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Compiling a Molecular Inventory for *Mycobacterium bovis* BCG at Two Growth Rates: Evidence for Growth Rate-Mediated Regulation of Ribosome Biosynthesis and Lipid Metabolism

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An experimental system of *Mycobacterium tuberculosis* growth in a carbon-limited chemostat has been established by the use of *Mycobacterium bovis* BCG as a model organism. For this model, carbon-limited chemostats with low concentrations of glycerol were used to simulate possible growth rates during different stages of tuberculosis. A doubling time of 23 h ($D = 0.03\ h^{-1}$) was adopted to represent cells during the acute phase of infection, whereas a lower dilution rate equivalent to a doubling time of 69 h ($D = 0.01\ h^{-1}$) was used to model mycobacterial persistence. This chemostat model allowed the specific response of the mycobacterial cell to carbon limitation at different growth rates to be elucidated. The macromolecular (RNA, DNA, carbohydrate, and lipid) and elemental (C, H, and N) compositions of the biomass were determined for steady-state cultures, revealing that carbohydrates and lipids comprised more than half of the dry mass of the BCG cell, with only a quarter of the dry weight consisting of protein and RNA. Consistent with studies of other bacteria, the specific growth rate impacts on the macromolecular content of BCG and the proportions of lipid, RNA, and protein increased significantly with the growth rate. The correlation of RNA content with the growth rate indicates that ribosome production in carbon-limited *M. bovis* BCG cells is subject to growth rate-dependent control. The results also clearly show that the proportion of lipids in the mycobacterial cell is very sensitive to changes in the growth rate, probably reflecting changes in the amounts of storage lipids. Finally, this study demonstrates the utility of the chemostat model of mycobacterial growth for functional genomic, physiology, and systems biology studies.

With three million people dying from tuberculosis (TB) annually, *Mycobacterium tuberculosis* remains a formidable pathogen. Tuberculosis ranks among the top 10 causes of global mortality and morbidity and is the leading cause of infectious disease (66). The ability of *M. tuberculosis* to adapt to and survive harsh environmental conditions in order to establish and maintain long-term infections within its human host is fundamental to this organism's success. Modification of the mycobacterial cell in response to changes in the environment is crucial to this adaptive process, but detailed information about how *M. tuberculosis* changes its macromolecular composition in response to its environment and growth rate is lacking.

The genome sequence of *M. tuberculosis* has been available since 1998 (9). Although information obtained from the genome sequence provided new and valuable insights into the biology of the tubercle bacillus, the genome itself provides few clues regarding how the pathogen responds to its environment by changing its cellular composition. One of the principal tasks of postgenomic biological studies of *M. tuberculosis* is to understand how the genome orchestrates the structure and dynamics of the cell in response to changes in the environment. This task requires an integration of information on the transcriptome (transcriptomics), the proteome (proteomics), and the metabolome (metabolomics). There is a need for an understanding of biological processes by integrating all three levels of cellular information (systems biology), and therefore the system under study must be defined. An inventory of the macromolecular contents of the mycobacterial cell under specific conditions will help to define the system under study and the reaction of this pathogen to the environment more precisely. This represents a first step towards a systems biology approach to understanding the TB bacillus.

Quantitative descriptions of the chemical composition of the mycobacterial cell are limited to a few studies that were performed many years ago (61, 63–65, 67). These data, in combination with standard mathematical models, have allowed mycobacterial researchers to make useful predictions concerning the chemical composition of an "average" mycobacterial cell (10–12). However, this recipe does not correspond to a defined physiological state, as the experiments were performed by the use of batch culture systems. A major limitation of batch cultures is that the cells are grown in an environment that is constantly changing with time as a result of bacterial metabolism. The excretion of by-products and depletion of nutrients from the medium result in an undefined environment, which has an unpredictable impact on the physiology and growth rate of the organism. In addition, batch cultivations yield no information about how the macromolecular content varies with the growth rate and specific environmental conditions and are therefore not amenable to systems biology approaches to modeling cellular physiology such as metabolic flux analysis (MFA) (48). MFA is a key technique in metabolic engineering, allowing metabolic phenotypes to be defined in terms of the carbon flux through a metabolic network by the use of mathematical

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modeling and computer simulation. Since it is becoming apparent that an intricate and controlled regulation of metabolic processes in response to the host environment is a critical component of the pathogenic strategy of *M. tuberculosis* (37), gaining information on the flux through the major pathways by MFA will be an important strategy for understanding the changes in metabolism related to the virulence of this organism.

Chemostat cultivation has made it possible to study the physiological impact on bacteria of specific nutritional conditions at different growth rates. This makes the chemostat an invaluable tool for investigating the macromolecular composition of the bacterial cell. The application of continuous culture technology to TB research has enormous potential. A chemostat can be used to investigate the effects of the growth rate and nutritional status of mycobacteria on gene expression, metabolism, and physiology. In addition, the chemostat provides a virtually unlimited source of mycobacterial cells in a defined state, which are ideal for functional genomic, physiology, and systems biology studies.

The low growth rate of pathogenic mycobacteria, problems associated with the clumping of bacilli, and safety considerations have all provided obstacles for researchers attempting chemostat cultures. As a result, only very few projects have utilized chemostats to grow this group of organisms (3, 27, 30, 35, 36). A continuous culture system has been described for *M. tuberculosis* and has recently been successfully employed to investigate the effects of 1% oxygen on the transcriptome of *M. tuberculosis* (3, 27). This study allowed the effect of oxygen limitation on the transcriptome of *M. tuberculosis* to be investigated at a constant growth rate and independently of any other environmental parameters (3). *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) has also been cultivated successfully in a bioreactor (16, 30, 42). These studies investigated mycobacterial growth at one specific growth rate. However, chemostats also provide researchers with the unique opportunity to investigate the effects of different growth rates on bacteria. A recent study investigating the transcriptional response of *Escherichia coli* demonstrated that the impact of low growth rates on bacteria may be far greater than the effects of nutrient limitation (24). Furthermore, the growth rate is a critical issue in the physiology and pathogenesis of *M. tuberculosis*. Firstly, the organism grows much slower than most other microbes that have been studied, with a doubling time that does not exceed one division every 16 h. Secondly, infection with *M. tuberculosis* is characterized by two distinct phases, i.e., an acute phase of the disease, in which the organism is assumed to be growing at or near its maximum rate, and a persistent asymptomatic infection, in which bacteria are either growing extremely slowly or existing in a nongrowing state. Dissecting the response of mycobacterial populations at different growth rates in a chemostat may therefore reveal important information about the physiological state of mycobacteria when growing in the host and thus offers an attractive approach to modeling persistence.

To overcome safety considerations when working with *M. tuberculosis*, we used the vaccine strain *M. bovis* BCG as a model organism for this study. The BCG vaccine originated from *M. bovis* attenuated by repeated passages in vitro and is therefore very closely related to *M. tuberculosis*. The genomes of *M. bovis* BCG and *M. tuberculosis* exhibit a high degree of homology, sharing 99.9% of their DNA (34). The principal genetic difference between the two organisms is that BCG contains several well-defined deletions (4). *M. bovis* BCG mimics *M. tuberculosis* in its ability to persist in the body and is also able to cause reactivation disease in the immunocompromised host (1, 33, 41, 43, 45, 56). Indeed, survival and persistence of the live BCG vaccine are necessary to elicit protective immunity. Researchers have demonstrated similarities between the physiological, molecular, and metabolic responses of *M. bovis* BCG and those of the tubercle bacillus (19, 25, 29, 40). Studying *M. bovis* BCG may help us to formulate hypotheses about the pathogenic mechanisms of TB, which can then be tested in *M. tuberculosis*.

It has been postulated that low growth rates and carbon limitation may be characteristics of *M. tuberculosis* in vivo. In an effort to mimic possible in vivo conditions, we developed a chemostat model system of mycobacterial growth, using *M. bovis* BCG as a surrogate for *M. tuberculosis*. The bacteria in this model were kept in a defined glycerol-limited environment and induced to replicate at known suboptimal growth rates. Therefore, this system was useful for modeling mycobacterial disease. Seminal experiments with *E. coli* have demonstrated that the size and composition of the bacterial cell are sensitive functions of the growth rate and that the composition of the environment is paramount for determining both the viability and the rate of bacterial growth (6, 15). To determine whether remodeling of the mycobacterial cell also occurs in response to changes in the growth rate, we investigated the macromolecular composition of *M. bovis* BCG in a chemostat model of mycobacterial growth. This is the first study to investigate the influence of specific growth rates in chemostat cultures on the physiological behavior of mycobacteria, and it provides valuable information to elucidate changes in metabolic flux distributions and cell adaptation.

MATERIALS AND METHODS

Bacterial strains. *M. bovis* BCG Pasteur, which was originally purchased from the American Type Culture Collection (ATCC 35748), was used for this study. Small aliquots of seed stocks were maintained in 10% (vol/vol) glycerol at −80°C.

Media. Middlebrook 7H11 agar and Middlebrook 7H9 broth containing 5% (vol/vol) oleic acid-albumin-dextrose-catalase enrichment medium supplement (Becton Dickenson) and 0.5% (vol/vol) glycerol were used to grow cultures from frozen stocks and for counting the numbers of culturable bacteria in chemostat samples. Brain heart infusion agar was used to assess culture purity (Becton Dickinson). Roisin's minimal medium was used for the cultivation of *M. bovis* BCG in continuous cultures (R. M. Owens, personal communication). The components of Roisin's minimal medium were as follows: KH_2PO_4, 1 g liter^{-1}; Na_2HPO_4, 2.5 g liter^{-1}; NH_4Cl, 5.9 g liter^{-1}; K_2SO_4, 2.0 g liter^{-1}; $ZnCl_2$, 0.08 mg liter^{-1}; $FeCl_3$, 0.4 mg liter^{-1}; $CuCl_2$, 0.02 mg liter^{-1}; $MnCl_2$, 0.02 mg liter^{-1}; $Na_2B_4O_7$, 0.02 mg liter^{-1}; NH_4MoO_4, 0.02 mg liter^{-1}; Tween 80, 0.2% (vol/vol); biotin, 0.5 mg liter^{-1}; glycerol, 5 ml liter^{-1}; and antifoam agent (Breox FMT 30), 0.05% (vol/vol). The pH was adjusted to pH 6.6, and the medium was sterilized by filtration through a 0.2-μm-pore-size Sartorius filter.

Continuous culture of *M. bovis* BCG. *M. bovis* BCG was grown in glycerol-limited steady-state cultures in 2-liter bioreactors fitted with a sealed titanium top plate. The culture system was operated as a chemostat by controlling nutrient addition from the medium reservoir by use of a Gilson Minipuls3 peristaltic pump, while a constant volume of 1 liter was maintained via an overflow tube attached to another pump (Watson Marlow). Medium delivery was monitored daily. The culture was stirred by use of an impeller set between 750 and 1,000 rpm. Culture conditions were continuously controlled by either a Biolab fermentation system (Braun) or the Voyager system (Adaptive Biosystems). The temperature was measured with a Biolab temperature probe and kept constantly at

37°C with a heating rod. The culture pH was monitored with an Ingold pH electrode (Broadley-James) and maintained at 6.6 by the automatic addition of either 1 M NaOH or 1 M HCl. The dissolved oxygen concentration was monitored with a polarographic oxygen electrode (Broadley-James) and was controlled at 70 to 100% by feedback control of the stirring rate. Air was pumped through a filter into the headplate at a constant rate of 0.1 liter min^{-1}. Effluent gas was passed through a cooling condenser to minimize evaporative loss and was analyzed continuously by infrared (CO_2) and electrochemical galvanic (O_2) analyzers (Adaptive Biosystems dual-gas tandem sensor).

Inoculum cultures were grown in 100 ml of modified Roisin's medium in glass flasks containing a Teflon-coated magnetic bar on a magnetic stirrer until late exponential phase (optical density at 600 nm [OD_{600}] = 1.0). These BCG precultures were transferred into the chemostat vessel through the inoculum port. After inoculation, each culture was allowed to grow batchwise until the OD_{600} reached approximately 1.0. Continuous culturing was then started at a known dilution rate of 0.03 h^{-1} (equivalent to a doubling time [t_d] of 24 h) or 0.01 h^{-1} (t_d = 69 h). Four volume changes were allowed to ensure that the cultures had reached a steady state, which was confirmed by constancy in oxygen consumption and in carbon dioxide and biomass production. Once the steady state was reached, the cells were harvested for analysis. In order to minimize the chances of a spontaneous mutant supplanting the original strain of BCG, we set up each chemostat culture independently.

Culture analyses. Culture samples were withdrawn from the chemostat by a tube submerged in the culture broth and were collected into a sterile bottle kept in an ice bath. The OD_{600} was recorded against a water reference by use of a Pharmacia Biotech Ultraspec 200 UV visible spectrophotometer. The biomass was determined according to the method described by Lynch and Bushell (32). Acid fastness was determined by use of a modified Ziehl-Neelson stain (BDH) according to the manufacturer's protocol. Viable counts were measured by plating 100-μl aliquots of decimal dilutions in sterile Ringer's solution plus 0.01% (vol/vol) Tween 80 onto Middlebrook 7H11 agar. Mycobacterial colonies were enumerated after 3 to 4 weeks of incubation at 37°C. The purity of the chemostat culture during the experiments was monitored by daily Gram staining and culturing of samples on brain heart infusion agar.

Chemical assays. Samples were collected on ice and harvested by centrifugation at 13,000 × *g* for 15 min at 4°C. The supernatant was filtered through a 0.2-μm-pore-size syringe filter and stored at −20°C until it was analyzed. The amounts of glycerol in the supernatant and in fresh medium were assayed by use of a commercial assay kit that employs a glycerokinase-coupled enzyme assay system (Boehringer Mannheim). Phosphate and ammonia were quantified by reflectometry (Rqflex; Merck). The cell pellet was washed twice with phosphate-buffered saline and then stored at −80°C. Prior to analysis, the samples were dried for at least 12 h in an Edwards Modulyo freeze dryer and then stored in an evacuated desiccator.

Macromolecular composition. The macromolecular composition of the cellular biomass was determined from freeze-dried cell pellets. Total carbohydrates were assayed by the phenol reaction, with glucose as a standard (17, 65). The total protein was extracted by the following two methods: (i) cells were boiled in 0.5 M sodium hydroxide for 10 min; and (ii) cells were resuspended in a protein solubilization buffer containing 9 M urea, 2% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 2% (wt/vol) SB 3–10 (3-[decylmethylammonio] propanesulfonate) and then transferred to 2-ml Ribolyser tubes containing 0.5 g of glass beads (150- to 212-μm diameter; Sigma-Aldrich). The mixture was vortexed for 30 s, cooled on ice for 10 min, and lysed by use of a Ribolyser (Hybaid) (four times for 30 s each at a speed setting of 6.5), with careful cooling between cycles. The cell-free supernatant was collected by centrifugation at 4,000 × *g* for 10 min at 14°C. The total cellular protein was measured by the Lowry method (31) and by use of a PlusOne 2-D Quant kit (Amersham). Bovine serum albumin was used as a standard and was included with each set of samples.

The extraction procedure for DNA was performed as described by Winder and Coughlan (60), and DNA was measured by the phenylalanine reaction, with calf thymus DNA (Sigma) as a control (13, 62). For RNA extraction, 20 ml of culture sample was rapidly withdrawn from the chemostat by a tube submerged in the culture broth and then directly injected into a sterile bottle containing 80 ml of GTC solution (5 M guanidinium thiocyanate, 0.5% [vol/vol] sodium *N*-lauryl sarcosine, 25 mM trisodium citrate, and 0.1 M dithiothreitol). RNAs were then prepared essentially as described by Stewart et al. (54). For the removal of contaminating DNAs, samples were digested with 2 U of DNase I for 30 min at 37°C (Ambion). The enzyme and divalent cations were then removed with a DNase inactivation reagent (Ambion). Total RNA concentrations were measured by use of a RiboGreen RNA quantification kit (Molecular Probes) according to the manufacturer's instructions and were expressed relative to the dry weight.

Lipid extractions were performed as described by Winder and Coughlan (60), with replacement of the final benzene treatment with an ethanol-ether (3:1) extraction at 60°C for 10 min. The carbon (C), hydrogen (H), and nitrogen (N) compositions of the dried biomass and of lipid extractions were determined by use of a CE-440 CHN elemental analyzer (Exeter Analytical). All chemical determinations were performed a minimum of three times for two independent chemostat cultivations. A standard error of the mean of <10% was achieved for all assays, with a mean standard error of 5.29%.

RESULTS

Chemostat culture. The first goal of this study was to develop and optimize conditions for growing BCG continuously in a fermentor. James and colleagues have successfully cultivated *M. tuberculosis* by using a complex nutrient-rich medium containing several carbon sources and an almost full complement of amino acids, but this medium is unsuitable for physiological studies due to difficulties in identifying the growth-limiting substrate (3, 27). For physiological studies with chemostat cultures, it is desirable to use defined media containing ingredients that can be easily monitored throughout the experiment. For this reason, Roisin's medium was investigated in this study as the base medium for the growth of *M. bovis* BCG. Roisin's medium has significant advantages over other media described for culturing mycobacteria in a chemostat, as it is inexpensive, minimal, and chemically defined and contains only one source of carbon, plus Tween 80 as a dispersal agent. In addition, carbon, nitrogen, and phosphate sources are easily measured by simple assays, and therefore the continuous cultivation of mycobacteria in this medium is amenable to other physiologically relevant studies such as metabolic flux analysis.

The medium composition was modified slightly for the cultivation of *M. bovis* BCG by the addition of the vitamin biotin, which is known to enhance the growth of this organism (2). Initial experiments investigated the ability of Roisin's medium to support the batch growth of *M. bovis* BCG in a bioreactor. Short lag phases and high growth rates were observed (data not presented). The results indicated that the maximal specific growth rate for BCG in Roisin's medium, calculated by measuring the growth rate during the log phase of growth, was approximately 0.033 h^{-1}, corresponding to a doubling time of 21 h. An analysis of the composition of the growth medium indicated that phosphate and ammonia remained in excess throughout the growth cycle, whereas glycerol was only present in trace amounts, confirming that Roisin's medium was a glycerol-limited culture medium. While the environmental milieu encountered by persisting *M. tuberculosis* remains relatively undefined and is being hotly debated, evidence suggests that the phagosomal environment is carbohydrate poor and therefore that Roisin's medium is an appropriate medium for modeling mycobacterial disease (37, 50).

Steady-state BCG cells grown at a low dilution rate of 0.01 h^{-1} (t_d = 69 h) were compared with cells growing at a higher growth rate of 0.03 h^{-1} (t_d = 23 h). A doubling time of 23 h was adopted in this study to represent the acute phase of infection. This growth rate has recently been demonstrated experimentally in the lungs of mice during early infection (52). The lower dilution rate of 0.01 h^{-1} was proposed to correspond to a persistent mycobacterial infection. The average growth char-

TABLE 1. Characteristics and composition of *M. bovis* BCG grown in continuous culture at two growth rates[a]

Characteristic	Units	Value for low growth rate	Value for high growth rate
Dilution rate	h^{-1}	0.01	0.03
Doubling time	h^{-1}	69.30	23.10
Optical density (600 nm)		1.91	0.83
CFU	10^6 ml^{-1}	163	4.64
Bacterial concentration	g of dry wt l^{-1}	2.2	0.32
Yield constant	g of bacteria g of $glycerol^{-1}$	0.30	0.36
Protein content	%	22.91	21.40
RNA content	%	1.28	3.58
DNA content	%	3.21	2.19
Carbohydrate content	%	29.20	21.91
Lipid content	%	33.90	44.82

[a] Values represent averaged data from two independent chemostat experiments. Cultures were assumed to be in the steady state when carbon dioxide and biomass production remained constant. All assays were performed a minimum of three times.

acteristics of BCG at these growth rates once the steady state had been reached (Table 1) were in good agreement with Monod's standard mathematical model for microbial population dynamics in a chemostat (for reviews of this model, see references 22, 28, and 39). There were 5.1 times as many cells per gram in cultures grown at the high growth rate (t_d = 23 h) as in cultures grown at the low growth rate (t_d = 69 h). This is consistent with the faster growing mycobacterial cells being much larger than slowly growing cells (15). The yield constant (defined as the mass of bacteria formed divided by the mass of substrate consumed) decreased at the higher dilution rate, as is usually observed (22).

The macromolecular composition of microbial cells has been shown to change with environmental conditions and the specific growth rate (6, 15). For this study, the macromolecular composition of BCG cells was experimentally determined at two different growth rates in carbon-limited chemostats (Table 1). A large percentage (63 to 67%) of the dry cell weight of BCG was found to be carbohydrates and lipids, whereas a much smaller percentage of the biomass was made up of proteins, RNAs, and DNA. The mycobacterial cell composition was therefore shown to be very different from that of *E. coli*. In *E. coli*, 75 to 91% of the dry weight is proteins and nucleic acids, with carbohydrates, lipids, soluble metabolites, and salts comprising the remaining dry mass (6). However, like the case for *E. coli*, we found that the sum of the dry weight percentages of protein, RNA, and DNA was independent of the growth rate (8).

The proportions of protein measured by two independent methods were identical and showed that the protein content of mycobacterial cells remains relatively constant, at 21 to 22% (wt/wt), and that it falls slightly as the growth rate increases. The validity of the protein data was further confirmed by the results of the elemental (CHN) analysis. This protein concentration is much lower than that predicted by Cox and Colston (53.70%) (10–12) but is of the same magnitude as the concentration reported for *M. tuberculosis* (67). These data also showed that the pools of RNAs and lipids increased with the specific growth rate, whereas the pool of carbohydrates decreased. The largest change was observed for the RNA component, reflecting the increasing concentration of ribosomes at higher growth rates (6). The sum of the proportions of proteins, RNAs, DNA, lipids, and carbohydrates corresponded to 90 to 93% of the total dry weight; the remaining 7 to 10% can be accounted for by soluble metabolites and salts (6).

Stoichiometric composition model for *M. bovis* BCG. To obtain a detailed inventory of the mycobacterial cell, we constructed a macromolecular composition model and calculated the elemental composition of the component macromolecules. This is a strategy which has been employed by others and is an important step in the development of a metabolic network such as those used for metabolic flux analysis (14). The average amino acid composition of the protein component was obtained from the Horizontal Gene Transfer Database (http://www.fut.es/~debb/HGT/) (20). RNA and DNA compositions were calculated by assuming a GC content of 65.6%, and the constituents of the average carbohydrate were calculated by the method of Winder et al. (9, 65). The elemental compositions of lipids were obtained experimentally by determining the CHN values for extracted lipids. Using the resulting composition model (Table 2), we calculated the elemental biomass compositions from the macromolecular biomass data and compared them to experimentally determined CHN data. At growth rates of 0.01 and 0.03 h^{-1}, compositions of $C_{1.000}H_{2.046}N_{0.109}O_{0.726}P_{0.005}S_{0.002}X$ (M_r = 27.43 g mol^{-1}) and $C_{1.000}H_{2.038}N_{0.107}O_{0.810}P_{0.007}S_{0.001}X$ (M_r = 28.93 g mol^{-1}), respectively, were calculated, with "X" denoting less important elements. These values are in good agreement with the experimentally determined values of $C_{1.000}H_{1.97}N_{0.114}$ (M_r = 26.15 g mol^{-1}) and $C_{1.000}H_{1.915}N_{0.1124}$ (M_r = 29.43 g mol^{-1}), thus validating the results.

The mycobacterial cell. To estimate the size and intracellular concentrations of components of the mycobacterial cell and also to facilitate comparisons with other published data, we needed to convert the macromolecular and elemental data from units per gram of dry weight to units per cell. However, since viable counts are a notoriously inaccurate measure of cell number for mycobacteria due to clumping, cell numbers were estimated by use of the quantity of DNA in the biomass (Table 1) (8, 11). All values needed for the calculations were experimentally determined except for the number of genomes per cell, which was calculated by using the equation $G = (t_d/\ln_2 C)\{[2(C + D)/td]/t_d - 2^{D/t_d}\}$ (8, 10, 23). This equation was derived to calculate the number of genomes in the cell during balanced growth in terms of the doubling time (t_d), the time taken to replicate the genome (C), and the time between the end of DNA synthesis and cell separation (D) (8). A C period of 10.33 h has been measured for *M. tuberculosis*, and although no data are available for the D period, it has been estimated to be approximately 6 h (10, 11, 23). The number of genomes per cell was calculated to be 1.12 at a dilution rate of 0.01 h^{-1} and 1.40 at a dilution rate of 0.03 h^{-1}.

The amount of RNA reflects the number of ribosomes in the cell, and therefore, in accordance with expectations, the RNA content per cell increased from 2.11 fg per cell at a dilution rate of 0.01 h^{-1} to 9.96 fg per cell at a dilution rate of 0.03 h^{-1} to accommodate the growth rate demand (Table 3). There are no data on the relative concentrations of mRNAs, tRNAs, and rRNAs in mycobacteria, but in *E. coli* the relative contents are

TABLE 2. Stoichiometric model of *M. bovis* BCG macromolecular compartments

Compartment	% of cell composition (wt/wt)
Amino acids[a]	
Ala	9.41
Arg	10.87
Asp	2.43
Asn	6.18
Cys	0.97
Glu	3.63
Gln	5.53
Gly	5.47
His	2.86
Ile	4.41
Leu	10.18
Lys	2.46
Met	2.27
Phe	3.83
Pro	5.43
Ser	4.71
Thr	5.62
Trp	2.45
Tyr	3.04
Val	8.25
DNA[b]	
dAMP	17.42
dCMP	30.81
dGMP	34.83
dTMP	16.95
RNA[b]	
AMP	18.53
CMP	31.12
GMP	34.98
UMP	16.37
Carbohydrates[c]	
Trehalose	7.78
Polysaccharide	4.67
Glucose	13.97
Mannose	8.14
Oligosacharide	0.12
6-*O*-methylglucose	0.83
Galactose	0.84
Arabinose	19.86
Ribose	1.55
Galactose	3.91
Glucosamine	32.73
Muramic acid	4.49
Glycerol	1.11
Lipids[d]	
Carbon	52.75
Hydrogen	1.64
Nitrogen	9.54
Oxygen	36.07

[a] The amino acid composition of an average *M. tuberculosis* protein was calculated from data on the http://www.fut.es/≈debb/HGT/website (20).
[b] RNA and DNA compositions were calculated for a GC content of 65.6% (9).
[c] The constituents of an average carbohydrate were calculated from the data of Winder and Rooney (65).
[d] The elemental lipid composition was determined experimentally by use of a CHN analyzer.

2, 15, and 83%, respectively (6). Although at very low growth rates the tRNA content increases at the expense of rRNA, rRNA levels are considered to be relatively constant at most growth rates (53). By using these values and the molecular weight of RNAs in an *M. tuberculosis* ribosome, we could estimate the number of ribosomes per cell (9). In addition, under steady-state growth conditions and assuming negligible or constant protein turnover, the ribosome efficiency could be shown to equal $\mu \cdot P/R$, where μ is the specific growth rate and P and R are the amount of cellular protein and the ribosome content (number of ribosomes), respectively (6, 10). According to such calculations, the slowly growing cells contained 687 ribosomes with an efficiency of 0.00055 fg of protein synthesized per ribosome per h, and the fast growing cells contained 3,880 ribosomes with an efficiency of 0.00056. The ribosomal efficiency was 21 times lower than the maximum efficiency reported for *E. coli* and appeared to be independent of the growth rate, a characteristic which has been reported for other bacteria (46, 49, 55)

TABLE 3. Macromolecular properties of carbon-limited *M. bovis* BCG cells cultured at two specific growth rates in a chemostat

Characteristic	Units	Value of low growth rate	Value at high growth rate
Dilution rate	h^{-1}	0.01	0.03
Doubling time (t_d)	h^{-1}	69.30	23.10
Genomes per cell[a]	No.	1.12	1.40
DNA per cell[b]	fg	5.35	6.73
RNA per cell	fg	2.24	9.96
Ribosomes per cell[c]	No.	686.58	3,879.61
Protein per cell	fg	37.74	72.07
Carbohydrate per cell	fg	49.06	60.93
Lipid per cell	fg	57.31	124.79
RNA/protein	Ratio	0.059	0.138
RNA/DNA	Ratio	0.419	1.480

[a] The number of genomes per cell was calculated by use of the formula described in the text, using the values $C = 10.33$ h and $D = 6$ h (8, 10, 23).
[b] The weight of the genome was calculated to be 4.79 fg (9).
[c] The number of ribosomes was calculated on the basis that rRNA comprises 83% of the total RNA and that each *M. bovis* BCG ribosome consists of 4,790 nucleotides (9, 12).

Lipids and carbohydrates make up a large proportion of the BCG cell. At a specific growth rate of 0.03 h^{-1}, the cells contained 124.80 and 57.31 fg of lipids and carbohydrates, respectively. Values of 54 fg of carbohydrate and 76 fg of lipid were reported by Winder and Rooney (65) for *M. bovis* BCG growing in batch cultures. Since our data show that the lipid content decreases with the growth rate, the differences in these observations are likely a reflection of the growth rate and the culture conditions of the experiments. The correlation between the growth rate and the lipid content of *M. bovis* BCG reflects a change in the pool of intracellular reserves, which may have particular significance for mycobacteria growing in the host.

DISCUSSION

A model experimental system for studying mycobacterial growth in carbon-limited chemostats was developed during this study, allowing for a complete description of changes in the cells' (macro)molecular composition in different physiological states. With this model, two growth rates were investigated to simulate possible growth rates at different stages of TB infection. A high growth rate of 0.03 h^{-1} ($t_d = 23$ h) was used as representative of cells during active disease, while a lower growth rate of 0.01 h^{-1} ($t_d = 69$ h) was proposed to correspond

to a persistent mycobacterial infection (52). This model provided ideal conditions for analyzing the physiology of mycobacteria at different growth rates, and these results provide the first reliable descriptions of the effects of the growth rate on the macromolecular and elemental composition of the mycobacterial cell. In addition, the stoichiometric model of macromolecular cellular composition constructed for this study allows the precursor requirements for *M. bovis* BCG to be predicted and is therefore an important first step in the development of a metabolic flux network for the tubercle bacillus.

In recognition of the importance of this topic, a theoretical framework was recently developed to examine the growth of *M. bovis* BCG by the use of macromolecular compositions from a limited amount of data reported in the 1970s (11, 12, 64). The general growth-related trends in the proportions of protein, RNA, and DNA for *M. bovis* BCG presented in this study follow a very similar pattern to the behavior predicted by the mathematical model (11). There are, however, significant discrepancies between the previous observations with batch-cultured *M. bovis* BCG and the results presented here (64). Most significantly, the amounts of protein and RNA in continuously grown cultures are less than those that were previously predicted, and as a consequence, the macromolecular composition equations derived by Cox (11) were found not to apply to *M. bovis* BCG cells grown in a chemostat.

It is difficult to interpret the significance of this discrepancy. The data used for the previous study were obtained from cells that were cultured for 6 days prior to sampling, and therefore, as observed by Winder and Rooney, the bacteria were not in the exponential growth phase (64). This compromises the validity and accuracy of the data. In contrast, bacterial cells at the steady state reached in the chemostat are physiologically homogenous, evenly distributed in a glycerol-limited environment, and all replicating at a controlled rate. Therefore, the overall macromolecular composition determined for the whole population can be averaged over all of the cells in the population.

The RNA-to-protein ratio is a rough measure of the physiological state of cells. The significance of this ratio lies in its close relationship to the ribosome synthesis rate, which is a measure of the metabolic activity that the cell devotes to ribosome synthesis. It has been demonstrated here that the RNA/protein ratio increases with the growth rate, a property that has been reported for other bacteria and that reflects changes in the rRNA concentrations of the cell. The notion that ribosomes function at a constant maximum efficiency and that an increase in the growth rate can only be achieved by increasing the ribosome concentration has become a central dogma in bacterial physiology. The relative constancy of the ribosome efficiencies calculated for *M. bovis* BCG is in accordance with this theory. Earlier studies by Winder and Rooney (64) suggested that BCG maintains a constant level of ribosome synthesis and may not be capable of altering cellular ribosome levels by regulating synthesis. Cross-species experiments have shown that the two promoters of the *rrn* operon of *M. tuberculosis* are both subject to growth rate-dependent control (GRDC) in the environment provided by *Mycobacterium smegmatis*, although other investigators have demonstrated that the rate of rRNA transcription initiation did not vary between the logarithmic and stationary phases of growth, leading them to conclude that GRDC of rRNA does not occur in *M. tuberculosis* (38, 57). The present study indicates that this is not the case, as the amount of RNA was altered significantly as a function of the growth rate, suggesting that the BCG *rrn* promoters are subject to GRDC under carbon-limiting conditions.

Mycobacteria dedicate large amounts of energy towards making lipids for construction of the cell wall and also for storage purposes. Lipids were the main component of the carbon-limited cells, and the fast growing cells contained twice as much lipid as slowly growing cells. Research has shown that the total lipid content both increases and decreases during the growth of mycobacterial cultures (7, 44). This is likely a consequence of changes in the availability of carbon, which can be converted into lipids. The lipid composition values obtained for chemostat-grown cells from carbon-limited cultures in this study are therefore likely to represent the minimum proportions of lipid required to maintain cellular metabolic and structural requirements at the imposed growth rate. The increased lipid composition observed at the high growth rate may reflect a buildup of energy reserves, which may be relevant to the survival of mycobacteria during persistence.

Nutrient limitation is likely a feature of the microenvironment of *M. tuberculosis* in vivo, as the growth substrates available are dependent on the host's metabolic activities. Although investigations of the intracellular survival of various auxotrophic mutants can give clues to the nutrient availability inside the host, the precise composition of each niche can only be guessed (26, 47). Carbon was chosen as the rate-limiting nutrient for this study because evidence suggests that the phagosomal environment is carbohydrate limited (50). The effect of starvation on mycobacterial cell physiology has previously been studied by simply resuspending cells in phosphate buffer (5). More recently, the effects of nutrient depletion on the transcriptome of *M. tuberculosis* have been investigated by the use of an extended stationary-phase culture in a bioreactor (21). This system allowed the pH, temperature, and dissolved oxygen tension to be kept constant throughout the experiments. Although this model goes some way toward addressing the problems associated with flask batch cultivations, the stationary phase of growth remains a very poorly defined condition. The mixture of cells in different states of growth and death leads to a fluctuating environment. Indeed, by the end of a 100-day culture in the extended stationary-phase model, 99% of the original culture had died, with a concomitant release of toxins and metabolites that would impact the physiological state of the surviving organisms (21). In contrast, the effect of carbon limitation can be studied in isolation in the chemostat model provided by this study. The specific response of the cell to carbon limitation can therefore be elucidated.

Another important issue to be addressed is the calculation of the distribution of metabolic fluxes under the different physiological conditions studied. MFA is a powerful methodology for the analysis of metabolic pathways. This technique has already proved to be an invaluable tool in metabolic engineering and has been used to successfully identify novel pathways in bacteria, providing an enormous potential for functional genomics (18). MFA calculates intracellular fluxes by using a combination of mathematical and experimental modeling. The minimum requirements for MFA are (i) the assembly of a metabolic flux network, (ii) the cultivation of the organism

under steady-state conditions, and (iii) specific measurements, including the macromolecular composition of the biomass and of extracellular metabolites. This study describes an appropriate system (i.e., chemostat cultures) for performing metabolic flux analysis and also provides data that may be used as input in a metabolic flux network. In addition, the stoichiometric model of macromolecular cellular composition has contributed to a metabolic flux matrix that is currently under development in our laboratory. MFA has never been attempted for *M. tuberculosis*, but given the importance that shifts in metabolism might have on the virulence of this pathogen, this technique may have a significant impact on mycobacterial research.

Models of persistence have focused on inducing a static state in which mycobacteria are not growing or dying; however, the state of bacterial growth at sites of latent TB is unknown (5, 58). Low growth rates and nutrient limitation may mimic the in vivo environment of persistent mycobacteria, and therefore studies such as the one presented here will reveal important clues about the physiology of persistence. This model may also be particularly useful for testing the sterilizing activities of new antitubercular drugs targeted towards persistent mycobacteria.

This study provides the macromolecular composition of glycerol-limited BCG cells at specific and well-defined growth rates. Extending these studies to include additional growth rates and other carbon sources would allow a more detailed understanding of how organisms structure their cells in response to the environment. It has been proposed that *M. tuberculosis* primarily metabolizes lipids in the host (51, 59). Investigating the physiological state of lipid-limited mycobacterial cells will therefore be an important extension of this work and may help to define the status of the *M. tuberculosis* cell in vivo.

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