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The *mvrA* locus of *Escherichia coli* does not encode a ferredoxin–NADP⁺ reductase

Ferredoxin–NADP⁺ reductases (FNRs) are FAD-containing enzymes found in bacteria, plastids and mitochondria that catalyse the reversible electron transfer between NADP(H) and ferredoxin or flavodoxin (1). They are involved in a wide variety of redox metabolic pathways in different tissues and organisms, functioning as a universal class of electronic transducers between obligatory one- and two-electron carriers (1). The isoform present in *Escherichia coli*, encoded by the *fpr* gene (2), is a member of the *soxRS* regulon, and is therefore involved in the concerted cell response towards superoxide intoxication (7).

In 1988, Morimyo (8) selected a number of mutagenized *E. coli* strains that were abnormally sensitive to the superoxide-propagating compound methyl viologen (MV). One of these strains was used as a host in complementation experiments to clone a gene (named *mvrA*) that was able to rescue the mutant cells from killing by MV (8). Further research showed that, despite some frameshift errors in the published sequence, the complementing gene was clearly related to *E. coli* FNR, raising the possibility that the *mvrA* locus could encode a ferredoxin–NADP⁺ reductase (2, 7). Indeed, expression of a plasmid-borne plant FNR restored the wild-type phenotype, including MV tolerance, to *mvrA* mutant bacteria (5), while *E. coli* cells in which the *fpr* gene had been knocked-out by insertional mutagenesis became hypersensitive to this redox cycling compound (3).

However, several observations challenged the validity of the previous assignment. Bianchi *et al.* (2) showed that the *mvrA* and *fpr* loci were located at different positions in the *E. coli* chromosome. Also, the phenotypes displayed by the two mutant strains were markedly different (3, 6, 8), although this discrepancy could be attributed to the different genetic backgrounds and mutagenesis procedures employed to generate them (3, 8). In order to solve this question, we undertook a reassessment of the relationship between these loci, by determining the expression and activity levels of FNR in *mvrA*

Fig. 1. Expression of ferredoxin–NADP⁺ reductase in different *E. coli* strains. FNR immunoblots were carried out on *E. coli* lysates as described in the text. Cells from strains MC48 (lanes 1 and 2), *mvrA* (lanes 3 and 4) and *fpr* (lanes 5 and 6) were challenged (lanes 2, 4 and 6) or not (lanes 1, 3 and 5) with 0.2 mM MV prior to rupture and analysis. Lane 7, 0.2 μ g *E. coli* FNR.

and *fpr* mutant cells under different growth regimes.

The genotypes and culture conditions of the *fpr*, *mvrA* and MC48 strains are described by Krapp *et al.* (6). To induce the *soxRS* response, bacterial cultures grown to the early exponential phase (OD_{550} ~0.3) were incubated for 1 h at 37 °C in Luria–Bertani broth containing 0.2 mM MV (7). Cells were then collected, washed and ruptured by sonic oscillation (6). The presence of FNR in the various *E. coli* strains was analysed by SDS-PAGE and immunoblotting (6). Bacterial lysates were separated on 12 % polyacrylamide gels, transferred to nitrocellulose membranes and immunodecorated with rabbit antisera raised against *E. coli* FNR (3). FNR activity in cleared lysates was determined by measuring the diaphorase reaction, namely, the ability to catalyse NADPH oxidation by artificial electron acceptors (1). In order to identify individual enzymes displaying diaphorase activity, *E. coli* extracts were resolved by electrophoresis in non-denaturing 10 % polyacrylamide gels, and stained for activity using nitro blue tetrazolium as electron acceptor (4).

The MC48 strain, from which the *mvrA* mutants were derived (8), was used as an FNR-proficient control. Lysates from these bacteria show a single protein species reacting toward FNR antisera (Fig. 1, lane 1), whose levels were increased ~20-fold upon a brief challenge with MV (Fig. 1, lane 2). FNR could not be detected in the soluble fractions obtained from *fpr* cells, even after MV treatment (Fig. 1, lanes 5 and 6), as expected from a bona fide FNR-deficient strain (3). At variance, the FNR patterns observed in *mvrA* mutant cells were similar to those displayed by the parental strain MC48 (Fig. 1, lanes 3 and 4), indicating that the enzyme was expressed in the mutant bacteria, and readily induced in the course of the *soxRS* response.

These experiments cannot rule out the possibility that the FNR expressed in *mvrA* cells might be inactive. Indeed, the procedure employed to generate the mutants, random

Fig. 2. Evaluation of the FNR-dependent diaphorase activity of *E. coli* lysates. Extracts were resolved by non-denaturing PAGE and analysed by activity staining (a) or FNR immunoblotting (b). *E. coli* cells from the MC48 (lanes 1 and 2), *mvrA* (lanes 3 and 4) and *fpr* strains (lane 5) had been treated (lanes 2, 4 and 5) or not (lanes 1 and 3) with MV as indicated in the text. The positions of the front dye and FNR are indicated by arrows on the left and right sides, respectively.

mutagenesis with ethyl methanesulfonate (8), is known to produce mostly point mutations that could inactivate the enzyme without affecting its expression. To assess this possibility, soluble extracts from the different *E. coli* strains were resolved in duplicate lanes by non-denaturing PAGE. Half of the gel was stained for diaphorase activity, and the replicate portion was immuno-blotted. The results are summarized in Fig. 2.

Four different molecular species displaying NADPH-dependent diaphorase activity could be resolved by this procedure (Fig. 2a, lane 1), presumably corresponding

to the four diaphorases identified by Liochev and co-workers using ion-exchange chromatography (7). Comparison of the patterns exhibited by *fpr* and MC48 lysates suggests that FNR corresponds to the band with the higher electrophoretic mobility (Fig. 2a, lanes 1, 2 and 5), as confirmed by reaction with FNR antisera (Fig. 2b, lane 2). A protein comigrating with FNR could be detected by activity staining in *mvrA* cells (Fig. 2a, lane 3). This protein was strongly expressed in response to MV exposition (Fig. 2a, lane 4), and was recognized by antibodies raised against *E. coli* FNR (Fig. 2b, lanes 3 and 4). Taken together, these results indicate that the *mvrA* mutant strain contains a fully active ferredoxin-NADP⁺ reductase.

The lesions that cause the *mvrA* phenotype are therefore not related to FNR inactivation, but their effects can be bypassed by expression of an active reductase from either bacterial (8) or eukaryotic origin (5, 6). Participation of FNR in the antioxidant response of *E. coli* is well documented (3, 5–7), but its mechanism of action remains obscure. The ability of this enzyme to suppress the deleterious effects of an unrelated mutation suggests that it may be playing a general protective role in the metabolic context of the stressed cell.

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