Partial Characterization of *Mycobacterium fortuitum* and *Mycobacterium smegmatis* Auxotrophs by Syntrophism Using *Bacillus subtilis*

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Syntrophism (cross-feeding) could be demonstrated between mutants of *Mycobacterium fortuitum* and *Mycobacterium smegmatis*, and previously characterized mutants of *Bacillus subtilis*, auxotrophic for arginine, histidine, lysine or phenylalanine. Based on this cross-feeding data, the possible site of blockage in the biosynthetic pathways of the mutants could be inferred.

INTRODUCTION

Most mycobacteria can be cultured in simple media and should therefore be capable of *de novo* biosynthesis of precursors of macromolecules. For a better understanding of the peculiarities of the genus *Mycobacterium*, it is necessary to study the relevant biosynthetic pathways. Not much information is available on the basic biology of *Mycobacterium*, owing to the lack of a well-worked-out system for genetic manipulation in this genus. Only recently have there been reports of concerted efforts for cloning genes from and into mycobacteria (Young *et al.*, 1985; Hopwood *et al.*, 1988; Snapper *et al.*, 1988). This approach would help, in no small way, our understanding of mycobacteria. However, while molecular cloning is a valuable tool, the importance of work on classical genetics cannot be over-emphasized.

Syntrophism or cross-feeding is a classical method in biochemical genetics for obtaining information on the affected sites in a biosynthetic pathway. Auxotrophs with a mutation at a particular step in a biosynthetic pathway cross-feed mutants blocked at an earlier stage in that pathway. Such an approach has so far not been reported for studying biosynthetic pathways in *Mycobacterium*. We isolated several auxotrophic mutants of *Mycobacterium* spp. (Subramanyam *et al.*, 1989) and in this work, we have characterized some of the mutants by syntrophism. Non-availability of well-characterized mutants of *Mycobacterium* prompted us to use mutants of *Bacillus subtilis*, which have well-characterized mutations and are easily available as reference strains.

METHODS

Organisms and media. The strains used in this study are listed in Table 1. Nutrient agar (NA), Middlebrook 7H9 broth base (MB) and agar were from HiMedia Laboratories, Bombay; amino acids and cytosine were from Loba-Chemie Indoaustran Co., Bombay. MB without ADC (albumin/dextrose/catalase) enrichment but with glucose (0.5% w/v), glycerol (0.2% v/v) and agar (1.5% w/v) was used as basal medium.

Syntrophism. To look for cross-feeding with reference to a particular amino acid, strains with similar amino acid requirements were tested against each other by streaking on basal medium supplemented with a suboptimal concentration ($1\ \mu\text{g ml}^{-1}$) of the amino acid. When using strains with multiple requirements, all the required nutrients, except the one under test, were provided ($20\ \mu\text{g ml}^{-1}$) in the basal medium. For example, when strain 1A8 was used for testing syntrophism of lysine auxotrophs, the basal medium was supplemented with $20\ \mu\text{g}$ aromatic amino acids ml^{-1} , and only $1\ \mu\text{g}$ lysine ml^{-1} . Because of the difference in growth rates, *B. subtilis* strains were inoculated 24 h after the mycobacteria. Three strains per plate were tested in different combinations. Plates were read after 2–4 d incubation at $37\ ^\circ\text{C}$. Because of the low concentration ($1\ \mu\text{g ml}^{-1}$) of the required amino acid in the media, auxotrophic mutants do not show appreciable growth except when cross-fed by a neighbouring strain. Thus, a better growth of mutant 'X' in the proximity of mutant 'Y' was recorded as positive cross-feeding – in this instance 'Y' cross-feeds 'X' (see Fig. 1).

Table 1. *Strains used*

Strain	Markers*	Source or derivation†
<i>B. subtilis</i>		
1A8	<i>trpC2 aroD120 lys-1</i>	BGSC
1A10	<i>trpC2 hisA1 thr-5</i>	BGSC
1A96	<i>trpC2 pheA1</i>	BGSC
1A510	<i>leuA8 arg15 thrA recE4 r⁻ m⁻ stp</i>	BGSC
<i>M. fortuitum</i>		
NIHJ1615	Prototroph	NIHJ
SM2	<i>arg-1</i>	NTG treatment of NIHJ1615
SM29	<i>arg-1 leu-2 his-1</i>	NTG treatment of SM2
<i>M. smegmatis</i>		
NIHJ1628	Prototroph	NIHJ
SM10	<i>lys-1</i>	NTG treatment of NIHJ1628
SM13	<i>leu-1</i>	NTG treatment of NIHJ1628
SM20	<i>lys-2</i>	NTG treatment of NIHJ1628
SM22	<i>arg-2</i>	NTG treatment of NIHJ1628
SM33	<i>leu-1 cyt-1 arg-3</i>	NTG treatment of SM13
SM42	<i>lys-1 phe-1</i>	NTG treatment of SM10

* Relevant characteristics of *B. subtilis* used as reference strains: *lys-1*, blocked in diaminopimelate decarboxylase; *hisA1*, histidine requirement; *pheA1*, blocked in prephenate dehydratase; *leuA8*, blocked in α -isopropylmalate synthase; *arg15*, arginine requirement.

† All *B. subtilis* strains were obtained from the *Bacillus* Genetic Stock Center (BGSC), The Ohio State University, USA; NIHJ1615 and NIHJ1628 were from the National Institute of Health (NIHJ), Japan; all strains with the prefix SM have been derived in our laboratory by mutagenesis of NIHJ strains with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG), as described elsewhere (Subramanyam *et al.*, 1989). (Briefly, bacteria were treated with 500 μ g NTG ml⁻¹ at 37 °C for 30 min, washed and plated on nutrient agar. Auxotrophs were identified by replica plating onto minimal medium. After three subcultures, only a fraction of the mutants retained their auxotrophic trait.)

RESULTS AND DISCUSSION

For *Mycobacterium*, there is no information at all on the biosynthesis of arginine, glycine, histidine, methionine, proline, serine and threonine (Ratledge, 1982). In the biosynthesis of lysine, only the final step has been studied (Willett, 1969). Regarding the biosynthesis of aromatic amino acids there have been only two reports, one showing the presence of tryptophan synthetase in *M. smegmatis* (Ratledge & Winder, 1961) and the other demonstrating all six enzymes of the common pathway in *M. phlei* (Yapo *et al.*, 1974).

Use of reference strains of bacteria such as *B. subtilis* offers great potential for understanding more about mycobacterial mutants. Both interspecies and intergeneric cross-feeding was observed amongst mutants of *M. fortuitum*, *M. smegmatis*, and *B. subtilis* auxotrophic for arginine, histidine, lysine and phenylalanine (Table 2). There was no cross-feeding amongst leucine auxotrophs.

Based on the cross-feeding data, the mutants could be arranged sequentially according to the possible site of mutation in the biosynthetic pathway (Table 3). In the pathway for arginine biosynthesis, by analogy with *Escherichia coli* (Vogel, 1970), SM2 is blocked in the final steps of formation of arginine, either at the argininosuccinate synthetase (*argG*) or at the argininosuccinase (*argH*) level; SM33 is suspected to be a mutant either at carbamoylphosphate formation or at the ornithine carbamoyltransferase (*argF*) locus, and SM22 at a locus preceding *argF*. These inferences are corroborated by the growth patterns of the mutants on basal medium supplemented with ornithine or citrulline, which are intermediates in arginine biosynthesis. SM22 grows equally well on ornithine, citrulline or arginine; SM33 grows on citrulline or arginine, but not on ornithine; and SM2 and 1A510 grow only on arginine but not on ornithine or citrulline.

In histidine biosynthesis, SM29 is expected to be blocked downstream of *hisA* since SM29 can cross-feed 1A10 which has a mutation at *hisA*. Similarly, cross-feeding data indicate that in

Fig. 1. Syntrophism (cross-feeding). Mutants 'X' (SM22), 'Y' (1A510) and 'Z' (SM2) were streaked on a minimal medium supplemented with only $1 \mu\text{g ml}^{-1}$ of the required amino acid (arginine). However, both 'Y' and 'Z' are able to cross-feed 'X'. 'X' shows good growth only in the proximity of the other two mutants.

Table 2. Cross-feeding between auxotrophs of *M. fortuitum*, *M. smegmatis* and *B. subtilis*

Cross-feeding with reference to each amino acid was examined as described in Methods by testing all combinations of the strains auxotrophic for that amino acid. Strains listed under column I cross-feed corresponding strains of column II on a medium supplemented with $1 \mu\text{g ml}^{-1}$ of the amino acid shown in column III. SM2 and SM29 are mutants of *M. fortuitum*; SM10, SM20, SM22, SM33 and SM42 are mutants of *M. smegmatis*; 1A8, 1A10, 1A96 and 1A510 are reference *B. subtilis* mutants. Combinations not shown in the Table were negative in cross-feeding experiments. All combinations were tested on four different occasions, with the same result.

I	II	III
SM2	SM22 and SM33	Arginine
SM33	SM22	Arginine
1A510	SM22 and SM33	Arginine
SM29	1A10	Histidine
SM20	SM10	Lysine
1A8	SM10 and SM20	Lysine
SM42	1A96	Phenylalanine

Table 3. Mutants arranged according to the possible site of mutation in the biosynthetic pathway based on cross-feeding data

Biosynthesis of:	Mutants*
Arginine	SM22→SM33→SM2 and 1A510†
Histidine	1A10→SM29
Lysine	SM10→SM20→1A8
Phenylalanine	1A96→SM42

* Relative positions from left-to-right indicate early-to-later steps in biosynthesis. SM2 and SM29 are *M. fortuitum* mutants; SM10, SM20, SM22, SM33 and SM42 are *M. smegmatis* mutants; 1A8, 1A10, 1A96 and 1A510 are reference *B. subtilis* mutants.

† SM2 and 1A510 probably each have a mutation at the same step.

lysine biosynthesis, SM10 and SM20 are blocked at two different points (SM10 at an earlier step than SM20), preceding the final step of L-lysine formation from *meso*-diaminopimelate, because 1A8, which is blocked at this final step, cross-feeds both SM20 and SM10, and SM20 in turn cross-feeds SM10. Since SM42 cross-feeds 1A96 which lacks prephenate dehydratase (the enzyme catalysing the penultimate step in the biosynthesis of phenylalanine), it can be inferred that SM42 is blocked at the very final step in phenylalanine biosynthesis.

Lack of cross-feeding among the leucine-requiring mutants suggests a common biosynthetic block, possibly at the level of α -isopropylmalate synthase, because 1A510 is a known mutant at this site (*leuA8*). It is important to note that several factors affect the results of cross-feeding experiments. The results might be misleading if, for instance, the strains being tested have other than point mutations in structural genes. Hence the results of cross-feeding experiments, particularly where only negative results are obtained, need to be interpreted with caution.

These findings indicate that using more reference strains of *Bacillus* and perhaps *E. coli*, it should be possible to study the biochemical genetics of *Mycobacterium*.

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