Investigation of the Essentiality of Glutamate Racemase in *Mycobacterium smegmatis*

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The mycobacterial cell wall frequently has been used as a target for drug development, and D-glutamate, synthesized by glutamate racemase (MurI), is an important component of peptidoglycan. While the essentiality of the *murI* gene has been shown in several bacterial species, including *Escherichia coli*, *Bacillus anthracis*, and *Streptococcus pneumoniae*, studies in mycobacteria have not yet provided definitive results. This study aimed to determine whether *murI* is indeed essential and can serve as a possible target for structure-aided drug design. We have achieved this goal by creating a Δ*murI* strain of *Mycobacterium smegmatis*, a close relative of *Mycobacterium tuberculosis*. The deletion of the *murI* gene in *M. smegmatis* could be achieved only in minimal medium supplemented with D-glutamate, demonstrating that MurI is essential for growth and that glutamate racemase is the only source of D-glutamate for peptidoglycan synthesis in *M. smegmatis*.

D-Glutamate is an essential component of the bacterial cell wall, and there are two enzymes in bacteria capable of synthesizing D-glutamate, glutamate racemase (MurI) and D-amino acid aminotransferase (DaaT). In *Escherichia coli* and *Streptococcus pneumoniae*, whose genomes contain only *murI*, genetic deletion studies have shown that MurI is essential for growth in the absence of external D-glutamate supplementation (1–4). Other bacteria, which contain DaaT, such as *Staphylococcus haemolyticus* and *Bacillus sphaericus*, are able to produce sufficient D-glutamate in the absence of glutamate racemase (5). There are also bacteria, including some other *Bacillus* species, that contain two copies of *murI*, as well as a copy of *daaT*, yet, at least in *B. subtilis*, *murI* is required for these bacteria to grow in both rich and minimal medium (6–8). MurI also has been implicated in several species as having a role in moderating the activity of DNA gyrase, which itself is an important drug target and an essential enzyme in bacteria (9–11). Therefore, the essentiality of *murI* is a complex question that appears to be species specific.

In mycobacteria, where the identification and validation of new targets for drug design is an ongoing priority, the essentiality of glutamate racemase is unclear. The inspection of the genome of *Mycobacterium tuberculosis* reveals only one copy of *murI* and no copy of *daaT* (12). Therefore, it seems likely that MurI is essential, as it is the only enzyme capable of D-glutamate biosynthesis in this organism. However, in a transposon mutagenesis study carried out in *M. tuberculosis*, *murI* was not identified as likely to be essential (13). However, further investigation of that particular strain revealed the transposon had integrated at the penultimate position of the gene sequence and may not have inactivated the gene. In support of this interpretation, a more recent investigation of this issue employing transposon mutagenesis coupled with deep sequencing did conclude that *murI* was likely to be an essential gene (14). Moreover, a different set of experiments investigating glutamate metabolism in *M. tuberculosis* found that a *murI* deletion strain grew in 7H9 media when supplemented with 2% glucose, a known osmoprotectant (15).

In order to study this issue more fully in mycobacteria, we have created a *murI* deletion in *Mycobacterium smegmatis*, a nonpathogenic relative of *M. tuberculosis* that has been used successfully as a drug development surrogate for pathogenic mycobacteria (16). Like *M. tuberculosis*, *M. smegmatis* contains only one copy of *murI* and no *daaT* gene. We have studied the growth characteristics of this *murI* deletion strain in minimal medium as well as in media containing osmotically active agents, and we report that in minimal medium the *murI* gene is indeed essential for growth in the absence of D-glutamate supplementation.

MATERIALS AND METHODS

Bacterial growth and culture conditions. The plasmids, strains, and oligonucleotides used in this study are listed in Table 1. *E. coli* was routinely cultured in LB containing 0.5% NaCl; *M. smegmatis* was cultured in LB medium with 0.05% Tween 80 (LBT). A low-salt version (0.05% NaCl) was used where specified. For solid media, 1.5% agar and 0.05% Tween were added. For the Δ*murI* strains, Sauton's minimal medium [0.1% L-asparagine, 0.2% citric acid, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.005% ammonium iron(III) citrate, 0.2% glycerol, 0.05% Tween 80], supplemented with 15 to 60 mM D-glutamate and adjusted to pH 7.4 with NaOH, was used. Sucrose (10%, wt/vol) also was included as needed during the knockout (KO) selection procedure. Unless otherwise stated, bacteria were grown at 37°C. During the *murI* deletion process, *M. smegmatis* harboring the gene replacement vector, pKKYL02, was grown at 28°C for temperature-sensitive vector propagation and then placed at 40°C for allelic exchange mutagenesis. All cloning procedures were performed in *E. coli* DH10B. Kanamycin, hygromycin, and gentamicin were used at final concentrations of 25, 50, and 5 μg/ml, respectively, for *M. smegmatis* mc²155 and 50, 100, and 7 μg/ml, respectively, for *E. coli*.

Mutagenesis vector construction. Following a method derived for use in this strain of *Mycobacterium* (17), a shuttle vector was created to delete *murI* by homologous recombination. Briefly, primers MurI^SM KO-1.3, -2.2, -3.1, and -4.2 were used to amplify the left and right flanks of *murI* from strain MB3360, generating two 1,080- and 1,043-bp amplicons. These flanking regions were ligated to an EcoRI- and HindIII-

TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant information or sequence	Restriction site	Reference and/or source
Escherichia coli			
DH10B	F⁻ *araD139* Δ(*ara, leu*)7697 Δ*lacX74 galU galK rpsL deoR* φ80d*lacZ*ΔM15 *endA1 nupG recA1 mcrA* Δ(*mrr hsdRMS mcrBC*)		30
MB3360	pET28-TEV; *M. tuberculosis murI* in NdeI and BamHI in DH10B		This study
Mycobacterium smegmatis			
mc²155 wild type	*ept-1*		31
KK104-10	mc²155 Δ*murI::aphA-3* Kanʳ; selected at 15 mM D-Glu		This study
KK104-11	mc²155 Δ*murI::aphA-3* Kanʳ; selected at 30 mM D-Glu		This study
KK104-12	mc²155 Δ*murI::aphA-3* Kanʳ; selected at 30 mM D-Glu		This study
KK104-13	mc²155 Δ*murI::aphA-3* Kanʳ; selected at 60 mM D-Glu		This study
KK104-16	mc²155 Δ*murI::aphA-3* Kanʳ; selected at 125 mM D-Glu		This study
KK104-19	mc²155 Δ*murI::aphA-3 murI⁺* Kanʳ; selected at 125 mM D-Glu		This study
KK104-09	KK104-10 harboring pKKYL03; Hygʳ		This study
Plasmids			
pBluescript II SK(+)	*E. coli* cloning vector; f1 Ori; Ampʳ		Stratagene
pDM3	Source of the mycobacterial *alr* promoter		18
pKKYL02	pPR23 harboring Δ*murI::aphA-3*; Kanʳ Gmʳ		This study
pKKYL03	pUHA267 expressing *murI*; Hygʳ		This study
pPR23	*E. coli*-mycobacterial shuttle vector; *oriM*(Ts) *sacB* Gmʳ		32
pUHA267	*E. coli*-mycobacterial shuttle vector; Hygʳ		AgResearch, Wallaceville, New Zealand; 19
Primers			
MurI^SM KO-3.1	5′-TTTAAAAAGCTTCCGAATTGGATTTGCTGCGA-3′	HindIII	This study
MurI^SM KO-4.2	5′-AGACTAGTGTCTTCTCGTCTGGACACGT-3′	SpeI	This study
MurI^SM KO-1.3	5′-CTCGTTCGACGCGGCCGCCCGCACCCGTGAACTCGTCA-3′	NotI	This study
MurI^SM KO-2.2	5′-AAATTTGAATTCGCGCGGATCTGCGGGATGGT-3′	EcoRI	This study
5alr2	5′-AAATTTCCATGGTGGGGCAGTACTACAACTTC-3′	KpnI	18
3alrpro	5′-GTGGTCTGCATATGCATAATCTCCGGCGCCCATT-3′	NdeI	This study
MurI^SM EX-1.0	5′-TTTAAACATATGAGCGATCGACTCGCGCC-3′	NdeI	This study
MurI^SM EX-2.1	5′-TTTAAAGGATCCGGTGCCAAGACATGCCCGGT-3′	BamHI	This study

treated kanamycin resistance cassette (*aphA-3*) and then inserted into the shuttle vector pPR23 using NotI and SpeI, creating plasmid pKKYL02. This plasmid carries a temperature-sensitive origin of replication, gentamicin resistance, and the sucrose sensitivity-conveying *sacB* gene for counterselection to be utilized in the screening of the double crossover event. Following the allelic exchange, Δ*murI* strains have 60% of the *murI* gene removed and are kanamycin resistant.

Selection of knockout strains and Southern blotting. The pKKYL02 gene replacement vector was transformed into *M. smegmatis* mc²155 via electroporation (0.2-cm-gap cuvette at 25 kV, 1 kΩ, and 25 μF). The transformants were grown in Sauton's medium supplemented with 15 mM D-glutamate at 28°C to allow propagation of the temperature-sensitive vector and allow for the gene crossover to take place. The culture then was plated on Sauton's medium supplemented with 15 mM D-glutamate, 10% sucrose, and kanamycin at 40°C to select for the *murI* deletion. After 5 days of growth, the colonies were streaked out on Sauton's medium containing gentamicin and 15 mM D-glutamate; the gentamicin sensitivity of these colonies verified the loss of the pKKYL02 vector backbone. The genotype of these putative Δ*murI* mutant strains was verified by Southern blotting in which genomic DNA was digested by SmaI, separated by agarose gel electrophoresis, and then blotted onto a nylon membrane. The radioactively labeled right flank then was used as a probe to detect homologous restriction fragments on the membrane by autoradiography (Fig. 1).

Construction of a complementation vector for Δ*murI* mutants. A vector expressing a copy of *murI* in *trans* was designed to complement the Δ*murI* mutant. The *murI* gene lies in an operon and its promoter is un-
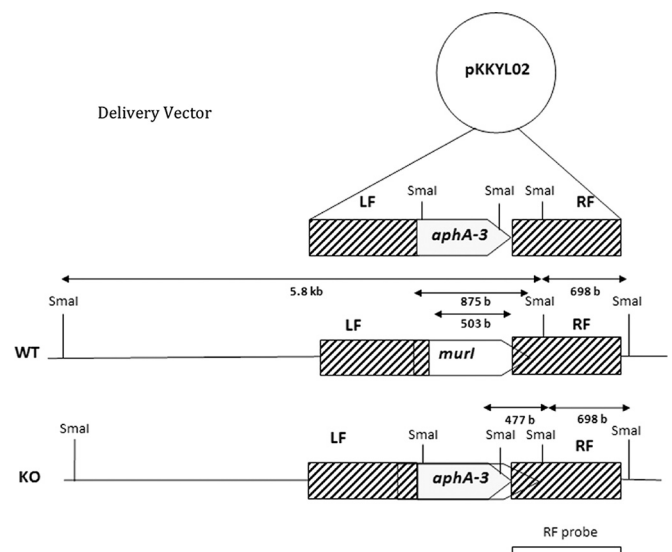

FIG 1 Vector map of the Δ*murI* mutant of *Mycobacterium smegmatis* mc²155. The construction of the delivery vector pKKYL02 was described in Materials and Methods. Homologous crossover at the left flank (LF) and right flank (RF) replaces 60% of *murI* with a nonpolar kanamycin resistance cassette (*aphA-3*). SmaI digestion sites utilized in the Southern blot are displayed. The right flank was used as a probe for hybridization.

known; therefore, the promoter of the *alr* gene from *M. smegmatis* was ligated to the *murI* gene (18). Primers MurI^{SM} EX-1.0 and MurI^{SM} EX-2.1 (Table 1) were used to amplify *murI*. The *alr* promoter from pDM3 was excised with NcoI and NdeI, ligated with *murI*, and then ligated into the *E. coli*-mycobacterial shuttle vector pUHA267 (19), forming plasmid vector pKKYL03.

RESULTS AND DISCUSSION

Selection of *murI* deletion mutants in *M. smegmatis* at 5 mM D,L-glutamate. The *murI* gene lies in an operon and overlaps MSMEG_4904 at its 5′ end by 4 bp. It is closely followed by *MSMEG_4902* at its 3′ end. To ensure that the disruption of the *murI* gene did not affect these two flanking genes, only 60% of the *murI* coding sequence was replaced with a nonpolar kanamycin cassette (20). However, both cysteines known to be essential for MurI activity were removed in this process (21, 22). The flanking DNA regions (1,080 bp and 1,043 bp, respectively) first were ligated with the kanamycin resistance-encoding *aphA-3* cassette and cloned in pBluescript II SK(+). The complete *murI::aphA-3* insert then was excised and ligated into the gene delivery vector pPR23 (Fig. 1). pPR23 contains a temperature-sensitive origin of replication that prohibits plasmid replication past 40°C, a genta-micin resistance cassette, and a sucrose sensitivity gene (*sacB*) for counterselection purposes. Thus, the initial selection attempt was carried out at 40°C in the presence of kanamycin and sucrose and 5 mM D,L-glutamate or 1 mM D-glutamate. Six colonies from this attempt were tested by pick and patch. However, these six colonies grew normally on LBT medium without D,L-glutamate supplementation. The selection was repeated, and all additional colonies tested by pick and patch remained independent of D-glutamate.

This result was consistent with the formation of a strain containing the expected *murI* disruption by *aphA-3* but that had also retained a wild-type (WT) copy of *murI*, either through gene duplication or because the plasmid had not properly integrated into the chromosome. Duplications in the *M. smegmatis* genome have previously been reported both in wild-type strains (23) and strains under genetic selection (17). If duplication of *murI* did occur during this experiment, it suggests that it was disadvantageous for the organism to lose its *murI* gene under the selection conditions containing 5 mM D,L-glutamate. Since D-glutamate is known to be poorly transported across bacterial membranes (24, 25), we concluded that larger amounts of D-glutamate supplementation likely were required for true Δ*murI* mutants to survive. This conclusion is consistent with studies in *E. coli* showing that a Δ*murI* strain could not be obtained until a mutant strain (WM335) was isolated that contained a mutation allowing for increased transport of D-glutamate across the plasma membrane (24, 26).

Disruption of the *murI* gene in *M. smegmatis* at high D-glutamate concentrations. To address the above-described hypothesis, the selection experiment was repeated in Sauton's minimal medium supplemented with 15 to 125 mM D-glutamate. Eight clones obtained under these conditions were chosen for screening by Southern blotting. Genomic DNA was extracted from these eight mutant colonies and from wild-type colonies and then digested by SmaI. For the wild type, hybridization with a probe for the right flank should have yielded bands at 5.8 kbp and 698 bp, respectively. For the Δ*murI* mutant, on the other hand, the 5.8-kbp band should disappear, leaving bands only at 698 bp and 477 bp. An incomplete removal of the gene replacement vector,
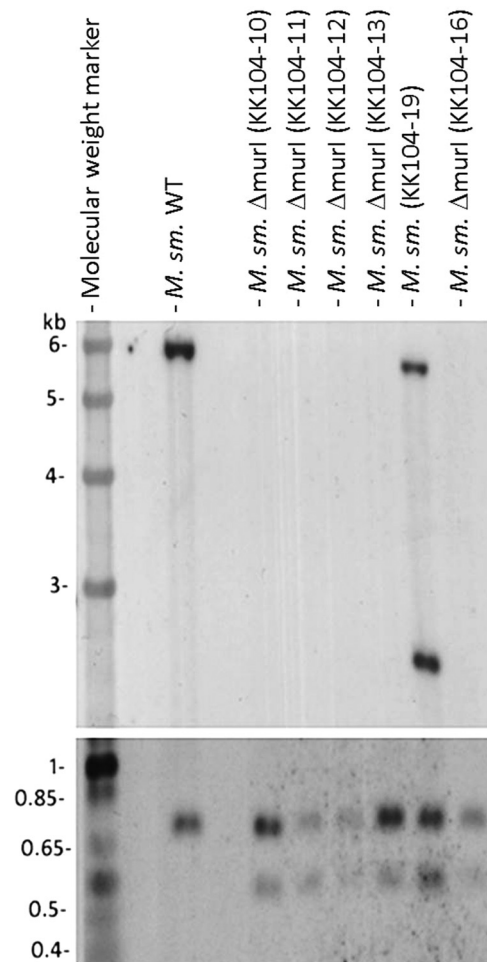

FIG 2 Southern blot of potential Δ*murI* mutants. Genomic DNA was isolated and digested with SmaI, and identical sample aliquots were loaded on low-percentage (top) and high-percentage (bottom) agarose gels. *M. sm.*, *M. smegmatis*.

pKKYL02, from the cell should yield bands at 477 bp and 2.5 kbp (Fig. 1).

As shown in Fig. 2, strains KK104-10 (selected at 15 mM D-glutamate), KK104-11 and KK104-12 (both selected at 30 mM D-glutamate), KK104-13 (selected at 60 mM D-glutamate), and KK104-16 (selected at 125 mM D-glutamate) all had undergone the double crossover event; therefore, they were genuine *murI* deletion mutants. Strain KK104-19 (selected at 125 mM D-glutamate) showed the typical wild-type band of 5.8 kbp and a typical vector band of 2.5 kbp, indicating incomplete gene disruption. When retested after the original selection, strain KK104-19 did not require any exogenous D-glutamate for growth on minimal medium. In addition, all six clones originally selected on 1 and 5 mM D-glutamate had maintained the two characteristic genomic DNA and vector DNA bands upon Southern hybridization (data not shown).

Phenotype of the Δ*murI* mutant strains on solid minimal medium. The phenotype of a representative selection of Δ*murI* mutants was characterized on solid Sauton's medium (Fig. 3). It was found that the Δ*murI* mutants KK104-10, KK104-11, KK104-12, KK104-13, and KK104-16 did not grow on Sauton's medium
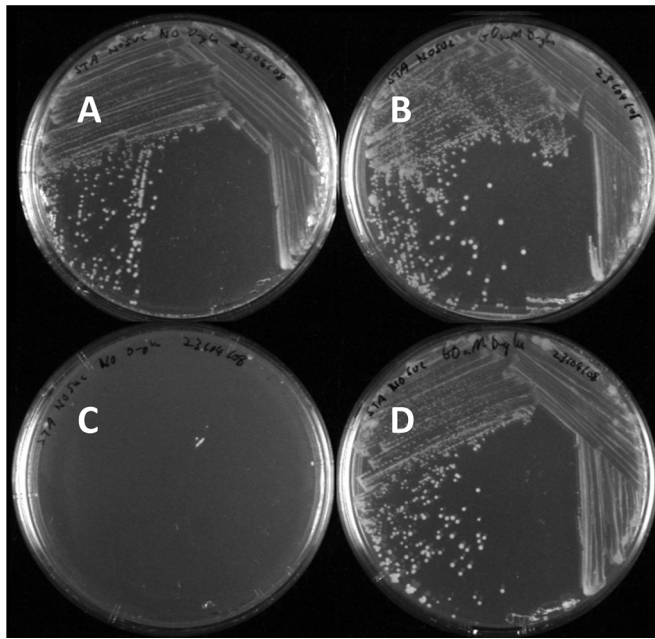
FIG 3 Phenotype of wild-type *M. smegmatis* cells grown on Sauton's medium in the absence (A) and presence (B) of 60 mM D-glutamate and Δ*murI* (KK104-10) mutant cells grown on Sauton's medium in the absence (C) and presence (D) of 60 mM D-glutamate.

FIG 4 Growth curves of the wild-type strain (◆) and the KK104-10 Δ*murI* strain (●), KK104-13 (Δ*murI*) strain (■), and K-09 (*murI* complemented in *trans*) strain (▲). The growth curve was performed in low-salt LB liquid broth without D-glutamate supplementation (A) and with 60 mM D-glutamate supplementation (B), and growth was recorded over 96 h.

in the absence of external D-glutamate supplementation. Below 5 mM D-glutamate, no appreciable growth was observed. With increasing D-glutamate supplementation, increased growth occurred. At 60 mM D-glutamate or higher, the Δ*murI* mutants grew indistinguishably from the wild-type strain. Strains that had undergone incomplete gene replacement events, such as KK104-19, did not require external D-glutamate supplementation to grow on solid Sauton's medium.

Growth of Δ*murI* mutant strains in other growth media. As described above, Δ*murI* mutant strains require D-glutamate supplementation for growth on Sauton's (agar) solid medium. These Δ*murI* deletion mutants also are unable to grow in low-salt LB broth without D-glutamate supplementation. However, supplementation of low-salt LB broth with either KCl, sucrose, or additional NaCl was found, at some concentrations, to complement the Δ*murI* phenotype. Using sucrose supplementation, minimal growth could be detected at 1 to 2% (wt/vol) sucrose supplementation, but 10% sucrose was required for wild-type-like growth. With NaCl supplementation, growth of the Δ*murI* mutant occurred at concentrations greater than 200 mM, but wild-type growth was never observed. Notably, LB solid medium containing 5% NaCl (855 mM) was able to support the growth of the Δ*murI* mutant.

These phenotypes have been observed in cell wall deletion mutants of other microorganisms and often are ascribed to osmotic stabilization effects (27, 28, and M. Benedik, personal communication). Strikingly, the Δ*murI* mutant does not require D-glutamate supplementation to grow in liquid Sauton's medium, although the growth rate is slower than that of the wild type. The difference in osmotic conditions between solid Sauton's medium and liquid medium is thought to contribute to these observations, with the salt and 0.2% glycerol present in liquid Sauton's medium allowing the Δ*murI* strain to grow in the absence of D-glutamate supplementation.
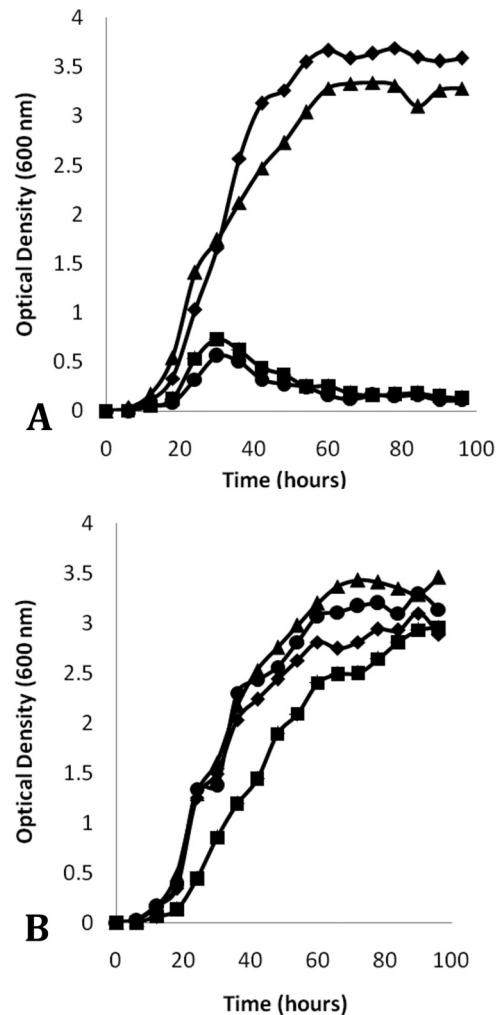
In order to demonstrate that the phenotype of the Δ*murI* strain was due to the lack of D-glutamate racemase (MurI), a copy of *murI* was provided in *trans* via the integrative shuttle vector pUHA267, forming strain KK-09 MurI⁺. This complementation allowed KKYL09 to grow normally in low-salt LB broth (Fig. 4), suggesting that the Δ*murI* strain phenotype in low-salt LB is due to insufficient D-glutamate.

Δ*murI* mutant cells display a morphology different from the wild-type cells. At the end of a growth curve in low-salt LB broth, a representative sample of stationary-phase cells of the Δ*murI* mutant strain was examined using electron microscopy and compared to the wild type. It was found that the Δ*murI* cells grown without D-glutamate supplementation grew to 2 to 3 times the size of wild-type cells (Fig. 5) and often displayed pear-shaped swellings at their polar ends, while in the presence of D-glutamate the phenotype was unchanged from that of the wild type (Fig. 5). A
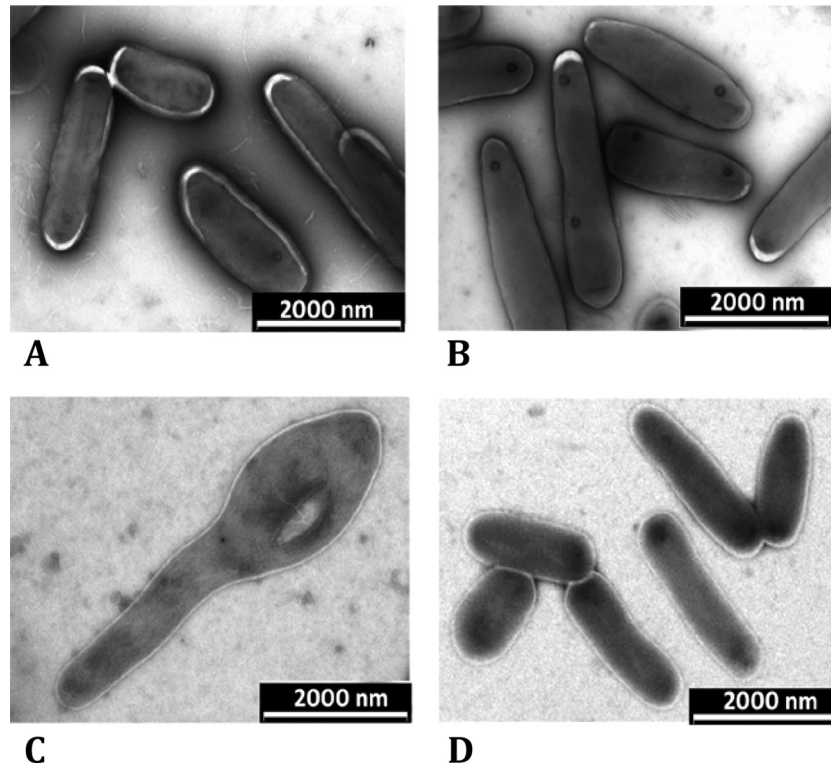
FIG 5 Electron microscopy of representative stationary-phase wild-type (WT) and mutant (KK104-10) *M. smegmatis* cells at the end of the growth curve in low-salt LB broth. Shown are the WT without D-glutamate (A) and with 60 mM D-glutamate supplementation (B) and KK104-10 mutants without D-glutamate (C) and with 60 mM D-glutamate supplementation (D).

similar phenotype also was reported for *M. smegmatis* Δ*alr* mutants, which grew to triple the length of wild-type strains (18).

Summary and conclusions. In this study, we demonstrate that the *murI* gene encoding glutamate racemase in *M. smegmatis* is essential for growth in Sauton's minimal liquid medium. This essentiality is overcome by adding >15 mM D-glutamate to this liquid growth medium. In other media containing osmoprotectants (salt, sugars, etc.), *murI* is nonessential, a result that is consistent with other studies on cell wall deletion mutants (27, 28). In fact, microorganisms that are completely lacking a cell well, such as L-forms of eubacteria, can be grown under iso-osmolar conditions (29). Our results also may help explain a recent report that *murI* is nonessential in *M. tuberculosis* (15). In this study, the deletion strain was studied only under one condition, 7H9 medium containing 2% glucose. It is tempting to propose that glucose was able to serve as an osmoprotectant, as we found in our studies on *M. smegmatis*. Certainly, our results show that *murI* deletion mutants do not grow in minimal medium. The relevance of these findings to human disease is difficult to ascertain, because it is unknown if conditions in the macrophage or a pulmonary granuloma would allow an organism with this cell wall defect to persist and to multiply. Studies with *murI* deletion mutants of *M. tuberculosis* are needed to investigate this possibility.

REFERENCES

1. **Baltz RH, Hoskins JA, Solenberg PJ, Treadway PJ.** November 1999. Method for knockout mutagenesis in *Streptococcus pneumoniae*. US patent 5,981,281.
2. **Hoffmann B, Messer W, Schwarz U.** 1972. Regulation of polar cap formation in the life cycle of *Escherichia coli*. J. Supramol. Struct. **1:**29–37. http://dx.doi.org/10.1002/jss.400010105.
3. **Lugtenberg EJ, Wijsman HJ, van Zaane D.** 1973. Properties of a D-glutamic acid-requiring mutant of *Escherichia coli*. J. Bacteriol. **114:**499–506.
4. **Song JH, Ko KS, Lee JY, Baek JY, Oh WS, Yoon HS, Jeong JY, Chun J.** 2005. Identification of essential genes in *Streptococcus pneumoniae* by allelic replacement mutagenesis. Mol. Cells **19:**365–374.
5. **Kimura K, Tran LS, Itoh Y.** 2004. Roles and regulation of the glutamate racemase isogenes, racE and yrpC, in *Bacillus subtilis*. Microbiology **150:**2911–2920. http://dx.doi.org/10.1099/mic.0.27045-0.
6. **Ashiuchi M, Soda K, Misono H.** 1999. Characterization of yrpC gene product of *Bacillus subtilis* IFO 3336 as glutamate racemase isozyme. Biosci. Biotechnol. Biochem. **63:**792–798. http://dx.doi.org/10.1271/bbb.63 .792.
7. **Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG, Bessières P, Bolotin A, Borchert S, Borriss R, Boursier L, Brans A, Braun M, Brignell SC, Bron S, Brouillet S, Bruschi CV, Caldwell B, Capuano V, Carter NM, Choi SK, Cordani JJ, Connerton IF, Cummings NJ, Daniel RA, Denziot F, Devine KM, Düsterhöft A, Ehrlich SD, Emmerson PT, Entian KD, Errington J, Fabret C, Ferrari E, Foulger D, Fritz C, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256. http://dx .doi.org/10.1038/36786.
8. **Shatalin KY, Neyfakh AA.** 2005. Efficient gene inactivation in *Bacillus anthracis*. FEMS Microbiol. Lett. **245:**315–319. http://dx.doi.org/10.1016 /j.femsle.2005.03.029.

9. **Ashiuchi M, Kuwana E, Komatsu K, Soda K, Misono H.** 2003. Differences in effects on DNA gyrase activity between two glutamate racemases of *Bacillus subtilis*, the poly-gamma-glutamate synthesis-linking Glr enzyme and the YrpC (MurI) isozyme. FEMS Microbiol. Lett. **223**:221–225. http://dx.doi.org/10.1016/S0378-1097(03)00381-1.

10. **Ashiuchi M, Kuwana E, Yamamoto T, Komatsu K, Soda K, Misono H.** 2002. Glutamate racemase is an endogenous DNA gyrase inhibitor. J. Biol. Chem. **277**:39070–39073. http://dx.doi.org/10.1074/jbc.C200253200.

11. **Sengupta S, Ghosh S, Nagaraja V.** 2008. Moonlighting function of glutamate racemase from *Mycobacterium tuberculosis*: racemization and DNA gyrase inhibition are two independent activities of the enzyme. Microbiology **154**:2796–2803. http://dx.doi.org/10.1099/mic.0.2008/020933-0.

12. **Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, III, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393**:537–544.

13. **Sassetti CM, Boyd DH, Rubin EJ.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol. Microbiol. **48**:77–84. http://dx.doi.org/10.1046/j.1365-2958.2003.03425.x.

14. **Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM.** 2011. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. PLoS Pathog. **7**:e1002251. http://dx.doi.org/10.1371/journal.ppat.1002251.

15. **Harth G, Maslesa-Galic S, Tullius MV, Horwitz MA.** 2005. All four Mycobacterium tuberculosis glnA genes encode glutamine synthetase activities but only GlnA1 is abundantly expressed and essential for bacterial homeostasis. Mol. Microbiol. **58**:1157–1172. http://dx.doi.org/10.1111/j.1365-2958.2005.04899.x.

16. **Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V.** 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science **307**:223–227. http://dx.doi.org/10.1126/science.1106753.

17. **Tran SL, Cook GM.** 2005. The F1Fo-ATP synthase of *Mycobacterium smegmatis* is essential for growth. J. Bacteriol. **187**:5023–5028. http://dx.doi.org/10.1128/JB.187.14.5023-5028.2005.

18. **Milligan DL, Tran SL, Strych U, Cook GM, Krause KL.** 2007. The alanine racemase of Mycobacterium smegmatis is essential for growth in the absence of D-alanine. J. Bacteriol. **189**:8381–8386. http://dx.doi.org/10.1128/JB.01201-07.

19. **Lee MH, Pascopella L, Jacobs WR, Jr, Hatfull GF.** 1991. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for Mycobacterium smegmatis, Mycobacterium tuberculosis, and bacille Calmette-Guerin. Proc. Natl. Acad. Sci. U. S. A. **88**:3111–3115.

20. **Menard R, Sansonetti PJ, Parsot C.** 1993. Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells. J. Bacteriol. **175**:5899–5906.

21. **Gallo KA, Knowles JR.** 1993. Purification, cloning, and cofactor independence of glutamate racemase from *Lactobacillus*. Biochemistry **32**:3981–3990.

22. **Gallo KA, Tanner ME, Knowles JR.** 1993. Mechanism of the reaction catalyzed by glutamate racemase. Biochemistry **32**:3991–3997.

23. **Springer B, Sander P, Sedlacek L, Hardt WD, Mizrahi V, Schar P, Bottger EC.** 2004. Lack of mismatch correction facilitates genome evolution in mycobacteria. Mol. Microbiol. **53**:1601–1609. http://dx.doi.org/10.1111/j.1365-2958.2004.04231.x.

24. **Doublet P, van Heijenoort J, Bohin JP, Mengin-Lecreulx D.** 1993. The murI gene of *Escherichia coli* is an essential gene that encodes a glutamate racemase activity. J. Bacteriol. **175**:2970–2979.

25. **Yabu K.** 1970. Amino acid transport in *Mycobacterium smegmatis*. J. Bacteriol. **102**:6–13.

26. **Dougherty TJ, Thanassi JA, Pucci MJ.** 1993. The *Escherichia coli* mutant requiring D-glutamic acid is the result of mutations in two distinct genetic loci. J. Bacteriol. **175**:111–116.

27. **Kriangkripipat T, Momany M.** 2009. Aspergillus nidulans protein O-mannosyltransferases play roles in cell wall integrity and developmental patterning. Eukaryot. Cell **8**:1475–1485. http://dx.doi.org/10.1128/EC.00040-09.

28. **Shen B, Hohmann S, Jensen RG, Bohnert H.** 1999. Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol. **121**:45–52.

29. **Dienes L, Sharp JT.** 1956. The role of high electrolyte concentration in the production and growth of L forms of bacteria. J. Bacteriol. **71**:208.

30. **Grant SG, Jessee J, Bloom FR, Hanahan D.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. U. S. A. **87**:4645–4649.

31. **Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4**:1911–1919.

32. **Pelicic V, Jackson M, Reyrat JM, Jacobs WR, Jr, Gicquel B, Guilhot C.** 1997. Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. U. S. A. **94**:10955–10960.