

Biosynthesis of Diaminopimelate, the Precursor of Lysine and a Component of Peptidoglycan, Is an Essential Function of *Mycobacterium smegmatis*

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Diaminopimelate (DAP) is a unique metabolite used for both the biosynthesis of lysine in bacteria and the construction of the peptidoglycan of many species of bacteria, including mycobacteria. DAP is synthesized by bacteria as part of the aspartate amino acid family, which includes methionine, threonine, isoleucine, and lysine. Aspartokinase, the first enzyme in this pathway, is encoded by the *ask* gene in mycobacteria. Previous attempts to disrupt this gene in *Mycobacterium smegmatis* were unsuccessful, even when the cells were supplied with all the members of the aspartate family, suggesting that unlike other bacteria, mycobacteria may have an absolute requirement for this pathway even when growing in rich medium containing DAP. The purpose of this study was to determine if the *ask* gene and the aspartate pathway are essential to *M. smegmatis*. This study describes a test for gene essentiality in mycobacteria, utilizing a counterselectable marker (streptomycin resistance) in conjunction with a specially constructed merodiploid strain. We have used this system to show that the *ask* gene could not be disrupted in wild-type *M. smegmatis*, using selective rich medium supplemented with DAP unless there was an extra copy of *ask* provided elsewhere in the chromosome. Disruption of *ask* was also possible in a lysine auxotroph incapable of converting DAP to lysine. The *ask* mutant, mc²1278 (*ask1::aph*), exhibits multiple auxotrophy (Met⁻, Thr⁻, DAP⁻, and Lys⁻) and is complemented by the *ask* gene. This is the first description of DAP auxotrophy in mycobacteria. The *ask* mutant lyses when deprived of DAP in culture, a characteristic which can be exploited for the reproducible preparation of protoplasts and mycobacterial extracts. The evidence presented here indicates that the aspartate pathway is essential to *M. smegmatis* and that DAP is the essential product of this pathway.

Globally, tuberculosis is the leading cause of death in adults due to an infectious organism (20). It is estimated that 90 million new tuberculosis cases resulting in 30 million deaths can be expected during the last decade of this century (51). The resurgence of tuberculosis in developing nations (62), the appearance of multidrug-resistant strains of *Mycobacterium tuberculosis* (62), and the problem of tuberculosis in the immunocompromised (28) call for further study of mycobacteria. More knowledge about the basic biology of mycobacteria is needed in order to develop a deeper understanding of the pathogenesis of mycobacterial diseases. Furthermore, identification of biological processes specifically essential for the growth and development of mycobacteria will allow the rational design of drugs to inhibit those processes.

The complex cell envelope of the mycobacteria is an outstanding feature of these organisms (13). The envelope is composed of a variety of complex lipids including the long-chain mycolic acids and unique polysaccharides such as arabinogalactan and arabinomannan (7). These components contribute to the hydrophobic nature of the mycobacterial cell surface (43) and the low permeability of the mycobacterial cell envelope (48) and play a role in the immunological responses of the host to mycobacterial infections (14). We are interested in the biosynthesis of the peptidoglycan, the innermost layer of the mycobacterial cell wall (12).

Peptidoglycan, present in virtually all bacteria, provides shape and structural integrity. The peptidoglycan of mycobacteria differs in a few respects from that of other bacteria. In most bacteria the glycan backbone of the peptidoglycan is comprised of *N*-acetylmuramic acid and *N*-acetylglucosamine (27). In mycobacteria the former is replaced by *N*-glycolylmuramic acid (4). The peptide portion of mycobacterial peptidoglycan is of the common A1 γ chemotype, consisting of L-Ala-D-Gln-*meso*-diaminopimelate (*meso*-DAP)-D-Ala (56), but the glutamyl and diaminopimelyl residues in the peptide are amidated (37). The peptidoglycan of *Mycobacterium leprae* differs from that of other mycobacteria in that the amino acid in position 1 of the peptide is glycine instead of L-alanine (21). As a whole, the mycobacterial peptidoglycan exhibits a high degree of interpeptide cross-linking, primarily through DAP: DAP cross-links in addition to the DAP:Ala cross-links more commonly seen in other bacteria (70). In relation to other components of the mycobacterial cell envelope, it is known that the mycolyl-arabinogalactan is covalently attached to the peptidoglycan via a unique disaccharide phosphodiester linkage, forming the mycolyl-arabinogalactan-peptidoglycan complex (8, 42).

We are studying DAP biosynthesis because it is central to the structure of the mycobacterial peptidoglycan. DAP is neither produced or required by humans, and thus the DAP biosynthetic pathway is an attractive target for the development of antibacterial drugs. DAP auxotrophs of virulent *M. tuberculosis* might prove to be attenuated and therefore potential live-vaccine strains.

DAP is synthesized by bacteria via the aspartate amino acid

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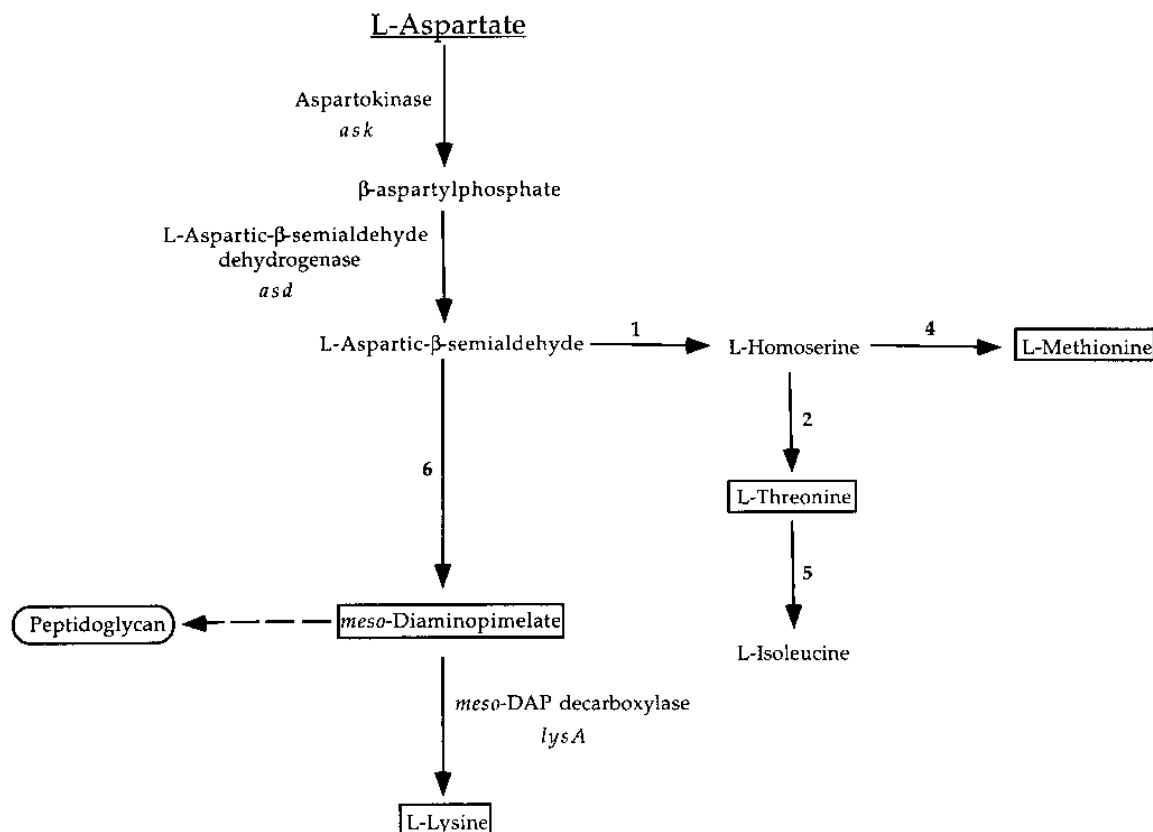


FIG. 1. The biosynthetic pathway for the aspartate family of amino acids. L-aspartate is the precursor for the synthesis of L-methionine, L-threonine, L-isoleucine, *meso*-DAP, and L-lysine. The first reaction in this pathway is the phosphorylation of L-aspartate by an aspartokinase enzyme, followed by the conversion of β -aspartylphosphate to L-aspartic- β -semialdehyde by a dehydrogenase. In *M. smegmatis*, the *ask* gene, encoding aspartokinase, is believed to be the promoter proximal gene in an operon with the *asd* gene encoding L-aspartic- β -semialdehyde dehydrogenase (18). L-Aspartic- β -semialdehyde is the branch point intermediate for the synthesis of the different amino acids in this family. A homoserine dehydrogenase activity (data not indicated) converts this intermediate to L-homoserine, which is used for L-methionine, L-threonine, and L-isoleucine synthesis. L-Aspartic- β -semialdehyde is diverted to DAP synthesis via dihydrodipicolinate synthase (data not indicated), and *meso*-DAP is the direct precursor for L-lysine. The number of steps in each branch of the pathway are shown in bold. The genes relevant to this study, *ask*, *asd*, and *lysA*, are indicated. This data is adapted from reference 69.

family pathway (Fig. 1) (69). This family is comprised of methionine, threonine, isoleucine, and lysine, amino acids whose carbon skeletons are primarily derived from aspartate. L,L-DAP, or its isomer *meso*-DAP, is an intermediate in this pathway used for peptidoglycan synthesis in some bacteria, while *meso*-DAP is the direct precursor to lysine in all bacteria (69). An aspartokinase enzyme, encoded by the *ask* gene in mycobacteria, catalyzes the first step in the aspartate family pathway (18). Previous attempts to disrupt the *ask* gene in *Mycobacterium smegmatis* were unsuccessful even when medium containing all the members of the aspartate family pathway was used, suggesting that *ask* may be essential (16). This is in contrast to the ability to obtain auxotrophic aspartokinase mutants in other species of bacteria (25, 68). We define the essentiality of *ask* in *M. smegmatis* by the inability to both disrupt the gene and recover viable mutants in rich medium supplemented with DAP. The purpose of this study was to determine if, and why, the *ask* gene is essential to *M. smegmatis*. We describe the construction of a counterselectable marker system for allelic exchange in mycobacteria and its application in a test for gene essentiality. Using this system we show that the *ask* gene is essential for viability in wild-type *M. smegmatis*. However, we could obtain the *ask* disruption in a *Lys*⁻ strain of *M. smegmatis* incapable of converting *meso*-DAP to lysine. This gen-

erated the first mycobacterial DAP auxotroph, a mutant which lyses after DAP deprivation.

MATERIALS AND METHODS

Bacterial strains and culture methods. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth, while *M. smegmatis* cultures were grown in LB broth containing 0.5% (wt/vol) Tween 80 (LBT). For growth on plates, both *E. coli* and *M. smegmatis* were grown on LB agar. All cultures were incubated at 37°C. When required, the following antibiotics were used at the specified concentrations; ampicillin (50 μ g/ml for *E. coli*), kanamycin A monosulfate (25 μ g/ml for *E. coli*; 10 μ g/ml for *M. smegmatis*), hygromycin B (50 μ g/ml for *E. coli*; 150 μ g/ml for *M. smegmatis*), and streptomycin sulfate (400 μ g/ml). Hygromycin B was purchased from Boehringer Mannheim (50 mg/ml in phosphate-buffered saline); all other antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.). The preparation of kanamycin A stock solutions was based on the activity per milligram (dry weight) of the antibiotic, as reported by the manufacturer. A mixture of L,L-, D,D-, and *meso*-DAP isomers (Sigma Chemical Co.) was added to the culture medium at a final concentration of 100 μ g/ml from a 10-mg/ml stock solution prepared in water. For "DAP-less death" experiments, cells were grown to mid-exponential phase (optical density at 600 nm [OD₆₀₀] of ~0.5) in LBT supplemented with kanamycin, streptomycin, and DAP. The culture was centrifuged, and the cell pellet was washed twice with LBT lacking DAP. The pellet was resuspended in a small volume of LBT and inoculated into fresh LBT supplemented with kanamycin and streptomycin with or without DAP. The cultures were incubated with gentle shaking (100 rpm), and the OD₆₀₀ was monitored over the course of several hours.

Plasmid construction. DNA manipulations were done essentially as previously

TABLE 1. Strains used in this study

Strain	Description	Reference
<i>E. coli</i> K-12 HB101	F ⁻ Δ(<i>gpt-proA</i>)62 <i>leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13</i>	11
<i>M. smegmatis</i>		
mc ² 155	<i>ept-1</i>	61
mc ² 1255	<i>ept-1 rpsL4</i>	45
P73	<i>lysA</i>	46
mc ² 1211	<i>lysA ept-4</i>	This work
mc ² 1212	<i>lysA ept-4 rpsL5</i>	This work
mc ² 1265	<i>ept-1 rpsL4 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] Sm ^s Km ^r	This work
mc ² 1266	<i>ept-1 rpsL4 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] <i>attB::pYUB412</i> Sm ^s Km ^r	This work
mc ² 1268	<i>ept-1 rpsL4 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] <i>attB::pYUB643</i> Sm ^s Km ^r	This work
mc ² 1269	<i>lysA ept-4 rpsL5 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] Sm ^s Km ^r	This work
mc ² 1270	<i>lysA⁺ ept-4 rpsL5</i>	This work
mc ² 1276	<i>lysA⁺ ept-4 rpsL5 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] Sm ^s Km ^r	This work
mc ² 1278	<i>lysA ept-4 rpsL5 ask1::aph</i>	This work
mc ² 1374	<i>ept-1 rpsL4 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] <i>attB::pYUB412</i> Sm ^r Km ^r	This work
mc ² 1375	<i>ept-1 rpsL4 ask attB::pYUB412</i> Sm ^r Km ^s	This work
mc ² 1376	<i>ept-1 rpsL4 ask1::aph attB::pYUB643</i> Sm ^r Km ^r	This work
mc ² 1377	<i>ept-1 rpsL4 ask attB::pYUB643</i> Sm ^r Km ^s	This work
mc ² 1385	<i>lysA ept-4 rpsL5 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] <i>attB::pYUB412</i> Sm ^s Km ^r	This work
mc ² 1386	<i>lysA ept-4 rpsL5 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] <i>attB::pYUB651</i> Sm ^s Km ^r	This work
mc ² 1387	<i>lysA⁺ ept-4 rpsL5 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] <i>attB::pYUB412</i> Sm ^s Km ^r	This work
mc ² 1388	<i>lysA⁺ ept-4 rpsL5 DUP1</i> [(ORF <i>x ask asd</i> ORF <i>y</i>)*pYUB608*(ORF <i>x ask1::aph asd</i> ORF <i>y</i>)] <i>attB::pYUB643</i> Sm ^s Km ^r	This work

described (2). Plasmids were constructed in *E. coli* HB101 and prepared by an alkaline lysis protocol (30). Plasmids used for recombination experiments in *M. smegmatis* were purified by using Qiagen (Chatsworth, Calif.) columns as recommended by the manufacturer. DNA fragments used for plasmid construction were purified by agarose gel electrophoresis and recovered by absorption to glass fines (GeneClean; Bio 101, Vista, Calif.). Plasmids used in this study are listed in Table 2. Plasmid pYUB412 is an integration-proficient vector used in this work (6). This vector has no mycobacterial origin of replication but instead has the mycobacteriophage L5 attachment site (*attP*) and the L5 integrase gene (*int*) (38). In addition, pYUB412 carries the *hyg* gene, conferring resistance to hygromycin. This vector efficiently integrates into the phage attachment site (*attB*) of the *M. smegmatis* chromosome and is stable (38).

Construction of *rpsL* suicide vector pYUB608. The wild-type *rpsL* gene of *M. smegmatis* was amplified from mc²155 genomic DNA with the GeneAmp PCR kit with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.). Oligonucleotides used as PCR primers were synthesized by the Albert Einstein College of Medicine oligonucleotide synthesis facility. The oligonucleotides used for amplification of *rpsL* were BJ-1313 (5'-atcggtacgaggatcc-ACAAGAGAAGC AACACACAG-3') and BJ-1314 (5'-tcgattagcggatcc-AGCAGGACCTTGTTCC ACGAG-3'). These primers were designed according to the previously reported

DNA sequence of the *rpsL* gene of *M. smegmatis* (35). The uppercase letters designate *rpsL*-specific flanking DNA sequences, while the lowercase letters designate the 5' extensions used to engineer unique *Bam*HI sites (underlined) at the ends of the PCR product. BJ-1313 is specific for sequences 31-bp upstream of the start codon of *rpsL*, while BJ-1314 is specific for sequences 93-bp downstream of the *rpsL* stop codon. The two primers amplify a 528-bp fragment containing the 372-bp *rpsL* gene. Reaction mixtures included approximately 100 ng of template DNA, 50 pmol of each primer, and 2.0 mM MgCl₂. Reaction mixtures were run on a Perkin-Elmer model 480 DNA thermal cycler with the following parameters: 94°C for 5 min (1 cycle) and 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (35 cycles). The identity of the 528-bp reaction product was confirmed by restriction endonuclease mapping. The product was cloned under the control of the *hsp60* promoter (via the engineered *Bam*HI sites) in the *E. coli*-mycobacteria shuttle vector pMV261, generating pYUB600. To construct pYUB608, the *rpsL* gene was removed from pYUB600 with *Mcs*I and *Cla*I and cloned into the *Hinc*II and *Cla*I sites of pYUB558 (Table 2).

Isolation and characterization of *M. smegmatis rpsL* mutants. The streptomycin counter selection system developed for this study required a streptomycin-resistant *M. smegmatis* strain with a mutation in the *rpsL* gene. Since *rpsL* mutants of *M. smegmatis* are resistant to high levels of streptomycin (>200

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pKSII ⁺	Ap ^r , high-copy number cloning vector, ColE1, unable to replicate in mycobacteria	Stratagene
pMV261	Km ^r , <i>E. coli</i> -mycobacteria shuttle vector, contains the <i>hsp60</i> promoter, ColE1, OriM	65
pET3d. <i>lysA</i>	<i>M. tuberculosis</i> Erdman <i>lysA</i> gene cloned into pET3d	1, 22
pYUB114	5-kb <i>Eco</i> RI fragment from <i>M. smegmatis</i> mc ² 6 containing ORF <i>x ask asd</i> ORF <i>y</i> cloned into the <i>Eco</i> RI site of pKSII ⁺	18
pYUB205	pYUB114 with a 1.2-kb <i>aph</i> cassette inserted into a <i>Pst</i> I site within the <i>ask</i> gene (<i>ask1::aph</i>), Km ^r	16
pYUB412	Ap ^r Hyg ^r , <i>E. coli</i> -mycobacteria shuttle vector, ColE1 origin, <i>int attP</i> nonreplicative but integration proficient in mycobacteria	6
pYUB558	pKSII ⁺ containing the major P _{left} promoter of mycobacteriophage L5	6
pYUB600	pMV261 containing the <i>M. smegmatis</i> mc ² 155 <i>rpsL</i> gene PCR product under control of the <i>hsp60</i> promoter	This work
pYUB608	465-bp <i>Msc</i> I- <i>Cla</i> I <i>rpsL</i> fragment from pYUB600 cloned into the <i>Hinc</i> II- <i>Cla</i> I sites of pYUB558, <i>rpsL</i> under control of the major P _{left} promoter of mycobacteriophage L5	This work
pYUB609	6.3-kb <i>Eco</i> RI fragment from pYUB205 containing ORF <i>x ask1::aph asd</i> ORF <i>y</i> cloned into the <i>Eco</i> RI site of pYUB608	This work
pYUB628	1.3-kb <i>Xho</i> I- <i>Bam</i> HI fragment from pET3d. <i>lysA</i> cloned into the <i>Pvu</i> II- <i>Bcl</i> I sites of pMV261, <i>lysA</i> under control of the <i>hsp60</i> promoter	This work
pYUB643	5-kb <i>Eco</i> RI fragment from pYUB114 containing ORF <i>x ask asd</i> ORF <i>y</i> cloned into the <i>Eco</i> RV site of pYUB412	This work
pYUB646	3.5-kb <i>Eco</i> RI- <i>Age</i> I fragment from pYUB114 containing ORF <i>x ask asd</i> cloned into the <i>Eco</i> RV site of pYUB412	This work
pYUB647	2.8-kb <i>Eco</i> RI- <i>Apa</i> LI fragment from pYUB114 containing ORF <i>x ask</i> cloned into the <i>Eco</i> RV site of pYUB412	This work
pYUB651	2.2-kb <i>Nor</i> I- <i>Ssp</i> I fragment from pYUB628 containing P _{hsp60} <i>lysA</i> cloned into the <i>Eco</i> RV site of pYUB412	This work

TABLE 3. Plasmid pYUB609 (ORF_x *ask1::aph asd* ORF_y)-chromosome recombination

Strain	Relevant genotype	Direct selection for Km ^r		Direct selection for Km ^r Sm ^r	
		No. of Km ^r recombinants (frequency of recombination) ^a	No. of Km ^r Sm ^r /no. of Km ^r clones (% of auxotrophy) ^b	No. of Km ^r Sm ^r recombinants (frequency of recombination) ^a	No. of auxotrophs/no. of Km ^r Sm ^r clones ^c
mc ² 1255	<i>ept-1 rpsL4</i>	271 (4.0 × 10 ⁻⁴)	2/94 (0)	13 (6.5 × 10 ⁻⁶)	0/13
mc ² 1212	<i>lysA ept-4 rpsL5</i>				
Expt 1		64 (3.2 × 10 ⁻⁵)	1/64 (100)	6 (3.0 × 10 ⁻⁶)	5/6
Expt 2		104 (7.0 × 10 ⁻⁵)	9/96 (100)	23 (1.5 × 10 ⁻⁵)	23/23
mc ² 1270	<i>lysA⁺ ept-4 rpsL5</i>				
Expt 1		616 (9.0 × 10 ⁻⁵)	22/150 (0)	165 (2.4 × 10 ⁻⁵)	0/125
Expt 2		500 (9.3 × 10 ⁻⁵)	15/150 (0)	133 (1.9 × 10 ⁻⁵)	0/125

^a Each strain was electroporated in duplicate with 1 µg of pYUB609 DNA. The number of recombinant clones obtained for each type of selection is shown, along with the recombination frequencies (in parentheses). These frequencies were calculated by dividing the number of clones obtained with pYUB609 by the number of Km^r transformants obtained with the replicating plasmid pMV261. In these experiments, the pMV261 electroporation efficiencies ranged from 1.5 × 10⁶ to 6.8 × 10⁶ kanamycin-resistant clones per 1 µg of input DNA.

^b Number of directly selected Km^r clones that are also Sm^r and the percentages of the Km^r Sm^r clones that are auxotrophic for Met, Thr, and DAP.

^c Number of directly selected Km^r Sm^r clones that are auxotrophic for Met, Thr, and DAP.

µg/ml) (35), a spontaneous streptomycin-resistant mutant of mc²155 (mc²1255) resistant to at least 500 µg of streptomycin per ml was isolated for use in these experiments. Plasmid pYUB600, bearing the wild-type *rpsL* gene expressed from the *hsp60* promoter, rendered the Sm^r mutant mc²1255 sensitive to streptomycin, proving that the mutation conferring streptomycin resistance in mc²1255 was in the *rpsL* gene and that the wild-type *rpsL* PCR product cloned in pYUB600 was functional. Subsequent experiments (data not shown) determined that the P_{hsp60} *rpsL* construct did not confer streptomycin sensitivity to mc²1255 when present in a single copy in the chromosome. This problem was overcome by cloning *rpsL* under the control of the major P_{left} promoter taken from mycobacteriophage L5 (47), as described above for the construction of pYUB608.

For this study we also characterized the Lys⁻ mutant strain P73. This strain is a nitroguanidine-generated mutant of *M. smegmatis* PM5 (46). The identity of the lesion in P73 resulting in the Lys⁻ phenotype is unknown; however, we believe it to be in the *lysA* gene. We isolated strain mc²1211, an efficient plasmid transformation (*ept*) mutant (61) of P73 and found that it could be complemented to Lys⁺ by pYUB628, a multicopy plasmid containing the *M. tuberculosis* *lysA* gene driven by the *hsp60* promoter (data not shown). Strain mc²1212, a spontaneous, high-level-Sm^r mutant of mc²1211 was isolated and used for this work. Introduction of the wild-type *rpsL* test plasmid pYUB600 into mc²1212 rendered the strain sensitive to streptomycin, showing that the Sm^r mutation of the strain was in the chromosomal *rpsL* gene.

Genetic nomenclature. When a suicide plasmid has integrated into the chromosome via homologous recombination between the chromosome and the DNA cloned in the plasmid, a direct-order duplication of the cloned genes results in the duplicated regions joined by the vector sequences of the plasmid. To describe such strains in an clear and unambiguous fashion, we have adopted the nomenclature used to describe chromosomal rearrangements in *Salmonella typhimurium* (29, 57). We use the designation *DUP* with a chromosome rearrangement number, followed by the genes involved in the duplication linked by the vector backbone of the suicide plasmid. For example, the integration of pYUB609 (containing the *ask1::aph asd* region cloned in a pYUB608 vector) into the *ask* region of the chromosome by a single crossover event downstream of *ask* is described as *DUP1* [(ORF_x *ask asd* ORF_y)*pYUB608*(ORF_x *ask1::aph asd* ORF_y). This designation clearly indicates the relative location of the mutant allele of interest and shows the type of vector used for construction of the strain.

Electroporation of *M. smegmatis*. Plasmid DNA was introduced into *M. smegmatis* by electroporation as previously described (17) except that the cells were grown in LBT and chilled in ice for 5 to 15 min prior to being washed. Freshly prepared cells were always used for recombination experiments, LBT supplemented with DAP was added to the cells following electroporation, and the mixtures were incubated for 4 h prior to being plated.

Southern hybridization analysis. Genomic DNA was prepared from *M. smegmatis* strains as previously described (31) except that glycine was added to the cultures at a final concentration of 1% (wt/vol) 3 h prior to harvest (5). Southern blotting was done by the alkali-denaturing procedure (39), and DNA was transferred to Biotrans nylon membranes (ICN, Irvine, Calif.) by the capillary method. Hybridization and detection were done with a chemiluminescent detection system (ECL, Amersham, United Kingdom) as recommended by the manufacturer, under high-stringency conditions for prehybridization and hybridization (0.1 M NaCl and 42°C). Washes were done at 42°C with primary wash buffer containing 6 M urea and 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RESULTS

Essentiality of *ask* in *M. smegmatis*. The pathway responsible for the biosynthesis of methionine, threonine, isoleucine, DAP, and lysine in bacteria is depicted in Fig. 1. In mycobacteria the first enzyme in this pathway is encoded by the *ask* gene (18). Earlier attempts at allelic exchange of the wild-type chromosomal *ask* gene with a disrupted *ask* allele in *M. smegmatis* to obtain mutants auxotrophic for Met, Thr, and DAP were unsuccessful, suggesting that disruption of *ask* is lethal to this organism even when the products of the aspartate pathway are present in the culture medium (16). Since DNA recombination in mycobacteria is poorly understood, and the failure to obtain a gene disruption is not an absolute measure of the essentiality of that gene, we sought to rigorously determine if *ask* is indeed essential. A counterselectable marker system was developed to facilitate allelic exchange in mycobacteria and used in a test of gene essentiality.

Our counterselectable marker system is based on the well-known phenomenon that streptomycin resistance mediated by mutations in the *rpsL* gene (encoding the S12 ribosomal protein subunit) is recessive to the wild-type *rpsL* gene (36). Counterselection schemes for allelic exchange utilizing *rpsL* have been successfully demonstrated for a variety of bacteria, including mycobacteria (26, 54, 55, 60). The counterselection system for allelic exchange described here used a strain with a chromosomal mutation in *rpsL* conferring streptomycin resistance (mc²1255) and the wild-type *rpsL* gene cloned in a suicide vector unable to replicate in mycobacteria (pYUB608). To construct the plasmid for allelic exchange of *ask*, a 6.2-kb DNA fragment containing the *ask* gene disrupted with a kanamycin resistance marker (*ask1::aph*) was cloned into the *rpsL* suicide vector pYUB608, yielding pYUB609. The 6.2-kb DNA fragment was derived from pYUB205, the same plasmid used in previous attempts to disrupt *ask* (16). In the earlier work, pYUB205 was believed to harbor a deletion of *asd* with the *aph* cassette replacing the deleted region (16). However, examination of pYUB205 in this study revealed instead that the *aph* cassette was inserted into a *Pst*I site within *ask*, in the opposite orientation with respect to the direction of *ask asd* transcription.

In initial experiments with pYUB609 and mc²1255 (Table 3), we were unable to isolate any Km^rSm^r *ask1::aph* auxotrophs, either by isolation of Km^r recombinants followed by screening

rpsL4 DUP1 [(ORF_x *ask asd* ORF_y)*pYUB608*(ORF_x *ask1::aph asd* ORF_y)] Km^r Sm^s

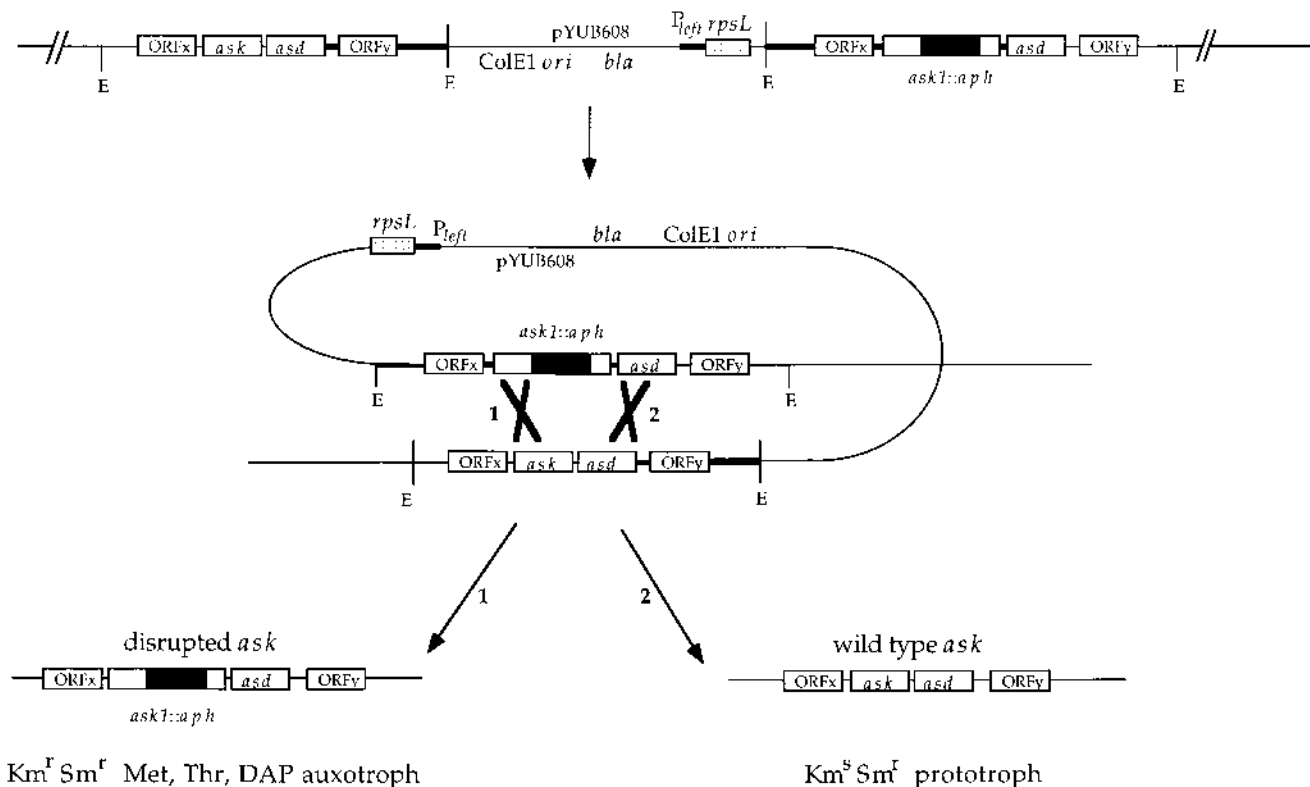


FIG. 2. Segregation of *DUP1* and recombination products. Homologous recombination between the direct-order repeats of *DUP1* will release the intervening DNA sequences, resulting in streptomycin-resistant recombinants. A crossover event upstream of the *ask1::aph* insertion (scenario 1) will result in a recombinant with a disrupted *ask* gene, while a crossover event downstream of the *ask1::aph* insertion (scenario 2) yields a recombinant with a wild-type *ask* gene.

for Km^rSm^r clones or by direct selection for Km^rSm^r recombinants. We then used an *ask asd* merodiploid strain to test whether the inability to disrupt *ask* is due to its essentiality or to the inability to achieve the proper DNA recombinations within the *ask* region of the chromosome. We chose to construct an *ask asd* merodiploid, instead of a strain merodiploid for only *ask*, since *ask* and *asd* are in an operon (18) and the *aph* insertion in the mutant *ask* allele of pYUB609 would likely have a polar effect on *asd* expression. A strain defective for both the *ask* and *asd* genes would be phenotypically indistinguishable from a strain with a mutation in either gene alone. Supplying an extra copy of the wild-type *ask* and *asd* genes elsewhere in the chromosome should allow for the exchange of wild-type *ask* at the normal chromosomal locus with the *ask1::aph* allele. The resultant *ask1::aph* recombinants would not be auxotrophs, but disruption of the gene could be confirmed by Southern analysis. For this experiment we characterized a single crossover recombinant clone from our earlier attempt to disrupt *ask* in mc²1255 using pYUB609 and streptomycin counterselection. This strain, mc²1265, is Sm^sKm^r and has pYUB609 integrated in the *ask asd* region of the chromosome with the rearrangement (*DUP1*) shown in Fig. 2. The insertion and orientation of pYUB609 in mc²1265 were confirmed by Southern analysis (Fig. 3, lane 3). An *ask asd* merodiploid strain was constructed with pYUB412, a vector capable of site-specific integration into the *M. smegmatis* chromosome (6, 38). A 5-kb DNA insert containing *ask asd* (Table 2) was

cloned into pYUB412, producing pYUB643. Strain mc²1268 was constructed by site-specific integration of the *ask asd* carrying plasmid pYUB643 (*hyg*) into the chromosome of strain mc²1265 (*rpsL4 DUP1* Sm^sKm^r). Strain mc²1266 is an isogenic control made by integration of the vector pYUB412 (*hyg*) into the chromosome of strain mc²1265. The different *ask asd* regions have different sizes and are easily distinguished from each other in Southern analysis. The strains mc²1268 and mc²1266 were used in an allelic exchange experiment described below.

The rationale of the essentiality test is as follows. The control strain mc²1266 has pYUB609 integrated into the *ask asd* region of the chromosome, resulting in direct-ordered repeats of the *ask asd* region (*DUP1*) and an Sm^s Km^r phenotype (Fig. 2). At a particular frequency, the repeats in *DUP1* will undergo homologous recombination with each other and the *ask* alleles will segregate (Fig. 2). Recombinants that retain the *ask1::aph* allele will be Sm^r Km^r mutants auxotrophic for Met, Thr, and DAP; recombinants with the wild-type gene will be Sm^rKm^s prototrophs. If *ask* is essential, only the wild-type *ask* Sm^rKm^s recombinants will be obtained. Strain mc²1268, the Sm^sKm^r *ask asd* merodiploid strain, has an extra copy of wild-type *ask* and *asd* located at *attB*; therefore both recombinant types should be seen. The frequency of the two recombinant classes (Sm^rKm^r and Sm^rKm^s) should be similar because the amount of homologous DNA flanking the *ask1::aph* allele in pYUB609 is similar (1.9 kb upstream and 3.0 kb downstream).

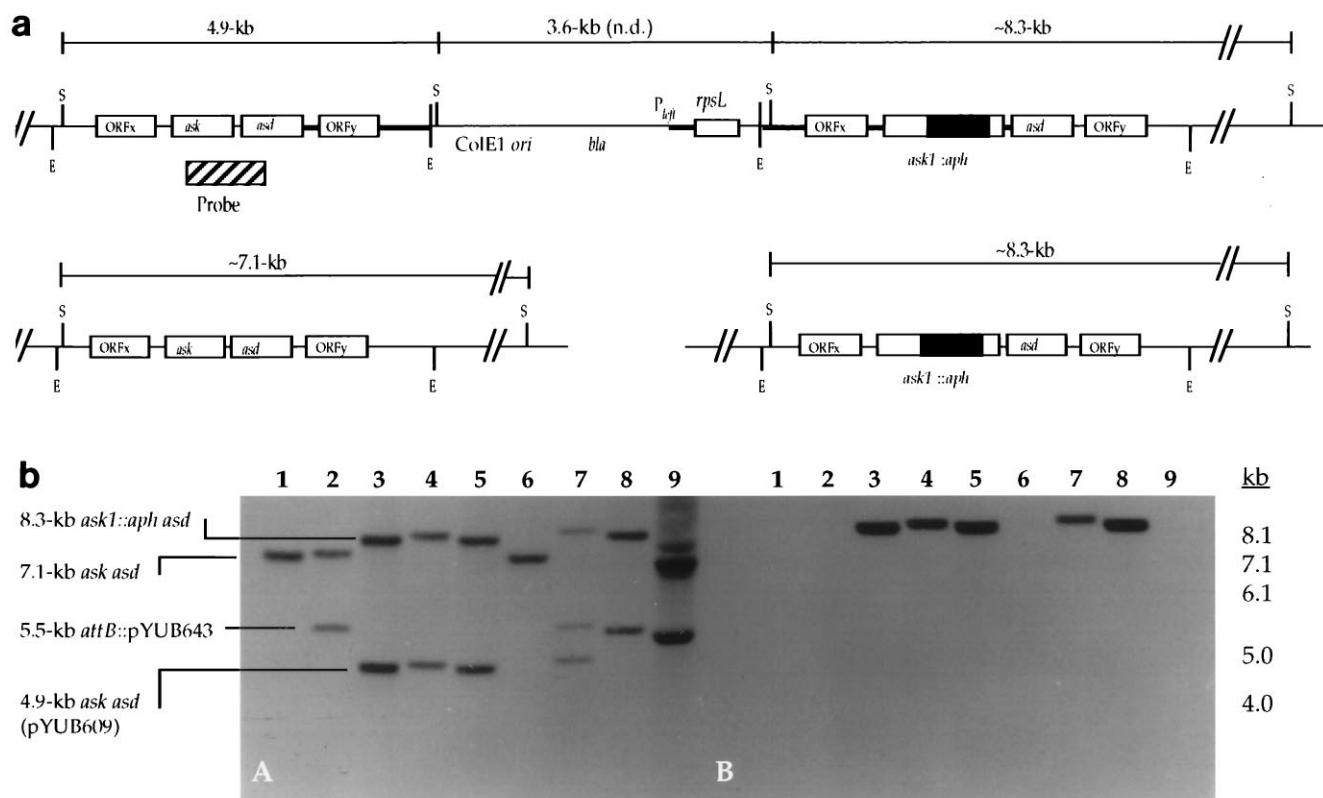


FIG. 3. Southern blot analysis of recombinants from *DUP1* segregation by *ask asd* merodiploid test. (a) Maps of pYUB609 integrated into *M. smegmatis* mc²1255 chromosome (*DUP1* [(ORFx *ask asd* ORFy)*pYUB608*(ORFx *ask1::aph asd* ORFy)] and recombination products resulting from *DUP1* segregation. E, *EcoRI*; S, *SacI* restriction endonuclease sites. (b, blot A) Southern blots of genomic DNAs from various *M. smegmatis* recombinants and parental strains, digested with *SacI*, and probed with a 919-bp *NcoI* DNA fragment (indicated by the hatched box in panel a). (b, blot B) The DNA digests shown in panel A were blotted and probed with the 1.2-kb *PstI aph* cassette from pYUB609. Note that the central 3.6-kb *SacI* fragment of the integrated pYUB609 is not detected (labeled n.d. in panel a) by these probes. The positions and identities of the fragments representing each copy of the *ask asd* region is indicated on the left, and the positions of the DNA size markers are indicated on the right (DNA markers, 1-kb ladder [GIBCO/BRL]). Lanes: 1, mc²1255 wild type; 2, mc²1255 *attB::pYUB643* (*ask asd*); 3, mc²1265 (*DUP1*); 4, mc²1266 (*DUP1 attB::pYUB412*); 5, mc²1374, an Sm^rKm^r derivative of mc²1266; 6, mc²1375, an Sm^r Km^s derivative of mc²1266; 7, mc²1268 (*DUP1 attB::pYUB643 ask asd*); 8, mc²1376, an Sm^r Km^r derivative of mc²1268; 9, mc²1377, an Sm^r Km^s derivative of mc²1268. The *SacI* digestion of DNA from mc²1377 was incomplete (lane 9).

To determine the recombination frequencies for this test, three separate cultures of each strain were grown to late logarithmic phase in LBT supplemented with hygromycin and DAP. The cultures were diluted and plated for the number of viable CFU per milliliter in LB-DAP medium either alone or supplemented with streptomycin or streptomycin and kanamycin. The results from these experiments revealed that the average frequency of streptomycin resistance was 10^{-3} for both strains, while the average frequency of Sm^rKm^r was 10^{-4} (Table 4, compare strain mc²1266 with strain mc²1268). We tested the phenotypes of 100 streptomycin-resistant clones from each culture for kanamycin resistance and relevant auxotrophy and also verified that each recombinant remained Sm^r (due to segregation of *DUP1*) and Hyg^r (due to the plasmid integrated at *attB*). As shown in Table 4, the strain lacking an additional copy of *ask* and *asd* at the *attB* locus (mc²1266) formed few Sm^rKm^r recombinants (3% \pm 1%), and none of these Sm^rKm^r recombinants had the expected auxotrophy of an *ask* mutant. The strain containing an addition copy of *ask* and *asd* at *attB* (mc²1268) frequently yielded Sm^rKm^r recombinants (50% \pm 4%), indicating that recombination at the *ask* locus readily occurs. As expected if *ask* is essential, mc²1268 yielded an equivalent distribution between Sm^rKm^r and Sm^rKm^s recombinants while virtually all the clones from the control strain mc²1266 were Sm^rKm^s. None of the directly selected Sm^rKm^r recombinants exhibited auxotrophy (Table 4). These findings

indicate that segregation of *DUP1* can yield an *ask* insertion mutant only if an extra copy of *ask asd* is present at another chromosomal site.

To confirm the genetic organization of the recombinants, Southern analysis was performed on the parental strains and the Sm^r recombinants (Fig. 3). The different *ask asd* regions in the chromosomes of the parent strains can be easily distinguished (Fig. 3a). Fig. 3b, blot A, is a collection of genomic DNAs digested with *SacI* and probed with an *ask asd* containing fragment of DNA. The first three lanes contain DNA from the parental strains and a control strain which is necessary for determining the appearance of each *ask asd* region. The wild-type strain mc²1255 (lane 1) shows a single band of ~7.1-kb corresponding to the wild-type *ask asd* region in the chromosome. Lane 2 is mc²1255 with *ask asd* of pYUB643 integrated at the *attB* site, showing the additional fragment of 5.5-kb, indicative of the *attB* copy. Lane 3 contains DNA from the strain mc²1265, which has the direct-order duplication *DUP1* [(ORFx *ask asd* ORFy)*pYUB608*(ORFx *ask1::asd* ORFy)]. For this strain, mc²1265, a 4.9-kb fragment corresponds to the wild-type *ask asd* copy, while the upper band at ~8.3 kb corresponds to the copy with the *ask1::aph* allele. The vector of plasmid pYUB609 has a *SacI* site near the copy of wild-type *ask asd*; this reduces the size of the *SacI* fragment of the wild-type *ask asd* region from ~7.1 kb to 4.9 kb (Fig. 3a). Lane 4 has a digest of strain mc²1266 (*DUP1 attB::pYUB412*), and

TABLE 4. Recombination products from *DUP1* [(ORF_x *ask asd* ORF_y)*pYUB608*(ORF_x *ask1::aph asd* ORF_y)] segregation

Strain	Relevant genotype	Direct selection for Sm ^r			Direct selection for Sm ^r Km ^r	
		Frequency of Sm ^r recombinants ^a	% of Sm ^r clones that are Km ^r ^b	No. of Sm ^r Km ^r /no. of Sm ^r clones (% of auxotrophy) ^c	Frequency of Sm ^r Km ^r recombinants ^a	No. of auxotrophs/no. of Sm ^r Km ^r clones ^d
mc ² 1266	<i>rpsL4 DUP1 attB::pYUB412</i>	4.0 ± 4.5	3.0 ± 1.0	9/300 (0)	0.12 ± 0.15	0/82
mc ² 1268	<i>rpsL4 DUP1 attB::pYUB643 (ask asd)</i>	1.0 ± 0.5	50 ± 4.0	152/300 (0)	0.58 ± 0.38	0/90
mc ² 1269	<i>lysA rpsL5 DUP1</i>	6.8 ± 7.4	13 ± 3.0	20/150 (95)	3.6 ± 2.6	70/75
mc ² 1276	<i>lysA⁺ rpsL5 DUP1</i>	7.0 ± 6.9	0.4 ± 0.7	1/275 (0)	0.34 ± 0.18	0/90
mc ² 1385	<i>lysA rpsL5 DUP1 attB::pYUB412</i>	12 ± 15	25 ± 1.4	25/100 (76)	0.10 ± 0.07	18/20
mc ² 1386	<i>lysA rpsL5 DUP1 attB::pYUB651 (lysA⁺)</i>	1.7 ± 1.9	14 ± 1.4	28/200 (0)	0.04 ± 0.01	0/20
mc ² 1387	<i>lysA⁺ rpsL5 DUP1 attB::pYUB412</i>	1.3 ± 0.8	3.0 ± 0	4/150 (0)	0.07 ± 0.06	0/10
mc ² 1388	<i>lysA⁺ rpsL5 DUP1 attB::pYUB643 (ask asd)</i>	1.4 ± 1.6	63 ± 20	101/150 (0)	1.9 ± 0.5	0/99

^a The frequency of Sm^r or Sm^r Km^r recombinants was calculated by dividing the CFU per milliliter obtained in LB DAP Sm or LB DAP Sm Km medium by the total viable CFU per milliliter obtained in LB DAP medium from two or three independent experiments (see the text). The averaged frequencies are reported above as $n(10^{-3}) \pm$ the standard deviation.

^b Averaged percentage of the directly selected Sm^r recombinants from multiple experiments that are also Km^r \pm the standard deviation.

^c Number of Sm^r Km^r recombinants obtained compared with the number of Sm^r recombinants screened, combined results for all experiments. The percentage of the Sm^r Km^r recombinants that are auxotrophic for Met, Thr, and DAP is given in parentheses.

^d Number of clones auxotrophic for Met, Thr, and DAP from the directly selected Sm^r Km^r recombinants, combined results for all experiments.

lane 7 has a digest of mc²1268, (*DUP1 attB::pYUB643 ask asd*), the *ask asd* merodiploid strain. The three different *ask asd* regions are clearly distinguishable from each other in lane 7. Lanes 5 and 6 represent recombinants derived from strain mc²1266 (*DUP1 attB::pYUB412*). Lane 5 shows that mc²1374, an Sm^rKm^r clone, has the same pattern as its parental strain, mc²1266 (lane 4), and therefore did not segregate *DUP1*. This recombinant may have been formed by a gene conversion event between the two *rpsL* genes resulting in two Sm^r alleles. A similar phenomenon of background streptomycin resistance was noted by other investigators utilizing this type of counter-selection system in *E. coli* (54). In contrast, lane 6 shows that mc²1375 is an Sm^rKm^r recombinant derived from mc²1266 (*DUP1 attB::pYUB412*) formed by segregation of *DUP1* and retention of the wild-type *ask* gene, as expected. A comparison of lanes 6 and 1 shows that recombinant mc²1375 has only the ~7.1-kb fragment that corresponds to the wild-type copy of *ask asd*. Strain mc²1268 (*DUP1 attB::pYUB643 ask asd*) should form two classes of Sm^r recombinants, depending on the *ask* allele that is retained after *DUP1* segregation. The Sm^rKm^r recombinants retain the *ask1::aph* allele, whereas the Sm^rKm^r recombinants retain the wild-type allele. Both recombinant classes have pYUB643 (*ask asd*) at *attB*. Lane 8 is mc²1376, an Sm^rKm^r derivative of mc²1268 which lacks the wild-type *ask asd*-specific ~7.1-kb fragment but has the ~8.3-kb fragment indicative of *ask1::aph asd*, as well as the copy of *ask asd* at the *attB* site (the 5.5-kb fragment). Lane 9 shows the pattern for mc²1377, an Sm^rKm^r derivative of mc²1268. This strain has the wild-type *ask asd*-specific fragment (~7.1 kb) and the 5.5-kb *ask asd* fragment at the *attB* site. To confirm the location of the *ask1::aph* alleles, the same genomic DNAs were probed with the *aph* gene (Fig. 3b, blot B). As expected, this probe hybridized only to the ~8.3-kb fragment of the Km^r strains (lanes 3, 4, 5, 7, and 8). In addition, these DNAs were probed with pKSII⁺ (the vector of pYUB608). No pKSII⁺-specific hybridization signals were seen with genomic DNA from mc²1375, mc²1376, and mc²1377, confirming that these recombinants had undergone *DUP1* segregation and lost the pYUB609 vector sequences (data not shown).

Disruption of *ask* can be obtained in a lysine auxotroph of *M. smegmatis*. There is no barrier to mutation of *ask* or *asd* (or

their homologs) in organisms such as *E. coli* and *S. typhimurium* (25, 68). Mutations within these genes results in strains auxotrophic for Met, Thr, and DAP. Mutants of *M. smegmatis* which are auxotrophic for Met, Thr, Ile, and Lys exist (41); therefore we focused on DAP as the essential metabolite of this pathway. Other workers have reported an inability to obtain DAP auxotrophs of the related bacterium *Corynebacterium glutamicum* (19). The explanation given for this phenomenon was that *C. glutamicum* is incapable of transporting DAP from the medium (19). An absolute inability to transport DAP would not appear to be the reason for the presumed essentiality of DAP synthesis in *M. smegmatis*, as mc²155 grows well in glucose-salts minimal medium with DAP as the sole nitrogen source (data not shown). Therefore, we sought another reason to explain why *ask* disruption and subsequent DAP auxotrophy would be lethal to *M. smegmatis*. Since *meso*-DAP is used for both lysine synthesis and peptidoglycan synthesis, we theorized that *ask* disruption is lethal to *M. smegmatis* because the extracellular *meso*-DAP taken up from the medium is converted to lysine, because of insufficient repression of *lysA*, the gene encoding DAP-decarboxylase (Fig. 1). This hypothesis is supported by reports that expression of *lysA* is constitutive in the corynebacteria, close relatives of the mycobacteria (40, 49). Wild-type mycobacterial cells may have a large intracellular DAP pool derived from endogenous synthesis, and the fraction is converted to lysine by a low but constitutive level of DAP-decarboxylase activity too small to affect peptidoglycan synthesis and cell integrity. However, a mutant with a newly disrupted *ask* gene, now dependent on exogenous DAP, may not be able to transport enough DAP to achieve an intracellular DAP pool as large as that in wild-type cells; thus, the basal level DAP-decarboxylase activity may divert a significant fraction of the pool away from peptidoglycan synthesis, leading to death of the cell.

We predict that an *M. smegmatis* Lys⁻ auxotroph unable to convert *meso*-DAP to lysine would accumulate a large intracellular pool of *meso*-DAP reserved for peptidoglycan synthesis and be permissive for *ask* disruption. To test this hypothesis we compared the ability to disrupt *ask* in strain mc²1212 (*lysA rpsL5*) and strain mc²1270 (*lysA⁺ rpsL5*). The latter strain is an isogenic, spontaneous Lys⁺ derivative of mc²1212. We at-

tempted allelic exchange of *ask* in these strains by two methods: either by direct selection of Km^r recombinants followed by identification of $Km^r Sm^r$ auxotrophs or by direct selection of $Km^r Sm^r$ recombinants and screening for auxotrophy. The two strains were electroporated with pYUB609 in duplicate with one transformation mixture plated onto LB-DAP-kanamycin medium and the other plated onto LB-DAP-kanamycin-streptomycin medium. As shown in Table 3, both strains yielded kanamycin-resistant recombinants at similar frequencies after direct selection on kanamycin medium. The number of Km^r clones that were also Sm^r was higher for the Lys^+ strain mc^21270 than for the Lys^- strain mc^21212 . However, only the Lys^- strain mc^21212 yielded mutants auxotrophic for Met, Thr, and DAP (Table 3). Likewise, direct selection for $Km^r Sm^r$ yielded similar frequencies for recombinants from both strains, but *ask* disruption was seen only in the Lys^- strain (Table 3).

To confirm these results, we compared the ability to effect *ask* disruption in isogenic Lys^+ and Lys^- strains which had integrated pYUB609 (ORF*x* *ask1::aph asd* ORF*y*) into the *ask* region of the chromosome. For these experiments, we isolated and examined a $Km^r Sm^r$ pYUB609 plasmid-chromosome recombinant of mc^21212 (Lys^-) and mc^21270 (Lys^+) from the experiment for which the results are shown in Table 3. Each recombinant has pYUB609 integrated in the same fashion as that previously described for the *ask asd* chromosomal rearrangement *DUP1* [(ORF*x* *ask asd* ORF*y*)*pYUB608*(ORF*x* *ask1::aph asd* ORF*y*)] (Fig. 2). The Lys^- strain with integrated pYUB609 is mc^21269 (*lysA rpsL5 DUP1*), while the Lys^+ strain with an integrated pYUB609 is mc^21276 (*lysA⁺ rpsL5 DUP1*). We tested to see whether the *ask1::aph* insertion could be retained in both strains following segregation of *DUP1*. Duplicate cultures of strain mc^21269 (*lysA rpsL5 DUP1*) and strain mc^21276 (*lysA⁺ rpsL5 DUP1*) were grown in LBT supplemented with DAP and plated out for viable CFU per milliliter, and colonies were tested for antibiotic resistance and auxotrophic phenotype (Table 4). As expected, the Lys^- strain mc^21269 yielded $Sm^r Km^r$ auxotrophic *ask* mutants by either direct selection for Sm^r recombinants or $Sm^r Km^r$ recombinants (Table 4). The Lys^+ strain formed Sm^r recombinants at a frequency comparable to that for the isogenic Lys^- mutant, but the frequency of $Sm^r Km^r$ recombinants was slightly less. None of the Lys^+ recombinants tested had an auxotrophic phenotype (Table 4).

To further examine the Lys^+ phenotype as a barrier to *ask* disruption, we constructed a derivative of mc^21212 that is Lys^+ due to the presence of a wild-type copy of *lysA*. The plasmid pYUB651 was constructed from the integrating vector pYUB412 and harbors the *lysA* gene of *M. tuberculosis* under control of the *hsp60* promoter. This plasmid was electroporated into strain mc^21269 (*lysA rpsL5 DUP1*), resulting in strain mc^21386 , which is phenotypically Lys^+ . Strain mc^21269 (*lysA rpsL5 DUP1*) containing pYUB412 served as the Lys^- control strain, mc^21385 . We examined the recombinants obtained from these two strains after *DUP1* segregation in duplicate experiments done in a manner similar to that for previous experiments (Table 4). We obtained $Km^r Sm^r$ *ask* auxotrophs only from the Lys^- control strain, mc^21385 (*lysA rpsL5 DUP1 attB::pYUB412*). No auxotrophs were obtained from experiments using the phenotypically Lys^+ strain mc^21386 (*lysA rpsL5 DUP1 attB::pYUB651*). The $Sm^r Km^r$ derivatives from both strains were patched onto defined medium (Middlebrook 7H9) containing hygromycin and the appropriate supplements with or without lysine to confirm the *Lys* phenotype of each clone. All clones were hygromycin resistant and maintained the *Lys* phenotype of their respective parental strain (data not shown).

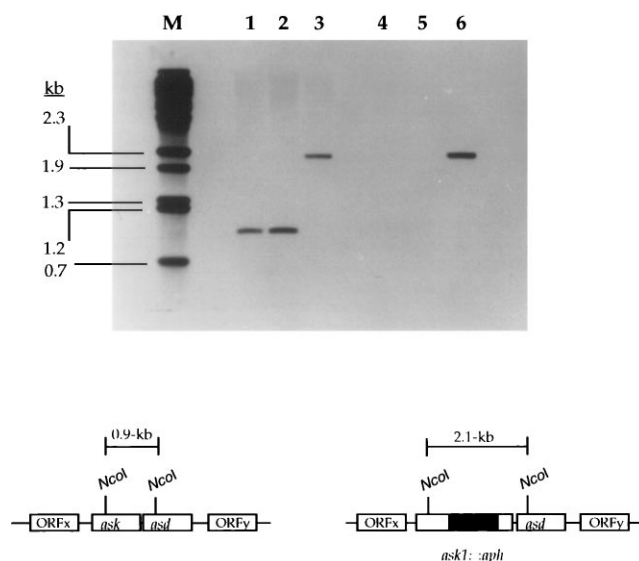


FIG. 4. Southern blot of *M. smegmatis* *ask1::aph* mutant mc^21278 . Genomic DNA (diagrammed below the blots) was digested with *NcoI* and probed with a 919-bp *NcoI* DNA fragment spanning the *aph* insertion point in *ask* (lanes 1 to 3) or with the 1.2-kb *PstI* *aph* cassette from pYUB609 (lanes 4 to 6). Lanes: M, λ -*Bst*EII kilobase markers (New England Biolabs, Beverly, Mass.). 1 and 4, mc^21255 *ask*; 2 and 5, mc^21212 *ask*; 3 and 6, mc^21278 *ask1::aph*.

To determine if *ask* could be physically disrupted in strain mc^21270 , the isogenic Lys^+ revertant of mc^21212 , we analyzed the products from *DUP1* segregation in strain mc^21276 (*lysA⁺ rpsL5 DUP1*), with or without an extra copy of *ask asd*. The experiment was essentially the same as that for the mc^21255 merodiploid test. Duplicate cultures of strain mc^21387 (*lysA⁺ rpsL5 DUP1 attB::pYUB412*) and strain mc^21388 (*lysA⁺ rpsL5 DUP1 attB::pYUB643 ask asd*) were grown to late logarithmic phase in LBT-DAP supplemented with hygromycin, plated onto various selective media, and screened for antibiotic resistance and auxotrophy. The results are shown in Table 4. From mc^21387 (*lysA⁺ rpsL5 DUP1 attB::pYUB412*) we obtained only 3% $Sm^r Km^r$ clones (none of which was an auxotroph) from the directly selected Sm^r clones. None of the directly selected $Sm^r Km^r$ recombinants was auxotrophic. However, from strain mc^21388 (*lysA⁺ rpsL5 DUP1 attB::pYUB643 ask asd*), directly selected Sm^r recombinants yielded $Sm^r Km^r$ clones at a frequency of $63\% \pm 20\%$ (Table 4). As for the wild-type strain mc^21255 , the Lys^+ revertant of mc^21212 appears permissive for *ask* disruption only when provided with an extra copy of *ask* and *asd*.

Characterization of *ask* mutant mc^21278 , a DAP auxotroph. One *ask1::aph* mutant obtained by direct selection for $Km^r Sm^r$ recombinants following electroporation of pYUB609 into mc^21212 (Table 3) was chosen for further study and designated mc^21278 . This strain is auxotrophic for Met, Thr, DAP, and *Lys* as expected for an *ask* mutation in this particular background. To confirm the allelic exchange of *ask*, mc^21278 was analyzed by Southern hybridization. Figure 4, lanes 1 to 3, shows genomic DNA prepared from mc^21255 , mc^21212 , and the *ask* mutant mc^21278 , digested with *NcoI* and probed with a 0.9-kb *NcoI* fragment spanning *ask* and *asd*. The fragment containing the wild-type gene is 0.9 kb in size, while the fragment bearing the *ask1::aph* allele is 2.1 kb. mc^21278 clearly shows the expected shift in size (+1.2 kb) for the disrupted *ask1::aph* allele (Fig. 4, lane 3). To confirm the identity of this fragment, the same *NcoI*-digested DNA samples were probed

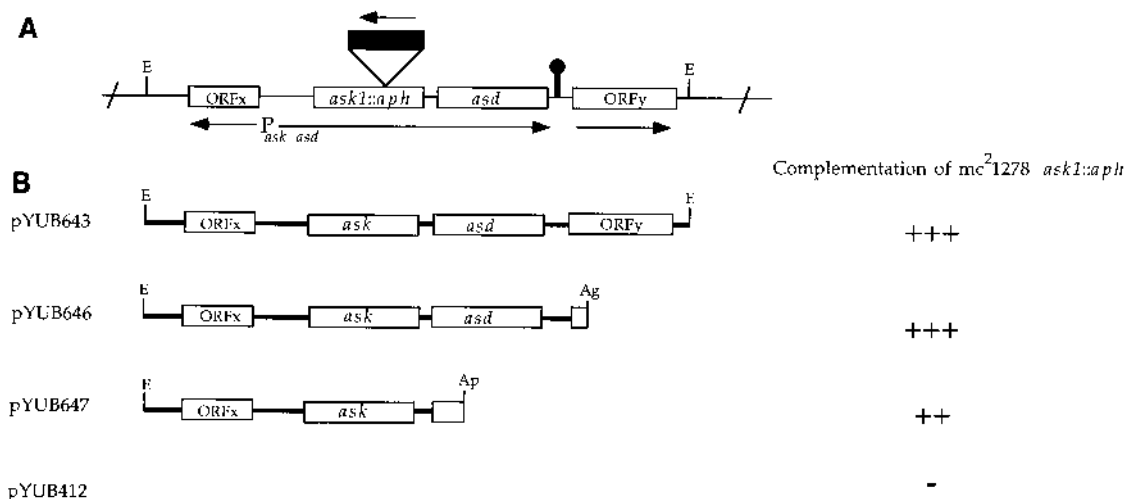


FIG. 5. Complementation tests of *mc²1278*. (A) Map of *ask1::aph asd* region of the *M. smegmatis mc²1278* chromosome. The direction of transcription of ORF_x, *ask asd*, and ORF_y is shown along with the *ask asd* promoter (*P_{ask asd}*). The *aph* cassette inserted into *ask* is indicated, with the direction of *aph* transcription opposite that of the *ask asd* operon. The putative terminator downstream of *asd* is also shown. (B) Maps of the various plasmid subclones in pYUB412, with results of the complementation test indicated on the right. The mutant was electroporated with the plasmids and transformants selected in LB-DAP medium containing kanamycin and hygromycin. The transformants were then patched into LB containing kanamycin and hygromycin medium with or without DAP and scored for the ability to grow in the absence of DAP. +++, abundant growth; ++, less abundant growth; -, no growth. E, *EcoRI*; Ag, *AgeI*; Ap, *ApaLI*.

with the *aph* cassette (Fig. 4, lanes 4 to 6). This probe hybridized only to the 2.1-kb fragment of *mc²1278* (Fig. 4, lane 6).

The transcriptional start site for the *ask* and *asd* genes of *M. smegmatis* has been mapped to a position 254 nucleotides upstream of the start codon of *ask* (18). There is a 22-bp inverted repeat downstream of *asd*, suggesting that the two genes constitute an operon. This operon structure is similar to that seen for the *ask asd* homologs of *C. glutamicum* and *Corynebacterium flavum* (23, 33). In *M. smegmatis*, there is a divergently transcribed ORF_x upstream of *ask*, while downstream of *asd* beyond the putative terminator and transcribed in the opposite direction is ORF_y (18). The functions of these two open reading frames are unknown. In *mc²1278*, the *aph* insertion in *ask* is oriented such that the direction of transcription of *aph* is opposite that of the *ask asd* operon. As mentioned earlier, this insertion may be polar after *asd* expression, making the *mc²1278* strain a double mutant; therefore, a complementation analysis of *mc²1278* was performed to determine the effect of the *aph* insertion on the operon (Fig. 5). As expected, the plasmid pYUB643 containing the full-length *EcoRI* fragment (ORF_x *ask asd* ORF_y) and plasmid pYUB646 (containing ORF_x *ask asd*) complement *mc²1278*. Interestingly, pYUB647, which contains only ORF_x and *ask*, also complemented the *ask1::aph* mutation. Although the growth of *mc²1278* with pYUB647 in medium lacking DAP was substantial, it was not as robust as that seen with pYUB643 or pYUB646.

DAP-less death of *mc²1278*. Since *meso*-DAP is required for lysine and peptidoglycan synthesis, it has the dual role of being important not only for protein synthesis but also for maintenance of cellular integrity. DAP auxotrophs of *E. coli* undergo DAP-less death within a few generations after DAP deprivation (44, 53). We tested to see if the *ask* mutant *mc²1278* would experience DAP-less death. A mid-exponential-phase culture of *mc²1278* was subcultured into media with or without DAP, and the OD of the cultures was monitored for several hours (Fig. 6). The culture containing DAP continued to grow exponentially while the culture without DAP similarly increased in density for almost 3 h (the approximate generation time of *M. smegmatis* in LBT), at which time the OD₆₀₀ began to plateau

then decrease rapidly. After an additional 3 hours, the OD₆₀₀ of the culture without DAP was below the initial OD value. After overnight incubation, the culture without DAP had cleared, while the culture with DAP had reached saturation. The saturated culture containing DAP was centrifuged, the cell pellet was washed and resuspended and diluted into fresh LBT Km Sm medium with or without DAP, and the OD₆₀₀ was monitored as described above. These cultures did not grow exponentially until approximately 5 h after subculture (Fig. 6).

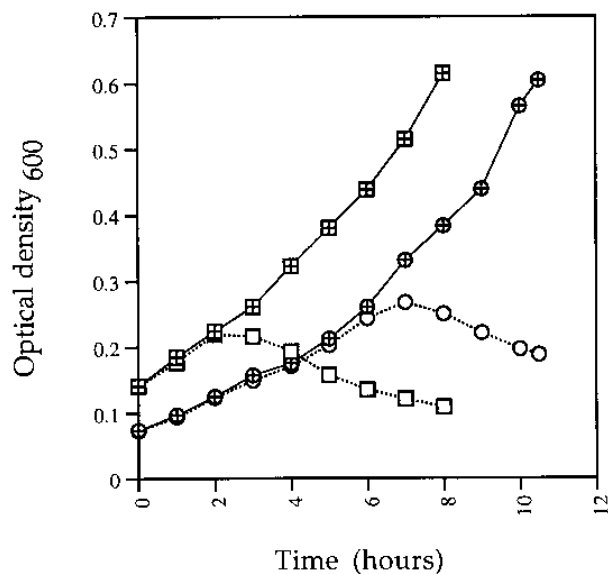


FIG. 6. DAP-less death of *mc²1278*. The *ask* mutant was grown to mid-exponential phase in LBT supplemented with 100 μ g of DAP per ml. The cells were washed and resuspended in fresh LBT with (hatched squares) or without (open squares) DAP. The OD of both cultures was monitored for 8 h. After incubation overnight, cells from the saturated culture containing DAP were washed and inoculated into LBT with (hatched circles) or without (open circles) DAP. The OD of these cultures was monitored for 10 h.

At approximately 3 h into the exponential phase of growth, the density of the culture lacking DAP began to decrease with the same kinetics seen previously, while the density of the culture containing DAP increased (Fig. 6). In a fashion similar to that of *E. coli*, this mycobacterial DAP auxotroph lyses when deprived of DAP. The onset of this phenomenon is most rapid in exponentially growing cultures.

DISCUSSION

We have investigated the essentiality of the biosynthetic pathway for the aspartate family of amino acids in *M. smegmatis*. We are interested in this pathway because one of its products, DAP, is a component of the peptidoglycan, an essential portion of the mycobacterial cell wall. Auxotrophic mutants with lesions in various genes within this pathway have been described for *E. coli* (68), *S. typhimurium* (25), *Shigella flexneri* (59), and *Bacillus megaterium* (24), to name a few. Previous attempts to disrupt *ask*, the first gene in this pathway, in *M. smegmatis* were unsuccessful (16). In the previous work, a suicide plasmid containing the *ask1::aph asd* fragment and a *lacZ* reporter gene was used for allelic exchange of *ask* in the *M. smegmatis* chromosome (16). Integration of this construct into the *ask* region via a single, homologous recombination event yielded a kanamycin-resistant prototrophic clone that was blue when plated in indicator medium. In that experiment, an *ask* mutant resulting from a secondary recombination event that removed the plasmid sequences (along with the *lacZ* gene) and left the *ask1::aph* allele in the chromosome could be identified by screening for kanamycin-resistant clones that were white in the indicator medium. Kanamycin-resistant, white clones arose at a frequency of $\sim 10^{-5}$ but were determined to be prototrophs (16). None of the clones had lost the integrated suicide plasmid. Instead, each clone had a novel mobile DNA element (*IS1096*) inserted into the *lacZ* gene (16).

The failure to obtain any *ask* mutants in the earlier work was suggestive, but not proof, that the gene was essential for *M. smegmatis* growing in fully supplemented medium. We took a similar approach for this determination, except that we used a counterselectable marker system (streptomycin resistance) instead of a counterscreenable marker system (β -galactosidase activity) and combined it with *ask* merodiploidy in a test of gene essentiality. The strength of the system lies in the ability to select against clones that retain the suicide vector sequences and selecting for those clones that have achieved allelic exchange or retained the wild-type allele. The ability to obtain all possible recombinants allows for the analysis of the distribution of phenotypes within the recombinant population. Streptomycin counterselection has been successfully used for allelic exchange in several species of bacteria, including *E. coli* (54), *Pseudomonas aeruginosa* (26), *Yersinia pestis* (60), and more recently, *M. smegmatis* (55). We developed our mycobacterial *rpsL* selection system concurrently with Sander et al. (55). The two systems differ in that we used the *rpsL* gene of *M. smegmatis* driven by a heterologous promoter, while the other workers used the *rpsL* gene of *Mycobacterium bovis* BCG under control its own promoter (55).

We have shown that the *ask* gene of *M. smegmatis* cannot be disrupted unless the strain is merodiploid for *ask* and *asd*. On the basis of the fact that *M. smegmatis* mutants auxotrophic for all the other amino acids of the aspartate family exist, we conclude that DAP must be the limiting metabolite preventing survival of an *ask* mutant. We believe that the essentiality of DAP synthesis results from the fact that DAP has the dual distinction of being both a component of the peptidoglycan and the direct precursor to lysine. We propose that transcrip-

tion of the *lysA* gene, encoding *meso*-DAP-decarboxylase, is not sufficiently repressed when the organism is growing in rich medium. In addition, we propose that the intracellular DAP pool derived from exogenous DAP may be less than that which is normally obtained from endogenous synthesis in wild-type cells. The effect of basal level DAP-decarboxylase activity on a large DAP pool present in wild-type cells would be small compared with the effect on a smaller pool present in a DAP auxotroph. Therefore, in the early development of an *ask* mutant, a significant fraction of the limited amount of DAP taken up into the cell is converted to lysine, resulting in an insufficient amount of DAP to support peptidoglycan synthesis.

In *E. coli*, an organism permissible for DAP auxotrophy, expression of the *lysA* gene is tightly repressed when the cells are growing in medium containing lysine (67). Regulation of *lysA* in *E. coli* is complex, with expression of the gene dependent on LysR, a transcriptional activator that is inactive in the presence of lysine (66, 67). Nothing is known about *lysA* regulation in mycobacteria. However, there are observations which support the view that the *lysA* gene of mycobacteria is constitutively expressed. The *lysA* gene is constitutively expressed in *C. glutamicum* (40), and in the related organism *Brevibacterium lactofermentum*, *lysA* transcription is only weakly repressed by lysine (49). In both organisms, the *lysA* gene is the second gene in an operon with *argS*, a gene encoding arginyl-tRNA synthetase (40, 49, 58). It is believed that the lack of complete repression of this operon is due to an absolute requirement for the *argS* gene product (49). A similar arrangement is seen for the *argS* and *lysA* genes of *M. tuberculosis* and *M. leprae* (1, 58, 64). We do not yet know the sequence of the *lysA* operon of *M. smegmatis*, but it is likely to be similar to those of the other operons. Furthermore, considering the evolutionary relationship between species of *Corynebacterium*, *Brevibacterium*, and *Mycobacterium*, it is likely that the expression of the *lysA* gene of mycobacteria is weakly, or not at all, repressed by lysine. If mycobacteria do not fully repress synthesis of DAP-decarboxylase and also cannot maintain a sufficient pool of intracellular DAP from exogenous DAP, it follows that obtaining a DAP auxotroph of *M. smegmatis* would require the use of a mutant without a functional *lysA* gene. This would allow the cell to utilize whatever amounts of DAP it can take up from the growth medium. In this view, an alternative method to achieve the same ends would be to use a mycobacterial mutant that transports DAP at a higher rate than do wild-type cells. Clearly, additional information is needed concerning the uptake and metabolism of DAP by the mycobacteria in order to obtain a better understanding of this phenomenon.

We believe that the Lys⁻ phenotype of mc²1212 was responsible for our ability to disrupt *ask* in this strain, since a spontaneous Lys⁺ revertant of mc²1212 and a derivative of mc²1212 that is functionally Lys⁺ because of the presence of a wild-type copy of the *M. tuberculosis lysA* gene were both nonpermissive for *ask* disruption. In our experiment utilizing the *M. tuberculosis lysA* gene for complementation of the Lys⁻ phenotype of mc²1212, the gene was under the control of the *hsp60* promoter and therefore was not subjected to normal *lysA* transcriptional regulation, if any. The purpose of that experiment was to rule out any differences between the two strains of *M. smegmatis* used in this study. However, demonstration that *lysA* expression from the constitutive *hsp60* promoter does not permit disruption of *ask* in that experiment is consistent with the view that constitutive expression of *lysA* could be the barrier to *ask* disruption in wild-type *M. smegmatis*. We are currently investigating the regulation of the *lysA* gene in mycobacteria in order to understand the role of this gene in DAP essentiality.

The *ask* mutant mc²1278 exhibited auxotrophy for Met, Thr, DAP, and Lys, as expected. The *aph* cassette insertion in *ask* does not appear to have a very strong polar effect on *ask* expression in mc²1278. The *aph* cassette is facing in the opposite direction with regard to the *asd* gene, and therefore the *aph* promoter cannot be driving *asd* transcription. The fact that the wild-type *ask* gene alone can complement the *ask1::aph* mutation in mc²1278 does not disprove the contention that the two genes are in an operon. The observation that pYUB647 (ORF_x *ask*) does not fully complement mc²1278 for wild-type levels of growth in medium lacking DAP may indicate that a weak promoter sequence upstream of *asd*, usually inactive in wild-type strains, may be driving expression of the gene in the mutant.

The conclusion that mc²1278 is defective for only *ask* and not for both *ask* and *asd* has an intriguing implication. There is widespread variability in the number and kinds of aspartokinase enzymes and genes seen in different bacteria. A single organism can produce one, two, or even three different aspartokinases. For example, *E. coli* has three genes encoding three different aspartokinases (50), and an aspartokinase null mutant of *E. coli* must have mutations in all three genes (68). Likewise, *Bacillus subtilis* has three aspartokinase isoenzymes, all of which appear to be produced from different genes (71). Other organisms, such as *Streptococcus bovis* (32) and *B. megaterium* (15), have two aspartokinase isoenzymes each, while organisms such as *C. glutamicum* (33) and *C. flavum* (23) appear to have only one aspartokinase. It has been demonstrated that the total aspartokinase activity from *M. smegmatis* sonicates can be differentiated into the activities of three different aspartokinase isoenzymes (63). These three isoenzymes can be distinguished from each other on the basis of the inhibition of their activity by different amino acids (63). Furthermore, the expression of these isoenzymes activities is modulated by the amino acid composition of the growth medium (63). The genetic data presented here reveal that there is but a single aspartokinase gene in *M. smegmatis*. Taken together with the biochemical data, these experiments suggest that in *M. smegmatis*, a single gene is responsible for the expression of three differentially regulated aspartokinase isoenzymes. The possibility of this unusual phenomenon will be investigated by further genetic and enzymological study of the *ask* mutant.

The gene encoding aspartokinase II of *B. subtilis* (9, 10) and the aspartokinase genes of *C. glutamicum* (34) and *C. flavum* (23) have been shown to possess an unusual character. Each gene gives rise to two proteins which result from translation of two in-phase ORFs within the gene. The larger polypeptide (the α subunit) results from translation of the entire ORF, while the smaller polypeptide (the β subunit) results from translational initiation at a codon within the larger ORF. It is believed that the aspartokinase enzymes from these organisms are composed of an $\alpha_2\beta_2$ heterodimer (10, 23, 34). Although the *M. smegmatis* aspartokinase amino acid sequence shows a high degree of homology with those of the corresponding corynebacterial proteins (18), it has not been conclusively demonstrated that the cloned *ask* gene of *M. smegmatis* directs production of a second polypeptide. However, biochemical data indicate that the aspartokinase isoenzymes of *M. smegmatis* do indeed have a two-subunit organization. In the studies by Sritharan et al. (63), each of the three aspartokinase isoenzyme activities of *M. smegmatis* was separated by gel filtration into two catalytically inactive subunits with sizes of 50 kDa (the α subunit) and 11 kDa (the β subunit). Translation of the full *ask* ORF of *M. smegmatis* gives an α subunit size of 46 kDa, while internal translational initiation at codon 240 (equivalent to the position for aspartokinase β subunit translation in

C. glutamicum) yields a β -subunit size of 18 kDa (18). These sizes are consistent with the reported subunit sizes of the purified aspartokinase isoenzymes of *M. smegmatis*. The *aph* insertion within the *ask* gene of mc²1278 is located downstream of the putative beta subunit translational start point; therefore, we predict that no functional subunits can be produced from this disrupted gene. These observations are consistent with the idea that a single *ask* gene in *M. smegmatis* produces two polypeptides that somehow combine to form three different aspartokinases.

Similar to *E. coli* DAP auxotrophs, mc²1278 underwent DAP-less death after DAP deprivation. Cell lysis occurred after one generation of exponential growth, during the time when cells are actively dividing and thus require continual peptidoglycan synthesis. This unique phenotype makes mc²1278 an extremely useful mutant. Preparation of lysates from mycobacteria for the purification of various macromolecules can be problematic because of the difficulties in breaking apart the cells. DAP auxotrophs of mycobacteria can be easily lysed by depriving the cells of DAP, thus facilitating the reproducible preparation of mycobacterial cell extracts. Furthermore, the observation that mycobacteria will lyse when deprived of DAP suggests that inhibitors of enzymes of the DAP biosynthetic pathway could be effective antimycobacterial agents. With the recent demonstrations of allelic exchange in slowly growing mycobacteria species such as *M. tuberculosis* (5) and *M. bovis* BCG (3, 52), it should be possible to construct similar auxotrophic mutants of these important species. Such mutants will be invaluable in the study of peptidoglycan and cell envelope biosynthesis in these organisms and will provide unique strains to be tested as potential live attenuated vaccines.

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