The common aromatic amino acid biosynthesis pathway is essential in *Mycobacterium tuberculosis*

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Attempts to construct *Mycobacterium tuberculosis* strains with a defect in the common aromatic amino acid biosynthesis pathway were made. In other bacteria the genes of this pathway (*aro*) can be disrupted in the presence of suitable media supplements. The genomic organization of the *aro* genes in *M. tuberculosis* reveals that there is one operon (*aroCKBQ*) and three isolated *aro* genes (*aroE*, *aroG* and *aroA*). The *aroK* gene was chosen as a target for disruption; this encodes shikimate kinase, which catalyses the fifth step in chorismate biosynthesis. Attempts to replace the wild-type *aroK* gene with a disrupted allele (*aroK*Δ::*hyg*) by a two-step homologous recombination procedure were unsuccessful in a wild-type strain. When a second functional copy of *aroK* was integrated into the chromosome, it was possible to isolate a strain carrying the disrupted gene. Excision of the L5-integrated copy of *aroK* by the L5 excisionase could not be achieved in the strain carrying the disrupted copy, but was possible in a strain carrying a wild-type copy. These results demonstrate that the chorismate pathway is essential for the viability of *M. tuberculosis*.

Keywords: chorismate biosynthesis, auxotrophs, gene replacement, bacteriophage L5, excisionase

INTRODUCTION

Tuberculosis (TB) poses a major worldwide public health problem (Dye *et al.*, 1999). The increasing prevalence of TB, the emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis* and the devastating effect of co-infection with HIV have highlighted the urgent need for new strategies and tools to control the disease. The available TB vaccine, the bacille Calmette–Guérin (BCG), is an attenuated strain of the closely related organism *Mycobacterium bovis*. Although widely used, its efficacy has been very variable in clinical trials conducted in different parts of the world (Fine, 2001). A new vaccine and new drugs are urgently needed to combat this devastating disease.

The shikimate biosynthetic pathway, in which 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) is con-

verted to chorismate, is required for synthesis of all aromatic amino acids, as well as other important metabolites in bacteria (Fig. 1; Pittard, 1987). Disruption of genes in this pathway has been successfully employed in a wide range of bacterial species to generate strains that are attenuated in models of infection (Günel-Ozcan *et al.*, 1997; Hoiseth & Stocker, 1981; Ingham *et al.*, 2002; Simmons *et al.*, 1997; Vaughan *et al.*, 1993). Such strains have been used as live attenuated vaccines (Dilts *et al.*, 2000). With this in mind, the *aroA*, *aroB* and *aroQ* genes were among the first *M. tuberculosis* genes to be cloned and sequenced, and proposed as attractive targets for gene disruption in order to generate a rationally attenuated vaccine to replace BCG (Garbe *et al.*, 1990, 1991).

We and others have demonstrated that *M. tuberculosis* strains with defects in amino acid biosynthesis pathways show a range of attenuation and can protect in challenge experiments (Hondalus *et al.*, 2000; Smith *et al.*, 2001). One of the genes we attempted to inactivate was *aroK* (Parish *et al.*, 1999). This gene encodes shikimate kinase which catalyses the fifth step in chorismate biosynthesis, in which shikimic acid is phosphorylated to form

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Abbreviations: BCG, bacille Calmette–Guérin; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate.

Fig. 1. Schematic outline of chorismate biosynthesis in *M. tuberculosis*. Enzyme names are given on the left with the corresponding genes on the right. Since there is some confusion over the naming of *aro* genes, for example *aroC* (chorismate synthase) has been designated as *aroF* in the annotated genome, we have also used the Rv numbering system from Cole *et al.* (1998). Chorismate is used in several biosynthetic pathways, represented by the dotted lines. Components of the *aro* supplement are shown in bold.

Table 1. Strains and plasmids used

Strain or plasmid	Description	Genotype/reference
<i>M. tuberculosis</i> strains		
H37Rv	Laboratory strain (ATCC 25618)	<i>aroK</i> ⁺
Aroma1	Single cross-over strain	<i>aroK</i> ⁺ <i>kan</i> <i>P</i> _{hsp60} - <i>sacB</i> <i>aroKΔ::hyg</i>
Aroma3	Single cross-over strain plus integrating vector	<i>aroK</i> ⁺ <i>kan</i> <i>P</i> _{hsp60} - <i>sacB</i> <i>aroKΔ::hyg</i> [<i>aroK</i> ⁺ int* <i>aacC1</i>]
Aroma5	Double cross-over strain (mutant) plus integrating vector	<i>aroKΔ::hyg</i> [<i>aroK</i> ⁺ int* <i>aacC1</i>]
Aroma6	Wild-type strain plus integrating vector	<i>aroK</i> ⁺ [<i>aroK</i> ⁺ int* <i>aacC1</i>]
Plasmids		
pAINT1	<i>aroK</i> ⁺ in the integrating vector – <i>attP</i> <i>int</i> <i>aacC1</i>	This study
pAROB14	Non-replicating delivery vector with disrupted gene <i>aroKΔ::hyg</i> <i>kan</i> <i>P</i> _{hsp60} - <i>sacB</i>	This study
pMV206	Control replicating vector, <i>kan</i>	Stover <i>et al.</i> (1991)
pJL28	<i>gp71-xis</i> in pMV206, <i>kan</i>	Parish <i>et al.</i> (2001)

* wild-type *aroK*⁺ integrated into the chromosome using the L5-based vector.

shikimate-3-phosphate (Fig. 1). Initial experiments were unsuccessful (Parish *et al.*, 1999) and we demonstrate here that this gene is essential in *M. tuberculosis*.

METHODS

Strains and plasmids. *M. tuberculosis* strains used in this study are shown in Table 1. *M. tuberculosis* was grown in Middlebrook 7H9 liquid containing 10% OADC (Becton Dickinson) and 0.05% w/v Tween 80 or on solid Middlebrook 7H10 agar containing 10% (v/v) OADC. Kanamycin was used at 20 µg ml⁻¹, hygromycin at 100 µg ml⁻¹ and gentamicin at 10 µg ml⁻¹ where appropriate. Aro supplement consisted of 40 µg ml⁻¹ each of L-tryptophan, L-phenylalanine and L-tyrosine and 250 µM each of *p*-hydroxybenzoate, *p*-amino-benzoic acid and 2,3-dihydroxybenzoate.

Bioinformatics. Preliminary sequence data for *Mycobacterium smegmatis* was obtained from The Institute for Genomic Research website at <http://www.tigr.org>. Data used were dated 9 Jan 2002. *M. bovis* and *Streptomyces coelicolor* sequence data were produced by the *M. bovis* and *S. coelicolor* Sequencing Groups at the Sanger Institute and can be obtained from <ftp://ftp.sanger.ac.uk/pub/pathogens/mb/> and ftp://ftp.sanger.ac.uk/pub/S_coelicolor/ respectively.

Construction of vectors. The *aroK*Δ::hyg disrupted allele from plasmid pAROB2 (Parish *et al.*, 1999) was cloned into p2NIL (Parish & Stoker, 2000a), which does not replicate in mycobacteria, to make pAROB11. This allele has a 324 bp *NruI* internal fragment of the gene replaced with the *hyg* gene. The vector carries 0.8 kb of the 3' end of the 1.2 kb *aroC* gene. The marker gene *P_{hsp60}-sacB* from pGOAL13 (Parish & Stoker, 2000a) was then cloned into the unique *PacI* site of pAROB11 to make the final non-replicating delivery vector pAROB14 (Fig. 2b). The wild-type *aroK*⁺ allele (Fig. 2a) was cloned as an *EcoRV* fragment from cosmid Y159 into pUC-Gm-INT (Mahenthiralingam *et al.*, 1998) which carries the L5 integrase (*int*) and attachment site (*attP*) and a gentamicin-resistance gene (*aacC1*), to make the integrating vector pAINT1 (Fig. 2c).

Isolation of single cross-over strain (Aroma1). Approximately 2 µg vector DNA (pAROB14) (Fig. 2b) was pretreated with UV light to stimulate homologous recombination and electroporated into *M. tuberculosis* (Hinds *et al.*, 1999; Parish *et al.*, 1999). Single cross-over strains were selected using hygromycin and kanamycin without aro supplement.

Isolation of double cross-over strains from Aroma1. The single cross-over strain (Aroma1) was streaked out onto media containing aro supplement but without antibiotics and incubated for 2 weeks at 37 °C. A loopful of cells was resuspended in liquid media by vortexing with 1 mm glass beads. Serial dilutions were plated onto 2% (w/v) sucrose plates plus aro supplement (and hygromycin where required). Plates were incubated at 37 °C for 4–6 weeks. Sucrose-resistant colonies were then patch-tested for kanamycin and hygromycin resistance on plates containing aro supplement.

Construction of merodiploid strain (Aroma3) and isolation of double cross-over strains. Aroma1 (single cross-over strain) was electroporated with the integrating vector pAINT1. Transformants carrying the vector were selected on gentamicin, kanamycin and hygromycin without aro supplement. An individual colony was picked for further manipulation (Aroma3). Isolation of double cross-over strains was carried out essentially as for the single cross-over strain. Briefly,

Fig. 2. Plasmid constructs. (a) Arrangement of genes in the *M. tuberculosis* chromosome, showing the regions used in the construction of pAROB14 and pAINT1. Relevant restriction sites are shown. White arrows, *aro* genes; black arrows, non-*aro* genes; grey box shows region replaced in *aroK*. AroP1, AroP2 and AroP3 are PCR primers used for characterizing strains (see Fig. 4). (b) pAROB14 delivery vector used for mutagenesis. The 2.8 kb *SphI* *M. tuberculosis* fragment indicated in (a) was used to construct the delivery vector. The internal 324 bp *NruI* fragment of *aroK* was replaced by the *hyg* gene. (c) pAINT1 vector used to complement mutants. The *EcoRV* fragment indicated in (a) was cloned into the L5-based integrating vector to make pAINT1.

Aroma3 was streaked out onto media containing aro supplement but without antibiotics and incubated for 2 weeks at 37 °C. A loopful of cells was resuspended in liquid media by vortexing with 1 mm glass beads. Serial dilutions were plated onto 2% (w/v) sucrose plates plus aro supplement (and hygromycin where required). Plates were incubated at 37 °C for 4–6 weeks. Sucrose-resistant colonies were then patch-tested for kanamycin and hygromycin resistance on plates containing aro supplement.

Excision of L5-integrated plasmids. Aroma5 and Aroma6 strains were electroporated with 0.5 µg plasmid pJL28 and plated onto kanamycin plus aro supplement to select for cells carrying the *xis* plasmid (Parish *et al.*, 2001). Kanamycin-resistant colonies were then patch-tested for gentamicin resistance to determine whether the integrated vector was still present.

RESULTS

Genetic organization of the *aro* genes

Analysis of the complete genome sequence of *M. tuberculosis* reveals the presence of seven *aro* genes predicted to be involved in the chorismate biosynthetic pathway (Cole *et al.*, 1998) (Fig. 1). Four of these (*aroCKBQ*; *Rv2540c*–*Rv2537c* using the Rv numbering system from Cole *et al.*, 1998) appear to be organized in an operon (Fig. 3) as they are found very close together; *aroC* and *aroK* are separated by three bases whereas *aroK/aroB* and *aroB/aroQ* have overlapping start and stop codons indicating that it is highly likely that they are co-transcribed. *aroE* (*Rv2552c*) is located 10 genes upstream of this operon, possibly in another operon with apparently unrelated genes. The other two *aro* genes are found elsewhere in the chromosome; *aroA*

(*Rv3227*) appears to be in an operon with a gene of unknown function (*Rv3228*) and *aroG* (*Rv2178c*) is probably monocistronic (Fig. 3a).

A comparison with other mycobacteria shows that the location and organization of *aroA* and *aroG* are conserved among the *M. tuberculosis* complex (*M. tuberculosis* and *M. bovis*), *Mycobacterium leprae* (Cole *et al.*, 2001) and *M. smegmatis*, but that the main *aro* operon is arranged differently (Fig. 3b). In *M. leprae*, *aroE* is part of the main operon, with the 10 intervening genes seen in *M. tuberculosis* missing. In *M. smegmatis*, *aroE* is separated from *aroC* by a single gene, encoding a putative membrane protein. However, unlike *M. tuberculosis* and *M. leprae*, *aroQ* is not part of this operon and is located elsewhere on the chromosome (Fig. 3c). The related actinomycete *S. coelicolor* has a similar arrangement to *M. smegmatis*, with *aroQ* being located separately, although it is flanked by different genes (not shown).

There is some confusion over the nomenclature of the *aro* genes. We have designated the chorismate synthase (*Rv2540c*) gene as *aroC* to avoid confusion (it is described as *aroF* in the original Sanger Centre annotation), since it is annotated as *aroC* in other bacteria

Fig. 3. Genetic organization of the *aro* genes in mycobacteria. White arrows, *aro* genes; black arrows, non-*aro* genes. (a) The location and arrangement of the seven *aro* genes in *M. tuberculosis*. (b) Organization of the region between *aroE* and *aroC* in *M. tuberculosis* (Mtb), *M. leprae* (Mlep) and *M. smegmatis* (Msm). The dotted lines indicate missing regions. (c) The location of the *aroQ* gene in *M. smegmatis*. Genes representing homologues of the *M. tuberculosis* genes are indicated in quote marks.

Fig. 4. PCR analysis of strains. (a) Analysis of single and double cross-over strains. PCR was carried out using primers AroP2 and AroP3, which flank the *hyg* insert in *aroK* and should amplify both the wild-type locus (0.5 kb) and the disrupted allele *aroK::hyg* (1.7 kb). However, they will not amplify the region in pAINT1 carrying the integrated copy, as AroP3 lies outside the region used for complementation. Lanes: 1, H37Rv; 2, Aroma5; 3, Aroma6; 4, Aroma3; 5, Aroma1. (b) Mapping of single cross overs (SCO). PCR was carried out using primers AroP1 and AroP3. AroP1 lies in *aroC* outside the insert used in the mutagenesis, while AroP3 lies in *aroB*, which is within this region, but on the far side of the mutagenized *aroK* (see Fig. 2). This thus differentiates between SCOs depending on whether the wild-type (WT) or the mutant gene is adjacent to AroP1. Amplification of the WT strain produced a 1.5 kb fragment, while amplification of the SCO produced a 2.7 kb band, indicating that the SCO had taken place on the right of *aroK* as depicted in Fig. 2. Note that the primers will not amplify the region in pAINT1 carrying the integrated copy, as AroP3 lies outside the region used for complementation. Lanes: 1, H37Rv; 2, Aroma5; 3, Aroma6; 4, Aroma3.

and in the well-characterized *Escherichia coli* pathway, *aroF* encodes one of the three DAHP synthases. The dehydroquinase synthase gene (*Rv2537c*) is designated as *aroD* in the annotation, but we prefer *aroQ* as named in the original characterization (Garbe *et al.*, 1991) to reflect the fact that it has homology to fungal catabolic 3-dehydroquinases (*aroQ*) rather than the prokaryotic biosynthetic 3-dehydroquinases (*aroD*).

Construction of a single cross-over strain (Aroma1)

We were interested in constructing mutants of *M. tuberculosis* for the *aro* pathway, since in other bacteria mutants are attenuated (Gunel-Ozcan *et al.*, 1997; Hoiseth & Stocker, 1981; Ingham *et al.*, 2002; Simmons *et al.*, 1997; Vaughan *et al.*, 1993). We have previously attempted to construct *aroK* double cross-over strains

where the chromosomal copy is replaced by a hygromycin-disrupted allele using a one-step strategy (Parish *et al.*, 1999). This was unsuccessful and demonstrated that there was a low frequency of recombination at this locus (Parish *et al.*, 1999). We therefore changed to a two-step strategy (Parish & Stoker, 2000a) and tried to isolate double cross-over strains from a single cross-over strain. A two-step strategy is more efficient when there is a low frequency of homologous recombination at a locus, since only one single cross-over is required at each step.

pARO14 (Fig. 2) was electroporated into *M. tuberculosis* and single cross-over strains selected using hygromycin and kanamycin. Using 2 µg DNA, only one single cross-over colony was obtained. PCR analysis (Fig. 4) and Southern blotting confirmed that it had the single cross-over genotype and demonstrated that recombination had occurred within the shorter 5' region of homology (1.0 kb as compared to 1.5 kb). In our previous work we had obtained a single cross-over strain where recombination had taken place within the longer 3' region (Parish *et al.*, 1999). This showed that recombination could occur on either side to generate viable strains. This strain (Aroma1) is not an *aro* mutant since it could be grown in the absence of *aro* supplement.

Second step selection for double cross-over strains from Aroma1

The single cross-over strain (Aroma1) was then used to try to isolate double cross-over strains. Aroma1 was streaked onto a fresh agar plate (containing *aro* supplement, but in the absence of antibiotics) to allow the second cross-over to occur. Since the delivery vector contained *P_{hsp60}-sacB*, the strain is sensitive to sucrose. Double cross-over strains can lead to wild-type or mutant cells, depending on the location of the second cross-over. We therefore plated the cells onto plates containing sucrose, hygromycin and *aro* supplement to select for the double cross-overs leading to the mutant allele. This confirmed that the strain was sucrose-sensitive since there was a reduction of approximately 10⁴ c.f.u. on plates containing sucrose as compared to those without sucrose. Colonies were then tested for kanamycin resistance to distinguish double cross-overs (*kan^S*) from spontaneous sucrose-resistant (*kan^R*) mutants. Sixty-seven colonies were tested and all were spontaneous sucrose-resistant, single cross-over strains. Thus, no mutant double cross-over strains were isolated. The absence of any mutants using this method suggested that the gene is essential.

We repeated the sucrose selection step in the absence of hygromycin to confirm that homologous recombination was occurring at this locus at a frequency that could be observed. Of 40 sucrose-resistant colonies tested, 32 were spontaneous sucrose-resistant strains (*kan^R hyg^R*) and eight were wild-type double cross-overs (*kan^S, hyg^S*). None were mutant double cross-overs (*kan^S, hyg^R*). This confirmed that we were seeing the second

Fig. 5. Excision of L5-integrated plasmids. (a) The genotype of the two strains with respect to *aroK* is shown. Each strain was transformed with the excisionase plasmid pJL28. (b) The expected transformants for each strain after electroporation with the replicating excisionase plasmid are shown. In each case, the L5-integrated vector should be lost. The *attB* integration site and the two sites present when a vector is integrated (*attL* and *attR*) are shown. *kan*, kanamycin-resistance gene; *hyg*, hygromycin-resistance gene; *gm*, gentamicin-resistance gene; *xis*, L5 excisionase gene; *int*, L5 integrase gene.

cross-over events, so the absence of mutants was not due to a complete lack of homologous recombination.

Construction of a merodiploid strain (Aroma3) and second step selection for double cross-over strains

We had already demonstrated the essentiality of another gene, *glnE*, by generating a merodiploid strain, which demonstrates that the lack of mutants isolated is not due to the lack of cross-overs leading to mutants, but rather to the viability of the mutants (Parish & Stoker, 2000b). We used a similar strategy to determine whether *aroK* is essential. First, we constructed a merodiploid strain (Aroma3) from the single cross-over strain Aroma1, which carried a second wild-type copy of the gene in a different region of the chromosome. An L5 phage-based integrating vector carrying an intact copy of *aroK* (pAINT1; Fig. 2) was electroporated into Aroma1. The construct was designed to carry the upstream region of *aroC* as well and therefore contained the natural promoter of the operon. Gentamicin-resistant colonies were isolated and the presence of the integrating plasmid confirmed by Southern analysis. We then plated Aroma3 onto sucrose plates to isolate double cross-over strains as we had done previously with Aroma1. No hygromycin was included, so that both wild-type and mutant double cross-over strains could be isolated. Out of 40 sucrose-resistant colonies tested, nine were wild-type double cross-over strains, one was a mutant double cross-over strain and the remaining 30 were spontaneous sucrose-resistant mutants (single cross-overs). The mu-

tant (Aroma5) was analysed by Southern blotting showing that in the mutant double cross-over strain, the wild-type *aroK* allele had indeed been replaced, whilst the L5-integrated copy was retained. These results demonstrated that homologous recombination was occurring at this locus to obtain both double cross-over strains although the frequency to give rise to the disrupted allele was low.

Excision of L5-integrated plasmid from double cross-over strain

Since the number of double cross-over strains obtained was very low in both experiments as compared to the background of spontaneous sucrose resistance, the results were not statistically significant. The bias of the second recombination event towards the wild-type strains means that obtaining enough double cross-over strains to obtain statistical significance would be extremely difficult and laborious. We therefore used a second approach to confirm that the gene was indeed essential (Fig. 5), in which the L5-integrated plasmid is efficiently excised using the L5 excisionase gene (Parish *et al.*, 2001).

We compared two strains; Aroma5 (*aroKΔ::hyg aroK⁺int aacC1*), described above, and a control strain Aroma6 (*aroK⁺ aroK⁺int aacC1*). We transformed both strains with plasmid pJL28, which replicates in mycobacteria and expresses the L5 excisionase gene *xis*. These cells were plated onto kanamycin and gentamicin to

Table 2. Transformation efficiencies

Strains were electroporated with 0.5–1.0 µg plasmid DNA. The number of transformants per microgramme of vector DNA is given.

Plasmid	Strain			
	Aroma 6		Aroma 5	
	Kanamycin	Kanamycin Gentamicin	Kanamycin	Kanamycin Gentamicin
pMV206	1×10^6	1×10^6	1×10^6	1×10^6
pJL28	1×10^6	2×10^3	2.7×10^3	2.4×10^3

select for strains carrying both the excisionase and L5-integrated plasmids (Table 2). We had previously shown that transforming pJL28 into a strain carrying an L5-integrated vector results in a 10^3 -fold reduction in cells carrying the L5-integrated vector. The small number of survivors are cells in which rearrangements have occurred, for example to inactivate the *xis* gene (Parish *et al.*, 2001). Thus, when pJL28 is transformed into Aroma6, excision of the integrating plasmid will lead to loss of one of two functional *aroK* genes and should not affect growth. We therefore expect that the transformation efficiency will be comparable to a control replicating vector, but that only 1 in 1000 transformants will retain the L5-integrated vector. However, if *aroK* is essential, in Aroma5 we should see a 10^3 -fold reduction in transformation efficiency using pJL28, as all cells in which the L5-integrated plasmid is excised will die and all the transformants should retain the L5-integrated vector.

Table 2 shows the results from the transformations. As expected, the efficiency of transformation of Aroma6 with pJL28 was comparable to the control vector pMV206. When plated onto kanamycin plus gentamicin, the number of colonies was 10^3 -fold lower, indicating that virtually all cells containing pJL28 had lost the integrating plasmid. With Aroma5, the transformation efficiency with the control plasmid was normal, but was reduced 10^2 - to 10^3 -fold with pJL28 (*P* value using Fisher's exact test $< 10^{-126}$). To confirm that the small number of surviving colonies with Aroma5 still contained the L5-integrated plasmid, 24 kanamycin-resistant transformants from each pJL28 transformation were patch-tested for gentamicin resistance. All Aroma6 transformants (24/24) were sensitive, while all Aroma5 transformants (24/24) were resistant, showing efficient excision of the L5-integrated vector from Aroma6 but not Aroma5. This confirms the essentiality of *aroK*, as 99.9% of transformants did not survive, and analysis showed the others still contained *aroK*.

DISCUSSION

The fact that we were unable to isolate strains containing only a disrupted allele of *aroK* demonstrate that this gene is essential for viability of *M. tuberculosis*. The

construction of a merodiploid strain containing an L5-integrated copy of *aroK* followed by use of the L5 excisionase allowed us to provide formal proof that the *aroK* gene is essential under the conditions employed. That is, homologous recombination leading to gene disruption could only occur in the presence of a functional copy of the gene and in such strains the L5-integrated copy could not be excised. The failure to obtain any *aro* mutants of *M. tuberculosis* had previously been attributed to the difficulty of obtaining successful gene replacement at all in slow-growing mycobacteria. We have now shown that in fact it is because the common aromatic amino acid biosynthesis pathway is essential.

We have used both one- (Parish *et al.*, 1999) and two-step recombination strategies to construct a mutant strain carrying a disrupted allele of *aroK*. In each approach, the disrupted allele was the same, but the delivery vector backbone was different. Using either vector we have seen a very low frequency of recombination as compared to other loci. Previous work has demonstrated that the frequency of recombination does vary greatly between loci (Parish *et al.*, 1999) and these data support the hypothesis that there is an inherently low frequency of recombination at the *aroK* locus. It is unlikely that the low frequency reflects an inability to get plasmids into the cells as the transformation efficiency using a replicating plasmid is 10^7 per µg DNA.

The possibility that the *aroK*Δ::*hyg* mutation results in a polar effect on downstream genes can be discounted. The *aroK* gene is found in a cluster of four closely linked shikimate pathway genes with the structure *aroC*–*aroK*–*aroB*–*aroQ*, which probably form a single transcription unit. Although the insertion of the *hyg* gene may interrupt transcription from the natural promoter, the *aroB* and *aroQ* genes should still be expressed from the *hyg* promoter. This is confirmed by the fact that the region used in the complementing integrated vector contained *aroC* and *aroK*, but not the two downstream genes. Thus the inability to obtain gene replacement cannot be attributed to disruption of the function of *aroB* and/or *aroQ*.

The end product of the common aromatic amino acid pathway is chorismate, the precursor for biosynthesis of

the aromatic amino acids tyrosine, tryptophan and phenylalanine, as well as folate and ubiquinone (Fig. 1). We expected that the aromatic supplements used would be sufficient to permit the growth of *aroK* mutants, as it is in other bacteria that have been studied. Fig. 1 shows the supplements added to the media in relation to the pathway. Several possibilities exist to explain the essentiality of this pathway. The first is that the supplements are not transported into the cell. We have already isolated a tryptophan auxotroph, which can be grown in the presence of L-tryptophan, showing that this amino acid is transported into the cell (Parish *et al.*, 1999). *M. tuberculosis* has a homologue of AroP2, an aromatic amino acid transporter (Cole *et al.*, 1998), so we would expect that the other aromatic amino acids are transported into the cell as well.

The second possibility is that *M. tuberculosis* relies on a constant supply of the final product of one or more downstream pathway(s) for its survival. It is feasible that there is another, as yet uncharacterized, branch from the aromatic pathway in mycobacteria. The end product of this pathway would have to be added to the supplements in order for *aroK* mutants to be viable. We also tried to isolate mutants in the presence of chorismate supplementation, but again were unsuccessful, suggesting that a branch point after chorismate itself is unlikely.

There are several differences between the well-characterized *E. coli* genes and the *M. tuberculosis* homologues. The first step of the pathway is catalysed by three enzymes in *E. coli*, each of which is independently regulated, whereas *M. tuberculosis* possesses only one DAHP synthase (*aroG*). *E. coli* also has two shikimate kinase genes (*aroK* and *aroL*), whilst *M. tuberculosis* only has one. Therefore there appears to be less redundancy in the *M. tuberculosis* pathway. Vinella *et al.* (1996) identified a second function for the *E. coli* *aroK* gene product that is distinct and unrelated to its shikimate kinase activity. Should the *M. tuberculosis* enzyme possess a similar activity it is possible that disruption of this activity would explain the inability of the bacteria to grow in the absence of a functional copy of the gene.

Analysis of the genetic organization of the *aro* genes between slow- and fast-growing mycobacteria reveals differences in the main *aro* operon. This may reflect differences in the regulation or the function of the pathway. We do not know if the common aromatic amino acid pathway is essential in all mycobacteria or whether this phenomenon is confined to *M. tuberculosis* alone.

The genes and pathways that are essential for the growth of *M. tuberculosis* make attractive drug targets since inhibiting their function may kill the organism. The chorismate pathway is one of great interest in that it makes a good target for drug development. The pathway is absent from the human host, thus the products cannot be gleaned from the host environment and inhibition of any of the mycobacterial enzymes is

unlikely to have a toxic side effect on the host. The evidence that this pathway is essential for *M. tuberculosis* even in the presence of exogenous supplements reinforces its attractiveness as a drug target. It is interesting to note that the *M. tuberculosis* shikimate kinase (AroK) has been crystallized (Gu *et al.*, 2001) and the structure of the dehydroquinase (AroQ) has been determined (Gourley *et al.*, 1999), opening the possibility of rational design of inhibitors.

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