SHORT COMMUNICATION

Cloning of the Aconitase Gene (acn) of Escherichia coli K12

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Lambda phages containing the aconitase gene (acn) of Escherichia coli K12 have been isolated by hybridization with an M13 probe containing part of the aconitase gene (citB) of Bacillus subtilis. Aconitase specific activities are amplified 5- to 18-fold in thermally induced λacn lysogens and threefold in a strain transformed with a plasmid derivative (pGS181).

INTRODUCTION

In Escherichia coli K12 the genes encoding all of the enzymes of the citric acid cycle except aconitase (aconitate hydratase, EC 4.2.1.3) have been located on the linkage map and cloned in phage or plasmid vectors. Nine of the genes are clustered at 16·7 min in a fully sequenced 13·1 kb segment of DNA which contains the citrate synthase (gltA), succinate dehydrogenase (sdhCDAB), 2-oxoglutarate dehydrogenase (sucA), dihydrolipoamide succinyltransferase (sucB) and succinyl-CoA synthetase (sucCD) genes (Spencer & Guest, 1982; Ner et al., 1983; Wood et al., 1984; Buck et al., 1985). The other genes are scattered throughout the linkage map. These include the recently cloned isocitrate and malate dehydrogenase genes (icd at 25·3 min, and mdh at 70·4 min; LaPorte et al., 1985; Sutherland & McAlister-Henn, 1985), and three fumarase genes (fuma and fume at 35·5 min; fumB at 93·5 min) plus the lipoamide dehydrogenase gene (lpd at 2·8 min), which have been cloned previously (Guest et al., 1985; Guest & Stephens, 1980).

Studies on the aconitase gene (acn) have been hindered by the fact that no E. coli mutants lacking aconitase activity have been characterized. This contrasts with the situation in Bacillus subtilis, where the aconitase gene (citB) was identified by Rutberg & Hoch (1970) during their analysis of citric acid cycle mutants. More recently, a fragment of DNA encoding the citB promoter and the N-terminal segment of the B. subtilis aconitase was cloned by virtue of its ability to repair the lesions of some, but not all, citB mutants (Rosenkrantz et al., 1985).

The B. subtilis clone has now been used to prepare a hybridization probe for isolating the acn gene of E. coli. As a result, several acn phages containing overlapping fragments of a unique segment of E. coli DNA have been characterized. Thermal induction of strains lysogenized by some of these λacn phages leads to 5- to 18-fold amplifications of aconitase activity, which supports the view that the aconitase gene has been cloned.

METHODS

Bacterial strains, bacteriophages and plasmids. The strains of Escherichia coli K12 used were: W3110 (trpR iclR, prototroph), C600 (thr-1 leuB6 thi-1 supE44 tonA21 lacYI, indicator strain for λ phages), JM101 (thi supE Δ(proAB–lac) F' traD36 proAB+ lacZΔM15 lacYI, transfection host for M13) and ED8641 (hsdR supE recA36 aux, transformation host). A λ gene bank in which Sau3A partially digested E. coli DNA replaces the central BamHI fragment of the vector pAPE11 (AsbHI–BamHI 2A cI857 nin5 chiA) was kindly provided by P. T. Emmerson (Arthur et al., 1982). The M13mp10 RF DNA was from Gibco-PL, and plasmid pMR41 containing

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the promoter and proximal segment of the aconitase gene (citB) of Bacillus subtilis was kindly provided by A. L. Sonenshein (Rosenkrantz et al., 1985).

**DNA preparation and in vitro manipulation.** The media and methods for the growth of *E. coli*, the propagation of λ and M13 phages, and the isolation of their DNAs have been listed previously, as have the general procedures for restriction analysis, ligation and transformation or transfection (Guest & Stephens, 1980; Guest et al., 1985; Sanger et al., 1980).

**Hybridization methods.** Single-stranded M13 DNA was labelled with [α-32P]dATP by synthesizing a complementary strand using a hybridization probe primer according to Hu & Messing (1982). Restriction fragments separated electrophoretically in agarose gels and λ plaques in agarose were transferred to nitrocellulose and treated as described by Maniatis et al. (1982). Hybridization was done in 6× SSC (1× SSC is 0-15 m-NaCl, 0-015 m-trisodium citrate, pH 7.0) at 65 °C for 16 h, and subsequently washed three times with 4× SSC (low stringency conditions).

**Induction of lysogens and aconitase assay.** Cultures in L broth (50 ml) were grown with shaking at 30 °C to OD600 = 0-7 and resuspended in the same volume of prewarmed medium at 42 °C (10 min) and then 37 °C. Samples (up to 10 ml) were washed and resuspended in 1 ml 40 mM-potassium phosphate buffer (pH 7·8), disrupted with an ultrasonic disintegrator (MSE, 150 W, 3×30 s), and clarified by centrifuging for 20 min at 13400 g (MSE MicroCentaur). Supernatant extracts were assayed for protein (Lowry method) and for aconitase by following the disappearance of cis-aconitate at 240 nm and 25 °C (Fansler & Lowenstein, 1969). Specific activities, reproducible to within ±5%, are expressed as μmol substrates transformed min−1 mg protein−1.

**Materials.** Restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment) and hybridization probe primer were purchased from BRL, New England Biolabs, Boehringer and Gibco-PL. 2'-Deoxyadenosine 5'-[α-32P]triphosphate (3 Ci mol−1, 110 GBq mol−1) was from Amersham.

**RESULTS AND DISCUSSION**

An M13 phage (M13mp10-KJ9) containing part of the *B. subtilis* aconitase gene (citB) that encodes 160 amino acid residues close to the amino-terminus was constructed by subcloning the 480 bp *PstI–EcoRI* fragment of pMR41 into the RF of M13mp10 (Fig. 1a). Using the single-stranded phage as a hybridization probe in Southern blot analyses revealed single hybridizing fragments in restriction digests of *E. coli* K12 total DNA (sizes in kb): ClaI, 7·2; SstI, 9·8; *PstI*, 10·8; *EcoRI*, 14·5; HindIII, 16·0; *BamHI*, 17·5; *BglII*, 17·5; *KpnI*, 17·5; *Xhol*, 20·0; *SphI*, 23·0. This indicates that *E. coli* contains a unique region, presumably in the aconitase gene (acn), which hybridizes with the *B. subtilis* citB gene. A λPE11–*E. coli* gene bank, constructed with a Sau3A partial digest of bacterial DNA, was accordingly screened for phages carrying the acn region, using plaque-hybridization with the same probe. Positive reactions were obtained with 45 out of 21800 plaques screened, and eight putative λacn phages were chosen for detailed analysis. They contain segments of *E. coli* DNA ranging in size from 8·2 to 13·7 kb and forming a partially overlapping series of inserts which, apart from λG172, are related to each other by sharing at least one restriction fragment with another member of the group (Fig. 1b). Southern blot analyses confirmed that all of the λacn phages (including λG172) contain a 350 bp Sau3A fragment which hybridizes with the citB probe and comigrates with an analogous fragment in total *E. coli* DNA. It would thus appear that the lack of colinearity between some of the λ-cloned segments is due to the rearrangement of partial Sau3A fragments during the construction of the gene bank. Southern blot analyses also confirmed that the site of homology with *E. coli* derived from the segments that occur in at least two λacn phages, is shown in Fig. 1b. The site of citB homology common to all of the phages must be within a 3·1 kb segment of the *SphI–SstI* fragment (Sp2–Ss1 in Fig. 1b).

Physiological evidence for the cloning of the acn gene was sought by constructing λacn lysogens of *E. coli* W3110 and assaying for aconitase following thermal induction. Three types of response were reproducibly observed: an early rise in aconitase activity with λG176, λG177 and λG178 lysogens; a delayed increase with λG165; and no significant increase before lysis with λG164, λG172, λG174 and λG179 (Fig. 2). The enzyme is amplified some 5- to 18-fold with four of the λacn phages. These presumably contain an active acn gene, but there is no unambiguous correlation between the positive response and a specific segment of cloned DNA. Nevertheless, it is possible that the early and late responses are related to the orientations of the
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Fig. 1. Restriction maps showing (a) the citB region of B. subtilis cloned in plasmid pMR41 and the segment sub-cloned for use as a hybridization probe (M13mp10-KJ9) and (b) the segments of E. coli DNA cloned in the λacn phages. In (a) the open arrow denotes the start point (0) and direction of synthesis of the citB transcript, and the hatched region represents the coding region of the B. subtilis aconitase. In (b) the segments of E. coli DNA (double lines) are flanked by phage DNA (single lines with arrowheads on the λ right arms). Restriction fragments that are shared by more than one insert, and thus contribute to the map deduced for the acn region, are stippled. The sizes (kb) of the inserts are included, and the aconitase responses of induced lysogens are indicated (+/-). The restriction sites are abbreviated: E, EcoRI; Na, NaeI; Nd, NdeI; Nr, NruI; P, PstI; Sa, SiaI; Sp, SphI; and Ss, SstI.

Fig. 2. Aconitase activities following thermo-induction of λacn lysogens. Cultures were assayed for aconitase activity (μmol min⁻¹ (mg protein)⁻¹) at intervals after induction as described in Methods. ●, λG176; ○, λG165; ■, λG164 and λ (phages giving analogous responses are shown in parentheses). The responses were reproducible (±20%) with duplicate cultures and with two independently isolated lysogens.

Inserts and the effects of the flanking λ promoters, the acn gene being polarized leftward in λG176-8 and rightward in λG165. Subcloning the 4-4 kb SphI–EcoRI fragment (Sp₂–E₁ in Fig. 1b) from λG176 into the corresponding sites of pBR322 generates a plasmid (pGS181) which amplifies aconitase threefold in ED8641 [3-4 compared with 1.2 μmol min⁻¹ (mg protein)⁻¹ with pBR322 and the plasmid-free strain]. This tentatively locates the acn gene in the Sp₂–E₁ fragment (Fig. 1b). It is not known why two of the phages containing this fragment (λG174 and λG179) are inactive, but it could be due to minor damage suffered during cloning, or to the effects of a strong converging promoter in the adjacent segment of bacterial DNA.

These observations indicate that E. coli possesses a unique segment of DNA sharing homology with part of the B. subtilis citB gene. The simplest interpretation of these findings is that it corresponds to the aconitase gene (acn) of E. coli. If so it means that the genes encoding all
of the citric acid cycle enzymes of E. coli have now been cloned. Future work will be aimed at locating the acn gene more precisely in the cloned DNA, identifying the gene product in transcription–translation systems, defining the nucleotide sequence of the structural gene and associated regulatory regions, and locating the acn gene in the E. coli linkage map.

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REFERENCES


