Two Active Forms of UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase in Gram-Positive Bacteria

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Gene sequences encoding the enzymes UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) from many bacterial sources were analyzed. It was shown that whereas gram-negative bacteria have only one *murA* gene, gram-positive bacteria have two distinct genes encoding these enzymes which have possibly arisen from gene duplication. The two *murA* genes of the gram-positive organism *Streptococcus pneumoniae* were studied further. Each of the *murA* genes was individually inactivated by allelic replacement. In each case, the organism was viable despite losing one of its *murA* genes. However, when attempts were made to construct a double-deletion strain, no mutants were obtained. This indicates that both genes encode active enzymes that can substitute for each other, but that the presence of a MurA function is essential to the organism. The two genes were further cloned and overexpressed, and the enzymes they encode were purified. Both enzymes catalyzed the transfer of enolpyruvate from phosphoenolpyruvate to UDP-*N*-acetylglucosamine, confirming they are both active UDP-*N*-acetylglucosamine enolpyruvyl transferases. The catalytic parameters of the two enzymes were similar, and they were both inhibited by the antibiotic fosfomycin.

UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) catalyzes the first committed step in bacterial cell wall biosynthesis (9, 20). The enzyme transfers an enolpyruvyl group from phosphoenolpyruvate (PEP) to UDP-*N*-acetylglucosamine (UDPAG) to form UDP-*N*-acetylglucosamine enolpyruvate (Fig. 1A). This is a precursor to UDP-*N*-acetylmuramate, an essential building block for the bacterial cell wall. MurA is inhibited by the antibiotic fosfomycin (Fig. 1B) (10), and because of its importance in peptidoglycan biosynthesis, it is of interest as a target for the design of novel antibacterial agents.

MurA (sometimes called MurZ) from *Escherichia coli* has been studied in great depth. There is one copy of the *murA* gene in *E. coli* (16), and this has been shown to be essential by gene deletion experiments (3). The *E. coli murA* gene has been overexpressed, and the MurA enzyme has been purified and kinetically characterized (16). The K_ms of UDPAG and PEP were reported to be 15 and 0.4 μM, respectively, while the k_{cat} was 3.8 s^{-1} (11). The crystal structure of the enzyme, either ligand free or complexed with UDPAG and fosfomycin, has been solved (21, 22). This shows the enzyme to be made up of two domains, with the active site located in a deep cavity between them, and identifies catalytically and structurally important residues. One catalytically important residue (Cys-115) has been investigated in detail. It was shown that this residue is the site of alkylation by fosfomycin, a finding that has been confirmed by kinetics and mutagenesis studies (Fig. 1B) (15, 27). Mutations in the cysteine residue (C115S) inactivated the enzyme, confirming that this is an important residue in catalysis (27). Another residue (Asp-305) is indicated by the structural data as being the general base involved in the deprotonation of the substrate UDPAG (22).

The importance of MurA as an antibacterial target led us to investigate the MurA enzymes from gram-positive pathogens. While there is only one *murA* gene in *E. coli*, we have identified in gram-positive bacteria two *murA* genes, which have arisen by a possible gene duplication. To investigate if the two genes present in the gram-positive bacteria both encode active enzymes, we performed an in-depth study of the two genes from the gram-positive bacterium *Streptococcus pneumoniae*. Gene deletion experiments were used to investigate the essentiality of the *murA* genes. The genes were then overexpressed, the enzymes that they encode were purified, and the biochemical activities of the enzymes were investigated.

MATERIALS AND METHODS

General materials. *S. pneumoniae* R6 is a nonencapsulated strain (13). pJS3 (2) was the kind gift of Sanford Lacks (Brookhaven National Laboratory). Biochemical reagents were from Sigma. Enzymes and reagents used in cloning were from Gibco BRL (Gaithersburg, Md.). DNA preparations were performed with assorted kits from Qiagen (Valencia, Calif.). Column chromatography was performed on an AKTA FPLC system (Pharmacia) with Pharmacia Hi-Load columns. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed on Novex (San Diego, Ca) 4 to 12% bis-Tris gels. The N-terminal amino acid sequence of the purified enzyme was deduced by automated Edman degradation with a Hewlett-Packard model G1000A sequencer. The molecular weight of the enzyme was determined by matrix-assisted laser-desorption ionization–time of flight (MALDI-TOF) mass spectrometry using a Voyager RP Biospectrometer (PerSeptive Biosystems, Framingham, Mass.).

Phylogenetic analysis. Homologous protein sequences were retrieved from public and proprietary genomic sequence databases using the software BLASTP and TBLASTN (1). The partial genome of *S. pneumoniae* type 4 from The Institute for Genome Research was used as the source of MurA1 and MurA2 described here. The proteins were aligned using the program CLUSTALW version 1.7 (23) with the BLOSUM62 (8) similarity matrix and gap opening and extension penalties of 10.0 and 0.05, respectively. The multiple sequence alignments were refined manually using the program SEQLAB of the Wisconsin Package, version 9.0 (Genetics Computer Group, Madison, Wis.).

Phylogenetic trees were constructed by maximum likelihood (ML), neighbor-joining (N-J), and maximum parsimony (MP) methods for each set of alignments. The software PUZZLE version 4.0 was used for ML tree construction (23). N-J trees were based on pairwise distances between amino acid sequences using the programs NEIGHBOR and PROTDIST of the PHYLIP 3.57c package (J. Felsenstein, PHYLIP [Phylogeny Inference Package] version 3.57c [http://evolution.genetics.washington.edu/phylip.html]). The "Dayhoff" program option was invoked in the latter program, which estimates based on the Dayhoff 120 matrix (6). The programs SEQBOOT and CONSENSE were used to estimate the confidence limits of branching points from 1,000 bootstrap replications. MP

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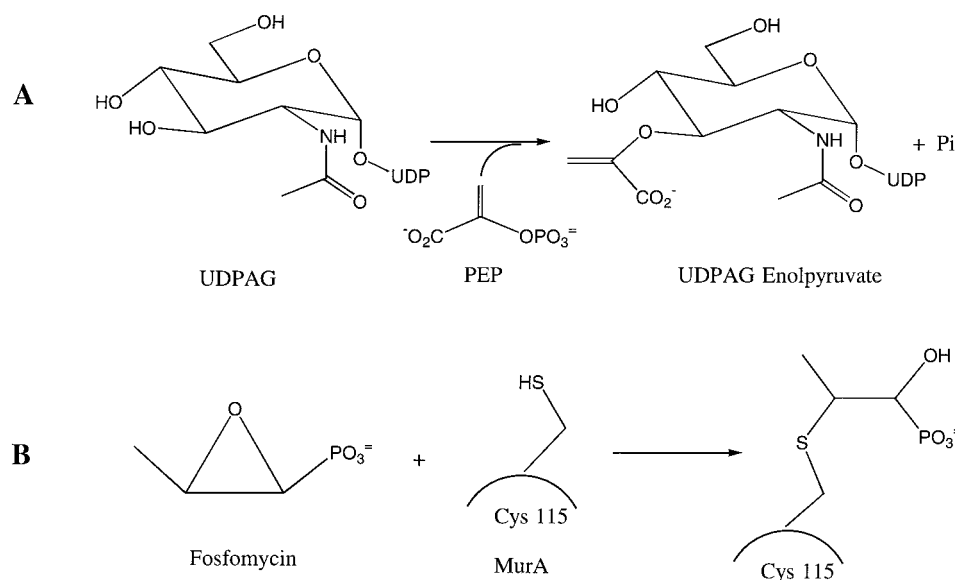
FIG. 1. (A) The enolpyruvyl transfer reaction catalyzed by MurA. (B) Inactivation of MurA by fosfomycin as a result of the covalent linkage between Cys-115 of MurA and fosfomycin.

analysis was done using the software package PAUP* (24). Given the large size of the data set, it was not possible to exhaustively search for the total number of minimal length trees. Instead, the numbers and lengths of minimal trees were estimated from 100 replicate random heuristic searches, while confidence limits of branch points were estimated by 1,000 bootstrap replications.

Generation of *S. pneumoniae* allelic replacement mutants. *S. pneumoniae* 0100993 chromosomal DNA was prepared as follows. Four milliliters of *S. pneumoniae* culture with an optical density at 650 nm of 0.6 to 0.7 was spun down, resuspended in 100 μl of 1 M Tris-HCl (pH 8)–0.25 M EDTA–5% sodium deoxycholate–40% glucose, and incubated at 37°C for 20 min; 10 μl of 10% (wt/vol) SDS was added, and the suspension was incubated at 37°C for a further 10 min. The mixture was phenol-chloroform extracted three times, followed by a single chloroform extraction. The DNA was precipitated with isopropanol, washed twice in 70% ethanol, and resuspended in water. Chromosomal DNA fragments flanking *murA1* and *murA2* were PCR amplified from chromosomal DNA and purified as previously described (19). These were then used to make antibiotic-resistant gene-targeting constructs as described in Results, which were used to transform *S. pneumoniae* R6 competent cells prepared according to published protocols. A total of 10^6 cells were incubated with 500 ng of allelic replacement cassette at 30°C for 30 min and then transferred to 37°C for 90 min to allow expression of antibiotic resistance (7). Cells were plated in AGCH medium (12) containing erythromycin (1 μg/ml) or chloramphenicol (2.5 μg/ml) as appropriate and incubated at 37°C for 36 h in 5% CO₂. Transformation efficiencies were 10^3 transformants per μg of control DNA.

If antibiotic-resistant *S. pneumoniae* R6 colonies were obtained, they were picked and grown overnight in Todd-Hewitt broth supplemented with 0.5% yeast extract. Chromosomal DNA was prepared and examined by Southern blot analysis and diagnostic PCR to verify that the appropriate chromosomal DNA rearrangement had occurred. In the former, flanking DNA fragments labeled using the ECL (enhanced chemiluminescence) system (Amersham Life Science Limited) were used as probes to chromosomal DNA restricted with appropriate enzymes and blotted using standard methodologies. In the latter, DNA primers designed to hybridize within *ermAM* or *cat* as appropriate were paired with primers hybridizing to distal chromosomal sequences to generate DNA amplification products of characteristic size.

Whole-cell antimicrobial activity. Whole-cell antimicrobial activity was determined by broth microdilution. Fosfomycin was dissolved in dimethyl sulfoxide and diluted 1:10 in water to produce a 256-μg/ml stock solution. Using a 96-well microtiter plate, a Microlab AT Plus 2 (Hamilton Co., Reno, Nev.) serially diluted 50 μl of the stock solution into cation-adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, Md.). After the fosfomycin was diluted, a 50-μl aliquot of the test isolate (~10^6 CFU/ml) was added to each well of the microtiter plate. The final test concentrations ranged from 0.06 to 64 μg/ml. Inoculated plates were incubated at 35°C in ambient air for 18 to 24 h. The MIC was determined as the lowest concentration of fosfomycin that inhibited visible growth.

Cloning and overexpression. The *murA1* and *murA2* genes were amplified by PCR from the genomic DNA of *S. pneumoniae* 100993. The *murA1* gene (1,281

bp) was amplified in two fragments: fragment A has *Nco*I and *Eco*RI restriction ends and was amplified using the oligonucleotides 5′-GGAAACGACCATGGA TAAAATTGTGGTTCAAGGTGG-3′ and 5′-CGCCTTTTGCAACTGTCATC AAGGCTG-3′. Fragment B has *Eco*RI and *Hin*dIII restriction ends and was amplified using the oligonucleotides 5′-GGGTGTTGAAGTAATTGAAGAAG ACG-3′ and 5′-GACCGATTCCTAGAGCCAGAGTACC-3′. Following the restriction digestion, the fragments were ligated into pET28a(+) (Novagen, Madison, Wis.), and the new construct was used to transform *E. coli* BL21(DE3) competent cells (Stratagene, La Jolla, Calif.). The expression of *murA1* was induced with 1 mM isopropylthio-β-D-galactoside (IPTG) at 18°C for 24 h.

The *murA2* gene was amplified with *Nde*I and *Hin*dIII restriction ends using the oligonucleotides 5′-CATATGAGAAAAATTGTTATCAATG-3′ and 5′-A AGCTTAATCCTCAACAAGTCTAATATC-3′. The PCR product of the *murA2* gene (1,260 bp) was first cloned into the cloning vector pCR-Blunt (Invitrogen, Carlsbad, Calif.), and the desired insert was cut from the plasmid with *Nde*I and *Hin*dIII. The insert was separated by 1% agarose gel, purified, and subsequently cloned into pET26b(+) (Novagen). The new construct was used to transform *E. coli* BL21(DE3). The expression of *murA2* was induced with 0.2 mM IPTG at 30°C for 3.5 h.

Enzyme purification. *E. coli* BL21(DE3) cells carrying the plasmid containing the *murA1* or *murA2* gene were grown, respectively, in 1 or 4 liters of LB medium containing kanamycin (0.1 mg/ml) and 2% (wt/vol) glucose. Gene expression was induced using the induction conditions described above. The harvested cells were resuspended in 100 mM Tris (pH 8.0)–5 mM dithiothreitol (DTT) and disrupted by sonication. For the MurA1 enzyme, the cell lysate was fractionated by ammonium sulfate precipitation (20, 40, and 70%), and the 70% ammonium sulfate supernatant was further purified on a Hi-Load (16/10) Q Sepharose column, equilibrated in 100 mM Tris (pH 8.0)–5 mM DTT. Protein was eluted with a gradient of 0 to 1 M KCl over 200 ml. For the MurA2 enzyme, the cell lysate was brought to 1.5 M ammonium sulfate, and the supernatant of this solution was purified on a Hi-Load (16/10) phenyl-Sepharose column, equilibrated in 100 mM Tris (pH 8)–5 mM DTT–1.5 M ammonium sulfate. Protein was eluted with a gradient of 1.5 to 0 M ammonium sulfate over 200 ml. MurA2 was further purified on a Hi-Load (16/10) Q Sepharose column under the same conditions as used for MurA1. MurA1 and MurA2 were identified by SDS-PAGE analyses and activity assays throughout the purification.

Enzyme assays. The activity of MurA1 and MurA2 was assayed by measuring the release of P_i from the UDPAG and PEP reaction, using a malachite green assay (14) in 50 mM HEPES (pH 7.5) at room temperature. A typical assay of 200 μl contained substrates and enzyme (MurA1 at 200 nM or MurA2 at 50 nM). For kinetic measurements, the assay was performed with a five-by-five array of various substrates on a half-area 96-well microtiter plate (Costar 3696; Corning Inc., Corning, N.Y.) with a SpectraMax Plus plate reader (Molecular Devices Corp., Sunnyvale, Calif.). Actual concentrations of the substrates are listed in the footnotes to Table 1. Enzyme activity during purification was assayed with both UDPAG and PEP fixed at 1 mM.
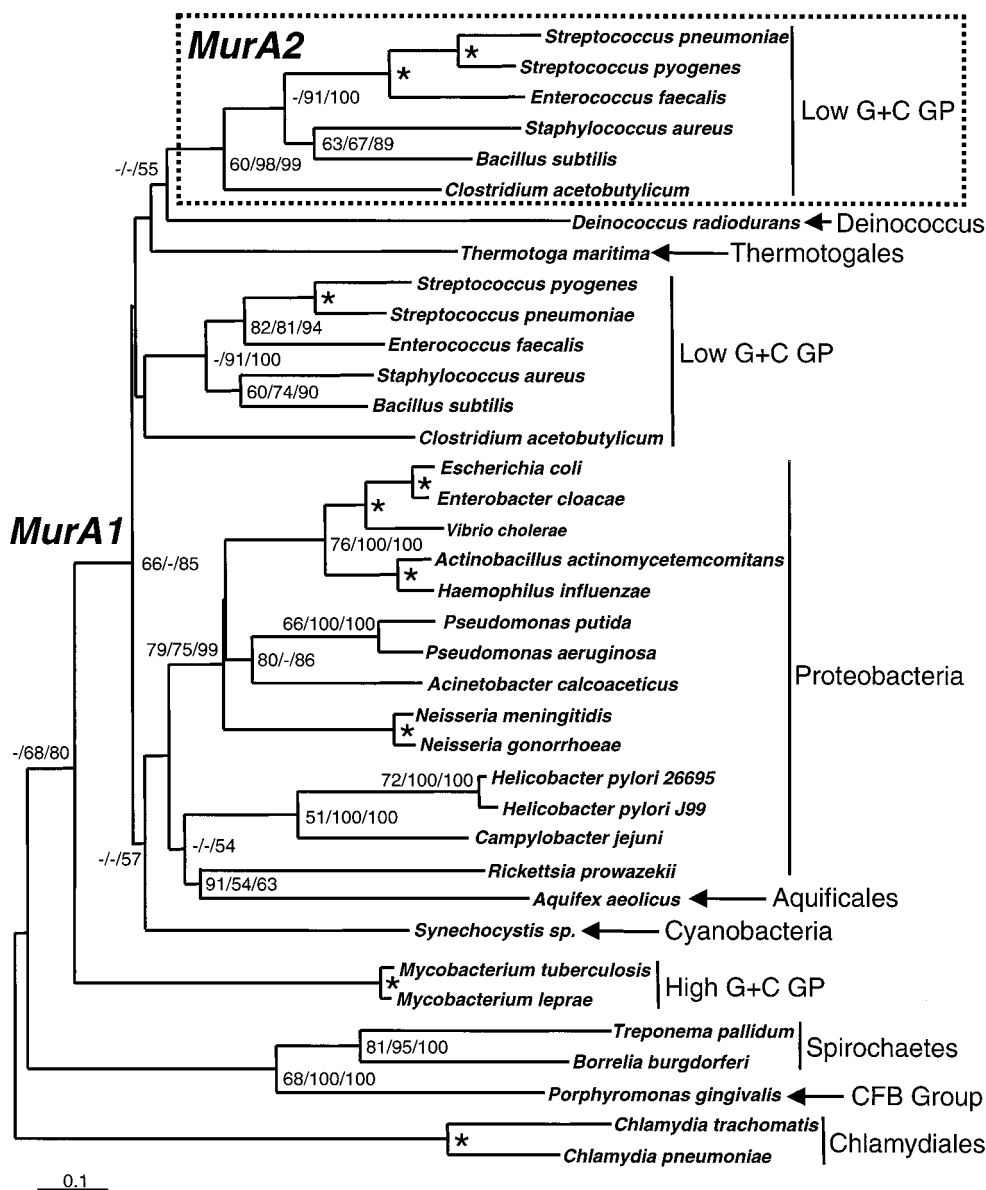
FIG. 2. Phylogenetic tree showing divergence of UDP-*N*-acetylglucosamine enolpyruvyl transferase isoforms (MurA1 and MurA2). Tree was constructed by the NJ method as implemented by the program NEIGHBOR of the PHYLIP 3.57c package (see Materials and Methods). The scale bar represents 0.1 expected amino acid replacements per site as estimated by the program PROTDIST using the Dayhoff PAM substitution matrix. Numbers at the branching points represent the percent occurrence in 1,000 ML puzzling steps and 1,000 random bootstrap replications of MP and NJ methods, respectively. ML and MP analyses were done with the programs PUZZLE version 4.0 (23) and PAUP* (24), respectively. Nodes where values by all three methods were 90% or greater are labeled with an asterisk. Values less than 50% are not shown or are indicated by a dash. Major taxonomic groups of bacteria (18) are indicated with abbreviations given for gram-positive bacteria (GP) and *Cytophaga-Flexibacter-Bacteroides* (CFB) group.

RESULTS

Sequence and structural comparisons of UDP-*N*-acetylglucosamine enolpyruvyl transferases. Database searches revealed that the gene encoding UDP-*N*-acetylglucosamine enolpyruvyl transferase exists widely in nearly all bacteria. However, in low-G+C gram-positive bacteria (*Bacillus subtilis*, *S. pneumoniae*, *S. pyogenes*, *Staphylococcus aureus*, and *Clostridium acetobutylicum*), two complete genes encoding UDP-*N*-acetylglucosamine enolpyruvyl transferase were found (Fig. 2). One of these genes (*murA1*) was more closely related to the *murA* genes found in gram-negative bacteria than the second gene copy (*murA2*). The extent of identical amino acids among

MurA1 proteins ranged from 32 to 96% (calculated over ungapped alignments), while that for MurA2 homologs ranged from 53 to 82%. The genus *Mycoplasma* lacked any open reading frames (ORFs) with significant homology to UDP-*N*-acetylglucosamine enolpyruvyl transferase, as would be expected for an organism that contains no peptidoglycan. Both genomes were thoroughly searched at the protein (BLASTP) and nucleotide (TBLASTN) levels using *B. subtilis* MurA1 and MurA2 (1).

The duplicate gene copies found in low-G+C gram-positive bacteria encoded roughly similar-sized polypeptides (419 to 436 amino acids in length). Comparisons to the crystal struc-
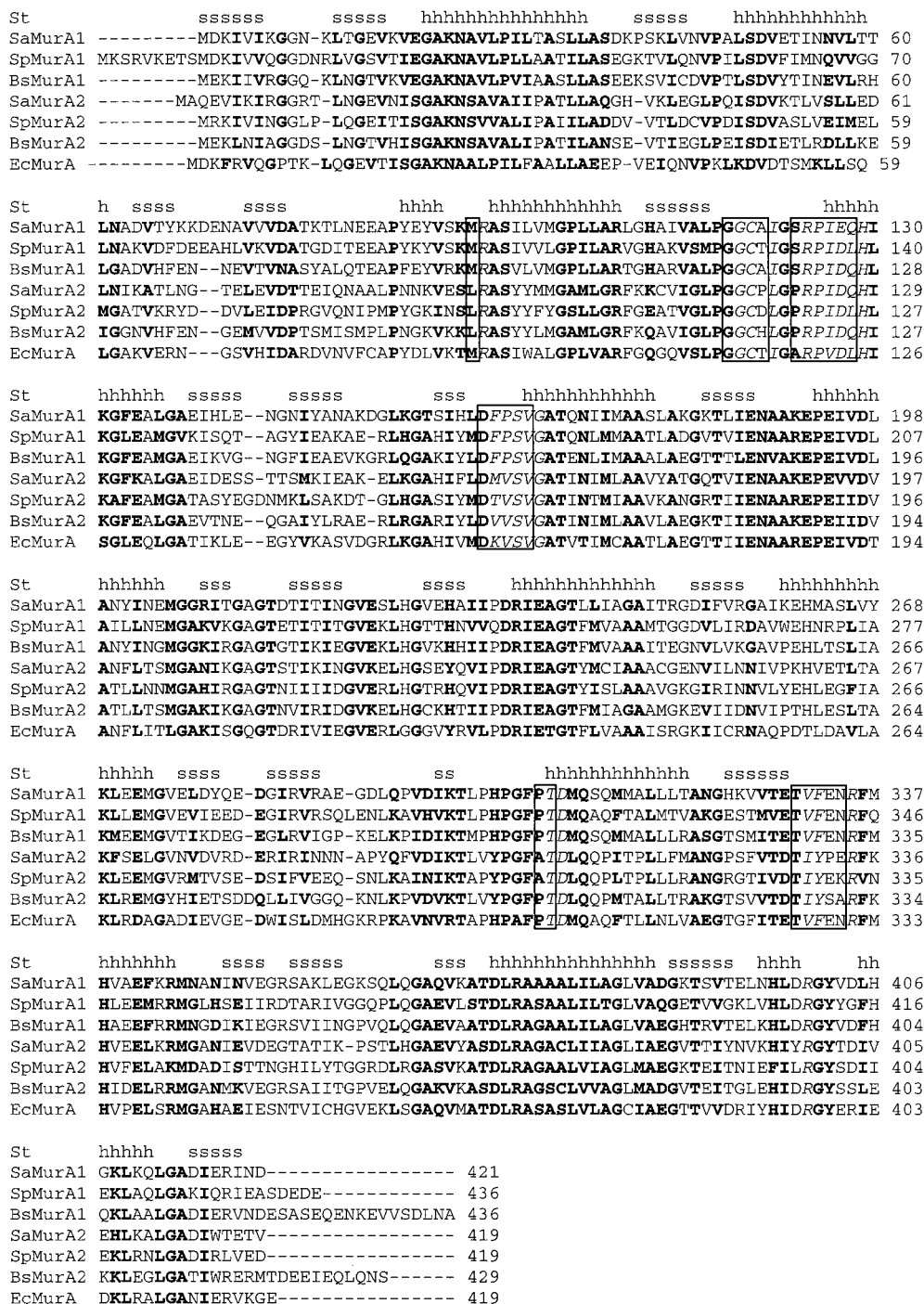
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St                sssss       sssss   hhhhhhhhhhhhhhh   sssss   hhhhhhhhhhh
SaMurA1 ---------MDKIVIKGGN-KLTGEVKVEGAKNAVLPILTASLLASDKPSKLVNVPALSDVETINNVLTT  60
SpMurA1 MKSRVKETSMDKIVVQGGDNRLVGSVTIEGAKNAVLPLLAATILASEGKTVLQNVPILSDVFIMNQVVGG  70
BsMurA1 ---------MEKIIVRGGQ-KLNGTVKVEGAKNAVLPVIAASLLASEEKSVICDVPTLSDVYTINEVLRH  60
SaMurA2 -------MAQEVIKIRGGRT-LNGEVNISGAKNSAVAIIPATLLAQGH-VKLEGLPQISDVKTLVSLLED  61
SpMurA2 ---------MRKIVINGGLP-LQGEITISGAKNSVVALIPAIILADDV-VTLDCVPDISDVASLVEIMEL  59
BsMurA2 ---------MEKLNIAGGDS-LNGTVHISGAKNSAVALIPATILANSE-VTIEGLPEISDIETLRDLLKE  59
EcMurA  ---------MDKFRVQGPTK-LQGEVTISGAKNAALPILFAALLAEEP-VEIQNVPKLKDVDTSMKLLSQ  59


St          h  ssss       ssss        hhhh    hhhhhhhhhhhh  ssssss         hhhhh
SaMurA1 LNADVTYKKDENAVVVDATKTLNEEAPYEYVSKMRASILVMGPLLARLGHAIVALPGGCAIGSRPIEQHI 130
SpMurA1 LNAKVDFDEEAHLVKVDATGDITEEAPYKYVSKMRASIVVLGPILARVGHAKVSMPGGCTIGSRPIDLHL 140
BsMurA1 LGADVHFEN--NEVTVNASYALQTEAPFEYVRKMRASVLVMGPLLARTGHARVALPGGCAIGSRPIDQHL 128
SaMurA2 LNIKATLNG--TELEVDTTEIQNAALPNNKVESLRASYYMGAMLGRFKKCVIGLPGGCPLGPRPIDQHI 129
SpMurA2 MGATVKRYD--DVLEIDPRGVQNIPMPYGKINSLRASYYFGSLLGRFGEATVGLPGGCDLGPRPIDLHL 127
BsMurA2 IGGNVHFEN--GEMVVDPTSMISMPLPNGKVKKLRASYYLMGAMLGRFKQAVIGLPGGCHLGPRPIDQHI 127
EcMurA  LGAKVERN---GSVHIDARDVNVFCAPYDLVKTMRASIWALGPLVARFGQGQVSLPGGCTIGARPVDLHI 126


St        hhhhhh  sssss     sssss     sss     hhhhhhhhhhhh   sssss    hhhhhh
SaMurA1 KGFEALGAEIHLE--NGNIYANAKDGLKGTSIHLDFPSVGATQNIIMAASLAKGKTLIENAAKEPEIVDL 198
SpMurA1 KGLEAMGVKISQT--AGYIEAKAE-RLHGAHIYMDFPSVGATQNLMMAATLADGVTVIENAAREPEIVDL 207
BsMurA1 KGFEAMGAEIKVG--NGFIEAEVKGRLQGAHIYLDFPSVGATENLIMAAALAEGTTTLENVAKEPEIVDL 196
SaMurA2 KGFKALGAEIDESS-TTSMKIEAK-ELKGAHIFLDMVSVGATINIMLAAVYATGQTVIENAAKEPEVVDV 197
SpMurA2 KAFEAMGATASYEGDNMKLSAKDT-GLHGASIYMDTVSVGATINTMIAAVKANGRTIIENAAREPEIIDV 196
BsMurA2 KGFEALGAEVTNE--QGAIYLRAE-RLRGARIYLDVVSVGATINIMLAAVLAEGKTIIENAAKEPEIIDV 194
EcMurA  SGLEQLGATIKLE--EGYVKASVDGRLKGAHIVMDKVSVGATVTIMCAATLAEGTTIIENAAREPEIVDT 194


St        hhhhhh   sss     sssss      ssss    hhhhhhhhhhhh   sssss    hhhhhhhh
SaMurA1 ANYINEMGGRITGAGTDTITINGVESLHGVEHAIIPDRIEAGTLLIAGAITRGDIFVRGAIKEHMASLVY 268
SpMurA1 AILLNEMGAKVKGAGTETITITGVEKLHGTTHNVVQDRIEAGTFMVAAAMTGGDVLIRDAVWEHNRPLIA 277
BsMurA1 ANYINGMGGKIRGAGTGTIKIEGVEKLHGVKHHIIPDRIEAGTFMVAAAITEGNVLVKGAVPEHLTSLIA 266
SaMurA2 ANFLTSMGANIKGAGTSTIKINGVELHGSEYQVIPDRIEAGTYMCIAAACGENVILNNIVPKHVETLTA 267
SpMurA2 ATLLNNMGAHIRGAGTNIIIIDGVERLHGTRHQVIPDRIEAGTYISLAAAVGKGIRINNVLYEHLEGFIA 266
BsMurA2 ATLLTSMGAKIKGAGTNVIRIDGVKELHGCKHTIIPDRIEAGTFMIAGAAMGKEVIIDNVIPTHLESLTA 264
EcMurA  ANFLITLGAKISGQGTDRIVIEGVERLGGGVYRVLPDRIETGTFLVAAAISRGKIICRNAQPDTLDAVLA 264


St        hhhhh  ssss     sssss         ss      hhhhhhhhhhhh    ssssss
SaMurA1 KLEEMGVELDYQE-DGIRVRAE-GDLQPVDIKTLPHPGFPTDMQSQMMALLLTANGHKVVTETVFENRFM 337
SpMurA1 KLLEMGVEVIEED-EGIRVRSQLENLKAVHVKTLPHPGFPTDMQAQFTALMTVAKGESTMVETVFENRFQ 346
BsMurA1 KMEEMGVTIKDEG-EGLRVIGP-KELKPIDIKTMPHPGFPTDMQSQMMALLLRASGTSMITETVFENRFM 335
SaMurA2 KFSELGVNVDVRD-ERIRINNN-APYQFVDIKTLVYPGFATDLQQPITPLLFMANGPSFVTDTIYPERFK 336
SpMurA2 KLEEMGVRMTVSE-DSIFVEEQ-SNLKAINIKTAPYPGFATDLQQPLTPLLLRANGRGTIVDTIYEKRVN 335
BsMurA2 KLREMGYHIETSDDQLLIVGGQ-KNLKPVDVKTLVYPGFPTDLQQPMTALLTRAKGTSVVTDTIYSARFK 334
EcMurA  KLRDAGADIEVGE-DWISLDMHGKRPKAVNVRTAPHPAFPTDMQAQFTLLNLVAEGTGFITETVFENRFM 333


St        hhhhhhh    ssss   sssss       sss  hhhhhhhhhhhhhhhh ssssss  hhhh    hh
SaMurA1 HVAEFKRMNANINVEGRSAKLEGKSQLQGAQVKATDLRAAAALILAGLVADGKTSVTELNHLDRGYVDLH 406
SpMurA1 HLEEMRRMGLHSEIIRDTARIVGGQPLQGAEVLSTDLRASAALILTGLVAQGETVVGKLVHLDRGYYGFH 416
BsMurA1 HAEEFRRMNGDIKIEGRSVIINGPVQLQGAEVAATDLRAGAALILAGLVAEGHTRVTELKHLDRGYVDFH 404
SaMurA2 HVEELKRMGANIEVDEGTATIK-PSTLHGAEVYASDLRAGACLIIAGLIAEGVTTIYNVKHIYRGYTDIV 405
SpMurA2 HVFELAKMDADISTTNGHILYTGGRDLRGASVKATDLRAGAALVIAGLMAEGKTEITNIEFILRGYSDII 404
BsMurA2 HIDELRRMGANMKVEGRSAIITGPVELQGAKVKASDLRAGSCLVVAGLMADGVTEITGLEHIDRGYSSLE 403
EcMurA  HVPELSRMGAHAEIESNTVICHGVEKLSGAQVMATDLRASASLVLAGCIAEGTTVVDRIYHIDRGYERIE 403


St        hhhhh    sssss
SaMurA1 GKLKQLGADIERIND---------------- 421
SpMurA1 EKLAQLGAKIQRIEASDEDE------------ 436
BsMurA1 QKLAALGADIERVNDESASEQENKEVVSDLNA 436
SaMurA2 EHLKALGADIWTETV---------------- 419
SpMurA2 EKLRNLGADIRLVED---------------- 419
BsMurA2 KKLEGLGATIWRERMTDEEIEQLQNS------ 429
EcMurA  DKLRALGANIERVKGE--------------- 419
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FIG. 3. Multiple sequence alignment of UDP-*N*-acetylglucosamine enolpyruvyl transferases (MurA1 and MurA2) from *B. subtilis* (Bs), *S. aureus* (Sa), *S. pneumoniae* (Sp), and *E. coli* (Ec). Shown are the locations of α helices (h) and β sheets (s) in the determined structure (St) of *E. coli* MurA (22). Highly conserved residues (from CLUSTALW output) are boldface, while residues of *E. coli* MurA involved in ligand interactions are in italics and boxed.

ture of *E. coli* MurA showed that major structural features and residues involved in ligand interactions were highly conserved among MurA1 and MurA2 proteins (Fig. 3). These structural similarities indicate that it is unlikely that one copy is a pseudogene.

To investigate if both genes encode active enzymes, we decided to study the two genes of *S. pneumoniae* more closely. The two MurA enzymes from this organism showed 45% iden-

tity to each other at the amino acid level. The genes were not close to each other in the *S. pneumoniae* genome and were not clustered with any other peptidoglycan synthesis enzymes. MurA1 is flanked by two small ORFs which might form a single transcriptional unit (operon). The upstream ORF is putative and unique to *S. pneumoniae*. The downstream ORF is 64 amino acids long and is homologous to EPUA protein, a possible membrane-bound nuclease required for DNA trans-

formation. MurA2 is upstream of a small hypothetical protein about 165 amino acids long which shows very low homology to *Bacillus licheniformis* hypothetical protein P20. There is no downstream linked ORF.

Essentiality. We first attempted to inactivate each *S. pneumoniae murA* gene by allelic replacement. Regions up- and downstream of each gene ranging from 406 to 555 bp were amplified by PCR and used to make constructs in which they flanked an antibiotic resistance gene. In the case of *murA1*, the constitutively expressed erythromycin resistance gene cassette *ermAM* from pAMβ1 was used (17), while the chloramphenicol resistance *cat* gene from pJS3 was used in the *murA2* construct (2). To minimize the potential polar effects of gene replacement, the PCR primers were chosen so that flanking genes and potential promoters would remain intact in the deletion mutant. In addition, the cassettes were designed so that the resistance gene was inserted in the same orientation as the target gene, to ensure transcription of the downstream region. This strategy was designed to generate mutants which were nonpolar (since there is no structural or transcriptional disruption of adjacent genes), stable in the absence of selective pressure (because there are no insertion site sequence duplications to allow spontaneous excision), and null (given that there can be no residual target gene activity).

The two allelic replacement constructs were used separately to transform *S. pneumoniae* R6, and mutants of both *murA1* and *murA2* were obtained. The structure of each mutant was confirmed by diagnostic PCR and Southern hybridization analysis. MICs for fosfomycin for the *S. pneumoniae* R6 *murA1* and *murA2* strains were unchanged compared to wild-type *S. pneumoniae* R6.

In an attempt to make a *murA1 murA2* double mutant, *murA2* allelic replacement construct DNA was transformed into competent cells of the *S. pneumoniae* R6 *murA1* mutant, but no transformants were obtained in multiple transformation experiments with positive allelic replacement and transformation controls. These results suggest that both *murA* genes encode active MurA enzymes, since elimination of only one still results in a viable organism.

Biochemical characterization of MurA1 and MurA2. MurA1 and MurA2 from *S. pneumoniae* were investigated further at the protein level. The *murA1* and *murA2* genes were overexpressed, and the enzymes that they encode, MurA1 and MurA2, respectively, were purified and kinetically characterized. Overexpression of the *murA1* and *murA2* genes in *E. coli* accounted for ca. 40 and 30% of total cell protein, and under the selected induction conditions, more than 50 and 40% of the MurA1 and MurA2 enzymes, respectively, remained soluble.

MurA1 was purified by ammonium sulfate fractionation and anion-exchange chromatography. An extra chromatography step on a hydrophobic interaction column was also included in the purification of MurA2. Approximately 33 mg of MurA1 and 21 mg of MurA2 were obtained from 1 and 4 liters of cultures, respectively, with the purity of the final preparations being 95% as judged by SDS-PAGE (Fig. 4). The identities of MurA1 and MurA2 were confirmed by N-terminal sequencing (N-terminal sequences being MDKIVVQGGD and MRKIVINGGL for MurA1 and MurA2, respectively) and MALDI-TOF mass spectrometry.

The catalytic activities of the two enzymes were then investigated. Both MurA1 and MurA2 were able to catalyze the transfer of enolpyruvate from PEP to UDPAG, demonstrating that they are both active UDP-*N*-acetylglucosamine enolpyruvyl transferases as expected from the sequence analysis and gene deletion experiments. The kinetic parameters for both enzymes were determined (Table 1). The K_m(UDPAG) and
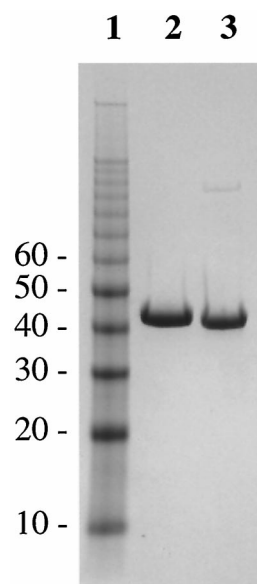

FIG. 4. SDS-PAGE analysis of purified MurA1 and MurA2. The experiment was performed on a Novex 4 to 12% bis-Tris gel with morpholineethanesulfonic acid running buffer. Lane 1, 10-kDa molecular mass ladder (with selected molecular masses [in kilodaltons] marked); lane 2, purified MurA1 after the Hi-Load Q column; lane 3, purified MurA2 after the Hi-Load Q column.

K_m(PEP) were two- and threefold lower for MurA2 than for MurA1, while k_{cat} was almost twice as high for MurA2 as for MurA1 (Table 1). More importantly, k_{cat}/K_m(UDPAG) and k_{cat}/K_m(PEP) values were four- and sixfold higher for MurA2 than for MurA1, indicating MurA2 is a more efficient enzyme than MurA1 under these assay conditions. Both enzymes were inhibited by the antibiotic fosfomycin, the 50% inhibitory concentrations for MurA1 and MurA2 being 2.3 and 14.9 μM, respectively.

DISCUSSION

We have shown by phylogenetic analysis that two distinct classes of UDP-*N*-acetylglucosamine enolpyruvyl transferase exist (Fig. 2). The first class of transferases, MurA1, occurs throughout all bacteria except gram-positive *Mycoplasma* spp. The second type of transferase, MurA2, exists as a duplicate gene copy in only the low-G+C gram-positive bacteria. A combination of sequence analysis, gene knockout experiments, and direct biochemical characterization was used to analyze

TABLE 1. Kinetic constants of *S. pneumoniae* MurA1 and MurA2

Protein	K_m(UDPAG) (μM)	K_m(PEP) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m(UDPAG) μM^{-1} s^{-1}	k_{cat}/K_m(PEP) (μM^{-1} s^{-1})
MurA1[a]	244 ± 20	37.0 ± 4.0	0.41 ± 0.01	0.0017	0.011
MurA2[b]	119 ± 16	11.1 ± 2.3	0.78 ± 0.03	0.0066	0.07

[a] Kinetic constants of MurA1 were obtained from a five-by-five array of various substrates ([UDPAG] = 100, 200, 500, 1,000, and 2,000 μM; [PEP] = 50, 65, 125, 250, and 500 μM). MurA1 was at 200 nM. The data were fitted into a ping-pong model in GraFit (version 4.09; Erithacus Software Limited).

[b] Kinetic constants of MurA2 were obtained from a five-by-five array of various substrates ([UDPAG] = 100, 140, 250, 500, and 1,000 μM; [PEP] = 20, 33.3, 50, 100, and 200 μM). MurA2 was at 50 nM. Data fitting was the same as for MurA1.

the two copies of *murA* found in these gram-positive organisms. Sequence analysis indicates both genes are complete and that the enzymes they encode contain the important catalytic residues previously identified in the *E. coli* MurA. Structurally, and probably functionally, MurA1 and MurA2 are highly similar. Gene deletion experiments further indicate that both genes must encode active enzymes since removal of either still results in a viable organism although this activity is clearly essential for viability. This suggests that the two enzymes have the same function and that one enzyme can substitute for the other.

To demonstrate conclusively that these two genes encode active enzymes, we overexpressed the two genes and purified the two proteins from *S. pneumoniae*. Both MurA enzymes are active and catalyze the reaction between UDPAG and PEP to give UDP-*N*-acetylglucosamine enolpyruvate and P_i. Both enzymes from *S. pneumoniae* appear to have higher K_ms for their substrates and lower k_{cat}s than the *E. coli* enzyme, indicating that at a kinetic level MurA1 more closely resembles MurA2 than its gram-negative counterpart. They are also both inhibited by the antibiotic fosfomycin. Fosfomycin possesses antibacterial activity against both gram-positive and gram-negative organisms. The majority of the data generated on the mode of action of this antibiotic has been focused on MurA from *E. coli* (for example, see references 11, 15, 16, and 26). In this study we have demonstrated that fosfomycin inhibits both MurA enzymes from *S. pneumoniae*, thus providing the first confirmatory evidence that MurA is also the antibacterial target of fosfomycin in this organism.

The existence of two active copies of *murA* gene in these gram-positive organisms raises the question of how the two copies arose. The phylogenetic tree suggests that *murA2* might have evolved from a duplication of the *murA1* gene in gram-positive bacteria, although the statistical support for this scenario, or any alternative, is weak. The branching of *Deinococcus radiodurans* and *Thermotoga maritima*, both not considered G+C gram-positive bacteria (18), among the low G+C gram-positive bacteria might be taken as negative evidence against the scenario of *murA2* evolution by gene duplication within the gram-positive bacteria. However, there is considerable debate about the phylogenetic placement of these bacterial taxa (Cavalier-Smith [5] proposed that *T. maritima* might be a gram-positive bacterium) and the clustering of *Deinococcus*/*Thermus* and *Thermotogales* among gram-positive bacteria has been observed in other protein phylogenies such as glutamine synthetase (4).

The phylogenetic and structural analysis does support the notion that MurA1 and MurA2 proteins are closely related and are likely to have evolved somewhere in the lineage leading to the low-G+C gram-positive bacteria. MurA1 and MurA2 proteins might have evolved from an even more ancient source as suggested by Psi-BLAST homology searches (1), which reveal significant similarities between MurA1/MurA2 and 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme in the shikimate pathway.

It is also unclear why a duplication of the enolpyruvyl transfer function has arisen and whether MurA1 and MurA2 carry out identical functions at a physiological level within the cell. The two enzymes catalyze the same reaction, which is the first committed step in peptidoglycan biosynthesis. The higher k_{cat}/K_m(UDPAG) and k_{cat}/K_m(PEP) values for MurA2 from *S. pneumoniae* (Table 1) indicate that MurA2 is a more efficient enzyme in utilizing both substrates under the assay conditions used here, but this may not be true in vivo. Both enzymes appear to be less efficient than their *E. coli* counterparts (11). It would be interesting to investigate the relative levels of

expression of the two *murA* genes within the cell, as this might shed more light on roles of their gene products in the peptidoglycan biosynthetic pathway. It is possible that although both MurA enzymes are active, only one *murA* gene is expressed and its expression product is utilized in *S. pneumoniae*.

It is interesting that the other genes encoding enzymes involved in peptidoglycan biosynthesis in gram-positive bacteria appear to be present as single copies. However, there is a further example in peptidoglycan biosynthesis of an enzyme activity that appears to have been duplicated: D-Ala-D-Ala ligase in gram-negative bacteria, which is encoded by the *ddlA* and *ddlB* genes. In this case again, the two purified enzymes show similar kinetic parameters and susceptibilities to inhibitors, and it is unclear why a duplication in activity has occurred (28).

MurA is potentially an important antibacterial target. The presence of two active copies of the enzyme in gram-positive pathogens illustrates the need for a MurA-directed antibacterial to inhibit both forms of the enzyme. This is clearly possible, as illustrated by fosfomycin, which inhibits both MurA1 and MurA2 and thus has antibacterial activity against *S. pneumoniae*. This observation and the fact that we have shown the enolpyruvyl transferase activity to be essential in this organism further validate the MurA enzyme as an antibacterial target.

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