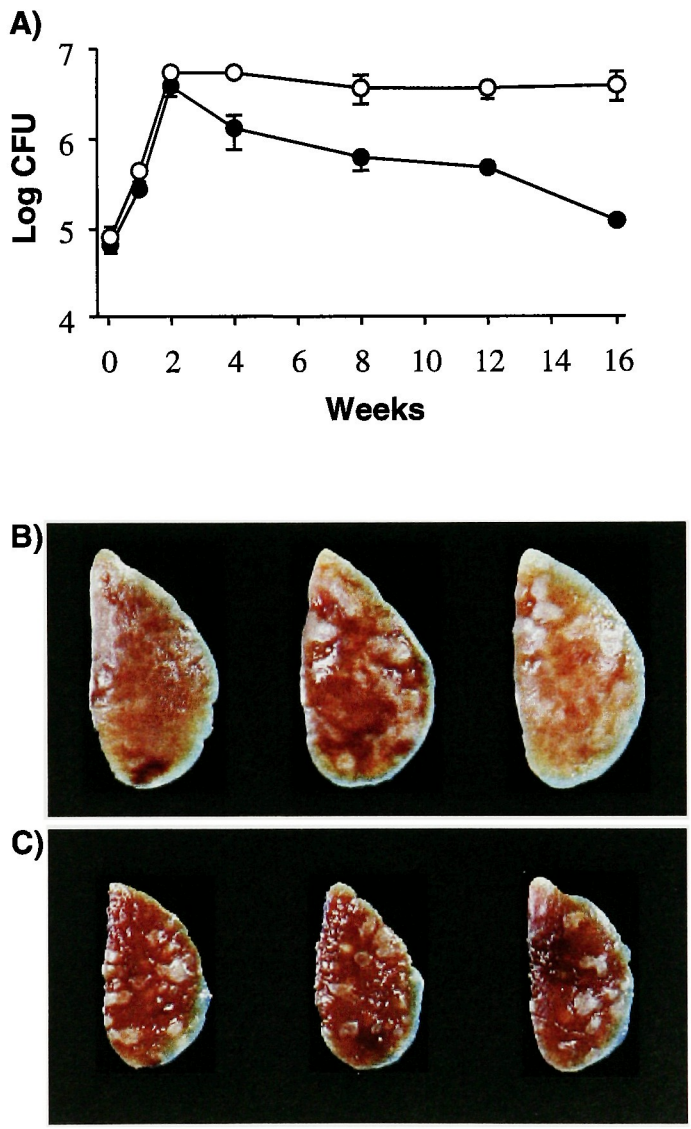


**Fig. 4.1. *icl1* is required for persistence and virulence of *M. tuberculosis* in mice.** (**A**) Bacterial loads in the lungs of B6X129F1 mice infected with $10^6$ *icl1*-deficient (filled circles) or wild-type (circles) MTB cells. (**B-C**) Lung pathology in B6X129F1 mice infected with (**B**) wild-type or (**C**) Δ*icl1* mutant bacteria at 16 weeks post-infection. (Data, courtesy of J. D. McKinney).

# PART II

# CHAPTER 5

## *Mycobacterium tuberculosis* Replication Dynamics *in vivo*

### INTRODUCTION

In the mouse model of TB a predominantly pulmonary infection is established after intravenous inoculation or exposure to aerosolized bacteria (North & Young, 2004). The acute phase of infection lasts a few wks and is characterized by exponential bacterial growth in the lungs. The transition to the chronic phase of infection is brought about by the emergence of acquired cell-mediated immunity (CMI), which is mediated by antigen-specific T cells and and immune-activated macrophage and results in the stabilization of bacterial loads in the infected organs (Flynn & Chan, 2001). A long-standing question has been whether this plateau in the bacterial growth curve during chronic infection represents a "static" equilibrium in which bacterial replication is slow or absent, or a "dynamic" equilibrium in which continued rapid bacterial replication is precisely balanced by an equally rapid rate of killing by the host immune response (Sever & Youmans, 1957). This issue has important implications for anti-mycobacterial drug therapy

because drugs that are currently used to treat TB are more effective against bacteria undergoing rapid growth and cell division (McKinney, 2000; Mitchison, 2004).

In a classic study, Rees and Hart (1961) sought to distinguish between "static" and "dynamic" models of the host-pathogen equilibrium in persistent TB by enumerating "viable counts" (CFU) and "total counts" (microscopically detectable acid-fast bacilli (AFB)) in the lungs of chronically infected mice. The authors reasoned that if the dynamic model were correct the viable counts and total counts should diverge over time due to progressive accumulation of dead bacilli, which would contribute to the total counts (AFB) but not to the viable counts (CFU). Instead, they found that the viable and total counts were remarkably congruent throughout the course of infection, a result that favored the static model. They acknowledged, however, that loss of acid-fast staining by dead bacilli could result in an underestimate of the true number of total counts. Loss of acid-fastness might also explain their observation that the total counts gradually declined in animals treated with the anti-mycobacterial drug isoniazid (INH), which is known to interfere with acid-fast staining (Middlebrook, 1952). It has also been suggested that acid-fast staining might be lost in tubercle bacilli present in latently infected human tissues (Seiler *et al.*, 2003).

In this chapter we describe experiments addressing this important aspect of the host-pathogen interface in persistent TB. First, we measured and compared *M. tuberculosis* viable counts (CFU), and total counts, represented by the number of

bacterial chromosome equivalents (CEQ), in the lungs of chronically infected mice. Our experimentally determined values were then compared with values obtained from an *in silico* model that was used to simulate bacterial growth curves (CFU accumulation) and to predict the extent of accompanying bacterial CEQ accumulation over time at different rates of bacterial cell division. Alternative scenarios were modeled in which the immune response was bacteriostatic, bactericidal, or both. Our results support the static equilibrium model, in which entry into the chronic phase of infection is accompanied by a marked reduction in the bacterial cell division rate. (The results discussed in this chapter are the result of a concerted effort by Wai-Tsing Chan, Jim Gomez, John McKinney, Juliano Timm, and the author and have been reported [Muñoz-Elías *et al.*, 2005]).

## RESULTS

### 5.1. Modeling the host-pathogen equilibrium in infected mice

In the mouse model of TB, the chronic phase of infection is characterized by progressive lung pathology despite relatively stable numbers of viable bacteria enumerated as colony-forming units (CFU) (Rhoades *et al.*, 1997). The plateau in the bacterial growth curve could represent a "static" equilibrium in which the rate of bacterial cell division is very slow, or a "dynamic" equilibrium in which rapid bacterial cell division continues unabated, balanced by an equal rate of bacterial

death effected by host immune mechanisms (Rees & Hart, 1961). The latter scenario would be akin to the highly dynamic equilibrium observed in HIV infection prior to the onset of AIDS (Simon and Ho, 2003). We reasoned that the two scenarios -static vs. dynamic- could be distinguished by comparing the "viable counts" (CFU) and "total counts" (bacterial chromosome equivalents (CEQ)) at various stages in chronically infected animals. The static model predicts that CFU and CEQ will not diverge over time, whereas the dynamic model predicts that bacterial CEQ will accumulate over time while CFU remain stable.

To illustrate these alternative scenarios, we devised a simple *in silico* model that can be used to generate CFU and CEQ curves representing a static equilibrium in which CMI is strictly bacteriostatic (Fig. 5.1A), or a dynamic equilibrium in which CMI is strictly bacteriocidal (Fig. 5.1B). The derivation of our model is described in the Materials and Methods.

In the "static" scenario, the model predicted that both viable counts (CFU) and total counts (CEQ) would remain stable and would not diverge over time (Fig. 5.1A). In the "dynamic" scenario, the model predicted that although viable counts would remain stable over time, the total counts would gradually rise due to the accumulation of dead bacteria; in the modeled experiment depicted in Fig. 5.1B, the CEQ/CFU ratio increased by ~ 100-fold between 2 wk and 16 wk post-infection. We also modeled semi-dynamic scenarios where CMI exerted both bacteriostatic and bacteriocidal effects resulting in stable numbers of CFU but gradual accumulation of CEQ. The semi-dynamic scenarios could reflect a partial

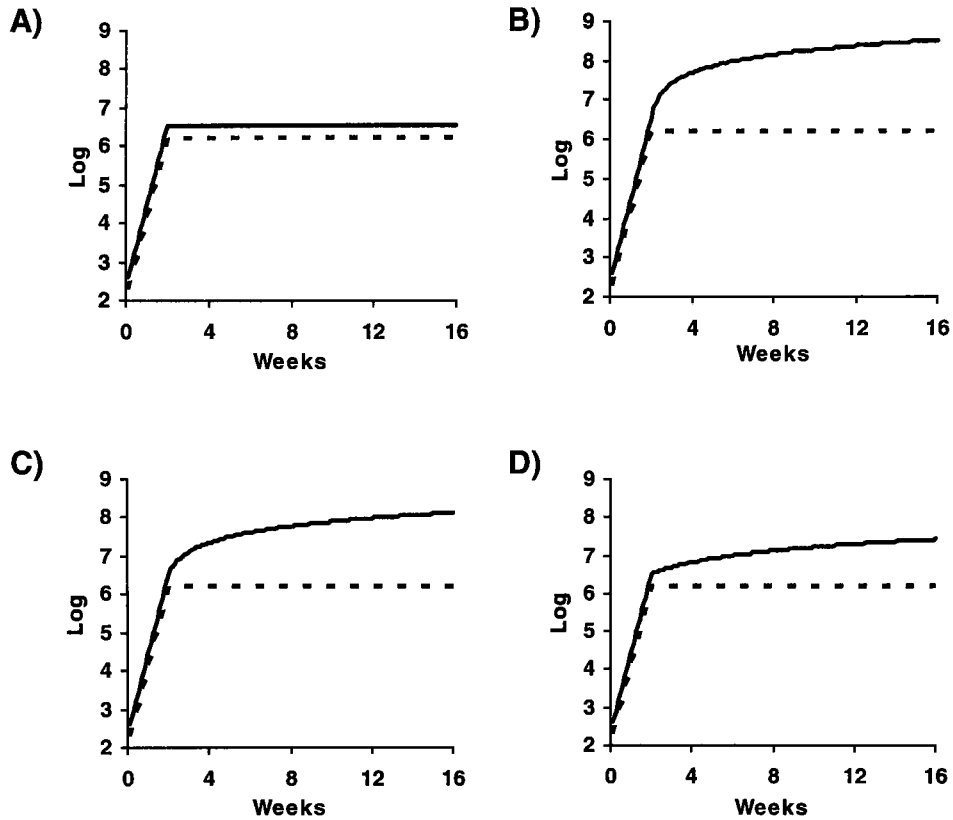


**Fig. 5.1. Modeling static and dynamic scenarios of the host-pathogen equilibrium in chronic murine TB.** Mice are infected with 200 CFU. Acute-phase infection (0-2 wk) is characterized by an exponential increase in viable counts (CFU) with a 24.6 h population doubling time. Chronic phase infection (2-16 wk) is characterized by stable viable counts. (**A**) Static scenario. Acquired immune response halts bacterial replication after the acute phase of infection. Viable counts (CFU, dotted lines) and total counts (CEQ, solid lines) do not diverge over time. (**B**) Dynamic scenario. Bacterial replication continues at an undiminished rate (24.6 h doubling time) throughout the chronic phase, but bactericidal acquired immune response kills 50% of the bacteria after each round of cell division. Balanced growth and death results in stable viable counts while total counts accumulate, resulting in rapid divergence of CEQ and CFU. (**C** & **D**) Semi-dynamic scenarios where the rate of bacterial cell division is reduced by (**C**) 50% or (**D**) 90% from the acute-phase rate, resulting in a more gradual divergence of CFU and CEQ.

effect of CMI on the entire bacterial population, or variable efficacy of CMI against different bacterial sub-populations (Fig. 5.1C, D); our model cannot distinguish between these possibilities. The semi-dynamic scenarios were modeled by varying the rate of bacterial cell division and death during the chronic phase, as described in the Materials and Methods. When the rate of cell division during the chronic phase was reduced by 50% (Fig. 5.1C) or 90% (Fig. 5.1D) as compared to the cell division rate during the acute phase, the CEQ/CFU ratio nonetheless rose appreciably—by ~ 40-fold (Fig. 5.1C) or ~ 8- fold (Fig. 5.1D), respectively—between 2 wk and 16 wk post-infection. Our model will also accommodate more complex scenarios, such as fluctuating rates of cell division or cell death during the course of infection (see Fig. 5.2). A key assumption is that non-viable bacteria (and their chromosomes) are not removed or degraded over time; here we provide experimental support for this assumption (see below), in agreement with evidence adduced previously by others (Rees & Hart, 1961).

## 5.2. Comparison of modeled and experimentally determined CFU and CEQ curves in infected mice

In order to generate experimental values for bacterial CFU and CEQ during the course of a murine infection, C57Bl/6 mice were aerosol-infected with ~200 CFU of *M. tuberculosis* (Erdman) and groups were sacrificed at selected time points for quantitative analysis of bacillary loads. Viable counts (CFU) were enumerated by

plate-culture of lung homogenates and total counts (CEQ) by quantitative real-time PCR (QPCR).

During the acute phase of infection (0-2 wk post-infection), the viable counts in the lungs increased exponentially with an average population doubling time of 24.6 hr (Fig. 5.2), which is typical in the mouse model of TB. During the chronic phase of infection (2-16 wk post-infection), there was a much slower rise in the viable counts with an average population doubling time of 1,676 hr (~ 70 d) (Fig. 5.2). The CEQ/CFU ratio showed little change during the chronic phase of infection (Fig. 5.2; inset numbers indicate CEQ/CFU ratios/time point), indicating that there was minimal accumulation of chromosomes from dead bacteria. These observations resemble the predicted curves generated by the "static" scenario in our model (Fig. 5.1A), and differ markedly from those of the "dynamic" scenario (Fig. 5.1B), and support the idea that the rate of bacterial cell division is very slow during the chronic phase of infection. In order to provide a more direct comparison between our modeled and experimental results, we modeled the "dynamic" and "static" scenarios using the growth rate constants derived from the experimental data depicted in Fig. 5.2, taking into account any changes in bacterial CFU between time points by varying the growth rates constants (K values) appropriately. The experimentally determined CEQ values (Fig. 5.2, open circles) diverged sharply from the modeled CEQ values in the "dynamic" scenario (Fig. 5.2, blue line) or the "semi-dynamic" scenario where replication of bacteria during the chronic phase was reduced by 50% (Fig. 5.2, green line), and fell between the

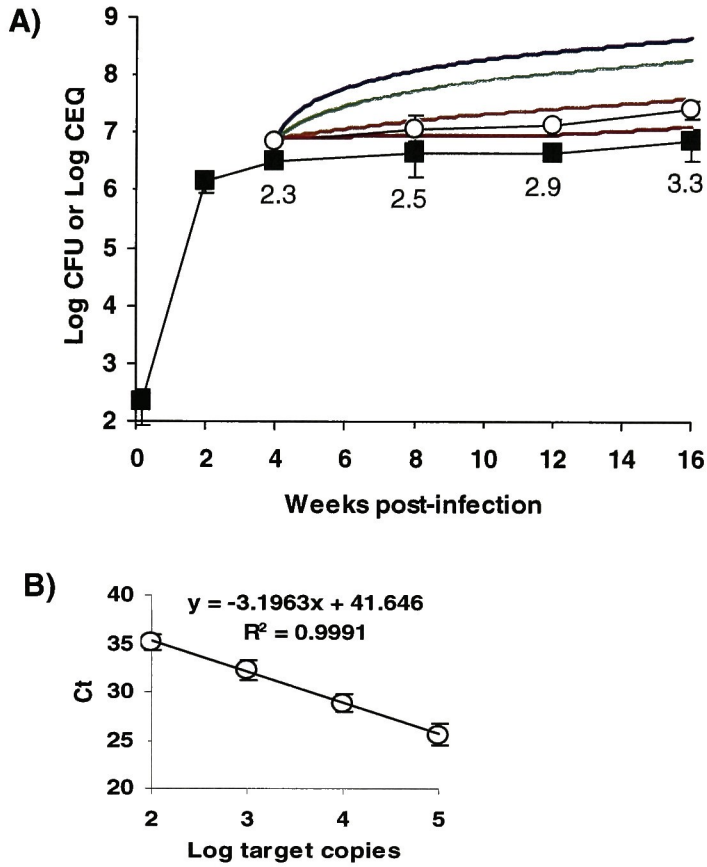


**Fig. 5.2. Comparison of modeled and experimentally determined CFU and CEQ curves in mice.** (**A**) Wild-type (C57Bl/6) mice were aerosol-infected with *M. tuberculosis* (~200 CFU/mouse). At indicated time points, mice were sacrificed and viable counts (CFU) were quantified by plating lung homogenates (closed squares); total counts (CEQ) were quantified by QPCR (open circles). Modeled CEQ values were derived using the static (red line) or dynamic (blue line) equilibrium scenarios, or semi-dynamic scenarios in which the rate of cell division during chronic infection was reduced by 50% (green line) or 90% (orange line) compared to acute infection. Symbols represent mean CFU or CEQ values (n = 4 mice/group); inset numbers = CEQ/CFU ratios at time point. *P* values for pairwise comparisons of the experimental and modeled CEQ values at 16 wk: experimental vs. dynamic or semi-dynamic (50%) scenarios, $P < 0.001$; experimental vs. static or semi-dynamic (10%) scenarios, $P > 0.05$. Results are representative of two experiments. (**B**) Standard curve for the *fadE15* primer-beacon set used to quantify bacterial CEQ in tissue homogenates. Threshold cycle (Ct) values (y-axis) were obtained by QPCR of target DNA (x-axis). Symbols (open circles) indicate mean Ct values of three independent experiments; error bars indicate STDEV. The trendline drawn through the data points indicates the linear regression curve, which was calculated using the least-squares method.

modeled CEQ values for the "static" scenario (Fig. 5.2, red line) and the "semidynamic" scenario where replication of bacteria during the chronic phase was reduced by 90% (Fig. 5.2, orange line). The parallel rise in experimental CFU and CEQ values between 2 and 16 wk post-infection indicates that stasis is not complete, although the doubling time of the bacterial population during the chronic phase is increased nearly 70-fold (1,676 hr) compared to the acute phase (24.6 hr). Thus, although it is likely that bacterial growth continues at a reduced rate during the chronic phase of infection, our results are not consistent with a model in which rapid bacterial cell division is balanced by equally rapid cell death.

## 5.3. Large numbers of bacterial chromosomes can be quantified in infected mice

We considered the possibility that our inability to detect a divergence of CFU and CEQ during the chronic phase of infection might be due to technical limitations in our methods for extraction and quantification of bacterial chromosomes from infected lungs. In order to confirm that there was no ceiling on the number of chromosomes per lung that we could accurately quantify, we enumerated CFU and CEQ in aerosol-infected IFN-γ deficient mice, which are unable to control replication of *M. tuberculosis* (Cooper *et al.*, 1993; Flynn *et al.*, 1993). By 4 wk post-infection, shortly before the IFN-$\gamma^{-/-}$ mice became moribund, the bacterial load in the lungs had reached ~$10^9$ CFU per mouse; the number of CEQ detected

by QPCR corresponded very closely to the number of CFU detected by plate culture (not shown). The close correspondence of CFU and CEQ spanning a range of > 5 $\log_{10}$ confirms that our inability to detect a divergence of bacterial CFU and CEQ trajectories in chronically infected mice (Fig. 5.2) was not due to a technical limitation of the QPCR assay.

### 5.4. Persistence of dead bacteria in mouse lungs

We considered the possibility that our inability to detect a substantial accumulation of bacterial chromosomes during the chronic phase of infection might be due to rapid removal or degradation of killed bacteria. In order to address this issue using QPCR to quantify bacterial chromosomes in the lungs, we assessed the impact of isoniazid (INH) therapy on the viable (CFU) and total (CEQ) bacterial counts in chronically infected C57BL/6 mice. Untreated controls showed relatively stable viable and total counts between 4 and 12 wk post-infection (Fig. 5.3B). In contrast, mice treated with INH for 8 wk (from 4 to 12 wk post-infection) showed a marked reduction in viable counts (3.6 $\log_{10}$), whereas the total counts remained stable (Fig. 5.3A). These observations indicate that non-viable tubercle bacilli (and their chromosomes) are not subject to rapid removal or degradation, in agreement with previous reports (de Wit *et al.*, 1995; Rees and Hart, 1961).

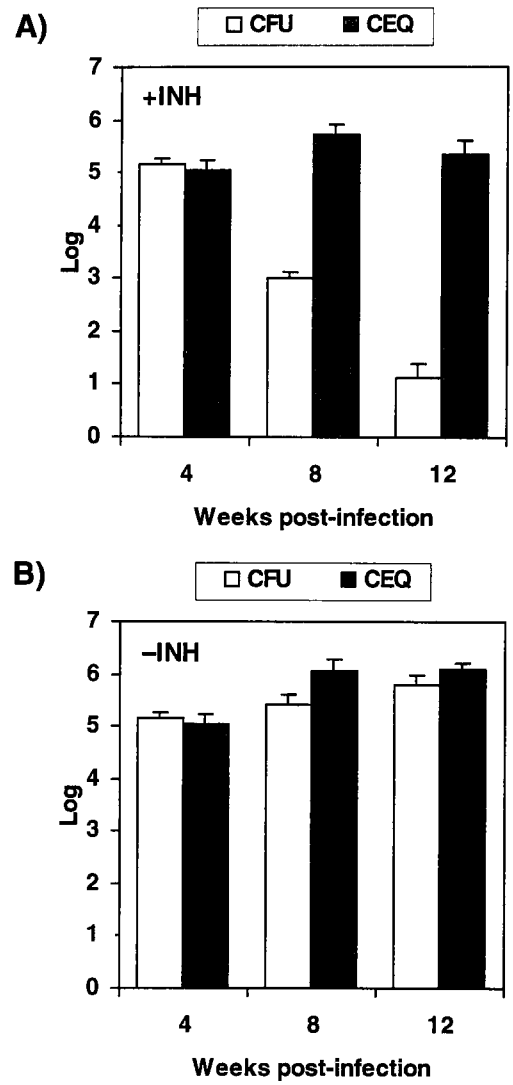


**Fig. 5.3. Chromosomes of non-viable *M. tuberculosis* in mouse lungs are not removed or degraded rapidly.** C57Bl/6 mice were infected i.v. with *M. tuberculosis* (~1 x $10^6$ CFU per mouse). One group (**A**) received INH (25 mg $kg^{-1}$ $d^{-1}$) for 8 wk starting at 4 wk post-infection; a control group (**B**) was left untreated. At 4 , 8, and 12 wk post-infection, mice from INH-treated and untreated groups were sacrificed and viable counts (CFU) were quantified by plating lung homogenates (open bars); total counts (CEQ) were quantified by QPCR (shaded bars). Bars represent mean CFU or CEQ values (n = 4 mice per group); error bars indicate STDEV from the means. In pairwise comparisons of CFU values in the INH-treated group for 4 wk vs. 8 wk or 4 wk vs. 12 wk post-infection, differences were highly significant ($P < 0.005$), while those of CEQ values in the INH-treated group for 4 wk vs. 8 wk or 4 wk vs. 12 wk post-infection were not ($P > 0.05$). Results are representative of two experiments.

# CHAPTER 6

## An Investigation into the Role of *icl1* and *icl2* in Carbon Metabolism and Pathogenesis of *Mycobacterium tuberculosis*

### INTRODUCTION

Microorganisms that invade, replicate, and persist within other host organisms must acquire and assimilate carbon substrates from their hosts' tissues. With few exceptions, little is known about the sources of nutrients or the metabolic pathways utilized by pathogens to use such nutrients during infection (Smith, 2000). Metabolic adaptations to tissue environments are almost certainly essential for the ability of pathogens to survive in the host in spite of acquired immunity. These adaptations might also contribute to the difficulty in eradicating microbes in the host despite prolonged therapy with drugs to which the pathogen, when tested *in vitro*, is susceptible (McDermott, 1958). *In vivo* drug tolerance is a major reason why tuberculosis (TB) therapy requires administration of multiple drugs for at least six months, resulting in high rates of patient non-adherence, treatment failure, and acquired drug resistance (Gomez & McKinney, 2004). Elucidation of the metabolic pathways required for growth and persistence of *M. tuberculosis* in the mammalian lung could suggest new avenues of intervention against this recalcitrant pathogen (Stewart *et al.,* 2003; Boshoff & Barry, 2005).

Several lines of evidence suggest that pathogenic mycobacteria primarily utilize fatty acids, rather than carbohydrates, as carbon substrates during infection (Chapter 3). Previously we reported that disruption of *icl1* in *M. tuberculosis* impairs the ability of the bacteria to survive at wildtype levels during the chronic phase of infection in mice, but has little effect on replication during the first two wks of the infection (McKinnney *et al.,* 2000; Chapter 3). The genome of *M. tuberculosis* has a gene homologous to *icl1*, termed *icl2* (MT1966, Rv1915/16), which encodes a second putative isocitrate lyase (ICL2) (Cole *et al.,* 1998). Here, we report that *icl1* and *icl2* provide overlapping and essential functions in growth and survival of *M. tuberculosis in vivo*. Deletion of *icl1* or *icl2* alone had little effect on bacterial catabolism of fatty acid substrates or multiplication in macrophages and mice. In contrast, bacteria lacking both genes were unable to grow in fatty acid-containing media or in macrophages, and were rapidly eliminated from the lungs of infected mice. Virulence of the Δ*icl*/Δ*icl2* strain was not restored in mice lacking macrophage activation pathways mediated by interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α), indicating that the essentiality of the gGC) at the onset of the infection is largely independent of the host immune response. Using a dual-specific ICL inhibitor, we adduce evidence that chemical inhibition of ICL activity might be a feasible and effective therapeutic approach.

# RESULTS

## 6.1. ICL isoenzymes of *M. tuberculosis*

Previously, we reported that isocitrate lyase (ICL1) facilitates persistence of *M. tuberculosis* in chronically infected mice and macrophages (McKinney *et al.,* 2000; Fig. 4.1). The genome sequence of a virulent clinical isolate of *M. tuberculosis* (CDC1551) identified a second *icl* gene (MT1966, Rv1915/16; here designated *icl2*) (Fleischmann *et al.,* 2002) with limited homology to *icl1* (MT0483, Rv0467); the predicted gene products are 27% identical overall, and differ significantly in size (ICL1 = 428 AA; ICL2 = 766 AA). Recombinant ICL2 displays isocitrate lyase activity *in vitro* although it is less active than ICL1 (Höner zu Bentrup *et al.,* 1999). Sequence alignments revealed three distinct groups of ICL proteins (Fig. 6.1), which differed in length and domain organization (Fig. 6.2A). Group I ICLs comprised eubacterial short ICLs, including *M. tuberculosis* ICL1. Group II ICLs included plant and fungal medium-length ICLs, which contained a central domain (Domain II) absent in group I. Group III ICLs, so far identified only in *Mycobacterium*, had a long C-terminal domain absent in groups I and II; they also contained a central region with significant homology to the central domain of the eukaryotic ICLs and were overall ~ 35% identical to them (Fig. 6.1&6.2). *In silico* modeling of the three-dimensional structure of ICL2, based on the X-ray crystal structures of ICL1 from *M. tuberculosis* (Sharma *et al.,*

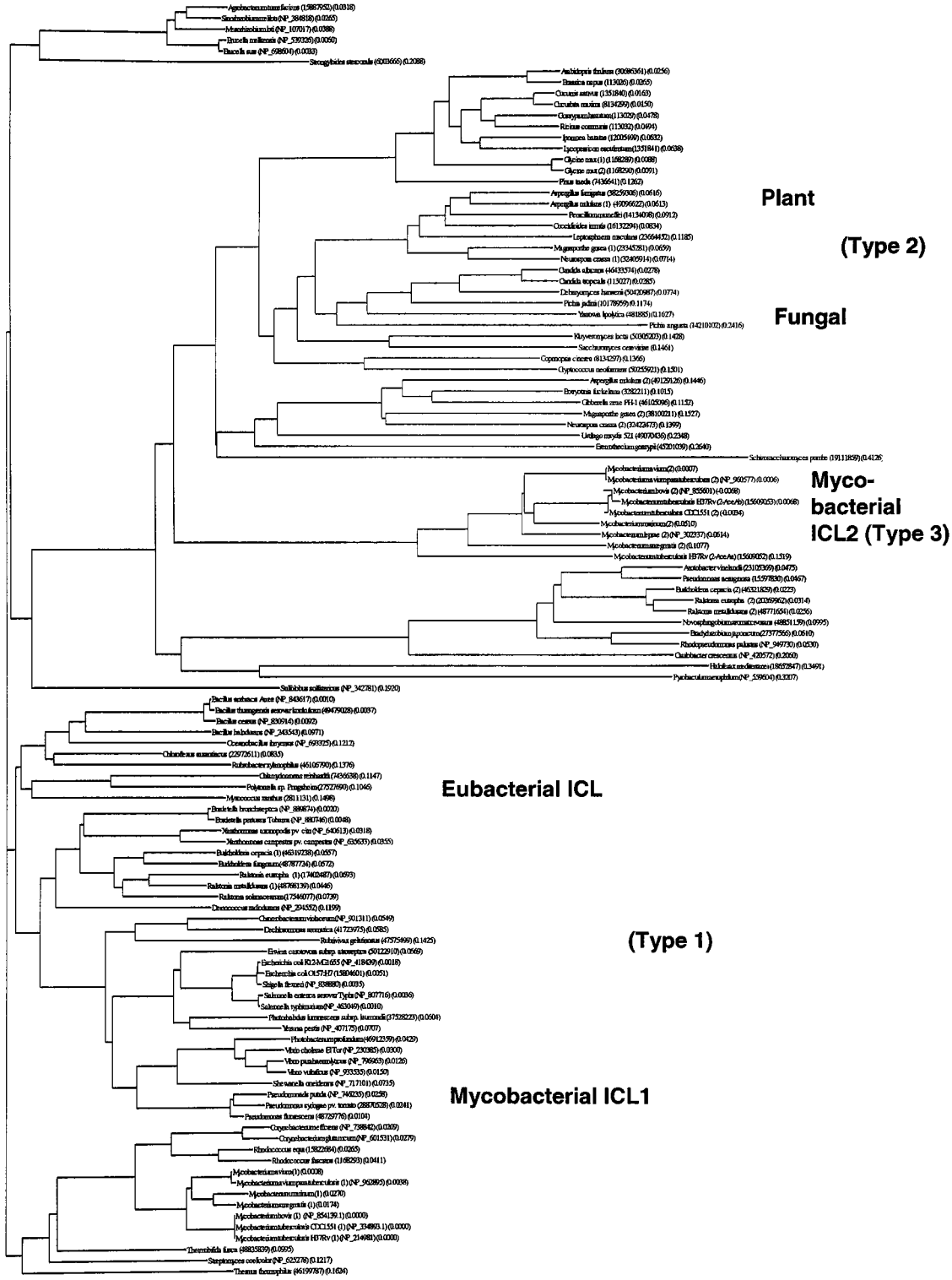


**Fig. 6.1. Isocitrate lyases dendrogram.** Sequences were obtained through the BLAST interface and genome databases (NCBI). Name is followed by accession number or unique gene identifier in parentheses, followed by number indicating distance to nearest neighbor. Tree was built using the clustal W algorithm of Vector NTI v.9.

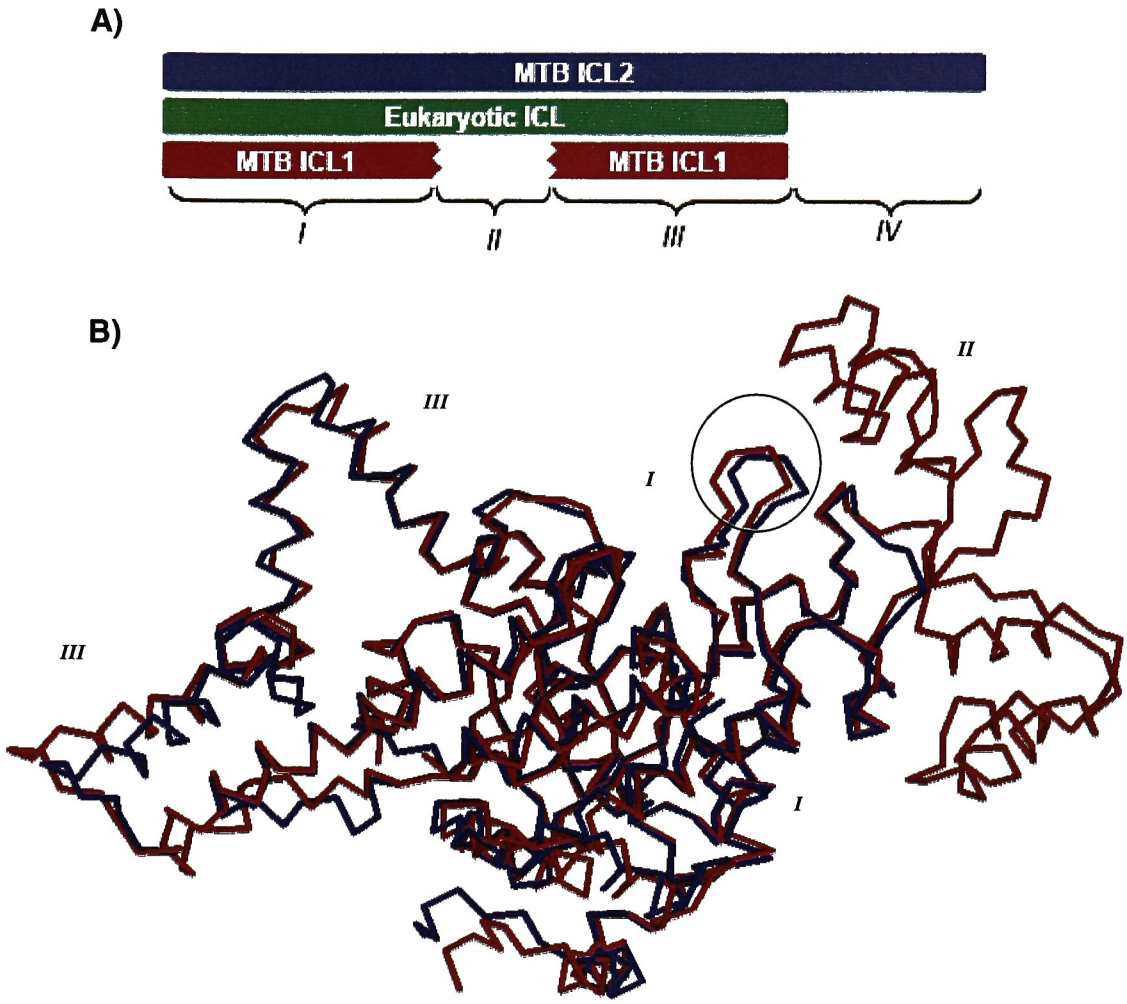


**Fig. 6.2 ICL1 and ICL2 are evolutionarily divergent structurally related proteins .** (**A**) Domain organization of isocitrate lyases in eubacteria and eukaryotes. Alignments were generated using the Conserved Domain Database (CDD v1.62, NCBI). Domains I and III are present in all ICLs. Domain I contains the conserved catalytic motif KKCGH. Domain II is present in fungal and plant ICLs and in mycobacterial ICL2, but absent in mycobacterial ICL1. Domain IV is unique to mycobacterial ICL2. (**B**) ICL1 and ICL2 are structurally related isocitrate lyases. Structural modeling of *M. tuberculosis* ICL2. Superimposition of the model of ICL2 monomer (red) and the X-ray crystal structure of an ICL1 monomer (blue). Encircled: ICL catalytic signature motif KKCGH (AA 193-203 in ICL1; AA 213-217 in ICL2). The ICL2 model was generated using MODELLER, based on the predicted AA sequence of ICL2 (GI: 50952459) and coordinates from the crystal structure of ICL1 (PDB 1F8M, GI: 15607608) (Sharma *et al.*, 2000). Domain II of ICL2 (AA 269-365) is absent in ICL1, and was modeled after the crystal structure of ICL from *Aspergillus nidulans* (GI: 49096622)24. Domain IV (not depicted) had no homology to any known sequences.

2000) and ICL from *Aspergillus nidulans* (Britton *et al.,* 2000), revealed a striking level of structural concordance (Fig. 6.2B). Significantly, the active site loops of ICL1 and ICL2, containing the conserved catalytic motif (KKCGH), were nearly superimposable (Fig. 6.2B, encircled). Thus, despite the limited homology between ICL1 and ICL2, conservation of tertiary structure and enzymatic activity suggested these enzymes might subsume overlapping biological roles.

## 6.2. Generation of *Mycobacterium tuberculosis icl* mutants

Microorganisms use the GC to replenish TCA cycle intermediates during growth on fatty acids because under these conditions the concentrations of PEP and pyruvate are insufficient to support anaplerosis via their conversion to oxaloacetate by PEP carboxylase (PPC) or pyruvate carboxylase (PCA), respectively (Fig. 3.6). To assess the role of the GC in mycobacterial pathogenesis and fatty acid metabolism, *M. tuberculosis* (Erdman) strains were constructed in which *icl1*, *icl2*, or both were deleted. The mutants were generated by allelic exchange as illustrated (Fig. 6.3A), using a replicative plasmid and sucrose counterselection as described previously (Pavelka & Jacobs, 1999). The *icl2* gene was disrupted by deletion of two internal MscI fragments and insertion of a kanamycin resistance cassette. The same construct was used to disrupt *icl2* in the chromosome of wild-type and Δ*icl1* mutant bacteria, thereby generating Δ*icl2* and

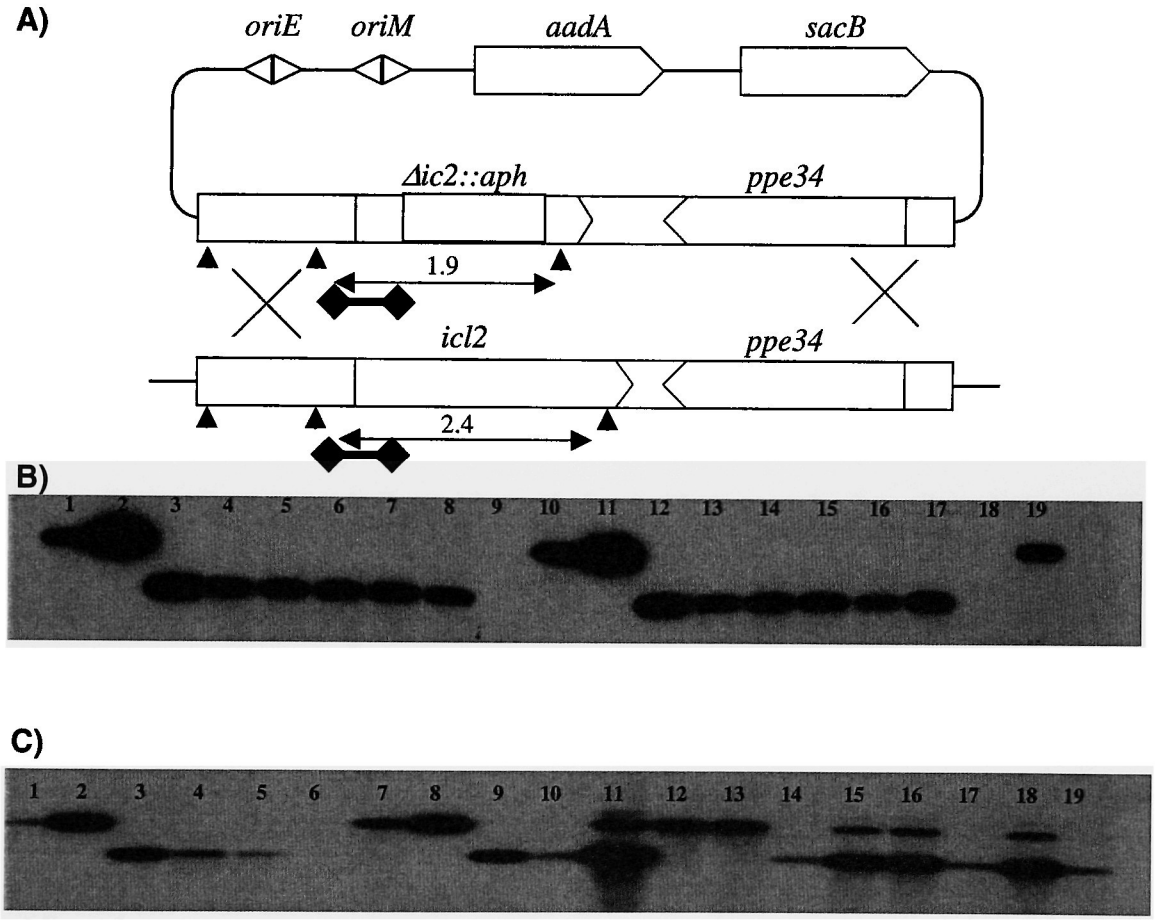


**Fig. 6.3. Disruption of *icl2* in the genome of wild-type *M. tuberculosis* (Erdman and H37Rv) & in the *icl1* mutant background (Erdman).** (**A**) Scheme for disruption of *icl2* in the chromosome of *M. tuberculosis* (Erdman). (**B**) Genomic DNA preparations from *M. tuberculosis* wild-type (Erdman) (lane 1), H37Rv (19), plasmid pEM027 carrying wild-type *icl2* (lanes 2 & 11), deletion construct pEM029 (lanes 3 & 12), mutant candidate clones in Erdman (lanes 4-8), in H37Rv (lanes12-17) and (**C**) Genomic DNA preparations from *M. tuberculosis* wild-type (lane 1), plasmid pEM027 carrying wild-type *icl2* (lanes 2&8), deletion construct pEM029 (lanes 3&9), Δ*icl1* (Erdman) (lane 7), Δ*icl2* (Erdman) (lane 4), Δ*icl2* (H37Rv) (lane 5), candidate clones for *icl2* disruption on the Δ*icl1* (Erdman) background (lanes 10-19) were digested with ApaLI, separated in 1% TBE agarose gels, denatured with 0.5 NaOH, transferred to Hybond N membrane, and probed with an 610 bp PCR fragment from pEM027 (indicated by diamond-headed marks) labeled with $^{32}P$ using a random priming labeling kit. Hybridization and washings were carried out under stringent conditions (65 C); specific hybridization was detected by autoradiography. See materials and methods for further details.

Δ*icl1*Δ*icl2* mutants, respectively. Disruptions were confirmed by Southern blot (Fig. 6.3B&C).

Disruption of *icl1* in *M. tuberculosis* (Erdman) and its complementation with an episomal plasmid carrying a wild-type copy of *icl1* gene were previously described (McKinney *et al.,* 2000). In the genome of *M. tuberculosis*, the ORF immediately downstream of *icl2* appears to be transcribed in the opposite orientation thus making it unlikely that the disruption of *icl2* would affect the expression of this downstream gene. However, another potential complication in the analysis of a mutant is that unintended mutations may be introduced elsewhere in the chromosome inadvertently during its construction. Thus, reversal of a mutant's phenotype by complementation with a functional copy of the gene that was disrupted is always desirable. Since in the Δ*icl1*/Δ*icl2* mutant strain we had generated *icl1* had been disrupted by deletion of part of the ORF and insertion of a hygromycin resistance cassette, while the *icl2* gene had been replaced by a kanamycin resistance cassette, complementing plasmids carrying a streptomycin resistance cassette and either *icl1* (pEM263-4/pICL1) or *icl2* (pEM3E4-8/pICL2) were constructed and transformed into the Δ*icl1*/Δ*icl2* (see Materials & Methods).

## 6.3. Characterization of *M. tuberculosis icl* mutants in standard media

*M. tuberculosis* lacking *icl1*, *icl2*, or both grew equally well and indistinguishably from wild-type bacteria in 7H9 broth in either rolling (Fig. 6.4A) or shaking bottles (Fig. 6.4B). Rolling bottles afforded better growth presumably because they allowed better aereation. The ability to form colonies on standard 7H10 plates was somewhat different for the different *icl* deficient strains. While the single Δ*icl2* mutant grew like wildtype (CFU in 3 wks), formation of a colony of a size comparable to that of wild-type bacteria at 3 wks by Δ*icl1* and Δ*icl1*/Δ*icl2* mutant bacteria required four and six wks of incubation, respectively (not shown).

## 6.4. Determining *in vitro* growth conditions to test utilization of C2 substrates

In most bacterial species studied so far, the GC is essential for growth solely when the only carbon substrates available are ones that enter central metabolism as acetyl-CoA (C2 substrates); such carbon sources are primarily fatty acids (Cronan & LaPorte, 1996). In order to assess the contribution of ICL1 and ICL2 to carbon metabolism in *M. tuberculosis*, we set sought to establish culture conditions permitting the assessment of the contribution of these enzymes to bacterial carbon metabolism.

Mycobacteria are notoriously slow-growing fastidious organisms (Wheeler, 1984; Höner zu Bentrup & Russell, 2001). *M. tuberculosis* can be

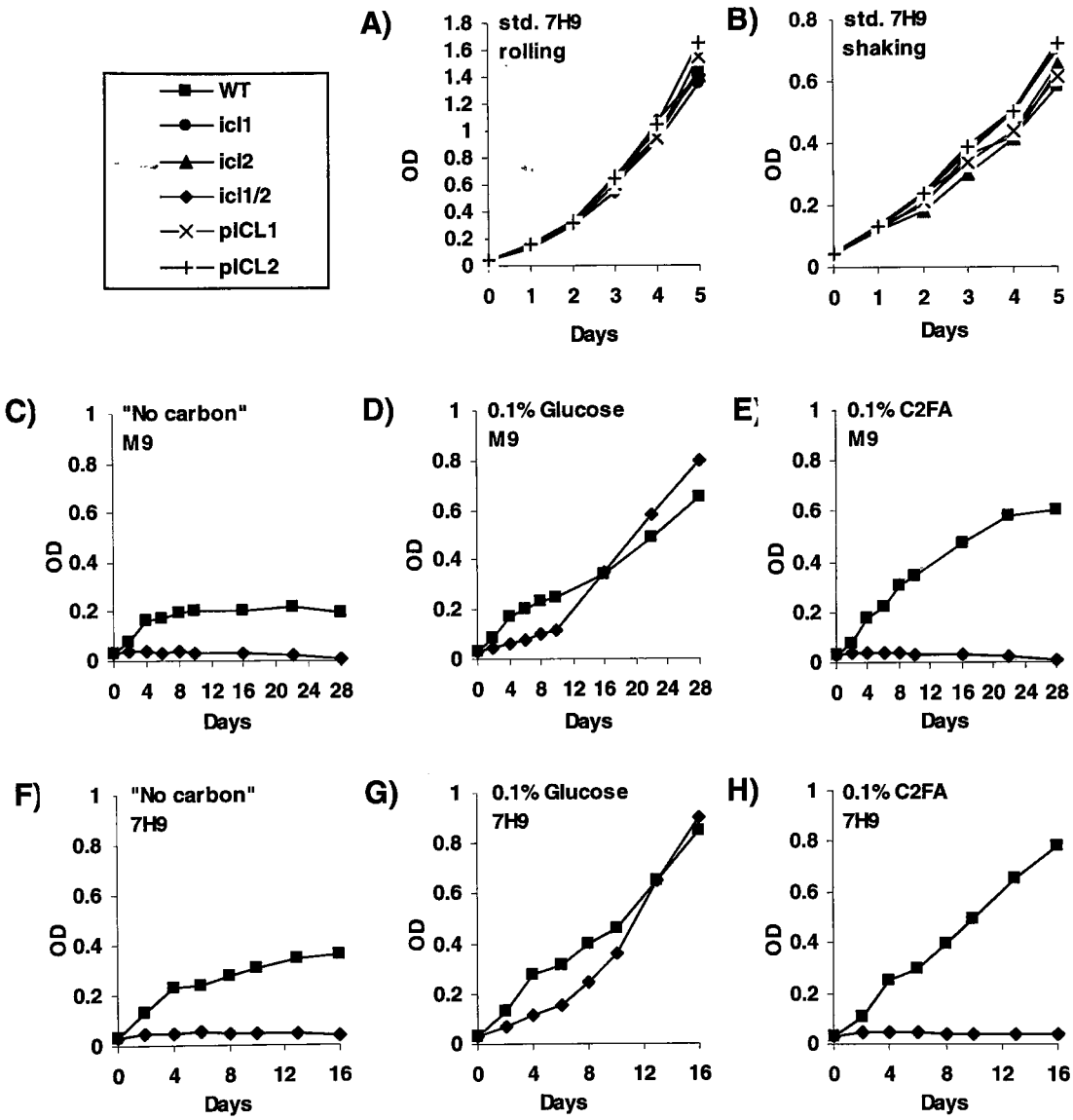


**Fig. 6.4. Characterization of *M. tuberculosis icl* mutant strains in standard 7H9 broth, M9-based and 7H9-based defined liquid media.** (**A-B**) *M. tuberculosis* (Erdman) bacteria were cultured in standard (std.) 7H9 broth: (**A**) in rolling bottles; (**B**) in shaking bottles. (**C-E**) M9-based defined media: (**C**) no carbon; (**D**) glucose; (**E**) acetate (C2FA). (**F-H**) 7H9-based defined media: (**F**) no carbon; (**G**) glucose; (**H**) acetate (C2FA). Both M9- & 7H9- based defined media contained Tween-80 (0.05%), Albumin fraction V (0.5%), and NaCl (0.08%). Growth was monitored by measuring culture turbidity ($OD_{600}$ nm) at the indicated time points. Results are representative of at least two experiments.

grown axenically, but its optimal cultivation requires relatively rich media (Dubos & Middlebrook, 1947). In liquid, *M. tuberculosis* is routinely grown in Middlebrook 7H9 powder (DIFCO)-based broth supplemented with "ADS" (w/v: 0.5% albumin, 0.2% glucose, 0.085% saline, 0.05% Tween-80, and 0.5% glycerol. On solid media, *M. tuberculosis* is grown on 7H10 agar (DIFCO)-based media supplemented with glycerol (0.5% w/v) and 10% OADC (DIFCO). OADC (DIFCO) is a commercial supplement containing oleic acid, albumin, glucose, and catalase at the same concentrations used by Middlebrook and Dubos (Dubos & Middlebrook, 1947), which are the same as those used in ADS for supplementation of 7H9 broth. In order to define conditions that would allow the study of carbon utilization of *M. tuberculosis* on solid media, we repeatedly attempted to use non-commercial (home-made) stocks of OADC to culture *M. tuberculosis*, but these media preparations consistently resulted in a drastic delay in colony formation (colonies took circa 8 wks to appear, not shown). This fact made studies of *M. tuberculosis* on defined solid media impractical.

Therefore, we took a two-pronged approach to study carbon utilization in mycobacteria. The first approach consisted in generating mutants in the same genes we were interested in studying in *M. tuberculosis*, namely GC genes, in *M. smegmatis*, a fast-growing saprophyte that is readily cultured in minimal media. The mutants were then complemented with the homologous *M. tuberculosis* genes to study their function as it pertains to carbon utilization. This work is reported in Chapter 9. The second approach was to find growth conditions in which a given

carbon substrate was the sole or at least the main carbon substrate present in liquid media for *M. tuberculosis* cultivation. Cultivation of *M. tuberculosis* in minimal M9 salts liquid media supplemented with different single carbon substrates resulted in very poor growth, clumping, and attachment of the tubercle bacilli to the walls of the culture flask (not shown). We next attempted to grow *M. tuberculosis* in M9 salts liquid media supplemented with albumin, saline and Tween-80 (same concentrations used for standard 7H9 broth) and a single carbon source at 0.1% (w/v). Encouragingly, M9-based media supplemented with either the short chain fatty acid acetate (C2FA) or glucose allowed significant growth (Fig. 6.4D&E). Some initial background growth was observed in media containing "no carbon;" this we attributed to the presence of Tween-80 (0.05%), which at low concentrations can be metabolized by mycobacteria (Saito *et al.*, 1983). Nonetheless, the growth obtained in M9-based media was very limited (the bacterial doubling time was ~ 5-6 days, compared to ~1 day in standard 7H9 broth). So we next replaced the M9 salts with 7H9 powder; 7H9 powder (DIFCO), in addition to basic salts, contains citrate (0.01%, w/v) and glutamate (0.05%, w/v), and traces of other elements that enhance bacterial growth. 7H9-based media supplemented with glucose (0.1%, w/v) or C2FA (0.1%, w/v) supported significantly better and faster growth of *M. tuberculosis* (doubling time 3-4 days) than similarly supplemented M9-based defined media (Fig. 6.4G&H).

## 6.5. Functional redundancy of *icl1* and *icl2* in fatty acid metabolism *in vitro*

Importantly, we observed that while both M9- and 7H9-based defined glucose media supported the growth of the Δ*icl1*/Δ*icl2* mutant (Fig. 6.4D&G), C2FA-supplemented media did not (Fig. 6.4E&H). Interestingly, the Δ*icl1*/Δ*icl2* mutant failed to grow even to background levels in M9- or 7H9-based media without added carbon, perhaps reflecting the inability of the mutant to grow on fatty acids derived from Tween-80 (Fig. 6.4C&F). These growth conditions were used to analyze the ability of the *icl* mutants to grow on different carbon substrates.

Deletion of *icl1* or *icl2* alone had little effect on bacterial replication kinetics in media containing glycerol (Fig. 6.5A), glucose (Fig. 6.5B), short-chain fatty acids (Fig. 6.5C-G), or long-chain fatty acids (Fig. 6.6A-E) as carbon sources. In contrast, deletion of both *icl1* and *icl2* eliminated growth on fatty acids, while having little effect on utilization of carbohydrates (Fig. 6.5&6.7). A slight but reproducible lag was observed for growth of the Δ*icl1*/Δ*icl2* strain in carbohydrate-containing media (Fig. 6.5B), suggesting carbon flux through the GC under these conditions in wild-type *M. tuberculosis*. A similar observation has been made in *Corynebacterium glutamicum* (Wendisch *et al.*, 2000). In the experiments shown in Fig. 6.5 & 6.7, growth was determined by monitoring the optical absorbance of the cultures, except for Fig. 6.6D, where the ability of the

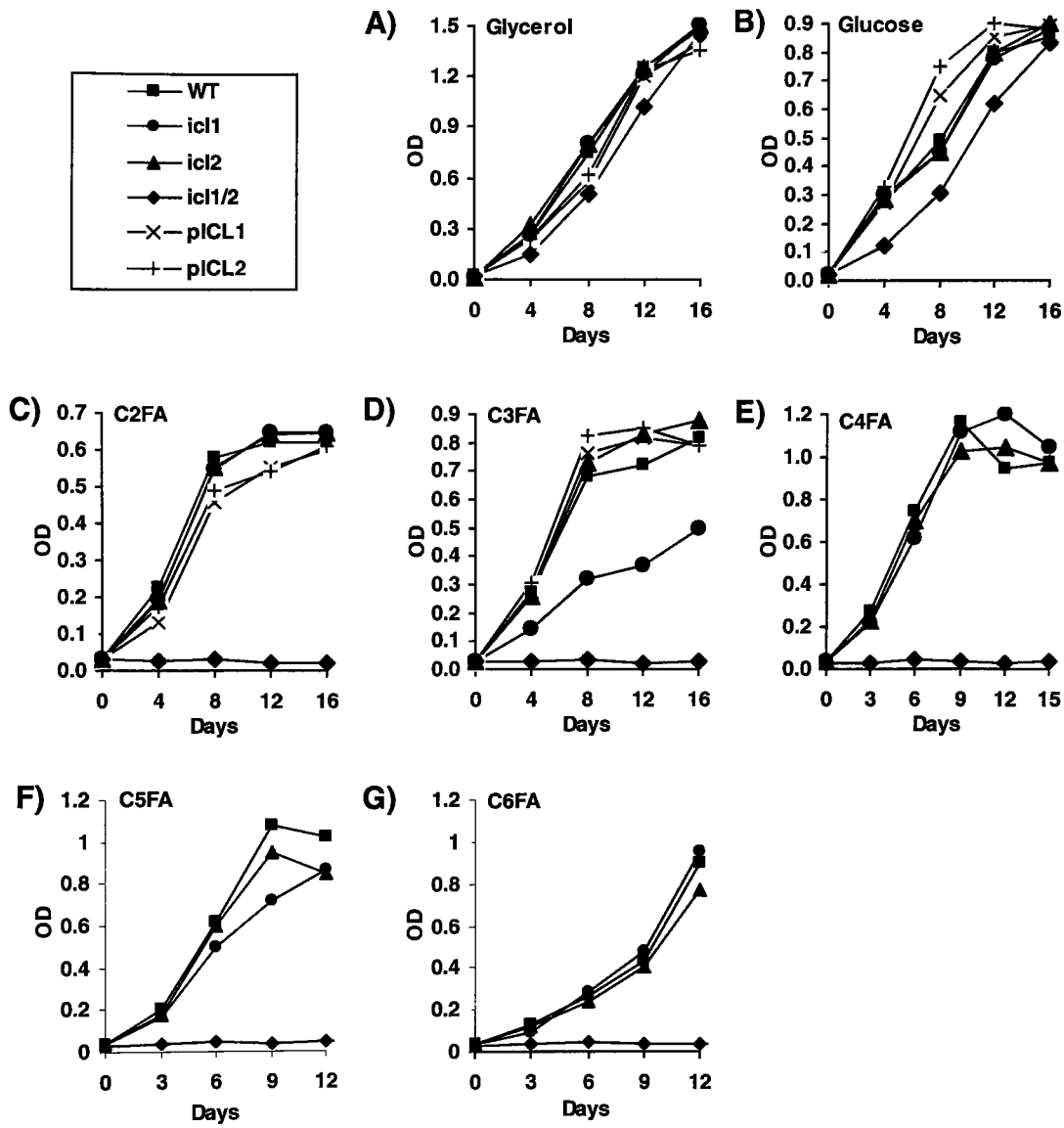


**Fig. 6.5. Overlapping roles of ICL1 and ICL2 in short chain fatty acid metabolism.** (**A-G**) *M. tuberculosis* (Erdman) bacteria were cultured in liquid media containing (0.1% w/v): (**A**) glycerol; (**B**) glucose; (**C**) acetate (C2FA); (**D**) propionate (C3FA); (**E**) butyrate (C4FA); (**F**) valerate (C5FA); (**G**) hexanoate (C6FA). Growth was monitored by measuring culture turbidity ($OD_{600}$ nm) at the indicated time points. Results are representative of at least two experiments.

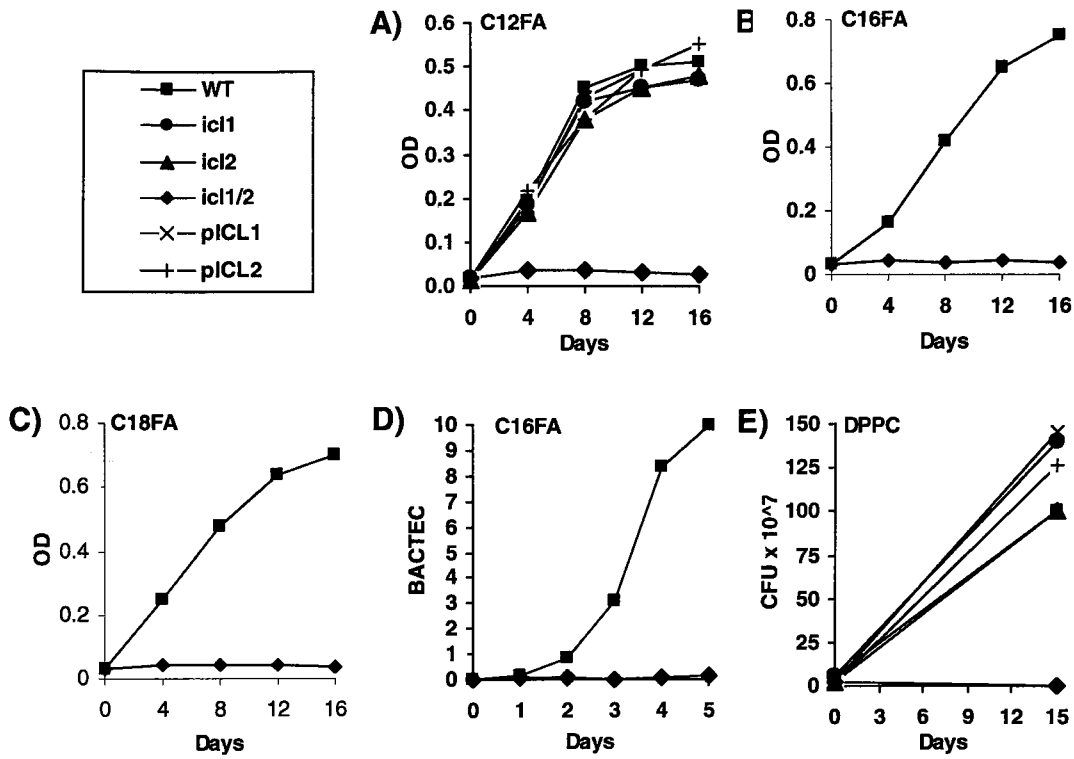


**Fig. 6.6. Overlapping roles of ICL1 and ICL2 in long-chain fatty acid metabolism.** (**A-C**) *M. tuberculosis* (Erdman) bacteria were cultured in liquid media containing (0.1% w/v): (**A**) polyoxyethylene sorbitan mono-laurate (C12FA); (**B**) polyoxyethylene sorbitan mono-palmitate (C16FA); (**C**) polyoxyethylene sorbitan mono-stearate (C18FA). Growth was monitored by measuring culture turbidity ($OD_{600}$ nm) at the indicated time points. (**D**) Growth on palmitate (C16FA) was measured by BACTEC, which quantifies [$^{14}$C]-$CO_2$ released by oxidation of [$^{14}$C]-palmitate. (**E**) Growth on dipalmitoyl-phosphatidylcholine (DPPC, C16FA), an abundant phospholipid in the lung, was determined by plating for CFU. Results are representative of at least two experiments.

bacteria to metabolize a C16FA was determined in a BACTEC apparatus in which the evolution of $CO_2$ from media containing radiolabeled palmitate is measured, and for Fig. 6.6E, where growth was determined by plating for CFU after the bacteria had been cultured for 15 days in media supplemented with dipalmitoyl-phosphatidylcholine (DPPC), a major phospholipid component of pulmonary surfactant (pulmonary surfactant is typically 90% lipids, 10% proteins) (Santangelo *et al.*, 1999).

## 6.6. ICL activities *in vitro* and *icl1* and *icl2* mRNA levels during infection

Next, we determined ICL activities present in extracts from bacteria grown on different carbon substrates to get an idea of the relative contribution of each of the isoenzymes to the overall ICL activity (Table 6.1). Extracts prepared from glucose-grown wild-type bacteria had basal levels of ICL activity, those from the single Δ*icl1* and Δ*icl2* mutants each had some activity as well, while Δ*icl1*Δ*icl2* extracts had no activity. The ICL activity in wild-type acetate extracts was about 10 fold higher compared to glucose, but unexpectedly, the activity in the Δ*icl1* mutant was barely detectable (similar to ICL levels in glucose), while that in the Δ*icl2* was higher than wild-type. Unexpectedly, growth on the C3 fatty acid propionate resulted in ICL activity which was ~10 fold higher than on acetate.

To get an idea of the regulation of ICL expression during infection, we next sought to determine the relative levels of mRNA for each of the *icl* homologs. Previously, Timm and colleagues (2003) reported up-regulation of *icl1* in *M. tuberculosis* (strain H37Rv) isolated from mouse lungs relative to bacteria grown *in vitro*. Confirming this previous observation, we found that *icl1* was highly up-regulated in bacteria isolated from mouse lungs that had been infected with *M. tuberculosis* Erdman, the strain used throughout

**Table 6.1. Isocitrate lyase activity in *M. tuberculosis icl* mutants**

| ***M. tuberculosis* strain** | **Glucose** | **Acetate** | **Propionate** |
|---|---|---|---|
| **Wildtype** | 2.10 +/- 0.11 | 26.20 +/- 2.48 | 406.47 +/- 28.25 |
| ***Δicl1*** | **0.35 +/- 0.16** | **0.54 +/-0.25** | **2.35 +/- 1.11** |
| ***Δicl2*** | 1.68 +/- 0.13 | 40.07 +/- 5.95 | 495.44 +/- 65.09 |
| ***Δicl1/icl2*** | **0.09 +/- 0.31** | **ND** | **ND** |

Bacteria were grown in defined 7H9 broth at 37 °C with shaking (65 rpm), and harvested at late log phase. Carbon substrates were used at 0.2 % w/v, each. Values correspond to specific activities (nmol/min/mg) in total cell-free extracts: isocitrate-stimulated activity using 1 mM isocitrate, 0.1 mM NADH at 25 °C. All strains are *M. tuberculosis* (Erdman). ND = not done. Data represent the average of 3 measurements from one set of extracts.

our studies. A peak in expression occurred at around 20 days post-infection, but relative high expression levels were maintained throughout (60 and 180 day time

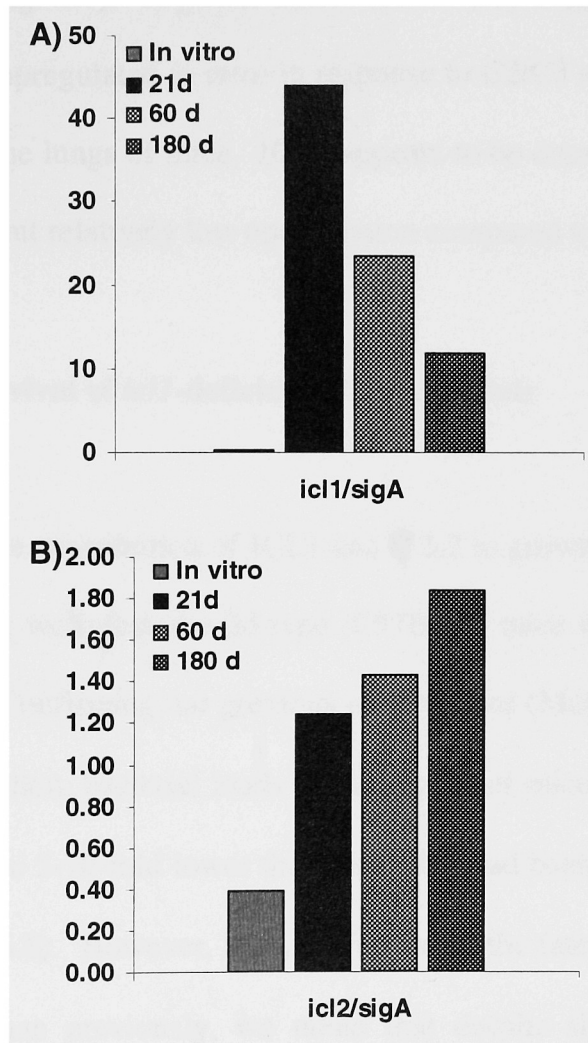


**Fig. 6.7. Expression of glyoxylate cycle enzymes during infection.** (**A**) *icl1* and (**B**) *icl2* mRNA levels in *M. tuberculosis* grown *in vitro* (gray bars), in mouse lungs at 21 d (black bars), 60 d (black and white checkered bars) and 180 d (black circles-filled bars). Bars represent ratios of *icl1 or icl2* levels relative to *sigA*, a gene whose expression remains relatively unchanged during the infection (Timm *et al.*, 2003). Number of transcript copies was determined by QRT-PCR using gene specific molecular beacons as detailed in materials and methods. Results are representative of least two experiments using samples from the same infection. Mice were infected i.v. with *M. tuberculosis* Erdman (1 x $10^6$ CFU/ mouse).

points) (Fig. 6.7). Expression of *icl2* was also higher *in vivo* compared to *in vitro*, but the levels of expression were significantly lower (~10 fold) than those of *icl1* at all time points (Fig. 6.7). Together these observations suggest that while ICL1 is inducible, being upregulated *in vitro* in response to C2/C3 substrates, and at the level of mRNA in the lungs of mice. ICL2 appears to be constitutive *in vitro*, and subjected to some, but relatively low upregulation compared to ICL1 *in vivo*.

## 6.7. Long-term survival of *icl1*-deficient *M. tuberculosis*

In order to assess the contribution of ICL1 and ICL2 to growth and survival of *M. tuberculosis in vivo*, we infected wild-type (C57BL/6) mice with bacteria lacking *icl1*, *icl2*, or both. Confirming our previous observations (McKinney *et al.*, 2000), by 4 wks post-infection, bacterial loads in the organs of mice infected with Δ*icl1* *M. tuberculosis* were 5-10 fold lower than those that had been infected with wild-type bacteria (Fig. 6.8). However, here, by following the fate of the Δ*icl1* mutant for much longer than previously, we noted that despite significant prolonged attenuation and reduced tissue pathology (Fig. 6.9), Δ*icl1* mutant bacteria were actually capable of long-term persistence in the lungs of mice (at around $10^5$ CFU/lung), and late in the infection resumed growth (compare CFU/lung at 16 vs 52 wk, post-infection), leading to pathology and death of the infected mice at circa 75 wks post-infection (Fig. 6.10). Mice infected with wild-type bacteria died at

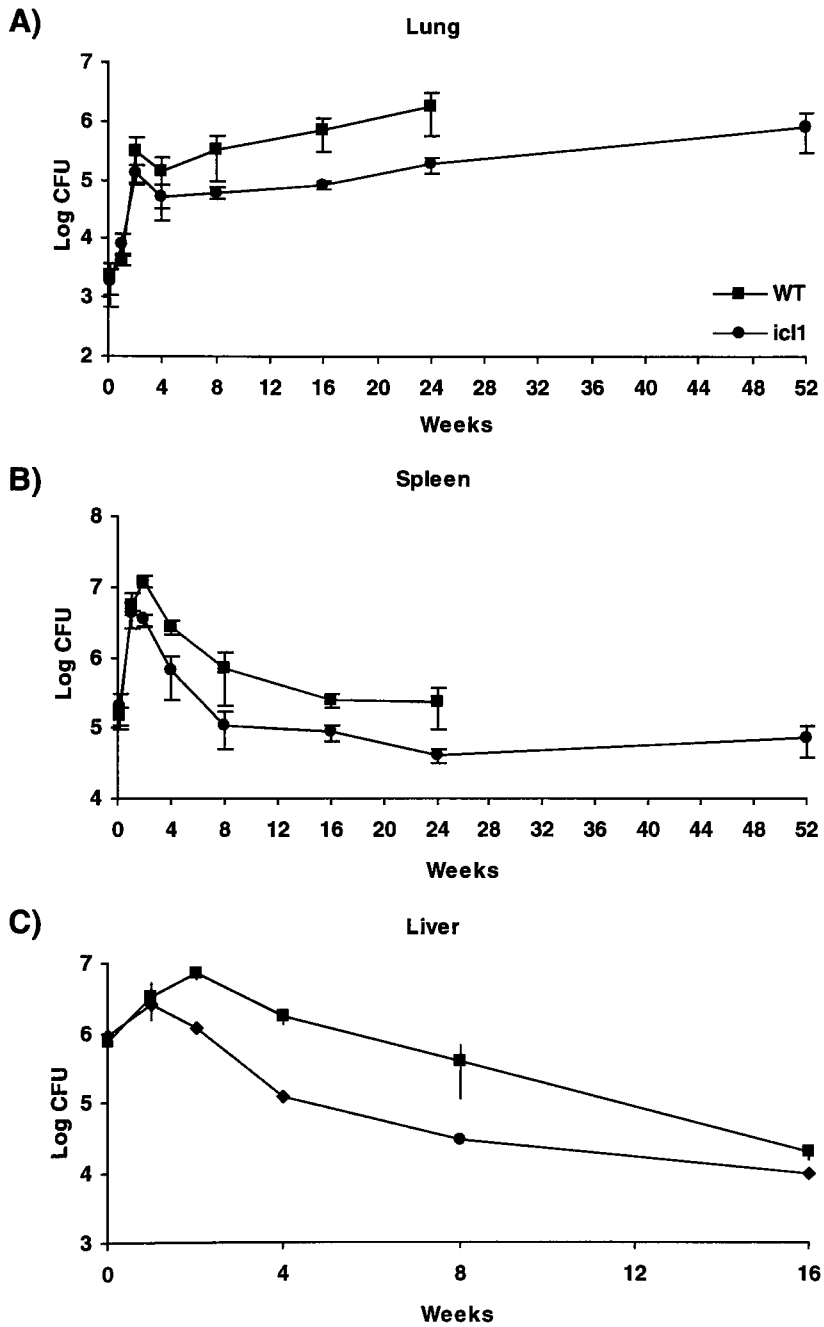


**Fig. 6.8. Loss of ICL1 attenuates *M. tuberculosis* in chronically infected mice.** Wild-type mice (C57BL/6) were infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1* mutant ($10^6$ CFU, i.v.). Bacteria in the (**A**) lung, (**B**) spleen, and (**C**) liver were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4-5).

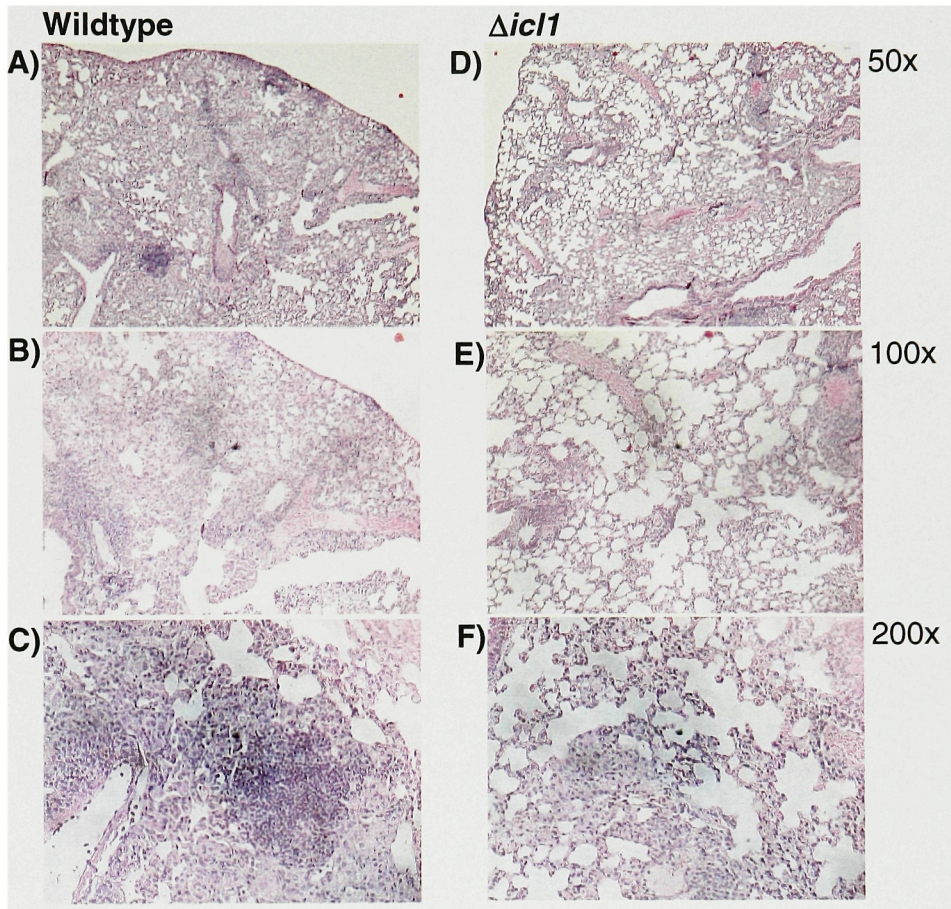


**Fig. 6.9. Lung pathology in mice infected with ICL1-deficient *M. tuberculosis*.**
Lung sections (H&E stain) at 16 wk post-infection in C57BL/6 mice infected with *M. tuberculosis* (Erdman) (~$5x10^5$, i.v.) (**A-C**) wild-type, (**D-F**) Δ*icl1*. Numbers on right margin indicate total magnification.

about 40 wks post-infection (Fig. 6.10), while uninfected mice lived longer than 104 wks (not shown). Based on our *in vitro* carbon utilization experiments indicating that the absence of either *icl* was not enough to impair growth on C2 substrates, we hypothesized that the observed ability of the Δ*icl1* mutant to persist in the organs of mice and resume growth late in the infection might be due to the presence of ICL2.

### 6.8. *icl2* is dispensable for *M. tuberculosis* growth and persistence in mice

To directly assess the contribution of ICL2 to *M. tuberculosis* pathogenesis, we evaluated the ability of the Δ*icl2* mutant to grow and persist in mice. Similarly to the Δ*icl1* mutant, the Δ*icl2* mutant replicated like wild-type during the first 2 wks of infection. However, in contrast to the Δ*icl1* mutant, bacteria lacking ICL2 remained at wild-type levels during the chronic phase of the infection (wk 2-16) (Fig. 6.11), and elicited comparable pathology (Fig. 6.12). The fact that deletion of each *icl* individually resulted in limited (Δ*icl1*) or no phenotype (Δ*icl2*) is consistent with the hypothesis of partial redundancy between the two homologs and our studies carbon utilization studies (Fig. 6.4-6.6).

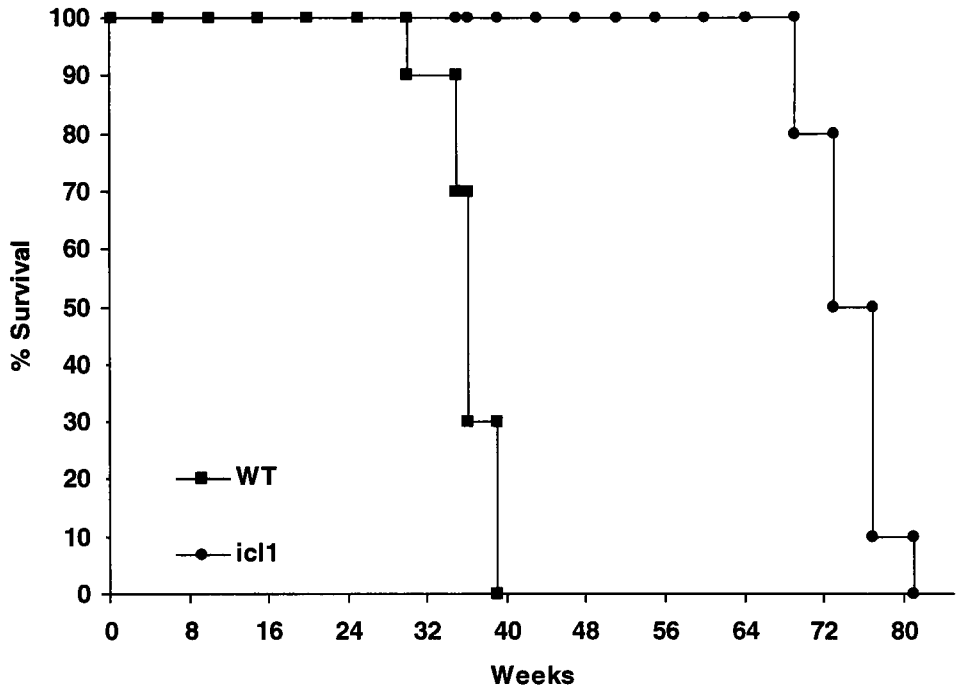


**Fig. 6.10. Survival of mice chronically infected with ICL1-deficient *M. tuberculosis*.** Time-to-death of wild-type (C57BL/6) mice infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1* mutant ($5x10^5$ CFU, i.v.). (n=10). (CFU data corresponding to this figure are shown in Fig. 6.9).

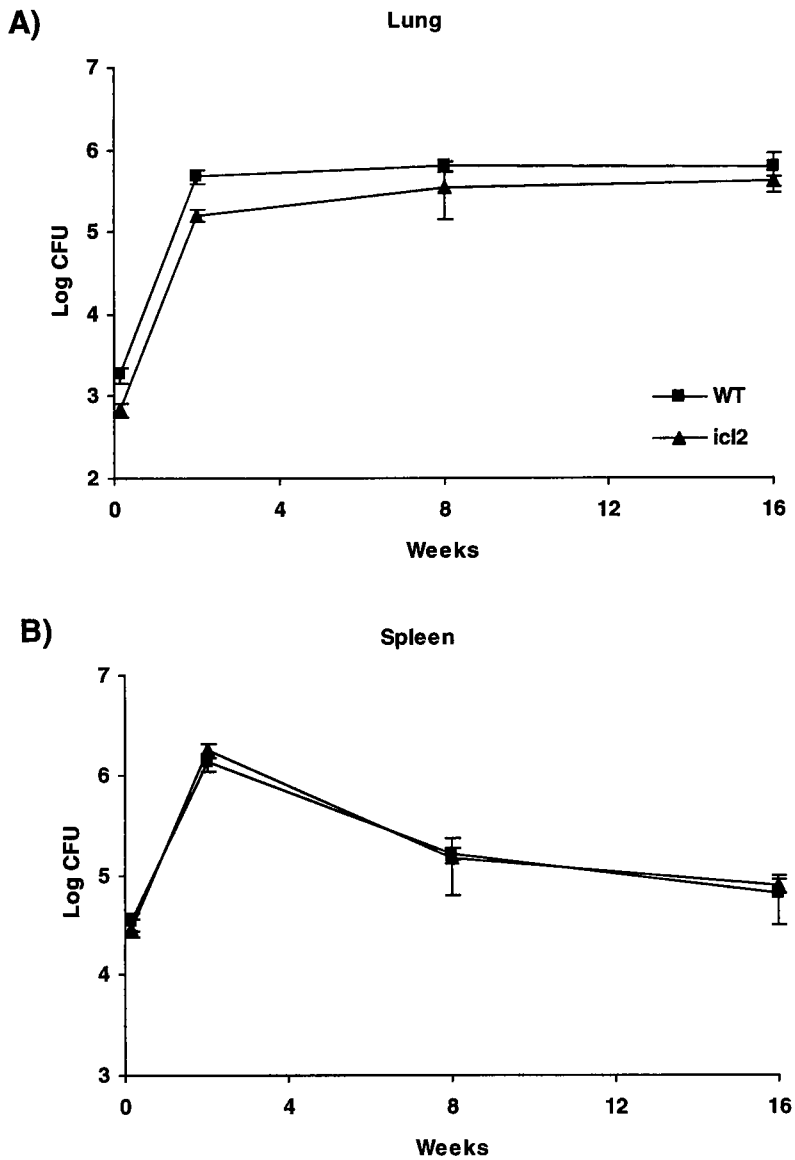


**Fig. 6.11. ICL2 is dispensable for *M. tuberculosis* growth and persistence in chronically infected mice.** Wild-type (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1* mutant ($5x10^5$ CFU, i.v.). Bacteria in the (**A**) lung and (**B**) spleen were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4-5).

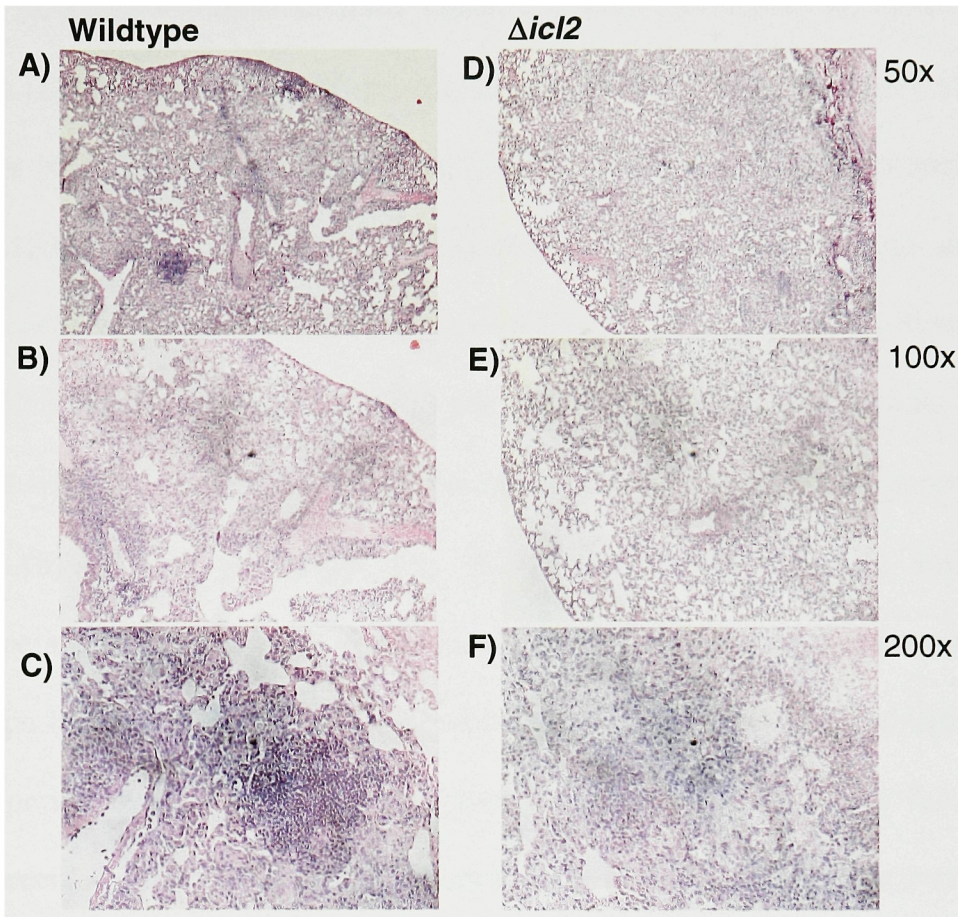


**Fig. 6.12. Lung pathology in mice infected with ICL2-deficient *M. tuberculosis*.**
Lung sections (H&E stain) at 16 wk post-infection in C57BL/6 mice infected with *M. tuberculosis* (Erdman) (~$5x10^5$, i.v) (**A-C**) wild-type, (**D-F**) Δ*icl2*.

## 6.9. Partial functional redundancy between *icl1* and *icl2 in vivo*

Indeed, in contrast to Δ*icl1* or Δ*icl2* mutant bacteria, Δ*icl1*/Δ*icl2 M. tuberculosis* mutants failed to replicate in lungs, spleens and livers of mice, and were cleared from the lungs by 4 wks post-infection, as seen in two independent experiments (Fig. 6.13A-C; Fig. 6.14 A&B). We observed no spontaneous relapse, and no bacilli could be recovered from any infected organ 48 wks post-infection (Fig. 6.13A-C; limit of detection = 2 CFU per organ) suggesting sterilization. Mice infected with the Δ*icl1*/Δ*icl2* mutant did not show any discernable splenomegaly (Fig. 6.15) or lung pathology (Fig. 6.16), and remained healthy until the experiment was terminated 2 years post-infection, whereas mice infected with wild-type bacteria uniformly succumbed between 8 and 9 months post-infection. The experiment was repeated a third time using the aerosol route of infection. Mice received 10 CFU/mouse as determined by plating lung homogenates for CFU at 24 hours post-infection. By 2 wks post-infection, Δ*icl1*/Δ*icl2* mutant bacteria had been eradicated from the lungs and had bacteria were unable to disseminate to the spleen as indicated by plating organ homogenates at 2 and 4 wks post-infection (limit of detection = 1 CFU/time-point). Complementation of the Δ*icl1*/Δ*icl2* mutant with an episomal plasmid carrying a wild-type copy of *icl1* restored bacterial growth in both lung and spleen (Fig. 6.17A&B), as well as lung tissue pathology (Fig. 6.18); late-stage persistence was largely, although not

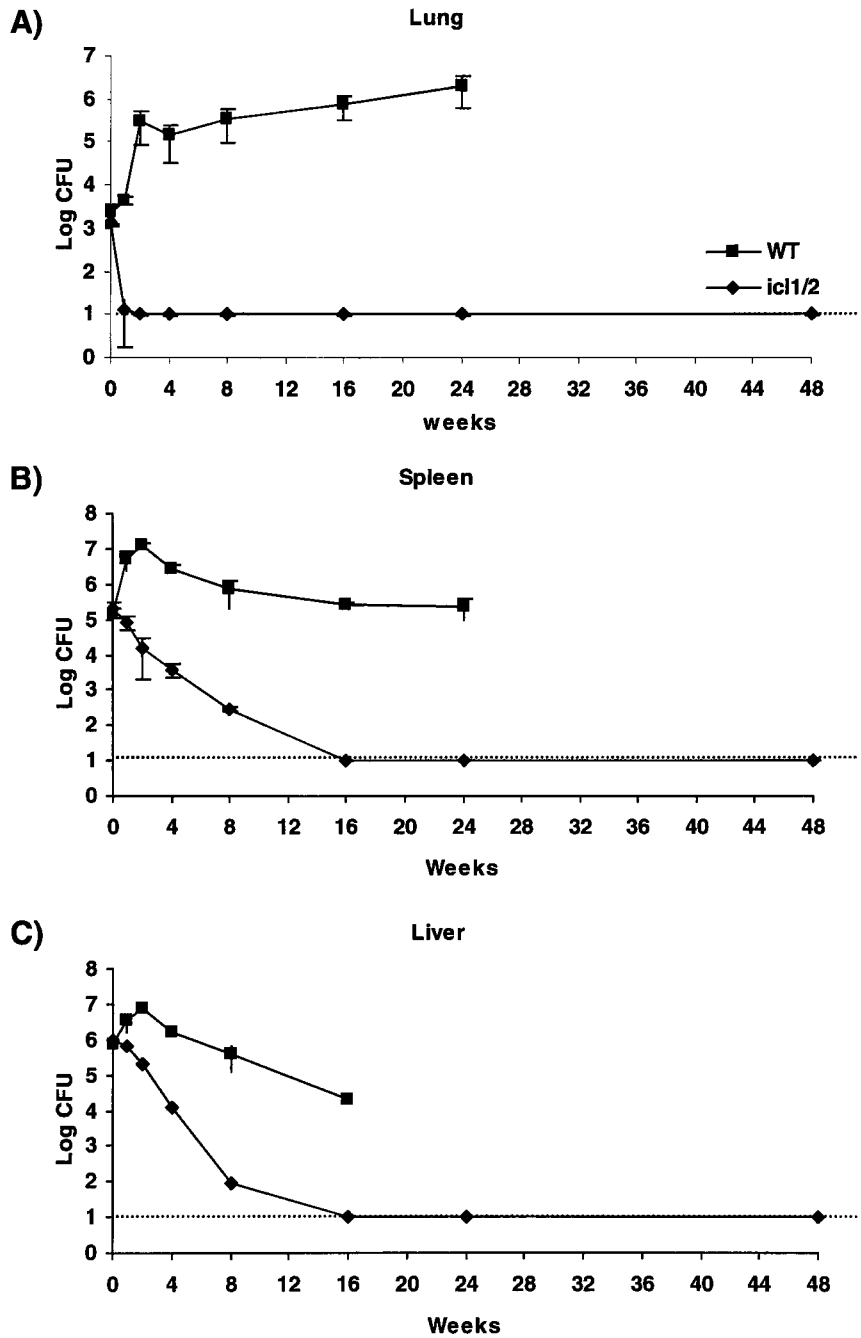


**Fig. 6.13. ICL1 and ICL2 are jointly required for growth and survival of *M. tuberculosis* in mice.** (**A-C**) Wild-type (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1*/Δ*icl2* (~$10^6$, i.v.). Bacterial loads in the (**A**) lung, (**B**) spleen, and (**C**) liver were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4-5). Limit of detection: 40 CFU/organ/mouse (dotted line), except for 48 wk time-point, 1 CFU.

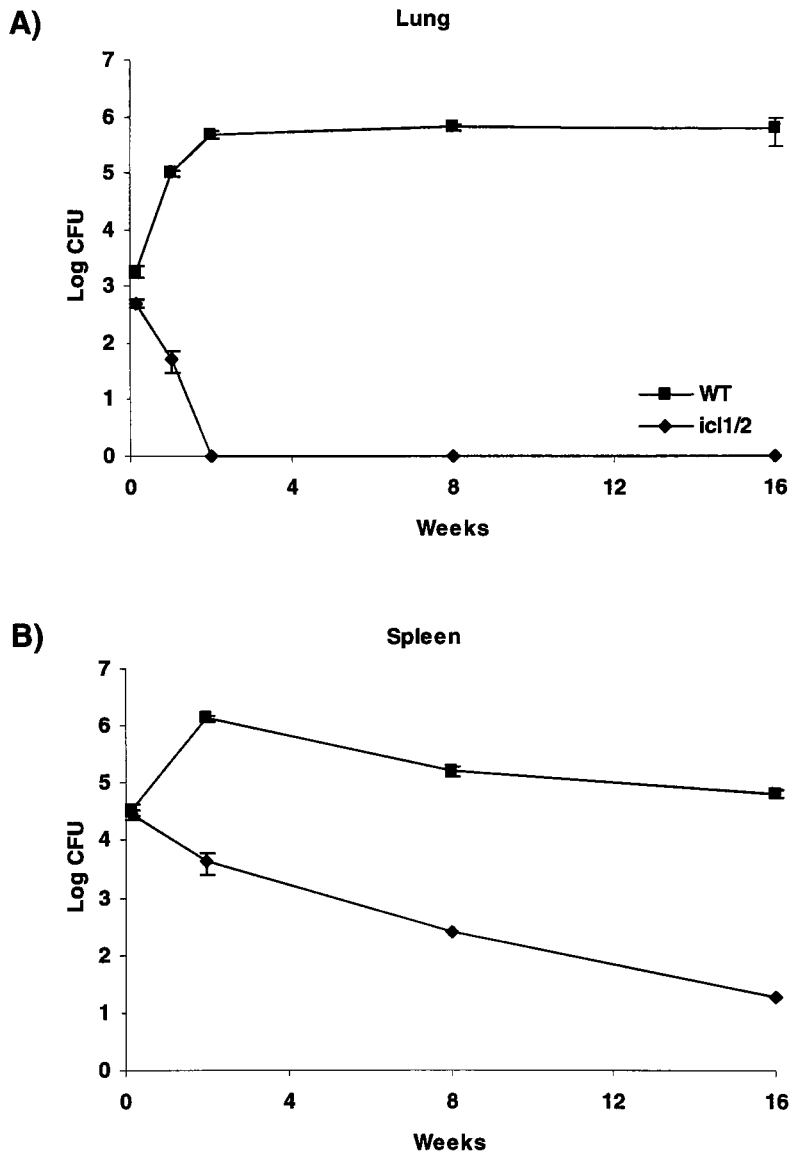


**Fig. 6.14. ICL1/ICL2-deficient bacteria are eradicated from mouse lungs within 4 weeks of infection.** (**A-B**) Wild-type (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) wild-type or *Δicl1/Δicl2* ($5x10^5$, i.v.). Bacterial loads in the (**A**) lung and (**B**) spleen were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4). This experiment only differs from the experiment shown in Fig. 6.13 in that a lower infection dose was used in this one. Limit of detection: 2 CFU/lung/mouse.

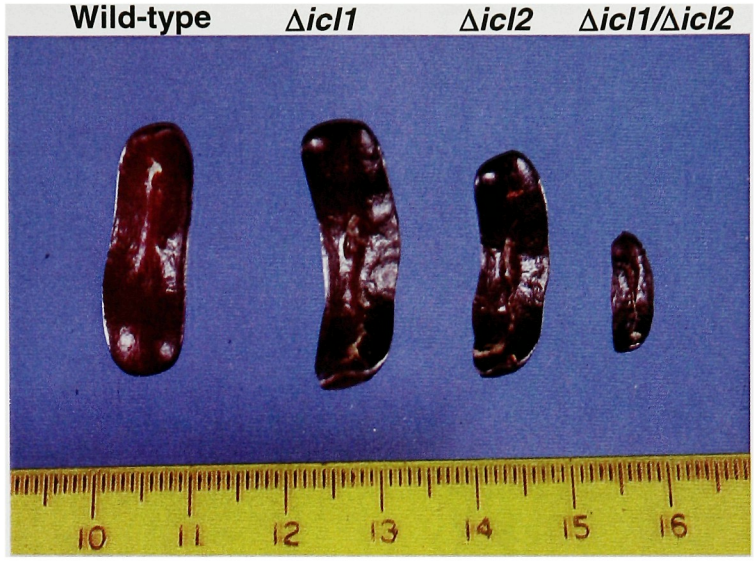


**Fig. 6.15. ICL1-/ICL2-deficient *M. tuberculosis* is avirulent in mice.** Spleens of C57BL/6 mice collected 2 weeks post-infection (~ $10^6$ CFU, i.v.) with wild-type (6.11 ± 0.15), Δ*icl1* (5.92 ± 0.12), Δ*icl2* (6.24 ± 0.08), or Δ*icl1*Δ*icl2* (3.58 ± 0.22) bacteria. Numbers in parentheses indicate $Log_{10}$ CFU ± STDEV at 2 wk post-infection. Scale is in cm.

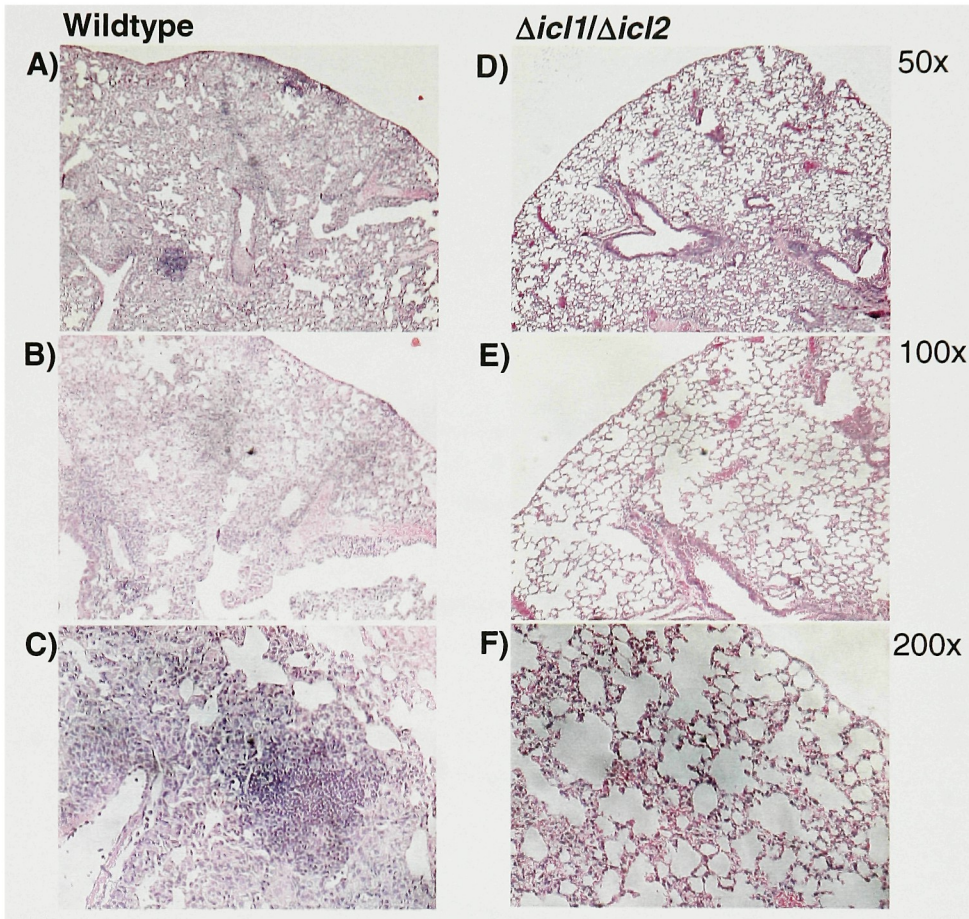


**Fig. 6.16. Lung pathology in mice infected with ICL1-/ICL2-deficient *M. tuberculosis*.**
Lung sections (H&E stain) at 16 wk post-infection in C57BL/6 mice infected with *M. tuberculosis* (~$5x10^5$, i.v.) (**A-C**) wild-type, (**D-F**) Δ*icl1*/Δ*icl2*.

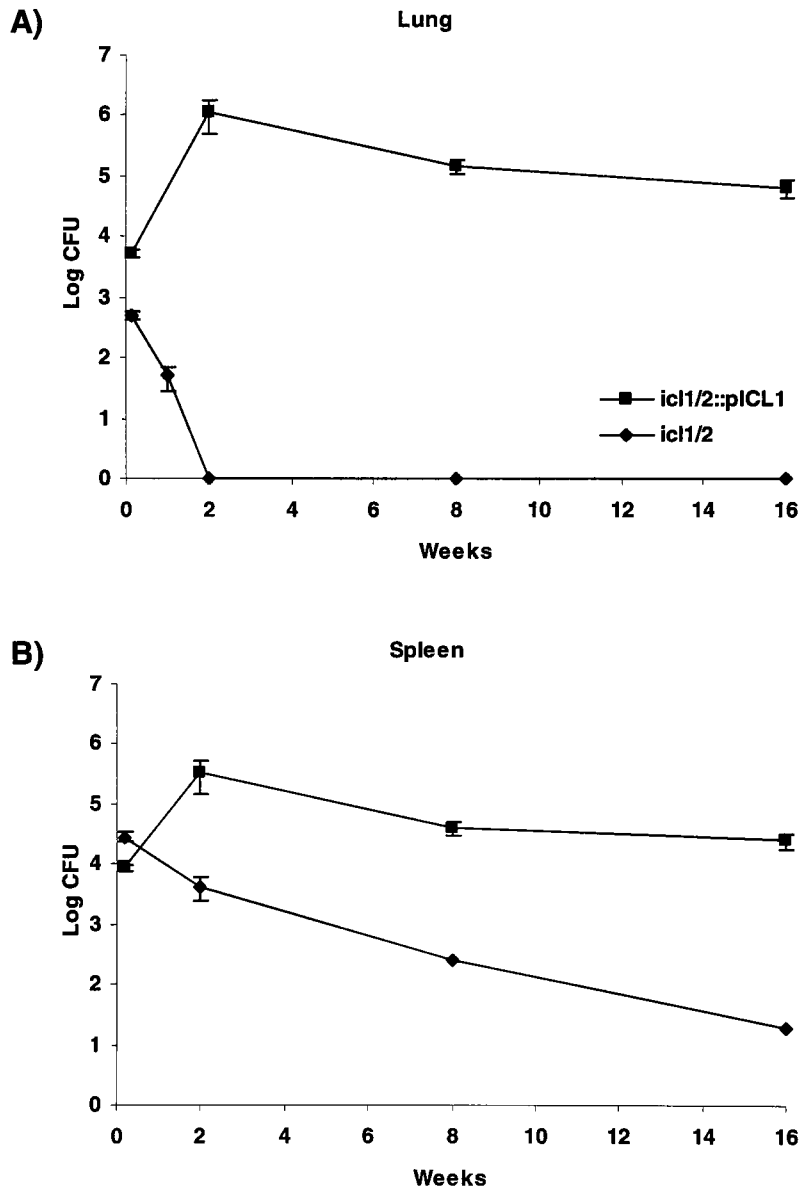


**Fig. 6.17. Growth of ICL1/ICL2-deficient *M. tuberculosis* in mice is restored by complementation with pICL1.** Wild-type (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) Δ*icl1*/Δ*icl2* or Δ*icl1*/Δ*icl2*::pICL1 ($5x10^5$ CFU, i.v.). Bacteria in the (**A**) lung and (**B**) spleen were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4-5).

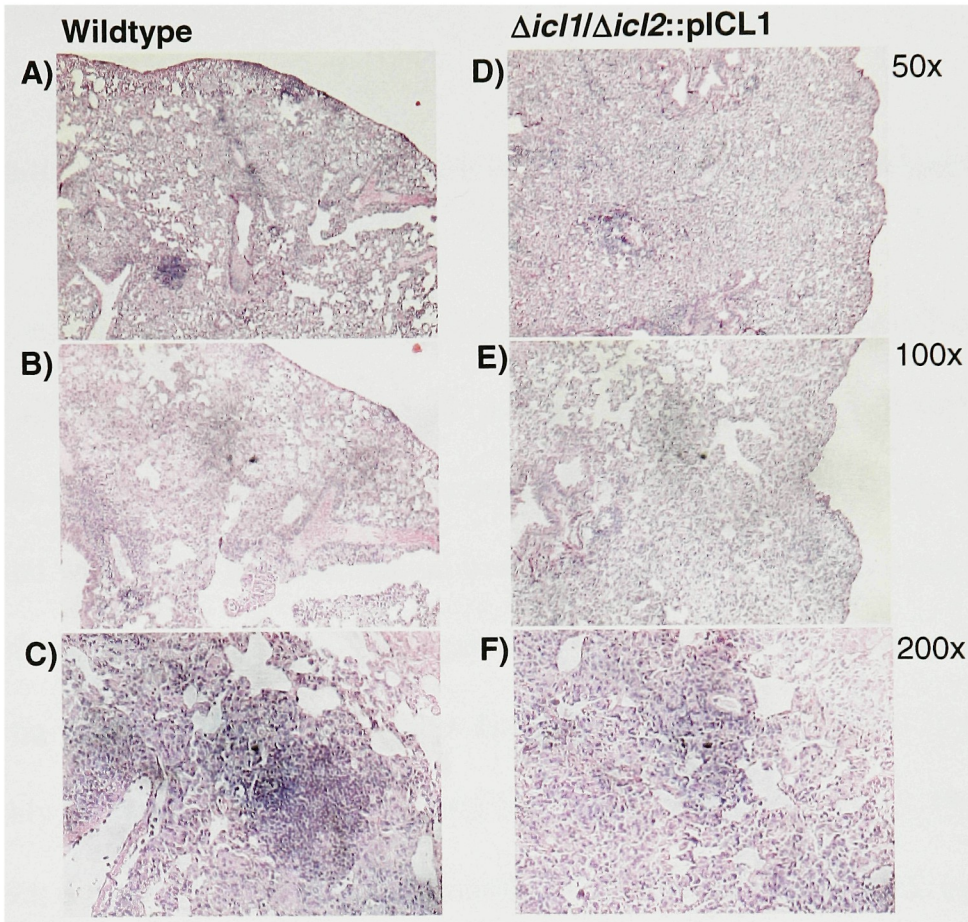


**Fig. 6.18. Lung pathology in mice infected with ICL1-/ICL2-deficient *M. tuberculosis* complemented with ICL1.** Lung sections (H&E stain) at 16 wk post-infection in C57BL/6 mice infected with *M. tuberculosis* (~$5x10^5$, i.v.) (**A-C**) wild-type, (**D-F**) Δ*icl1*/Δ*icl2*::pICL1.

completely, restored as well. An *in vivo* experiment with the Δ*icl1*/Δ*icl2* mutant that had been complemented with a plasmid carrying a wild-type copy of *icl2* was not performed.

### 6.10. Δ*icl1*/Δ*icl2 M. tuberculosis* mutant is avirulent in immune-deficient mice

TB case fatality rates are higher in AIDS patients than in HIV-negative individuals despite appropriate anti-mycobacterial therapy, suggesting that antimicrobials might be less efficacious in immune-compromised hosts (El-Sadr *et al.*, 2001). We asked what effect ICL inactivation would have on growth and survival of *M. tuberculosis* in immuno-deficient hosts by infecting mouse strains lacking interferon-γ (IFN-$\gamma^{-/-}$) or tumor necrosis factor-α receptor 1 (TNF-α-$R1^{-/-}$), which are highly susceptible to *M. tuberculosis* infection (Cooper *et al.*, 1993; Flynn *et al.*, 1993; Flynn *et al.*, 1995). Importantly, we found that Δ*icl1*/Δ*icl2* bacteria were incapable of replicating in IFN-$\gamma^{-/-}$ mice (Fig. 6.19) or TNF-α$RI^{-/-}$ mice (Fig. 6.20). These experiments suggest that targeting ICLs might be effective even in immune-compromised individuals.

Previously we had seen that the attenuation of Δ*icl1* bacteria in C57BL/6 mice was largely reversed in IFN-$\gamma^{-/-}$ mice as well as in mice deficient in other key components of the immune response, suggesting that the *in vivo* requirement for ICL activity might be triggered by T-cell-mediated activation of macrophages

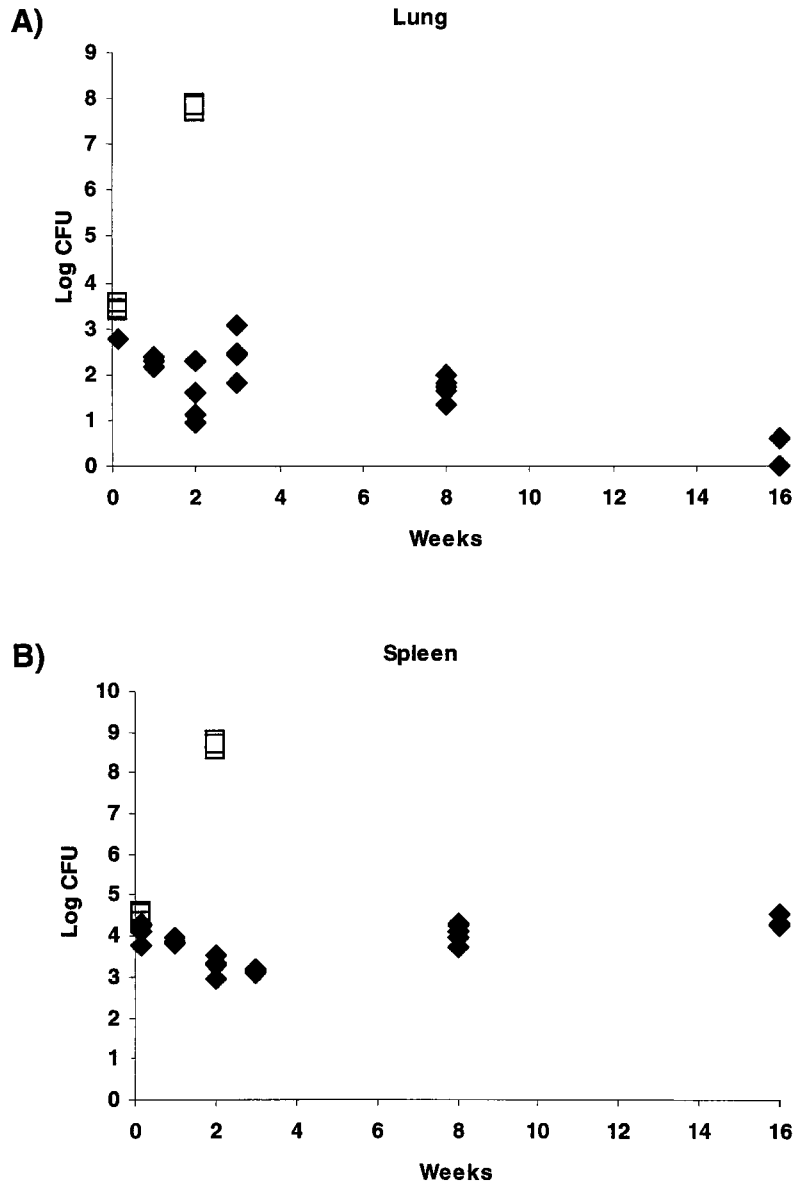


**Fig. 6.19. ICL1/ICL2-deficient *M. tuberculosis* is avirulent in immune-deficient (IFN-γ$^{-/-}$) mice.** IFN-γ-deficient (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1*/Δ*icl2* ($10^6$ CFU, i.v.). Bacteria in the (**A**) lung and (**B**) spleen were enumerated by plating organ homogenates for CFU at indicated time-points. Individual symbols represent CFU in the organs of individual mice (n=3-5).

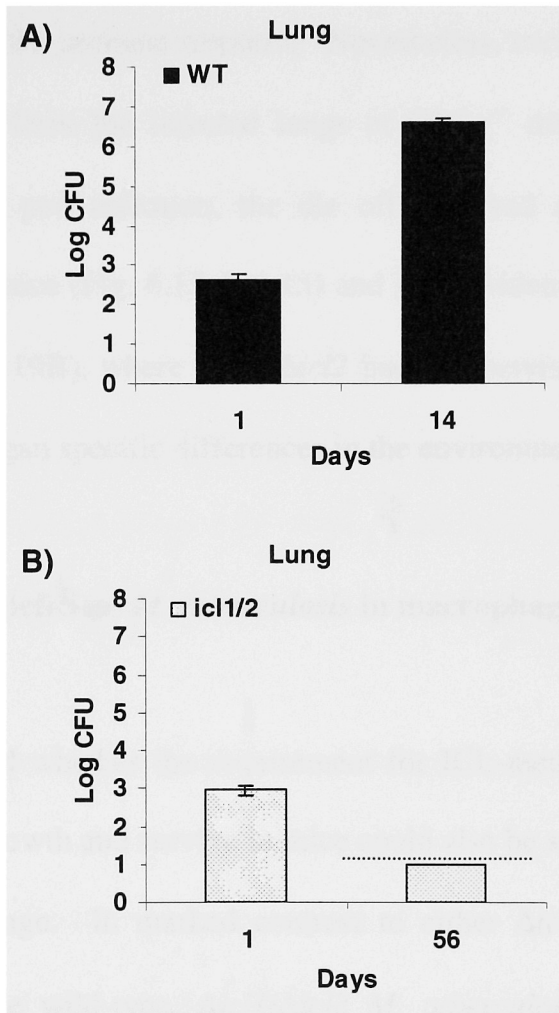


**Fig. 6.20. ICL1-/ICL2-deficient *M. tuberculosis* is avirulent in immune-deficient (TNF-αRI$^{-/-}$) mice.** TNF-αRI-deficient (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) (**A**) wild-type or (**B**) *Δicl1/Δicl2* ($5x10^5$ CFU, i.v.). Bacteria in the lung were enumerated by plating organ homogenates for CFU at indicated time-points. Limit of detection at 56 days: 40 CFU/organ (indicated by dotted line). Error bars indicate STDEV for each group (n = 4).

(Chapter 7; McKinney *et al.*, 2000). Our results with Δ*icl1*/Δ*icl2* mutant bacteria are largely inconsistent with this idea and suggest that dependence of *M. tuberculosis* on ICL activity for early growth *in vivo* is largely independent of these components of the immune response. Nonetheless, even though clearance of Δ*icl1*/Δ*icl2* bacteria from the infected lungs of IFN-$\gamma^{-/-}$ mice (Fig. 6.19A) was observed by wk 16 post-infection, the die off occurred more slowly than in immune-competent mice (Fig. 6.13 & 6.15) and little evidence of it was observed in the spleen (Fig. 6.19B), where Δ*icl1*/Δ*icl2* bacteria persisted without growing, perhaps reflecting organ specific differences in the environment of the infection.

### 6.11. Growth of *icl*-deficient *M. tuberculosis* in macrophages

We next investigated whether the requirement for ICL-mediated metabolism by *M. tuberculosis* to growth and survive in mice could also be seen at the level of the parasitized macrophage. In marked contrast to either Δ*icl1* or Δ*icl2* bacteria, which replicated like wild-type, Δ*icl1*/Δ*icl2* *M. tuberculosis* was incapable of intracellular replication in resting macrophages (Fig. 6.21).

Stimulation by mycobacterial products and certain cytokines activates macrophage effector mechanisms including phagocyte-oxidase-mediated generation of ROI, inducible nitric oxide (NOS2)-mediated generation of RNI, and LRG47-mediated phagolysosomal fusion and acidification (Nathan & Shiloh,

2000; Russell, 2001; MacMicking, 2005). To determine the impact of host cell activation on the survival of ICL1-/ICL2-deficient *M. tuberculosis*, bone marrow-derived macrophages were activated with IFN-γ (100 U mL$^{-}$) prior to and during intracellular infection. While macrophage activation was bacteriostatic to intracellular wild-type *M. tuberculosis* as expected, it was markedly bactericidal to Δ*icl1*/Δ*icl2 M. tuberculosis* (Fig. 6.21). *M. tuberculosis* lacking *icl1*, but not *icl2*-deficient bacteria also were slightly attenuated for growth and survival within macrophages (Fig. 6.21). Complementation of the Δ*icl1*/Δ*icl2* mutant with wild-type copies of *icl1* (Fig. 6.22 A&B) or *icl2* (Fig. 6.22 C&D) restored replication in non-activated macrophages (Fig. 6.22 A&C) and survival in IFN-γ-activated macrophages (Fig. 6.22 B&D).

## 6.12. Growth of *icl*-deficient *M. tuberculosis* in human macrophages

To extend our observations to human cells, human blood monocyte-derived macrophages were infected *ex-vivo*. Similarly to wild-type, Δ*icl1* and Δ*icl2 M. tuberculosis* readily replicated in human primary macrophages as determined by CFU (Fig. 6.23) and 4',6'-diamino-2-phenylindole (DAPI)-staining of intracellular bacteria (Fig. 6.24). In contrast, Δ*icl1*/Δ*icl2* bacteria were incapable of any intracellular replication (Fig. 6.23-6.25). These data suggest that *M. tuberculosis* might depend on the GC during infection of its natural host.

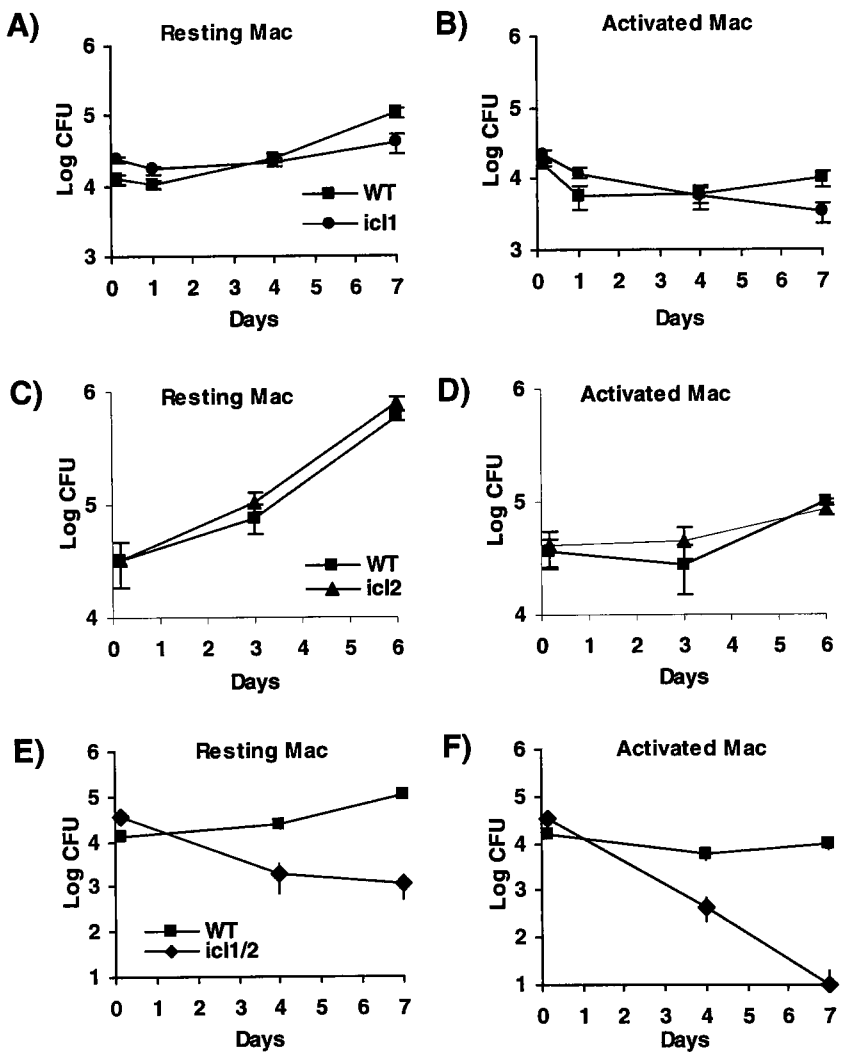


**Fig. 6.21. Replication and survival of *icl* mutants of *M. tuberculosis* in murine macrophages.** Non-activated (**A,C,E**) and IFN-γ-activated (**B,D,F**) macrophages were infected with *M. tuberculosis* wild-type, Δ*icl1*, Δ*icl2* or Δ*icl1*/Δ*icl2* as indicated (MOI 2:1). At the indicated time points, cells were lysed (n = 4 wells) and plated to enumerate CFU. Symbols represent means; error bars indicate STDEV. Results are representative of two experiments.

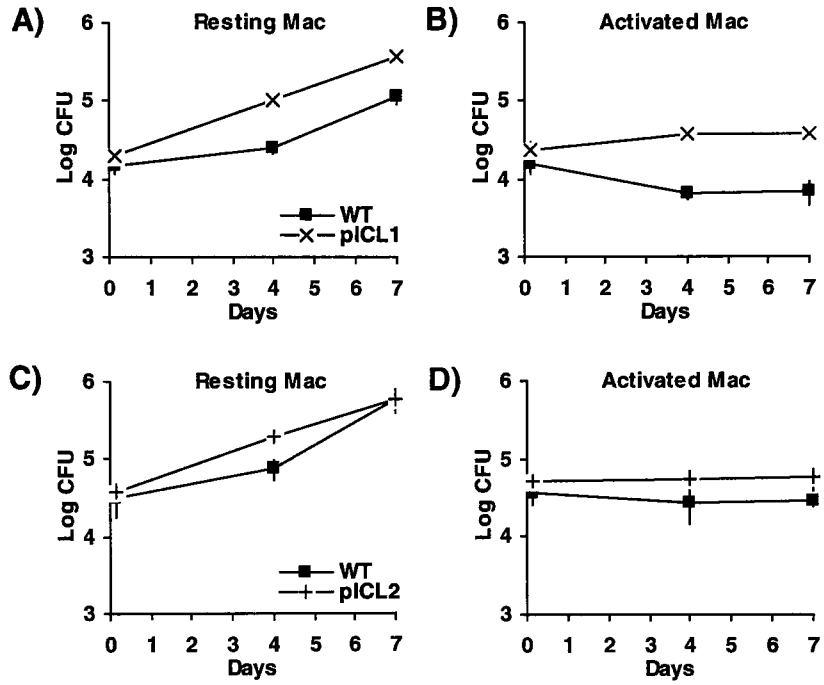


**Fig. 6.22. Replication and survival of *M. tuberculosis* Δ*icl1*/Δ*icl2* complemented with pICL1 or pICL2 in murine macrophages.** Murine bone marrow-derived macrophages, (**A,C,**) non-activated or (**B,D**) IFN-γ-activated were infected with *M. tuberculosis* wild-type, Δ*icl1*/Δ*icl2*::pICL1 or Δ*icl1*/Δ*icl2*::pICL2 strains as indicated (MOI 2:1). At indicated time points, cells were lysed (n = 4 wells) and plated for CFU enumeration. Symbols represent means; error bars indicate STDEV. Results are representative of two experiments.

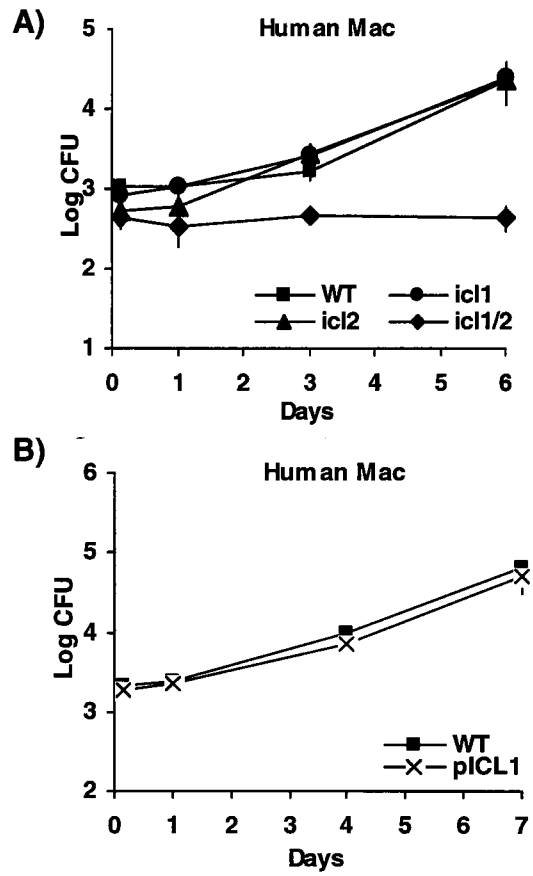


**Fig. 6.23. Replication and survival of *icl M. tuberculosis* mutants in human macrophages. (A-B)** Human blood monocyte-derived macrophages were infected with *M. tuberculosis* wild-type, Δ*icl1*, Δ*icl2*, Δ*icl1*/Δ*icl2* or Δ*icl1*/Δ*icl2*::pICL1 strains as indicated (MOI 1:1). At indicated time points, cells were lysed (n = 4 wells) and plated to enumerate CFU. Symbols represent means; error bars indicate STDEV. Results are representative of two experiments.

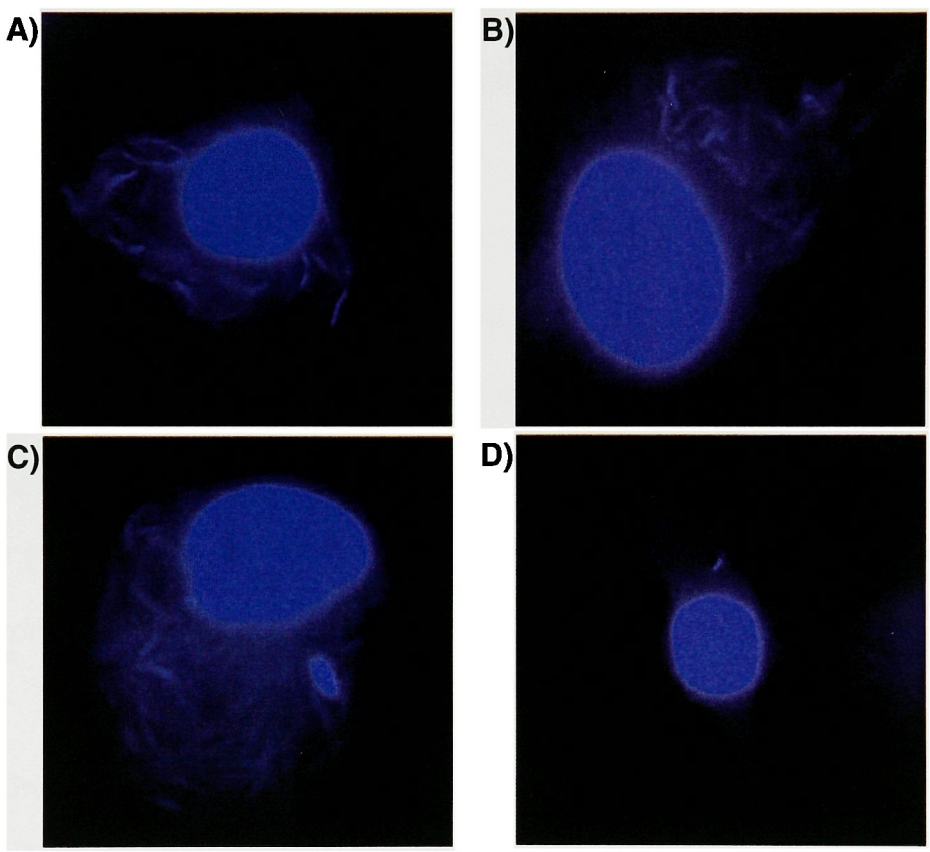


**Fig. 6.24. *M. tuberculosis icl* mutants in human macrophages .** (**A-D**) Human blood monocyte-derived macrophages seeded on round cover slips were infected with *M. tuberculosis* (Erdman) (**A**) wild-type, (**B**) Δ*icl1*, (**C**) Δ*icl2*, (**D**) Δ*icl1*/Δ*icl2*; (MOI 1:1). At 6 days post-infection, bacteria were fixed and stained with DAPI. Results are representative of two experiments.

Together, these observations suggest that fatty acids might be the principle carbon source used by mycobacteria to grow and survive intracellularly.

## 6.13. The ICL inhibitor 3-nitropropionate blocks replication of *M. tuberculosis* on fatty acids and in macrophages

Our demonstration that ICL activity is essential for replication and survival of *M. tuberculosis in vivo* suggested that chemical inhibition of ICL1 and ICL2 might be an effective therapeutic strategy. We therefore tested the ability of the prototype ICL inhibitor 3-nitropropionate (3-NP) (Schloss & Cleland, 1982; Höner zu Bentrup *et al.*, 1999; Sharma *et al.*, 2000) to block growth of *M. tuberculosis* in media containing specific carbon substrates (Fig. 6.25) and in macrophages (Fig. 6.26).

Growth of *M. tuberculosis* on glycerol (Fig. 6.25A) or glucose (Fig. 6.25B) was only slightly affected by the addition of 0.1 mM 3-NP, comparable to the slightly reduced growth kinetics of the Δ*icl1*/Δ*icl2* strain under these conditions. In contrast, 3-NP prevented growth of *M. tuberculosis* on fatty acids (Fig. 6.25C-F). When *M. tuberculosis* was grown in murine macrophages (Fig. 6.26), addition of 3-NP resulted in inhibition of intracellular replication (58-fold reduction in CFU at day 7) (Fig. 6.26B), comparable to the growth inhibition resulting from deletion of *icl1* and *icl2* (54-fold reduction in CFU at day 7) (Fig. 6.26A). Inhibition of mycobacterial growth in macrophages by 3-NP was dose-dependent

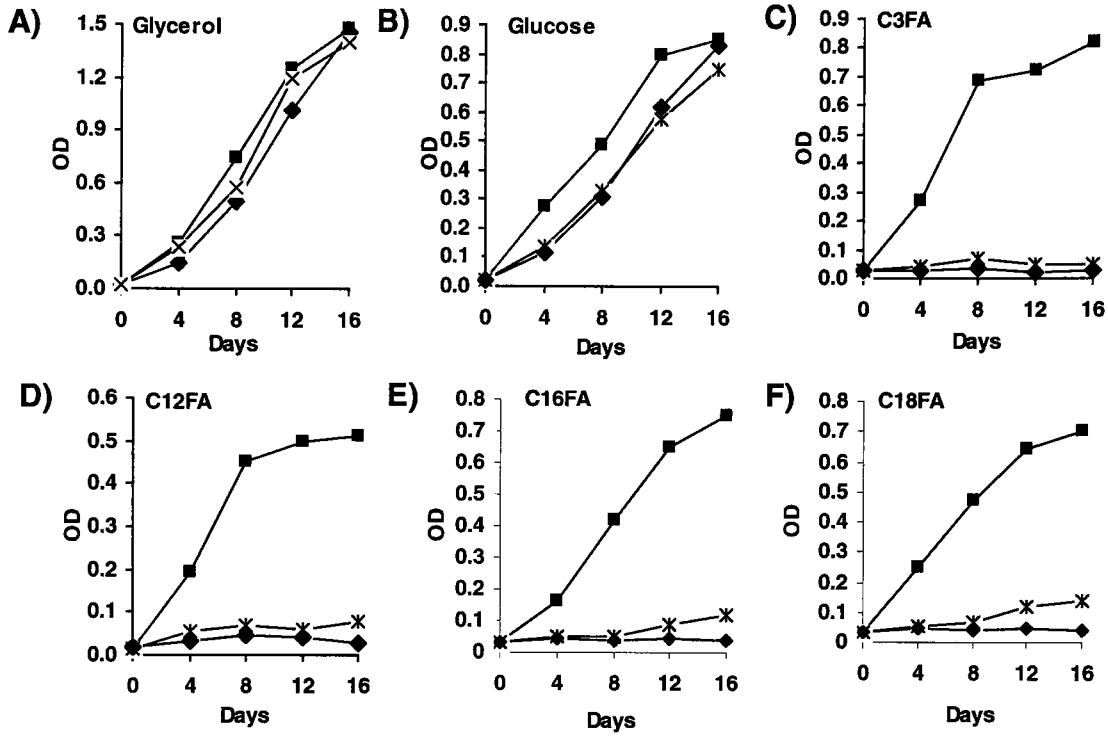


**Fig. 6.25. Chemical inhibition of ICL blocks growth of *M. tuberculosis* on fatty acids.** Wild-type *M. tuberculosis* (Erdman) bacteria were cultured in liquid media containing (0.1% w/v) (**A**) glycerol; (**B**) glucose; (**C**) propionate (C3FA); (**D**) polyoxyethylene sorbitan mono-laurate (C12FA); (**E**) polyoxyethylene sorbitan mono-palmitate (C16FA); (**F**) polyoxyethylene sorbitan mono-stearate (C18FA) with (asterisks) or without (filled squares) the ICL inhibitor 3-NP (0.1 mM). Growth was monitored by measuring $OD_{600}$ nm at the indicated time points. Wild-type bacteria: squares; ICL1/ICL2-deficient bacteria: diamonds; ICL1/ICL2-deficient bacteria complemented with episomal plasmid carrying ICL1: asterisks. Results are representative of three experiments.

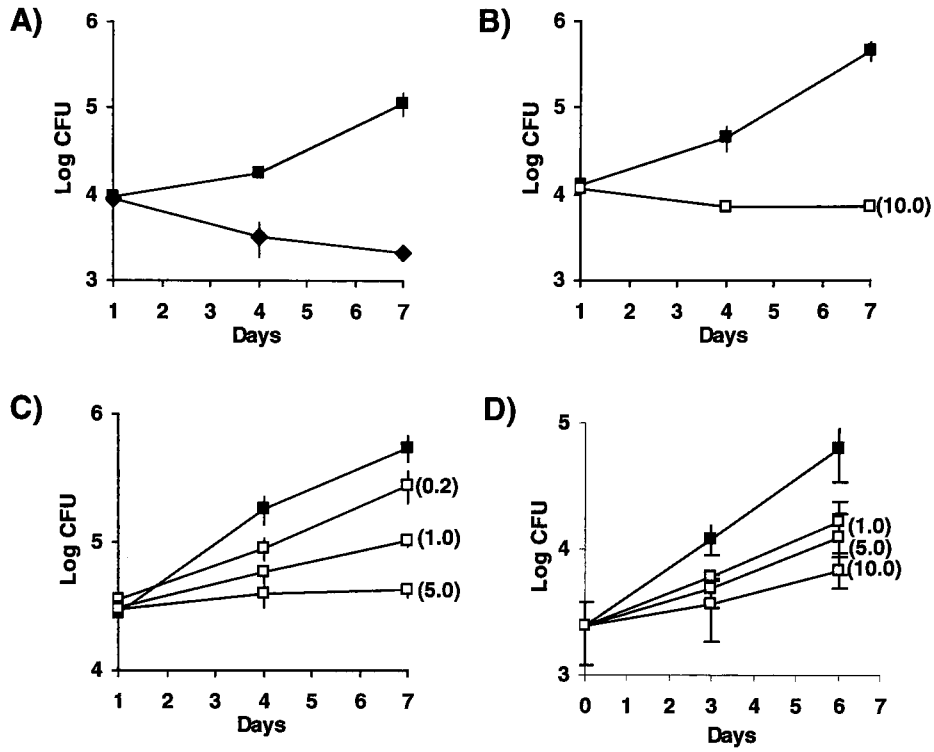


**Fig. 6.26. Chemical inhibition of ICL blocks intracellular growth of *M. tuberculosis* in macrophages.** Growth in murine bone marrow-derived macrophages (**A-C**) and in human blood monocyte-derived macrophages (**D**). Macrophages were infected with wild-type (squares) without (filled), with (empty) 3-NP, or Δ*icl1*/Δ*icl2* mutant *M. tuberculosis* (diamonds) After 4 hr, cells were washed to remove non-cell-associated bacteria and incubated in fresh medium with or without 3-NP (numbers in parentheses indicate mM concentrations). At the indicated time points cells were lysed (n = 4 wells) and plated to enumerate CFU. Symbols represent means; error bars indicate STDEV. Results are representative of two experiments.

(Fig. 6.26C). Growth of *M. tuberculosis* in human macrophages was also inhibited by 3-NP in a dose-dependent manner (Fig. 6.26D), although inhibition was somewhat less effective in human as compared to murine macrophages, perhaps due to differences in the uptake or metabolism of the compound.

Consistent with our interpretation that growth inhibition by 3-NP was due to inhibition of ICL *per se*, bacteria that over-expressed ICL1 from a plasmid (~6 fold higher expression under *in vitro*-inducing growth conditions) were less sensitive to growth inhibition by 3-NP than wild-type bacteria *in vitro* (Fig. 6.27A&B) and *ex-vivo* during growth in murine macrophages (Fig. 6.27C). These results provide a proof of concept for the potential development of ICL inhibitors as antimycobacterial drugs.

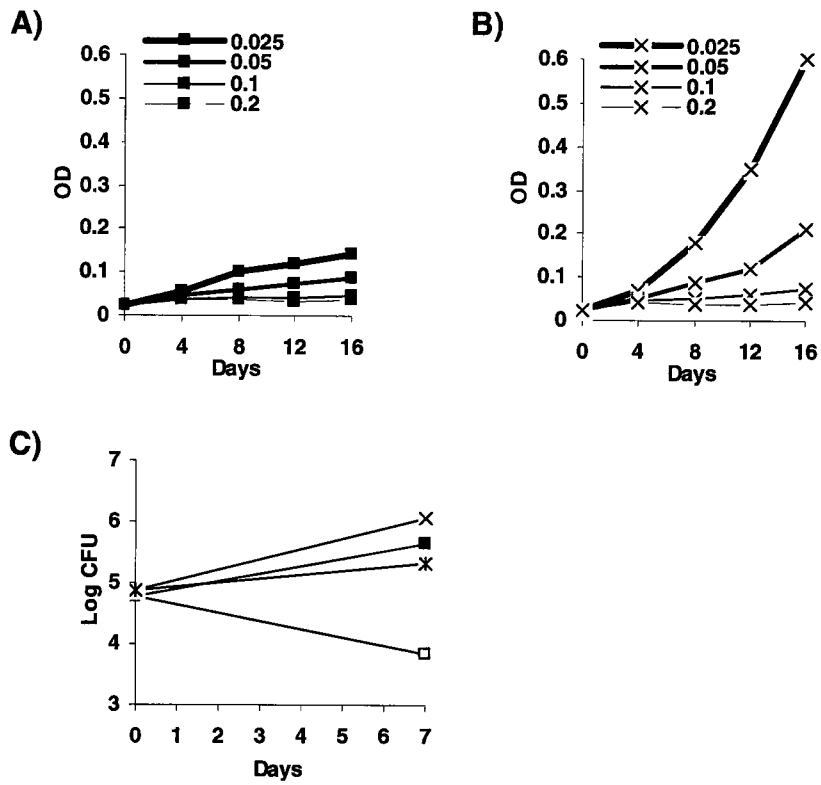


**Fig. 6.27. *M. tuberculosis* strain over-expressing ICL is resistant to growth inhibition by 3-nitropropionate.** *M. tuberculosis* (**A**) wild-type or (**B**) ICL1-over-expressing bacteria were grown in liquid media containing (0.1% w/v) propionate (C3FA) in the presence of increasing concentrations of 3-NP (as indicated, numbers in mM). Growth was monitored by measuring $OD_{600}$ nm at the indicated time points. (**C**) Bone marrow-derived mouse macrophages were infected with *M. tuberculosis* wild-type without (filled squares) or with (empty squares) 3-NP (10 mM) or with an ICL1-over-expressing strain without (x) or with (*) 3-NP (10 mM). (A& B) results are representative of two experiments. (C) results of one experiment.

# CHAPTER 7

## A link between the Immune Response, Bacterial Metabolism, and Persistence of *M. tuberculosis* in the Host

### INTRODUCTION

The immune system has evolved a number of strategies to curtail the replication and survival of intracellular pathogens. One important strategy involves a remodeling of the infected area so as to isolate it from healthy tissue as it occurs during granuloma formation. As this occurs, the host cells that harbor the infecting bacteria are also altered (activated) or even killed in an effort to limit the growth of the bacteria within them. Activation of effector mechanisms within cells involves the production of noxious molecules such as reactive oxygen and nitrogen intermediates (ROI and RNI), which are thought to directly block or kill the infecting bacteria.

In dealing with *M. tuberculosis,* our immune system engages a number of mechanisms of defense (Flynn and Chan, 2001; Nathan & Ehrt, 2003; MacMicking 2005). In addition to the production of ROI and RNI, during activation of infected cells other changes in the chemical composition of the intracellular space the pathogen inhabits take place. Macrophage activation via

interferon gamma (IFN-γ) leads to increased phagolysosomal fusion and acidification, which at least in part is mediated by IFN-γ-induced GTPase LRG47 (MacMicking *et al.,* 2003; MacMicking, 2005). Although some evidence indicates that the mycobacterial vacuole in macrophages is devoid of certain amino acids (Smith *et al.,* 2001; Parish & Stoker, 2002), and limiting in some essential elements such as iron and magnesium (Buchmeier *et al.,* 2000; Rodriguez & Smith, 2003), little is known about the carbon substrate vacuolar contents or how the concentrations of any of these elements or carbon substrates might be influenced by immune-mediated activation of the macrophage.

We showed previously that *M. tuberculosis* required ICL to grow and survive in mice from the onset of the infection, suggesting that the ability to metabolize C2 substrates during infection is essential for this pathogen. Our data also showed that either of the two isoenzymes could provide enough activity for growth and survival during the acute phase of the infection (~wk 0-2). However, during the persistent phase of the infection, Δ*icl1* mutant bacteria were unable to maintain wild-type levels of bacterial loads in the lung, while Δ*icl2* mutant bacteria behaved like wild-type (Chapter 6; McKinney *et al.,* 2000; Muñoz-Elías & McKinney, 2005). Thus, the functional overlap between the two homologs is only partial: loss of *icl2* is fully compensated by *icl1* during both acute and persistent phases of the infection, while loss of *icl1* is only fully compensated by *icl2* during the acute phase of the infection.

The fact that the *in vivo* phenotype of the Δ*icl1* mutant coincided with the time at which acquired immunity to *M. tuberculosis* normally emerges in the mouse model of tuberculosis suggested a causal link. Can acquired immunity bring about changes in the infection milieu that prompt *M. tuberculosis* to rely on ICL to a greater degree during the persistent phase of the infection than during the acute phase? To shed light on this question, we carried out a series of experiments analyzing the ability of ICL1-deficient *M. tuberculosis* to grow in mice deficient in several known anti-tubercular pathways (Flynn & Chan, 2001; North & Jung, 2004; MacMicking, 2005).

Here we show that the host immune response is largely responsible for the attenuation phenotype of ICL1-deficient bacteria during the chronic phase of the infection in immune-competent mice. While T cells, IFN-γ, and TNF-α played a role in bringing about this phenotype, the downstream effectors inducible nitric oxide (NOS2) and LRG47 were only partially involved, and phagocyte oxidase (PHOX) was completely dispensable. Our results suggest that in an immune-competent host, the emergence of acquired immunity might cause *M. tuberculosis* to increase its reliance on the GC, implicating carbon restriction as an antimycobacterial mechanism specific to persistence.

## RESULTS

### 7.1. *icl1*-deficient *M. tuberculosis* regains virulence in T cell-deficient mice

The adaptive immune response to *M. tuberculosis* infection is largely mediated by antigen-specific $CD4^+$ and $CD8^+$ T cells, which exert their protective effects via secretion of the macrophage-activating cytokine IFN-γ (Flynn & Chan, 2001; North & Jung, 2004). Thus, we started evaluating our hypothesis by testing the ability of Δ*icl1* bacteria to grow in Rag1-deficient mice (Mombaerts *et al.,* 1992), which were expected to be highly susceptible to *M. tuberculosis* infection.

The attenuation of the Δ*icl1* mutant seen in wild-type mice was largely reversed in $Rag1^{-/-}$ mice. Δ*icl1* mutant bacteria replicated similarly to wild-type bacteria, and were almost as virulent (Fig. 7.1) (mean time-to-death for $Rag1^{-/-}$ mice infected with Δ*icl1* mutant and wild-type bacteria were 34 and 19 days, respectively). Thus, the persistence-specific attenuation of Δ*icl1* mutant bacteria appears to be driven by an interaction with components of the host's acquired immune response.

## 7.2. IFN-γ-dependent mechanisms contribute to killing of *icl1*-deficient bacteria

Studies using mice deficient in $CD4^+$ and $CD8^+$ T cells have established that the contribution of $CD4^+$ T cells to the protective immune response to *M. tuberculosis* infection is more crucial than that of $CD8^+$ T cells (Caruso *et al.,* 1999; Serbina &

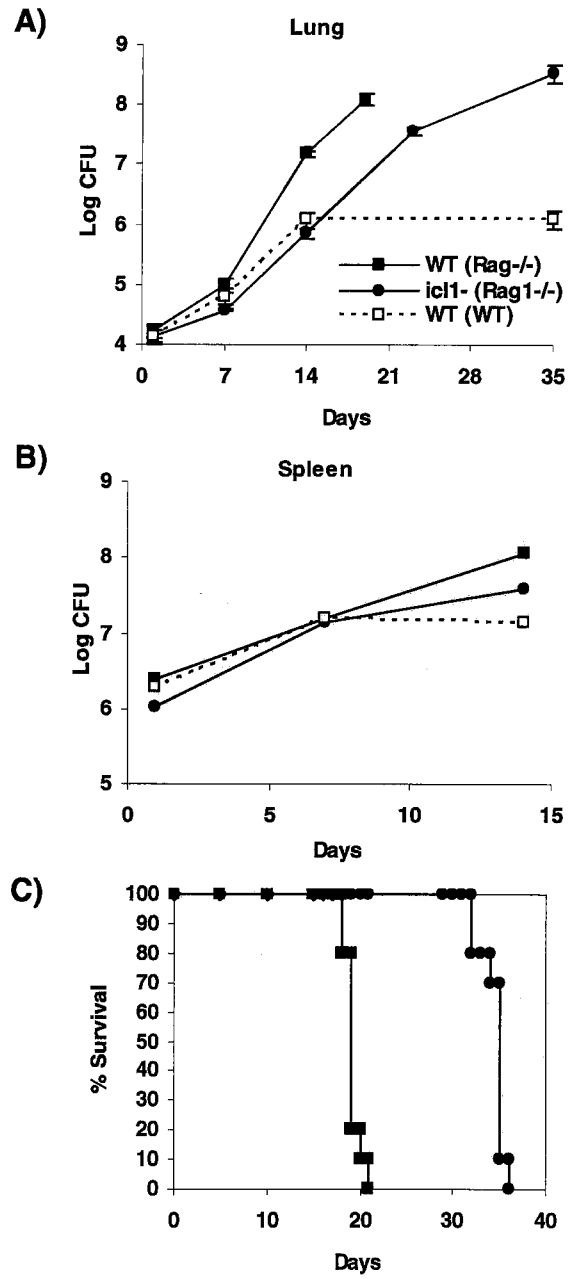


**Fig. 7.1. ICL1-deficient *M. tuberculosis* regains virulent in T cell-deficient mice.** (**A-B**) $Rag1^{-/-}$ mice are highly susceptible to *M. tuberculosis* infection. Wild-type mice (dotted lines, empty squares) and $Rag1^{-/-}$ mice (solid lines/filled squares) were infected with *M. tuberculosis* (Erdman, ~5 x $10^6$ CFU, i.v.) . (**A-C**). Δ*icl1* mutant reverts to virulence in $Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice were infected with wild-type (solid lines/filled squares) or Δ*icl1* mutant bacteria (solid lines/filled circles). Bacteria in the (**A**) lung and (**B**) spleen were enumerated by plating for CFU at indicated time-points. Error bars indicate STDEV (n=4). **C**) Survival of $Rag1^{-/-}$ mice infected with wild-type (squares) or Δ*icl1* mutant bacteria (circles) (n=7).

Flynn, 1999; Serbina *et al.,* 2001). $CD4^+$ T cells contribute to anti-tubercular immunity largely through the production of IFN-γ, which turns on several anti-mycobacterial defense mechanisms (Flynn & Chan, 2001; North & Jung, 2004; MacMicking, 2005). Therefore, we next asked whether Δ*icl1* bacteira could also "revert" to virulence in mice lacking IFN-γ.

Similarly to our observations in $Rag1^{-/-}$ mice, Δ*icl1* bacteria showed a clear reversal toward virulence in IFN-$\gamma^{-/-}$ mice, growing in all organs analyzed similarly to wild-type bacteria (Fig. 7.2A-C), and killing these mice by ~33 days post infection (Fig. 7.2D). As expected, IFN-$\gamma^{-/-}$ mice infected with wild-type *M. tuberculosis* succumbed by ~17 days post-infection (Fig. 7.2D).

The reversal of the Δ*icl1* mutant we saw in both $Rag1^{-/-}$ and IFN-$\gamma^{-/-}$ (Fig. 7.1&7.2) was not complete. While wild-type bacteria grew unabated in the organs of both of these mice, the Δ*icl1* mutant noticeably slowed down at about 7-10 days post-infection (Fig. 7.1&7.2). This difference was underscored by the delayed kinetics with which mice infected with Δ*icl1* bacteria succumbed to infection compared to those infected with wild-type bacteria (Fig. 7.1C&7.2D). These observations suggest that other host factors which are T cell/IFN-γ-independent, likely contribute to the attenuation of Δ*icl1 M. tuberculosis* in an immune-competent wild-type host.

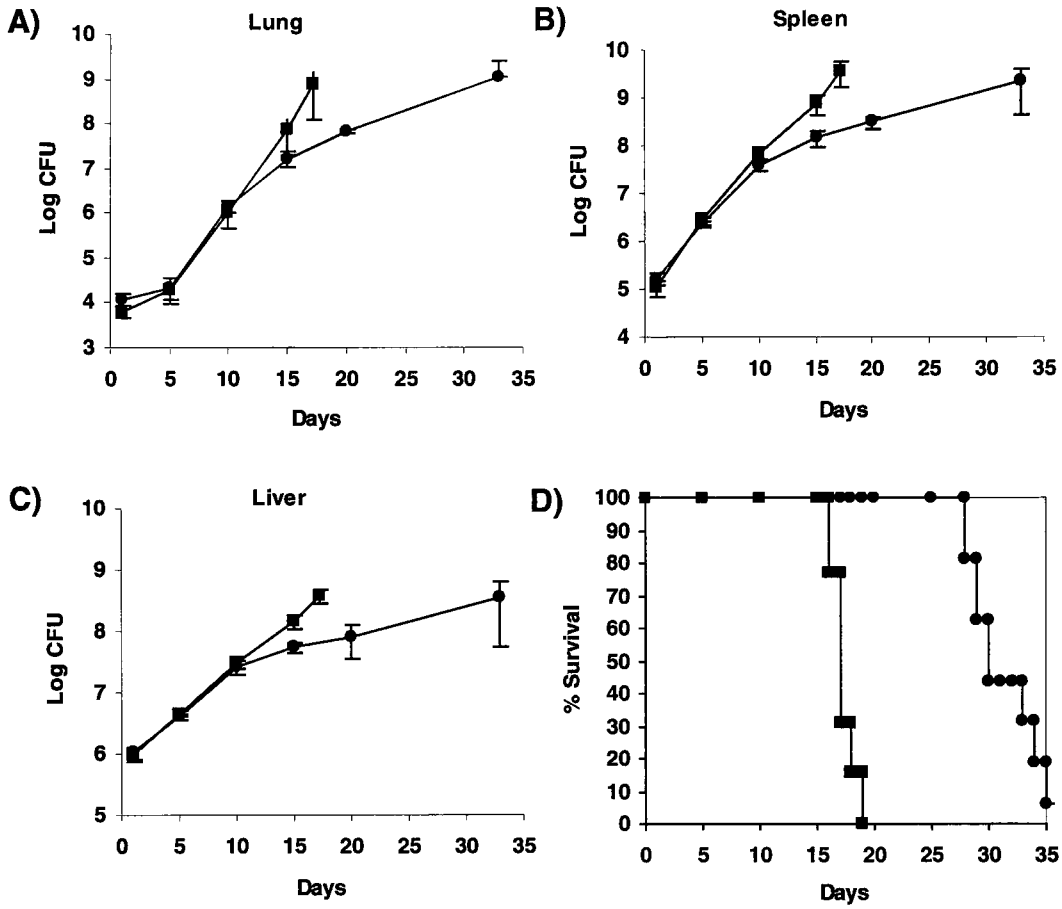


**Fig. 7.2. ICL1-deficient *M. tuberculosis* regains virulence in IFN-γ$^{-/-}$ mice.** IFN-γ$^{-/-}$ mice (C57Bl/6) were infected with *M. tuberculosis* (Erdman) wild-type (squares) or Δ*icl1* (circles) bacteria (0.5-1 x $10^6$ CFU, i.v.). Bacteria in the (**A**) lung, (**B**) spleen, and (**C**) liver were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4). **D**) Survival (n=10).

## 7.3. Immune effector mechanisms that contribute to the control of *icl1*-deficient *M. tuberculosis*

We next turned our attention to effector mechanisms downstream of IFN-γ that could be responsible for the persistence-specific attenuation of ICL1-deficient *M. tuberculosis* observed in wild-type mice (McKinney *et al.,* 2000; Muñoz-Elías & McKinney, 2005). IFN-γ induces expression of $Ca^{++}$-independent nitric oxide synthase (NOS2) through the JAK/STAT pathway (Xie *et al.,* 1992; Nathan & Xie, 1994; Darnell, 1997). Nitric oxide and other reactive nitrogen intermediates (RNI) are key components in the host's anti-tubercular arsenal (MacMicking *et al.,* 1997; Nathan & Shiloh, 2000). To address the contribution of this host defense pathway to the attenuation of Δ*icl1 M. tuberculosis*, we infected $NOS2^{-/-}$ mice with wild-type or Δ*icl1* bacteria and monitored bacterial growth and survival in their lungs. Unexpectedly, $NOS2^{-/-}$ mice were capable of stabilizing Δ*icl1* mutant bacterial loads in the lungs at about ~$10^6$ CFU from day 20-70 post-infection, only losing control of the infection after ~112 days (Fig. 7.3A-C). In contrast, wild-type bacteria multiplied to about ~$10^9$ CFU per lung in only ~40 days in these mice, in accordance with previous results (MacMicking *et al.,* 1997; Scanga *et al.,* 2001). Moreover, while $NOS2^{-/-}$ mice infected with Δ*icl1* mutant bacteria survived up to ~ 135 days, those infected with wild-type bacteria succumbed within ~40 days (Fig. 7.3D).

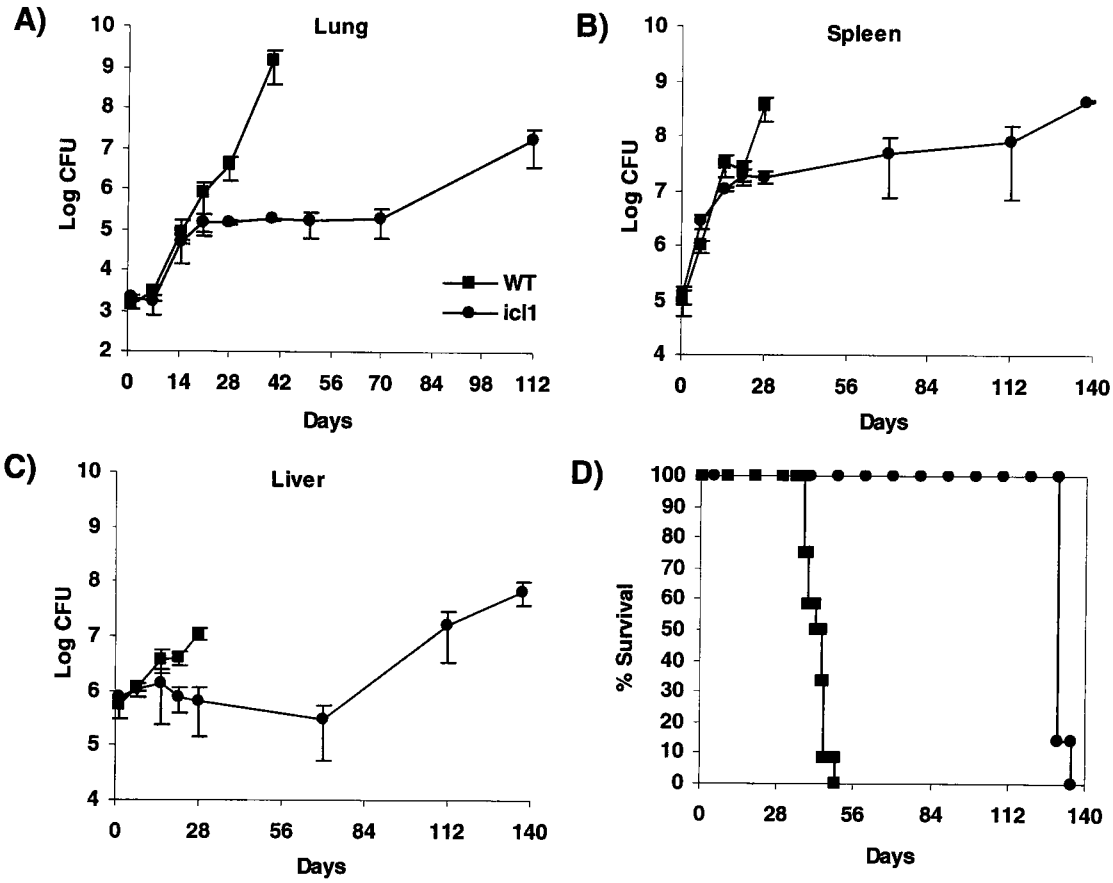


**Fig. 7.3. Nitric oxide synthase contributes to the attenuation of ICL1-deficient *M. tuberculosis*.** (**A-D**) NOS2$^{-/-}$ (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1* ($5x10^5$ CFU, i.v.). Bacteria in the (**A**) lung, (**B**) spleen, and (**C**) liver were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4). (**D**) Survival (n=8).

The fact that Δ*icl1* mutant bacteria did revert to some degree in NOS2$^{-/-}$ mice – a slight increase in bacterial loads in both lungs and spleens was observed- suggests that NOS2, presumably via RNI production, does contribute to the phenotype of the Δ*icl1* mutant during the persistence phase of the infection in wild-type mice. However, that the Δ*icl1* mutant's reversal was not nearly as dramatic as that observed in IFN-γ-deficient mice suggests that other IFN-γ-dependent mechanisms exist that are capable of inhibiting the growth of the mutant, but do not impair wild-type bacteria to the same degree.

Another IFN-γ-dependent mechanism that plays a key role in the control of *M. tuberculosis* infection is the LRG47 GTPase-mediated increase in lysosomal fusion and acidification of the *M. tuberculosis* vacuole (MacMicking *et al.,* 2003). When LRG47$^{-/-}$ mice were infected with the Δ*icl1* mutant, some reversal of the mutant's persistence defect was observed. However, similarly to NOS2$^{-/-}$ mice, LRG47$^{-/-}$ mice were also capable of controlling the growth of the Δ*icl1* mutant and, in fact, did so more efficiently than NOS2$^{-/-}$ mice (N. Dhar & J.D. MacMicking, personal communication).

Mice lacking phagocyte oxidase (PHOX) are highly susceptible to infections by several fungi and bacteria including intracellular *Salmonellae* spp (Nathan & Shiloh, 2000). However, *M. tuberculosis* is relatively resistant to ROI *ex vivo* (Manca *et al.,* 1999), and gp91$^{(PHOX-/-)}$ mice control *M. tuberculosis* infection as efficiently as their wild-type counterparts (Nathan & Shiloh, 2000; Ng

*et al.,* 2004). Nevertheless, we considered it possible that lower ICL activity in the ICL1-deficient strain might interfere with the bacteria's ability to generate effective defenses against ROI, and make them more susceptible to a host-mediated mechanism to which wild-type bacteria are normally resistant. To test this hypothesis, we monitored the growth of Δ*icl1* mutant bacteria in gp91$^{(PHOX-/-)}$ mice.

Interestingly, we failed to see any reversal of the Δ*icl1* mutant's attenuation, and instead observed a slight increase in its attenuation. PHOX-deficient mice killed Δ*icl1* mutant bacteria more efficiently than wild-type mice (Fig. 7.4A-C). The bacterial requirement for ICL1 in these mice might even appear earlier on because only 2 wks into the infection, the lung bacterial loads of the Δ*icl1* mutant were about 10X lower than wild-type bacteria (Fig. 7.4A); further, the Δ*icl1* mutant only replicated slightly in the spleen (Fig. 7.4B) and not at all in the liver (Fig. 7.4C) during the acute phase. These data argue that the phenotype of the Δ*icl1* mutant in wild-type mice is unlikely to be driven by ROI.

## 7.4. TNF-αRI-mediated immunity contributes to the attenuation of *icl1*-deficient bacteria

The cytokine tumor necrosis factor alpha (TNF-α) is a major player in the immune response to *M. tuberculosis* infection (Flynn *et al.,* 1995; Adams *et al.,* 1995;

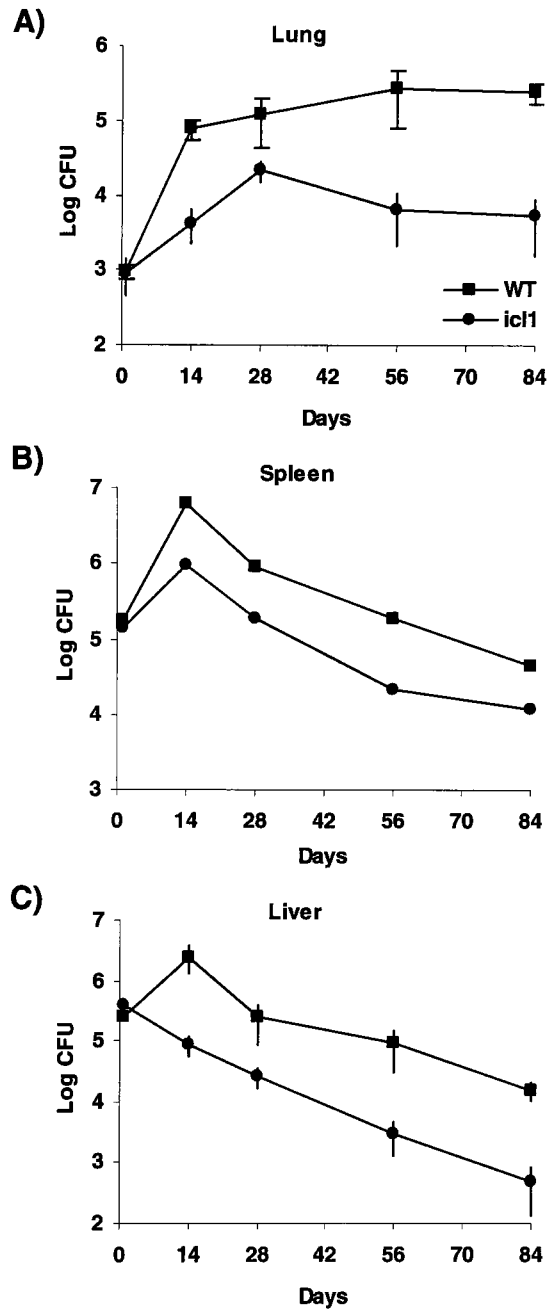


**Fig. 7.4. Phagocyte oxidase is dispensable for control of ICL1-deficient *M. tuberculosis*.** (**A-C**) gp91$^{phox-/-}$ (C57Bl/6) mice were infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1* bacteria (~5x10$^5$ CFU, i.v.). Bacteria in the (**A**) lung, (**B**) spleen, and (**C**) liver were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4).

Flynn & Chan, 2001). Together with IFN-γ, TNF-α synergistically activates macrophages inducing production of higher levels of RNI and ROI, which results in higher anti-mycobactericidal activity (Flesch & Kaufman, 1990; Chan *et al.,* 1992; Flynn & Chan, 2001). However, TNF-α exerts its antimycobacterial effects through both iNOS-dependent and independent mechanisms (Flynn *et al.,* 1995; Adams *et al.,* 1995; Bean *et al.,* 1999; Ehlers *et al.,* 1999; Bekker *et al.,* 2001). Since we had found that NOS2 and LRG47 appeared to be only partially responsible for the attenuation of the Δ*icl1* mutant, we next asked whether TNF-α-mediated mechanisms might be involved.

To this end, we evaluated the ability of the Δ*icl1* mutant to grow in mice lacking TNF-αRI. Signaling through this receptor is thought to mediate most of the anti-tubercular effects attributed to this cytokine (Algood *et al.,* 2003). Surprisingly, we found that the attenuation of Δ*icl1* mutant bacteria was almost completely reversed in these mice (Fig. 7.5A-C). Growth of the Δ*icl1* mutant was essentially indistinguishable from that of wild-type bacteria, and only a slight delay in death in the mice infected with the Δ*icl1* mutant compared to wild-type bacteria was observed (Fig. 7.5D). This experiment was carried out with slightly higher dose of bacteria than previous ones (~2-3 x $10^6$ CFU/mouse versus ~ 5 to 10 x $10^5$ CFU/mouse by i.v.), thus it is possible that the high number of bacteria overwhelmed the immune response and prevented discerning any residual attenuation of the Δ*icl1* mutant in the experiment shown in Fig. 7.5. In fact, in a

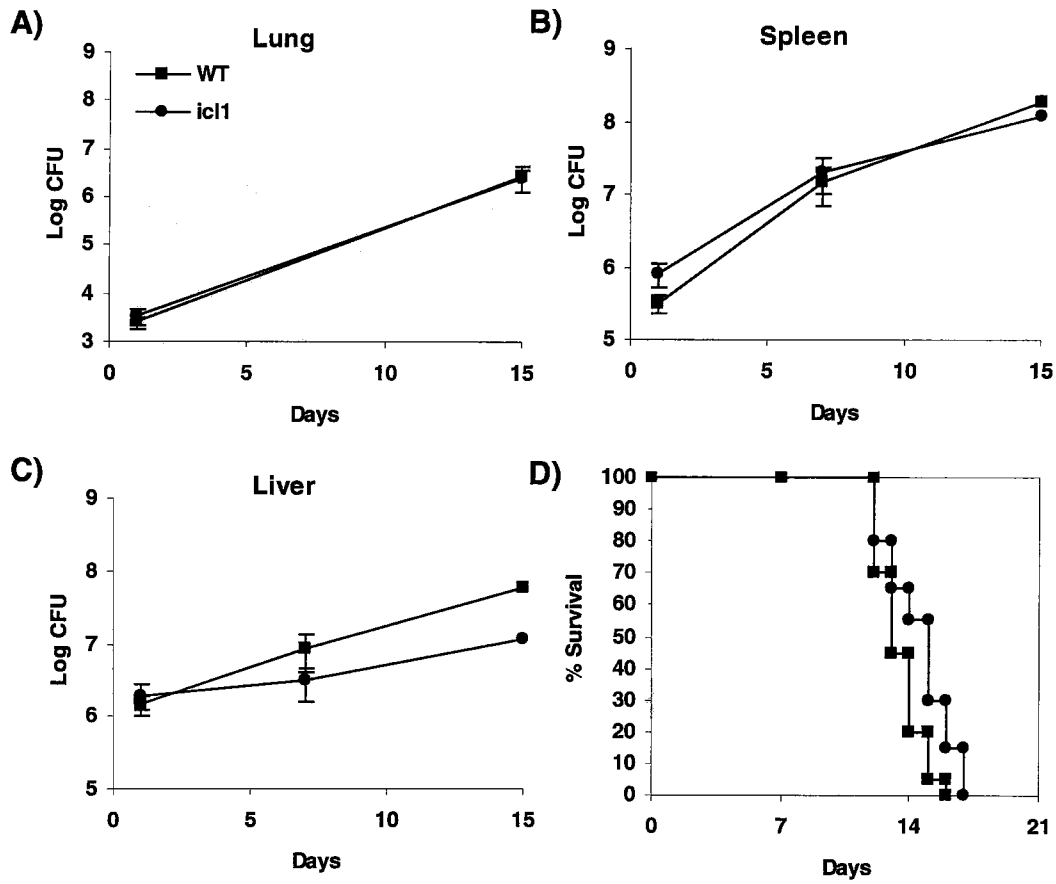


**Fig. 7.5. TNF-αRI-deficient mice fail to control the growth of ICL1-deficient *M. tuberculosis*.** (**A-D**) TNF-αRI(p55$^{-/-}$) (C57BL/6) mice were infected with *M. tuberculosis* (Erdman) wild-type or Δ*icl1* (~2x10$^6$ CFU, i.v.). Bacteria in the (**A**) lung, (**B**) spleen, (**C**) liver were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4). (**D**) Survival (n=10).

second experiment in which bacteria were administered by aerosol (~100 CFU), the reversal of the phenotype of the Δ*icl1* mutant was less dramatic (not shown) and was more reminiscent of the pattern of growth we saw in IFN-γ-deficient mice (Fig. 7.2 & Dahr & MacMicking, personal communication). Nonetheless, these data together suggest that TNF-α is a major factor contributing to the immune response that causes *M. tuberculosis* to rely on ICL1-mediated metabolism during infection.

# CHAPTER 8

## Assessing the Potential of Inhibiting ICL1 in Enhancing Chemotherapy of Tuberculosis in Mice

### INTRODUCTION

During the persistent phase of the infection in the mouse model of chronic tuberculosis (TB) bacilli are thought to undergo limited replication (Rees and Hart, 1961; Muñoz-Elías *et al.,* 2005; Chapter 5). Not surprisingly, drugs like isoniazid (INH) that preferentially kill replicating bacteria are relatively less efficient when administered to infected mice late rather than early in the infection (McCune *et al.,* 1956). Nonetheless, even if *M. tuberculosis* is only replicating slowly during infection, it must maintain some metabolic activity in order to support basic cell functions, and to counteract the constant onslaught of host immune effectors. The type of metabolism characterizing *M. tuberculosis* during persistence might be different from that in periods of rapid bacterial growth, such as during the acute phase of the infection.

Largely based on the fact that antibiotics have dramatically different anti-tubercular effects depending on when during the infection and in what combination they are given when treating TB, it has been proposed that the

physiology/metabolism of the tubercle bacillus in the lung might be radically different from that of bacteria growing *in vitro* and that different populations of bacteria might exist, which differ metabolically/physiologically from each other as well (Mitchison, 1979, 1980). These phenotypic changes might contribute to the ability of drug-susceptible bacteria to persist *in vivo* despite adequate chemotherapy, a phenomenon known as *in vivo* drug tolerance and a major obstacle to the eradication of TB (McDermott, 1958).

Based on our finding that ICL1-deficient bacteria are specifically attenuated during persistence in immuno-competent mice (McKinney *et al.,* 2000; Chapters 6&7), and that expression of *icl1* is highly induced throughout the infection in mouse lungs (Timm *et al.,* 2003; Fig. 6.7), we hypothesized that ICL1-mediated metabolism might be particularly relevant to the persistent phase of the infection. Thus, we set out to test the effect that lack of ICL1 alone would have on the treatment of chronically infected mice, and on the ability of ICL1-deficient bacteria to reactivate from "latency" in a chemotherapy-induced model of latency in mice.

## RESULTS

### 8.1. An additive anti-mycobacterial effect between INH and lack of ICL1

The early anti-tubercular bactericidal activity of isoniazid (INH) is attributed to its efficacy in killing actively multiplying bacilli (McKinney, 2000; Mitchison, 2004). It is thought that INH's initial rate of killing subsequently declines because residual organisms replicate and metabolize more slowly or differently (Mitchison, 2004; Boshoff & Barry, 2005). Since our results with the Δ*icl1* mutant suggested that the GC might be required during both the acute and chronic phases of the infection, we asked whether treatment of INH and lack of ICL1 alone would synergize in killing bacteria specifically during the persistent phase of the infection. This would be akin to assessing the effect that a potential inhibitor of ICL1 would have on TB chemotherapy if co-administered with INH. To this end we infected mice (C57BL/6) with Δ*icl1* mutant or wild-type bacteria and compared the *in vivo* susceptibility of the two strains to INH treatment. Mice were inoculated intravenously with wild-type or Δ*icl1* bacteria and the infection was allowed to proceed normally for 4 wks (Fig. 8.1). At that point groups of mice infected with either strain were placed in monotherapy with INH (25 mg/kg/day) (Canetti, 1960) for the next 8 wks; every 2 wks, 4 mice from each group (treated and untreated, infected with either strain) were sacrificed and the numbers of bacteria in their organs were determined by plating for CFU. As expected, by wk 4 post infection, the counts for the Δ*icl1* mutant in the lung were already about 5 fold lower than those of wild-type bacteria and remained between ~0.75 and 1

$\log_{10}$ lower than wild-type throughout the experiment in lungs, spleens, and livers (Fig. 8.1A & Fig. 8.2A&B).

INH treatment sharply reduced bacterial loads in the lungs (2 log drop/4 wks) (Fig. 8.1A), spleens and livers (Fig. 8.2A-B) of mice infected with wild-type bacteria during the initial 4 wks of the infection; a similar but more pronounced drop in the census of the Δ*icl1* mutant (2.5 log drop/4 wks) was observed during this period. Interestingly, the killing kinetics over this early period of chemotherapy indicated an additive bactericidal effect between INH treatment and ICL1 deficiency (Fig. 8.1A&B): By the end of the second wk of INH treatment, 99% of Δ*icl1* bacteria had been killed compared to ~90 % of wild-type bacteria. This enhancement of killing suggests that the eventual use of ICL1-specific inhibitors in the treatment of TB might not interfere with the action of bactericidal drugs like INH, an important consideration when evaluating the potential of a bacterial target for antibiotic development (Mitchison, 1979; 1980; Nathan, 2004).

## 8.2. Lack of ICL1 does not reduce *M. tuberculosis in vivo* drug tolerance

That ICL1-deficiency led to increased susceptibility to killing by INH during the early phase of chemotherapy was consistent with the idea that ICL-mediated metabolism is essential for *M. tuberculosis* throughout the infection (Chapter 6). However, the increased susceptibility of ICL1-deficient bacteria to INH we observed during the first 4 wks of treatment was not seen later on in the infection:

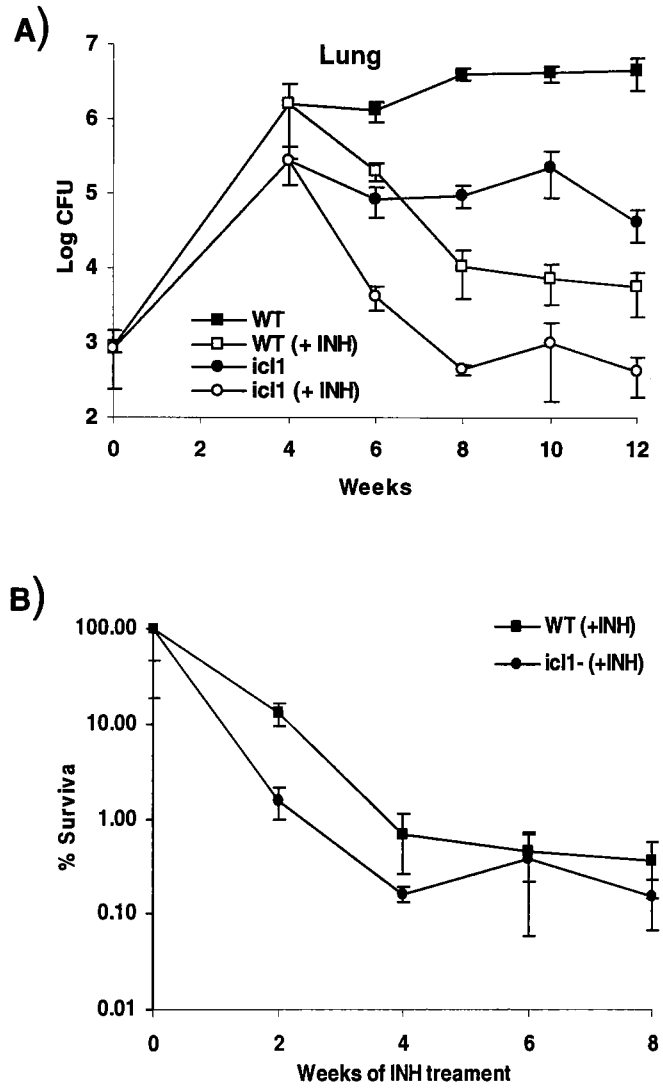


**Fig. 8.1. Isoniazid therapy of mice infected with *icl1*-deficient *M. tuberculosis*.** Widltype (C57BL/6) mice infected with wild-type or Δ*icl1 M. tuberculosis* (Erdman) (~$10^6$ CFU, i.v.). Beginning on week 4 post infection, mice were treated with INH (25 mg/kg/day) for 8 weeks. Bacteria in the (**A**) lung were enumerated by plating organ homogenates for CFU at indicated time-points. (**B**) Data from INH-treated mice from (**A**) plotted as % survival. Error bars indicate STDEV for each group (n=4).

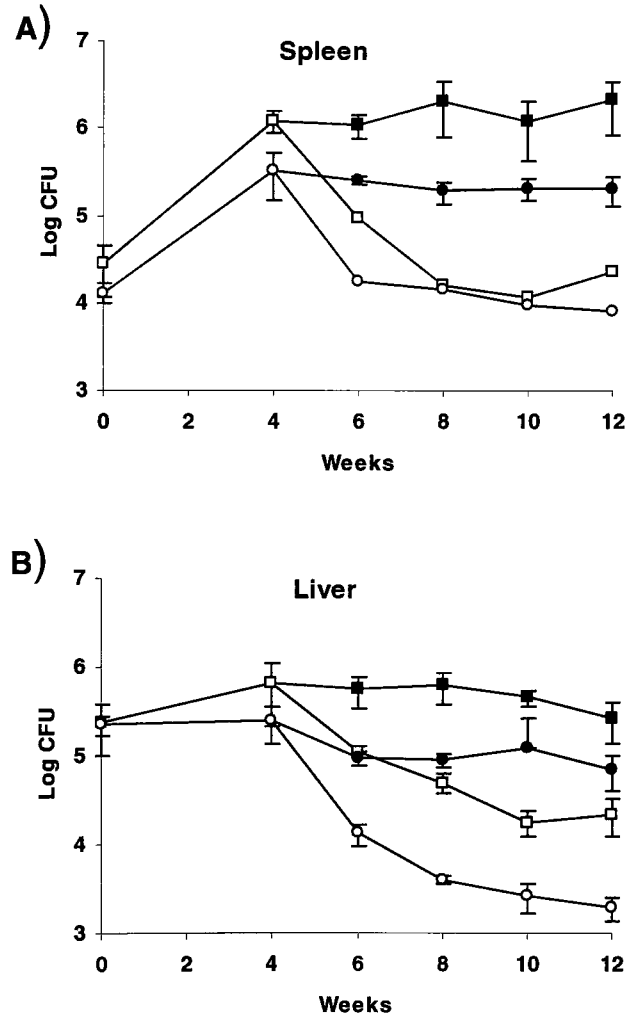


**Fig. 8.2. Isoniazid therapy of mice infected with *icl1*-deficient *M. tuberculosis*.** Wild-type (C57BL/6) mice infected with wild-type or Δ*icl1* *M. tuberculosis* (Erdman) (~$10^6$ CFU, i.v.). Beginning on week 4 post infection, mice were treated with INH (25 mg/kg/day) for 8 weeks. Bacteria in the (**A**) spleen and (**B**) liver were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4).

INH treatment during wks 4-8 did not further significantly reduce bacterial loads of either wild-type or ICL1-deficient bacteria (Fig. 8.1&8.2). Importantly, bacteria surviving these 8 wks of INH treatment were largely drug susceptible because only 0.25% of wild-type and no Δ*icl1* mutant bacteria found in the lungs after the 8 wks of chemotherapy were resistant to INH (INH, 0.5 μg/mL; limit of detection, 1 CFU/lung). Thus, the great majority of these bacteria belonged to the category McDermott (1958) termed "*in vivo* drug tolerant."

That the lack of ICL1 did not significantly enhance the ability of INH to eradicate the bacteria from the organs of mice suggests that if *in vivo* INH-tolerant bacteria depend on C2-metabolism for persistence, ICL2 is capable of providing enough ICL activity to the bacteria so that they can persist on C2 substrates.

### 8.3. A Potential role for ICL1 during reactivation from latency

We next considered the possibility that *M. tuberculosis* dependence on ICL1 during persistence might pertain to the bacterial population(s) responsible for latency. Latency in humans is the most common outcome of *M. tuberculosis* infection and the result of a partially effective immune response. Since no good animal model of such scenario exists apart from the monkey (Capuano *et al.,* 2003; Flynn *et al.,* 2003), we decided to use a modified version of the "Cornell model" of drug-induced latency (See Chapter 6) to test the possibility that ICL1-mediated metabolism might be important for the establishment and/or reactivation

from latency. To this end we adapted a recently described aerosol model of drug induced latency in which mice are treated with INH and rifampin (RIF) starting at 2 wks post-infection and then treated with a NOS2 inhibitor to induce reactivation (Botha *et al.*, 2002). Since we had observed that IFN-γ-dependent/NOS2-independent factor(s) could, at least transiently, keep Δ*icl1* bacteria in check during the persistent phase of the infection (Chapter 7), we decided to carry out the drug-induced latency study in $NOS2^{-/-}$ mice. In preliminary experiments, we confirmed that Δ*icl1* bacteria were attenuated during the persistence phase of the infection compared to wild-type bacteria when given via the respiratory route (Fig. 8.4 C), confirming our previous results via the intravenous route. $NOS2^{-/-}$ mice were infected by aerosol with either wild-type or Δ*icl1* bacteria (~30 CFU/mouse, aerosol) and at 2 wks post-infection, a group of mice infected with each strain was put on an 8-wk long INH-RIF combination. Bacterial counts were determined throughout, at 1-wk intervals during the first 4 wks of treatment, and at 2-wk intervals from wks 6-10. 8 wks of chemotherapy resulted in drastic reductions in wild-type and Δ*icl1* mutant bacterial loads in the lungs (CFU decreased from $10^6$ to ~ 50 CFU, and in some mice, to zero) (Fig. 8.3). The killing rates of wild-type and Δ*icl1* bacteria during antibiotic treatment phase were indistinguishable. This observation suggests that NOS2 might contribute to the slightly increased early killing of Δ*icl1* mutant we had observed before (Fig. 8.1A & B). However, since in that experiment only INH was administered, the two experiments cannot be

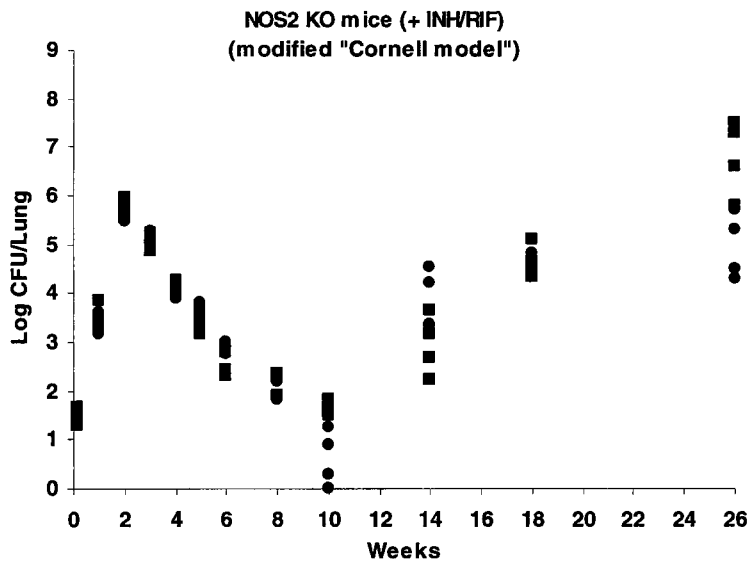


**Fig. 8.3. Evaluating the role of ICL1 in the drug-induced model of latent tuberculosis in mice (Modified "Cornell Model").** NOS2$^{-/-}$ (C57BL/6) mice infected by aerosol with wild-type or Δ*icl1* *M. tuberculosis* (Erdman, ~30 CFU/mouse) received isoniazid plus rifampin (25 mg/kg/day, each) from week 2-10 post infection (drugs were given in drinking water and withdrawn 24 hr before each time-point). Bacteria in the lungs were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4).

compared directly.

In the models of drug-induced latency, if one assumes that the bacteria remaining after treatment are in a state akin to "latency," then the relapse phase is equivalent to reactivation from "latency." If ICL1-mediated metabolism were important for the survival of bacteria in this state "latent" state, the Δ*icl1* mutant would be expected to survive less well, perhaps be incapable of reactivating from such state. As expected based on previous reports (Botha *et al.*, 2002; Flynn *et al.*, 1998; Scanga *et al.*, 1999), NOS2-deficiency hasten the kinetics of reactivation significantly. Only 4 wks after drug withdrawal, wild-type bacterial numbers had bounced back one thousand fold and by 8 wks had climbed to about $10^5$ CFU per lung (Fig. 8.3). Interestingly, growth of wild-type bacteria during this reactivation phase was clearly slower than earlier in the acute phase of the infection. During a similar time interval in $NOS2^{-/-}$ mice, wild-type bacterial loads increased from ~30 to about $10^9$ CFU per lung, which killed the mice within 9 wks of infection (Fig. 8.4 A&B). These observations suggest the existence of NOS2-independent mechanism(s) that may contribute to the control of *M. tuberculosis* during persistent infection and that perhaps together with NOS2 play a role in maintaining latency, as proposed before (Flynn *et al.*, 1998).

During this initial 8 wks after drug withdrawal, ICL1-deficient bacteria reactivated with indistinguishable kinetics from wild-type (Fig. 8.3). Nevertheless, over the ensuing 8 wks (wks 18-26 post infection), while wild-type

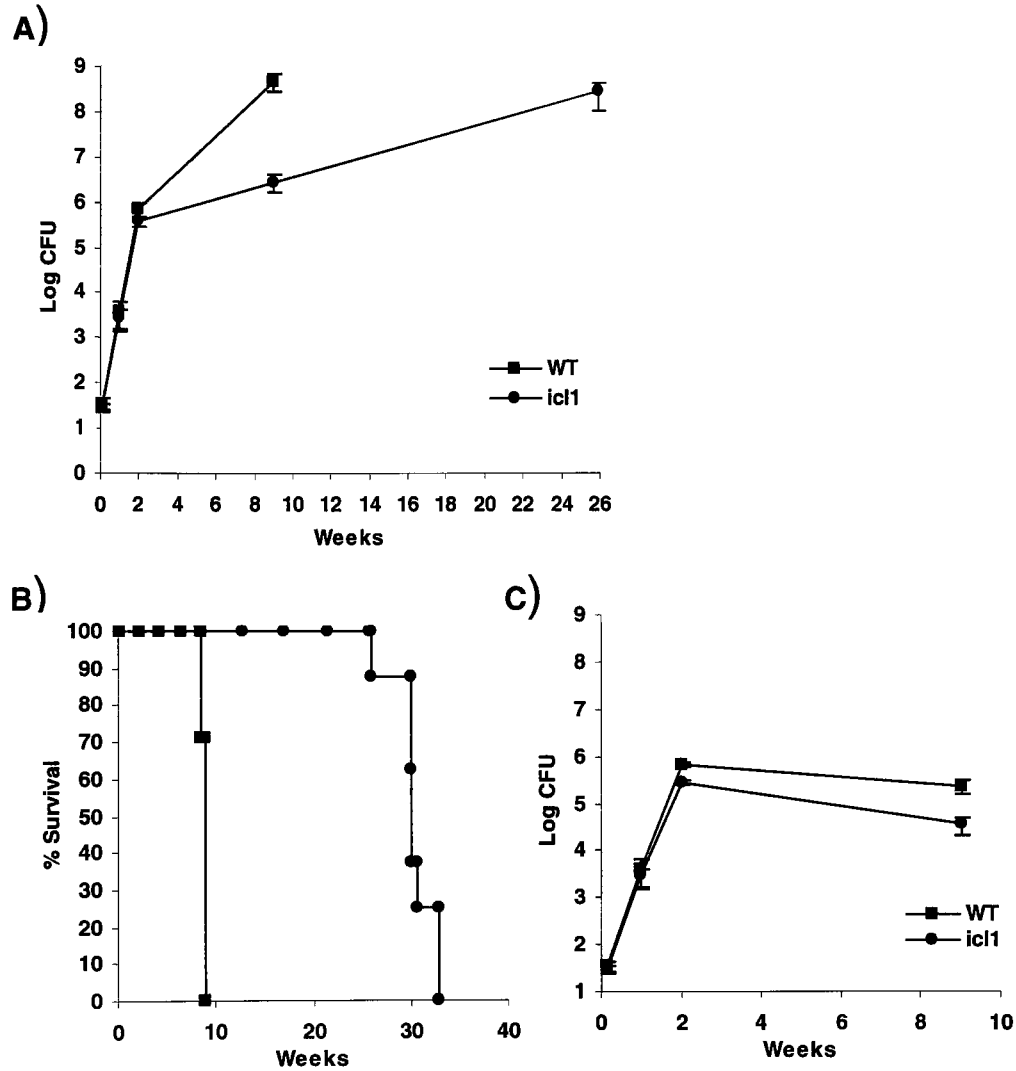


**Fig. 8.4. Comparison of growth of *M. tuberculosis icl1*-deficient mutant in wild-type and NOS2$^{-/-}$ mice.** (**A&B**) NOS2$^{-/-}$ and (**C**) wildtype (C57BL/6) mice were infected by aerosol with wild-type (squares) or Δ*icl1* (circles) *M. tuberculosis* (Erdman, ~30 CFU/mouse). Bacteria in the lungs were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4). (**B**) Survival of NOS2$^{-/-}$ mice infected with wild-type or Δ*icl1* mutant (n=8).

bacteria continued to grow, reaching between $10^7$-$10^8$ CFU per lung, Δ*icl1* mutant bacteria hovered at around $10^5$ CFU per lung (Fig. 8.3). In addition, reactivated wild-type bacteria killed infected mice between wk 30-34 post-infection, while Δ*icl1* mutant bacteria allowed much longer survival (mice started to die at 38 wks post-infection and some mice survived up to 54 wks post-infection) (not shown). Bacterial CFU during the survival part of the experiment were not determined. These data suggest that NOS2-independent antimycobacterial mechanism(s) exist that can more effectively control ICL1-deficient bacteria compared to wild-type, and these might be important during reactivation from "latency." It is unclear if this mechanism(s) is the same that transiently controlled the growth of Δ*icl1* mutant bacteria in the standard mouse model of chronic TB also using NOS2-deficient mice (Fig. 8.4A; see also Chapter 7).

# CHAPTER 9

# The Role of the Glyoxylate Cycle in the Growth of Mycobacteria on Fatty Acids & Carbohydrates

## INTRODUCTION

Previously, we reported that Δ*icl1*/Δ*icl2 M. tuberculosis* bacteria are unable to replicate *in vivo* and are rapidly cleared from the lungs of mice (Chapter 6; Muñoz-Elías & McKinney, 2005). As expected based on what is known about the canonical role of the GC in carbon metabolism, Δ*icl1*/Δ*icl2* bacteria were unable to grow on C2 substrates, but unexpectedly, they were also incapable of growth on propionate and other C3FAs. β-oxidation of odd-chain fatty acids generates both acetyl-CoA ($C_2$) and propionyl-CoA ($C_3$); the latter can be metabolized via one of several possible oxidative pathways (Halarnkar & Blomquist, 1989). Biochemical and functional genetic evidence indicates that several bacteria including *E. coli* (Textor *et al.,* 1997), *S. typhimurium* (Horswill & Escalante-Semerena, 1997; 1999) and *C. glutamicum* (Claes *et al.,* 2002), catabolize propionate via the methylcitrate cycle (MCC), whose role in $C_3$ substrate catabolism was discovered in fungi three decades ago (Tabuchi & Uchiyama, 1975) (Chapter 3).

The *M. tuberculosis* genome has homologs of methylcitrate synthase (*prpC*) and methylcitrate dehydratase (*prpD*), while methylisocitrate lyase (MICL, *prpB*), which catalyzes the breakdown of methylisocitrate into pyruvate and succinate, is apparently missing. However, ICLs show limited homology to MICLs and can have weak MICL activity, i.e. use methylcitrate as a substrate (Luttik *et al.*, 2000), while MICLs show negligible activity on isocitrate (Brock *et al.*, 2001). These observations led us to consider the possibility that ICL1, ICL2, or both might substitute for MICL in the MCC of mycobacteria, thus explaining the inability of the Δ*icl1/icl2 M. tuberculosis* mutant to grow on $C_3$ substrates (Chapter 6). Alternatively, the mutant's phenotype could be interpreted to mean that the GC is required for anaplerosis during growth on $C_3$ substrates, as apparently is the case in *E. coli* (Textor *et al.*, 2003).

To differentiate between these two possibilities, we first turned our attention to the fast-growing avirulent saprophyte *M. smegmatis*, which has genes putatively encoding both, an intact GC (*icl1*, *icl2*, *glcB*), and an intact MCC, including all three *prp* genes in an operon (*prpDBC*). Here, we report the generation and characterization of *M. smegmatis* Δ*icl1*, Δ*icl2*, and Δ*icl1*/Δ*icl2* mutant strains. In parallel studies, we have directly studied the contributions of the *prp* genes to *M. smegmatis* (A. Upton, personal communication) and *M. tuberculosis* $C_3$ metabolism and pathogenesis (Chapter 6).

## RESULTS

## 9.1. Cloning and disruption of glyoxylate cycle genes in *M. smegmatis*

By performing BLAST database searches of the unfinished genome sequence of *M. smegmatis* (tigr.org) with the amino acid sequences of *M. tuberculosis* ICL1, ICL2 and MLS, we identified contigs encompassing *icl1, icl2* and *glcB* putative open reading frames. The predicted *icl1* gene product encodes a 428 amino acid protein that is 95, 93, 93, 92 and 92 % identical to the predicted sequences of ICL1 from *M. marinum, M. avium, M. avium paratuberculosis, M. bovis*, and *M. tuberculosis*, respectively; the predicted product of the *icl2* gene encodes a 769 amino acid product that is 80, 80, 78, 77, 77, and 77 % identical to ICL2 from *M. avium, M. avium paratuberculosis*, *M. marinum*, *M. leprae*, *M. bovis*, and *M. tuberculosis*, respectively. The amino acid identity between the *M. smegmatis* ICL1 and ICL2 isoenzymes is ~27% (over the entire length of the protein), which is similar to that between all mycobacterial ICL1 and ICL2 protein sequences (not shown).

## 9.2. Generation of Δ*icl1*, Δ*icl2*, Δ*icl1*/Δ*icl2 M. smegmatis* mutants

To assess the function of the *icl* genes in *M. smegmatis* carbon metabolism, we constructed in-frame unmarked Δ*icl1*, Δ*icl2*, Δ*icl1*/Δ*icl2* deletion mutants. These were generated by allelic exchanged delivered via suicide plasmids as described (Pavelka & Jabcobs, 1999); see materials and methods for details. The Δ*icl1*

mutant was generated and kindly provided by L. Merkov. All chromosomal disruptions were confirmed by S. blot analysis (not shown).

### 9.3. Carbon utilization of Δ*icl1*, Δ*icl2*, Δ*icl1*/Δ*icl2 M. smegmatis* mutants

Disruption of either *icl1* or *icl2* singly or jointly did not affect the ability of *M. smegmatis* to grow axenically in standard 7H9 broth, 7H10 plates (not shown) or minimal M9 liquid media supplemented with either glycerol (not shown) or glucose (Fig. 9.1A,D) as sole carbon source. As expected, Δ*icl1*/Δ*icl2 M. smegmatis* bacteria did not grow on even-chain (C2) fatty acids (Fig. 9.1B-D). In contrast to the single Δ*icl2* mutant, which grew similarly to wild-type on all C2 fatty acids tested, the single Δ*icl1* mutant did not (Fig. 9.1B&C). Previously, we had made a similar observation using a chemical putative *icl1* mutant (McKinney *et al.*, 2000) that has been confirmed to have a point mutation in *icl1* (A. Upton, personal communication). The finding that in *M. smegmatis* deletion of *icl1* alone abrogated growth on C2 substrates contrasts with our carbon utilization experiments in *M. tuberculosis* where deletion of *icl1* alone did not abrogate growth on C2 substrates (Chapter 6; Muñoz-McKinney, 2005).

One advantage of working with *M. smegmatis* as opposed to the more fastidious *M. tuberculosis* is that carbon utilization studies can be done under more

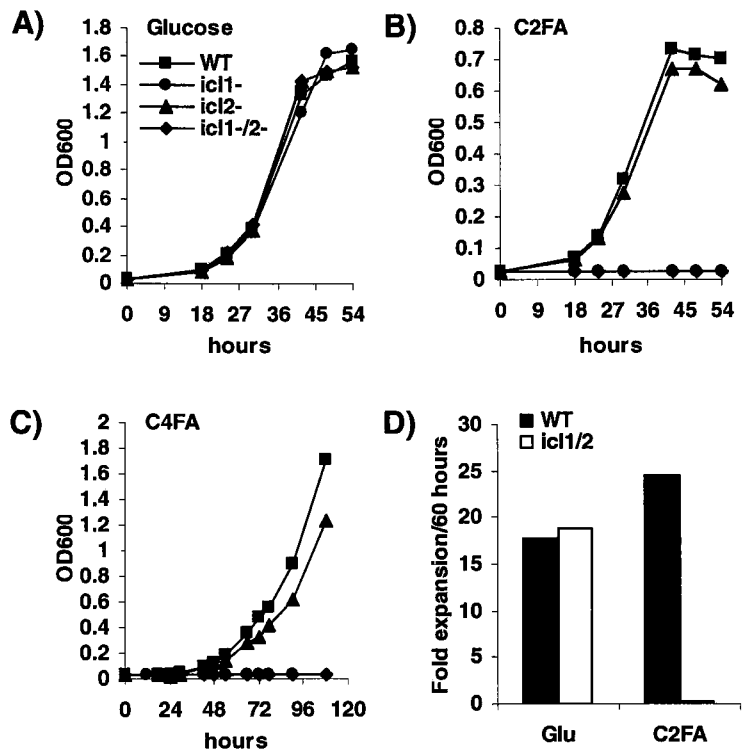


**Fig. 9.1. Non-overlapping roles of ICL1 and ICL2 in *M. smegmatis* fatty acid metabolism.** (**A-D**) Bacteria were cultured in minimal M9 liquid media containing (0.1% w/v): (**A**) glucose; (**B**) acetate (C2FA); (**C**) butyrate (C4FA). (**A-C**) Growth was monitored by measuring culture turbidity (OD600 nm) at the indicated time points. (**D**) *M. smegmatis* wild-type (filled columns) or Δ*icl1*/Δ*icl2* mutant (empty columns) were grown in minimal M9 liquid media supplemented with (0.1% w/v) Glu (glucose) or C2FA (acetate) for 60h and then plated for CFU. Results are representative of two experiments.

stringent conditions with minimal M9 salts and single carbon substrates on solid media. Echoing our results in liquid media, all *icl* mutants grew on glucose plates (Fig. 9.2). However, while wild-type and *icl2*-deficient bacteria grew on all C2 substrates tested, including acetate, butyrate, and palmitate, neither Δ*icl1* nor Δ*icl1*/Δ*icl2* mutant bacteria were capable of growth on C2 substrates (Fig. 9.2).

## 9.4. Complementation of *M. smegmatis icl* mutants with *M. tuberculosis* genes

Both *icl1* and *icl2* genes from *M. smegmatis* provided separately restored the ability of the Δ*icl1*/Δ*icl2 M. smegmatis* mutant to grow on acetate (Fig. 9.3B&C) and other C2 substrates (not shown). Importantly, either *icl1* or *icl2* from *M. tuberculosis* also restored the ability of the *M. smegmatis* Δ*icl1*/Δ*icl2* mutant to grow on C2 substrates (Fig. 9.3D&E). That the Δ*icl1*/Δ*icl2*::pICL2 strain grew on C2 substrates (Fig. 9.3B&E), while the Δ*icl1* single mutant did not (Fig. 9.1&9.2), might be due to higher ICL activity in the complemented strain resulting from the fact that the episomal plasmid used to complement it was medium-copy.

## 9.5. Dispensability of *icls* for odd-chain fatty metabolism in *M. smegmatis*

The observation that *M. tuberculosis* Δ*icl1*/Δ*icl2* mutant bacteria were unable to grow on propionate and that ICL activity was highly up-regulated in total protein

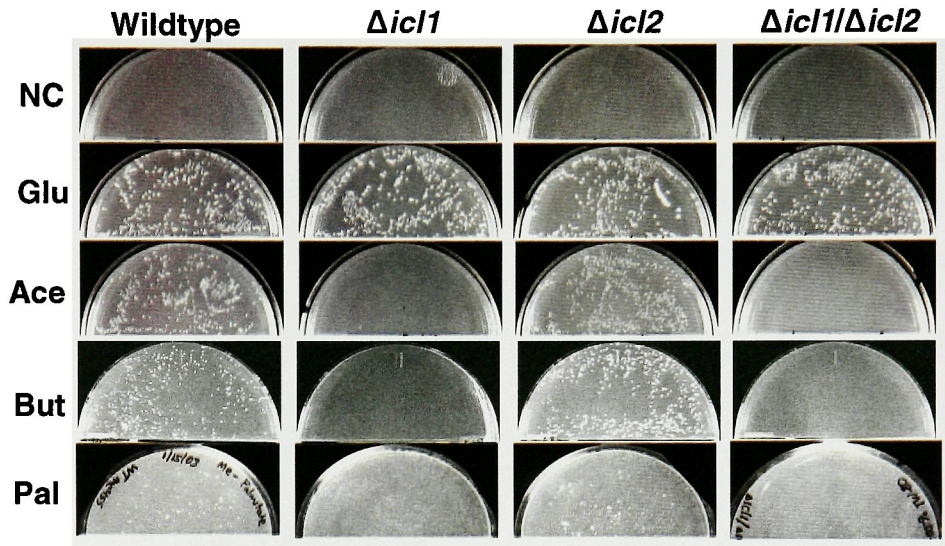


**Fig. 9.2. Growth of *icl* mutants of *M. smegmatis* on minimal solid media.** *M. smegmatis* wild-type, Δ*icl1*, Δ*icl2* or Δ*icl1*/Δ*icl2* strains were grown in 7H9 broth to late log phase, serially diluted in PBS and plated on minimal M9 solid media supplemented with 0.1% (w/v) carbon substrates. NC: no carbon; Glu: glucose; Ace: acetate; But: butyrate; Pal: methyl-palmitate. Plates were incubated at 37 °C for 5 days. Results are representative of three experiments.

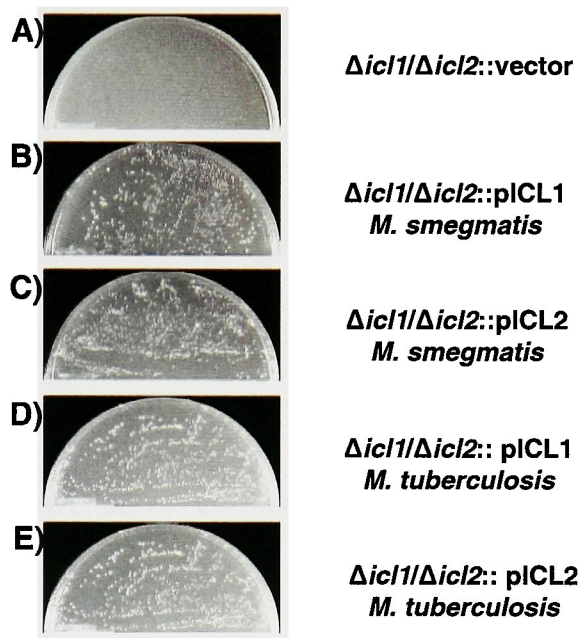


**Fig. 9.3.** ***M. tuberculosis icl1* or *icl2* genes restore ability to grow on acetate to ICL-deficient *M. smegmatis*.** *M. smegmatis* Δ*icl1*/Δ*icl2* mutant was transformed with (**A**) empty vector; (**B**) *icl1* (*M. smegmatis*); (**C**) *icl2* (*M. smegmatis*); (**D**) *icl1* (*M. tuberculosis*); (**E**) *icl2* (*M. tuberculosis*). Bacteria were grown in 7H9 broth to late log phase and plated on minimal M9 solid media supplemented with 0.1% (w/v) acetate and incubated at 37 °C for 5 days.

extracts of *M. tuberculosis* grown on propionate compared to glucose (Table 6.1), suggested that ICLs might play a dual role in mycobacterial metabolism, perhaps functioning as methylisocitrate lyases (MICL) in the methyl citrate cycle (MCC), in addition to the GC. Alternatively, *M. tuberculosis* might require ICLs for growth on C3 substrates because the anaplerotic function of the GC might be needed under such growth conditions.

Since *M. smegmatis* has at least one putative *prpB* gene encoding a dedicated MICL, Δ*icl1*/Δ*icl2 M. smegmatis* mutants should only be impaired for growth on C3 substrates if the GC is required for C3 metabolism. Thus, we tested the ability of the different *M. smegmatis icl* mutants to grow on C3 substrates as sole carbon sources. Wild-type *M. smegmatis* grew very efficiently on the C3 fatty acid propionate. In sharp contrast to our findings with *M. tuberculosis*, *icl* genes appeared completely dispensable for growth of *M. smegmatis* on C3 substrates. Wild-type, Δ*icl1*, Δ*icl2* and Δ*icl1*/Δ*icl2 M. smegmatis* mutants grew indistinguishably from each other in glucose (Fig. 9.4A), propionate (Fig. 9.4B) or valerate (Fig. 9.4C) liquid media, or propionate plates (Fig. 9.4D).

These observations suggest that if the MCC is required for C3 utilization by *M. smegmatis*, MICL encoded by *prpB* is probably sufficient for catalyzing this step of the pathway. They could also mean that the GC is not required for C3 metabolism in mycobacteria, and that replenishment of TCA cycle intermediates during growth on C3 substrates likely takes place via conversion of pyruvate, the product of the MCC, to oxaloacetate by pyruvate carboxylase (PCA). This

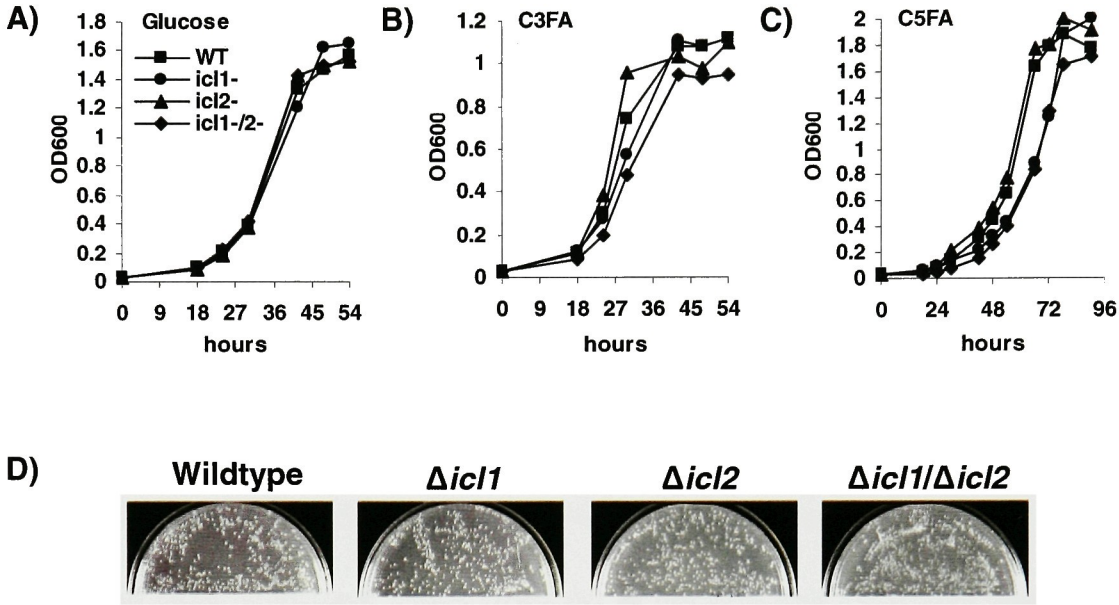


**Fig. 9.4. *icl* genes are dispensable for odd-chain fatty acid metabolism in *M. smegmatis*.** (**A-C**) Bacteria were cultured in minimal M9 liquid media containing (0.1% w/v): (**A**) glucose; (**B**) propionate (C3FA); (**C**) valerate (C5FA). Growth was monitored by measuring culture turbidity ($OD_{600}$ nm) at the indicated time points. (**D**). *M. smegmatis* wild-type, Δ*icl1*, Δ*icl2* or Δ*icl1*/Δ*icl2* strains were grown in 7H9 broth to late log phase and plated on minimal M9 solid media supplemented with (0.1% w/v) propionate (C3FA). Plates were incubated at 37 °C for 5 days. Results are representative of three experiments.

suggestion is further supported by the finding that a mutant of *glcB*, encoding malate synthase (second step of the GC), grew indistinguishably from *M. smegmatis* wild-type on C3 substrates (L. Merkov, personal communication).

## 9.6. ICL activity during growth on C2/C3 substrates

In *M. tuberculosis*, *icl1* and *icl2* are separately dispensable but jointly required for growth on C2 substrates (Chapter 6). Interestingly, while *M. tuberculosis* extracts from Δ*icl2* mutant bacteria had ICL activity levels that were comparable or higher than those from wild-type, those from Δ*icl1* bacteria had only very low basal levels of activity (Table 6.1). To study the regulation of ICLs in *M. smegmatis*, we grew the different *icl* mutants on different C2 and C3 substrates to late-log phase and prepared cell-free extracts for analysis of ICL activity. Extracts from wild-type *M. smegmatis* grown on glucose had considerable levels of ICL activity, which increased about 5 fold when the bacteria were grown in glucose plus acetate (Table 9.1). Extracts from dextrose-grown Δ*icl1* mutant had about 14% of the activity of extracts from glucose-grown wild-type cells. However, extracts from glucose/acetate-grown Δ*icl1* mutant had almost no activity. Interestingly, and reminiscent of our observations in *M. tuberculosis*, all extracts prepared from the Δ*icl2* mutant had ICL levels that were higher (~2X) than those of wild-type. No

**Table 9.1. Isocitrate Lyase Activity of *M. smegmatis icl* mutants**

| Strain | Glu | Glu/Ace | Ace | Pro | But |
|---|---|---|---|---|---|
| **Wild-type** | 17.13 (3.40) | 78.88 (8.79) | 39.33 (15.20) | 35.99 (4.03) | 56.68 (2.73) |
| ***Δicl1/Δicl2*** | 0.071 (0.12) | 0.154 (0.24) | ND | 0.75 (1.07) | ND |
| ***Δicl1*** | 2.38 (1.09) | 0.94 (0.77) | ND | 0.63 ( 0.49) | ND |
| ***Δicl2*** | 20.53 (1.29) | 128.43 (21.53) | 64.55 (13.77) | 69.07 ( 7.44) | 90.88 ( 0.47) |

Bacteria were grown in minimal M9 broth at 37 °C with shaking (100 rpm), and harvested at late log phase. Carbon substrates (0.1 % w/v, each): Glu = glucose; Ace = acetate; Pro = propionate; But = butyrate. Data represent the average of 2 independent cultures from which 3 measurements were made. Total protein: isocitrate stimulated specific activity (nmol/min/mg) using 1 mM isocitrate, 0.1 mM NADH at 25 °C (Data were kindly provided by A. Upton).

ICL activity was detected in extracts from *Δicl1/Δicl2* bacteria grown on glucose or glucose plus acetate. Extracts of acetate-, propionate-, or butyrate-grown wild-type *M. smegmatis* bacteria had 2-3 fold higher levels of ICL activity than extracts from glucose-grown bacteria (Table 9.1).

## 9.7. A role for the glyoxylate cycle in carbon utilization in mixed substrates

Under standard conditions, *E. coli* requires the GC only when C2 substrates are the sole carbon source (Kornberg, 1966; Cronan & LaPorte, 1996). However, our finding that extracts from *M. smegmatis* grown on a mixture of glucose and acetate had considerably higher ICL activity than extracts from glucose-grown bacteria,

and similar observations made in *M. avium* (Höner zu Bentrup *et al.,* 1999), suggest mycobacteria might employ the GC in mixed substrates. Indeed, we found that *M. smegmatis* Δ*icl1*/Δ*icl2* had a significant lag during growth on 1:1 mixtures of acetate and glucose (not shown). We sought to extent these observations to *M. tuberculosis* by assessing the ability of *icl*-deficient *M. tuberculosis* to grow on mixed carbon substrates. While wild-type *M. tuberculosis* grew on C2 substrates alone, glucose alone, or mixtures of the two, unexpectedly, the Δ*icl1*/Δ*icl2* mutant was unable to grow on glucose plus any C2/C3 substrate tested including acetate (Fig. 9.5A), propionate (Fig. 9.5B), C12FA (Fig. 9.5C), and C16FA (Fig. 9.5D). These experiments suggest in order to grow on mixed substrates *M. tuberculosis* requires ICL. Thus, the GC requirement by *M. tuberculosis* during infection might not indicate absolute inaccessibility to carbohydrates.

## 9.8. Inhibition of *M. tuberculosis* ICL1 or ICL2 in whole cell assays

The absence of ICL orthologs in mammals should facilitate the eventual development of GC inhibitors as drugs for the treatment of TB. Using the ICL inhibitor 3-nitropropionate (3-NP) (Schloss and Cleland, 1982; Höner zu Bentrup *et al.,* 1999; Sharma *et al.,* 2000), we provided evidence suggesting the feasibility of blocking ICL1 and ICL2 in *M. tuberculosis*, and that such inhibition prevented bacterial growth on fatty acids and in macrophages (Chapter 6). Screening for

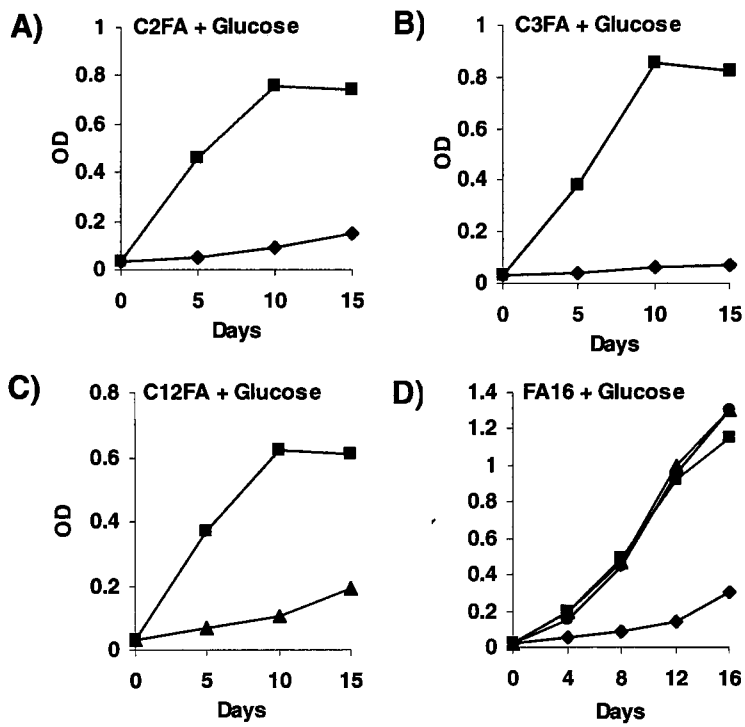


**Fig. 9.5. *M. tuberculosis* relies on glyoxylate cycle ICL for growth on fatty acid/carbohydrates mixed substrates.** Bacteria were cultured at in liquid media containing (0.1% w/v each): (**A**) acetate (C2FA) + glucose; (**B**) propionate (C3FA) + glucose; (**C**) polyoxyethylene sorbitan mono-laurate + glucose; (**D**) polyoxyethylene sorbitan mono-palmitate + glucose. Growth was monitored by measuring culture turbidity (OD600nm) at the indicated time points. Results are representative of two experiments.

ICL inhibitors would be greatly facilitated if initial testing could be done in *M. smegmatis*. Here, we show that Δ*icl1*/Δ*icl2 M. smegmatis* mutant strains carrying the heterologous *icl1* or *icl2 M. tuberculosis* paralogs can be used for such purpose. Addition of 3-NP to C2FA, but not to glucose minimal liquid media, prevented the growth of wild-type *M. smegmatis,* as well as that of Δ*icl1*/Δ*icl2 M. smegmatis* carrying either *icl1* or *icl2* genes from *M. tuberculosis* (Fig. 9.6). Similarly, addition of 3-NP to M9 plates abrogated bacterial growth on C2FA but not on glucose (Fig. 9.6).

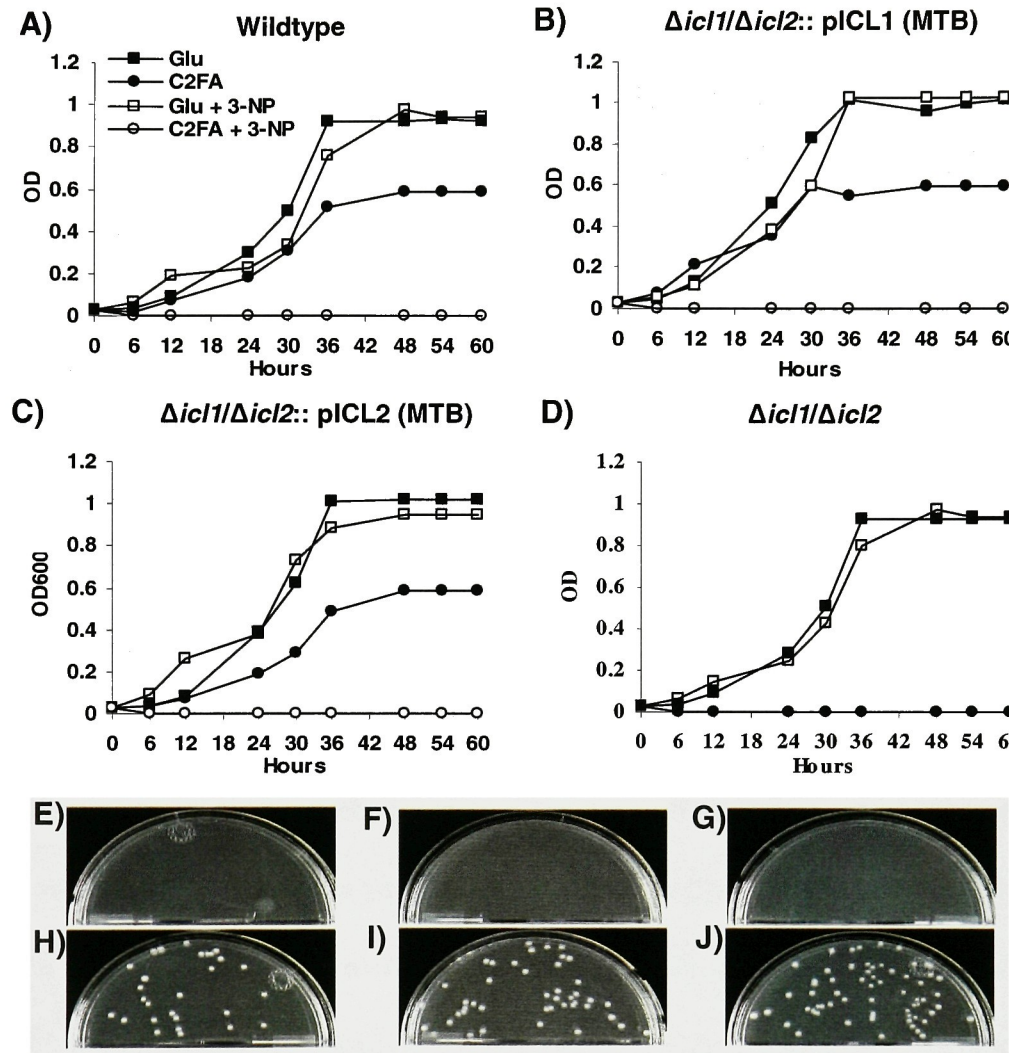


**Fig. 9.6. Whole cell screening assay for dual inhibitors of *M. tuberculosis* ICL1 and ICL2 isoenzymes.** (**A-D**) ICL inhibitor 3-nitropropionate (3-NP) (empty symbols) inhibits growth of *M. smegmatis* (**A**) wild-type, (**B**) Δ*icl1* Δ*icl2::*pICL1 (*M. tuberculosis)*, **C**) Δ*icl1*/Δ*icl2::*pICL2 (*M. tuberculosis*) on acetate (circles) but not on glucose (squares), (**D**) *M. smegmatis* Δ*icl1*/Δ*icl2* mutant. Bacteria were cultured in minimal M9 broth supplemented with (0.1% w/v) glucose (Glu) (filled squares), glucose plus 3-NP (empty squares), C2FA (acetate) (filled circles) or C2FA (acetate) plus 3-NP (empty circles). Growth was monitored by measuring $OD_{600}$ nm at the indicated time points (**E-J**) Plate assay. Growth inhibition of *M. smegmatis* (**E&H**) wild-type, (**F&I**) Δ*icl1* /Δicl2::pICL1 *(M.* tuberculosis) (**G&J**) Δ*icl1* /Δicl2::pICL2 (*M. tuberculosis*) by 3-NP on (0.1% w/v) (**E-G**) acetate but not on (**H-J**) dextrose, as carbon source in minimal M9 plates. 3-NP was included in the medium at 5 mM. Results are representative of three experiments.

# CHAPTER 10

## The Methyl Citrate Cycle in *M. tuberculosis*

### INTRODUCTION

In the foregoing chapter (Chapter 9), we presented evidence that ICL1 and ICL2 are dispensable for growth of *M. smegmatis* on C3 substrates, ruling out an essential role for ICLs in the methylcitrate cycle (MCC) in this species (Fig. 10.1). Since *M. smegmatis* has a *prpB* homolog that putatively encodes a canonical methylisocitrate lyase (MICL), these findings are not surprising. Furthermore, since *glcB*, encoding malate synthase, is also dispensable for growth on C3 substrates (L. Merkov, personal communication), the GC is apparently not required for *M. smegmatis* growth under these conditions either. In contrast, *M. tuberculosis icl* genes are jointly required for both C2FA and C3FA metabolism (Chapter 6). The *M. tuberculosis* genome has homologs of methylcitrate synthase (*prpC*) and methylcitrate dehydratase (*prpD*), while methylisocitrate lyase (MICL, *prpB*), which catalyzes the breakdown of methylisocitrate into pyruvate and succinate, is apparently missing. However, ICLs show limited homology to MICLs and can have weak MICL activity, i.e. use methylisocitrate as a substrate (Luttik *et al.*, 2000), while MICLs show negligible activity on isocitrate (Brock *et*

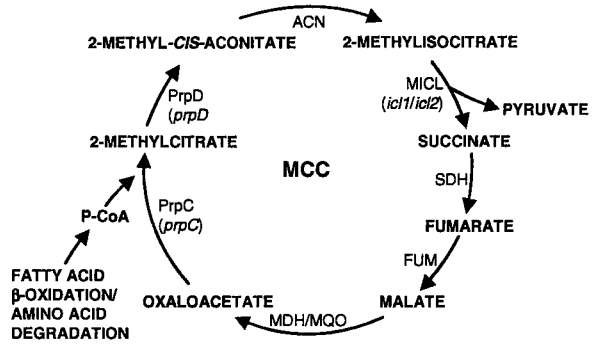


**Fig. 10.1. Methyl citrate cycle (MCC) in *M. tuberculosis*.** Even- and odd-chain fatty acids are degraded to acetyl-CoA (C2) and propionyl-CoA (C3), respectively, via the beta-oxidation pathway. Propionyl-CoA can be metabolized through the methylcitrate cycle. The glyoxylate cycle permits the net synthesis of C4 intermediates, which can serve directly as biosynthetic precursors or can be further metabolized to C3 precursors. Glyoxylate cycle specific enzymes: isocitrate lyase 1 (ICL1), isocitrate lyase 2 (ICL2) and malate synthase (MLS). ICL1 and ICL2 could function as methylisocitrate lyases in the methylcitrate cycle together with the methylcitrate cycle-specific enzymes 2-methylcitrate synthase (GLTA1) and 2-methylcitrate dehydratase (MCD). Glyoxylate cycle enzymes in common with the TCA cycle: Citrate synthase I (GLTA2); citrate synthase II (CITA), aconitase (ACN), succinate dehydrogenase (SDH), fumarate (FUM), malate dehydrogenase (MDH) and malate:quinone oxidoreductase (MQO). Phosphoenol pyruvate (PEP) can be produced by decarboxylation of oxaloacetate by PEP carboxykinase (PCK). Pyruvate can be generated from malate by malic enzyme (MEZ), from PEP by pyruvate kinase (PYK), or through the MCC by the action of ICL1/ICL2 on 2-methylisocitrate producing succinate and pyruvate. Gene names are in italics.

*al.,* 2001). These observations led us to consider the possibility that ICL1, ICL2, or both might substitute for MICL in the MCC of mycobacteria, thus explaining the inability of the Δ*icl1/icl2 M. tuberculosis* mutant to grow on $C_3$ substrates (Chapter 6). Alternatively, the mutant's phenotype could be interpreted to mean that the GC is required for anaplerosis during growth on $C_3$ substrates, as apparently is the case in *E. coli* and Ralstonia (Textor *et al.,* 1997; Wang *et al.,* 2003a).

Here we present evidence indicating that ICLs are induced during growth on propionate and are bifunctional enzymes that can use both isocitrate (ICA) and methylisocitrate MICA) as substrates. The ability of ICL1 to function as a MICL was superior to that of ICL2, since even overexpression of ICL2 resulted in only low levels of MICL activity. Furthermore, ICLs appeared to function together with methylcitrate synthase and methylcitrate dehydratase encoded by *prpC* and *prpD M. tuberculosis* genes, respectively, to form a functional MCC, which we is required by *M. tuberculosis* to grow on odd chain fatty acids *in vitro*. A requirement for the MCC by *M. tuberculosis* during infection was suggested by the inability of a Δ*prpDC* mutant to grow in murine macrophages, but was not supported by *in vivo* evidence indicating the dispensability of these genes for growth and persistence in mice. The work presented in this chapter is the result of collaborative work with A. Upton in the laboratory.

## RESULTS

## 10.1. *icl* genes of *M. tuberculosis* encode isocitrate/methylisocitrate lyases

To test the hypothesis that one or both of the *icl* genes in *M. tuberculosis* encoded a bifunctional ICL/MICL, we cultured *M. tuberculosis* (Erdman) wild-type and Δ*icl1*/Δ*icl2* bacteria in defined media containing glucose or propionate and measured ICL and MICL activity in cell-free extracts. MICA was not available commercially and was chemically synthesized starting with mesaconic acid by modifying previously published methods (Brock *et al.*, 2001; Plaut *et al.*, 1975); this was done in collaboration with J. Cherian and T. Kapoor at Rockefeller University (see materials & methods).

Extracts prepared from wild-type *M. tuberculosis* demonstrated ICL and MICL activity, both of which where much higher in extracts from bacterial cultures grown on propionate (Fig. 10.2A) compared to glucose (not shown). Extracts from wild-type bacteria grown on propionate had very high activity on ICA and high, though significantly lower (3 fold), activity on MICA (Fig. 10.2A). The Δ*icl1*/Δ*icl2* mutant grown on glucose had no detectable activity (not shown). The finding that propionate extracts prepared from the Δ*icl1* mutant (which, though impaired, grew sufficiently to allow preparation of cell-free extracts) had very low levels of activity (Fig. 10.2A&B), while propionate extracts from the Δ*icl2* mutant had ICL and MICL levels of activity comparable to those from

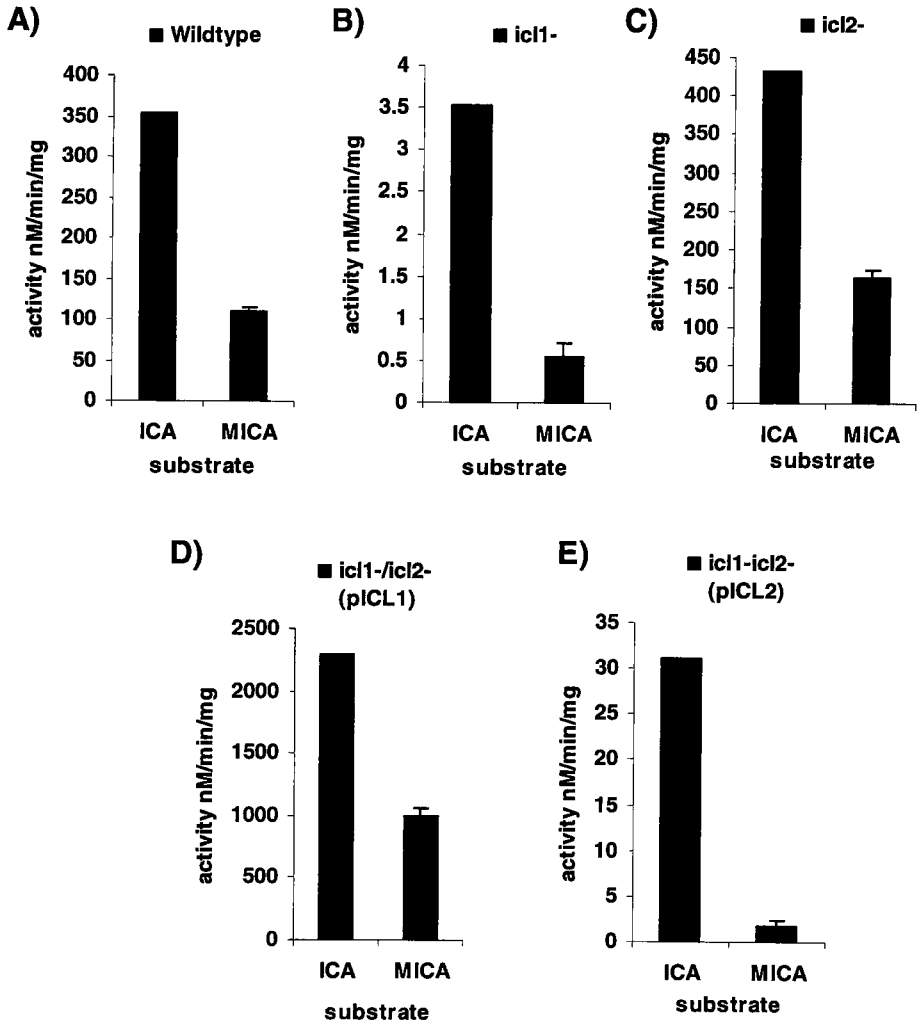


**Fig. 10.2. ICL and MICL activities of propionate-grown *M. tuberculosis* cell-free extracts.** (**A-E**) *M. tuberculosis* (Erdman) bacteria were grown at 37 °C with shaking to late log phase in liquid media containing (0.2% w/v) propionate (C3FA). Cell-free extracts were assayed for activity on isocitrate (ICA) or methylisocitrate (MICA) as indicated. (**A**) Wild-type, (**B**) Δ*icl1*, (**C**) Δicl2, (**D**) Δ*icl1*/Δ*icl2* transformed with an episomal plasmid carrying *icl1*, (**E**) Δ*icl1*/Δ*icl2* transformed with an episomal plasmid carrying *icl2*. Assays were done in triplicates from two set of extracts. Date are from one experiment.

wild-type (Fig. 10.2A&C) suggested that ICL2 was not as efficient as ICL1 as a MICL. In addition, propionate extracts of a strain overexpressing *icl1* from an episomal multicopy plasmid on an Δ*icl1*Δ*icl2* background had markedly higher levels of expression of both ICL (~6.5 fold) and MICL activity (~9 fold) compared to wild-type (Fig. 10.2A&D), while overexpression of *icl2* in the same background resulted in a large increase in ICL actitvity (10 fold higher than in extracts of the Δ*icl1* mutant) (Fig. 10.2D), but a minimal increase in MICL activity (Fig. 10.2E). These data indicate that at least *in vitro*, ICL1 is a bifunctional ICL/MICL enzyme, and suggest that it might function in the MCC cycle of *M. tuberculosis* in that capacity. The ability of ICL2 to function as a MICL is more limited.

## 10.2. Cloning and disruption of the putativte methylcitrate cycle genes in *M. tuberculosis*

The inability of the Δ*icl1*/Δ*icl2* *M. tuberculosis* mutant to use propionate as substrate and the lack of MICL and ICL activities in extracts from this strain (grown on glucose), together with the induction of ICL and MICL activities during growth on propionate, suggested that *M. tuberculosis* might require the MCC and the MICL function encoded by *icl1* and/or *icl2* genes for propionate metabolism. However, the possibility remained that in *M. tuberculosis*, unlike in *M. smegmatis*, the GC was required for anaplerosis during propionate metabolism either via the

MCC or another pathway, and the inability of the Δ*icl1*/Δ*icl2* mutant to grow on propionate was due to anaplerotic deficiency rather than impairment of propionate metabolism *per se*. To distinguish between these scenarios, we constructed a strain of *M. tuberculosis* (Erdman) in which the genes unique to the MCC, *prpD* and *prpC*, were deleted. Homologs of *prpD* and *prpC* are found together in the *M. tuberculosis* genome such that the final codon of *prpD* overlaps the first of *prpC*. Although annotated as a 526 amino acid protein in the genome (H37Rv), ORF analysis of the *prpDC* region revealed 3 possible start sites for *prpD* that would give rise to proteins of 526, 505 or 502 amino acids in length. The 3 possible peptides, conceptually translated, were aligned using BLASTP to all PRPD peptide sequences available in NCBI databases, and the most probable was chosen based on similarity to enzymes reported to be functional (Horswill & Escalante-Semerena, 1999). This ORF, which encoded the shortest PRPD peptide (502AA), was defined as the *prpD* gene sequence for the present study.

*M. tuberculosis* with an in-frame unmarked deletion in the *prpD* and *prpC* ORFs was generated by homologous recombination such that the wild-type *prpDC* locus was replaced with sequence encoding a 9AA peptide. The deletion was confirmed by PCR analysis and Southern blot (not shown). For complementation studies of the Δ*prpDC* mutant, the strain was transformed with an integrating plasmid derived from pMV361 (Stover *et al.,* 1991) carrying a wild-type copy of the putative *prpDC* operon.

## 10.3. The methylcitrate cycle is required for propionate metabolism and growth of *M. tuberculosis* on odd-chain fatty acids

Deleting *prpDC* had no effect on bacterial growth on glucose or glycerol (Fig. 10.3A&B). Unlike the parental strain, the Δ*prpDC* mutant was unable to grow with propionate or any other odd-chain fatty acid given as substrate (Fig. 10.3). Unlike the Δ*icl1*/Δ*icl2* mutant (Chapter 6), the Δ*prpDC* mutant exhibited growth on even chain fatty acids that was comparable to that of the parental strain (Fig. 10.3). Complementation of the Δ*prpDC* mutant with an integrating plasmid carrying the wild-type putative *prpDC* operon largely restored growth on propionate media. Thus, the *prpDC* genes and hence the MCC are required specifically for *in vitro* growth on odd-chain fatty acids by *M. tuberculosis*. The ability of this mutant to grow using C2 carbon sources indicates that the MCC is not required for growth on C2 substrates as expected. Together these results suggest that ICL1 and perhaps ICL2 might function as MICL in the MCC.

## 10.4. The methylcitrate cycle is required for survival and replication of *M. tuberculosis* in resting and activated murine macrophages

We next investigated whether the requirement by *M. tuberculosis* for the MCC could be seen at the level of the parasitized macrophage. In marked contrast to *M.*

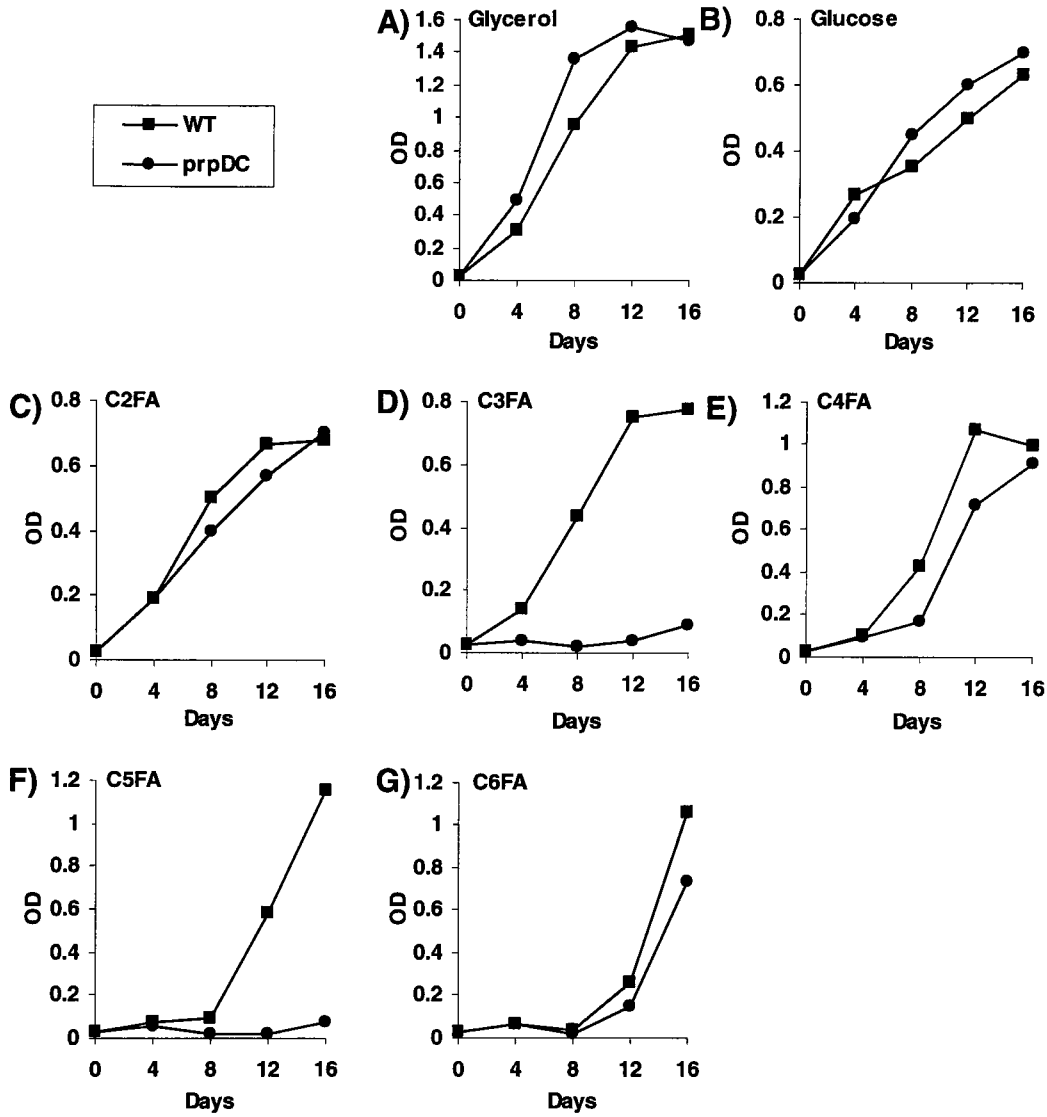


**Fig. 10.3. Indispensable role of *prpC* and *prpD* in C3 fatty acid metabolism.** (**A-G**) *M. tuberculosis* bacteria were cultured at 37 °C in liquid media containing (0.1% w/v): (**A**) glycerol; (**B**) glucose; (**C**) acetate (C2FA); (**D**) propionate (C3FA); (**E**) butyrate (C4FA); (**F**) valerate (C5FA); (**G**) hexanoate (C6FA). Growth was monitored by measuring culture turbidity ($OD_{60}$ nm) at the indicated time points. Results are representative of two experiments with similar results.

*tuberculosis* wild-type bacteria which grew nearly one log over the 7 day time course of an infection of bone marrow-derived murine macrophages, Δ*prpDC* mutant bacteria were incapable of any growth (Fig. 10.4A). To determine the impact of host cell activation on the survival of MCC-deficient bacteria, macrophages were activated with IFN-γ (100 U mL$^{-}$) prior and during intracellular infection. While macrophage activation was bacteriostatic to intracellular wild-type *M. tuberculosis*, it was slightly bactericidal to Δ*prpDC* bacteria (Fig. 10.4B). Complementation of the Δ*prpDC* mutant strain with a copy of wild-type *prpDC* genes restored growth in resting macrophages and wild-type levels of survival in activated macrophages (Fig. 10.4C&D).

## 10.5. The methylcitrate cycle is dispensable for growth and persistence of *M. tuberculosis* in the mouse model of chronic tuberculosis.

Since our data suggested that ICL1 and to a lesser extent ICL2 could function as a MICL, we considered the possibility that the *in vivo* phenotype of the *icl1/icl2* or single Δ*icl1* mutants could be due to their function as a MICL instead of or in addition to their role as ICLs. To directly address this question and the contribution of the MCC to the pathogenesis of *M. tuberculosis in vivo*, we evaluated the ability of the Δ*prpDC* mutant to grow and persist in mice. Unlike

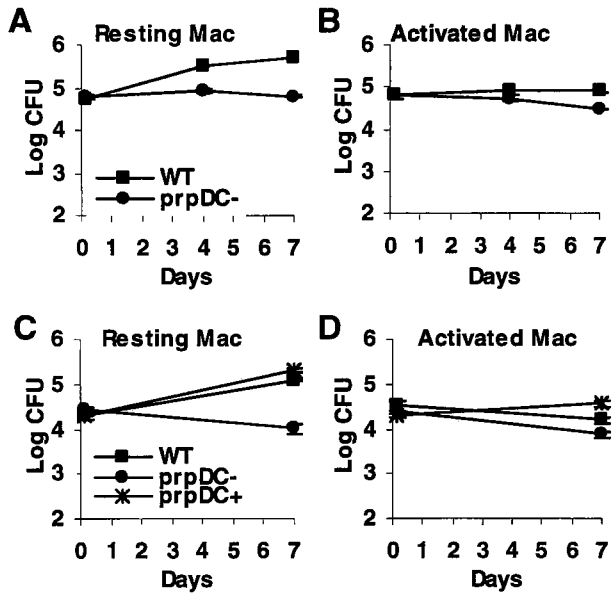


**Fig. 10.4. Methylcitrate cycle genes *prpC* and *prpD* are required for intracellular replication of *M. tuberculosis*.** (**A-D**) Murine bone marrow-derived macrophages (**A&C**) non-activated or (**B&D**) IFN-γ-activated (100 U/mL) were infected with (**A-D**) wild-type, Δ*prpD*/Δ*prpC*, or (C&D) Δ*prpD*/Δ*prpC*::PprpDC+ *M. tuberculosis* (Erdman), (MOI 2:1). Monolayers were incubated at 37 °C in 5% $CO_2$. At indicated time points, cells were lysed (n = 4 wells) and lysates were plated to enumerate CFU. Symbols represent means; error bars indicate STDEV. This experiment was done twice.

Δ*icl1*/Δ*icl2* mutant bacteria which are impaired for growth from the onset of the infection, or Δ*icl1* mutant bacteria, which become impaired at around 2-3 wks post-infection, Δ*prpDC* mutant bacteria replicated and persisted indistinguishably from wild-type bacteria throughout the infection in both lungs (Fig. 10.5A) and spleens (Fig. 10.5B), and elicited comparable pathology (Fig. 10.6). Thus, the requirement for ICL during infection appears to be due to a requirement for a functional GC rather than a MCC.

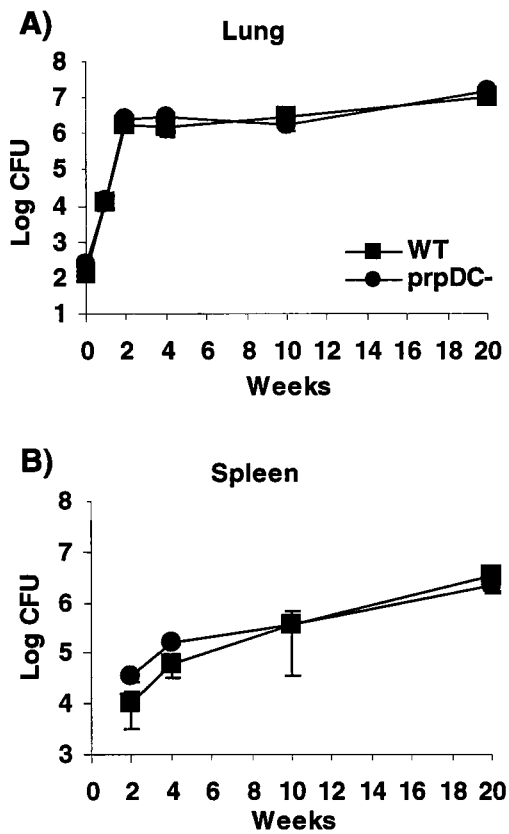


**Fig. 10.5. *prpC* and *prpD* are dispensable for growth and persistence of *M. tuberculosis* in mice.** (**A&B**) Wild-type (C57BL/6) mice were infected with wild-type or Δ*prpDC M. tuberculosis* (Erdman) (~100 CFU, aerosol). Bacteria in the (**A**) lung and (**B**) spleen were enumerated by plating organ homogenates for CFU at indicated time-points. Error bars indicate STDEV for each group (n=4). Experiment was done once.

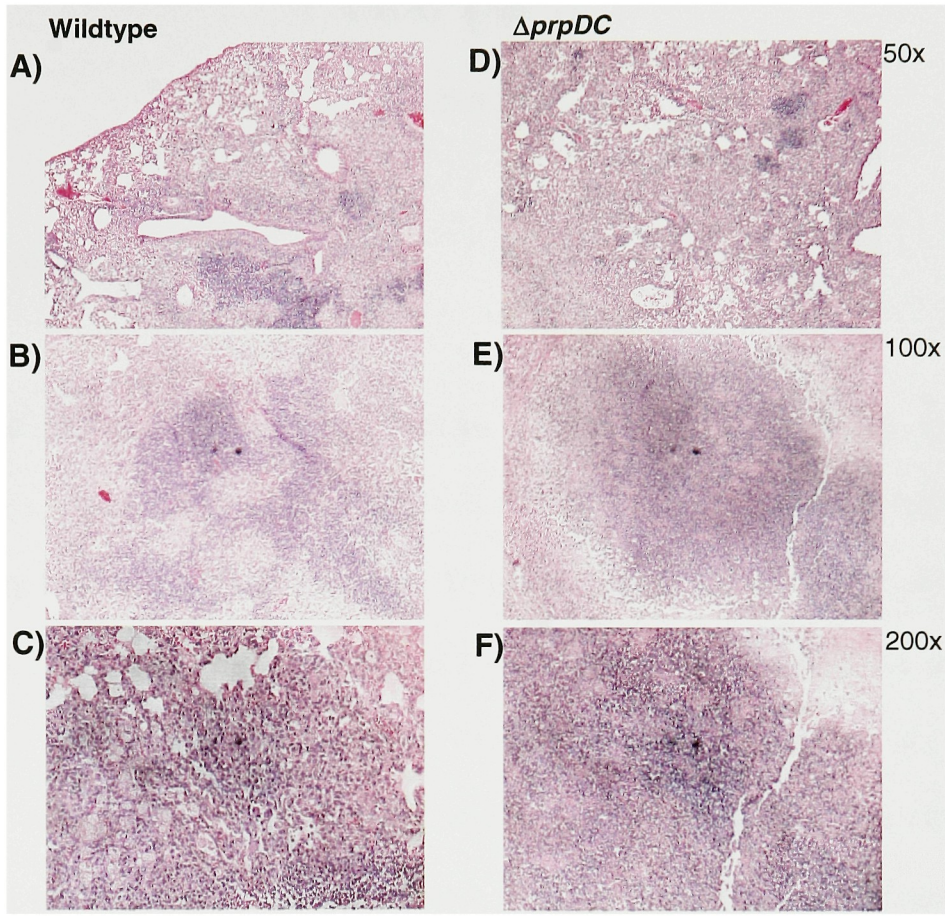


**Fig. 10.6. Lung pathology in mice infected with Δ*prpDC M. tuberculosis*.** (**A**-**F**) Histopathology of H&E (haematoxylin-eosin)-stained lungs from C57BL/6 mice infected with *M. tuberculosis* (Erdman) (~100 CFU, respiratory route) at 10 weeks post-infection. Bacterial strains: (**A**-**C**) wild-type, (**D**-**F**) Δ*prpDC*. Corresponding CFUshown in Fig. 10.5.

# PART III

# CHAPTER 11

# Discussion

## INTRODUCTION

Some of the most devastating diseases known to man, including syphilis, gonorrhea, typhoid fever, and tuberculosis (TB) are caused by bacteria capable of persisting in their hosts often for life. To our detriment, we know little about the interactions between hosts and pathogens that allow the establishment of such persistent infections (Muñoz-Elías & McKinney, 2002; Stewart *et al.,* 2003; Monack *et al.,* 2004). Regardless of whether a persistent infection is clinically active or latent, the establishment of such infection means that the pathogen will most likely succeed in being transmitted to a new host, even if it must wait, sometimes, for a lifetime. Investigation into the nature of bacteria during infection, including the bacterial characteristic *in vivo* physiology and elicited host immune responses, and the way pathogens evade and counteract these defenses successfully, might provide a foundation on which to base efforts toward developing new drugs and vaccines against these foes.

Persistence of *M. tuberculosis* in the host is the result of the pathogen's interaction with a partially effective immune response, i.e., one that controls

bacterial growth but fails to eradicate the infection (Flynn & Chan, 2001; Nathan & Ehrt, 2003; North & Jung, 2004). In order to persist, *M. tuberculosis* must adapt to environmental conditions that are complex, and at least in the case of chronic active disease, constantly changing as a result of lesion-specific varying degrees of ongoing inflammation, cellular infiltration, tissue destruction, and further bacterial replication and dissemination (Vandiviere *et al.,* 1956; Wayne & Salkin, 1956; Kaplan *et al.,* 2003). The bacterial factors involved in survival *in vivo* have begun to be identified through a combination of reverse genetics (Glickman & Jacobs, 2001; Gomez & McKinney, 2004; Boshoff & Barry, 2005) and differential gene expression in macrophages of murine and human origin (Dubnau & Smith., 2003; Schnappinger *et al.,* 2003; Voskuil *et al.,* 2003).

Bacterial gene expression studies of mouse lung-grown bacteria suggest that the adaptation of *M. tuberculosis* to the *in vivo* environment involves the differential expression of genes responsive to iron limitation, specific carbon metabolism, and cellular hypoxia (Shi *et al.,* 2003; Timm *et al.,* 2003); limited studies with infected human samples suggest that some of these adaptations might also occur in the human lung (Timm *et al.,* 2003).

Despite having preoccupied the mind of early investigators of tuberculosis during the first half of the 20$^{th}$ century, two major aspects of the *in vivo* biology of *M. tuberculosis* are still poorly understood: 1) the replicational status of *M. tuberculosis* during active, and latent infection, and 2) the metabolic activity of *M. tuberculosis* during growth and survival in the host. The work I have carried out

over the last few years, and which I have presented in this thesis, was performed with the intention of shedding some light on these two major issues.

### 11.1. The replication status of *M. tuberculosis* in experimentally infected mice

Bacteriologic and pathologic studies of tuberculous human and animal lesions indicate that *M. tuberculosis* in the lung resides in different types of lesions, which differ from each other in terms of cellular organization, degree of inflammation and necrosis, as well as apparent bacterial burden (Lurie & Zappasodi, 1942; Medlar, 1948; Medlar *et al.,* 1952; Dannenberg & Rook, 1994a; Kaplan *et al.,* 2003). The replicational status of the *M. tuberculosis* in such lesions has not been determined, but it is thought to vary between active replication and stasis. Replication is believed to occur on the surface of open cavities and to take place intracellularly within macrophages there (Kaplan *et al.,* 2003), and within the softened center of liquefied cavities, where bacteria might multiply extracellularly (Lurie, 1964; Dannenberg & Rook, 1994a; Grossett, 2003). In Chapter 5, we presented data from experiments performed to explore this question in the mouse model of chronic TB. It has been established that immediately after *M. tuberculosis* enters the host, robust bacterial multiplication is necessary to ensure the establishment of the infection and the steady plateau of viable bacteria that characterizes the persistent phase of the infection in lungs of mice (Orme, 1994). However, little is known about growth rates during this phase of the infection. As

first suggested by Severs and Youmans (1956), fluctuations in bacterial viable populations can be readily measured, but these presumably represent the net result of multiplication and destruction by host defenses. In a pioneering work, Rees and Hart (1961) addressed this question by tallying total bacterial counts determined by staining bacterial bodies and comparing these to viable counts (CFU). Based on the finding that total and viable bacterial counts overlapped throughout the persistent phase of infection in mice, they concluded that persistent bacteria were not actively replicating, and favored a model of "static" equilibrium. However, in those experiments, the lack of accumulation of bacterial bodies that was observed may have been due to loss of acid-fast staining of dead bacterial bodies, a phenomenon that has been reported by other investigators (McCune *et al.,* 1956; Middlebrook, 1952). Thus, we revisited this question.

Viable counts (CFU) obtained from mouse lungs at intervals during the chronic phase of a mouse infection were compared with total counts determined by quantitative real time PCR. Amplification of chromosome equivalents (CEQ) was performed directly from infected lungs by adapting a recently developed real time PCR technique used for the precise molecular quantification of mRNA (Manganelli *et al.,* 1999; Timm *et al.,* 2003). Remarkably, we found that at all points analyzed (4, 8, 12 and 16 weeks post-infection) CFU and CEQ curves closely matched each other. If constant bacterial turnover during persistence occurred, we would have detected the resulting accumulation of bacterial genomes (CEQ), assuming that the genomes were not removed/degraded soon after the

bacteria were killed. Since we did not observe significant build-up of dead bacilli in any of the chronically infected mouse lungs analyzed, we conclude that most bacilli in the persistent phase of infection are likely undergoing little or no replication.

The assumption that genomes of killed bacteria are relatively stable was based on control experiments where genomes from dead bacteria were shown to linger in infected lungs (Fig. 5.3). INH therapy started at the onset of the persistence phase (4 weeks post-infection) relatively rapidly reduced viable bacterial counts (CFU) by ~2 and 4 $\log_{10}$ by 4 and 8 weeks of treatment, respectively. In contrast, CEQ did not diminish after 4 weeks of treatment compared to those in lungs of concomitantly infected but untreated mice, and did so only slightly after 8 weeks of treatment (Fig. 5.3). The waxy coat of the tubercle bacillus, made up of unique glycol-lipids (Brennan, 2003), likely contributes to the permanence of bacterial bodies and their DNA content (de Wit *et al.,* 1995; Rees and Hart, 1961; Seiler *et al.,* 2003).

It is possible that killing of bacilli by immune mechanisms as opposed to drugs as done here, may lead to genome degradation. However, this possibility could not be tested using the mouse model of chronic TB because the immune response against *M. tuberculosis* infection is not bactericidal, but bacteriostatic. Also, our results also do not rule out the possibility that the stable number of viable counts in the lungs of chronically infected mice is due to replication *in situ*

balanced by trafficking of organisms out of the lungs, resulting in failure of both CFU and CEQ to accumulate over time.

A key question in the context of any persistent infection is the replication status of the pathogen over time. Dynamic pathogens such as the human immunodeficiency virus (HIV) and hepatitis C virus (HCV) replicate at very high rates throughout the course of infection. In persistently HIV- and HCV-infected individuals a dynamic equilibrium is achieved in which a relatively stable viral load is maintained by balanced production and immune-mediated destruction of virions (Pawlotsky, 2004; Simon & Ho, 2003). Viral persistence is also facilitated by antigenic variation, involving sequential emergence of variants that temporarily escape surveillance by the adaptive immune response. Antigenic variation is an essential persistence strategy of many slower-replicating pathogens as well, including parasites and bacteria; well-characterized examples include African trypanosomes, *Plasmodium falciparum* and *Borrelia* spp. These pathogens display oscillating population kinetics *in vivo*, representing sequential waves of antigenic variants that (temporarily) escape destruction by the adaptive immune response (Deitsch *et al.,* 1997; Reviewed in Chapter 1).

Although antigenic variation is a common feature of many persistent pathogens, there is little evidence to support a role for antigenic variation in *M. tuberculosis* persistence in chronically infected individuals (Cole, 2002). We propose that *M. tuberculosis* represents a third persistence strategy, where exponential replication of the organism during the acute phase of infection is

followed by a relatively static equilibrium, a state of slow- or nonreplicating persistence, during the chronic phase. The hypothesis that persistent mycobacteria are in a slow- or non-replicating state is consistent with several observations: (i) the induction of a gene expression program that is characteristic of non-replicating bacilli during the chronic phase of murine infection (Shi *et al.,* 2003); (ii) the relatively poor efficacy of drugs that target cell wall synthesis (such as INH) when administered to chronically infected mice as compared to acutely infected mice (McCune *et al.,* 1956); (iii) the increased thermal stress resistance of *M. tuberculosis* (characteristic of stationary-phase cells) in chronically infected mice as compared to acutely infected mice (Wallace, 1961); (iv) the lack of accumulation of microscopically visible AFB in chronically infected mice (Rees and Hart, 1961); (v) the lack of accumulation of bacterial chromosomes in chronically infected mice we described here (Muñoz-Elías *et al.,* 2005). Kanai (1966) provided direct proof of the ability of non-replicating *M. tuberculosis* to survive for extended periods *in vivo* by showing that continued administration of streptomycin was not necessary for persistence of a streptomycin-growth dependent strain of *M. tuberculosis* in chronically infected mice.

The ability of *M. tuberculosis* to persist without replicating does not imply that bacterial replication is entirely absent in chronically infected individuals or animals, nor does it imply that persistent bacteria are metabolically inactive. Indeed, slow fluctuations in the bacterial load over time, which are typically observed in chronically infected mice, presumably reflect slow or intermittent

replication of at least some bacilli (Sever & Youmans, 1957), and the dramatic increase in bacterial load in late-stage infected mice (Orme, 1988) can only be explained in terms of bacterial replication. Evidence from the *M. marinum*-leopard frog model also supports the idea that chronic mycobacterial infections involve some ongoing bacterial cell division (Bouley *et al.,* 2001). What seems increasingly likely, however, is that *M. tuberculosis* persistence in chronically infected mice does not represent a highly dynamic equilibrium in which rapid bacterial growth is balanced by equally rapid immune-mediated killing, but rather a relatively static equilibrium in which the rate of bacterial cell division is very slow. It is unknown to what extent this conclusion can be extrapolated to humans with chronic TB or latent TB infection. Studies on TB lesions in humans suggest that the replicative state of the bacteria vary depending on the histopathological characteristics of the individual lesions (Kaplan *et al.,* 2003; Medlar *et al.,* 1952; Vandiviere *et al.,* 1956; Wayne & Salkin, 1956).

The replicative status of bacilli during persistent infection has important implications for the development of more effective interventions against persistent *M. tuberculosis* infections. Treatment of TB is notoriously difficult and protracted, requiring a minimum of 6-9 months of multi-drug therapy to prevent post-therapy relapse (El-Sadr *et al.,* 2001). Most of the drugs currently used to treat TB are known to target cellular processes involved in cell growth, such as cell wall biogenesis and DNA replication; consequently, these drugs are less effective against bacteria that are not undergoing rapid cell division (McKinney,

2000; Stewart *et al.,* 2003; Mitchison, 2004; Boshoff and Barry, 2005). Elucidation of the mechanisms required for persistence of *M. tuberculosis* in a slow- or non-replicating state in chronically infected individuals with active tuberculosis or latent infection might suggest new strategies for targeting persistent bacteria.

## 11.2. The Glyoxylate Cycle and the Pathogenesis of *M. tuberculosis*

All pathogens must acquire nutrients from their hosts in order to replicate and survive long enough to ensure their transmission to a new host. Most facultative intracellular bacteria grow in specialized phagosome-derived vacuoles (Sinai & Joiner, 1997); only a few species can escape the phagosomal compartment and grow freely in the cytoplasm. It is not clear whether facultative vacuolar pathogens do not grow in the cytosol simply because they lack the machinery to break free from their vacuoles. Some evidence suggests that even if they reached the cytosol, these pathogens would not be able to grow in it readily, suggesting that the cytosol is not a very permissive compartment for growth and that special adaptations are required to occupy this niche. By microinjecting several bacterial species into the cytosol of host cells, Goetz and colleagues (2001) found that pathogens that normally reside in a vacuole, e.g., *Salmonella*, could not freely replicate in the cytosol, while those that infect the cytosol as part of their normal life cycles such as *Shigella* or *Listeria* could. *M. marinum*, a mycobacterial

pathogen of fish, apparently can escape the phagosome and move around in the cytosol, however, it is unclear whether it can replicate in this compartment (Stamm *et al.,* 2003).

The high level of specialization required by a pathogen to successfully inhabit a given niche likely involves mechanisms that allow it to acquire and metabolize the nutrients available in that environment. With few exceptions, the substrates pathogens utilize during infection and the mechanisms involved in their acquisition and assimilation are unknown (Reviewed in Chapter 4).

Carbohydrates comprise the principal carbon source for bacteria occupying such diverse niches as the intestinal lumen (Peekhaus & Conway, 1998; Chang *et al.,* 2004), the cytoplasm of infected host cells (Chico-Calero *et al.,* 2002), and perhaps the modified vacuoles within macrophages (Eriksson *et al.,* 2003). In contrast, pathogenic mycobacteria isolated from lung tissues preferred to oxidize fatty acids rather than carbohydrates (Segal & Bloch, 1956). Of the activities required for fatty acid catabolism, GC enzymes ICL and MLS were demonstrated in extracts of both slow and fast growing mycobacteria including *M. tuberculosis* and *M. leprae* (Murthy *et al.,* 1973; Kannan *et al.,* 1985; Bharadwaj *et al.,* 1987; Wheeler & Ratledge, 1988). That these activities were found in *in vivo* grown bacteria, as well as upregulated specifically in stationary phase "old" cultures, led some of these early investigators to suggest that GC-mediated metabolism might be the basis of alternate types of metabolism employed by *M. tuberculosis* to

persist in the host and to avoid eradication by drugs (Murthy *et al.,* 1973; Kannan *et al.,* 1985; Bharadwaj *et al.,* 1987; Gupta & Katoch, 1997).

More recently, mycobacterial genes and enzymes involved in fatty acid utilization including the GC and fatty acid β-oxidation have been shown to be up-regulated during infection of mice (Timm *et al.,* 2003; Dubnau *et al.,* 2005), and macrophages of mouse and human origin (Graham & Clark-Curtiss, 1999; Dubnau & Smith, 2003; Schnappinger *et al.,* 2003). A central role for fatty acid metabolism is also suggested by the extensive duplication of genes involved in lipid breakdown and assimilation in *M. tuberculosis*, *M. bovis* and *M. leprae* (Cole 1998; Cole *et al.,* 2001; Fleischmann *et al.,* 2002; Garnier *et al.,* 2003). Induction of multiple genes encoding analogous functions in β-oxidation (multiple *fadDs*, *fadBs* and *fadAs*) suggests that these might be involved in the degradation of different types of C2 substrates, as it occurs in mammalian cells (Kunau *et al.,* 1995).

The *M. tuberculosis* genome encodes only two homologous genes encoding isocitrate lyases which catalyze the first step in the GC required for anaplerosis during growth on fatty acid substrates. Previously, we reported that *icl1* is dispensable for acute-phase growth but essential for persistence at wild-type levels during the chronic phase of infection in *M. tuberculosis*-infected mice, and proposed that *M. tuberculosis* might switch to fatty acid catabolism at the transition from the acute to the chronic phase of infection (McKinney *et al.,* 2000; Chapter 4).

**On the essential role of isocitrate lyase (ICL) in *M. tuberculosis in vivo* growth and survival.** In Chapter 6, we present evidence that ICL1 and ICL2 provide overlapping and essential roles in metabolism of *M. tuberculosis* during infection. Deletion of *icl1* or *icl2* alone did not impair replication in macrophages, or in the lungs of mice. In contrast, deletion of both *icl1* and *icl2* abrogated growth, and led to rapid clearance of the *Δicl1/Δicl2* mutant bacteria from the lungs of mice. Our new findings that ICL activity is required from the onset of the infection indicate that ICL-mediated metabolism and fatty acid catabolism have a more fundamental role during infection than we had previously envisioned.

Although the essentiality of the GC for growth and survival of *M. tuberculosis* in the host suggests primacy of fatty acid metabolism, this does not rule out the possibility that *M. tuberculosis* might also have access to carbohydrates. Irrespective of the carbon source, anaplerosis is necessary to replenish intermediates of the TCA cycle, such as oxaloacetate , succinyl-CoA, and α-ketoglutarate, which are siphoned off to biosynthetic pathways (Fig. 11.1; See also Chapter 3). However, the anaplerotic pathways bacteria employ depend on carbon availability and growth conditions (Kornberg & Krebs, 1957; Kornberg & Sadler, 1960; Ornston & Ornston, 1969; Cronan & LaPorte, 1996; Holms, 1996; Fischer & Sauer, 2003). For instance, in batch culture in excess glucose medium, *E. coli* replenishes oxaloacetate by carboxylation of phosphoenolpyruvate (PEP) or pyruvate, via PEP carboxylase (PPC), or pyruvate carboxylase (PCA), respectively (Anderson & Wood, 1969; Peng & Shimizu,

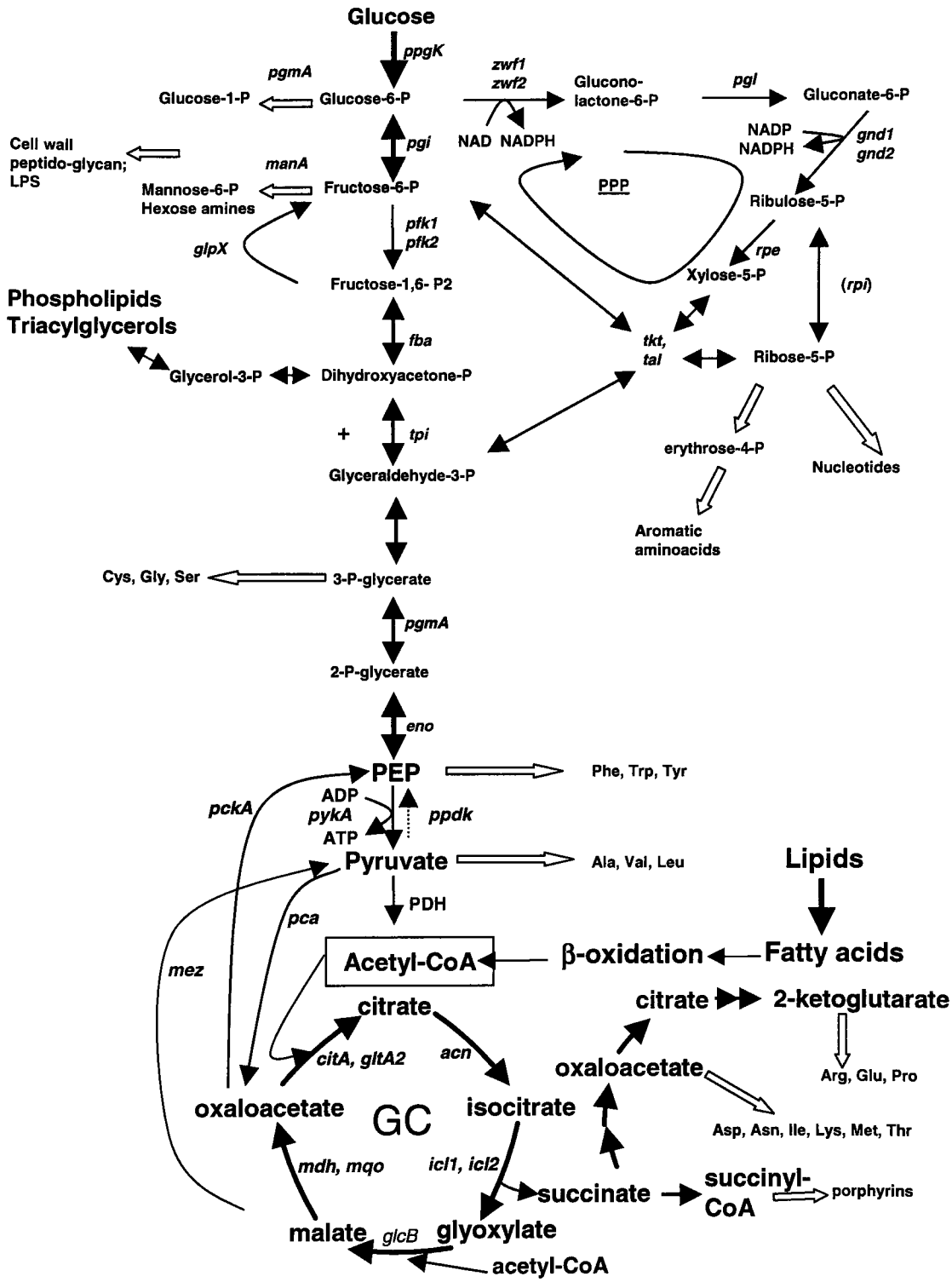


**Fig. 11.1. Intermediary metabolism in *M. tuberculosis*.** It is proposed that in *M. tuberculosis* the Glyoxylate Cycle (GC) plays a central role in intermediary metabolism where it keeps the balance between anaplerotic and biosynthetic reactions of central carbon metabolism.

2003). However, during slow growth in glucose-limited medium, *E. coli* employs the GC for C4 anaplerosis (Emmerling *et al.*, 2002; Fischer & Sauer, 2003). Likewise, the GC is the predominant anaplerotic pathway during growth of *C. glutamicum* on mixed substrates (glucose and short-chain fatty acid) (Wendisch *et al.*, 2000). Interestingly, *A. nidulans* growing on carbohydrate and acetate mixtures maintains ICL activity comparable to that seen during growth on acetate alone, which is much higher than on carbohydrates alone (Armitt *et al.*, 1970).

Studies of carbon flux in nonmodel bacteria suggest that during growth on glucose bacteria rely on shunting of malate, which can be generated by the GC, to pyruvate and then to oxaloacetate, as preferred way of replenishing TCA cycle intermediates. A potential role the GC in anaplerosis during growth on glucose was suggested long ago based on the fact that the slow growth on glucose of a Δ*pfk E. coli* mutant -likely the result of reduced carbon flow into glycolysis- could be compensated by constitutive expression of *icl* (Vinopal & Fraenkel, 1974) probably by restoring PEP concentrations to optimal levels (Kornberg & Smith, 1970). Thus, the GC might be required for *M. tuberculosis* metabolism *in vivo*, regardless of whether carbohydrate concentrations are limiting or not.

**Carbon utilization studies of Mycobacteria.** Though studies of carbon metabolism, mostly utilizing $^{14}$C-labeled carbon substrates, indicate that *M. tuberculosis* and other mycobacteria readily respire a variety of carbon substrates including including glucose, glucose 6-phosphate, 6-phosphogluconate, pyruvate, citrate, succinate, glutamate, and palmitate (Wheeler, 1984), few, if any studies

have actually documented carbon substrate-specific growth. Carbon utilization/metabolic studies in mycobacteria have been greatly limited by our inability to grow these slow-growing fastidious bacilli in defined minimal media (Kondo & kanai, 1976; Katoch *et al.*, 1987). Even in the standard relatively rich Middlebrook/Dubos media the tubercle bacillus has a doubling time of ~ 20 hours.

Our initial experiments on defined media supplemented with different C2/C3 fatty acid substrates indicate that deletion of the first step of the GC results in an inability to grow on C2 substrates. Unexpectedly, however, the mutants are also impaired on combinations of carbohydrates and fatty acids (Chapter 9), indicating that *in vitro M. tuberculosis* relies on the GC for fatty acid metabolism even in the presence of carbohydrates. That *M. tuberculosis* lacking both ICL1 and ICL2 shows a slight lag during growth on glucose suggests that some carbon might flow through the GC even when glucose is the major carbon substrate present (Chapter 6). We cannot rule out that the slight phenotype of the Δ*icl1*/Δ*icl2* mutant observed is due to the presence of Tween-80 (0.05 w/v) in the media, which is included to prevent clumping. However, we see no such lag during growth of the Δ*icl1*/Δ*icl2* on glycerol media. Furthermore, we have observed that the Δ*icl1*/Δ*icl2 M. smegmatis* mutant is also impaired for growth on mixed substrates where it shows a lag phase compared to dextrose or glycerol media (not shown).

The central role of the GC in *M. tuberculosis* metabolism is further suggested by the apparent absence of PEP carboxylase (PPC) (Cole *et al.,* 1998; Fleischman *et al.,* 2002). PPC is the canonical enzyme used for C4 anaplerosis from sugars in prokaryotes (Anderson & Wood, 1969; Peng *et al.,* 2004) (Fig. 11.1). Adaptation to low concentrations of glycolytic substrates might also involve reduced flux of carbon through glycolysis to conserve glucose-6-P and its derivatives for biosynthetic pathways (Fig. 11.1). In agreement, genes encoding a putative high-affinity carbohydrate transporter (*lpqY*, *sugA-C*) and a glucokinase (*ppgK*) are important for *in vivo* growth of *M. tuberculosis* (Sassetti & Rubin, 2003), whereas the glycolytic steps catalyzed by triosephosphate isomerase (*tpi*), glyceraldehyde 3-phosphate dehydrogenase (*gap*), and phosphoglycerate kinase (*pgk*), which are important for *in vitro* growth on standard 7H10 plates supplemented with glucose (Sassetti *et al.,* 2003), might be dispensable *in vivo* (Sassetti & Rubin, 2003). These observations suggest that during infection, PEP, which is required for the biosynthesis of aromatic amino acids and the peptidoglycan precursor UDP-N-acetylmuramic acid, might be generated not by glycolysis but by conversion of oxaloacetate to PEP by PEP carboxykinase (PCK) (Fig. 11.1). Consistent with this idea, expression of *pckA* encoding PCK (Fig. 11.1) is upregulated in the lungs of *M. tuberculosis*-infected mice (Timm *et al.,* 2003). Further, *M. bovis pckA* mutants are highly attenuated in guinea pigs (Collins *et al.,* 2002), and an *M. bovis* BCG *pckA* mutant is attenuated in SCID mice and in murine macrophages (Liu *et al.,* 2003b).

PEP not consumed in biosynthesis could flow into gluconeogenesis to provide essential precursors such as glucose-6-P. Alternatively, it could be further oxidized to $CO_2$ through the PEP-glyoxylate cycle (Fischer & Sauer, 2003) (Fig. 11.2A), a pathway described similar to one described long ago as the dicarboxylic acid cycle (Kornberg & Sadler, 1960). The PEP-GC has been shown to operate in *E. coli* cells when these bacteria are shifted to slow growth in very low levels of glucose (<0.002%) – below concentrations that exert cAMP-mediated catabolite repression (Notley-McRobb *et al.,* 1997; Matin & Matin, 1982). Under these conditions, up to 80% of the cell's oxaloacetate is provided by the GC. During this shift, *E. coli* cells drastically downregulate PPC (~12 fold) and IDH (~4 fold), while upregulating ICL (~15 fold) and PCK (~10 fold). Other than the TCA cycle, the PEP-GC cycle is the only known energy-yielding pathway capable of complete oxidation of PEP to $CO_2$ (Fischer & Sauer, 2003) (Fig. 11.2A). If the major carbon substrate available to the cell is acetyl-CoA (as during growth on fatty acids), this routing of carbon would allow the oxidation of acetyl-CoA to $CO_2$, while also providing PEP and pyruvate for biosynthesis. Under these conditions reactions of the TCA cycle might be needed mostly to supply glutamtate (Kornberg & Sadler, 1960). Alternatively, the pyruvate produced from PEP might instead be recycled back to oxaloacetate, as it occurs during the operation of the pyruvate shunt in *B. subtilis* (Diesterhaft & Freese, 1973) and several other less studied bacterial species (Fuhrer *et al.,* 2005).

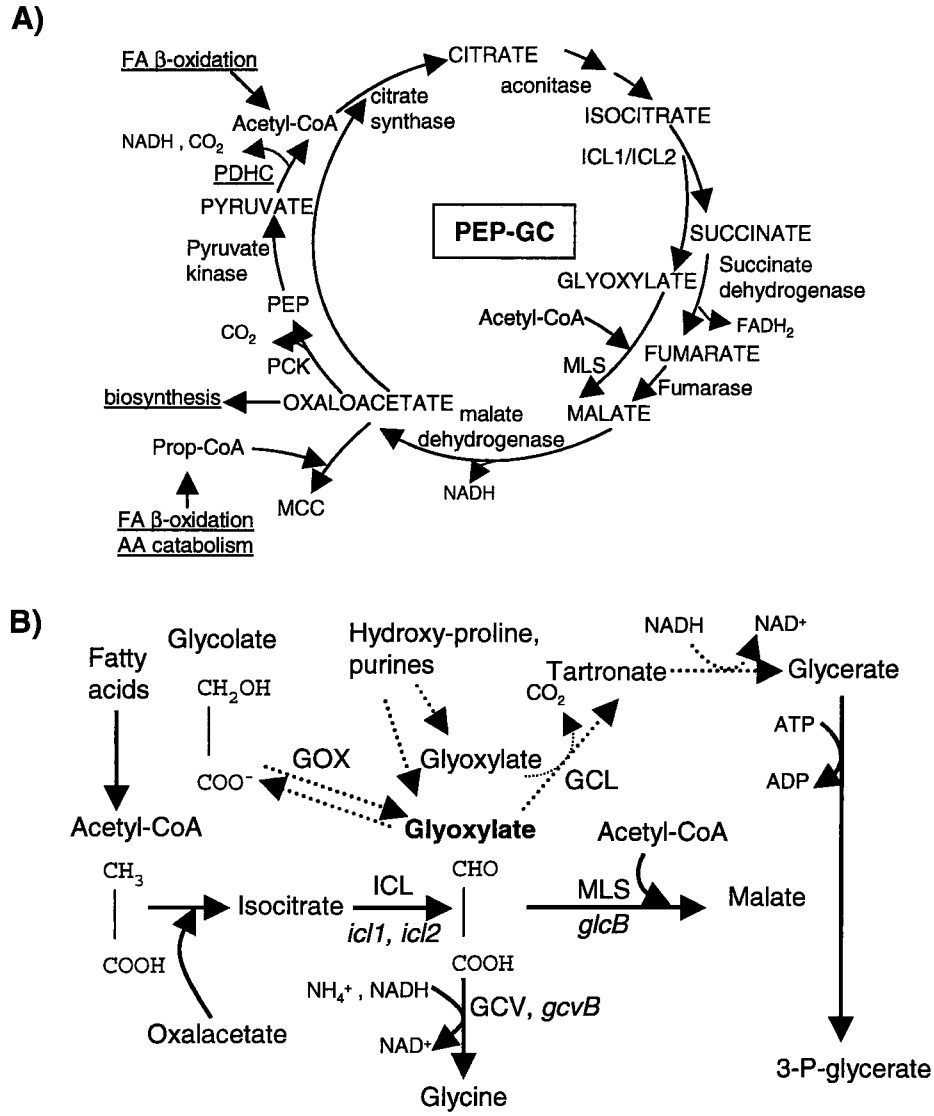


**Fig. 11.2. The PEP-GC cycle and the metabolism of glyoxylate.** (**A**) PEP-GC cycle. Catabolism of carbohydrates and fatty acids yields acetyl-CoA that feeds into the PEP-GC cycle and or the TCA cycle (not shown). GC cycle reactions ICL and MLS together with phosphoenol pyruvate carboxykinase (PCK), pyruvate kinase (PYK) and the pyruvate dehydrogenase complex (PDHC) together allow the oxidation of acetyl-CoA/glyoxylate to $CO_2$. (**B**) Reactions converging on glyoxylate (Glx). Glx can be a substrate for MLS, glycine dehydrogenase (GCV), or Glx carboligase (GCL). Dotted lines indicate reactions which based on BLASTP searches are probably absent in *M. tuberculosis*.

**Potential sources of fatty acids for *M. tuberculosis* during infection.** The sources and chain-lengths of fatty acids utilized by *M. tuberculosis* during infection are unknown. A possible supply is long-chain phospholipids freed from cell (phagosomal) membranes by phospholipases. In *C. neoformans*, extracellular phospholipases, which avidly digest naturally occurring phospholipids of the lung surfactant such as dipalmitoyl phosphatidylcholine and phosphatidyl glycerol (Santangelo *et al.,* 1999), are required for virulence (Cox *et al.,* 2001). In accord with this idea, four *M. tuberculosis* genes encoding cell wall-associated phospholipase C isoforms are up-regulated during infection of macrophages and are jointly essential for normal growth in the lungs of mice (Raynaud *et al.,* 2002).

In addition to phospholipids, Triacylglycerols (TAGs) present in most mammalian cells are also a potential exogenous source of carbon that could be used by *M. tuberculosis* during infection. TAGs are highly reduced molecules with very high energy content, e.g., 1 gram of triacylglycerol can provide as much as 6 times the energy found in 1 gram of glycogen, and are well suited for energy storage. TAGs could also constitute an internal lipid reserve which *M. tuberculosis* might draw upon when external supplies of carbon become limiting. Though TAGs are common in eukaryotes, their occurrence in prokaryotes is rare. In fact, the only bacteria known to produce and store large amounts of TAGs are members of the actynomycete family which includes Mycobacterium, Rhodococcus and Nocardia, among other genera (Alvarez & Steinbuchel, 2002). Remarkably, fatty acids from endogenous TAGs can make up to 78% of the dry

weight of a Rhodococcus cell (Alvarez *et al.,* 1996). Recent evidence suggests that *M. tuberculosis* encodes several functional TAG synthases (*tgs*) (Daniel *et al.,* 2004). These synthases are upregulated when *M. tuberculosis* bacteria are subjected to an *in vitro* model of persistence in which bacteria enter a nonreplicative state induced by slow oxygen reduction and exposure to low nitric oxide (Daniel *et al.,* 2004). Interestingly, Nocardia species initiate synthesis and accumulation of TAGs during late log phase and continue accumulating the compounds throughout stationary phase for as long as carbon sources are available. The *in vitro* model used by Daniel and colleagues (Voskuil *et al.,* 2003) and stationary phase *in vitro* growth conditions are known to elicit *M. tuberculosis* gene expression patterns that significantly overlap (Muttucumaru *et al.,* 2004; Voskuil *et al.,* 2004). Thus, the stimulus for TAG synthesis might be carbon substrate availability and/or replication rates rather than levels of oxygen in the flasks of the "non-replicational persistence" model (Wayne & Sohaskey, 2001) used by Daniel and colleagues (2004).

When external carbon sources become unavailable, Rhodococcus species readily begin to mobilize their internal reserves of TAGs, eventually breaking down up to 90% of such reserves (Alvarez *et al.,* 2000). The initial breakdown of TAGs requires serine hydrolases known as lipases (Gupta *et al.,* 2004), and *M. tuberculosis* has circa twenty genes that could encode such functions *(lipC-lipW)*. Although these genes are not induced during the initial 24-48 hr of infection in macrophages (Schnappinger *et al.,* 2003), this might be expected if TAGs are

needed only during the persistent phase of the infection after external carbon substrates become unavailable. Fatty acids released from TAGs are likely oxidized via β-oxidation, in much the same way it occurs during early development of some plant seedlings. For instance, *A. thaliana* seedlings lacking a peroximal 3-ketoacyl-thiolase do not grow normally because they cannot oxidize TAG-derived long chain fatty acids, which end up accumulating in their cells. Interestingly, postgerminative growth and seedling establishment in oilseed plants is dependent not only on β-oxidation but also on the GC. Seedlings lacking ICL are impaired during seedling establishment (Eastmond *et al.,* 2000), and ICL is upregulated specifically during the first days of seedling development (Runquist & Kruger, 1999). Thus, utilization of fatty acids resulting from TAG hydrolysis might well require a functional GC regardless of whether glycerol or other carbohydrates are present.

It is tempting to speculate that the phenotype of Δ*icl1 M. tuberculosis* mutant might result from an increased bacterial reliance on these stored sources of fatty acids during the persistence phase of the infection. At the beginning of the infection mixed substrates might be available requiring limited carbon flux through the GC, which our data suggests could be provided by either ICL1 or ICL2. Later on, as the bacteria switch entirely to fatty acids, their reliance on ICL might increase accordingly. That *M. tuberculosis* experiences nutritional stress during persistence is suggested by the finding that a mutant lacking *relA* – a gene

of the stringent response to starvation – is severely impaired for persistence (Dahl *et al.,* 2003).

**The methylcitrate cycle of *M. tuberculosis.*** β-oxidation of odd-chain fatty acids generates both acetyl-CoA (C2) and propionyl-CoA (C3). Propionyl-CoA can be metabolized via several routes including the citramalate, methylmalonyl-CoA, and methylcitrate pathways (Halarnkar & Blomquist, 1989). Mounting evidence indicates that the methylcitric acid cycle (MCC) (Fig. 10.1) is required for propionate catabolism in several eubacteria and fungi (Tabuchi & Uchiyama, 1975; Horswill & Escalante-Semerena, 1999; Brock *et al.,* 2001). In all these species, a dedicated MICL is present, which cannot function as an ICL (e.g. has no activity on isocitrate) (Luttik *et al.,* 2000; Brock *et al.,* 2001). The *M. tuberculosis* genome contains homologs of the enzymes catalyzing the first two steps of the MCC. These are methylcitrate synthase (*prpC*, Rv1131) and methylcitrate dehydratase (*prpD*, Rv1130), and they are up-regulated during infection of macrophages and *in vivo* (Schnappinger *et al.,* 2003).

Since a methylisocitrate lyase (MICL, *prpB*) homolog is apparently absent from *M. tuberculosis*, and ICL1 and ICL2 are essential for growth of *M. tuberculosis* on propionate as the sole carbon source (Chapter 6), we postulated that ICL1 and ICL2 might subsume this function. ICLs and MICLs share ~25% sequence homology and while all predicted ICLs have the catalytic site residues K[KQ]CGH, which constitute a signature motif , methylisocitrate lyase enzymes (*prpB*) have the signature sequence KRCGH at their catalytic site; mycobacterial

ICL1 and ICL2 predicted proteins have the motif KKCGH. Nonetheless, ICL from *S. cerevisiae* has weak MICL (Luttik *et al.,* 2000). Here, we provide evidence that ICLs and in particular ICL1 can function as MICL (Chapter 10). The idea that *prpB* is indeed missing, and that ICL1 and/or ICL2 substitute for this enzyme in *M. tuberculosis* is suggested by the lack of MICL activity in extracts from the Δ*icl1*/Δ*icl2* mutant. Furthermore, an Δ*icl2* mutant and a strain overexpressing ICL1 had normal and elevated levels of MICL, respectively, while a Δ*icl1* mutant extracts had negligible activity on methylisocitrate.

Some bacteria, including *E. coli* and *Ralstonia eutropha*, which require the MCC for propionate metabolism and have a dedicated MICL (*prpB*), depend on the GC presumably for anaplerosis (Textor *et al.,* 1997; Bramer & Steinbuchel, 2001). However, *Salmonella* mutants lacking ICL or MLS are not impaired for growth on propionate media (Horswill & Escalante-Semerena, 1999). In these cases, anaplerosis during MCC-mediated propionate metabolism is probably carried out by PCA, or perhaps via PPS together with PPC, rather than the GC. Data presented in Chapter 9 indicating that the GC is dispensable for growth on propionate by *M. smegmatis*, which unlike *M. tuberculosis* encodes a dedicated MICL (*prpB*), suggest that mycobacteria, similarly to *Salmonella*, does not require the GC for anaplerosis on propionate, and instead likely carry out anaplerosis via PCA (Mukhopadhyay & Purwantini, 2000) during growth on C3 subtrates.

Experiments directly assessing the role of the MCC in *M. tuberculosis* C3FA utilization and pathogenesis are presented in Chapter 10. Here we provide

evidence that metabolism and growth on C3 substrates such as propionate and valerate by *M. tuberculosis* requires the genes putatively encoding methylcitrate synthase (PRPC) and methylcitrate hydratase (PRPD). The requirement for this pathway *in vivo* is less clear. In an ongoing experiment, the Δ*prpDC M. tuberculosis* mutant showed no difference compared to wild-type up until wk 10 post-infection in both bacterial burden and pathology (Chapter 10). However, the Δ*prpDC* mutant was impaired for growth intracellularly in mouse primary macrophages. These contrasting findings underscore the fact that significant differences exist between the metabolic requirements of bacteria depending on whether they are grown *in vitro, ex-vivo*, or *in vivo*.

It is possible that C3FAs are used as carbon sources during growth in macrophages in tissue culture, but that *in vivo* other substrates become available, thus lessening bacterial dependence on C3 substrates. Alternatively, during infection *M. tuberculosis* might be able to turn on alternative pathways of C3 metabolism, which it fails to engage during growth in the macrophage model. *M. tuberculosis* has several putative propionyl-CoA carboxylases (*accD* genes), and methylmalonyl-CoA mutase genes (*mutA* and *mutB*), which may allow propionyl-CoA utilization via the methyl-malonate-CoA pathway. Several of these putative propionyl-CoA carboxylases are found in the vicinity of *fadD* and *fadE* genes, suggesting they might be involved in C2/C3 catabolic pathways.

**Potential of ICL1 and ICL2 as drug targets.** The complete reliance on ICL for replication of *M. tuberculosis in vivo* suggests that inhibiting both ICLs

might have potent therapeutic activity. Experiments presented in Chapter 8 in which the effect of blocking only one ICL (ICL1) in the context of chemotherapy with isoniazid, in the mouse model of chronic TB, and with isoniazid and rifampin, in a modified Cornell model of drug-induced latency, indicate that inhibition of ICL1 alone would not significantly improve currently available drug regimens. Thus, both ICL1 and ICL2 will likely need to be blocked by any future ICL-inhibiting drug for it to be effective. The feasibility of blocking ICL during infection as a therapeutic approach is supported by our demonstration that the prototype ICL inhibitor 3-nitropropionate (Schloss & Cleland, 1982; Sharma *et al.,* 2000), which inhibits both ICL1 and ICL2 in the tube (Höner zu Bentrup *et al.,* 1999), effectively impedes replication of *M. tuberculosis* on fatty acids and in macrophages (Chapter 6). *M. smegmatis* Δ*icl1*/Δ*icl2* mutant bacteria expressing wildtype *M. tuberculosis icl1* and *icl2* genes have been constructed and their usefulness as tools for testing potential inhibitors of ICLs is supported by data presented in Chapter 9. These strains are currently being employed to test lead compounds identified in high-through put screens that are being conducted by collaborators in industry (GlaxoSmithKline).

The importance of ICL and the GC as a target for therapeutics development is enhanced by findings implicating the GC in the pathogenesis of several other microorganisms including the bacteria *Rhodococcus fascians* (Vereecke *et al.,* 2002), *S. typhimurium* (Fang *et al.,* 2005) and the fungi *Leptosphaeria maculans* (Idnurm & Howlett,, 2002); *Magnaporthe grisea* (Wang *et al.,* 2003b), and

*Candida albicans* (Lorenz & Fink, 2001). Thus, although the GC is probably not universally required for pathogens' survival in the host, as suggested by findings in *C. neoformans* (Rude *et al.,* 2002) and *Y. pestis* (Sebbane *et al.,* 2004), a growing number of organisms are being identified which at some point in their infection cycle require the GC for growth or survival during infection.

## 11.3. Future Studies

**The role of ICL in *M. tuberculosis* persistence *in vivo*.** The fact that either ICL1 or ICL2 can sustain the metabolism of *M. tuberculosis* early in the infection, even though ICL2 is likely providing considerably less activity than ICL1, together with our previous observation that Δ*icl1* mutant bacteria are attenuated specifically during the persistent phase of the infection (McKinney *et al.,* 2000), and, in particular, that this attenuation results from an interaction with the immune response (Chapter 7), suggest that at the onset of persistence, the infection milieu changes in a way that forces *M. tuberculosis* to increase its reliance on ICL-mediated metabolism. One way in which this could happen is by immune-mediated restriction in access to carbohydrates. However, the phenotype of the Δ*icl1* mutant, though stage-specific in appearance, is not a demonstration that the function of ICL1 is specifically required during persistence. The function of ICLs could be performed early in the infection but the effect of lacking such function may not manifest itself until later. The possibility that ICLs are

dispensable for *M. tuberculosis* remains and ought to be explored. To address this issue, conditionally ICL-expressing strains of *M. tuberculosis* are being built using Δ*icl1*/Δ*icl2* mutant bacteria and recently developed methods of conditional expression of *M. tuberculosis* genes (Blokpoel *et al.,* 2205; Ehrt *et al.,* 2005).

**The role of the fatty acid β-oxidation cycle in *M. tuberculosis* metabolism in vivo.** If lipid-derived fatty acids are indeed the main carbon substrates employed by *M. tuberculosis* in the host, regardless of the source, the pathways involved in their oxidation should be essential for the growth and survival of the bacteria during infection. As discussed in detail in Chapters 3 and 4, testing this prediction might be difficult because of the potential for redundancy in many of the steps of β-oxidation. A survey of these putative homologous genes indicates that the group with the lowest number of homologues is that of the ketoacyl thiolases encoded by the *fadA* genes, of which there are six. Though the oxidation of 3-hydroxyacyl-CoAs might only involve the products of 3 annotated *fadBs*, the presence of some 30 or so, other short-chain hydroxyacyl dehydrogenases, which though closer in homology to eukaryotic enzymes, might nevertheless functionally overlap with the canonical FADBs (Maggio-Hall & Keller, 2004), makes this group a less attractive target.

**The role of malate synthase in *M. tuberculosis* metabolism *in vivo*.** We hypothesize that the joint essentiality of ICL1/ICL2 *in vivo* is attributable to their canonical function in the GC. However, it is possible that ICL-mediated cleavage of isocitrate into succinate and glyoxylate functions together with other

pathways capable of oxidizing the generated glyoxylate. In bacteria such as *P. denitrificans*, glyoxylate is converted to oxaloacetate via the beta-hydroxy aspartate pathway in which glyoxylate is first converted to glycine through a reductive amination reaction catalyzed by glycine dehydrogenase (GCV), followed by condensation with another glyoxylate to form erythro-beta-hydroxy aspartate, which is then dehydrated to oxaloacetate (Liu *et al.,* 2003a). Another route for the metabolism of glyoxylate through a glycine intermediate could involve a serine-glyoxylate aminotransferase pathway, which would lead to formation of glycerate and sugars. This routing of carbon occurs in MLS-deficient seedlings (Cornath *et al.,* 2004) and is analogous to the glycerate pathway (See Chapter 3). In this pathway, two glyoxylates are condensed by glyoxylate carboligase (GCL) to form tartronate, ultimately generating glycerate (Fig. 11.2B). It is not known whether any of these pathways operate in *M. tuberculosis* since no clear homologs of the enzymes involved can be found in its genome. However, the reductive amination of glyoxylate to glycine catalyzed by glycine dehydrogenase has been demonstrated in extracts of *M. tuberculosis* H37Ra (Goldman & Wagner, 1962). This reaction not only would allow the metabolism of glyoxylate but would also aid in the regeneration of $NAD^+$, which might be important if bacteria experience hypoxic conditions *in vivo* (Wayne *et al.,* 1982; Boshoff & Barry, 2005).

In *E. coli*, the genes encoding ICL (*aceA*) and MLS (*glcB*) are in an operon (*aceBAK*). In contrast, in *M. tuberculosis* they are not in the proximity of each

other in the chromosome, and while *icl1* is upregulated *in vivo, glcB* is not (Timm *et al.,* 2003; Fig. 6.7); furthermore while ICL is massively upregulated *in vitro* on C2 substrates, MLS is not. Though these differences might not be important, and constitutive levels of MLS might be sufficient for the function of the GC, they could also be a hint that other pathways involved in the metabolism of glyoxylate might be operative. Two types of malate synthase enzymes have been described: type A and type G. Most micro-organisms have only one type but it is not uncommon to find species that have both. In *E. coli*, which has both, MLS-A is induced by acetate, while MLS-G is induced by glycolate (Molina *et al.,* 1994). However, there is cross-induction of the two enzymes by acetate and glycolate (Pellicer *et al.,* 1999). Mycobacteria have only one MLS and it is closer to the MLS-G type, encoded by *glcB*.

To study the role of MLS in *M. tuberculosis*, deletion of the single-copy gene *glcB* in *M. tuberculosis* was attempted. Two different strategies for gene disruption in *M. tuberculosis* - plasmid-mediated (Pavelka & Jacobs, 1999) and phage-mediated (Bardarov *et al.,* 1997) homologous recombination - were used in separate attempts to make marked deletions in *glcB* in both Erdman and H37Rv *M. tuberculosis* strains. All of these attempts were unsuccessful. We considered the possibility that disruption of *glcB* could have polar effects on downstream genes, and therefore, tried to generate the mutant using unmarked in-frame deletion constructs with different flanking lengths (pEM304-306 series). In all cases, homologous recombination was obtained, but resolution of the plasmid after

sucrose counterselection always resulted in regeneration of the wild-type locus. These results suggest that *glcB* might be essential. If this is the case, this could be formally shown by obtaining the mutation in a merodiploid strain in which the chromosomal deletion is facilitated by the presence of a second wild-type copy either episomally or integrated into the chromosome. Efforts are ongoing to do just that. If *glcB* indeed proves to be essential *in vitro*, determining the role of MLS in the biology of *M. tuberculosis* might still be feasible by adapting methodologies used for conditional gene expression (Blokpoel *et al.*, 2205; Ehrt *et al.*, 2005).

**Determining the contributions of glycolysis and gluconeogenesis to *M. tuberculosis* metabolism during infection.** Based on our data, we hypothesize that carbohydrates are not available, or available in only in low concentrations to *M. tuberculosis* during infection, and furthermore suggest that their availability might change overtime, decreasing during the chronic phase of the infection. However, several alternative scenarios are possible, and thus it will be important to evaluate the role of sugar metabolism-specific pathways in infection.

If carbohydrates are completely inaccessible, then the ability to catabolize carbohydrates (glycolysis) should be dispensable, while the ability to synthesize carbohydrates de novo (gluconeogenesis) should be essential. If this scenario is correct, then the functions of PCK and GLPX ought to be essential, while those of PPGK/GLK and PFK1/2 ought to be dispensable for bacterial growth *in vivo*.

Alternatively, if some carbohydrate is available, perhaps enough to provide glucose-6-P and related biosynthetic precursors, but not enough to be used for energy, and most of the carbon and energy is derived from fatty acid breakdown, then it is possible that the upper and lower branches of glycolysis/gluconeogenesis are 'disconnected'. In this scenario, only enzymes in the upper part of glycolysis would be required (PGI, PFKs, FBA, GLPX) as well as sugar transporters (PPGK), but not enzymes that connect the upper part of glycolysis to PEP (TPI, GAP, PGK, GPMs). PCK would be needed to provide PEP. It is possible that both scenarios hold true at different times during the infection. Early on, glycolysis might play a role while carbohydrates are available, but as the bacteria lose access to them, bacteria might have to switch to split C2/carbohydrate metabolism (no connection between GC/TCA and upper part of glycolysis). Later still, they may become strictly lypolytic and gluconeogenic.

Lastly, *M. tuberculosis* could have access unimpeded access to sugars, perhaps low levels, and similarly to *E. coli* growing on very low levels of glucose/carbohydrates rely on the GC to do so. In this scenario, the requirement for ICL would be due to its role in oxidation of sugar- instead of fatty acid-derived acetyl-CoA. PCK would still be needed for PEP generation (Fischer & Sauer, 2003), but GLPX ought to be dispensable. Furthermore, PPGK/GLK and all glycolytic activities ought to be needed as well.

The finding that *M. bovis* lacks a functional PYK (Garnier *et al.*, 2003; Keating *et al.*, 2005) has been cited as evidence that glycolysis is likely

dispensable in pathogenic mycobacteria for growth *in vivo* since *M. bovis* causes disease in bovines and humans. However, it is possible that in this species the loss of PYK has been compensated by upregulation of either MEZ or PCA, or both. In order to evaluate the role of glycolysis in mycobacterial metabolism both PFK and PYK need to be lacking because PYK could be needed for the metabolism of PEP that has been produced via PCK.

As detailed in Chapter 3, bacteria have enormous metabolic flexibility. In *E. coli*, deletion of *ppc* results in the upregulation of the GC for anaplerosis during growth on glucose, and a mutation on PFK can be compensated by a mutation that upregulates the GC as well. Examples of this sort abound. Studies of gene expression in different carbon sources have now been carried out on a global scale in *E. coli* and *C. glutamicum* grown in specific carbon sources (acetate vs dextrose); similar studies in *M. tuberculosis* ought to be feasible using the growth conditions we have established (See Chapter 6 and Chapter 12 (Materials & Methods)), and might be informative in aiding our understanding of carbon flux in *M. tuberculosis.* Carbon flux analysis with radiolabeled carbon substrates would also greatly enhance our understanding of mycobacterial metabolism. As we have learned, metabolism is less well understood than sometimes assumed.

## 11.4. Conclusions

We ignore much about the metabolic pathways specifically employed by microorganisms to use the nutrients available in the environment of the infection, a prerequisite for pathogenesis. Uniquely, mycobacterial genomes encode an unprecedented number of putative fatty acid metabolism genes including several paralogs of fatty acid β-oxidation and two genes encoding isocitrate lyases (ICL) – an enzyme generally needed when fatty acids are the principle sources of carbon and energy available to bacteria. While single Δ*icl1* and Δ*icl2* mutants are capable of growth on $C_2$ substrates, intracellularly in both mouse and human macrophages, and in the lungs of mice during the acute phase of infection, a Δ*icl1*/Δ*icl2* mutant is incapable of growth on $C_2$ substrates, or in macrophages, and is avirulent, being cleared from lungs within 2 weeks of infection. This functional overlap uncovered between ICL1 and ICL2 makes developing dual specificity inhibitors a desirable goal. That this is feasible is suggested by the high conservation between the proteins' active sites revealed by *in silico* structural modeling of ICL2, and by the abrogation of wild-type *M. tuberculosis* growth on $C_2$ substrates and in macrophages by an ICL inhibitor. These findings establish an essential requirement for ICL from the onset of infection. A role for the GC during persistence of *M. tuberculosis* remains an attractive possibility that is supported by the phenotype of the single *icl1* mutant and gene expression data, as well as by a considerable body of data from other groups. The potential of the GC as a novel

target for drug development is enhanced by its apparent absence in mammalian cells, and by emerging evidence that the pathway is important for virulence in various bacterial and fungal pathogens. These include major causes of human morbidity, such as *Candida albicans*, for which the existing therapeutic options are suboptimal. The lengthy chemotherapeutic regimens prescribed to treat TB and the emergence of rapidly spreading multiple-drug resistant *M. tuberculosis* makes finding more effective drugs an imperative. A novel drug targeting both rapidly dividing bacteria and slow growing or non-replicating 'persisters' would likely reduce the number of antibiotics prescribed, shorten duration of treatment, improve compliance, and contribute to controlling the current TB epidemic.

# PART IV

# CHAPTER 12

# Materials and Methods

## 12.1. Bacterial strains and growth conditions

**Standard media.** *M. tuberculosis* Erdman and *M. smegmatis* $mc^2155$ were stored at -80°C in 15% glycerol. Bacteria were grown with aeration at 37°C in Middlebrook 7H9 broth (DIFCO) containing 10% OADC (DIFCO), 0.5% glycerol, and 0.05% Tween-80, or on Middlebrook 7H10 agar (DIFCO) containing 10% OADC and 0.5% glycerol. Antibiotics (Sigma) were hygromycin (50 $\mu g\ ml^{-1}$), kanamycin (25 $\mu g\ ml^{-1}$) and streptomycin (20 $\mu g\ ml^{-1}$).

**Defined media.** Carbon-source defined media (CDM) contained (w/v): 0.47% Middlebrook 7H9 powder (DIFCO), 0.5% albumin, 0.085% NaCl, and 0.05% Tween-80. Carbon substrates were used at 0.1% (w/v) or as indicated. pH was adjusted to 6.8. Growth curves were done as follows: bacteria were inoculated into standard 7H9 media and grown to late log phase (OD600 1-2). These cultures were used to inoculate dextrose-CDM cultures at an initial OD600 ~ 0.025; these were grown for 7-10 days till late log phase (OD600 0.8-1) and then

used to inoculate "experimental" CDM supplemented with the desired carbon substrates.

In one set of experiments, M9 salts (DIFCO) supplemented with (0.1 mM) CaCl2, (2mM) MgSO4, 0.5% albumin, 0.085% NaCl and 0.05% Tween-80 were used. However, CDM supplemented with glucose (0.1%, w/v) or C2FA (0.1%, w/v) supported significantly better and faster growth of *M. tuberculosis* (doubling time 3-4 days) than similarly supplemented M9-based defined media (~6 day dobling time). *M. tuberculosis* extracts were prepared from CDM (7H9-based) where carbon substrates were used at (w/v) 0.2%.

Minimal media for carbon utilization experiments with *M. smegmatis*: M9 salts (DIFCO), (0.1 mM) CaCl2, (2mM) MgSO4 without (liquid) or with (solid) agar (15 g $L^{-1}$). Carbon substrates were used at 0.1% (w/v). *M. smegmatis* extracts were made from minimal M9 liquid media where carbon substrate concentrations were (w/v) 0.1%. Distilled water was used in all cases. Media were sterilized by either by autoclaving (solid) or filtering (0.2 micron) (liquid).

**BACTEC.** Degradation of [14C]-palmitate to [14C]-CO2 was measured using the BACTEC system (Becton Dickenson) per the manufacturer's instructions.

## 12.2. Mouse infections/animal models

**Mouse strains.** All mice used were either male or female (3-5 wks of age) on the C57BL/6 background and were purchased from Jackson Laboratories. Mice were housed under specific pathogen free conditions at Rockefeller University, and were routinely tested and found to be free of common mouse pathogens. All experiments with live mice were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by Rockefeller University's Institutional Animal Care and Use Committee.

**Mouse infections.** Bacteria were grown to mid-log phase ($OD_{600}$ ~ 0.5) in 7H9 broth, collected by centrifugation, washed with PBST, adjusted to ~ 1 x $10^7$ CFU $ml^{-1}$, and sonicated briefly in a cup-horn sonicator to disrupt aggregates. Mice were infected by injection of 0.1 ml (~ 1 x $10^6$ CFU) of the bacterial suspension into a lateral tail vein.

For respiratory infections, bacteria were grown in 7H9 broth to mid-log phase (OD600 ~0.5), collected by centrifugation, washed once with PBST, centrifuged at low speed for 5 min to remove clumps and the supernatant was suspended at ~106-108 CFU ml-1 (adjusted by OD600), depending on the dosage required (101-103 CFU per mouse); aerosol exposure of mice was carried out for 15 min, followed by a 20 min purge with room air, using a custom-built aerosol exposure chamber (University of Wisconsin, Madison), as described (MacMicking *et al.,* 2003)

**Chemotherapy of mice.** Where appropriate, *M. tuberculosis*-infected mice received isoniazid (0.1 mg/mL ~ 25 mg per kg body weight per day) or isoniazid plus rifampin (0.1 mg/mL ~ 25mg/kg/day) delivered *ad libitum* in the drinking water. Drugs were replaced by fresh water 24 h before mice were sacrificed at each time-point.

## 12.3. Bacterial quantification

**Quantification of *M. tuberculosis* colony forming units (CFU) in organs from infected mice.** Infected mice were euthanized by $CO_2$ overdose (n = 4-5 per group). M. *tuberculosis* CFU were enumerated by homogenizing the left lung of each mouse in 5 ml PBST using a Polytron tissue homogenizer, plating the diluted homogenates on 7H10 agar, and scoring colonies after 3-4 wk (or 6-8 wk, in the case of Δ*icl1* Δ*icl2* bacteria) at 37°C. Portions of tissue were fixed in phosphate-buffered formalin (10%) for histopathology. Histopathology staining was performed at Cornell University Pathology Laboratory (New York, NY).

**Quantification of *M. tuberculosis* CEQ in lungs of infected mice.** *M. tuberculosis* chromosomal DNA was prepared by homogenizing the right lung of each mouse in 5 mL PBST, collecting the bacteria by centrifugation at 3000 *g* for 10 min at 4 °C, resuspending the bacterial pellet in 1 mL TE, adding 0.25 ml warm (70 °C) phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma) and 0.25 mL

zirconia-silica beads (Biospec Products), and breaking the bacteria in a bead-beater (Biospec Products). Cell debris was removed by centrifugation at 12,000 *g* for 10 min, 50 µl 5 M NaCl was added to the clarified supernatant, extraction with phenol/chloroform/isoamyl alcohol was repeated, and genomic DNA was precipitated by addition of isopropanol. The precipitated genomic DNA from a single infected mouse was ethanol-washed, air-dried, resuspended in 300 µL of water, and stored at -20 °C. Quantitative PCR (QPCR) reactions were carried out as described (Timm *et al.,* 2003). Each reaction was in a volume of 50 µL containing 1x Taqman buffer A (Perkin Elmer), 4 mM MgCl2, 0.25 mM dNTPs, 2.5 units of AmpliTaq Gold polymerase (Perkin-Elmer), 0.5 µM of the *fadE15* primers, 0.3 µM of the *fadE15* molecular beacon, and the template DNA. Typically, 1-2 µl of template DNA (representing 0.33-0.67% of the total genomic DNA extracted from a single mouse) was used per reaction. In some cases (where bacterial loads were very high), template DNAs were diluted before amplification in order to obtain QPCR threshold cycle (Ct) values in the range 25-35; control reactions confirmed that dilution of the template had no effect on the linearity of amplification (data not shown). The set of primers and the molecular beacon used to quantify bacterial chromosomes had the following sequences (hybridizing to sequences in the *fadE15* gene): FAM-5'-GCAGCC CGCATGACCATCGGAGTTAAGT GGCTGC-3' (molecular beacon); 5'-GATTGCGCAGATGTTCACGG-3' (forward primer); 5'-GCCCTGCACCCGCTCCTTGG-3' (reverse primer). Real-time QPCR reactions

were run and analyzed on an ABI 7900 machine per the manufacturer's instructions. PCR conditions were: Amplitaq Gold activation step 95 °C (10 m), followed by 40 cycles of 95 °C (30 s), 57 °C (30 s, data collection), 72 °C (30 s). Standard curves were generated using serial dilutions of *M. tuberculosis* genomic DNA of known concentration. Quantitative analysis of the data was carried out as described (Timm *et al.*, 2003).

## 12.4. Tissue culture.

### 12.4.1. Infection of bone-marrow-derived murine macrophages with *M. tuberculosis.*

**Isolation of cells.** Bone marrow cells were flushed from mouse femurs and cultured for 7 days in DMEM (Cellgro) containing 0.58 g $l^{-1}$ L-glutamine, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (HIFBS; Hyclone), 10 mM Hepes 7.55, 100 U $ml^{-1}$ penicillin G, 100 μg $ml^{-1}$ streptomycin, and 20% L929 cell-conditioned medium (Ehrt *et al.*, 2001). Adherent cells were harvested, seeded in 12-well plates (2 x $10^5$ cells per well), and incubated at 37 °C, 5% $CO_2$. Where indicated, macrophages were activated by addition of 50 U $ml^{-1}$ rIFN-γ (R&D) to the medium 16 h prior to infection and thereafter.

**Bacterial Infection.** Bacteria were grown to mid-log phase ($OD_{600}$ ~ 0.5) in 7H9 broth, washed in PBS containing 0.05% Tween-80 (PBST), and centrifuged 5

min at low speed to remove aggregates. Macrophage monolayers were infected at an MOI of 2:1 in DMEM containing 0.58 g $l^{-1}$ L-glutamine, 1 mM sodium pyruvate, 5% HIFBS, and 5% horse serum (Gibco); after 4 h monolayers were washed to remove non-cell-associated bacteria, and incubated (37°C, 5% $CO_2$) with medium changes every 36 h. Where indicated, 3-NP was included in the medium from 4 h post-infection onwards; addition of 3-NP had no detectable effect on adherence or viability (trypan blue exclusion) of macrophages. At the indicated time points, infected monolayers were washed, lysed with 0.5% Triton X-100, and cell-associated bacteria were enumerated by plating lysates on 7H10 agar and scoring CFU after 3-4 wks incubation at 37 °C.

**12.4.2. Infection of human blood monocyte-derived macrophages with *M. tuberculosis*.** Monocyte/lymphocytes were purified from whole human blood (New York, Blood Center) by the Ficoll-plaque differential centrifugation method as per manufacturer's instructions (Pharmacia Biotech). Cells were seeded in tissue culture 12- well plates (Corning) at a density of 2.4 million cells/well and cultured for 2 h in 10% PHS-RPMI = RPMI-1640 (Life Technologies) containing 10% heat inactivated pool human serum (Labquib, Ltd.), 10 mM Hepes, and gentamicin (20 μg/mL). Non-adherent cells were removed by 4 washes with plain RPMI (37 °C). Adherent monocytes were cultured for 1 wk in 10% PHS-RPMI. Before bacterial infections, monolayers were washed 3X with 37 °C PBS.

Infections of human cells with *M. tuberculosis* were carried out identically to those in murine macrophages, except that 10% PHS-RPMI was used throughout the experiments.

**12.4.3. DAPI Staining of macrophages and intracellular Bacteria.** Human primary monocytes derived as described above were seeded on round cover slips fitted in the bottom of 24-well plates. At desired-time points, monolayers were washed 3X with cold PBS, and 500 μL of 4% paraformaldehyde in 1X PBS was added per well. After fixing for , wells were washed 3X with PBS and 300 μL of 4',6'-diamino-2-phenylindole (DAPI stain (1μg/mL final concentration in PBS) were added per well. The wells were then incubated in the dark for ~30 min at 4 °C. Wells were then washed 3X with PBS and the cover slip was mounted on a microscope slide for observation using a fluorescent microscope.

**12.5. Molecular microbiology**

**12.5.1. Construction and complementation of *icl* mutants in *M. tuberculosis*.** For disruption of *icl2*, a KpnI fragment containing *icl2* was sub-cloned into pBSKS- (Stratagene) to generate pEM027-14. A 1.8 kbp MscI fragment internal to *icl2* was replaced with an *aph* kanamycin resistance (Kmr) cassette (1.3 kbp BamHI fragment from pUC4) to generate pEM028; the deletion encompassed AA 2-612 including the catalytic motif KKCGH (AA 213-217). The KpnI fragment

containing the *icl2::aph* allele was cloned into the unique SalI site of pEM025, an *E. coli-Mycobacterium* episomal shuttle vector containing the pAL5000 replication origin (Ranes *et al.* 1990), *aadA* streptomycin resistance ($Sm^R$) marker (1.9 kb DraI fragment from pHP4547), and *sacB* counter-selection marker to generate pEM029-1. The *icl2::aph* allele was recombined into the *M. tuberculosis* chromosome by selection of pEM029-1 ($Km^R$) transformants followed by counter-selection on 5% sucrose as described (Pavelka & Jacobs 1999). The *Δicl1/Δicl2* mutant was generated by disrupting *icl2* in the chromosome of the previously generated *Δicl1* (*icl1::hpt*) strain (McKinney *et al.,* 2000). Replacement of the wild-type *icl2* gene with the *icl2::aph* allele was confirmed by PCR analysis and Southern blot (Fig. 6.3). pICL1 (pEM263-4) was constructed by sub-cloning a 1.6 kb SpeI fragment containing *icl1* into the HpaI site of pEM262, an episomal vector derived from the shuttle vector pMV261 (Stover *et al.,* 1991) by replacing the $Km^R$ cassette with the *aadA* $Sm^R$ cassette. pICL2 (pEM3E4-8) was constructed by inserting a 6.1 kbp KpnI fragment (from an *M. tuberculosis* Erdman genomic library cosmid, gift of M. Glickman, Sloan Kettering Institute) containing *icl2* into the HpaI site of pEM262.

**12.5.2. Construction and complementation of *icl M. smegmatis* mutants.** *M. smegmatis* (strain $mc^2155$) mutant strains with in-frame unmarked deletions in *icl1* or *icl2* or both ORFs were generated by homologous recombination using suicide constructs based on vectors pJG1100 and pJG1001, respectively (gift of J.

Gomez). For disruption of *icl1* (carried out by L. Merkov), a 2.3kb fragment containing the entire icl1 gene of *M. smegmatis* was amplified by PCR from genomic DNA using primers smicl1upxhoI: 5-CTCGAGCTTCGACCACATGAACAACG-3; smicl1dnxhoI: 5-CTCGAGGATCTTCATGATCGGGATGC-3. The PCR product was subcloned into pCR2.1 (to give pLM217s) and sequenced. A 2.3kb EcoRI fragment containing the PCR product was subcloned into EcoRI digested pBSKS (pBluescript) to give pLM218s. pLM218s was digested with SfoI to remove an 855 bp fragment internal to the *icl1* gene (removes 285 AAs from the ICL protein, including the catalytic site, and results in an in frame deletion). The other (5.4kb) SfoI fragment from pLM218s was religated to give pLM219s, which was then digested with XhoI to excise the 1.5kb band containing the truncated icl1 gene. This 1.5kb fragment was subcloned into XhoI digested pJG1100 vector to create pLM220s, which was electroporated into smegmatis.

For disruption of *icl2,* a 3.9kb AgeI fragment from cosmid pEM353 (*M. smegmatis* cosmid genomic library was a gift of B. Subramanian) carrying *icl2* was cloned into pSL301 to generate pEM701, which was EcoRV-digested to obtain a 3.5kb fragment carrying *icl2*, which was then cloned into the PmeI site of pJG1001 to generate pEM902. pEM902 was BstEII-digested to remove a 660-bp fragment, which generated an unmarked in-frame deletion after relegation into pEM903; The deletion encompasses AA 307 to 526 of the *icl2* ORF. pEM903 was electroporated into wild-type *M. smegmatis*. Counter selection was performed

as described (Pavelka & Jacobs, 1999). A Δ*icl1*/Δ*icl2 M. smegmatis* mutant was generated by transforming the Δ*icl2* mutant with pML220s (described above).

### 12.5.3. Construction and complementation of Δ*prpDC M. tuberculosis* mutants.

**Disruption of the *prpdc* ORFs.** *M. tuberculosis* (strain Erdman) with an in-frame unmarked deletion in the *prpd* and *prpc* ORFs was generated by homologous recombination using a suicide construct based on the vector pJG1111. This vector was derived from pJG1004, a derivative of the kanamycin and ampicillin resistant pMV261 (Stover *et al.,* 1991) into which hygromycin resistance and sucrose sensitivity cassettes had been inserted (Gomez & Bishai, 2000). Additional restriction sites (SpeI, BstBI, SspI, AvrII, NdeI, XhoI, and AflII) were added between the pJG1004 PacI and AscI sites via a PCR-generated polylinker, to generate pJG1100. A 39894 bp SmaI/NruI fragment excised from pGOAL19 (Parish & Stoker, 2000) containing the *E. coli lacZ* gene under the control of the *M. tuberculosis* Antigen 85 promoter was cloned into the unique PmeI site of pJG1100 to generate pJG1111 by L. Merkov. 1 kb regions upstream and downstream of the putative *prpDC* ORFs were PCR-amplified (with a *M. tuberculosis* genomic library cosmid as template), using primers designed to introduce the restriction sites PacI/AvrII into the 5'/3' termini of the upstream region (primers used were ttaattaactgctctgcggcacggtg and cctaggatgcatcaacat

aatccg) and AvrII/AscI sites into the 5'/3' termini of the downstream region (using primers cctaggggcaccttctagtcttat and ggcgcgccagcagaccatgatcgtcg). The resulting PCR products were subjected to restriction digestion and cloned together into the PacI and AscI sites of pJG1111 such that the 2 insert fragments were ligated to each other at their AvrII sites (the 3' terminus of the upstream region to the 5' terminus of the downstream region). Homologous recombination into the *M. tuberculosis* chromosome would result in the replacement *prpDC* with an ORF encoding a fusion protein consisting 9 AA. This construct was recombined into the *M. tuberculosis* genome by selection of white, hygromycin and kanamycin resistant transformants followed by counter-selection on 5% sucrose as described (Pavelka & Jacobs, 1999). Replacement of *prpDC* with the sequence encoding the 9aa peptide was confirmed by PCR analysis and Southern blot (not shown).

## 12.6. Gene expression analysis by real time RT-PCR

RNA preparation from infected tissues was done as described (Timm *et al.*, 2003). Bacteria were from mouse lungs of wild-type (C57BL/6) mice infected with *M. tuberculosis* Erdman (1 x $10^6$ CFU/mouse, i.v.) or from cultures grown in 7H9 following standard conditions and harvested at mid-log phase.

**12.6.1. Primers and Molecular Beacons.** Primers with similar melting temperatures (60–66°C) were designed by using PRIMER 3 software (Rozen &

Skaletsky, 2000). Primers were tested in PCRs with 100 Erdman genome equivalents as template and the amplification products were evaluated by gel electrophoresis. Molecular beacons (Tyagi & Kramer, 1996) were designed as described (www.molecular-beacons.org); The beacon for *icl2* was purified by J. Timm at PHRI, NY. Standard curves were generated by using serial dilutions of Erdman genomic DNA as template. The sequences of the beacon and primers for *icl2* gene expression analysis were the following:

Beacon: 5'-GCAGGCTGACGATGCCGCGGTGCTGGGCCTGC-3'

RT primer: 5'-CGGATCAGGTTGCGTACGTG-3'

Primer1: 5'-GCTCCAGCACCGAAGATCC-3'

Primer 2: 5' CTGTCGCTCGCTCATCTGC-3'

(Those for *icl1* have been reported before (Timm *et al.*, 2003)).

**12.6.2. Reverse Transcription (RT) and QRT-PCR.** QRT-PCRs were as described (Manganelli *et al.*, 2001). Mock reactions (no RT) were done on each RNA sample to rule out DNA contamination. Control reactions with uninfected murine and human tissues were performed to rule out cross-hybridization. RT reactions were performed with Klenow DNA polymerase from *Carboxydothermus hydrogenoformans* (Roche Applied Science): annealing, 95°C (1 min), 65 °C (3 min), and 57 °C (3 min); polymerization, 60 °C (30 min); RT inactivation, 95°C (5 min). Real-time PCRs were run and were analyzed on an ABI7900 real-time PCR machine. PCR conditions: AmpliTaq Gold activation, 95 °C (10 min); 15 cycles of

95 °C (30 sec), 65 °C (0.5° stepdown each cycle, 30 sec), and 72°C (30 sec); 25 cycles of 95°C (30 sec), 57°C (30 sec), and 72°C (30 sec).

**12.6.3. Normalization of Transcripts.** For each RNA sample, the control transcripts (*sigA* mRNA) and target mRNAs were reverse-transcribed together in one reaction, and the resulting cDNAs were quantified by real-time PCR. The target cDNA was normalized internally to the *sigA* cDNA levels in the same sample and expressed as (Target mRNA)/(*sigA*).

## 12.7. Biochemical procedures

**12.7.1. Preparation of total cell extracts from mycobacterial cultures** Cultures of the different strains of *M. tuberculosis* (100-150 mL in roller bottles; OD600 = 0.7-1) or *M. smegmatis* (30 mL in shaking flasks; OD600 = 0.7-1.2) were grown to late-log phase in 7H9-based CLDM broth (*M. tuberculosis*) or M9-broth (*M. smegmatis*) supplemented as indicated above. Bacteria were washed three times in PBS-Tween80 (0.05%) and resuspended in MOPS buffer (50mM morpholinepropane sulfonate, 5mM MgCl2, pH 6.8, supplemented with a protease inhibitor cocktail (Sigma P-8465)) in 1/75$^{th}$ vol. of culture (*M. tuberculosis*) or 1/20$^{th}$ vol. of culture (*M. smegmatis*). Bacterial cells were disrupted by bead beating (high power) for 3 cycles of 1 min pulses followed by 30 seconds of rest (on ice). The cell-debris was removed by centrifugation, the extracts were filtered

(*M. tuberculosis*) (0.45 micron low-protein binding SpinX", Corning) and the concentration of protein in the supernatants was measured using a Biorad (*M .* smegmatis) or BCA (M .tuberculosis) protein assay kit (Pharmacia).

**12.7.2. Isocitrate lyase assay.** ICL activity was measured by following the lactate dehydrogenase (LDH) mediated reduction of glyoxylate to glycolate, with concomitant oxidation of NADH (Warren *et al.,* 1970). Reactions were performed at room temperature in a 1ml assay volume containing: cell-free extract (10-100 μg total protein in 5-50 μL), 0.1 mM NADH and 7U LDH (Roche), in 50 mM MOPS/5 mM MgCl2 buffer. Reactions were preincubated for 5 min at room temperature before being started by the addition of 1mM threo-D-L-isocitrate (Sigma). Isocitrate-stimulated NADH oxidation was determined spectrophotometrically at 340nm using the standard extinction coefficient 6.22 mM-1 for a path length of 1cm.

**12.7.3. Methylisocitrate lyase assay.** Me-ICL activity was measured in analogous fashion by following LDH mediated reduction of pyruvate to lactate, with concomitant oxidation of NADH in a 1 mL reaction containing: cell-free extract (10-100 μg total protein in 5-50 μL), 2 mM DTT, 0.2 mM NADH and 7U LDH (Roche) in 50 mM MOPS/5mM MgCl2 buffer. Reactions were started by addition of 1 mM threo-D-L-2-methylisocitrate.

**12.7.4. Synthesis of threo-D-L-2-methylisocitrate.** The following procedure is based on those described in Plaut *et al.*, 1975 and Brock *et al.*, 2001, with modifications.

**Step 1. Trans-epoxymethylsuccinic acid.** Mesaconic acid (10.0 gm, 76.8 mmol) was suspended in water (40 mL) and NaOH (6.1 g, 153.7 mmol) was added portionwise. The resulting solution was cooled to 0 °C and $Na_2WO_4.2H_2O$ (0.760 g, 2.3 mmol) was added, followed by dropwise addition of $H_2O_2$ (30% aqueous solution, 9.6 mL, 84.55 mmol). After stirring at 0 °C for 1 h, the mixture was heated at 65 °C. After 8 h, the reaction mixture was allowed to cool to RT and evaporated to dryness. The residue was partitioned between water (10 mL), ethyl acetate (100 mL) and ether (100 mL). Conc. $H_2SO_4$ (8 mL) dissolved in ether (70 mL) was added and the mixture stirred at RT for 3 h. The organic layer was separated, dried ($Na_2SO_4$) and evaporated to obtain the epoxide as a white powder (9.0 g).

$^1$H NMR (Acetone-$d_6$, 400 MHz): δ 1.58 (s, 3 H), 3.84 (s, 1 H).

**Step 2. Dimethyl trans-epoxymethylsuccinate.** $SO_2Cl_2$ (5.6 mL, 75.3 mmol) was added to a solution of the epoxide (5 g, 34.2 mmol) in anhydrous MeOH (50 mL) at 0 °C and the mixture stirred at RT for 8 h. The reaction mixture was concentrated in vaccuo and the residue partitioned between ether and sat. aq. $NaHCO_3$. The ether layer was separated, dried and evaporated to afford the dimethyl ester (5.2 g) which was used directly for the next reaction.

$^1$H NMR ($CDCl_3$, 400 MHz): δ 1.63 (s, 3 H), 3.79, (s, 3 H), 3.83 (m, 4 H).

**Step 3. 2-Methyl-5-oxo-tetrahydrofuran-2,3,4-tricarbomethoxytetrahydrofuran.** Sodium metal (0.69 g, 28.7 mmol) was added to previously cooled anhydrous MeOH (15 mL) under argon atmosphere at 0 °C stirring until the metal had dissolved. Dimethyl malonate (3.2 mL, 28.7 mmol) was added to the sodium methoxide thus formed and the resulting mixture stirred at RT until a white precipitate formed (20-30 min). Epoxide obtained in step II (5.0 g, 28.7mmol) was added and the mixture stirred for 3 days at RT. Conc. HCl was added and the stirring continued for another 2 h. The solid formed (NaCl) was filtered, the filtrate concentrated to afford crude lactone which was direcly used for the next reaction.

**Step 4. 2-Methyl-5-oxo-tetrahydrofuran-2,3-dicarboxylic acid.** To the crude furan obtained (1.5 g) was added 6 N HCl (50 mL) and the mixture refluxed for 3 h. The reaction mixture was concentrated and to remove traces of HCl the residue was redissolved in water and evaporated. The brown residue thus obtained was dissolved in water and treated with activated charcoal at 60 °C. After 30 min, the mixture was filtered and the filtrate evaporated to afford colourless crude lactone.

$^1$H NMR (Acetone-$d_6$, 400 MHz): δ 1.78 (s, 3 H), 2.78 – 2.84 (m, 1 H), 3.02 – 3.08 (m, 1 H), 3.60 – 3.63 (m, 1 H), 11.61 (S, 2H).

**Step 5. Methyl Isocitrate.** Water (10 mL) and NaOH (0.95g, 23.9 mmol) were added to the compound obtained in the previous step (1.5 g) and the mixture heated at 80 °C. At the end of 12 h, the solution was neutralised with Amberlite

H+ resin at pH 7. After the removal of the solvent, the residue was redissolved in 10 mL of water and treated with activated charcoal. The mixture was filtered and the filtrate evaporated to afford the methyl isocitrate.

$^1$H NMR ($D_2O$, 400 MHz): δ 1.32 (s, 3 H), 2.55-2.67 (m, 2 H), 3.14-3.16 (m, 1 H).

**12.7.5. Southern Blots.** ~2 μg of genomic DNA from the different strains analyzed were digested with restriction enzymes (NEB), separated in 1% TBE agarose gels, denatured with 0.5 NaOH, transferred to Hybond N membrane (Amersham Labs.), and probed with inserts/PCR products from appropriate plasmids/genomic DNA, labeled with $^{32}$P using a random priming labeling kit (Boehringer Mannheim). Stringent conditions were used for hybridization (2 h at 65° C) and washings (20 min in 2X SSC, 0.1%(w/v) SDS at RT; 2X 15 min in 1X (1st) and 0.1X (2nd) SSC, 0.1% (w/v) SDS at 65° in rolling in oven). Specific hybridization was detected by autoradiography.

## 12.8. Computer modeling and bioinformatics

**12.8.1. Sequence Alignments.** Guide tree of isocitrate lyases amino acid sequences generated using Vector NTI Advance 9.0 AlignX, which uses the Clustal W algorithm (Thompson *et al.,* 1994) to create an alignment from which a tree is generated using the Neighbor Joining method (Saitou & Nei, 1987).

**12.8.2. Bioinformatics and modeling.** Nucleotide sequences of *icl1* (MT0483) and *icl2* (MT1966) (http://www.tigr.org) were conceptually translated; protein domains were identified with Pfam (http://www.sanger.ac.uk/Software/Pfam/) and domain alignments were generated with CDD v1.62 (NCBI). Protein sequences were aligned with PSI-BLAST (NCBI), and an ICL2 structure model was generated with MODELLER (http://salilab.org/modeller/modeller.html) by threading on the structures of ICL1 (Sharma *et al.* 2000) and *A. nidulans* ICL (Britton *et al.* 2000).

**12.8.3. Static and dynamic models of host-pathogen equilibrium.** A simple *in silico* model of *in vivo* bacterial growth dynamics and host-pathogen equilibrium was generated using Microsoft Excel (spreadsheet available upon request). Figures referred to in this section are those in chapter 5. The following input parameters were selected for generating the curves depicted in Fig. 5.1: inoculum = 200 CFU; acute phase = 0-2 wk post-infection; chronic phase = 2-16 wk post-infection; replication rate (population doubling time) during acute phase growth = 24.6 h. These input parameters were selected in order to match the experimentally determined parameters shown in Fig. 5.2A, but the model will accommodate any set of input parameters. In our model (Fig. 5.1), the curves depicting viable CFU incorporate the potentially bactericidal impact of the immune response by calculating pre- and post-killing CFU values on a daily basis. Different types of equilibria (from fully static to fully dynamic or semi-dynamic scenarios) can be

modeled by varying the growth rate and the killing rate during the chronic phase of infection; in our specific case, the chronic phase was defined as 2-16 wk post-infection, although these parameters can also be varied as appropriate. "Pre-killing" CFU values (CFUpre) on each successive day were calculated according to the formula CFUpre = CFUi•eK•T, where CFUi is the number of CFU on the preceding day (postkilling), K is the growth rate constant (in d-1) before adjustment for host-mediated killing, and T = 1 d. "Post-killing" CFU values (CFUpost) on each successive day were calculated according to the formula CFUpost = CFUi•eK'•T, where CFUi is the number of CFU on the preceding day (postkilling), K' is the growth rate constant (in d-1) after adjustment for immune-mediated killing, and T = 1 d. The "static" scenario (Fig. 5.1A) was modeled by setting K at 0 d-1 (∞doubling time) during the chronic phase of infection (2-16 w). The "dynamic" scenario (Fig. 5.1B) was modeled by setting K at 0.6764 d-1 (24.6 h doubling time, identical to the acute-phase rate). The "partially dynamic" scenarios, with bacterial cell division rates reduced by 50% (Fig. 5.1C) or 90% (Fig. 5.1D) compared to the acute-phase rate, were modeled by setting K at 0.3382 d-1 (49.2 h) or 0.0676 d6 (246.0 h), respectively. For illustrative purposes, the curves in Fig. 5.1 were generated by setting K' = 0 during the chronic phase of infection (2-16 w) in order to maintain a stable plateau in CFU numbers; more complex scenarios (rising or falling CFU over time) can be modeled by independently varying K and K' (as in Fig. 5.2A). Numbers of total chromosome equivalents (tCEQ) on each successive day were calculated by summing the CEQ

from viable bacteria (vCEQ) with those from dead bacteria (dCEQ), according to the formula tCEQ = vCEQ + dCEQ, where vCEQ = CFUpost, and dCEQ = Sum of (CFUpre – CFUpost) for each successive day inclusive of the current day. The chromosomal ploidy of *M. tuberculosis* under different growth conditions is unknown; in *M. tuberculosis*-infected mice the experimentally determined bacterial CEQ/CFU ratio at 2 wk post-infection is typically 1-3 (Fig. 5.2, 5.3, and 5.4; our unpublished results). For illustrative purposes, a ploidy value of 2 was arbitrarily used to generate the curves in Fig. 5.1 in order to avoid overlap of the CFU and CEQ curves in Fig. 5.1A. The ploidy value was conservatively set at 1 for the modeled curves depicted in Fig. 5.2A; higher ploidy values would lead to a more rapid divergence of the CEQ and CFU curves.

### 12.9. Statistical analysis

Data are represented as means; error bars indicate standard deviations from the means. Student's *t*-test (two-tailed) was used to evaluate differences in bacterial CFU and CEQ obtained from infected mouse lungs or from *in silico* modeling. Trendlines were computed using the least-squares method.

# PART V

# CHAPTER 13

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