Comparison of the UDP- N-Acetylmuramate: L-Alanine Ligase Enzymes from Mycobacterium tuberculosis and Mycobacterium leprae

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In the peptidoglycan of Mycobacterium leprae, L-alanine of the side chain is replaced by glycine. When expressed in Escherichia coli, MurC (UDP-N-acetylmuramate:L-alanine ligase) of M. leprae showed \( K_m \) and \( V_{max} \) for L-alanine and glycine similar to those of Mycobacterium tuberculosis MurC, suggesting that another explanation should be sought for the presence of glycine.

Some chemical differences exist in the peptidoglycan of Mycobacterium spp. compared to other bacteria (3). Mycobacterial muramic acid is thought to be glycolylated instead of acetylated (14). In the case of Mycobacterium leprae, the first amino acid in the tetrapeptide side chain of the peptidoglycan is Gly instead of L-Ala (5), as found in Mycobacterium tuberculosis and many other bacteria, implying that the M. leprae genome may encode a unique UDP-N-acetylmuramate:L-Ala/Gly (UDP-MurNAc:L-Ala/Gly) ligase (MurC) specific for the addition of Gly to UDP-MurNAc. These special structural features of mycobacterial peptidoglycan suggest the presence of unique enzymes that could be exploited as drug targets.

murC genes of Mycobacterium. The complete sequence of the open reading frame of MLCB268.01c was revealed from the assembled genome sequence of M. leprae; it corresponds to bp 1084518 to 1086003. The resulting protein contains 595 amino acid residues with a theoretical molecular mass of 51 kDa, very similar to M. tuberculosis MurC (about 79% identity) but with only \( \sim 34\% \) identity to E. coli MurC. Both Rv2152c (M. tuberculosis) and MLCB268.01 (M. leprae) are found within the mra cluster and contain eight of nine invariant amino acids (2) that align perfectly with known MurCs from other organisms (Fig. 1). However, the MurC homologs found outside the mra clusters (Rv3712 and MLCB2407.24c) have only \( \sim 22\% \) identity with the putative MurCs found within the clusters, and four of the nine invariant amino acids either are absent or did not align.

Cloning, expression, and purification of UDP-N-MurNAc:L-Ala ligase (MurC). Rv2152c and Rv3712 were amplified from M. tuberculosis H37Rv genomic DNA and cloned into the pET29a vector (Novagen, Madison, Wis.) (16), yielding pSM201 and pSM203, respectively. The M. leprae MLCB268.01 and MLCB2407.24c genes were amplified from M. leprae genomic DNA and cloned into pET28a and pET29a, respectively (16, 17). E. coli BL21(DE3) harboring plasmid pSM201, pSM203, pSM206, or pSM208 was grown in Luria-Bertani broth containing kanamycin, induced with isopropyl-\( \beta \)-D-thiogalactopyranoside, lysed by sonication on ice, and centrifuged at 30,000 \( \times g \) for 30 min (17). The resulting supernatant containing the soluble His-tagged fusion proteins were loaded on a nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) column (18) which was washed with 20 mM Tris-HCl (pH 8.0)–10 mM

E. coli 39 LANEYQISSGLDAPHPVTQVIWNLGATIYFN 117 RFRHGLIAIAFHSTTTTT
M. leprae 30 LLDRGGILGSQAKERSVRHARGARLIRIG 113 DGTITTMVAPFHTTTTT
M. tuberculosis 30 LLDRGGILGSQAKERSVRHARGARLIRIG 113 AGRTTIIAMYTHTTTTT

E. coli 165 LGHRYLIAADESDDLFHLPQMAVVTIADNADDFTY 285 TINAPGRFNAALAAA
M. leprae 161 HGLGACFVAADEGDGLLEETPTPNVAVTNIDSHLDPFT 288 RLPGVGRFNADAGLA
M. tuberculosis 161 HGSQDCFVADEGDGLLEETPTPNVAVTNIDSHLDPFT 290 RLSVPGRFNAADAGLA

E. coli 287 ALESQFOCTGJRF 346 AMLVVDYQHHPETVDATIKAAR
M. leprae 319 DGLAGFQGRVITF 347 VRTEYDIYAHFPEISATMIRAFF
M. tuberculosis 321 DGLAGFQGRVITF 346 VRTEYDIYAHFPEISATMIRAFF

FIG. 1. Alignment of amino acid residues of MurC from M. tuberculosis, M. leprae, and E. coli, showing conserved residues (highlighted).

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MgCl₂–2 mM β-mercaptoethanol–30 mM imidazole (pH 8.0) and 0.5 M NaCl, and the His-tagged proteins were eluted from the column with buffer containing 300 mM imidazole (pH 7.5) (18). Protein-containing fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) to show a high degree of purification (Fig. 2).

**Assay for UDP-MurNAc:Ala ligase (MurC).** Purified fractions were pooled, and imidazole was removed by dialysis. The UDP-MurNAc:Ala and UDP-MurNAc:Gly ligase activities were assayed as described by Liger et al. (12). For this purpose, UDP-MurNAc was prepared by a two-step coupled enzymatic conversion of UDP-GlcNAc to UDP-MurNAc according to Jin et al. (9) and identified through negative-ion fast atom bombardment-mass spectroscopy (FAB-MS) (the expected mass of 679 was observed) and nuclear magnetic resonance (NMR) (300 MHz). The following signals were clearly identified by 1H NMR spectroscopy in heavy water at 300 MHz: d7.94 (doublet, j = 8.1 Hz, H-6; uracyl), d 5.96 (doublet, j = 4.5 Hz, H-1; ribosyl), d 5.96 (doublet, j = 8.1 Hz, H-5; uracyl), d 5.60 broadened doublet (j = 4.2 Hz, H-1; muramyl), d 2.03 (singlet, methyl; N-acetyl muramyl), and d 1.32 (doublet, j = 6.6 Hz, methyl; lactyl-muramyl). Reaction mixtures contained 100 mM Tris-HCl (pH 8.6), 25 mM (NH₄)₂SO₄, 20 mM MgCl₂, 2 mM β-mercaptoethanol, 1 mM UDP-MurNAc, 2 mM ATP, 50 μM L-[14C]Ala (specific activity, 164 mCi/mmol) (ICN Radiochemicals, Irvine, Calif.) or [14C]Gly (46.87 mCi/mmol) (NEN Life Science Products, Boston, Mass.), and a predetermined amount of crude cell lysate or purified enzyme in a 25-μl reaction mix. Reactions were conducted under conditions in which product formation was linear with respect to both time and protein concentration. Reactions were stopped by the addition of 10 μl of glacial acetic acid and briefly centrifuged, and 3.5 μl of the supernatant was applied to a silica gel thin-layer chromatography plate which was developed in isobutrylic
acid–1 M ammonium hydroxide (5:3) to separate the reaction product from unreacted amino acids. Radioactivity was measured using Bioscan Imaging Scanner System 200-IBM (Bioscan Inc., Washington, D.C.). The proportion of counts of substrate and product compared to the total counts applied to the plate was used to calculate enzyme activity.

The purified proteins arising from cloned Rv2152c and MLCB268.01c showed good ligase activity using both t-Ala and Gly as substrates (Fig. 3 and 4). The products of the ligase reactions were also analyzed by MS; UDP-MurNAc-t-Ala gave the expected molecular weight of 750, and the UDP-MurNAc-Gly gave the expected molecular weight of 736. The \( K_m \) and \( V_{max} \) of both Rv2152c and MLCB268.01c were determined in the presence of either Gly or t-Ala (Fig. 3 and 4). Rv2152c showed an apparent \( K_m \) of 38 \( \mu \)M and a \( V_{max} \) of 220 pmol/mg/min for Gly. When assayed with various amounts of t-Ala, this enzyme showed an apparent \( K_m \) of 14 \( \mu \)M and a \( V_{max} \) of 1,200 pmol/mg/min. Even though the \( K_m \) values for both of these substrates were similar, the \( V_{max} \) for t-Ala was found to be much greater than that seen for Gly, indicating better catalysis with t-Ala as the substrate. Similar results were obtained with MLCB268.01c; this \( M. \) leprae enzyme had an apparent \( K_m \) of 25 \( \mu \)M and a \( V_{max} \) of 76 pmol/mg/min for Gly, and, when assayed with various amounts of t-Ala, this enzyme showed an apparent \( K_m \) of 10 \( \mu \)M and a \( V_{max} \) of 460 pmol/mg/min.

Nonactive MurC homologs outside the \( mra \) cluster. The MurC homologues Rv3712 and MLCB2407.24c were also expressed in \( E. \) coli as C-terminally His-tagged proteins using the

![FIG. 4. Effect of amino acid concentration on the rate of UDP-MurNAc-[\( ^{14}C \)]Gly (A) or UDP-MurNAc-[\( ^{14}C \)]L-Ala (B) biosynthesis by purified MLCB268.01c from \( M. \) leprae. The apparent \( K_m \) and \( V_{max} \) values were derived from a double-reciprocal plot of these data (inset).](http://jb.asm.org/)

![FIG. 5. Comparison of the chromosomal organization of the \( mra \) clusters in \( M. \) tuberculosis, \( M. \) leprae, and \( E. \) coli.](http://jb.asm.org/)
T7 expression system, resulting in soluble proteins capable of being purified by Ni-NTA column chromatography. However, the purified proteins as well as the crude cell lysate from the overproducing E. coli cells showed no significant ligase activity when tested.

Molecular organization of the M. leprae, M. tuberculosis, and E. coli mra clusters. In general, the basic genetic organization of the mra gene cluster responsible for peptidoglycan biosynthesis (19) of M. tuberculosis, M. leprae, and E. coli is similar except for four additional open reading frames in the M. tuberculosis mra cluster between phpB and murE (Fig. 5). Clearly, Rv2152c from M. tuberculosis and MLCB268.01c from M. leprae encode the MurC enzymes of their respective species, in that the E. coli-overexpressed enzymes were enzymatically active, indicating that they underwent proper folding even when expressed in a nonhomologous system. The properties of these two ligases are very similar. The calculated apparent \( K_m \) for Gly of both ligases was found to be much lower than the reported \( K_m \) value of \( \approx 2.5 \) to 10 mM for Gly of E. coli MurC (7, 12). The mycobacterial MurCs also had similar \( K_m \) values for L-Ala, and in both cases, this value was slightly lower than the \( K_m \) for Gly. However, the apparent \( V_{\max} \) for L-Ala is much higher than that for Gly in both cases, suggesting better catalysis of L-Ala ligase activity.

The other two open reading frames (Rv3712 and MLCB2407.24c) that show homology to E. coli murC do not appear to encode any ligase activity, probably due to the absence of four of the nine invariant amino acids found in bona fide members of the MurC enzyme family. Therefore it can be concluded that M. tuberculosis and M. leprae, like other bacteria, have only one such ligase. Thus, the presence of a Gly-specific ligase can apparently be ruled out as the reason for the specific occurrence of Gly instead of L-Ala in the peptidoglycan are observed (15). From the data presented here, it can be hypothesized that, in M. leprae, the incorporation of Gly into peptidoglycan is due to a combination of the substrate specificity of the MurC and the nature of the intracellular environment.

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