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Regulation of Central Metabolism Genes of *Mycobacterium tuberculosis* by Parallel Feed-Forward Loops Controlled by Sigma Factor E (σ^E)$^{\nabla}$†

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Cells respond to external stimuli through networks of regulatory interactions. The human pathogen *Mycobacterium tuberculosis* responds to stress encountered during infection by arresting multiplication and implementing critical metabolic changes that lead to or sustain the nonreplicative state. Much of this differentiation program is recapitulated when *M. tuberculosis* cultures are subjected to gradual oxygen depletion *in vitro*. Here we report that hypoxic induction of critical central metabolism genes in the glyoxylate shunt (*icl1*) and in the methylcitrate cycle (*gltA1*) involves both global and local regulators. The global regulators are accessory sigma factors σ^B for *icl1* and σ^E for *gltA1*. The local regulators are the products of two paralogous genes mapping at positions adjacent to the corresponding effector gene or operon. We call these genes *lrpI* and *lrpG* (for local regulatory protein of *icl1* and *gltA1*). We also found that (i) each sigma factor controls the corresponding local regulator, (ii) both global and local regulators are required for effector gene induction, and (iii) the occurrence of sigma factor control of effector gene induction is independent of its control over the corresponding local regulator. Together, these data indicate that induction of *icl1* and *gltA1* utilizes parallel feed-forward loops with an AND input function. Both feed-forward loops are affected by σ^E, since this sigma factor is part of the *gltA1* loop and controls *sigB* in the *icl1* loop. Feed-forward loops may critically contribute to the cellular developmental program associated with *M. tuberculosis* dormancy.

Bacteria have evolved complex strategies to survive stress encountered in their habitats. Much of the complexity resides in the structure of the regulatory network that controls the stress response. Work with model microorganisms has shown that the stress response usually requires the integration of multiple signal-processing pathways by information-processing units that control downstream cascades, ultimately regulating effector gene expression (2, 13, 24). Understanding such complexity is facilitated by dissecting the regulatory network into smaller, relatively autonomous units that are amenable to experimental manipulation. These units are often identified as highly recurring network motifs (28). One such motif, termed a feed-forward loop (FFL), is defined by a transcription factor, X, that regulates a second transcription factor, Y, and by joint regulation by X and Y of a third gene/operon, Z (14). The relationships between the structure and dynamics of FFLs have been previously investigated, primarily in *Escherichia coli* studies (14, 28), and may have functional implications. For example, coherent type 1 FFLs, in which all regulatory interactions have a positive sign (activation), can filter noise and protect against brief input fluctuations (15). How network motifs are implicated in the virulence and adaptation of pathogenic bacteria is unknown. Here we report that parallel FFLs (each composed of a master regulator, a local regulator, and a central metabolism gene) are utilized in the stress response of two critical central metabolism genes of the human pathogen *Mycobacterium tuberculosis*.

M. tuberculosis is an intracellular pathogen that responds to the stress generated by the host immune response by entering a nonreplicating state associated with a chronic, asymptomatic (latent) infection. Latent infection progresses to disease when the immune response is depressed and tubercle bacilli resume growth. The response of *M. tuberculosis* to immunity-mediated stress includes changes in bacterial central metabolism, which have typically been revealed by transcriptional analyses of tubercle bacilli during mouse lung infection (see, for example, references 29, 30, 31, and 34). Two metabolic pathways that are involved in lipid catabolism, the glyoxylate shunt and the methylcitrate cycle, have been previously investigated in detail (19, 21, 22). Both pathways utilize the product of *icl1* (*aceA*, or *rv0467* according to the *M. tuberculosis* H_{37}Rv numbering system devised by Cole et al. [3]), which exhibits isocitrate lyase activity in the glyoxylate shunt and 2-methyl-isocitrate lyase activity in the methylcitrate cycle (10). Two additional enzymes, methylcitrate dehydratase and methylcitrate synthase, which are encoded by the *gltA1* operon (also named *prpDC* and *rv1130–rv1131*), are involved in the methylcitrate cycle. Upregulation of *icl1* and *gltA1* occurs when tubercle bacilli respond to expression of adaptive immunity in the lungs of infected mice (31, 34). Tubercle bacilli isolated from sputum of tuberculosis patients also show increased expression of *icl1* relative to cultures growing exponentially *in vitro* (8). Thus, induction of these pathways is relevant to the bacterial state *in vivo*.

Regulation of *icl1* and *gltA1* involves accessory sigma factors (27), which direct the bacterial RNA polymerase holoenzyme to specific genes during particular growth phases or under

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pMP167	*Mycobacterium*/*E. coli* shuttle vector with *groEL* promoter; Apr^r	4
pRv0465c	*rv0465c* gene locus cloned into pMP167 with PvuII and EcoRI	This study
pRv1129c	*rv1129c* gene locus cloned into pMP167 with PvuII and EcoRI	This study

particular stress conditions (11). Gene expression data determined by microarray hybridization show that *icl1* and *gltA1* are among the genes regulated by σ^E under conditions of detergent-mediated, cell-surface stress (17). σ^E also regulates *sigB* (19), and it is regulated by *sigH* under conditions of heat and oxidative stress (16, 26). Moreover, the *gltA1* operon is regulated by σ^B when tubercle bacilli are subjected to diamide stress (7). Thus, it is likely that multiple accessory sigma factors are involved in the regulation of *icl1* and *gltA1*. How these sigma factors interact to regulate the two effector genes (and whether additional transcriptional factors are involved) has not been established.

In the present work, we used a model of gradual oxygen starvation of *M. tuberculosis* cultures *in vitro* (35) to examine regulatory interactions that control expression of *icl1* and *gltA1* in response to bacteriostatic treatment. This *in vitro* model recapitulates transcriptional changes seen in tubercle bacilli during mouse lung infection (see, for example, references 29, 30, and 31). We found that induction of *icl1* and *gltA1* during hypoxic stress is regulated by two parallel, σ^E-controlled FFLs that include a global regulator (an accessory sigma factor) and a local regulator.

MATERIALS AND METHODS

Bacterial strains, reagents and media, and growth conditions. *M. tuberculosis* mutants with knockouts in *sigE* and *sigB*, complemented *sigE* and *sigB* mutants, and transposon-insertion mutants in gene loci *rv1129c* and *rv0465c* were previously reported (see references 7 and 17 and http://webhost.nts.jhu.edu/target). The gene numbering of the *M. tuberculosis* genome is presented according to the system of Cole et al. (3). *M. tuberculosis* cultures were grown in Middlebrook (MB) 7H9 (liquid medium) or 7H10 (solid medium) (Difco) supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% ADN (2% glucose, 5% bovine serum albumin [BSA; Sigma], 0.15 M NaCl). Aerated liquid cultures of *M. tuberculosis* were grown in 25-ml tubes at 37°C with magnetic-bar stirring at 450 rpm. Plates were incubated at 37°C in sealed plastic bags. Hypoxic cultures of *M. tuberculosis* were grown in Dubos Tween-albumin broth (Becton Dickinson). *Escherichia coli* XL1 Blue was grown in Luria-Bertani broth (LB; Difco), and on LB agar plates at 37°C.

Construction of *rv0465c*- and *rv1129*-complementing plasmids. Fragments containing the coding region of *rv0465c* or *rv1129c* were amplified by PCR from *M. tuberculosis* genomic DNA (see Table S1 in the supplemental material for PCR primers) and cloned under the control of the constitutive *groEL* promoter in the replicative apramycin (Apr) resistance shuttle plasmid pMP167 (4). The resulting plasmids were named pRv0465c and pRv1129c (Table 1). *E. coli* transformants were selected on LB agar plates containing 40 μg/ml apramycin. Recombinant plasmids were transferred to *M. tuberculosis* by electroporation. Transformants were selected on Middlebrook 7H10 agar plates containing 40 μg/ml apramycin after three weeks of incubation at 37°C.

Gradual oxygen depletion. *M. tuberculosis* cultures were grown in Dubos Tween-albumin broth at 37°C as described by Wayne and Hayes (35). In brief, mid-log cultures were diluted to an optical density at 580 nm (OD_{580}) of 0.004 and subjected to slow stirring with a magnetic stirring bar in sealed tubes with a ratio of headspace air to medium of 0.5. Growth was monitored by turbidity measurement and CFU enumeration. At selected times, cells were harvested by centrifugation and quickly frozen in a dry-ice/alcohol bath for subsequent RNA extraction.

Enumeration of bacterial transcripts. RNA extraction, reverse transcription, and real-time quantitative PCR (qPCR) were performed as previously described (see reference 29 and http://www.phri.org/research/res_pigennaro.asp). Briefly, bacterial cell pellets were resuspended in 1 ml of TRI reagent (Molecular Research Center, Cincinnati, OH) and disrupted by rapid mechanical lysis. Total RNA was purified in TRI reagent according to the manufacturer's instructions and stored at −80°C. Reverse transcription was performed with random hexameric primers and ThermoScript reverse transcriptase (Invitrogen, Carlsbad, CA). Enumeration of *M. tuberculosis* mRNAs was carried out by qPCR using gene-specific primers, molecular beacons, and AmpliTaq Gold polymerase (Applied Biosystems) in a Stratagene Mx4000 thermal cycler (Agilent Technologies). Nucleotide sequences of PCR primers and molecular beacons are listed in Table S2 in the supplemental material. The *M. tuberculosis* 16S rRNA copy number was used as a normalization factor to enumerate bacterial transcripts per cell, as previously described (29).

RESULTS

The hypoxic response of *icl1* and *gltA1*. When *M. tuberculosis* cultures are subjected to gradual oxygen depletion, they stop growing after approximately 3 days of incubation (35) (Fig. 1A). We used this growth arrest model to investigate the expression of *ic11* and *gltA1*. Transcript enumeration by qPCR showed that both genes were induced: *icl1* peaked at day 3 and *gltA1* at day 4 of oxygen depletion (5- and 10-fold induction relative to mid-log growth, respectively) (Fig. 1B and C). Thus, *icl1* and *gltA1* are transiently upregulated during gradual O_2 depletion.

Sigma factor requirements for the hypoxic response of *gltA1* and *icl1*. As noted above, it is likely that multiple accessory sigma factors are involved in the regulation of *icl1* and *gltA1*. One accessory sigma factor candidate is σ^E, since both *icl1* and *gltA1* were previously described as responding to sodium dodecyl sulfate (SDS)-mediated stress in a *sigE*-dependent fashion (17). When *sigE* transcript levels were examined during hypoxia, we observed a gradual induction (4- to 5-fold increase in copy numbers at day 5 relative to mid-log growth) (Fig. 2A), which indicates a transcriptional response of *sigE* to hypoxia. We then tested the role of *sigE* in the *icl1* and *gltA1* hypoxic response by enumerating *icl1* and *gltA1* transcripts in a *sigE* knockout mutant. Upregulation of *icl1* was reduced by the *sigE* mutation (48% decrease at day 3; $P < 0.05$ for the Student *t* test) (Fig. 2B), while that of *gltA1* was nearly abolished (Fig. 2C).

The partial effect of *sigE* inactivation on *icl1* induction suggested that additional sigma factors might be involved in the network regulating *icl1*. A potential candidate was *sigB*, since *sigB* contains a *sigE*-dependent promoter (17, 32) and because *sigB* levels are decreased in a *sigE* mutant during logarithmic growth, SDS-mediated stress (17), and hypoxia (Fig. 2D). We found that a *sigB* knockout mutation nearly abolished induction of *icl1* (but not of *gltA1*) during hypoxia (Fig. 2 B and C). Thus, induction of *gltA1* requires a functional *sigE*, whereas induction of *icl1* requires *sigB* function. Given the effect of *sigE* on *sigB* expression, the weaker effect on *icl1* induction of *sigE* loss of function relative to that of *sigB* suggests that *sigE* might control the *icl1* response via *sigB*.

Two local regulatory factors and their requirement for the hypoxic response of *gltA1* and *icl1*. Among the gene loci regulated by σ^E in SDS-stressed cultures are *rv0465c* and *rv1129c* (17). These two gene loci are annotated as transcription factors

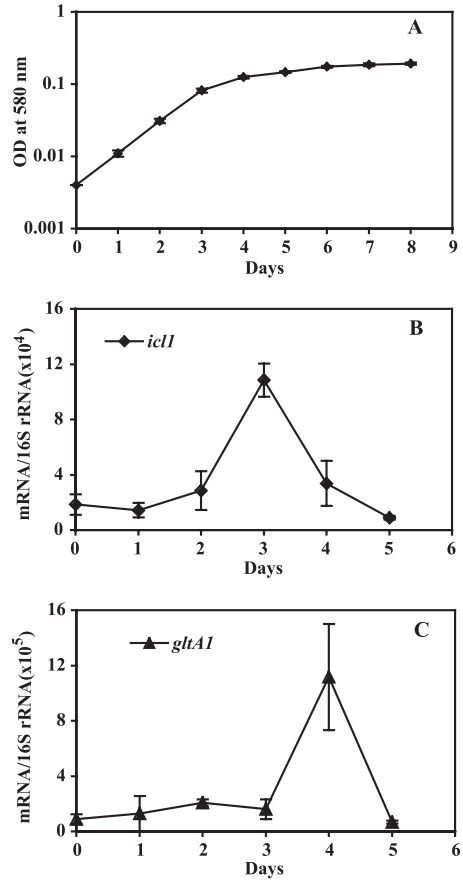

FIG. 1. The hypoxic response of *icl1* and *gltA1*. Cultures of *M. tuberculosis* were subjected to gradual O_2 depletion (35). (A) Growth curve. In the Wayne model, cultures of *M. tuberculosis* continue to multiply for approximately 70 h and then stop growing (microaerophilia). After this time, the additional slow rise in absorbance is due to cell wall enlargement. Anaerobiosis ensues at around 200 h, with no further cell enlargement. Bacterial growth was monitored by measuring OD at 580 nm. (B and C) Enumeration of *icl1* and *gltA1* transcripts. Cultures subjected to gradual O_2 depletion were harvested daily, RNA was extracted, and bacterial transcripts were enumerated by qPCR. Transcript copy numbers were normalized to 16S rRNA (29, 30). Three independent experiments were carried out, and means (± standard deviations [SD]) were calculated for normalized mRNA copy numbers at each time point. The results shown represent normalized copy numbers of mRNA for *icl1* and *gltA1* at each time point (one gene per panel, as indicated). Measurements performed using *M. tuberculosis* H_{37}Rv and *M. tuberculosis* CDC1551 gave indistinguishable results (see Fig. S1 in the supplemental material), indicating that the hypoxic response of *icl1* and *gltA1* is conserved among different *M. tuberculosis* strains.

(http://genolist.pasteur.fr/TubercuList/) and map at positions adjacent to (but on the opposite strand from) *icl1* (*rv0467*) and *gltA1* (the *gltA1* operon includes *rv1130* and *rv1131*, which encode two enzymes of the methylcitrate cycle [22]), respectively. The deduced amino acid sequences share 47% identity and a three-domain architecture that includes DNA-binding motifs (Fig. 3). These observations raised the possibility that *rv0465c* and *rv1129c* are paralogous gene loci involved in the regulation of the adjacent effector genes. When we measured the relative levels of abundance of *rv0465c* and *rv1129c* transcripts during hypoxia, we found that each gene was upregu-

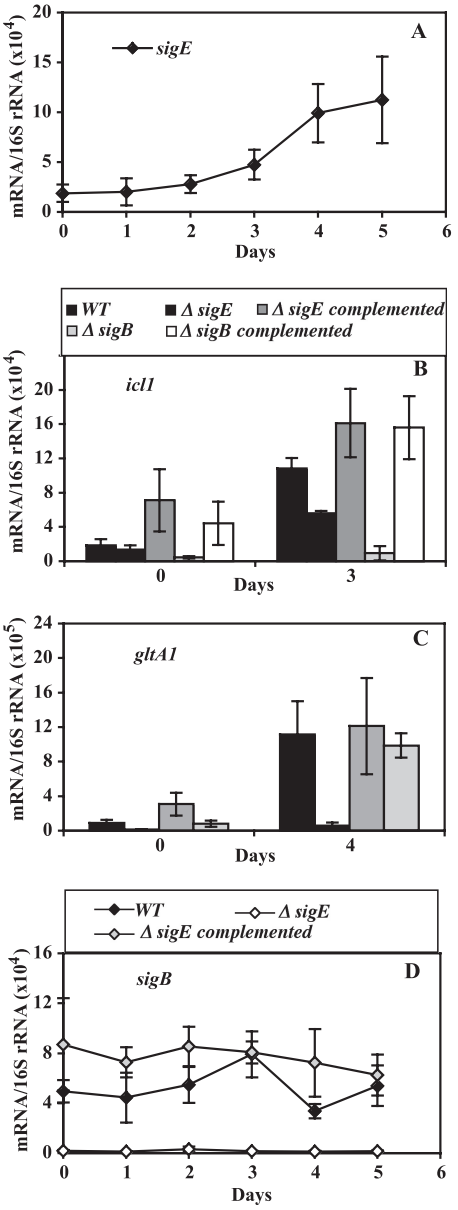

FIG. 2. Effect of *sigE* and *sigB* inactivation on the hypoxic response of *icl1* and *gltA1*. Wild-type and mutant cultures were subjected to gradual O_2 depletion, cells were harvested, RNA was extracted, and bacterial transcripts were enumerated by qPCR, as described in the Fig. 1 legend. In all panels, means (± SD) for each time point were calculated from data from three independent experiments. For the duration of the treatment, CFU counts of wild-type and mutant strains were indistinguishable (see Fig. S2 in the supplemental material). Each panel presents data for one gene. (A) The hypoxic response of *sigE*. The results shown represent normalized copy numbers of the *sigE* transcript in wild-type cultures during 5 days of a hypoxic time course. (B and C) The hypoxic response of effector genes. The results shown represent normalized copy numbers of *icl1* (B) and *gltA1* (C) at mid-log growth (day 0) and at the day of peak gene activation (day 3 for *icl1* and day 4 for *gltA1*) in wild-type, *sigE* and *sigB* mutant, and complemented mutant strains. (D) The hypoxic response of *sigB*. The results shown represent normalized copy numbers of the *sigB* transcript in wild-type, *sigE* mutant, and complemented *sigE* mutant cultures during 5 days of a hypoxic time course.

Rv0465c aa no. 10-64	187-310	311-469	475-end
Rv1129c aa no. 22-76	199-322	323-479	486-end

FIG. 3. Architecture of the proteins encoded by *rv0465c* and *rv1129c*. The deduced amino acid sequences of Rv0465c and Rv1129c were interrogated against an integrated database of protein domains and functional sites with the InterProScan package (http://www.ebi.ac.uk/Tools/InterProScan/). The analysis revealed that the two proteins belong to a protein family sharing a three-domain architecture: HTH_3 is a helix-turn-helix, DNA-binding motif; DUF955 (IPR010359) is a domain of unknown function; and DUF2083 (IPR018653) is annotated as a predicted transcriptional regulator found in the XRE family of prokaryotic transcriptional regulatory proteins (http://dbtbs.hgc.jp/ver1/tfactable.html). Rv0465c and Rv1129c are members of a ubiquitous protein family and are widely distributed in *Mycobacteriaceae* (see Fig. S3 in the supplemental material).

lated at the same time as the corresponding adjacent effector gene (Fig. 4A and B; compare with Fig. 1B and C). When we used qPCR to assess the hypoxic response of *icl1* and *gltA1* in transposon-insertion mutants of *rv0465c* and *rv1129c*, we found that the absence of *rv0465c* and *rv1129c* abolished induction of *icl1* and *gltA1*, respectively (Fig. 4C and D). Thus, *rv0465c* and *rv1129c* are required for the hypoxic response of the adjacent effector gene. Moreover, their activity is specific to the target, since deletion of either regulator had little if any effect on the expression of the distant target gene.

Interactions between sigma factors and *rv0465c* and *rv1129c* in the hypoxic response of *icl1* and *gltA1*. We tested the requirement for *sigE* and *sigB* in the induction of *rv0465c* and *rv1129c* during hypoxic stress. Induction of *rv0465c* was abolished by a *sigB* mutation and was decreased almost 50% by a *sigE* mutation (Fig. 5A), which are results comparable with those obtained for *icl1* induction with these two mutant strains (Fig. 2B). Similarly, induction of *rv1129c* was abolished by a *sigE* mutation and unaffected by a *sigB* mutation (Fig. 5B), as seen with *gltA1* (Fig. 2C). Thus, induction of the local regulators *rv0465c* and *rv1129c* in hypoxic cultures required functional *sigB* and *sigE*, respectively, which is similar to the results seen with induction of the corresponding effector genes. To fully examine the regulatory relationships between local regulators and sigma factors, we also tested the effect of mutations in *rv0465c* and *rv1129c* on the hypoxic response of *sigE* and *sigB*. We found no effect caused by the local regulators on these sigma factors (Fig. 5C and D), indicating that the local regulators exert no feedback regulation on the master regulators (i.e., the sigma factors).

We next asked whether the observed dependence of effector gene induction on the sigma factor (*sigB* for *icl1* and *sigE* for *gltA1*) reflected a requirement for *rv0465c* and *rv1129c* induction. To address this question, we examined the sigma factor requirement for induction of the effector gene in the presence or absence of a constitutively expressed copy of the corresponding local regulator. We introduced a plasmid expressing *rv0465c* (or *rv1129c*) controlled by the strong constitutive *groEL* promoter in a *sigB* (or *sigE*) knockout mutant and measured the transcript abundance of *icl1* (or *gltA1*). Ectopic expression of the local regulator failed to bypass the relevant sigma factor requirement for *icl1* or *gltA1* induction in hypoxic cultures (Fig. 6A and B). That the plasmids used in these experiments expressed functional Rv0465c and Rv1129c proteins was demonstrated by the ability of the same plasmids to complement the corresponding mutations (Fig. 4C and D). Thus, the requirement for a sigma factor to induce *icl1* and *gltA1* is independent of the regulation of the local regulator by the same sigma factor.

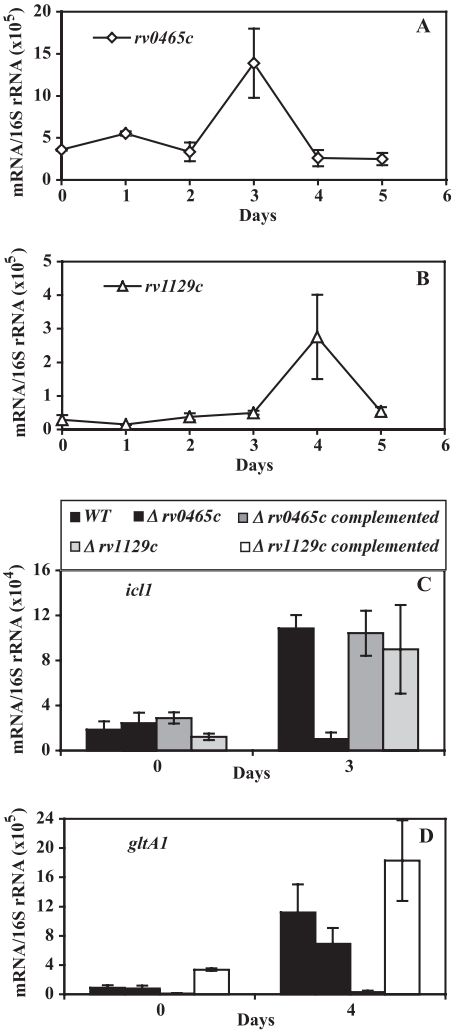

FIG. 4. Characterization of adjacent regulatory factors. O_2-depleted culture growth, RNA extraction, and mRNA enumeration by qPCR were conducted as described in the Fig. 1 legend. For the duration of the treatment, CFU counts of wild-type and mutant strains were indistinguishable (see Fig. S2 in the supplemental material). Each panel presents data for one gene. (A and B) The hypoxic response of *rv0465c* and *rv1129c*. The results shown represent normalized copy numbers of mRNA for *rv0465c* and *rv1129c* at each time point. (C and D) The hypoxic response of effector genes in wild-type and *rv0465c* and *rv1129c* transposon-insertion mutant strains. The results shown represent normalized copy numbers of *icl1* and *gltA1* at mid-log growth (day 0) and the day of peak gene activation (day 3 for *icl1* and day 4 for *gltA1*) in wild-type and *rv0465c* and *rv1129c* mutant strains and the corresponding complemented mutant strains. Means (± SD) for each time point were calculated from data from three independent experiments.

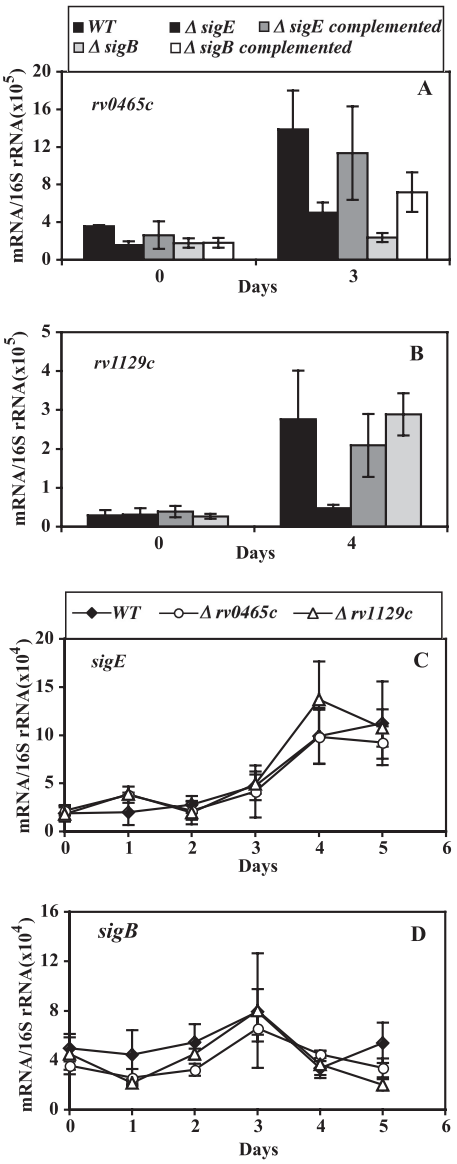

FIG. 5. Regulatory interactions between *sigE*, *sigB*, *rv0465c*, and *rv1129c*. O_2-depleted cultures, daily harvesting, and mRNA enumeration by qPCR were conducted as described in the Fig. 1 legend. Means (± SD) for each time point were calculated from data from three independent experiments. Each panel presents data for one gene. (A and B) Effect of *sigE* and *sigB* inactivation on the hypoxic response of *rv0465c* and *rv1129c*. Transcripts were enumerated and data are presented as described in the Fig. 2B and C legends. (C and D) Effect of *rv0465c* and *rv1129c* inactivation on the hypoxic response of *sigE* and *sigB*. Transcripts were enumerated and data are presented as described in the Fig. 2A to D legends.

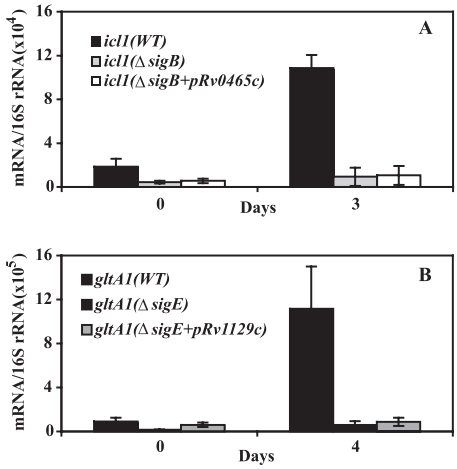

FIG. 6. Effect of constitutive expression of local regulators on *icl1* and *gltA1* hypoxic response in sigma factor mutants. Derivatives of plasmid pMP167 expressing *rv0465c* or *rv1129c* under the control of the constitutive *groEL* promoter (indicated as pRv0465c and pRv1129c) were introduced in *sigB* or *sigE* knockout mutant strains. Cultures were harvested at daily intervals, and mRNA enumeration by qPCR was conducted as described in the Fig. 1 legend. Each panel presents data for one gene (panel A, *icl1*; panel B, *gltA1*). The results shown represent the mid-log time point and the time points corresponding to peak gene induction (day 3 for *icl1* and day 4 for *gltA1*).

DISCUSSION

In the present report, we provide genetic evidence that the response to stress of two central metabolism genes of *M. tuberculosis*, *icl1* and *gltA1*, is controlled by a global regulator and by a regulator located near the effector gene/operon. The global regulators are sigma factors, σ^B for *icl1* and σ^E for *gltA1*. The local regulators are the products of *rv0465c* and *rv1129c*. To underscore their role in regulating adjacently located effector genes, we name these genes *lrpI* (local regulatory protein of *icl1*, or *rv0465c*) and *lrpG* (local regulatory protein of *gltA1*, or *rv1129c*). The data also show that (i) induction of either effector requires both global and local regulators and (ii) the global regulator controls both local regulator and effector. These regulatory interactions are not explained by a simple cascade in which the global regulator controlled the local regulator that in turn induced the effector operon, because high-level, constitutive expression of the local regulator failed to bypass the requirement for the global regulator. In contrast, the data demonstrate the existence of nested feed-forward loops (5, 28) in which an accessory sigma factor regulates a second, local regulator and both factors jointly regulate the target gene/operon (*icl1* or *gltA1*) (Fig. 7). Both FFLs appear to be of coherent type 1 (all regulatory interactions have a positive sign) and exhibit AND logic (both global and local regulators are needed to induce expression of the effector gene) (14).

Three-node, coherent type 1 FFLs do not fully account for the observed behavior of *icl1* and *gltA1* in response to hypoxia. A coherent type 1 FFL is expected *per se* to filter noise and reject transient input stimuli (14), whereas it should maintain the response for persistent input stimuli. However, even though hypoxia persisted in our experimental setting and *sigE* transcript levels gradually increased (at least through day 5; Fig. 2A), the hypoxic induction of *gltA1* and *icl1* was transient (Fig. 1B and C), with both transcripts returning to near-basal levels by day 5. Thus, the downturn of the *icl1* and *gltA1* hypoxic response strongly implies the existence of yet-unrevealed downregulatory interactions. One candidate for downregulation is the two-component system *mprAB* (36), since an *mprAB* mutant exhibits elevated levels of *gltA1* (but not of *icl1*) at day 5 of hypoxia, the time when *gltA1* is downregulated in

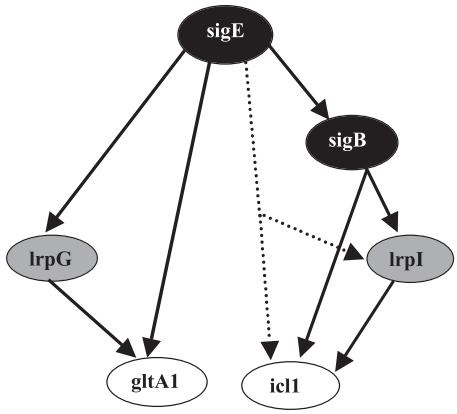

FIG. 7. Feed-forward loops controlling *icl1* and *gltA1* hypoxic induction. The figure shows the regulatory interactions revealed in the present work. The *sigE-sigB* interaction is taken from the literature (17, 32). The colors of the ovals containing the gene names represent gene classes in the network structure as follows: black, global regulators; gray, local regulators; white, effector genes. The dotted line indicates a potential direct interaction between *sigE* and *lrpI*/*icl1*, a possibility that cannot be excluded by our genetic data.

wild-type cells (data not shown). This result is consistent with the increased abundance of *gltA1* (but not *icl1*) transcripts seen in an *mprA* mutant relative to wild-type cells during SDS stress (23). Another possibility is that, as expression levels of *lrpI* and *lrpG* increase, the corresponding gene products become repressors, thus turning the FFLs from coherent to incoherent (many transcription factors function both as activators and repressors, for example, the bacteriophage lambda repressor [25]). The idea of dual regulation by the *lrp* genes is supported by the recent observation that the product of *rv0465c* (*lrpI*) represses *icl1* during growth of *M. tuberculosis* on glucose (20). Thus, studying gene expression in the context of network structure should help unravel additional regulatory interactions and reconstruct increasingly complex networks.

The gene network revealed by our genetic work requires biochemical characterization to assess protein-DNA interactions at the regulatory sequences of the genes in the network. Published data agree with our genetic results, since a σ^E recognition sequence is found upstream of the *gltA1* operon (17), the product of *rv0465c* binds to DNA upstream of *icl1* (20), and we have mapped a *sigA* or *sigB* promoter (it is not possible to discriminate between these two consensus sequences [27]) upstream of *icl1* (R. Manganelli and M. L. Gennaro, unpublished data). In particular, while our data clearly show that the effect of *sigB* on *lrpI* and *icl1* expression is more pronounced than that of *sigE*, we cannot exclude the possibility of a direct interaction between *sigE* and *lrpI* or *icl1* (as sketched in Fig. 7).

The dynamics and the regulation of the *icl1* and *gltA1* stress response revealed by our work fit well with the physiology of *M. tuberculosis* dormancy. The involvement of *sigE* as a common regulatory node may reflect a requirement for coordinated expression of *icl1* and *gltA1*, since the products of these two genes participate in the methylcitrate cycle (22), a metabolic pathway for the assimilation of propionyl coenzyme A (CoA) generated during lipid catabolism. Moreover, *icl1* and *gltA1* are critical for the metabolic remodeling associated with stress-induced growth arrest of *M. tuberculosis* (31); thus, it seems appropriate that their upregulation should be controlled by network structures, such as coherent FFLs, which reject transient input signals (15). Additional regulatory interactions may have evolved to turn off expression of *icl1* and *gltA1*, even when the input stimulus persists, to help implement the overall metabolic shutdown associated with dormancy. Indeed, the transient nature of *icl1* and *gltA1* induction should be physiologically relevant, since it is also seen during mouse lung infection (31). Moreover, peak induction of *icl1* precedes that of *gltA1* in the murine model as well (31), thus providing another similarity between the *in vivo* and *in vitro* situations. A more general consideration is that a role for FFLs in *M. tuberculosis* dormancy is consistent with the notion that these network structures are involved in cellular differentiation processes in other microorganisms, such as *Bacillus subtilis* sporulation (6).

The general stress response in bacteria, which affords cross-protection against multiple stresses, typically entails global regulators (reviewed in reference 13). We propose σ^E as a central regulator of the *M. tuberculosis* stress response. The *sigE* gene is induced during growth in human macrophages and in response to various stress conditions *in vitro*, such as detergent- and vancomycin-mediated cell surface stress, pH stress, heat shock, oxidative stress (reviewed in reference 27), and hypoxia (this work). Various signal-processing and stress-responsive factors, such as the *mprAB* two-component system (36) in *M. tuberculosis* and *M. smegmatis* (12, 33) and the accessory sigma factor σ^H (16, 26), regulate *sigE* transcription during heat and oxidative stress. We postulate that σ^E acts as an information-processing unit that connects upstream signaling systems to downstream networks that regulate effector functions. A critical role for σ^E in the response of *M. tuberculosis* to stress is consistent with the observations that *sigE* knockout mutants were severely attenuated for growth in one mouse model (18) and for virulence in another (1) and that they also grew poorly in macrophages and in dendritic cells (9, 18).

The genetic findings reported above open the way to future research to characterize protein-DNA interactions involved in the FFLs that regulate *icl1* and *gltA1* and to identify additional regulatory interactions implicated in the observed gene expression patterns. We intend also to examine whether the FFL motif applies to *icl1* and *gltA1* regulation under different sets of stress conditions and whether it is utilized to regulate the stress response of other metabolic genes of *M. tuberculosis*. Moreover, we plan to address the nature of the physiological signal(s) activating and subsequently repressing the response of these FFLs, which we expect to show to be related to stress rather than associated with changes of carbon source (31). Reconstructing networks regulating the stress response should help to identify nodes that are critical to the *M. tuberculosis* survival program and to target them with drugs and vaccines.

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