Characterization of the Product of the Cloned fdhF Gene of Escherichia coli

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Random insertions of mini-MudI1734 lac gene fusion bacteriophage were constructed on plasmid pLW06, which carries the fdhF gene coding for benzyl-viologen-linked formate dehydrogenase. They allowed us to limit the size of the gene to a 3 kb fragment and to define its direction of transcription. The identification of the fdhF product as a 85 kDa protein was achieved after expression of derivative plasmids in a maxicell system.

INTRODUCTION

The facultative anaerobic bacterium Escherichia coli K12 can derive energy for growth from the mixed acid fermentation of glucose, which generates formic acid in addition to succinic, lactic and acetic acids. Acidification of the medium results in the synthesis of the formate hydrogenlyase system that converts formate to carbon dioxide and molecular hydrogen (Peck & Gest, 1957). It consists of a benzyl-viologen-linked formate dehydrogenase (EC 1.2.1.-; FDH-BV) which catalyses the oxidation of formate (Ruiz-Herrera & Alvarez, 1972) and of the hydrogenase 3 responsible for the evolution of hydrogen (Sawers et al., 1985). The first step of this pathway involves the product of the structural gene fdhF. It is located at 93 min on the E. coli linkage map (Pecher et al., 1985) and is transcribed counterclockwise on the chromosome (Wu & Mandrand-Berthelot, 1987). Its expression is induced by formate and repressed by nitrate and trimethylamine N-oxide. It is also sensitive to positive control exerted by several fdh and hyd genes participating in the formate hydrogenlyase route (Wu & Mandrand-Berthelot, 1987).

We have previously cloned the fdhF gene into the plasmid vector pBR322. It was isolated on a 5-5 kb BamHI–PvuII DNA fragment (Fig. 1) (Wu & Mandrand-Berthelot, 1986a). Here we report a more precise location of the fdhF gene and its expression in maxicells.

METHODS

Bacterial strains, phage, plasmids and culture media. The strains of E. coli K12 used were: HY15 (proA23 trp-30 his-51 lac-28 rpsL10I fdhF15), S148F [ara-2 xyl malB mtl rpsL Δ(fdhF148-mel)], FD710 [araD139 Δ(lac) U169 thi rpsL Δ(fdhF71)], a heat-induction deletion derivative of mutant strain FD71, fdhF::MudI(ApR, lac) (Wu & Mandrand-Berthelot, 1986a) and CSR603 (thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 wcrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 λ− supE44) (Sancar et al., 1979) provided by B. Bachmann. Mini-MudI1734 bacteriophage lysates were obtained by induction at 42°C of the Mucts and mini-MudI lysogenic strain POII1734TR (Castilho et al., 1984). Plasmids pLW06 and pLW10 (Wu & Mandrand-Berthelot, 1986a) are pBR322 (bla+, tet+) derivatives (Bolivar et al., 1977).

Media and growth conditions were as described by Wu & Mandrand-Berthelot (1986b). When needed, ampicillin and kanamycin were added to final concentrations of 50 μg ml⁻¹ and 20 μg ml⁻¹ respectively.

Insertions of mini-MudI on plasmid pLW06. Random insertions of mini-MudI1734 were constructed by the procedure of Castilho et al. (1984). Strain POII1734TR, lysogenic for both Mucts and mini-MudI1734 (KmR, lac), was transformed by pLW06 (ApR). One of the ApR transformants selected at 30°C was used to produce a Mu lysate by heat induction. In this lysate, some Mu particles contained a MudI1734 inserted on pLW06. They were recovered by transducing mutant HY15 and selecting ApR KmR clones. Transductants were then checked for

Abbreviation: FDH-BV, benzyl-viologen-linked formate dehydrogenase

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FDH-BV activity by dye overlay test (Mandrand-Berthelot et al., 1978). They were also scored for β-galactosidase activity: clones were grown anaerobically on glucose plates, treated with toluene and covered with a solution of 4 mg o-nitrophenyl β-D-galactoside ml⁻¹ (Miller, 1972). To determine the location and orientation of the insertions, the resulting fusion plasmids were cleaved by EcoRI, HindIII, BII and PvuII restriction endonucleases.

In vitro manipulation and maxicell technique. Procedures for isolation of DNA, restriction analysis, ligation and transformation have been listed previously (Wu & Mandrand-Berthelot, 1986a). Plasmid-containing cultures were labelled by the 'maxicell' procedure of Sancar et al. (1979). Identical patterns were obtained with anaerobically or aerobically grown cells.

Enzyme assays. FDH-BV activity was scored on MacConkey/formate/fumarate medium (Wu & Mandrand-Berthelot, 1986b) and by dye overlay screening (Mandrand-Berthelot et al., 1978). FDH-BV and β-galactosidase activities were assayed spectrophotometrically as described earlier (Wu & Mandrand-Berthelot, 1986b).

RESULTS AND DISCUSSION

Mini-Mu insertions into pLWO6 and location of the fdhF promoter–operator region

We have previously reported the cloning of the fdhF gene in plasmid pBR322 (Wu & Mandrand-Berthelot, 1986a). In order to determine more precisely its position on the pLWO6 map and the location of its operator–promoter sites, we took advantage of the use of mini-MuIll1734 bacteriophage to select random Mu insertions and lac gene fusions in this plasmid, according to the method of Castilho et al. (1984) (see Methods). A total of 231 ApRKmR clones were recovered by transducing strain HY 15 fdhF with a mini-MuIll1734 lysate induced from strain POII1734TR transformed by pLWO6; 44 of these clones were devoid of FDH-BV activity. This result indicated that plasmid pLWO6 had lost its complementation ability due to the integration of miniMuIll into the fdhF gene. If Mu insertions are distributed at random around the plasmid and do not affect the ori region and the bla gene, the percentage of FDH-BV- insertions (19%) allows us to estimate the size of the fdhF gene at about 2 kb. Among the 44 FDH-BV- transductants, 7 exhibited β-galactosidase activity as detected by an in situ dye test on anaerobically grown colonies. Therefore, in these latter strains, MudIIlac transcription–translation fusions occurred in the proper orientation and in the correct reading phase from the promoter of fdhF. Moreover, the frequency of Lac+ insertions observed in the fdhF gene (16%) agreed well with that expected for MudII translation fusions (Castilho et al., 1984).

The plasmid content of the 44 FDH-BV- clones and of 17 FDH-BV+ clones was extracted, purified and digested by BglII, EcoRI, HindIII and PvuII in order to determine the exact position and the orientation of the insertion (Fig. 1). The 44 FDH-BV- insertions were distributed on a 2.5 kb DNA segment within which no FDH-BV+ insertion was observed. The two nearest FDH-BV+ insertions bordering this fragment were located at approximately 0.1 kb from the left side (810) and 0.6 kb from the right side (472). As a consequence, the size of the fdhF gene was assumed to be smaller than 3.2 kb. All the Lac+ FDH-BV- clones carried insertions orientated in the same direction, corresponding to a transcription of gene fdhF from the BamHI-2 site to the BamHI-1 site. In addition, insertions 830 (Lac+, FDH-BV+) and 472 (Lac-, FDH-BV+) allowed us to locate the fdhF promoter–operator region between these two sites on a 1.2 kb fragment (Fig. 1). According to the previously reported orientation of transcription of fdhF from melA to malB on the E. coli chromosome (Wu & Mandrand-Berthelot, 1987), the BamHI-1–PvuII-1–PvuII-2–BamHI-2 restriction map order of pLWO6 must lie clockwise on the genome.

In a previous paper (Wu & Mandrand-Berthelot, 1986a) we reported that plasmid pLW10, which carries the 5.5 kb BamHI-1–PvuII-2 insert (Fig. 1), was able to complement fdhF mutations in strains HY15 (fdhF15), S148F [Δ(fdhF148–mel)] and FD710 (ΔfdhF71). This finding suggested that these mutations might affect the same gene fdhF. However, pLW10 is large enough to complement several mutations lying in closely linked genes. In order to solve this problem, the complementation pattern of plasmid pLWO6 harbouring various MudII insertions was analysed in strains HY15, S148F and FD710 (Fig. 1). Mu insertion 827 prevented the ability of plasmid pLWO6 to complement all these mutants. As this insertion was located at the
beginning of gene fdhF it could induce a polar effect on distal genes within the same operon, but this possibility was ruled out by examination of the effect caused by the two other Mu integrations situated upstream (472) and downstream (810), outside gene fdhF in plasmid pLWO6 (Fig. 1). Both still permitted the complementation of those three mutations, supporting the notion that they belong to the same gene.

At the time this work was achieved, we became aware of a publication from Böck's laboratory relating the cloning and the determination of the nucleotide sequence of the fdhF gene (Zinoni et al., 1986). The open reading frame encoding the 80 kDa selenopolypeptide of FDH-BV was shown to reside on a DNA region containing a 1.4 kb KpnI–BglII restriction fragment. Plasmid pLWO6 was also found to contain the 0.27 kb KpnI–KpnI, 0.87 kb KpnI–BglII and 0.24 kb BglII–BglII fragments lying on this restriction fragment, indicating the identity of the two independently cloned regions. Moreover the size and the accurate localization of the open reading frame derived from a 6.2 kb BamHI insert corresponding to that drawn in Fig. 1 were compatible with the limits of the fdhF gene determined by mini-Mu transposition mutagenesis.

Expression of the cloned fdhF gene

To identify the fdhF gene product, plasmids pLWO6, pLW10 and pLWO6 carrying the Mu 827 insertion (Lac+) were introduced and expressed in the maxicell strain CSR603. Plasmid pBR322, the vector used for constructing pLWO6, was included as a control. In all cases, two major proteins with molecular masses of 29 and 31 kDa were observed (Fig. 2). They represented the mature β-lactamase and its precursor form respectively. The pLWO6 and pLW10 patterns contained an additional specific protein of apparent molecular mass 85 kDa, which is likely to represent the polypeptide encoded by fdhF. Final evidence for this suggestion came from the observation that the Mu 827 insertion into the fdhF gene eliminated the 85 kDa band from the pLW06-encoded proteins. Instead, a polypeptide with a molecular mass of about
138 kDa was detected. It was attributed to the synthesis of a hybrid β-galactosidase resulting from Mu lac gene fusion in plasmid pLWO6 and containing the N-terminal part of the FDH-BV polypeptide. These results are in good agreement with the 80 kDa molecular mass previously demonstrated for the selenopolypeptide of FDH-BV, following selenium labelling of either a truncated polypeptide synthesized from an fdhF::MudI mutant (Pecher et al., 1985) or wild-type, deleted and fused proteins encoded by various fdhF plasmids (Zinoni et al., 1986).

As we reported above, β-galactosidase activity was detected in seven derivatives of plasmid pLWO6 carrying mini-Mu lac insertions located at different sites in the fdhF gene (Fig. 1). The regulation of β-galactosidase synthesis was analysed in three of them (Table 1: p469, p827 and p830). In all cases, β-galactosidase activity was induced under anaerobic conditions. It was enhanced upon addition of formate and repressed by nitrate, demonstrating that transcriptional and translational signals belonged to the fdhF gene. As a control, plasmid p810, which carries a mini-Mu Lac+ insertion outside the fdhF gene, did not show any significant difference in β-galactosidase activity under the various growth conditions tested (Table 1). In addition, β-galactosidase activities measured in fusion plasmid p469 were about four times lower than those found in plasmids p827 and p830. This difference could be due to the fact that an increase in the size of the FDH-BV part of the chimeric protein reduces the stability of the quaternary structure of β-galactosidase. From our results, it appears that the expression of insertions in the multicopy plasmids was similar to that reported for a single-copy chromosomal fusion (Wu & Mandrand-Berthelot, 1987). This observation correlates with the absence of enhancement of FDH-BV activity by plasmid pLW06 (Wu & Mandrand-Berthelot, 1986a), which could be attributed to the strict control of the fdhF expression by regulatory molecules.
Table 1. Expression of mini-Mu lac gene fusions in plasmid pLW06

<table>
<thead>
<tr>
<th>Mu lac fusion plasmid</th>
<th>Aerobic growth + formate (30 mM)</th>
<th>Anaerobic growth No effector + Formate (30 mM) + Nitrate (100 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p469</td>
<td>17</td>
<td>46  88  54</td>
</tr>
<tr>
<td>p827</td>
<td>11</td>
<td>225 368 36</td>
</tr>
<tr>
<td>p830</td>
<td>22</td>
<td>216 357 44</td>
</tr>
<tr>
<td>p810</td>
<td>42</td>
<td>66  59  42</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>12  12  11</td>
</tr>
</tbody>
</table>

The hybrid proteins encoded by the fdhF-lacZ gene of plasmids p827, p830, and p469 would be expected to have a molecular mass greater than the monomer of β-galactosidase (116.5 kDa) and should be easily identified from crude extracts in polyacrylamide gels. Strain HY15 carrying fusion plasmid p827 produced a polypeptide with a molecular mass (135 kDa) close to that detected in the maxicell experiment under anaerobic conditions in the presence of formate (data not shown). Since its synthesis was repressed by nitrate, we concluded that it was the product of the fdhF-lacZ fusion. We observed a similar size for the hybrid β-galactosidase isolated from fusion plasmid p830, although these two insertions were separated by a distance of 0.4 kb (Fig. 1). This finding indicated that large-size hybrid fdhF-lacZ proteins were not very stable.

Experiments are in progress to purify hybrid β-galactosidase proteins encoded by fdhF-lacZ fusions in order to compare the N-terminal amino acid sequence with that deduced from the nucleotide sequence of gene fdhF (Zinoni et al., 1986). Moreover their purification could allow us to raise antisera against the FDH-BV part of the hybrid protein, which would be a useful tool to follow purification of the labile FDH-BV enzyme.

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