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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Leeuwenhoek Lecture, 1995. Adaptation to life without oxygen

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SUMMARY

The Earth was populated by anaerobic organisms for at least a thousand million years before the atmosphere became oxygenated and aerobes could evolve. Many bacteria like *Escherichia coli* retain the ability to grow under both aerobic and anaerobic conditions. Recent studies have revealed some global regulatory mechanisms for activating or repressing the expression of relevant genes in response to oxygen availability. These mechanisms ensure that the appropriate metabolic mode is adopted when bacteria switch between aerobic and anaerobic environments.

1. INTRODUCTION

I have long admired the work of Antoni van Leeuwenhoek and shared the obvious pleasure he derived from exploring the hitherto unseen world of microbiology. This amateur scientist, born in Delft in 1632, possessed remarkable powers of observation and the ability to construct simple microscopes of unrivalled quality. He duly became the Father of Microbiology and I am deeply honoured to be invited to give this lecture in his name.

Like many of my generation, I first encountered Antoni van Leeuwenhoek in Paul de Kruif's excellent book *Microbe Hunters* (de Kruif 1926) and later read translations of his numerous letters to the Royal Society in Clifford Dobell's fascinating account of his life and works (Dobell 1960).

He is still widely revered, particularly in Delft, where you step from the railway station into van Leeuwenhoeksingel and can take the Antoni van Leeuwenhoek walking or cycling trails, which include a visit to his tomb in the Oude Kerk. The journal of the Netherlands Microbiology Society is named after him and until fairly recently his portrait was used as a logo by the American Society for Microbiology.

With his simple microscopes he was, in 1676, the first to see a variety of different bacteria, when trying to discover why pepper is hot. In 1680 he was the first to observe the growth and gas-producing fermentations of anaerobic bacteria (letter 32, quoted by Dobell 1960), and according to Beijerinck's reconstruction experiments one of the observed species was probably *Escherichia coli*. These observations were almost two centuries before Pasteur rediscovered 'la vie sans l'air' (Pasteur 1861). Indeed, it now seems incredible that the existence of microorganisms was known for such a long time before their roles in fermentation, putrefaction and disease were established. One wonders whether anyone today could be recording observations whose significance will not be appreciated until the 22nd century.

2. METABOLIC EVOLUTION AND THE CITRIC ACID CYCLE

The topic of my lecture is 'Adaptation to life without oxygen' and you might ask what is novel about that? After all, we believe that life on Earth had its origins 3.5 billion (3.5×10^9) years ago in the completely anaerobic environment of our cooling planet. The earliest cellular organisms probably obtained metabolic energy by *fermenting* the rich mixture of organic compounds that had accumulated over countless years of chemical and physical activity in a previously sterile world. They may also have used primitive forms of *anaerobic respiration* in which the oxidation of organic substrates, and the oxidation of ferrous salts to ferric sulphide (pyrite) by hydrogen sulphide, are coupled to the reduction of elemental sulphur via an ancestral proton translocating ATPase. Later, the evolution of light-sensitive porphyrins allowed the Sun's radiant energy to be harnessed, initially by anaerobic non-oxygenic phototrophic organisms resembling present day photosyntgetic bacteria. The stage was then set for the most monumental step in biological evolution. This was the emergence of the oxygenic phototrophs (or cyanobacteria), which occurred over 2 billion years ago, when the second light reaction was incorporated into photosynthesis. During the next billion years these organisms created an oxygenated atmosphere in which *aerobic respiration* could evolve and become the most energetically favourable metabolic mode adopted by a vast array of extremely successful and increasingly complex organisms.

However, despite conferring enormous energetic benefits, the use of oxygen poses serious threats. This is because the byproducts of its reduction to water are extremely toxic. The stepwise reduction of oxygen

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generates the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($HO^{\bullet}$) and finally the hydroxyl anion (HO^-) or water. Moreover, formation of the hydroxyl radical, the most toxic and most short-lived intermediate, is catalysed by iron in the Haber–Weiss reaction ($O_2^- + H_2O_2 \rightarrow HO^{\bullet} + HO^- + O_2$). Iron is the fourth most abundant element in the Earth's crust, and it is widely recruited by living organisms as a component of respiratory enzymes and other proteins. So, to use aerobic respiration, or even to survive under aerobic conditions, an organism needs mechanisms for removing the toxic intermediates, e.g. via superoxide dismutase, catalase and peroxidases, or for replacing or repairing the damage wrought by these cytotoxic agents on their enzymes, nucleic acids and other cellular components (Storz *et al.* 1990; Demple 1991). It should also be appreciated that despite the Earth's oxygenation there remains a plethora of anaerobic and microaerobic habitats occupied by a diverse array of anaerobic organisms which grow and compete successfully for the nutritional resources they afford.

Of special interest to me are the facultative anaerobes which can grow under aerobic and anaerobic conditions. They are widely distributed among the Bacteria, Archaea and Eukarya, where representatives use different combinations of fermentation, anaerobic respiration and phototrophy as alternative energy-generating metabolic modes (Unden *et al.* 1994). Carbohydrates and amino acids are fermented and oxides of nitrogen, sulphur, sulphoxides and fumarate are the most widely respired alternatives to oxygen. Representative organisms include the enteric bacteria and species of *Rhodobacter*, *Pseudomonas*, *Bradyrhizobium*, *Bacillus* and *Halobium*, the most versatile being *Rhodobacter* and *Halobium* species which can use all three modes of anaerobic metabolism. My chosen facultative organism is *Escherichia coli*. It inhabits the anaerobic intestine of man and other mammals but during the natural course of events, is excreted into an aerobic environment at a rate of 2×10^{10} per person per day (100 million per gram of faeces). *E. coli* is of course a modern organism and we do not know whether its ancestors have been continuously facultative from a specific time in the Earth's oxygenation, or whether either capacity for aerobic or anaerobic growth has been lost and regained on one or more occasions during its evolution. However, it seems very likely that during evolution its genome has been much modified by mutation, duplication and the incorporation of genetic material from other organisms, in order to adapt to changing environments and stresses.

I shall illustrate this from some features of the citric acid cycle (CAC). The CAC catalyses the total oxidation of acetyl units derived from other catabolic pathways, and it is the major energy-generating pathway in aerobic heterotrophs as well as an important source of precursors for cellular biosynthesis (figure 1). However, under anaerobic conditions (or even under aerobic conditions with excess carbohydrate, where abundant energy is provided by glycolysis), the CAC is converted into a branched or non-cyclic form, largely by repression of the 2-oxoglutarate dehydrogenase complex (ODHC), succinyl-CoA synthetase (SCS) and succinate dehydrogenase (SDH). In consequence, carbon flows at a much reduced rate through two routes. The oxidative route provides 2-oxoglutarate for biosynthetic purposes, and the reductive route (including a diversion via aspartate) leads to succinate and at the same time serves as an electron sink for balancing unlinked oxidative reactions. Under these conditions the acetyl units are converted to ethanol

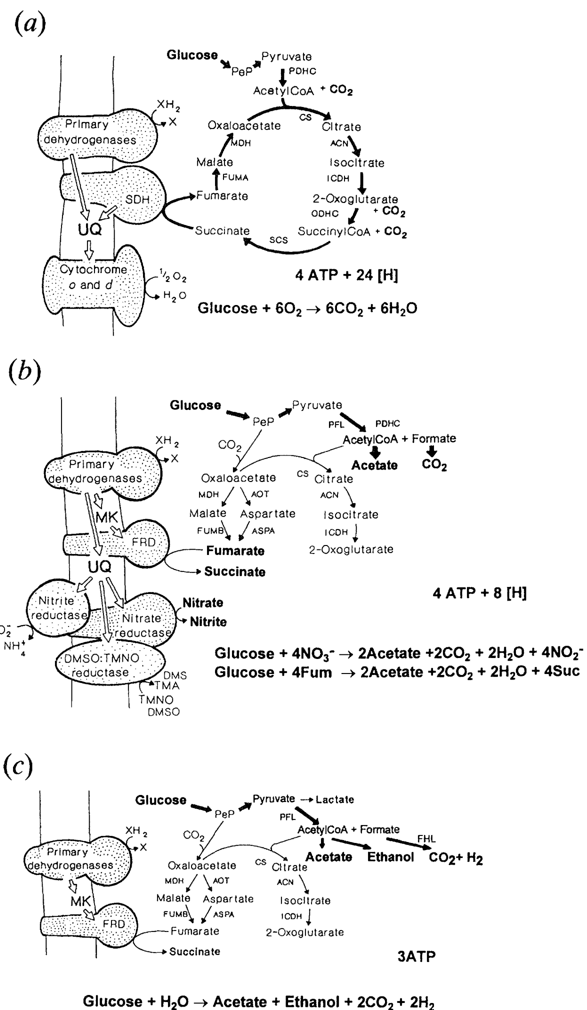

Figure 1. The metabolic modes of *E. coli.* The central pathways of carbon and energy metabolism are illustrated for (*a*) aerobic respiration, (*b*) anaerobic respiration and (*c*) fermentation. Cyclic and non-cyclic forms of the citric acid cycle of *E. coli.* The cyclic form of the citric acid cycle operates under aerobic conditions with limiting glucose whereas the non-cyclic or branched form operates under anaerobic conditions or with excess glucose. Where more than one enzyme catalyses the same reaction, only that which functions primarily in the relevant pathway is shown. Some isoenzymes with different or unidentified functions are not shown: the third fumarase (FUMC), the second aconitase (ACNB) and a second citrate synthase (CSB). Other abbreviations are: aspartase (ASP), aspartate-oxaloacetate aminotransferase (AOT), formate hydrogenlyase (FHL), fumarate reductase (FRD); isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH), menaquinone (MK), succinyl-CoA synthetase (SCS), succinate dehydrogenase (SDH), 2-oxoglutarate dehydrogenase complex (ODHC), pyruvate dehydrogenase complex (PDHC), pyruvate formate-lyase (PFL); and ubiquinone (UQ).

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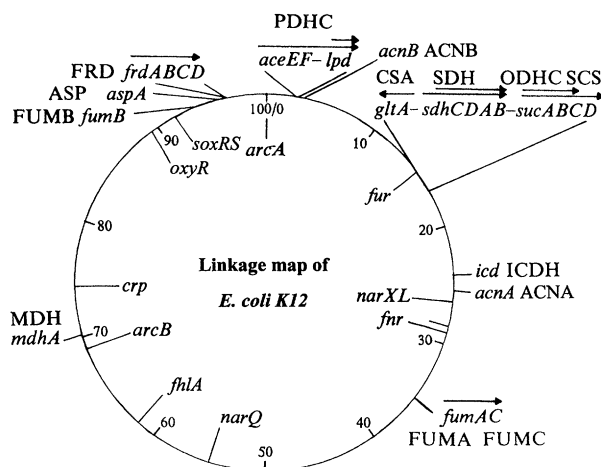

Figure 2. Circular linkage map of *E. coli* showing the positions and transcriptional relations of citric acid cycle and related genes (outside markers) and relevant transcription regulator genes (inside markers).

and/or acetate. About thirty years ago, when it was popular to assemble all known metabolic reactions on a metabolic map, the central position occupied by the CAC seemed to signify that it might have arisen very early in metabolic evolution. Moreover, I like to think that the origin of the CAC is recapitulated in its transition from the non-cyclical repressed form to the aerobic cyclical form. The interconversions of di- and tricarboxylic acids in the oxidative and reductive routes could represent some of the earliest metabolic transformations performed by primitive organisms on our nutrient-rich but anaerobic planet. After oxygenation, these routes might have been linked and the flow reversed in the reductive route, to generate an oxidative metabolic cycle (Gest 1981). Fumarate reductase could likewise have been the terminal reductase of a primitive proton-translocating electron transport chain.

Another interesting feature of the CAC that has emerged since the advent of gene cloning and reverse genetics (the ability to replace chromosomal genes by *in vitro* disrupted variants) is the fact that several steps are catalysed by more than one genetically distinct enzyme (figure 2). For example, there are analogous membrane-bound flavoprotein complexes catalysing the interconversion of succinate and fumarate; SDH is the aerobic enzyme which is subject to anaerobic and catabolite repression, whereas FRD is derepressed anaerobically to function in the reductive branch of the anaerobic cycle. Likewise, there are two almost identical fumarases (FUMA and FUMB) catalysing the interconversion of fumarate and malate (Guest 1992). These are iron–sulphur containing enzymes that are so unstable in air that their existence was not detected until the corresponding genes were cloned. Here, FUMA is the aerobic CAC enzyme, subject to anaerobic and catabolite repression (and iron-dependent activation), whereas FUMB is induced under anaerobic conditions. The stable fumarase (FUMC) is an entirely different type of enzyme, closely resembling the eukaryotic mitochondrial enzymes and unrelated to FUMA and FUMB. This enzyme is synthesized under both aerobic and anaerobic conditions, but it is specifically induced by oxidative stress (Liochev & Fridovich 1992; Gruer & Guest 1994).

More recently, the gene encoding an aconitase (ACNA) was cloned, sequenced and then inactivated in the chromosome to generate an *acnA* mutant, whereupon it was found that *E. coli* possesses a second iron–sulphur containing aconitase, ACNB (Gruer & Guest 1994). Again, the pattern of anaerobic and catabolite repression suggests that ACNA is an aerobic CAC enzyme. It is subject to iron-dependent activation and induction during oxidative stress. The regulatory responses associated with ACNB, which is only distantly related to ACNA, have still to be defined. There is also evidence for a second citrate synthase (CSB) resembling the dimeric mitochondrial enzymes rather than the hexameric *gltA* gene product (CSA). The newly discovered enzyme appears to be expressed by the reactivation of a cryptic gene and it is not yet known whether it has a specific function (Patton *et al.* 1993).

In some cases it is clear how gene duplication has provided opportunities for functional specialization and the acquisition of independent regulatory mechanisms tailored to their metabolic roles, e.g. SDH/FRD and FUMA/FUMB. In others, it appears that distantly related genes have been recruited for specific purposes, e.g. FUMC and possibly ACNB and CSB. It would also appear that the retention of two or more differentially regulated genes with isofunctional products offers advantages over the constitutive expression or mutliple regulation of one gene encoding a single all-purpose enzyme. However, this scenario consists sharply with that for lipoamide dehydrogenase. Here a single *lpd* gene supplies lipoamide dehydrogenase components to the differentially regulated pyruvate and 2-oxoglutarate dehydrogenase complexes and the glycine cleavage system, with which it is coregulated. In this case there seems to have been insufficient need for functional specialization of the enzyme to favour gene duplication (Guest *et al.* 1989). Students often regard the CAC as a dreary bit of old-fashioned biochemistry. However, to me it has been full of unexpected findings relating to metabolic and chromosomal evolution and to questions concerning the relative merits of multifactorial regulation of single genes versus the independent regulation of multiple genes.

The remainder of this lecture will be devoted to considering the mechanisms of selective gene expression in *E. coli*, with special reference to the response to anaerobiosis and the FNR-mediated system.

3. THE *E. COLI* CHROMOSOME AND SELECTIVE GENE EXPRESSION

The *E. coli* chromosome is a circular DNA molecule containing 4.66 Mb.p. or about 4700 genes, and it can be replicated every 40 min in growing bacteria. About 60% of the nucleotide sequence has been defined, but only 35% by a systematic approach (F. R. Blattner and K. E. Rudd, personal communications). Most of the DNA encodes proteins or RNA products and in a

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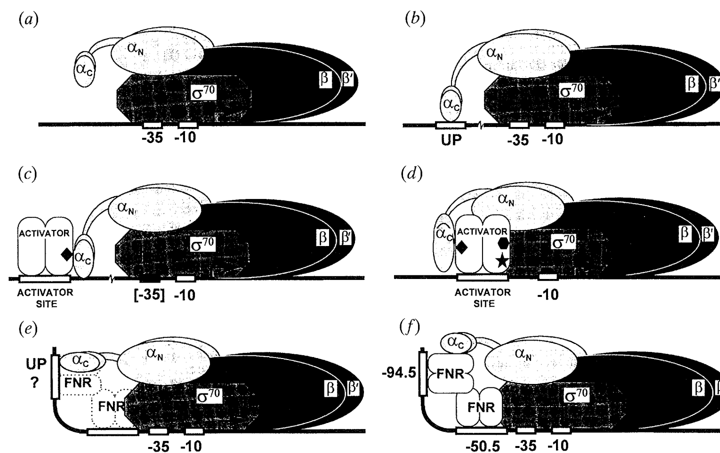

Figure 3. RNA polymerase and selective gene expression in *E. coli*. The subunits and promoter elements involved in transcription activation are shown: (*a*) basal, (*b*) UP-element activated, (*c*) activator-dependent (class I) and (*d*) activator-dependent (class II). A possible mechanism for transcription repression by FNR is illustrated for the *ndh* gene: (*e*) aerobic induced and (*f*) anaerobic repressed. The closed symbols (diamond, hexagon and star) denote different sites of subunit–regulator interaction. Based on Busby & Ebright (1994).

representative 1 Mb segment 40% of the genes have known functions and 6% (1 in 17) are identifiable as regulatory genes (genes that control the expression of other genes). It is hoped that the complete sequence will be known at sometime in 1996. So far, approximately 1720 genes have been identified by different criteria and, of 164 that encode regulators, 43 have been designated broad or global regulators (Riley 1993). Gene expression involves DNA transcription by RNA polymerase (RNAP) followed immediately by translation of the elongating mRNA by the ribosomal protein-synthesizing machinery, or mRNA processing into other forms of RNA. In *E. coli*, gene expression is mainly regulated at the transcriptional level by activating or repressing the formation of mRNA transcripts by RNAP.

The RNAP holoenzyme is assembled from four types of subunit, $\alpha_2\beta\beta'\sigma^{70}$ (figure 3). It binds to specific sequences (promoters) located upstream of the coding regions where the formation of an effective complex and the corresponding level of gene expression are determined by the binding specificity and strength (Busby & Ebright 1994). The binding specificity is largely conferred by the σ^{70} subunit interacting with two hexameric elements located at −35 and −10 relative to the transcription start site: TTGACA and TATAAT, usually separated by 17 base pairs. In extremely strong promoters there is an additional interaction between the C-terminal domain of the α subunit (α_C), which is tethered to its N-terminal domain (α_N) by a flexible linker, and an UP element or *ca.* 20 b.p. AT-rich sequence located upstream of the −35 sequence (figure 3*b*). Promoter strength depends upon the presence (or absence) and quality of the −35, −10 and UP elements.

This basic model provides plenty of scope for regulating the expression of specific genes or groups of genes in response to different stimuli, e.g. by replacing existing components or by adding further components to the transcription complex. Thus, the transcription specificity of RNAP can be reprogrammed by replacing σ^{70} with an alternative σ factor that recognizes a different promoter sequence. Examples of such sigma factors (and their binding site consensuses, where established) include: σ^{54} or σ^{N} (T**GG**CAC-5bp-TT**GC**A) which mediates the response to nitrogen limitation; σ^{38} or σ^{S} for genes that are expressed in stationary phase; and σ^{32} or σ^{H} (TCTCNCCCTT-GAA—(13 to 17 b.p.)—CCCCATNTA) and σ^{24} or σ^{E} for genes that are induced at high temperatures. Alternatively, an otherwise silent or poor promoter can be activated by a transcription regulator whose binding site overlaps or lies upstream of the −35 element. When such an activator is bound at an upstream site it is thought to interact with α_c in such a way as to compensate for the lack of an UP element or good −35 and −10 sequences (figure 3*c*), whereas at an overlapping site the activator can make effective contact with the transcription complex via the σ subunit (figure 3*d*).

Many target-specific regulators responding to different stimuli have been identified. They are designated specific regulators if they control a single gene, an operon or a regulon (a collection of functionally related transcriptional units), in response to a specific substrate or metabolite. Two well known examples (with coeffectors) are AraC (arabinose) and LacI (β-galactosides). The regulators may be designated global, broad or pleiotropic regulators if they control a modulon (a family of gene, operons and regulons) in response to a common stimulus, in addition to any other specific regulation that may apply. Global regulators may control as many as 70 transcriptional units in response to different stimuli. Some examples include: CRP, carbon limitation; PhoB, phosphate limitation; SoxRS, superoxide stress; OxyR, peroxide stress; OmpR, osmotic shock. Depending on the positions of their binding sites and the way in which they interact with RNAP, global regulators can function either positively or negatively, i.e. to activate or repress gene expression. In the latter case their functions overlap those of the negatively acting specific regulators (repressors), which bind to specific sites (operators) in the promoter regions and in

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consequence block transcription, occlude the promoter or displace the polymerase unless released by the corresponding coeffectors.

In recent years it has become increasingly apparent that the expression of a single gene can be extremely complex, not least because it may belong to several independent modulons, each with its own regulator, but also because there are further regulatory tiers or interconnections that control expression of the regulatory genes themselves. Furthermore, it should be appreciated that these specific and global controls are superimposed on more general or universal controls stemming from the effects of osmolarity, anaerobiosis, pH and temperature on DNA structure. So the ultimate pattern of gene expression obtaining in a given environment is the product of a highly complex, finely tuned and rapidly reacting network of interacting factors capable of sensing and responding to an organism's needs in a rapidly changing environment.

4. ANAEROBIC GENE EXPRESSION IN *E. COLI*: THE METABOLIC ARENA

As already indicated, *E. coli* is a metabolically versatile chemoheterotroph which can use a variety of substrates to support growth under aerobic and anaerobic conditions. It derives energy from respiratory or fermentative processes. Examples of some basic modes of glucose metabolism are summarized in figure 1. Under aerobic conditions *E. coli* uses the proton-translocating aerobic respiratory chain with oxygen as the ultimate electron acceptor (figure 1*a*). Pyruvate is oxidized by the pyruvate-inducible pyruvate dehydrogenase complex (PDHC) and, when glucose is not provided in excess, the resulting acetyl units are completely combusted to carbon dioxide (CO_2) via the CAC. This generates an abundance of utilizable energy: 4 ATP + 24 reducing equivalents per mole of glucose.

Under anaerobic conditions, oxygen can be replaced by alternative electron acceptors such as fumarate, nitrate, nitrite, dimethylsulphoxide (DMSO), trimethylamine-*N*-oxide (TMAO) and tetrahydrothiophen-1-oxide, and the corresponding anaerobic respiratory chains, each with a specific terminal reductase, are induced (figure 1*b*). Here the PDHC is partially repressed and inhibited; so the conversion of pyruvate to acetyl-CoA increasingly depends on the induction and activation of pyruvate formate-lyase (PFL). The substrate is not totally combusted because the CAC is reduced to its non-cyclic form and the energy yield is accordingly limited to 4 ATP + 8 reducing equivalents per mole of glucose. The major product is acetate and the formate generated by PFL is oxidized to CO_2 by different formate dehydrogenases depending on the ultimate electron acceptor, e.g. FDH_N with nitrate.

Then, in the absence of an exogenous electron acceptor, energy is obtained by the mixed acid fermentation (figure 1*c*). This involves the redox-balanced dismutation of the substrate with the formation of acetate plus CO_2, ethanol plus hydrogen (H_2), and small amounts of succinate, lactate and formate. Here, acetyl-CoA is generated largely by PFL together with formate. Half of the acetyl-CoA is reduced to ethanol to achieve redox balance, and formate is converted to H_2 plus CO_2 by the formate hydrogenlyase system (FHL) which is induced in response to the accumulation of formate. This mixture of H_2 plus CO_2 is undoubtedly what van Leeuwenhoek saw 'escaping with force' when he opened a sample of pepper and water that had been sealed in a glass vial for several days. The energy yield from the mixed acid fermentation is only 3 ATP per mole of glucose, mainly derived by substrate-level phosphorylation during glycolysis and during the conversion of acetyl-CoA to acetate.

The switch between different metabolic modes is accompanied by very significant changes in enzyme synthesis. This in turn is controlled by transcription regulators which respond to the availability of oxygen or alternative electron acceptors. Furthermore, these regulatory mechanisms ensure that, in a given environment, the most energetically favourable process is used. So, aerobic respiration is preferred to nitrate, nitrite and fumarate respiration, nitrate respiration is preferred to fumarate and DMSO respiration and it seems likely that fumarate respiration is preferred to hydrogen-evolving fermentation where pyruvate and acetyl-CoA serve as endogenous electron acceptors. This hierarchy reflects the energy yields (ATP + H), the midpoint redox potentials of the electron acceptors (E_0', mV) and the free energies of the corresponding metabolic reactions ($\Delta G_0'$, kJ (mol glucose)$^{-1}$). The respective values for relevant electron acceptors are: aerobic respiration (4 ATP + 24 H), oxygen (+820, −2830); anaerobic respiration (4 ATP + 8 H), nitrate (+430, −858), nitrite (+374, ?), DMSO (+160, −650), TMAO (+130, −625) and fumarate (+30, −550); fermentation (3 ATP), acetyl (−412, −218).

It seems unlikely that *E. coli* could have evolved a regulator capable of sensing and responding across the entire redox or free-energy range, even though it would provide a single all-purpose mechanism for exploiting the metabolic opportunities offered by different redox environments. Such a versatile regulator would probably exceed the functional capacity or structural and design constraints of a single protein. Instead, *E. coli* imposes its metabolic priorities by making very effective use of four or five independent transcription regulators. Furthermore, these regulators illustrate the basic mechanisms of the single- and two-component regulatory systems that are widely used for selective gene expression in *E. coli* and other bacteria.

5. ANAEROBIC GENE EXPRESSION IN *E. COLI*: THE TRANSCRIPTION REGULATORS

(*a*) *The ArcBA sensor–regulator system*

The ArcBA systen has been characterized by Lin and coworkers who isolated two classes of mutant that fail to repress SDH and other aerobic enzymes under anaerobic conditions (Iuchi & Lin 1988; Iuchi *et al.* 1990). The corresponding genes were given the *arc* designation to denote their lack of *a*erobic *r*espiration

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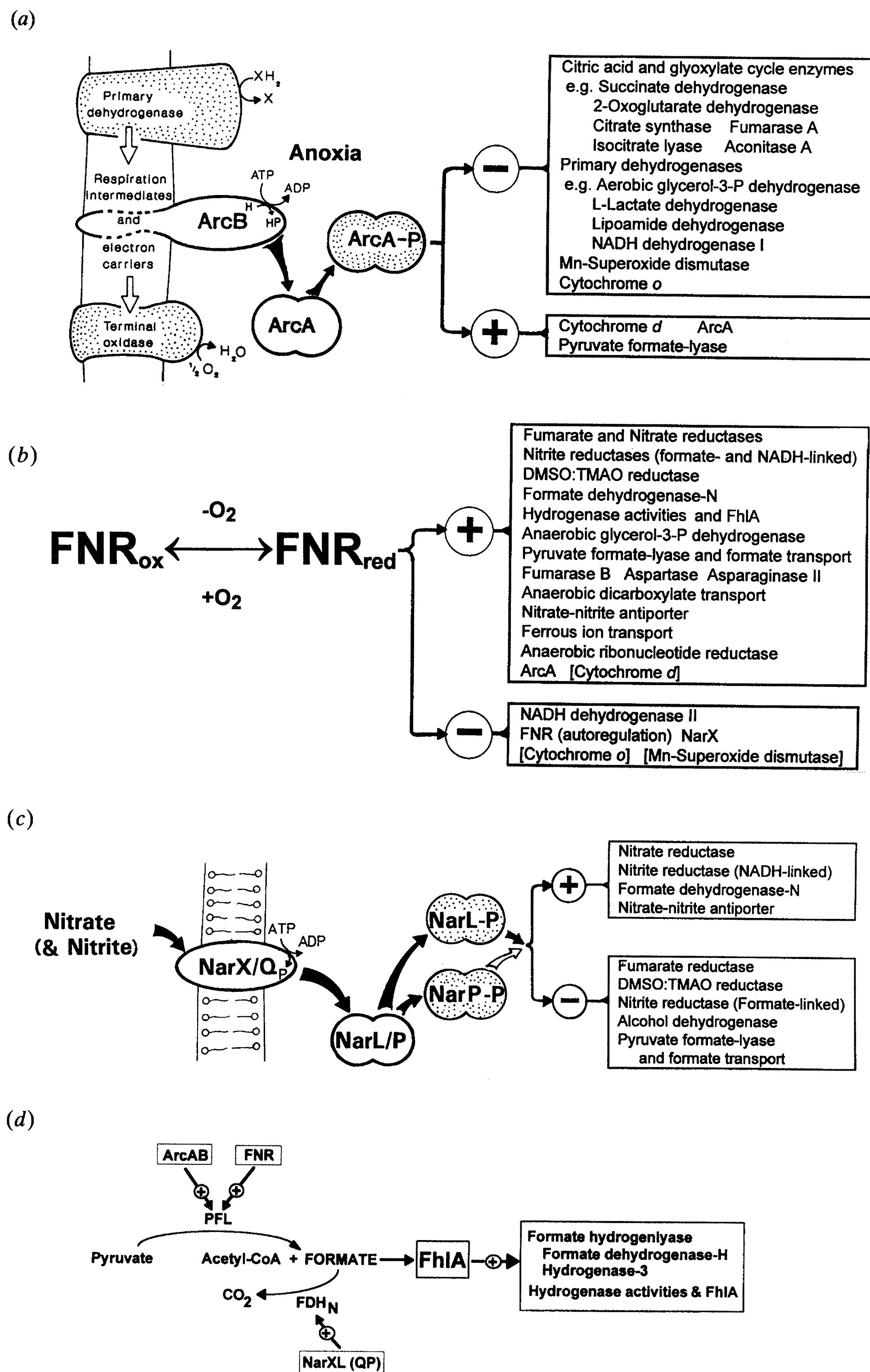

Figure 4. Transcription regulators that control the metabolic priorities of *E. coli* and some representative target functions. (*a*) The ArcBA sensor–regulator system, (*b*) the FNR system, (*c*) the NarXL and NarQP sensor–regulator systems, (*d*) FhLA, the formate regulator. The regulators respond directly or indirectly to oxygen, nitrate and nitrite or formate and either activate (+) or repress (−) the corresponding genes. The responses shown in (*c*) are essentially those mediated by the NarXL pair with nitrate; different responses are evoked by nitrite, or are mediated by alternative sensor–regulator pairs than with nitrate or nitrite (Rabin & Stewart 1993).

control, and they were shown to encode the membrane-bound sensor (ArcB) and cytoplasmic regulator (ArcA) components of a two-component signal-transducing system (figure 4*a*). The two-component system is widely used in bacteria for regulating gene expression in response to environmental changes and stresses, and it has recently been found in yeast and other eukaryotes (Stock *et al.* 1989). The sensors are characterized by the presence of an N-terminal signal-recognition or input domain, linked to a histidine autokinase transmitter module that becomes phosphorylated in response to the primary signal. The signal is then transmitted to

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the regulator by the phosphorylation of a conserved aspartyl residue in its N-terminal receiver module. The receiver possesses transphosphorylase, phosphatase and dephosphatase activities, and it is linked to an output domain that performs the transcription regulatory function. The structural organization varies in different systems and it is interesting that there is a limited and presumably intentional (or tuned) degree of 'cross-talk' between sensors and regulators of different specificities (Wanner 1992). Other examples of this type of regulator include: EnvZ–OmpR, osmoregulation of porin synthesis; CheA–CheB/Y, chemotaxis; and PhoR–PhoM, phosphate limitation.

The precise nature of the anaerobic signal sensed by the ArcBA system is not known although it seems likely that ArcB responds either to anaerobic stress in the aerobic respiratory chain or to the accumulation of metabolic intermediates and products like pyruvate, acetate, lactate and NADP (Iuchi & Lin 1993; Iuchi *et al.* 1994). It is also apparent that the input and transmitter modules of ArcB are covalently linked to a pair of receiver and output domains, such that phosphorylation of ArcA is preceded by two cycles of autophosphorylation and transphorylation in ArcB (Iuchi & Lin 1993). Another plausible possibility is that ArcA might be activated by direct phosphorylation by acetylphosphate. Acetylphosphate could serve as an excellent internal metabolic reporter, and it is already known to phosphorylate some response regulators (McClearly *et al.* 1993; Wanner & Wilmes-Riesenberg 1992). The phosphorylated form of ArcA functions as an anaerobic repressor of the CAC and respiratory activities (figure 4*a*). It also functions as an anaerobic activator of the microaerobic cytochrome oxidase (CYD), pyruvate formate-lyase (PFL) and its own expression. A recent survey has indicated that the ArcA modulon contains 18 negatively regulated and eight positively regulated genes or operons, based on transcriptional and enzymic criteria (Lynch & Lin 1995). However, it has not yet been established whether ArcA-mediated gene expression involves a site-specific interaction between ArcA and promoter DNA, or some other mechanism. This is because the relevant promoters appear not to contain an obvious binding-site consensus, and clean footprints have yet to be obtained.

(*b*) *The FNR system*

FNR represents a one-component system in which the sensory and regulatory functions are combined in a single protein (Spiro & Guest 1990; Unden *et al.* 1994; Guest *et al.* 1995). Under anaerobic conditions, FNR activates the fumarate, nitrate, nitrite and DMSO reductases and a host of other anaerobic functions (figure 4*b*). It even potentiates the effects of ArcA by enhancing ArcA synthesis. This indirect effect of FNR has led to some members of the *arc* modulon being assigned to the *fnr* modulon, but in physiological terms it means that the initial effects of anaerobiosis mediated by ArcA are intensified by FNR as anaerobiosis persists or deepens. FNR also functions as an anaerobic repressor of the non-proton-translocating NADH dehydrogenase II and it negatively autoregulates its own synthesis. A recent survey has indicated that the FNR modulon contains 29 transcriptional units (70 genes) of which 22 are positively regulated and 7 are negatively regulated (Guest *et al.* 1995). It is interesting that PFL is activated by both FNR and ArcA, whereas the microaerobic cytochrome *d* is activated by ArcA but repressed by FNR, and cytochrome *o* is anaerobically repressed by both of the regulators. The existence of two regulators each responding to different anaerobic signals (FNR at $E_0' > +400$ mV; ArcA to $\Delta\mu_{H^+}$ or metabolites) and functionally linked insofar as one is a member of the alternate modulon offers considerable advantages in coordinating and fine-tuning gene expression in response to graded environmental shifts across the redox range. Further details of the FNR system will be discussed below.

(*c*) *The NarXL and NarQP sensor–regulator systems*

Next come the NarXL and NarQP sensor–regulator systems (figure 4*c*), which respond to exogenous nitrate and nitrite with subtly different effector and target specificities (Stewart 1988, 1993; Rabin & Stewart 1993). Thus, in response to nitrate, NarL activates the respiratory nitrate reductase, the NADH-dependent nitrite reductase, formate dehydrogenase$_N$ and the nitrite transporter (together with FNR), but represses the fumarate and DMSO reductases, the anaerobic dicarboxylic transport systems and alcohol dehydrogenase. Different regulatory consequences are mediated by NarL in response to nitrite (e.g. repression of the formate-linked nitrite reductase) and by NarP in response to nitrate and nitrite (Rabin & Stewart 1993). Nevertheless, it is clear that once oxygen has been consumed the positive and negative effects of the NarXL and NarQP systems establish the priority for nitrate and nitrite utilization over that of fumarate and DMSO.

(*d*) *FhlA, the formate regulator*

Finally, reference must be made to the single-component formate regulator, FhlA (Lutz *et al.* 1991; Rossmann *et al.* 1991; Sauter *et al.* 1992). It responds to the accumulation of formate during fermentation by activating the synthesis of the H_2 plus CO_2-evolving formate hydrogenylase, FHL (figure 4*d*). Formate, derived from pyruvate by the action of PFL, accumulates during fermentation but not in other metabolic modes, because it is rapidly oxidized to CO_2 at the expense of the exogenous electron acceptor. The FhlA modulon encodes the specific formate dehydrogenase and hydrogenase components of FHL, some other hydrogenases and the positive autoregulated *fhlA* gene. It is also interesting that FhlA-dependent genes require σ^{54}-RNAP for their transcription rather than σ^{70}-RNAP.

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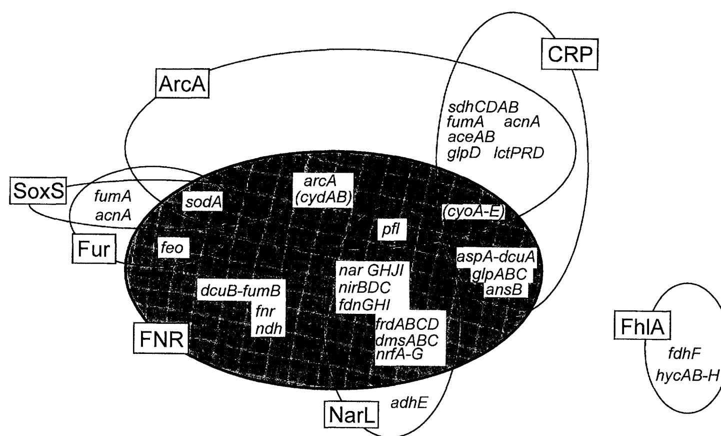

Figure 5. Overlapping modulons in *E. coli*. Each modulon, or family of genes and operons controlled by a global regulator, is enclosed by a labelled ellipse. The diagram illustrates how members of one modulon such as the FNR modulon (shaded ellipse) can be members of other modulons and thus subject to multifactorial transcription control. Adapted from Iuchi & Lin (1993).

(*e*) *Overlapping modulons and multifactorial control*

The mechanisms described above show how the basic metabolic modes are imposed at the transcriptional level in response to environmental factors. It is also apparent that many central metabolic genes are controlled by more than one global regulator, i.e. they are subject to dual or multifactorial control, and thus belong to more than one modulon (figure 5). For example, members of the FNR modulon may belong to the CRP (carbon catabolism), Fur (iron uptake) and SoxS (redox stress) modulons as well as to the NarL and ArcA modulons. Particularly interesting is the Mn-superoxide dismutase gene (*sodA*) which is activated by the SoxRS system in response to superoxide stress but repressed by ArcA and FNR (directly or via *arcA* activation) under anaerobic conditions where superoxide generation is less intense (Compan & Touati 1993, 1994).

The transcription of a single promoter can thus be subject to the positive or negative influences of a variety of regulators, each responding to different environmental stimuli. The existence of such regulatory networks extends the opportunities for applying further priorities with respect to e.g. carbon, nitrogen and phosphate metabolism, and for modulating or fine-tuning the level of gene expression to suit a wide range of conditions. They add a further regulatory tier on top of the primary controls imposed by specific regulators and the general controls stemming from the underlying effects of anaerobicity and osmolarity on DNA supercoiling. Furthermore, it would appear that each regulator has an independent binding site, and that the ultimate activity of a promoter depends on the relative affinities and efficacies of each regulator interacting with the transcription complex, although the activity of one regulator (e.g. NarL) may require that another (e.g. FNR) is functional.

6. FNR AND ANAEROBIC GENE EXPRESSION

The remainder of this lecture will be devoted to discussion of the nature of the FNR protein, site-specific DNA binding and transcription regulation by FNR, the mystery of redox-sensing, and some general points about the CRP–FNR family of transcription regulators. The FNR system was first identified in studies with some pleiotropic mutants that failed to use fumarate and nitrate as terminal electron acceptors (Spiro & Guest 1990). The corresponding gene was assigned the symbol *fnr* to denote the defects in *f*umarate and *n*itrate *r*eduction stemming from its inactivation. The first significant clues about its function came from the nucleotide sequence of the *fnr* gene. This established that its product, FNR, is a transcription regulator closely resembling CRP, the cyclic AMP receptor protein (also known as CAP, the catabolite gene activator protein). Sequence comparisons and secondary structure prediction indicated that FNR retains all of the structural elements in the CRP monomer (figure 6). These include: the helix–turn–helix motif in the DNA-binding domain, where specificity-conferring interactions occur between the amino acid side chains in the DNA-recognition helix and nucleotide base pairs in the major groove of the DNA duplex; the series of β-strands in the sensory domain (but not the residues that interact with cAMP); and the large helix forming the dimer interface in CRP. It was therefore predicted that FNR is a site-specific DNA-binding protein and a global transcription regulator controlling anaerobic gene expression in response to anaerobiosis or some unknown coeffector that signals oxygen starvation. It was likewise predicted that FNR operates the master switch that ensures the preferential use of oxygen over other electron acceptors, just like CRP ensures the preferential use of glucose relative to lactose and other carbohydrates. Much subsequent work has been devoted to investigating and for the most part confirming, these predictions. However, FNR poses some special problems and it is significantly different from CRP.

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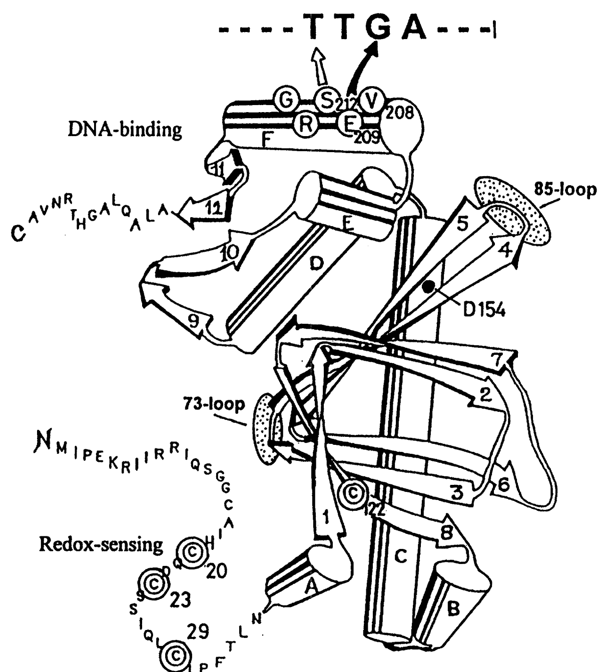

Figure 6. Model predicted for the FNR monomer based on the known structure of CRP. The essential cysteine residues in the putative iron-binding redox-sensing domain are encircled, and the proposed conserved and discriminatory interactions between residues in the DNA-recognition helix (α_F) and specific base pairs in the FNR-site core motif, relative to those established for CRP, are denoted by black and white arrows (respectively). Regions of FNR that are thought to make activating contacts with RNA polymerase are the 85-loop which contacts σ when FNR is bound at −41.5 (class II promoters) and the 73-loop which contacts α_C when FNR is bound at −41.5 and sites further upstream (class II and class I promoters).

(*a*) *The FNR protein*

The FNR protein can be amplified in *E. coli* to 20% of soluble protein (figure 7) and purified to near homogeneity in a single chromatographic step (Green *et al.* 1995). The purified protein is monomeric (M_r 28000 by gel filtration and 30000 by SDS–PAGE; 27965 predicted) unlike CRP, which is dimeric, although FNR appears to be dimeric when bound to DNA. FNR differs from CRP in possessing a cysteine-rich N-terminal extension that contains three of the four essential cysteine residues, C20, C23, C29 and C122 (figure 6). However, nine apparently non-essential N-terminal residues are removed by periplasmic or membrane-bound proteases when FNR is isolated from some strains of *E. coli*. Another notable feature of FNR is the presence of up to 1.1 atoms of iron per FNR monomer (Green *et al.* 1991). Furthermore, the iron content is inversely related to the protein sulphydryl content and it is lowered to 0.54 and 0.05 atoms per mole in two of the cysteine-substituted proteins (C23G and C122A, respectively), which suggests that some of the cysteine residues serve as iron ligands (Green *et al.* 1991, 1993). More recently it has been found that the iron content can be increased to as much as 2.7 atoms per FNR monomer by incubation with ferrous iron and reducing agents, and this is accompanied by a size shift to M_r 40000 (indicating the presence of dimers) and absorption maxima at 315 and 420 nm ($\epsilon_{315} = 3700\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) similar to those of iron–sulphur proteins (Green *et al.* 1995). It would thus appear that FNR contains a redox-sensitive iron–sulphur centre.

There is also evidence for two species of FNR both in the isolated protein and in intact bacteria (Green *et al.* 1993). The minor or oxidized component (FNR_{27}) is converted to the major form (FNR_{30}) when treated with β-mercaptoethanol, apparently by reduction of an intramolecular disulphide bond or some other link between C122 and one of the N-terminal cysteine residues. The proportion of the oxidized form increases from 14% in anaerobic bacteria to 40% during aerobic growth, which may be physiologically significant especially as the transition is accompanied by a conformational change that affects electrophoretic mobility. It is also interesting that by using electrospray mass spectrometry a modified component 78 ± 7 Da larger than predicted has been detected in wild-type and mutant proteins, but not in the C122A protein

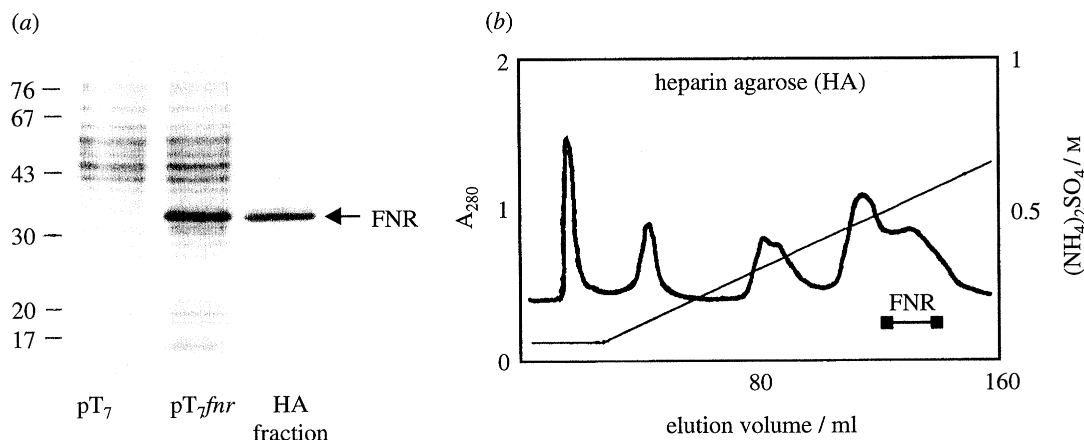

Figure 7. Amplification and purification of the FNR protein. (*a*) SDS–PAGE analysis of soluble proteins from IPTG-induced cultures of *E. coli* strains containing pT_7 *fnr*, an expression plasmid in which of the *fnr* gene is cloned downstream of an IPTG-regulated pT_7 promoter or pT_7, the expression vector alone, and heparin–agarose purified FNR. The positions of molecular mass markers ($M_r \times 10^{-3}$) are indicated. (*b*) Heparin–agarose elution profile for the amplified extract with a bar to denote fractions containing highly enriched FNR (Green *et al.* 1995).

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(Green *et al.* 1993). This could signify the presence of a covalently bound redox-signalling coeffector bound at C122 unless it represents the remnants of an iron–sulphur centre.

(*b*) *DNA binding*

Purified FNR binds to DNA containing partially palindromic sequences conforming to a consensus that resembles the CRP binding-site consensus:

FNR site A–A–**T**TGAT– – – – –ATCA**A**T– – –;

CRP site AA–T**G**TGA– – – – – – –TCA**C**A–TT.

Thus, FNR sites contain a well conserved **T**TGA half-site motif compared with the **G**TGA motif in CRP sites. Moreover, the two sites can be functionally interconverted (*in vivo* and *in vitro*) simply by interchanging **T** for **G** and **A** for **C** at the critical positions in each ahlf site (reviewed by Spiro & Guest 1990). The binding specificities of the two proteins can likewise be interconverted by exchanging key residues in the DNA-binding faces of the recognition helices (α_F) of the respective proteins: **VE**– –**S** in FNR (figure 6) for **RE**– –G at the corresponding sites in CRP, and *vice versa* (Spiro & Guest 1987; Spiro *et al.* 1990). Two specificity-conferring interactions have been proposed: a conserved interaction between E209 and the G–C base pair in the FNR half-site motif (figure 6), analogous to an established interaction between E181 in CRP and the same G–C base pair in the CRP site; and a discriminatory interaction between S212 and the critical T–A base pair in the FNR site, which replaces that between R180 and the critical G–C base pair in the CRP site. Genetically engineered hybrids are capable of regulating catabolite-sensitive promoters (e.g. the *lac* promoter) in response to anaerobiosis or FNR-regulated genes (such as the semisynthetic *FFmelR* promoter) in response to cAMP (glucose starvation). The ensuing metabolic derangements amply confirm the earlier predictions that FNR has close structural and functional relations with CRP.

DNase I footprints with several positively regulated (*FFmelR*, *frdA*, *pflP6*, *fdnG*, *narG*, *nirB*, *ansB*) and negatively regulated (*fnr*, *ndh*) promoters have shown that FNR specifically protects regions of 23–33 b.p. that overlap the predicted sites, and that two monomers are bound at each site (Sharrocks *et al.* 1991; Green *et al.* 1995). Representative FNR-regulated promoter regions are illustrated in figure 8. All of the positively regulated genes lack −35 sequences but have potential FNR sites centred at −40 to −50, where FNR is bound. Some promoters contain another potential site but in *nirB* and *ansB* the extra site is neither essential nor protected by FNR. In contrast, negatively regulated promoters contain good −35 sequences and two FNR-protected sites (figure 8). Significantly, the upstream sites (centred at −94 to −107) are particularly important for repression because their deletion severely impairs FNR-mediated repression (Green & Guest 1994; Takahashi *et al.* 1994). It is also interesting that in the positively regulated *fdnG* promoter, where there are two FNR-protected sites, that centred at −42.5 is essential for activation, whereas the site at −97.5 is thought to be involved in down-regulating FDH_N synthesis in the absence of nitrate (Li & Stewart 1992). A far-upstream site would therefore appear to be specifically associated with FNR-mediated repression.

Footprinting studies have shown that apo-FNR is almost as effective as Fe-FNR and that anaerobiosis (or reducing agents) is not essential for site-specific DNA protection. However, DNA binding is impaired with mutant FNR proteins lacking an essential cysteine residue. In the *ndh* promoter this impairment is particularly apparent at the rather poor (but important) upstream FNR site, but hardly detectable at the better −50.5 site, and the C122A-substituted protein, which has the lowest iron content, is the most impaired (Green *et al.* 1993). The binding affinity would thus seem to depend on the qualities of both the site and the protein, and the iron content is important in marginal situations.

(*c*) *Transcription regulation by FNR*

The demonstration that FNR activates *FFmelR*-mediated transcription and represses *ndh* transcription *in vitro* provided the first tangible evidence that FNR could be purified in a functional form (Sharrocks *et al.* 1991). It has been further shown that Fe-FNR (FNR with a high iron content) is essential for both activities, but inactive apo-FNR can be reactivated by pre-incubation with ferrous ions and a reducing agent such as β-mercaptoethanol (Green & Guest 1993*a*, *b*). Fe-FNR is also essential for open complex formation both *in vitro* and *in vivo* (Green & Guest 1993*b*). It can thus be concluded that FNR binds iron, which is essential for *in vitro* transcription regulation but not for site-specific DNA binding.

The mechanism envisaged for transcription activation by FNR corresponds to that shown in figure 3*d*. There is no −35 sequence, instead two FNR monomers centred in the −40 to −50 region make activating contacts with α_C and σ^{70} in RNAP, under anaerobic conditions. This is similar to the mechanism proposed for CRP activation of class I promoters. FNR is less active when its binding site is relocated to that found in many class II promoters, −61.5 in *FF+20melR* (Bell *et al.* 1990). Here, transcription activation seems to involve contact between the downstream subunit of the FNR pair and α_C alone, as illustrated in figure 3*c*. Two potential contact sites have been identified by mutational analysis: one in the 85-loop situated in the β_4–β_5 turn (figure 6) of the upstream subunit is required for activation from the −41.5 position (classII), whereas the 73-loop in the β_3–β_4 turn (figure 6) is important for activation from both the −41.5 and the −61.5 positions (class II and class I) (Williams *et al.* 1991; Bell & Busby 1994).

Repressors generally bind to DNA in such a way as to affect promoter binding by RNAP or its activation. However, a different mechanism is envisaged for transcription repression by FNR (Green & Guest 1994). This is exemplified by the *ndh* promoter which contains a −35 sequence and two FNR sites where FNR binds without displacing RNAP (figure 3*e*) but

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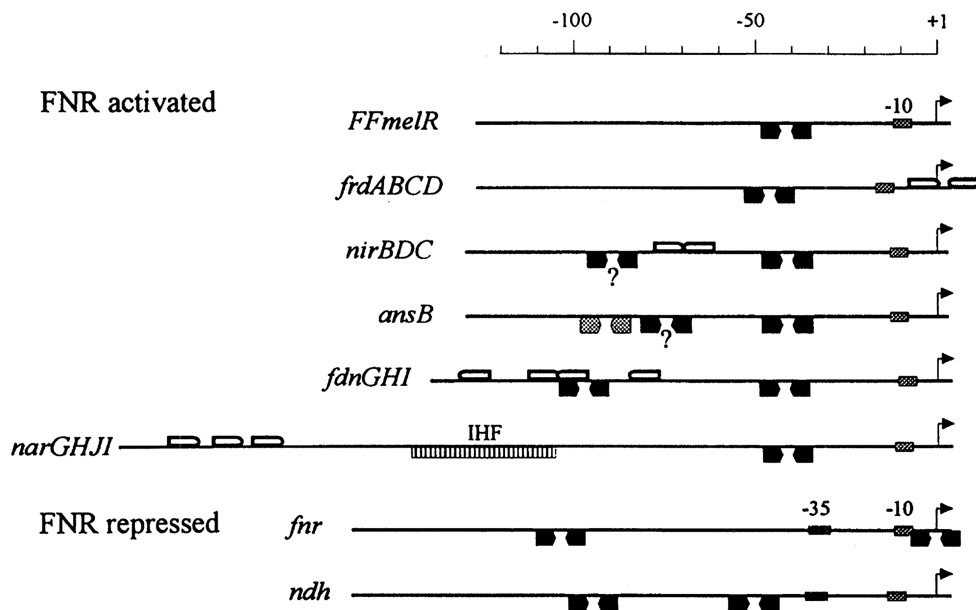

Figure 8. Promoter regions of representative FNR-regulated transcriptional units. Regulator binding sites for FNR (■), CRP (▩) and NarL (▭) and consensus −35 and −10 hexamers (▧) are located relative to the transcription start sites (arrows). The question marks denote two predicted FNR sites where FNR binding cannot be detected.

nevertheless inhibits transcription under anaerobic conditions (figure 3*f*). Binding at the site centred at −94.5 is particularly important for repression and the mechanism seems to involve direct interaction between RNAP and FNR at the upstream site and/or the abolition of essential upstream contacts between RNAP and DNA (figure 3*f*). The latter could involve displacing α_C from an UP element in or near the FNR site; indeed UP elements and FNR sites have common features. There is clearly much to be learned about FNR-mediated transcription regulation, particularly with respect to the novel features associated with the upstream sites that are needed for repression.

(*d*) *The mystery of redox-sensing and the sensory mechanisms of related regulators*

This brings me to the question of how FNR senses anaerobiosis and transmits the information to the transcription complex. It is a mystery because Fe-FNR isolated from aerobically grown bacteria can activate and repress transcription without any need for an anaerobic stimulus. This suggests that at least a fraction of the purified protein retains the active anaerobic conformation. It also indicates that FNR neither senses nor is inactivated by oxygen *per se*, as does the observation that the transcriptional switch can be mediated by ferricyanide (E'_0, +520 mV) in the complete absence of oxygen (Unden *et al.* 1990).

Several mechanisms have been proposed for the sensing and response to anaerobiosis by FNR (Guest *et al.* 1995). Since each commands some experimental support, it seems likely that the ultimate mechanism will reconcile elements from the various possibilities. Early observations that iron chelators mimic oxygen in their effects on FNR-regulated gene expression and the reactivities of FNR sulphydryl groups strongly support a role for this element in the sensory pathway. The

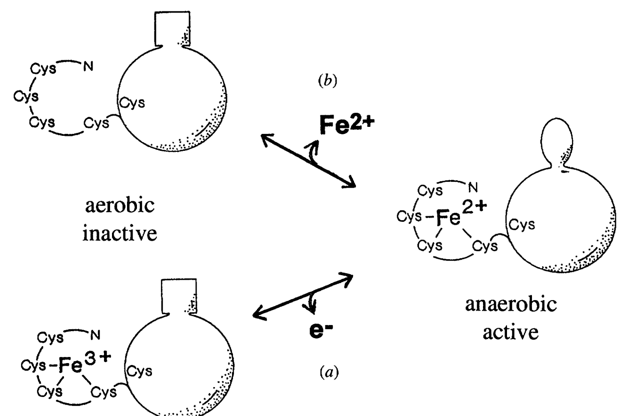

Figure 9. Possible modes of redox sensing and activation of FNR. Anaerobic activation could involve (*a*) a ferric–ferrous redox-cycling mechanism or (*b*) the reversible uptake and release of ferrous iron, accompanied by conformational changes that regulate RNA polymerase activity. Only one subunit of the dimeric DNA-bound form is shown. It now seems likely that the functional form contains two 4Fe–4S clusters per dimer (Khoroshilova *et al.* 1995).

ferric–ferrous redox-cycling mechanism predicts that the N-terminal segment forms part of an iron-binding redox-sensing domain, in which the reduction of a cysteine-bound iron cofactor converts FNR into the active anaerobic conformation that makes effective contacts with RNAP (figure 9*a*). According to this model, FNR could sense the intracellular redox state either directly or via some agent such as the ferrous iron concentration. Alternatively, the anaerobic switch could be mediated by the reversible *uptake and release of ferrous iron* (figure 9*b*). Such a mechanism is consistent with the variable iron content of FNR, its increased sulphydryl reactivity in aerobic bacteria and the reversible assembly of a polynuclear iron–sulphur centre. The reactivation of apo-FNR by ferrous ions

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under reducing conditions provides good *in vitro* support for this mechanism. Other potential mechanisms include: *monomer–dimer transition*, with or without the reduction or reductive modification of an intracellular disulphide bond; and covalent or non-covalent interaction between FNR and an unidentified *redox-signalling coeffector*.

Of prime importance are studies with two classes of *fnr** mutants that partially activate anaerobic gene expression under aerobic conditions (Kiley & Reznikoff 1991; Melville & Gunsalus 1990). One class has single alterations in the proposed redox-sensing domain (D22G, D22S, L28H or an insertion of a serine residue after residue 17) which could mimic the effects of cofactor reduction or stabilize the iron cofactor so that it is re-tuned to a higher aerobic range. In the other class, a residue at the dimer interface is altered (e.g. D154A) and this produces a dimeric form of FNR which is known to have a higher affinity for DNA because it retards target DNA in gel-shift assays (Lazazzera *et al.* 1993; Ziegelhoffer & Kiley 1995). Such a protein may adopt the conformation that is normally induced earlier in the sensory pathway by iron uptake or reduction of the iron cofactor. Very recently, a double mutant protein (L28H, D154A) has been shown to contain at least one $[3Fe\ 4S]^+$ centre per dimer (not present in the D154A protein); it also has a higher dimer content and a much enhanced DNA-binding affinity than the D154A protein (Khoroshilova *et al.* 1995). It was further observed that DNA binding is lowered by exposure to oxygen but restored by reconstituting the iron–sulphur centre *in vitro*, thus providing a plausible mechanism for regulating transcription activity in the wild-type protein.

Despite the recent advances, there is still much to be learned about redox-signalling and signal transduction in the transcription complex. It will be important to establish the relation between iron–sulphur cluster assembly and dimer formation, the number of iron–sulphur centres per dimer, the respective iron ligands, and whether the fully assembled cluster is the redox sensor or whether activation depends on the incorporation of a fourth iron atom into each cluster. The FNR protein is clearly a very labile dimeric protein containing an inherently unstable iron–sulphur cluster which may be reassembled in a manner analogous to that observed in the reactivation of aconitase and the iron–sulphur containing fumarases (FUMA and FUMB). Another feature deserving attention is the role of FNR in aerobic gene expression. There are at least eight proteins whose aerobic synthesis requires FNR (Sawers *et al.* 1988). Since apo-FNR and Fe-FNR exhibit site-specific DNA binding under aerobic conditions, it is conceivable that they can serve as activators in transcriptional complexes that possess the appropriate geometry.

Several other redox-responsive or related regulatory systems have been characterized. Closest to FNR is the NifA protein of *Bradyrhizobium japonicum* which mediates the redox- and iron-dependent regulation of N_2 fixation (Hennecke 1990). The response to superoxide stress mediated by the two-stage SoxRS system in *E. coli* is initiated by SoxR, which has recently been shown to contain an iron–sulphur centre that is essential for transcription activation but not DNA binding (Hildago & Demple 1994). Interestingly, the SoxR iron–sulphur centre differs from that of FNR in being sensitive to β-mercaptoethanol rather than requiring reducing conditions for maintaining its integrity. Transcription activation by the peroxide stress regulator (OxyR) differs in being switched on by a peroxide-dependent oxidation of a cysteine residue (Kullick *et al.* 1995). Then, another group of regulators uses haem as an oxygen-sensing cofactor. These include the FixL sensory component of the FixLJ two-component system, which contains a haemoglobin-like domain and controls N_2 fixation in *Rhizobium meliloti*, both directly and via another regulator, FixK (Gilles-Gonzales *et al.* 1991). The way in which ArcB senses anaerobiosis is not understood, nor is it known whether the haemoglobin-like protein (Hmp) of *E. coli* performs an oxygen-sensing function (Poole *et al.* 1994).

(*e*) *The CRP–FNR family*

Some 30 members of the CRP–FNR family of regulators have so far been found in almost as many bacterial species. They control a diverse range of genes in response to changes in nitrogen, sulphur, carbon and oxygen supply, but the binding sites, target operons and regulatory stimuli have not been identified in all cases. About 20 members compose a group of FNR homologues that have regulatory functions in anaerobic respiration, nitrogen fixation, bioluminescence, arginine fermentation and aromatic acid degradation in photosynthetic bacteria, haemolysin synthesis and virulence (Spiro 1994; Guest *et al.* 1995). Among these, it is significant that most of the redox-responsive regulators have four conserved cysteine residues (three near the N-terminus and one located centrally). Most have DNA-recognition helices that contain the –**E**––**SR** motif associated with FNR-site recognition rather than the **RE**–––R motif associated with CRP-site specificity. However, there are some interesting variations in domain organization and the sensory and target specificities. For example, FixK of *Rhizobium meliloti*, which functions in a nitrogen-fixing regulatory cascade, lacks the cysteine-rich N-terminal segment, the central cysteine residue of FNR and the cAMP binding residues of CRP, but retains an FNR-site binding specificity. It is also interesting that the regulators are not confined to Gram-negative bacteria. *Lactobacillus casei* and *Lactococcus lactis* each contain FNR-like regulatory proteins (FLP) whose target genes and sensor specificities are now being sought. They possess FNR-like DNA-recognition motifs but retain only two conserved cysteine residues (one N-terminal and one central). Likewise, *B. subtilis* contains an FNR protein associated with nitrate respiration (P. Glaser, personal communication). Here the cysteine-rich sensory cluster is located near the C-terminus and there is a CRP-like recognition motif. The degree of structural variation and functional versatility exhibited by the CRP–FNR family thus provides a notable example of how a basic molecular framework can be successfully adapted to control diverse regulatory responses.

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7. CONCLUDING REMARKS

Forty years ago when basic metabolic pathways were being defined, one had the impression that the channelling of metabolic intermediates through catabolic and anabolic routes was predetermined at the enzyme–substrate level. Once primed with substrate, metabolism seemed to proceed automatically, being perturbed only at the periphery by simple regulatory responses to specific primary substrates or preformed end products. However, it is now abundantly clear that the pattern of metabolism adopted by an organism is the product of a complex network of interacting and overlapping regulatory processes that sense and respond to an extensive array of environmental stimuli. This provides an organism with an effective and competitive strategy for adapting to, and making best use of, a given environment. In terms of adaptation to oxygen limitation we have seen how two major regulators, ArcA and FNR, modulate enzyme synthesis. Even so, we do not understand exactly how either senses anaerobiosis, how the phosphorylated form of ArcA interacts with the transcription complex, nor how their functions are integrated into the overall regulatory framework. The present era of nucleotide sequencing has let us see into the hidden depths of a cellular organism, and within months the primary structures of all its catalytic and regulatory components will be known. Like van Leeuwenhoek, we shall have witnessed and documented their presence but as yet not fully understood their functions or overall significance. At least the scope of the problem will have been delimited, and we should not have to wait as long as two centuries before our understanding of the full extent and coordination of life's chemistry is complete.

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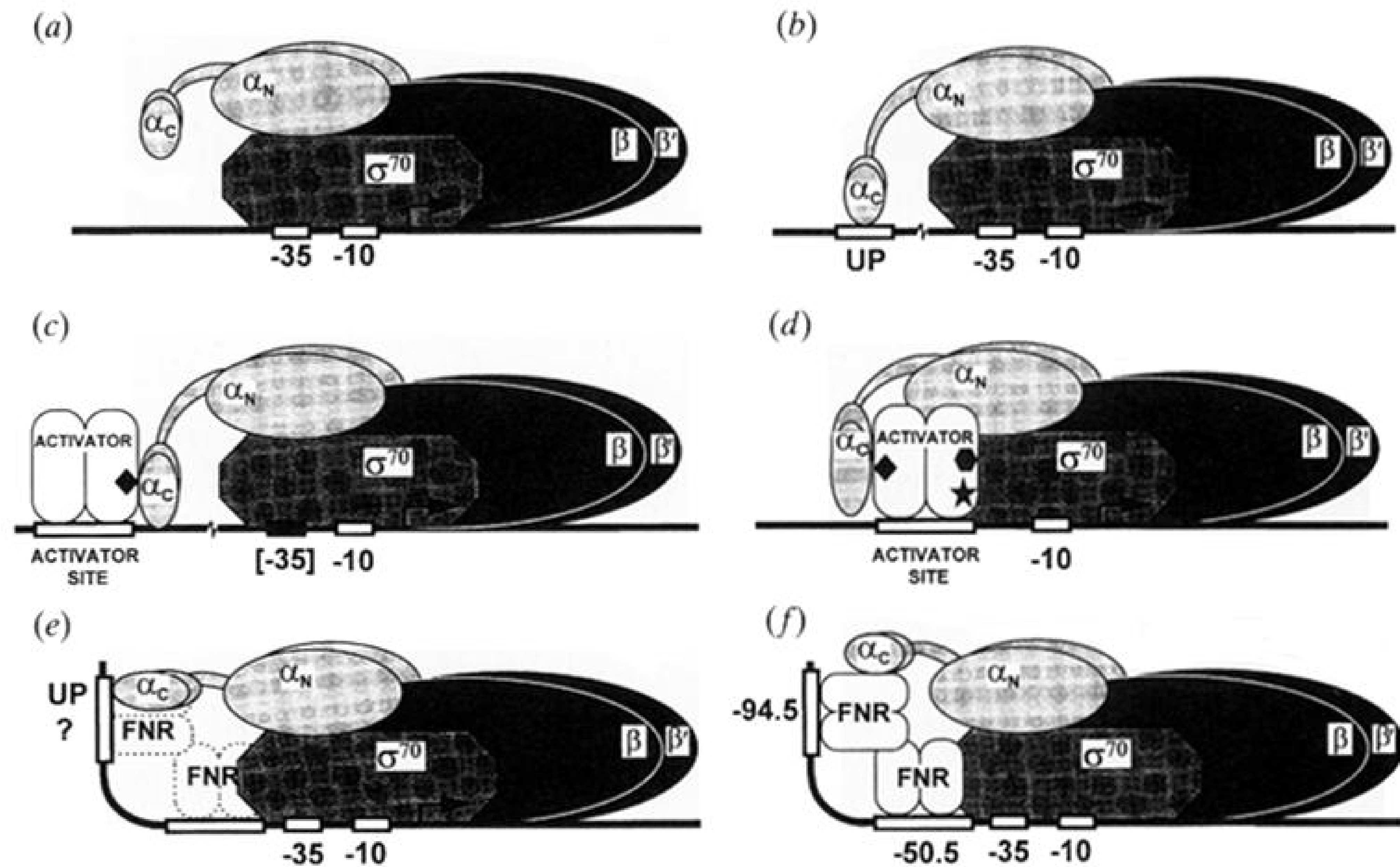

gure 3. RNA polymerase and selective gene expression in *E. coli*. The subunits and promoter elements involved in anscription activation are shown: (*a*) basal, (*b*) UP-element activated, (*c*) activator-dependent (class I) and (*d*) tivator-dependent (class II). A possible mechanism for transcription repression by FNR is illustrated for the *ndh* ne: (*e*) aerobic induced and (*f*) anaerobic repressed. The closed symbols (diamond, hexagon and star) denote fferent sites of subunit–regulator interaction. Based on Busby & Ebright (1994).

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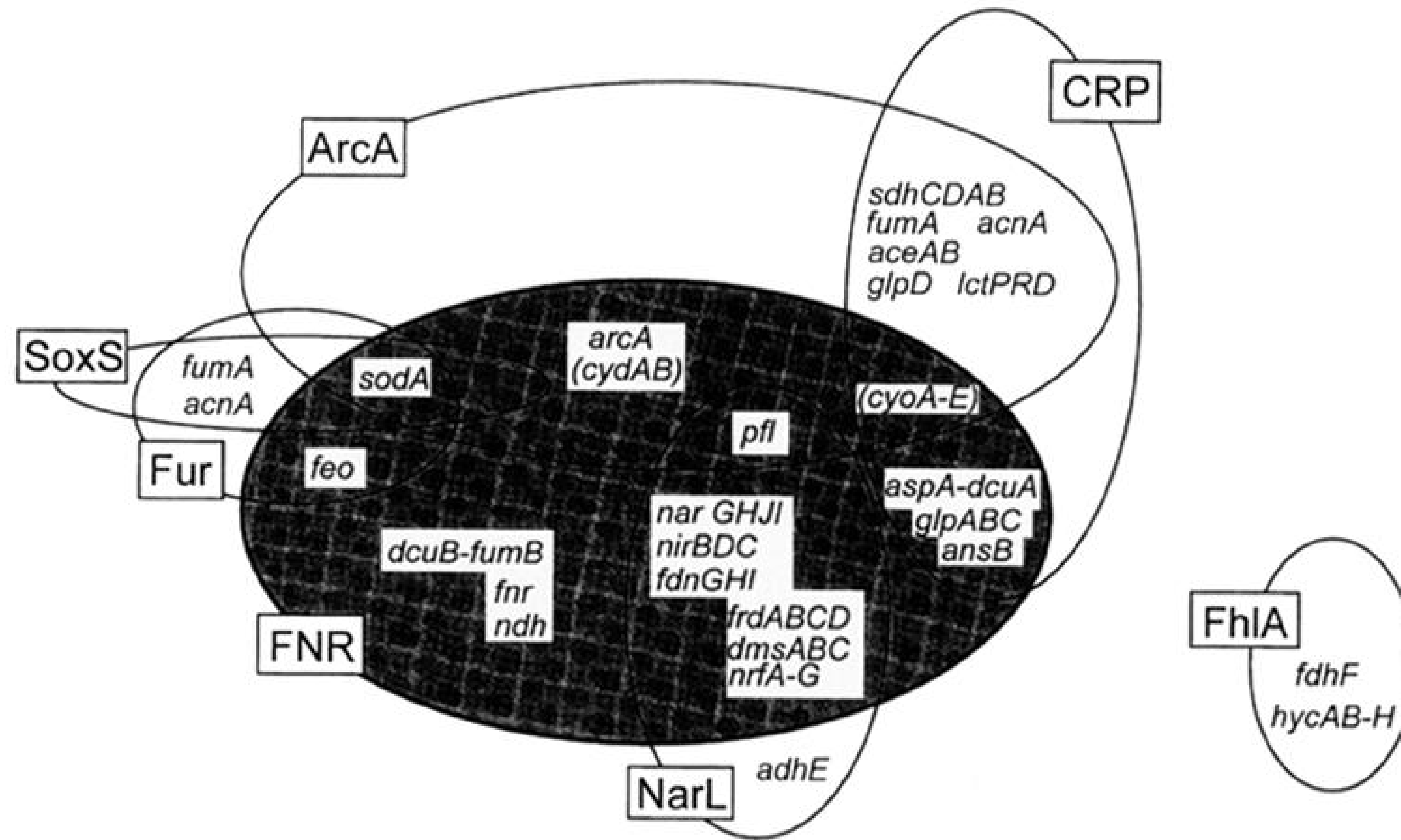
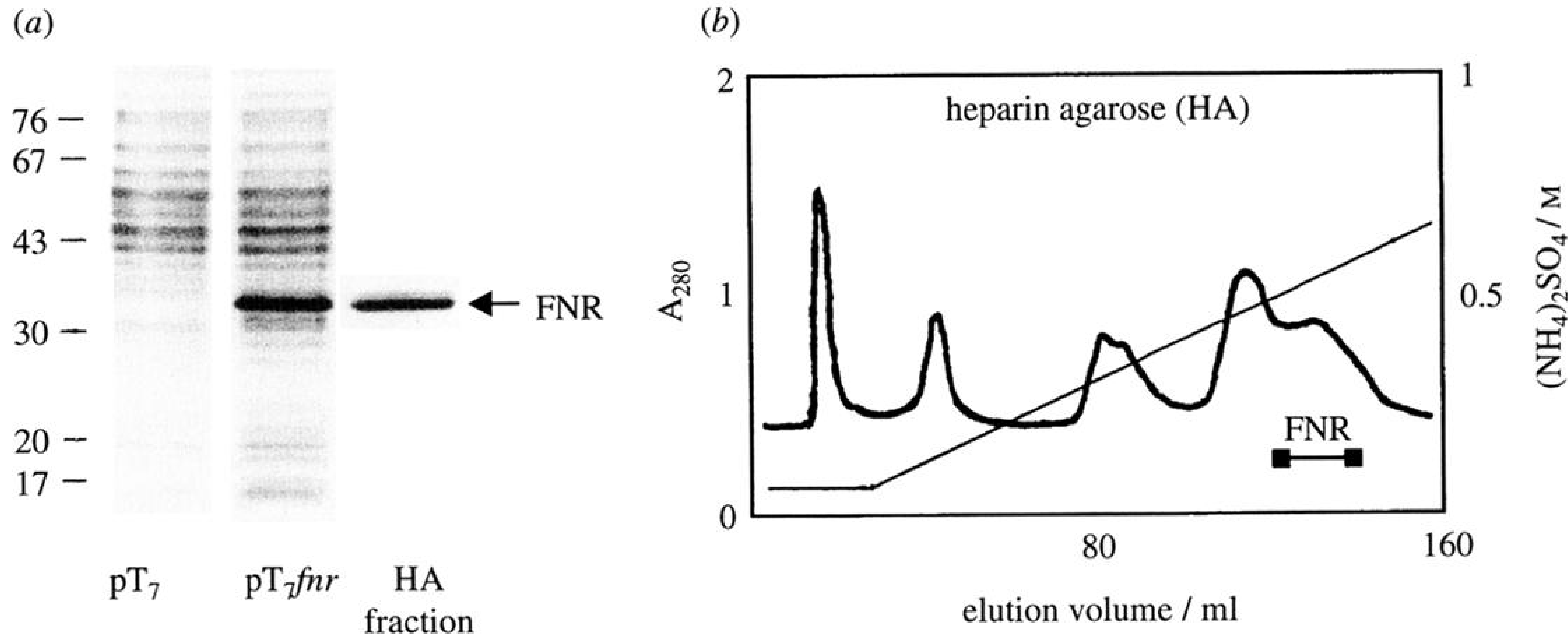


igure 5. Overlapping modulons in *E. coli*. Each modulon, or family of genes and operons controlled by a global gulator, is enclosed by a labelled ellipse. The diagram illustrates how members of one modulon such as the FNR odulon (shaded ellipse) can be members of other modulons and thus subject to multifactorial transcription control. dapted from Iuchi & Lin (1993).



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gure 7. Amplification and purification of the FNR protein. (*a*) SDS–PAGE analysis of soluble proteins from IPTG-duced cultures of *E. coli* strains containing pT_7 *fnr*, an expression plasmid in which of the *fnr* gene is cloned ›wnstream of an IPTG-regulated pT_7 promoter or pT_7, the expression vector alone, and heparin–agarose purified NR. The positions of molecular mass markers ($M_r \times 10^{-3}$) are indicated. (*b*) Heparin–agarose elution profile for the nplified extract with a bar to denote fractions containing highly enriched FNR (Green *et al*. 1995).