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 *fumC* 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 aconitase 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 lacY1*, indicator strain for λ phages), JM101 (*thi supE Δ(proAB-lac)/F' traD36 proAB⁺ lacZΔM15 lacI^q*, transfection host for M13) and ED8641 (*hsdR supE recA56 aux*, transformation host). A λ gene bank in which *Sau3A* partially digested *E. coli* DNA replaces the central *Bam*HI fragment of the vector λ PE11 (*λsbhIΔ1° srIA1-2Δ cl857 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 - 32 P]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 $6\times$ SSC ($1\times$ 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 $4\times$ SSC (low stringency conditions).

Induction of lysogens and aconitase assay. Cultures in L broth (50 ml) were grown with shaking at 30 °C to OD₆₅₀ = 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 $\pm 5\%$, are expressed as μ mol 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 - 32 P]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 *Pst*I-*Eco*RI fragment of pMR41 into the RF of M13mp10 (Fig. 1*a*). 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): *Cla*I, 7.2; *Sst*I, 9.8; *Pst*I, 10.8; *Eco*RI, 14.5; *Hind*III, 16.0; *Bam*HI, 17.5; *Bgl*II, 17.5; *Kpn*I, 17.5; *Xho*I, 20.0; *Sph*I, 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 λ PE11-*E. coli* gene bank, constructed with a *Sau*3A 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 *lacn* 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 λ G172, are related to each other by sharing at least one restriction fragment with another member of the group (Fig. 1*b*). Southern blot analyses confirmed that all of the *lacn* phages (including λ G172) contain a 350 bp *Sau*3A 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 *Sau*3A fragments during the construction of the gene bank. Southern blot analyses also confirmed that the site of homology with *citB* resides in a 4.4 kb *Sph*I-*Eco*RI fragment in total *E. coli* DNA and in the analogous fragments of five of the *lacn* phages. A map of the *acn* region of *E. coli* derived from the segments that occur in at least two *lacn* phages, is shown in Fig. 1*b*. The site of *citB* homology common to all of the phages must be within a 3.1 kb segment of the *Sph*I-*Sst*I fragment (Sp₂-Ss₁ in Fig. 1*b*).

Physiological evidence for the cloning of the *acn* gene was sought by constructing *lacn* 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 the λ G176, λ G177 and λ G178 lysogens; a delayed increase with λ G165; and no significant increase before lysis with λ G164, λ G172, λ G174 and λ G179 (Fig. 2). The enzyme is amplified some 5- to 18-fold with four of the *lacn* 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

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 λ *acn* phages. In (a) the open arrow denotes the start point (○) 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, *SalI*; Sp, *SphI*; and Ss, *SsrI*.

Fig. 2. Aconitase activities following thermo-induction of λ *acn* lysogens. Cultures were assayed for aconitase activity [μ mol min⁻¹ (mg protein)⁻¹] at intervals after induction as described in Methods. ●, λ G176; ○, λ G165; ■, λ G164 and λ^- (phages giving analogous responses are shown in parentheses). The responses were reproducible ($\pm 20\%$) 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 λ G176-8 and rightward in λ G165. Subcloning the 4.4 kb *SphI*-*EcoRI* fragment (Sp₂-E₁ in Fig. 1b) from λ G176 into the corresponding sites of pBR322 generates a plasmid (pGS181) which amplifies aconitase threefold in ED8641 [3.4 compared with 1.2 μ mol 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 (λ G174 and λ G179) 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

- ARTHUR, H. M., BRAMHILL, D., EASTLAKE, P. B. & EMMERSON, P. T. (1982). Cloning of the *uvrD* gene of *E. coli* and identification of the product. *Gene* **19**, 285–295.
- BUCK, D., SPENCER, M. E. & GUEST, J. R. (1985). Primary structure of the succinyl-CoA synthetase of *Escherichia coli*. *Biochemistry* **24**, 6245–6252.
- FANSLER, B. & LOWENSTEIN, J. M. (1969). Aconitase from pig heart. *Methods in Enzymology* **13**, 26–30.
- GUEST, J. R. & STEPHENS, P. E. (1980). Molecular cloning of the pyruvate dehydrogenase complex genes of *Escherichia coli*. *Journal of General Microbiology* **121**, 277–292.
- GUEST, J. R., MILES, J. S., ROBERTS, R. E. & WOODS, S. A. (1985). The fumarase genes of *E. coli*: location of the *fumB* gene and discovery of a new gene (*fumC*). *Journal of General Microbiology* **131**, 2971–2984.
- HU, N. & MESSING, J. (1982). The making of strand-specific M13 probes. *Gene* **17**, 271–277.
- LAPORTE, D. C., THORSNESS, P. E. & KOSHLAND, D. E. (1985). Compensatory phosphorylation of isocitrate dehydrogenase: a mechanism for adaptation to the intracellular environment. *Journal of Biological Chemistry* **260**, 10563–10568.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NER, S. S., BHAYANA, V., BELL, A. W., GILES, I. G., DUCKWORTH, H. W. & BLOXHAM, D. P. (1983). Complete sequence of the *gltA* gene encoding citrate synthase in *Escherichia coli*. *Biochemistry* **22**, 5243–5249.
- ROSENKRANTZ, M. S., DINGMAN, D. W. & SONENSHEIN, A. L. (1985). *Bacillus subtilis citB* gene is regulated synergistically by glucose and glutamine. *Journal of Bacteriology* **164**, 155–164.
- RUTBERG, B. & HOCH, J. A. (1970). Citric acid cycle: gene-enzyme relationships in *Bacillus subtilis*. *Journal of Bacteriology* **104**, 826–833.
- SANGER, F., COULSON, A. R., BARRELL, B. G., SMITH, A. J. H. & ROE, B. A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *Journal of Molecular Biology* **143**, 161–178.
- SPENCER, M. E. & GUEST, J. R. (1982). Molecular cloning of four tricarboxylic acid cycle genes of *Escherichia coli*. *Journal of Bacteriology* **151**, 542–552.
- SUTHERLAND, P. & MCALISTER-HENN, L. (1985). Isolation and expression of the *Escherichia coli* gene encoding malate dehydrogenase. *Journal of Bacteriology* **163**, 1074–1079.
- WOOD, D., DARLISON, M. G., WILDE, R. J. & GUEST, J. R. (1984). Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of *Escherichia coli*. *Biochemical Journal* **222**, 519–534.