Threonine Inhibition of Growth of *Bacillus subtilis*: Positive Selection for Isoleucine Auxotrophy

By DAVID H. LAMB* AND KENNETH F. BOTT

*Curriculum in Genetics, and Department of Bacteriology and Immunology,
University of North Carolina, Chapel Hill, North Carolina 27514, U.S.A.*

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INTRODUCTION

Inhibition of microbial growth has often been used as a probe to study the biochemical genetics of autotrophic (Rittenberg, 1969) and heterotrophic (see Ingram & Jensen, 1973) micro-organisms. To gain insight into the metabolic regulation of growth and sporulation, we have studied the inhibition of *Bacillus subtilis* by threonine. We found that 8.4 mM-threonine prevented growth and sporulation by restricting valine synthesis and proposed that this restriction was due to an excess of 2-oxobutyrate (derived from threonine) which competed with pyruvate for the active site of acetohydroxyacid synthase (EC 4.1.3.18) thus restricting the flow of carbon into valine (Lamb & Bott, 1978, 1979). In support of this 'substrate competition' model, we found that most mutants selected for resistance to threonine were auxotrophic for isoleucine and that one of these auxotrophs appeared to be altered in threonine dehydratase (EC 4.2.1.16). It is possible that an isoleucine requirement in the absence of a simultaneous valine requirement is due to an altered threonine dehydratase or an altered isoleucyl-tRNA synthetase (EC 6.1.1.5). Here we report that the majority of threonine-resistant mutants found were auxotrophic for isoleucine irrespective of whether isoleucine was included in the selective medium. In addition, all of the strains auxotrophic for isoleucine that we examined were altered in threonine dehydratase activity.

METHODS

Bacteria and isolation of mutants. The wild-type strain used was *B. subtilis* 168 (*trpC*). All mutants were spontaneously occurring variants and were isolated as described previously (Lamb & Bott, 1979). Briefly, mutant selection consisted of growing bacteria to mid-exponential phase in Penassay broth (Difco), harvesting by centrifugation, washing once and plating on minimal agar (Spizizen, 1958) which contained (filter-sterilized and aseptically added) threonine (20 mg ml⁻¹) and leucine (4 mg ml⁻¹). Resistant colonies were streaked to give single colonies on tryptose blood agar base (Difco) and a single colony was transferred to selective medium. Clones that survived two such cycles of purification were studied further. L-Threonine (*allo*-free) was A grade from Calbiochem.

Enzyme assays. Threonine dehydratase was assayed by the method of Umbarger & Brown (1957). Protein was determined by the method of Lowry *et al.* (1951).

Transformation. Transformations and related procedures have been described by Wilson & Bott (1968). Genetic linkage between the determinant of the Ile⁻ phenotype of threonine-resistant mutants and the 168 derivative Mu8u5u1 (*ile*, *leu*, *met*, *str^R*) was measured by the recombination index method of Ephrati-Elizur *et al.* (1961). Strain Mu8u5u1 served as recipient for all experiments and selection for Ile⁺ transformants was made on minimal agar (Spizizen, 1958).

RESULTS AND DISCUSSION

The term 'threonine resistance' is used to indicate inhibition of growth by a combination of threonine and leucine. Threonine alone caused only transient inhibition of growth in a glucose/salts minimal medium. Consequently, leucine is required for selection of

* Present address: Department of Biological Sciences, Purdue University, Life Sciences, West Lafayette, Indiana 47907, U.S.A.

Table 1. *Threonine dehydratase activity and genetic linkage of several threonine-resistant mutants*

Strain	Specific activity of threonine dehydratase*	Genetic linkage†
168	4.34	—
Thl6	0.31	0.09
Thl10	0.29	0.18
Thl11	0.33	ND
Thl17	0.38	0.07
Thl18	0.21	0.06

ND, Not done.

* Specific activities are expressed as μmol 2-oxobutyrates h^{-1} (mg protein) $^{-1}$.

† Values indicate the recombination index between the *Ile*⁻ phenotypes of the mutant strains and strain Mu8u5u1. In *B. subtilis*, values of 0.1 or less are consistent with the location of two mutations in the same or adjacent genes (see Piggot & Coote, 1976).

'threonine-resistant' variants and mutants are designated Thl to indicate this. The term 'threonine resistance' appears to be appropriate, however, since leucine merely potentiates the inhibition by threonine but does not alter it qualitatively. This conclusion is supported by the observations that leucine alone does not inhibit growth, valine addition reverses inhibition by threonine plus leucine as it does inhibition by threonine alone (Lamb & Bott, 1979), and two Thl^a mutants [Thl12 (*ile*) and Thl13 (*ile*⁺)] show increased resistance to inhibition of growth by threonine alone (data not shown).

Mutants resistant to threonine appeared spontaneously at a frequency of approximately 10^{-6} . Most of these (75 %) were simultaneously auxotrophic for isoleucine. The frequency of isoleucine auxotrophs was not altered if isoleucine was omitted from the selection medium. This observation can probably be explained by the findings of Skarstedt & Greer (1973) who demonstrated that the threonine synthase (EC 4.2.99.2) of *B. subtilis* has an associated threonine dehydratase activity. This associated activity would make possible the indirect suppression of mutations in the major biosynthetic threonine dehydratase when large intracellular concentrations of threonine were present.

The finding that high threonine concentrations masked the isoleucine requirement of these strains is consistent, therefore, with the idea that most, if not all, of these strains were defective in threonine dehydratase. To examine this possibility further, several auxotrophic resistant strains were characterized in more detail (Table 1). All mutants examined had low threonine dehydratase activity compared with the parental strain. These mutations also showed close genetic linkage to a known threonine dehydratase mutation (Barat *et al.*, 1965). We conclude that most mutations to threonine resistance are due to alterations in threonine dehydratase. This supports our earlier conclusion that conversion of threonine to 2-oxobutyrates is essential for inhibition of *B. subtilis* by threonine. However, we cannot yet formally eliminate the alternative (but, in our opinion, less likely) possibility that some or all of these mutations conferring isoleucine auxotrophy are regulatory mutations that prevent the synthesis of normal levels of threonine dehydratase. In this context, we have observed that of 22 threonine-resistant mutants selected for the presence of a halo of background growth on selective plates (which often indicates that the resistant strain is overproducing and excreting a compound that can reverse the original inhibition), three were auxotrophic for isoleucine.

The above results imply that selection for threonine resistance may be used as a positive selection for alterations in the *B. subtilis* threonine dehydratase. This observation may be of more than passing interest since recent work has suggested that this enzyme may play an autoregulatory role in the regulation of isoleucine-valine synthesis in *B. cereus* (Raimond & Fargette, 1977) and in a variety of other micro-organisms (Goldberger, 1974; Calhoun &

Hatfield, 1975). The possibility that the *B. subtilis* threonine dehydratase may play a regulatory role in isoleucine–valine synthesis has not been reported. Leibovici & Anagnostopoulos (1969) showed that an alteration in the threonine dehydratase of *B. subtilis* conferred sensitivity to valine. This is similar to the findings of Levinthal *et al.* (1973) who showed that a mutation to leucine sensitivity in *Escherichia coli* was due to an altered threonine dehydratase and that this strain also showed altered regulation of isoleucine–valine synthesis. These observations, taken together, are consistent with the possibility that threonine dehydratase may play a regulatory role in isoleucine–valine synthesis in *B. subtilis* but are not, in themselves, sufficient to prove that such a role exists.

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