Genetic control of manno(fructo)kinase activity in *Escherichia coli*

Andrew A. Sproul, Linda T. M. Lambourne, Jims Jean-Jacques D, and Hans L. Kornberg*

Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215

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Mutants of *Escherichia coli* unable to use fructose by means of the phosphoenolpyruvate/glycose phosphotransferase system mutate further to permit growth on that ketose by derepression of a manno(fructo)kinase (Mak$^+$ phenotype) present in only trace amounts in the parent organisms (Mak-o phenotype). The *mak* gene was located at min 8.8 on the *E. coli* linkage map as an ORF designated *yajF*, of hitherto unknown function; it specifies a deduced polypeptide of 344 aa. The derepression of Mak activity was associated with a single base change at position 71 (codon 24) of the gene, where GCC (alanine) in Mak-o has been changed to GAC (aspartate) in Mak$^+$. By cloning selected portions of the total 1,032-bp *mak* gene into a plasmid that also carried a temperature-sensitive promoter, we showed that the mutation resided in a 117-bp region that does not specify sequences necessary for Mak activity but was located 46 bp upstream of a 915-bp portion that does. Mak$^+$ and Mak-o strains differ greatly in the heat stability of the enzyme: at 61°C, *mak-o* cloned into a *mak-o* recipient loses 50% of its activity in approximately 6 min, whereas it takes over 30 min to achieve a similar reduction in the activity of *mak*$^+$ cloned into a *mak-o* strain. However, the Mak activity of the cloned fragment specifying the enzyme without the regulatory region lost activity with a half-life of 29 min irrespective of whether it was derived from a *mak*$^+$ or a *mak-o* donor, which indicates that the A24D mutation contributes to the high enzyme activity of Mak$^+$ mutants by serving to protect Mak from denaturation.

Enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* grow readily on a variety of hexoses, including fructose, as their sole source of carbon. Many of these sugars are taken up from the medium and concomitantly phosphorylated by the concerted action of specific membrane-spanning proteins and the phosphoenolpyruvate/glycose phosphotransferase system (PTS) (1); mutants devoid of PTS activity fail to grow on such sugars. However, further mutants of *S. typhimurium* (2) and *E. coli* (3) have been described that grow on fructose even though they are unable to use other PT sugars. In the latter organisms, fructose has been shown (3) to enter the cells by facilitated diffusion by means of an isoform of the membrane-spanning protein PtsG, which is normally the primary port for the PT-dependent entry of glucose; the internalized ketose subsequently is phosphorylated to fructose 6-phosphate by ATP and a fructo(manno)kinase (4).

In the present paper, we report the location and sequence of the gene specifying manno(fructo)kinase activity (Mak) on the linkage map (5) of *E. coli*-K12 and describe a mutation affecting the preferential synthesis and heat stability of this enzyme.

Materials and Methods

The strains of *E. coli* used in this work have been described (3). They were grown aerobically at either 30°C or 39°C, in liquid culture consisting of LB broth (25 g of LB base per liter of water) or in defined media (6), or on such media solidified with 2% agar. Procedures used for high-frequency recombination-mediated conjugations and phage-mediated transductions have been described (7). Manipulations of DNA fragments and agarose gel electrophoresis were performed as described (8). Genomic DNA was prepared with the aid of a QIAmp Tissue kit (Qiagen, Chatsworth, CA), following the manufacturer's instructions; VENT polymerase (New England Biolabs) was used in PCRs, and DNA thus amplified was gel-purified with a QIAquick Gel Extraction kit (Qiagen). Plasmid DNA was isolated and purified with a Wizard Plus SV Miniprep Purification system (Promega); restriction enzymes and T_4 DNA ligase were from New England Biolabs and used as recommended by the supplier.

Mak activity was measured in extracts of cells that had been disrupted by sonic oscillation and clarified by centrifugation for 5 min at 9,000 × *g*, by the procedure described in ref. 4.

Sequencing reactions were carried out with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer), as recommended by the manufacturer. Reaction products were purified on Centri-Sep spin columns (Princeton Separations, Adelphia, NJ). Samples were analyzed by the Boston University Sequencing Facility on an ABI 377XL DNA sequencer (Perkin–Elmer).

Results

Location of the Gene Specifying Mak Activity on the *E. coli* Genome. We have shown (3) that a mutant of *E. coli* (HK 1691) in which the normal PTS-linked modes of fructose utilization were abolished gave rise to a further mutant (HK 1787) that grew on fructose by a novel route, in which fructose entered the cells by facilitated diffusion, followed by its phosphorylation to fructose 6-phosphate in an ATP-linked reaction catalyzed by a highly active manno(fructo)kinase (4). We also showed (3) that the facilitated diffusion of fructose was effected by an isoform (PtsG-F) of the principal glucose transporter, the normally PTS-linked protein PtsG, in which a valine had been replaced by a phenylalanine (V12F), the gene specified by *ptsG* maps at min 24.9 on the *E. coli* linkage map (5). However, the gene specifying Mak activity had not been located on the *E. coli* genome.

To determine this location, phages were grown on the Mak$^+$ mutant HK 1787 and were used to infect a Mak-o recipient (HK 2140) that contained the PtsG-F isoform and carried deletions in *srlA*, *fruK* and *manXYZ*, as well as point mutations in *fruA* and *mtlA;* fructose-positive transductants were selected on medium containing this ketose at 40 mM. One of these F^- streptomycin-resistant transductants (HK 2148), which was found to be Mak$^+$, was crossed with a variety of high-frequency recombination (Hfr) strains, each of which was streptomycin-sensitive and contained the tetracycline resistance transposon Tn10 integrated at known sites and transferred early on (9); this crossing made it possible to select exconjugants on plates containing both streptomycin and tetracycline after transfer of relatively small regions of the genome. The greatest number of recombinants that had lost the ability to grow on fructose but that had retained the V12F mutation were obtained when the Hfr donor was strain BW 6160 (point of origin, approximately min 8, clockwise); such

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Abbreviation: PTS, phosphotransferase system.

*To whom reprint requests should be addressed at: The University Professors, 745 Commonwealth Avenue, Boston, MA 02215. E-mail: hlk@bu.edu.

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exconjugants had less than 10% of the Mak activity of strain HK 1787.

The gene specifying this enzyme was located more precisely by phage-mediated transductions. In these, phages P1 were grown on *E. coli* strains carrying Tn10 integrated at known sites (10) and were crossed with the Mak$^+$ strain HK 2148. The highest degree of cotransduction of tetracycline resistance and fructose negativity was found when the regions transduced were close to min 7.9 (12% linkage) and min 9.3 (39% linkage). When phages, grown on a transductant that had received Tn10 at min 9.3 but that had remained Mak$^+$, were crossed with the Mak-o strain HK2140, 44% of the resultant tetracycline-resistant transductants grew on fructose and contained a 30- to 40-fold enhanced Mak activity. This finding suggested that the *mak* gene was located close to *araJ*, which had been located at this position (11). This hypothesis was confirmed by crossing phages, grown on either of two different *E. coli* mutants in which the kanamycin-resistance transposon Tn9 was integrated into *araJ* (11), with Mak$^+$ recipients: in each case, over 90% of the kanamycin-resistant transductants had also lost over 90% of their Mak activity. However, as a significant number of such kanamycin-resistant transductants had retained the *mak*$^+$ gene, it was likely that Mak activity was specified not by *araJ* itself but by an ORF, such as *yajF*, very close to it.

Sequence Analysis. DNA prepared from the *yajF* region of a fructose-positive kannamycin-resistant transductant (HK 2193) and from its fructose-negative counterpart (HK 2194), amplified by PCR, was sequenced completely. The only difference detected between the sequence of *yajF* from *mak-o* [which was identical to the Blattner *yajF* sequence (12)] and that from *mak*$^+$ strains was that GAC in codon 24 of the former had been changed to GCC in the latter. This change in bp 71 would alter the identity of the deduced amino acid at position 24 of the Mak protein from alanine in Mak-o to aspartate in Mak$^+$. This A24D mutation was found also in the original Mak$^+$ strain HK 1787 and was absent in its parent Mak-o strain HK 1691.

Consequences of the A24D Mutation. To determine the effects of the A24D change on the properties of the Mak system, the whole of the ORF, and some portions thereof, were amplified by PCR from genomic DNA of Mak-o and Mak$^+$ cells and cloned into the pGSB7 plasmid (13), replacing the *ptsG* in that plasmid with the amplified material. This plasmid carries a temperature-sensitive promoter, which is inactive at or below 30°C and is active at 37°C-42°C. The various constructs derived from the otherwise isogenic Mak-o and Mak$^+$ strains were transformed into the identical Mak-o recipient; they are shown schematically in Fig. 1.

The Mak activity of construct C, derived from a Mak$^+$ donor and transformed into Mak-o cells (designated C$^+$/A-o), was low and independent of the temperature at which the transformed cells were grown (Table 1); moreover, the same result was obtained with the analogous construct from Mak-o cells (designated C-o/A-o). This finding showed that the region up to and including bp 269 did not specify Mak activity, irrespective of whether the A24D mutation was present in the cloned fragment.

In contrast, the whole *mak* region amplified and cloned into a Mak-o recipient from either Mak$^+$ or Mak-o donors (Fig. 1, construct A) gave rise to Mak activities much greater than exhibited even by Mak$^+$ organisms (Table 1), which can be

Table 1. Mak activities of chromosomal and plasmid extracts

Construct	Growth temperature, °C	Mak activity, nmol/min/mg·protein
Mak-o	39	0.26
Mak$^+$	39	17
A-o/A-o	30	60
A-o/A-o	39	380
A$^+$/A-o	30	390
A$^+$/A-o	39	350

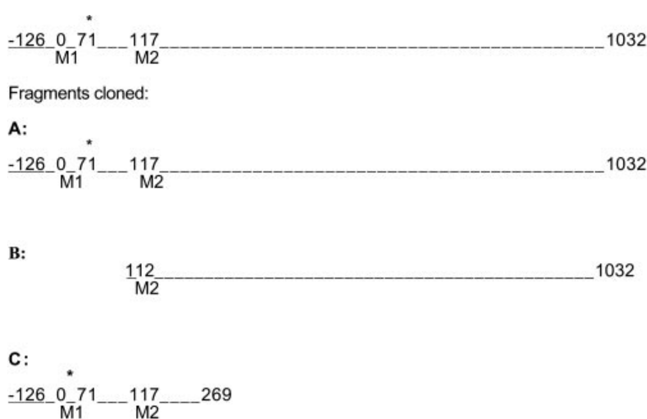

Fig. 1. The *mak* gene and its regions, cloned into pGSB7. The cloned regions comprised bp −126 to +1032, i.e., the whole *mak* gene and 132 bp upstream of the translational start site (*A*); bp 112 to bp 1032, including the codon specifying a second *met* but excluding bp 71 (*B*); bp −126 to bp +269, including the regulatory site (bp71) and codons for both *met1* and *met2* but truncating the *mak* gene (*C*). The location of the mutation at bp 71 is marked by *. The size of each construct was confirmed by restriction mapping and by sequencing of both strands of the cloned DNA. M1 and M2 indicate the locations of two methionines that act as starts of the regulatory (M1) and enzymic (M2) transcription sites of the *mak* gene.

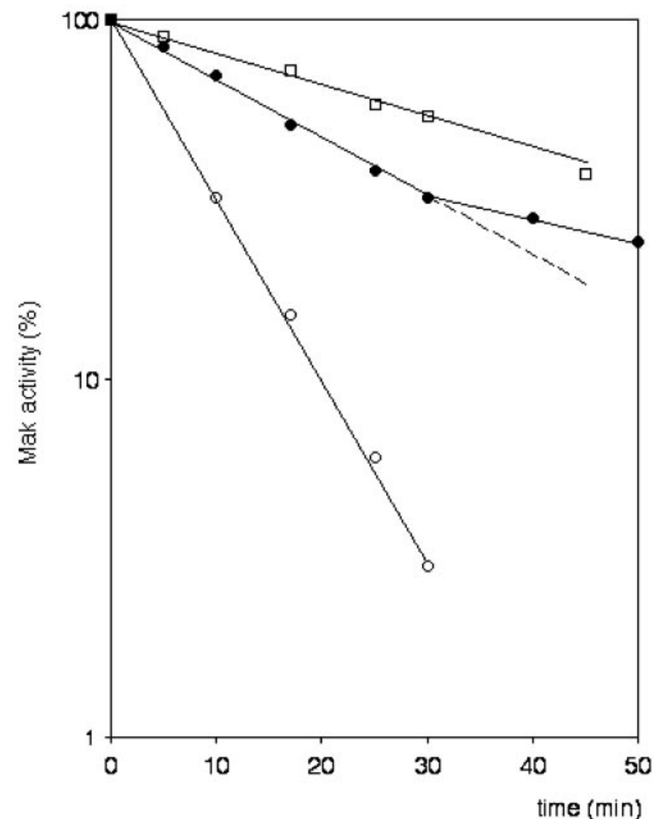

Fig. 2. Stability of Mak activity at 61°C expressed by cloned *mak*$^+$ (A$^+$/A-o; Π), *mak-o* (A-o/A-o; o), and a 1:1 mixture of the two (●).

ascribed at least in part to the presence of the plasmid in multiple copies. However, when (after growth at 30°C) the plasmid promoter was inactive and Mak was expressed solely under the influence of the chromosomal promoter, the Mak activity of (A-o/A-o) transformed cells was considerably lower than it was in the same organism after growth at 39°C when both promoters were active. On the other hand (A^+/A-o), cells exhibited very high Mak activities both after growth at 30°C and at 39°C, which indicated that the A24D mutation led to an increase in the amount of the enzyme elaborated and/or the properties of that enzyme.

To distinguish between these alternatives, two types of experiment were performed. In one, the K_m values for fructose and for 2-deoxyglucose were measured in extracts containing the whole *mak* gene from either *mak-o* or mak^+ cells cloned into plasmid pGSB7 and transformed cloned into *mak-o* recipients (Fig. 1, construct A): no differences were detected between these parameters. In a second experiment, region B (Fig. 1) was thus cloned and transformed. As expected, the presence of either construct (B^+/A-o) or (B-o/A-o) enabled the recipient cells to grow on fructose at 39°C, when the plasmid promoter was active, and to exhibit high Mak activity, but neither construct elicited Mak activity at 30°C, when the plasmid promoter was inactive. That neither of the B constructs carried the A24D mutation at bp 71 supports the view that the enzymic activity is wholly specified by the stretch of DNA downstream of the mutation at bp 71 and is encompassed by the region bp 122 to bp 1032.

Another striking consequence of the A24D mutation is that it profoundly affects the heat stability of Mak (Fig. 2). Although the activity of this enzyme in extracts of Mak-o cells is too low to permit precise measurement, it sufficed to show that such extracts lost virtually all of their Mak activity when kept for 30 min at 61°C, whereas the enzymic activity of extracts derived from Mak^+ cells was reduced by only approximately 50% under these conditions. This difference was strikingly exhibited also by Mak-o cells containing the cloned DNA fragments: at 61°C, constructs (A-o/A-o) lost 50% of their Mak activity in 6–8 min, whereas it took 30 min to effect a similar reduction in the constructs (A^+/A-o), containing the whole mak^+ gene (Fig. 2). Similarly, both (B^+/A-o) and (B-o/A-o) constructs, in which the plasmid-borne promoter controlled the expression of the DNA region specifying only the enzymic activity, lost Mak activity at 61°C at identical rates of 29 min, which further supports the view that the properties of the Mak enzyme per se are the same, irrespective of whether it is present in derepressed and repressed cells. It appears therefore that the replacement of alanine by aspartate at position 24 of the polypeptide exerts a regulatory effect on the Mak activity expressed accompanied by a significant protection against heat denaturation. The mechanisms of these effects remain to be elucidated.

It is of interest that, although no "normal" substrate for the Mak activity in *mak-o* cells has yet been found, there are a number of proteins in various microorganisms that exhibit considerable degrees of sequence similarity with *mak* DNA. In particular, a family of proteins (designated the ROK family, an acronym for "repressor, ORF, kinase") has been described (14) that includes XylR xylose repressors from bacilli and kinases that catalyze the phosphorylation of fructose from *Zymomonas mobilis* (15) and *Streptococcus mutans* (16) and of glucose from *Streptomyces coelicolor* (17). However, to the best of our knowledge, the only homologous protein in *E. coli* whose physiological role has been established is the *N*-acetylglucosamine repressor NagC (18).

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