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# Evolution of an *Escherichia coli* Protein with Increased Resistance to Oxidative Stress*

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**L-1,2-Propanediol:NAD$^+$ 1-oxidoreductase of *Escherichia coli* is encoded by the *fucO* gene, a member of the regulon specifying dissimilation of L-fucose. The enzyme normally functions during fermentative growth to regenerate NAD from NADH by reducing the metabolic intermediate L-lactaldehyde to propanediol which is excreted. During aerobic growth L-lactaldehyde is converted to L-lactate and thence to the central metabolite pyruvate. The wasteful excretion of propanediol is minimized by oxidative inactivation of the oxidoreductase, an $Fe^{2+}$-dependent enzyme which is subject to metal-catalyzed oxidation (MCO). Mutants acquiring the ability to grow aerobically on propanediol as sole carbon and energy source can be readily selected. These mutants express the *fucO* gene constitutively, as a result of an IS*5* insertion in the promoter region. In this study we show that continued selection for aerobic growth on propanediol resulted in mutations in the oxidoreductase conferring increased resistance to MCO. In two independent mutants, the resistance of the protein was respectively conferred by an $Ile^7 \rightarrow Leu$ and a $Leu^8 \rightarrow Val$ substitution near the NAD-binding consensus amino acid sequence. A site-directed mutant protein with both substitutions showed an MCO resistance greater than either mutant protein with a single amino acid change.**

Oxidative damage to macromolecules is an inescapable price for the evolution of aerobic metabolism. The co-evolution of protective agents, both catalytic (enzymes such as superoxide dismutases and catalases) and stoichiometric (antioxidants such as glutathione and $\alpha$-tocopherol), can at best reduce the magnitude of damage. Evolution of active mechanisms of repair apparently is limited only for DNA, probably because of chemical feasibility and strong selective pressure. For other kinds of damaged macromolecules clearing by turnover seems to be the only option. A possible exception is the repair of oxidized methionine residues on the surface of proteins by a specific sulfoxide reductase (1). The replacement strategy seems satisfactory for the perpetuation of unicellular organisms, although it is not always available or adequate for multicellular organisms. For instance, accumulation of oxidatively damaged proteins is often associated with senescence and various disease states (2–4).

A significant fraction of protein damages is thought to result from the metal-catalyzed oxidation system (MCO)[1] mediated by reactive species such as hydrogen peroxide, as outlined in Reactions 1–4 (2, 4, 5).

$$NAD(P)H + H^+ + O_2 \xrightarrow{MCO} H_2O_2 + NAD(P)^+$$

$$NAD(P)H + 2Fe^{3+} \xrightarrow{MCO} 2Fe^{2+} + NAD(P)^+ + H^+$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + HO^{\cdot}$$

$$HO^{\cdot} + \text{protein} \longrightarrow \text{Covalently damaged protein}$$

REACTIONS 1–4

$H_2O_2$ is formed routinely by monooxygenation reactions and occasionally by autoxidation of flavo-dehydrogenases, when an element in the electron transport chain is rate-limiting. Cationic iron is maintained in the ferrous state by reducing compounds, such as NADH or NADPH. The presence of $H_2O_2$ and $Fe^{2+}$ generates, by the Fenton reaction, a highly reactive $HO^{\cdot}$ (hydroxyl radical) which can covalently attack an amino acid residue.

When the iron is bound to a protein, the $H_2O_2$-dependent redox cycling of $Fe^{2+}$ to $Fe^{3+}$ is thought to proceed in a "cage," thus allowing the hydroxyl radical to extract an H atom from a local amino acid residue, before diffusing into the surrounding medium (6–9). Such a model would account for the limited number of amino acid residues that are susceptible to the damage, with each protein exhibiting a distinctive target signature of residues. The model is also supported by the evidence of substrate protection against oxidative damage (6, 8, 10). Although Arg, Cys, His, Lys, Met, and Pro residues are most susceptible to metal-catalyzed destruction, only the oxidation of Arg, Pro, His, and Lys has been reported to result in the formation of a carbonyl derivative which provides a means for monitoring the protein oxidation process (2, 11).

L-1,2-Propanediol:NAD$^+$ 1-oxidoreductase of *Escherichia coli* is an $Fe^{2+}$-dependent enzyme that normally functions as a reductase in a fermentation pathway for the dissimilation of L-fucose or L-rhamnose (12). This enzyme, inducible by either of the methyl pentoses, is inactivated during aerobic growth (9,

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[1] The abbreviations used are: MCO, metal catalyzed oxidation; kb, kilobase pair(s).

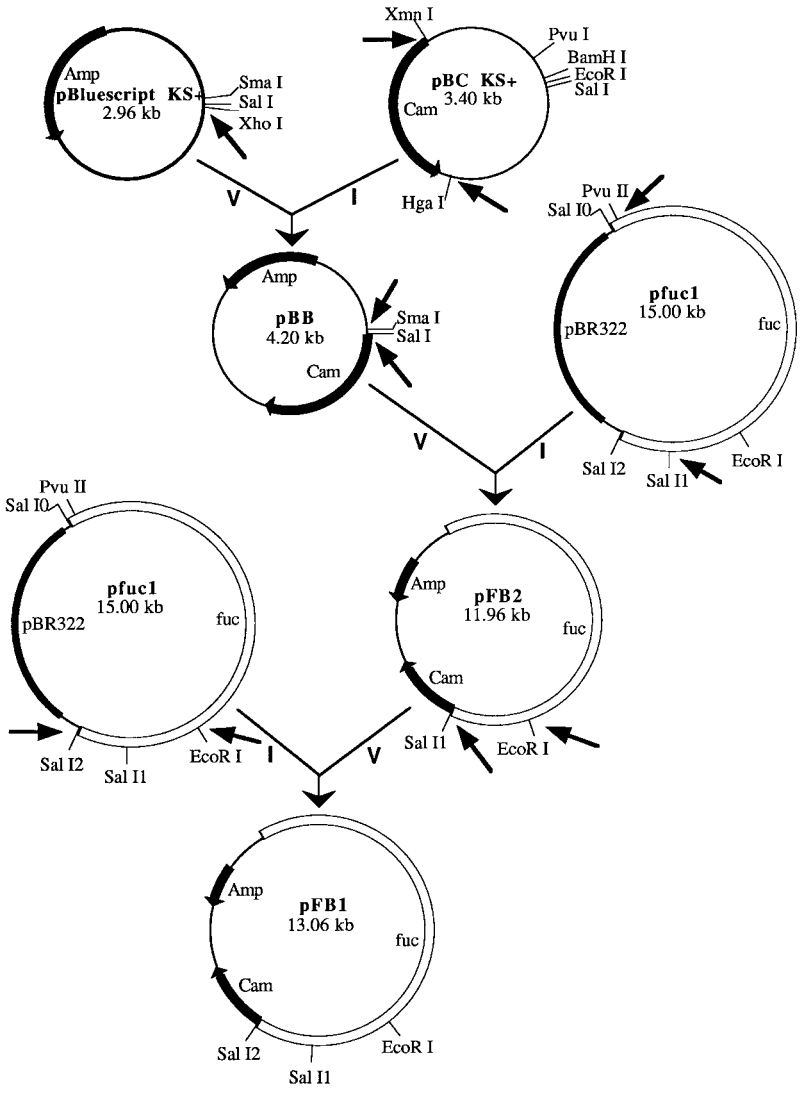


FIG. 1. **Cloning of the *fuc*$^+$ regulon into the shuffling vector.** *V*, vector fragment; *I*, insert fragment. *Arrows* indicate the digestion sites used in generating the fragments for ligations. See "Experimental Procedures."

13, 14). In this study we tried to find out whether repeated selection of mutants that utilize this enzyme exclusively as a dehydrogenase for aerobic growth, on propanediol as the sole carbon and energy source, would result in an altered protein resistant to oxidative inactivation.

## EXPERIMENTAL PROCEDURES

*Sequencing the fucO Gene of Strains ECL1, ECL3, ECL56, ECL421, ECL430, and ECL459*—The oligonucleotide primers 5′-CGGATCCG-GCATTATCACATCAG and 5′-CGAATTCAAGAGTAATTTCGTAAAGC flanking the coding region of *fucO* (15, 16) were synthesized and used to amplify by polymerase chain reaction the gene of each strain. A sample of each amplified product was digested with restriction enzymes to give five overlapping fragments that were then subcloned into pBluescript vectors (Stratagene, La Jolla, CA). Each of these fragments was sequenced for both strands with the T3 and T7 primers (Stratagene, La Jolla, CA) by the dideoxy method.

*Selecting an E. coli Strain with Complete Deletion of the Fuc Sequence*—Cells with deletions in the *fuc* locus were selected from strain ECL330 bearing a *fuc*::Mu d1-amp$^r$ insertion by growth at a nonpermissive temperature (42 °C) that allows the growth of only Mu dl$^-$ cells. Clones with a Mu dl$^-$ Amp$^s$ Fuc$^-$ phenotype were identified by growth at 42 °C on MacConkey/fucose medium and then purified by growing at 37 °C on the same medium. The deletion ranges in these cells were screened by Southern blots probed with a set of *fuc* fragments spanning the entire region of the *fuc* regulon (17). A strain completely deleted in the *fuc* regulon was thus identified and designated as ECL733.

Cells of the Δ*fuc* strain ECL733 were used as hosts for introducing by λ vectors single copies of the *fuc* regulon, each bearing a distinctive *fucO* allele. Detailed procedures are described below.

*Generating Mutant Alleles of fucO*—The *fucO*$^{\text{Ile-7}\rightarrow\text{Leu}}$ and *fucO*$^{\text{Leu-8}\rightarrow\text{Val}}$ mutations were regenerated by site-directed mutagenesis (18) of the wild-type sequence. A *fucO* allele with the above two mutations combined, *fucO*$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$, was created in parallel. A 5-kb *Sal*I$^o$-*Bam*HI fragment cut from pfuc16 (19) containing the wild-type *fucO* allele was subcloned into an M13 mp18 vector (Stratagene, La Jolla, CA), and the DNA was used to transfect CJ236 cells (Bio-Rad). Single-stranded M13 DNA was then prepared from the cells, hybridized to the oligonucleotide primers containing the desired point mutations (ordered from Oligos Etc., Inc., Guilford, CT), and used as template for synthesizing the second DNA strand. The duplex products were used to transfect XL1 cells (Stratagene, La Jolla, CA). A plaque from each transfection was purified, and a *fucO* fragment in the phage DNA was sequenced to confirm the nucleotide substitution(s). The phage DNA containing the mutated *fucO* alleles was respectively designated ϕFM5/*O*$^{\text{Ile-7}\rightarrow\text{Leu}}$, ϕFM5/*O*$^{\text{Leu-8}\rightarrow\text{Val}}$, and ϕFM5/*O*$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$.

*Cloning the Entire Wild-type Fuc Regulon into a Shuffling Vector*—As shown in Fig. 1, a modified pBluescript plasmid was used as an intermediary shuffling vector for carrying *fuc* sequences onto λ vector. The modification was made by inserting a *cam*$^r$ gene cut from a pBC KS$^+$ plasmid (Stratagene, La Jolla, CA) into the pBluescript polylinker region, to place the cloned *fuc* sequence close to a selectable marker for subsequent insertion into the chromosome via λ vectors (see below). The resulting plasmid was designated pBB. The wild-type *fuc* regulon (about 9 kb) was cloned into pBB in a two-step procedure as follows: step 1, an approximately 8-kb *fuc* fragment *Pvu*II-*Sal*I$^1$ was cut from pfuc1 containing the entire *fuc* regulon (19) and inserted into pBB; and step 2, the remaining 1-kb *fuc* fragment Sa1I$^1$-Sa1I$^2$ was appended to the insertion by substituting the *Eco*RI-*Sal*I$^1$ fragment in the first insertion with the *Eco*RI-*Sal*I$^2$ fragment cut from pfuc1. The resulting

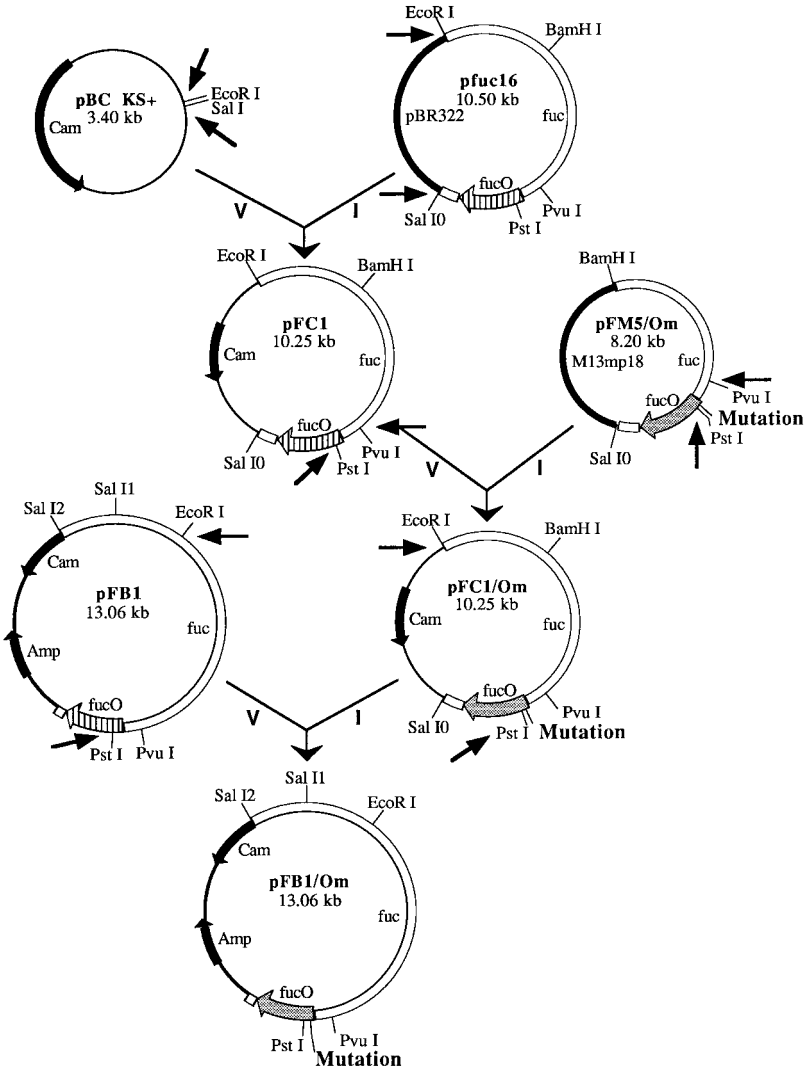


FIG. 2. **Cloning *fuc* regulons with wild-type and mutated *fucO* alleles into shuffling vectors.** Various *fuc* regulons bearing different *fucO* alleles were cloned into pFB1 vectors. *Om* represents $O^{Ile\text{-}7\rightarrow Leu}$, $O^{Leu\text{-}8\rightarrow Val}$, or $O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$. *V*, vector segment; *I*, insert fragment. *Arrows* indicate the digestion sites used in generating the fragments for ligations. See "Experimental Procedures."

plasmid was designated pFB1. For the propagation of various plasmids, XL1 cells were used.

*Cloning Fuc Regulons with the Mutated fucO Alleles into the Shuffling Vector*—The mutant *fuc* regulons were cloned into the shuffling vector by substituting the wild-type *fucO* sequence in pFB1 with the mutated counterparts. This procedure was carried out in eight steps, illustrated in a condensed manner in Fig. 2. Step 1: a pBC KS$^+$ plasmid was modified by destroying the single *Pvu*I restriction site with the Klenow enzyme and ligase, resulting in a second shuffling vector pBC$^*$ (not shown). Step 2: a 7-kb *fuc* fragment *Sal*I$^0$-*Eco*RI was cut from pfuc16 and inserted into pBC$^*$, resulting in pFC1. Step 3: a 2.2-kb fragment *Bam*HI-*Bam*HI$^{linker}$ was deleted from pFC1 to eliminate a *Pst*I site in the pBC polylinker region, resulting in pFC2 (not shown). Step 4: the 0.5-kb *fuc0* fragment *Pst*I-*Pvu*I in pFC2 was cut off and substituted with a corresponding fragment bearing one of the *fucO* mutations cut from $\phi$FM5/$O^{Ile\text{-}7\rightarrow Leu}$, $\phi$FM5/$O^{Leu\text{-}8\rightarrow Val}$, or $\phi$FM5/$O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$, resulting in pFC2/$O^{Ile\text{-}7\rightarrow Leu}$, pFC2/$O^{Leu\text{-}8\rightarrow Val}$, or pFC2/$O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$ (not shown). Step 5: the *Pst*I-*Pvu*I fragments in pFC2/$O^{Ile\text{-}7\rightarrow Leu}$, pFC2/$O^{Leu\text{-}8\rightarrow Val}$, and pFC2/$O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$ were sequenced to confirm the correct substitutions. Step 6: the *Bam*HI-*Bam*HI$^{linker}$ fragment from pFC1 was inserted back into pFC2/$O^{Ile\text{-}7\rightarrow Leu}$, pFC2/$O^{Leu\text{-}8\rightarrow Val}$, and pFC2/$O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$, resulting in plasmids pFC1/$O^{Ile\text{-}7\rightarrow Leu}$, pFC1/$O^{Leu\text{-}8\rightarrow Val}$, and pFC1/$O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$. Step 7: the 5-kb *fuc* fragment *Pst*I-*Eco*RI in pFB1 was cut off and substituted with a corresponding fragment cut from pFC1/$O^{Ile\text{-}7\rightarrow Leu}$, pFC1/$O^{Leu\text{-}8\rightarrow Val}$, or pFC1/$O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$, resulting in pFB1/$O^{Ile\text{-}7\rightarrow Leu}$, pFB1/$O^{Leu\text{-}8\rightarrow Val}$, or pFB1/$O^{Ile7\text{-}\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$, plasmid bearing full-length *fuc* regulon. Step 8: the *Pst*I-*Pvu*I fragments in pFB1/$O^{Ile\text{-}7\rightarrow Leu}$, pFB1/$O^{Leu\text{-}8\rightarrow Val}$, and pFB1/$O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$ were sequenced to confirm the correct *fucO* mutation status.

*Inserting Wild-type and Mutant Fuc Regulons into Host Chromosomes via λ Vectors*—The plasmids pFB1, pFB1/$O^{Ile\text{-}7\rightarrow Leu}$, pFB1/$O^{Leu\text{-}8\rightarrow Val}$, and pFB1/$O^{Ile\text{-}7\rightarrow Leu,\ Leu8\rightarrow Val}$ were digested with restriction enzymes *Apa*I and *Xba*I, yielding 11-kb fragments each containing a full-length *fuc* regulon and the *cam*$^r$ gene. These fragments were then ligated with wild-type λ DNA cut with the same enzymes. The ligation mixtures were packaged with the Gigapack Gold Lambda packaging extract (Stratagene, La Jolla, CA) and used to infect the Δ*fuc* strain ECL733. Cells bearing the foreign sequences on the chromosome were selected as λ lysogens growing on chloramphenicol and MacConkey/fucose plates. Single copy *fuc* regulon insertions were confirmed by Southern blots probed with *fuc* fragments at both ends of the *fuc* sequence. The strains bearing the wild-type and mutant *fuc* regulons at the *att* site are designated ECL734, ECL735, ECL736, and ECL737.

*Growth Conditions and Preparation of Cell Extracts*—Cells were grown aerobically as described previously (20) on Luria broth or 0.5% casein acid hydrolysate. Anaerobic cultures were grown as described previously (20) in 1% casein acid hydrolysate supplemented with 1 mM pyruvate. Where indicated, L-fucose was added as inducer at 10 mM concentration for aerobic growth and 20 mM for anaerobic growth. For enzyme assays, cells were harvested at the end of the exponential phase, and cell extracts were prepared as described previously (20) in 10 mM Tris-HCl buffer, pH 7.5. For enzyme purification, the extracts were prepared using a 50 mM Tris-HCl buffer, pH 7.5, containing 2.5 mM NAD.

*Enzyme Purification*—Propanediol oxidoreductase was purified from extracts of cells grown anaerobically in Luria broth plus L-fucose by the method of Cabiscol *et al.* (9). Enzyme purity was assessed by electrophoresis performed according to Laemmli (21) using 10% acrylamide as resolving gel. Proteins were stained with Coomassie Blue R-250.

*Enzyme Activity Assays*—Propanediol oxidoreductase was routinely

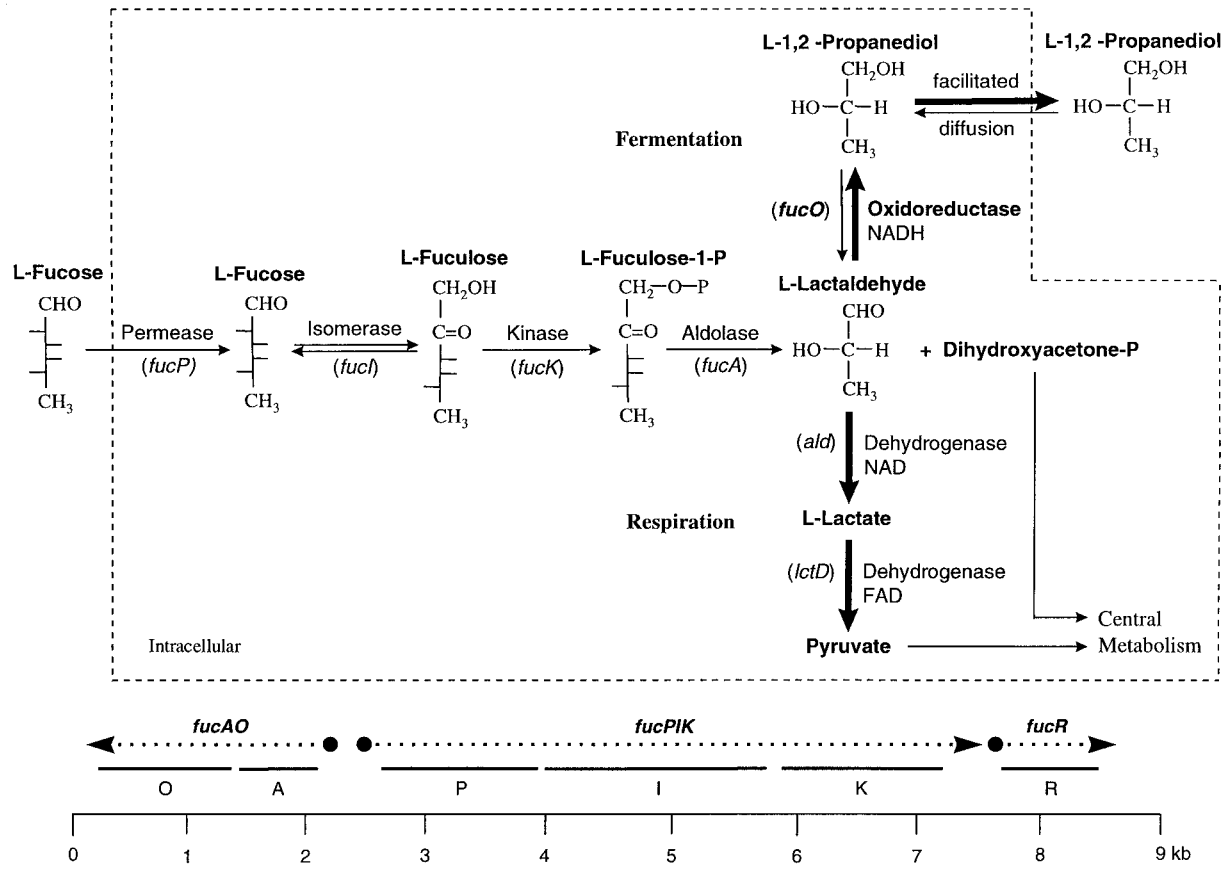


FIG. 3. **Metabolic pathway and genetic organization of the L-fucose system.** Utilization of the methylpentose depends on L-fucose permease (encoded by *fucP*), L-fucose isomerase (encoded by *fucI*), L-fuculose kinase (encoded by *fucP*), L-fuculose-1-phosphate aldolase (encoded by *fucA*), and L-1,2-propanediol:$NAD^+$ oxidoreductase. Under fermentative conditions, the oxidoreductase serves to reduce L-lactaldehyde to propanediol which appears to be excreted via a facilitator (35, 56). Under aerobic or other respiratory conditions, L-lactaldehyde is oxidized to L-lactate by an $NAD^+$-dependent oxidoreductase (41) encoded by the *ald* gene at min 31.2 (68–70). L-Lactate is further oxidized to pyruvate by a flavo-dehydrogenase encoded by *lctD* (71, 72). Determination of the metabolic flow of L-lactaldehyde by respiratory growth conditions (*heavy arrows*) depends on transcriptional control of *ald* (73, 74) and post-translational inactivation of the *fucO* gene products (15, 42, 43, 71). The *fuc* genes, located at min 60 of the chromosome are organized in two divergent operons, *FucAO*, *fucPIK*, under the positive control of *fucR* (17, 29). Propanediol oxidoreductase and L-lactaldehyde dehydrogenase are also required for the dissimilation of L-rhamnose (12, 20, 75).

assayed by its NADH-dependent glycolaldehyde reduction to ethylene glycol (22). Glycolaldehyde, readily available commercially, was shown to be an alternative substrate for the enzyme (23). In experiments testing enzyme protection by NAD or propanediol, the enzyme was assayed by NAD-dependent propanediol dehydrogenation (20). Protein concentration was determined by the Bradford method (24) using bovine serum albumin as standard. Immuno-quantification of propanediol oxidoreductase protein was carried out by Laurell rocket immunoelectrophoresis (25) according to a calibration curve (not shown) derived by using the propanediol oxidoreductase purified from strain ECL1. Antibodies were obtained as described (26).

*Testing Thermal Stability*—Stock solutions of purified propanediol oxidoreductases (0.5 mg/ml) in 50 mM Tris-HCl buffer at pH 7.5 were kept at 4 °C. At time 0, 0.5 ml of each solution was transferred to tubes preincubated at different temperatures, with or without supplementation with 50 mM DL-1,2-propanediol or 1 mM NAD. Enzyme activities were assayed at various intervals. Thermal stability of the enzyme in crude extract (0.5-ml samples containing 8 mg/ml of total protein) was tested under similar conditions.

*Testing Oxidative Inactivation by NADH*—Purified propanediol oxidoreductases (0.5 mg/ml) were incubated at 20 °C in 50 mM Tris-HCl buffer, pH 7.5, in the presence of 0.5 mM NADH. Enzyme activities were assayed at various intervals.

*Testing Oxidative Inactivation by Ascorbate Plus Iron*—Purified propanediol oxidoreductases (0.5 mg/ml) were preincubated at 20 °C for 5 min with 50 mM 1,2-propanediol, followed by addition of 15 $\mu$M $FeCl_3$ and 30 mM ascorbate according to Levine *et al.* (27). Enzyme activities were assayed at various intervals.

*Immunodetection of Protein Carbonyl Groups*—Dinitrophenylhydrazine derivatization of protein carbonyl groups resulting from oxidation was performed according to Levine *et al.* (28). Electrophoresis was performed using 10% acrylamide as resolving gel. After electrophoresis the proteins were transferred to polyvinylidene difluoride membranes by semi-dry blotting. Immuno-detection of protein-bound dinitrophenylhydrazones was performed according to Levine *et al.* (27). The primary antibody was a polyclonal rabbit preparation (Dako, V0401, Denmark). The secondary antibody was goat anti-rabbit conjugated with alkaline phosphatase (Tropix, Bedford, MA).

*Metal Analyses*—Atomic absorption spectroscopy measurements were conducted with a Jobin-Yvon spectrometer, JY-38. Samples were submitted to high performance liquid chromatography gel filtration in a Protein Pak 125 (Waters) prior to metal analysis to eliminate reagents and metals not bound to the enzyme. The eluent used was MilliQ water (resistivity greater that 18 MΩ). Fractions were collected in metal-free polypropylene tubes.

*Fourth Derivative Spectra*—Absorption spectra and their fourth derivative were taken on a Shimadzu UV-160A spectrophotometer, using a derivative interval of 2.4 nm, a slit width of 2 nm, and a scan rate of 80 nm/min. Purified enzymes were dissolved in 50 mM Tris-HCl buffer, pH 7.5.

## RESULTS

*Wild-type and Propanediol-positive Strains and Comparison of Their fucO DNA Sequences*—Propanediol oxidoreductase is encoded by the *fucO* gene, a member of the *fuc* regulon specifying the utilization of L-fucose. The genes of the *fuc* regulon are organized in two divergent operons, *fucPIK* and *fucAO* (Fig. 3). Induction of the operons requires the activator FucR (17, 29) and its effector, L-fuculose-1-phosphate (30). L-1,2-Propanediol, a product of L-fucose fermentation, cannot serve aerobically as

TABLE I
*Bacterial strains, plasmids, and phages used in this study*

| Cell or plasmid | Derived from | Relevant property/genotype | Ref./Source |
|---|---|---|---|
| Strains | | | |
| ECL1 | HfrC | HfrC *phoA8 relA1 tonA22* $T2^r$ (λ) | 76 |
| ECL3 | ECL1 | HfrC *fucAO*(Con) *fucPIK*(Non)[a] | 33 |
| ECL56 | ECL3 | HfrC *fucAO*(Con) *fucPIK*(Con) *crp-201* | 15 |
| ECL116 | YMC9 | $F^-$ Δ*lacU169 thi endA hsdR* | 43 |
| ECL330 | ECL116 | $F^-$ φ[*fucO-Mu d1 cts* ($Ap^r$ *lacZYA*)] | Y.-M. Chen 43 |
| ECL421 | ECL1 | HfrC *fucAO*(Con) *fucPIK*(Non) | 32 15 |
| ECL430 | ECL3 | HfrC | 35 |
| ECL459 | ECL421 | HfrC *fucAO*(Con) *fucPIK*(Con) | 15 |
| ECL733 | ECL330 | $F^-$ Δ*fuc* | This study |
| ECL734 | ECL733 | $F^-$ Δ*fuc* λ ($Cam^r$ $fucAO^+$ $fucPIK^+$ $fucR^+$) | This study |
| ECL735 | ECL733 | $F^-$ Δ*fuc* λ ($Cam^r$ $fucA^+O^{Ile\text{-}7\rightarrow Leu}$ $fucPIK^+$ $fucR^+$) | This study |
| ECL736 | ECL733 | $F^-$ Δ*fuc* λ ($Cam^r$ $fucA^+O^{Leu\text{-}8\rightarrow Val}$ $fucPIK^+$ $fucR^+$) | This study |
| ECL737 | ECL733 | $F^-$ Δ*fuc* λ ($Cam^r$ $fucA^+O^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$ $fucPIK^+$ $fucR^+$) | This study |
| Plasmids | | | |
| pfuc1 | | pBR322 ($fuc^+$) | 19 |
| pfuc16 | | pBR322 (*fuc* $SalI_0$-*Eco*RI) | 19 |

[a] (Con) denotes constitutive expression and (Non) denotes noninducibility. These phenotypes resulted from an insertion of an IS5 element at a precise position in the intergenic region of the divergently transcribed *fucAO* and *fucPIK* operons. The wild-type operons, under the positive control of *fucR*, are inducible by fuculose 1-phosphate. Thus, *fucO* expression would not be inducible by L-propanediol in the culture medium (Refs. 15, 33, and 75 and Z. Lu, unpublished data).

a sole carbon and energy source, because the 3-carbon compound fails to induce the *fuc* regulon expression. If *fucO* is expressed constitutively at an adequate level, the cell should be able to grow on propanediol by converting it to pyruvate via L-lactaldehyde and L-lactate (Fig. 3).

Mutants as described above were actually isolated and previously reported. Strain ECL3 was isolated from wild-type strain ECL1 after 100 generations of selection on propanediol, attaining a doubling time of about 90 min (31). Strain ECL421 was isolated from another wild-type clone after a similar selection for 140 doublings (32). In both mutants, an IS5 insertion occurred at precisely the same location in the region between the two diverging operons, causing constitutive activation of *fucAO* and noninducibility of *fucPIK* (15, 33).[2]

Since strains ECL3 and ECL421 were isolated after prolonged selection on propanediol, mutations in the coding region of *fucO* conferring resistance to oxidative damage might also have taken place. Indeed, it was found that the ECL3 enzyme exhibited a decreased thermal stability (34). To determine the exact nature of the mutations and their phenotypic consequences, the *fucO* alleles of the wild-type and the two mutant strains were amplified by polymerase chain reaction for gene cloning and sequencing. For comparison, the *fucO* alleles of three other derivative strains were included in the procedure as follows: ECL430, selected from ECL3 for improved propanediol-scavenging power (35); ECL56, a $Fuc^+$ revertant of ECL3 generated by an unlinked mutation (33, 36); and ECL459, the corresponding $Fuc^+$ revertant of ECL421 (15). A single nucleotide substitution, an A to C change converting the N-terminal $Ile^7$ to $Leu^7$, was found in the *fucO* of strains ECL3, ECL56, and ECL430. Another single nucleotide substitution, a C to G change converting the N-terminal $Leu^8$ to $Val^8$, was found in the *fucO* of strains ECL421 and ECL459 (data not shown).

*Reconstruction of the fucO Mutations in an Otherwise Wild-type Background*—To find out whether the two different single *fucO* mutations conferred resistance of the enzyme to aerobic inactivation, we tested plasmid-borne wild-type and mutant *fucO* alleles in a standard wild-type background. Preliminary activity assays of the oxidoreductase were not satisfactorily reproducible. The difficulty seems to be attributable to variations in the plasmid copy number.

To improve the reproducibility of enzyme activity assays, we decided to use strain ECL733 with a complete deletion of the *fuc* regulon to host a λ-borne *fuc* regulon with one of the *fucO* alleles. First, each of the two *fucO* mutations was re-created by site-directed mutagenesis in a wild-type *fuc* sequence to ensure that the mutation detected was sufficient to account for any observable phenotypic difference. In addition, a double mutation, $Ile^7 \rightarrow Leu$ and $Leu^8 \rightarrow Val$, was created in parallel to discover whether there is an additive effect of the single mutations. A complete *fuc* regulon was then engineered to bear the wild-type *fucO* or one of the re-created mutant *fucO* alleles, inserted into λ DNA, and packaged *in vitro* to infect the Δ*fuc* strain ECL733. The purified λ lysogens were used for further study.

*Expression of fucO Genes*—The λ lysogens ECL734 ($fucO^+$), ECL735 ($fucO^{Ile\text{-}7\rightarrow Leu}$), ECL736 ($fucO^{Leu\text{-}8\rightarrow Val}$), and ECL737 ($fucO^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$) and the wild-type nonlysogen, ECL1, were grown aerobically or anaerobically on casein acid hydrolysate in the presence of L-fucose as inducer of the *fuc* regulon. Extracts from each culture were assayed for propanediol oxidoreductase activity (Table I). The specific activities of propanediol oxidoreductase (units/mg enzyme protein) in extracts of anaerobically grown cells were all about the same, irrespective of the nature of the *fuc* allele. In contrast, the specific activity of the enzyme in extracts of aerobically grown cells was dependent on the *fucO* allele: $FucO^+ < FucO^{Ile\text{-}7\rightarrow Leu} < FucO^{Leu\text{-}8\rightarrow Val} < FucO^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$. It should be mentioned that if the inducer was not added, there was no detectable oxidoreductase activity in extracts prepared from the different strains, even when the cells were grown anaerobically (not shown), indicating that transcriptions were from the same *fuc* promoter. The data taken together indicate that a single amino acid substitution was sufficient to confer significant resistance of the protein to oxidative damage and that the double amino acid substitutions have an additive protective effect. On the other hand, the amino acid substitutions do not appear to have a significant effect on the catalytic property of the enzymes, at least when assayed by the reduction of the substrate analog glycolaldehyde (Table II).

*Thermal Inactivation of Wild-type and Mutant Propanediol*

[2] Z. Lu, unpublished observations.

TABLE II
*Propanediol oxidoreductase activities of wild-type and fucO mutant strains grown aerobically and anaerobically*

| Strain | Propanediol oxidoreductase specific activity[a] | |
|---|---|---|
| | Aerobic | Anaerobic |
| | *units/mg enzyme protein* | |
| ECL1 (*fucO*$^{+}$) | 4.9 | 28 |
| ECL734 (*fucO*$^{+}$) | 5.5 | 24 |
| ECL735 (*fucO*$^{\text{Ile-7}\rightarrow\text{Leu}}$) | 8.8 | 27 |
| ECL736 (*fucO*$^{\text{Leu-8}\rightarrow\text{Val}}$) | 12 | 27 |
| ECL737 (*fucO*$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$) | 14 | 25 |

[a] Enzyme activity was assayed by glycolaldehyde-dependent NADH oxidation, and the amount of enzyme protein was determined by Laurell rocket immunoelectrophoresis.

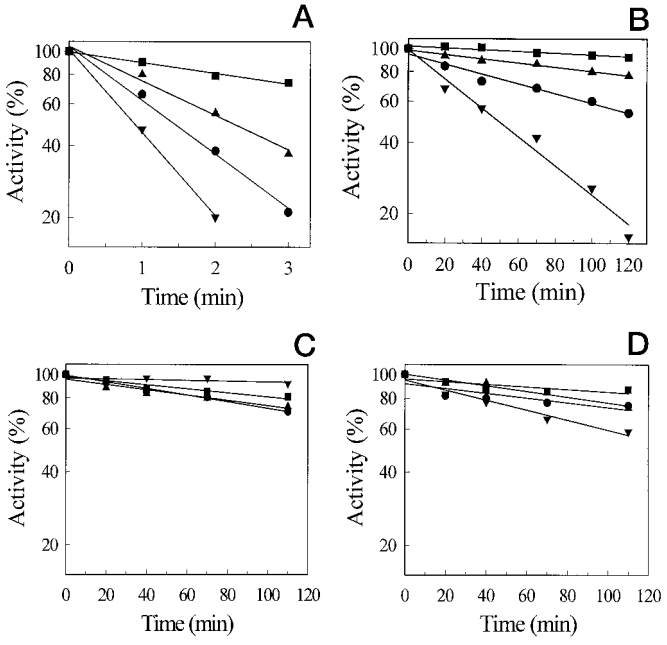


FIG. 4. **Thermal inactivation of mutant and wild-type propanediol oxidoreductases.** Purified FucO$^{+}$) (■), FucO$^{\text{Ile-7}\rightarrow\text{Leu}}$ (▲), FucO$^{\text{Leu-8}\rightarrow\text{Val}}$) (●), and FucO$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$) (▼) were tested for stability under various conditions. *A*, 50 °C; *B*, 20 °C; *C*, 20 °C in the presence of 50 mM DL-1,2-propanediol; and *D*, 20 °C in the presence of 1 mM NAD. At the indicated times samples were withdrawn for enzyme activity assay by the rate of glycolaldehyde-dependent oxidation of NADH (*A* and *B*) or by the rate of 1,2-propanediol-dependent reduction of NAD (*C* and *D*). In each case, the specific activity of the enzyme at 0 time was expressed as 100%.

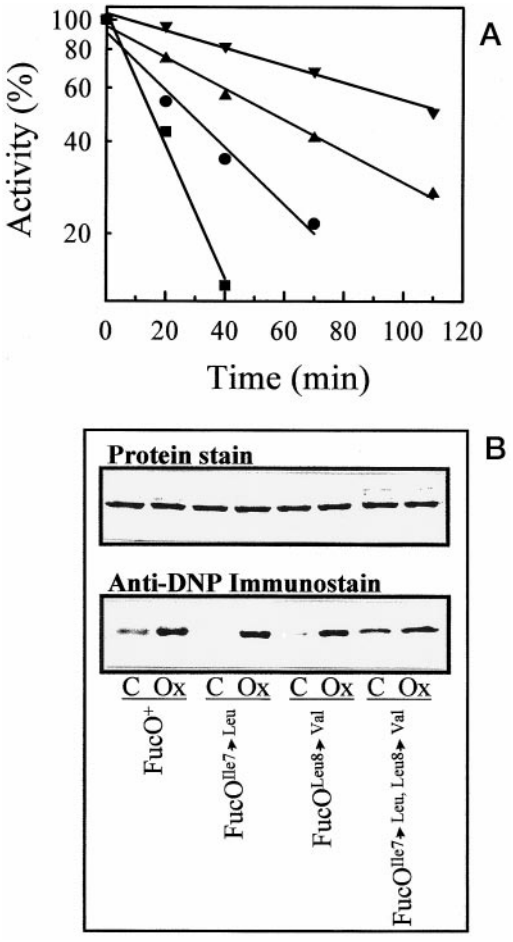


FIG. 5. **Oxidative inactivation of purified wild-type and mutant propanediol oxidoreductases.** *A*, Fuc$^{+}$ (■), FucO$^{\text{Ile-7}\rightarrow\text{Leu}}$ (▲), FucO$^{\text{Leu-8}\rightarrow\text{Val}}$ (●), and FucO$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$ (▼) were incubated at 20 °C with 0.5 mM NADH. At the indicated times samples were withdrawn for assay of enzyme activity which was expressed as percent of the 0 time value. *B*, Western blot of oxidized enzymes. After 40 min of incubation with NADH, samples were taken and processed for immunodetection of protein carbonyl groups produced during oxidation. Samples of unoxidized control proteins were taken before NADH was added for incubation. *C*, control protein; *Ox*, NADH-treated protein.

*Oxidoreductase Enzymes*—Additional evidence of structural alteration of the mutant propanediol oxidoreductase was their decreased thermal stability. When purified wild-type and mutant propanediol oxidoreductases were incubated at two different temperatures at pH 7, the activity decay rate of the enzymes followed the same order: at 50 °C, FucO$^{+}$ ($t_{½}$ = 6.6 min), FucO$^{\text{Ile-7}\rightarrow\text{Leu}}$ ($t_{½}$ = 2.3 min), FucO$^{\text{Leu-8}\rightarrow\text{Val}}$ ($t_{½}$ = 1.5 min), and FucO$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$ ($t_{½}$ = 0.9 min) (Fig. 4*A*); at 20 °C, FucO$^{+}$ ($t_{½}$ = 840 min), FucO$^{\text{Ile-7}\rightarrow\text{Leu}}$ ($t_{½}$ = 340 min), FucO$^{\text{Leu-8}\rightarrow\text{Val}}$ ($t_{½}$ = 140 min), and FucO$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$ ($t_{½}$ = 48 min) (Fig. 4*B*). It is, however, not clear from these experiments whether the loss of activity resulted from irreversible denaturation of the protein or the loss of the cofactor $Fe^{2+}$.

It is well known that the presence of the coenzyme or a substrate can stabilize enzymes from thermal inactivation. This seems to apply to the three mutant enzymes in the presence of 1 mM NAD (Fig. 4*C*) or 50 mM DL-1,2-propanediol (Fig. 4*D*).

*Oxidative Inactivation of Purified Propanediol Oxidoreductase Enzymes by NADH*—Since propanediol oxidoreductase is an iron enzyme, the presence of both molecular oxygen and NADH is expected to result in an intrinsically catalyzed Fenton reaction that damages the protein. Purified wild-type and mutant enzymes were incubated for 120 min in the presence of 0.5 mM NADH at 20 °C under air. As shown in Fig. 5*A*, FucO$^{+}$ was rapidly inactivated ($t_{½}$ = 15 min). In contrast, the mutant proteins were substantially more stable: FucO$^{\text{Ile-7}\rightarrow\text{Leu}}$ ($t_{½}$ = 28 min), FucO$^{\text{Leu-8}\rightarrow\text{Val}}$ ($t_{½}$ = 54 min), and FucO$^{\text{Ile-7}\rightarrow\text{Leu, Leu-8}\rightarrow\text{Val}}$ ($t_{½}$ = 110 min). The possibility that differences in sensitivity of the enzymes are attributable to the disparities in the amount of iron bound was excluded by the finding that the metal contents of the four different purified enzymes were equal, as determined by atomic absorption spectroscopy. The iron-dependent Fenton reaction as the cause of the observed enzyme inactivation was suggested by the NADH dependence of oxidative protein damage, as revealed by immunochemical assays for the carbonyl groups generated (Fig. 5*B*).

*Oxidative Inactivation of Purified Propanediol Oxidoreductase Enzymes by Ascorbate and $Fe^{3+}$*—Other evidence of the susceptibility of propanediol oxidoreductase to damage by Fenton reaction was demonstrated by incubation of the protein in the presence of ferric chloride and ascorbate, instead of NADH, as the reductant (28). All four enzymes were incubated at 20 °C

for 120 min in the presence of 50 mM DL-1,2-propanediol to stabilize the proteins against simple thermal inactivation. The relative rates of inactivation of the four enzymes were consistent with those observed during incubation with NADH (Fig. 5*A*): FucO$^{+}$ ($t_{½}$ = 70 min), FucO$^{Ile\text{-}7\rightarrow Leu}$ ($t_{½}$ = 110 min), FucO$^{Leu\text{-}8\rightarrow Val}$ ($t_{½}$ = 260 min), and FucO$^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$ ($t_{½}$ = 430 min) (Fig. 6).

*Analysis of Fourth Derivative Ultraviolet Spectra of Wild-type and Mutant Enzymes*—On the basis of crystallographic data on the highly conserved folding motifs of NAD-dependent oxidoreductases, it can be predicted that the amino acid substitutions found in the mutant FucO proteins are close to the $\alpha$A-$\beta$2 turn of the mononucleotide-binding motif (or Rossmann fold) where Tyr$^{31}$ is located (17, 37).

Since alterations in the chemical environments of aromatic amino acid residues produce changes in their fourth derivative spectra (38), we thought that the Tyr$^{31}$ in FucO might serve as a reporter for conformational changes in the NAD binding domain. The spectra obtained from these wild-type and mutant FucO proteins were therefore compared (Fig. 7). The peaks in the range of 270–300 nm correspond to the absorption of tyrosine and tryptophan in the proteins; the main peak expected of Tyr is at 282 nm, whereas that of Trp is at 290 nm (39, 40).

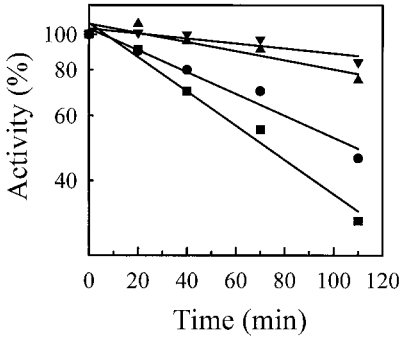


FIG. 6. **Inactivation of pure propanediol oxidoreductase by ascorbate and iron.** Purified FucO$^{+}$) (■), FucO$^{Ile\text{-}7\rightarrow Leu}$ (▲), FucO$^{Leu\text{-}8\rightarrow Val}$) (●), and FucO$^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$ (▼) were oxidized at 20 °C with ascorbate and iron in the presence of 1,2-propanediol. At the indicated times samples were taken to assay enzyme activity which was expressed as percent of the initial activity before incubation with ascorbate and iron was started.

Although the data showed no apparent variation for the Trp spectra, clear differences in the Tyr peak can be discerned. In a control experiment, wild-type and mutant proteins denatured by incubation in 6 M guanidine chloride displayed no difference in the fourth derivative spectra (data not shown). It is therefore tempting to suppose that Tyr$^{31}$ is responsible for the alteration of the peak.

## DISCUSSION

Although early work on propanediol oxidoreductase showed that $Fe^{2+}$ activates the apoenzyme (41) and that the protein induced during aerobic growth lacked catalytic activity (42, 43), MCO of the enzyme protein was not suspected until a hint was provided by the work on $Fe^{2+}$-catalyzed inactivation of *E. coli* glutamine synthetase (6, 7, 44–46). In the case of FucO, the $Fe^{2+}$ is at the catalytic center which binds the protein to NADH for fermentative reduction of L-lactaldehyde. During aerobic metabolism when the enzyme serves no physiological role, the generation of $H_2O_2$ allows the $Fe^{2+}$ to catalyze a Fenton reaction, and the highly reactive $OH^{\cdot}$ formed is likely to diffuse a distance of the order of its mean free path, which is only a few times of the free radical's radius, before hitting a target amino acid residue (*i.e.* diffusion-controlled encounter). A frequent occurrence is the destruction of the side chain of a conserved His$^{277}$, 10 residues away from the proposed metal-binding site, His$^{263}$-*X*-*X*-*X*-His$^{267}$, causing a decrease in the apparent affinity of the protein for NAD (13).

It is remarkable that the relatively conservative hydrophobic amino acid substitutions, Ile$^{7}$ → Leu and/or Leu$^{8}$ → Val, near the NAD-binding sequence Gly$^{15}$-Arg-Gly-Ala-Val-Gly$^{20}$ of FucO (15, 16, 47) could confer significant protective effects against MCO damage. This resistance, however, is achieved at a price of decrease in protein stability. The loss of stability might be attributable to "cavity creation" associated with diminished hydrophobic interactions. In the case of T4 lysozyme a Leu → Ala substitution raised the $-\Delta G$ of the folded form of the enzyme by 1.9 kcal/mol; increases as high as 6.2 kcal/mol have been observed in hydrophobic amino acid substitutions in other proteins (48).

Interestingly, the alcohol oxidoreductase II of *Zymomonas mobilis*, an enzyme highly homologous to FucO (16), is damaged by MCO in a similar way (9). Similar cases of enzyme inactivation by MCO were observed in studies of *Klebsiella*

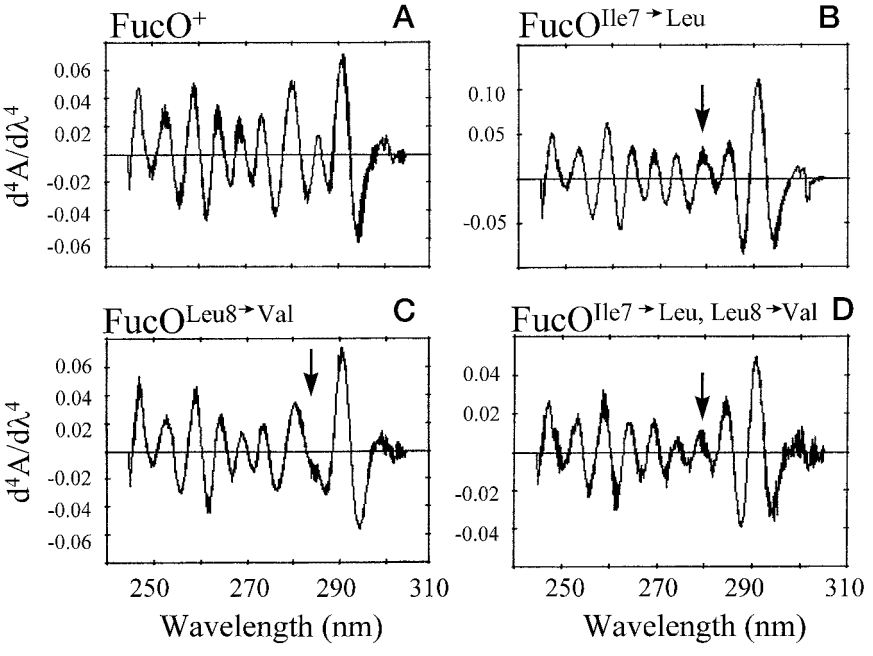


FIG. 7. **Fourth derivative spectra representation of the wild-type and mutant FucO proteins.** The plot taken from a Shimadzu UV 160A spectrophotometer shows the spectra of purified propanediol oxidoreductase from strains: *A*, Fuc$^{+}$ (0.7 mg/m); *B*, FucO$^{Ile\text{-}7\rightarrow Leu}$ (0.9 mg/ml); *C*, FucO$^{Leu\text{-}8\rightarrow Val}$ (0.75 mg/ml); and *D*, FucO$^{Ile\text{-}7\rightarrow Leu,\ Leu\text{-}8\rightarrow Val}$ (0.4 mg/ml). The spectra were taken between 240 and 320 nm. *Arrows* indicate the main differences observed between mutant and wild-type proteins.

*pneumoniae*. Glycerol:NAD 2-oxidoreductase, which serves for the utilization of glycerol under fermentative conditions, is inactivated during aerobic metabolism (49). The initial step involves the MCO-caused loss of apparent affinity for NAD (50, 51). 1,3-Propanediol:NAD 1-oxidoreductase (disposing of NADH by reduction of 3-hydroxypropionaldehyde during fermentative growth on glycerol) and ethanol:NAD oxidoreductase (disposing of NADH by reduction of acetyl-CoA during sugar fermentation) seem to be likewise inactivated (52, 53).

Although protein turnover necessitated by MCO is metabolically costly (44, 54, 55), inactivation of certain enzymes during aerobic respiration can be beneficial in the balance. In the case of FucO, the continued presence of a catalytically active protein during aerobic utilization of L-fucose or L-rhamnose would wastefully deplete both L-lactaldehyde and NADH (56, 57). Viewed from this angle, the bound $Fe^{+2}$ might be regarded as an adaptive self-destruct mechanism for facilitating the transition from fermentative to aerobic metabolism. The same reasoning should apply to ethanol oxidoreductase. In the case of glycerol oxidoreductase, the rapid inactivation of the enzyme would facilitate the shift from the relatively ineffective anaerobic substrate capturing pathway initiated by the NAD-coupled oxidoreductase to the more avid aerobic substrate scavenging pathway initiated by the ATP-driven kinase (58), a kinetic advantage predictable by the Haldane equation relating $K_{eq}$ to the $k_{cat}/K_m$.

In contrast to NAD(P) enzymes that are involved in fermentative metabolism, those that are needed for both aerobic and anaerobic metabolism, such as glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, and malate dehydrogenase of *K. pneumoniae*, are resistant to inactivation by oxidative metabolism (53). The same is true for the *E. coli* NAD-linked L-lactaldehyde dehydrogenase that is required only for aerobic substrate utilization (14). These resistant enzymes are probably dependent on $Zn^{2+}$ instead of $Fe^{2+}$ or are metal-independent (see below). An interesting case is the glucose-6-phosphate dehydrogenase in *Leuconostoc mesenteroides*, which is thought to mediate only anaerobic glucose catabolism by a pentose pathway (59); it is $Fe^{2+}$-dependent and subject to MCO inactivation (8).

On the basis of amino acid sequence homology, NAD(P)-dependent alcohol dehydrogenases (oxidoreductases) fall into the following three families: (i) long chain $Zn^{2+}$-dependent, (ii) short chain $Zn^{2+}$-independent, and (iii) $Fe^{2+}$-activated (60, 61). *Zymomonas mobilis*, an $O_2$-tolerant and obligately ethanologenic anaerobe (62), has two alcohol oxidoreductases, one is $Fe^{2+}$-dependent and the other dependent on $Zn^{2+}$ (16, 63–65). As one would expect, the $Zn^{2+}$ enzyme is MCO-resistant, but the $Fe^{2+}$ enzyme is susceptible to MCO damage (66). It was suggested that having two enzymes with different metal ion requirement would be a nutritional insurance (67). The question of whether the $Zn^{2+}$ enzyme plays the major role in aerobic ethanologenesis and the $Fe^{2+}$ enzyme in anaerobic ethanologenesis has not been addressed. The yeast, *Saccharomyces cerevisiae*, also possesses both $Zn^{2+}$ and $Mn^{2+}$ alcohol dehydrogenases (60). The physiological and/or evolutionary basis for employing both metal ions has yet to be explored.

In light of the fact that no $Fe^{2+}$-dependent oxidoreductase is known to have an aerobic function, it is tempting to suggest that such enzymes evolved early when the global environment was highly reducing and the supply of ferrous iron was abundant. With the emergence of photosynthesis and attendent accumulation of $O_2$, aerobic metabolism developed, and iron became mostly sequestered as insoluble $Fe^{3+}$ compounds. $Fe^{2+}$-dependent oxidoreductases were gradually supplanted by $Zn^{2+}$-dependent ones. Those oxidoreductases that persisted as $Fe^{2+}$ enzymes did so either because there was a lack of selective pressure to switch to $Zn^{2+}$ or because retention of $Fe^{2+}$ actually provided the cell with an adaptive mechanism for thrifty shift from anaerobic to aerobic metabolism.

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# Evolution of an *Escherichia coli* Protein with Increased Resistance to Oxidative Stress

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