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Nitrate Respiration in Relation to Facultative Metabolism in Enterobacteria

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INTRODUCTION

Nitrogen metabolism has attracted attention from a variety of viewpoints. The most familiar is that of nutrition, and the importance of nitrogen assimilation for biosynthetic purposes is well documented. Nitrate is an important source of assimilable nitrogen, and many species of plants, fungi, and bacteria can incorporate nitrate through the combined action of assimilatory nitrate reductase and assimilatory nitrite reductase. Typically, these soluble enzymes are induced by nitrate or nitrite and are also subject to general regulation by ammonia, as in nitrogen metabolite repression in fungi (74, 92, 141).

A separate process allows many bacteria to derive energy from nitrate respiration. Respiratory nitrate reductase, like cytochrome oxidase, is the terminal component in an electron transport chain, and it is invariably membrane bound. Respiratory nitrate reductase accepts electrons from substrate oxidation to reduce nitrate (NO_3^-) to nitrite (NO_2^-). Much of the resultant energy is conserved as proton motive force (PMF), which in turn is used for adenosine 5′-triphosphate (ATP) generation, solute transport, and other cellular processes. Respiratory nitrate reductase is induced by nitrate in the absence of oxygen. Thus, nitrate respiration is completely distinct from nitrate assimilation, in both function and regulation.

In members of *Enterobacteriaceae*, the product of nitrate reduction, nitrite, can be reduced to ammonia by either of two nitrite reductases. Nitrite reduction can be used to regenerate oxidized nicotinamide cofactor, or it can serve to form PMF (see next section).

In some other bacteria, nitrite is reduced to gaseous compounds (NO, nitric oxide; N_2O, nitrous oxide; N_2, dinitrogen) in a process termed denitrification. Each step of denitrification can be coupled to energy conservation. Members of the *Enterobacteriaceae* are not true denitrifiers, although they do produce small amounts of nitrous oxide (338, 357).

Nitrate respiration is an important process in anaerobic metabolism, which is becoming an increasingly popular subject for microbiological research. Recent information suggests new avenues for research, and I believe that the physiological role of nitrate respiration deserves an updated perspective. I limit my treatment to nitrate respiration as it has been studied in members of the *Enterobacteriaceae*, in hopes of making it more accessible for both those who study nitrate metabolism in other organisms and those who study other aspects of facultative metabolism. I emphasize studies

on nitrate reductase physiology, genetics, and regulation, and I consider selected aspects of nitrate reductase structure, enzymology, and biosynthesis. Others have reviewed nitrate reductase (382), denitrification (214, 303), nitrate assimilation (92, 141), and respiration of enteric bacteria (13, 164).

I use "nitrate reductase" to mean "respiratory nitrate reductase" for the remainder of this review. I use "enteric bacteria" to mean "members of the *Enterobacteriaceae*."

Organisms

Escherichia coli, the type species of the *Enterobacteriaceae*, is well known to biologists. However, the *Enterobacteriaceae* includes many distinct species. Studies on some of these relatives of *E. coli* have provided a significant portion of the literature on facultative metabolism. Inevitably, discussions of the enteric bacteria tend to be cast in terms of *E. coli*, but I hope to emphasize those instances in which species differences may have influenced results or interpretations.

Salmonella typhimurium has long been an important subject of bacterial genetics and also of many genetic studies on anaerobic metabolism.

Aerobacter aerogenes is often used for studies on bacterial physiology; many important observations on nitrate reductase have been made with this organism. About 15 years ago, a taxonomic reclassification designated nonmotile *A. aerogenes* strains as *Klebsiella pneumoniae*. However, the guidelines were confusing, and many investigators renamed their strains as *K. aerogenes* (e.g., see reference 381). To further confuse the issue, more recent classification has defined indole-positive *Klebsiellae* as *K. oxytoca* (293). Thus, many strains termed "*K. aerogenes*" are more properly designated as *K. pneumoniae*, whereas strain M5al, termed "*K. pneumoniae*" and widely used in studies of dinitrogen fixation, is properly designated *K. oxytoca* (293). With this shifting taxonomy, confusion seems inevitable and is compounded by the fact that, in contrast to strains K-12 and LT2 of *E. coli* and *S. typhimurium*, respectively, no one strain of *A. aerogenes* was adopted for use by most laboratories (e.g., see references 151, 310, 384, and 391). For this review, I consider *A. aerogenes*, *K. aerogenes*, *K. oxytoca*, and *K. pneumoniae* to be synonyms and refer to all four as *K. pneumoniae*.

Citrobacter freundii is very closely related to *E. coli* and has been the subject of some work on anaerobic metabolism.

Proteus mirabilis is a more distant relative of *E. coli*. The Southamer laboratory switched from *K. pneumoniae* to *P. mirabilis* in the late 1960s, partly in hopes of using transduction to examine the genetic basis of anaerobic respiration (80). Unfortunately, this potential for genetic analysis has not been realized (e.g., see reference 59).

The family *Enterobacteriaceae* is a large group of bacteria, and its members are found in a variety of ecological niches (40). All members are facultative anaerobes: they grow well in the absence of oxygen when fermentable substrates are available, and they also grow with oxidizable substrates when environmental conditions allow for respiration. All enteric bacteria respire oxygen, nitrate, and fumarate, and most species can use other terminal electron acceptors as well.

Bacterial systematics is such that clear distinctions between "species" are sometimes difficult to determine. Brenner (40) has used deoxyribonucleic acid (DNA) relatedness groupings to extensively classify members of the *Enterobacteriaceae*. These groupings indicate the evolutionary relationships between different genera: thus, *Escherichia*, *Salmonella*, and *Klebsiella* are closely related, while *Proteus* is more distantly related. These relationships are also revealed by comparing the genetic maps of these species; the gene order in *Proteus* has diverged from the well-conserved order found in *Escherichia*, *Salmonella*, and *Klebsiella* (325, 334).

Historical Perspective

Nitrate reduction was discovered early in the history of microbiology, and the role of denitrification in energy production was recognized near the turn of the century. Payne recounts this early period in the study of nitrogen metabolism (303). By 1925, Quastel had demonstrated that nitrate can substitute for oxygen in a nondenitrifying bacterium, *E. coli*, allowing it to grow anaerobically on nonfermentable substrates such as lactate (320, 321; reviewed in reference 134). The landmark theory of biochemical unity provided the means for understanding this type of energy conservation as a series of coupled hydrogen (electron) transfers (210). Soon, Stickland and colleagues established that nitrate reductase is distinct from aerobic respiratory enzymes and that it interacts with other components of the respiratory chain through then-unidentified carriers (134, 370).

After World War II, Pollock included "nitratase" in his studies of enzymatic adaptation (315), and other early observations were refined with the demonstration that oxygen suppresses both the formation and the in vivo activity of nitrate reductase (310). Thus, nitrate reductase became an attractive model system for studying respiration: it is not essential for growth under most conditions, and its formation can be controlled by relatively simple changes in growth conditions.

At about this time, Egami, Sato, and colleagues began a pioneering series of biochemical studies on *E. coli* nitrate reductase. They soon showed that cytochrome *b* is a mediator of electron flow to nitrate reductase and that a variety of compounds (including formate, reduced nicotinamide adenine dinucleotide [NADH], lactate, and glycerol-3-phosphate) are electron donors for nitrate reduction (168; reviewed in references 337 and 398). They also attempted the first purification of nitrate reductase, but were hampered by the particulate nature of the enzyme. Their procedures yielded "particles" containing formate dehydrogenase, nitrate reductase, cytochrome, and quinone (162, 169, 388). They were able to partially solubilize nitrate reductase and characterized it as an iron-molybdenum (Fe-Mo) protein (388). Finally, they showed that formate dehydrogenase is induced during anaerobic growth on nitrate, reinforcing the idea that formate is an important electron donor for nitrate reduction (169). Many of the conclusions reached by this group have provided a foundation for present-day studies of nitrate respiration.

During this time, denitrification had also attracted attention (211). The best studied of the denitrifying bacteria are obligate respirers that fail to grow anaerobically in the absence of the appropriate nitrogen oxides. In the view of the Delft school (211, 412), the enteric bacteria simply used nitrate reduction as another hydrogen-accepting pathway during fermentation, with little impact on growth ("incidental dissimilatory nitrate reduction"). Kluyver and Verhoeven defined denitrification, an essential process for anaerobic growth, as "true dissimilatory nitrate reduction" (211, 412). Egami and colleagues argued that the enteric type of nitrate reduction was also a true respiratory process (337,

386, 389; reviewed in reference 387). (This point had been raised nearly a quarter century before by the work of Quastel and Stickland; see above). By 1965, growth yield measurements had reinforced the conviction that enteric-type nitrate reduction provides a major source of energy for anaerobic growth (152). Today, most investigators use the terms "respiratory nitrate reduction" and "dissimilatory nitrate reduction" as synonyms, without regard to the fate of nitrite.

Genetic analyses of nitrate reduction began with the discovery that chlorate (ClO_3^-) reduction is associated with nitrate reductase (144). Piéchaud et al. (313) and DeGroot and Stouthamer (80, 371) selected chlorate-resistant (*chl*) mutants of various enteric bacteria and found that many mutants had lost not only nitrate reductase, but also other anaerobic enzymes such as formate dehydrogenase and formate-hydrogen lyase. Further, they found that many different genes could mutate to give a chlorate-resistant (Chl^r) phenotype, suggesting that control of nitrate respiration might be complex.

During this period, Green et al. championed Green's "structural protein" hypothesis to explain the organization of membrane-bound respiratory proteins (133), arguing that membranes consisted largely of structural (noncatalytic) proteins and that respiratory enzymes were assembled onto these structures in specific rigid complexes. The isolation of a formate dehydrogenase-nitrate reductase "complex" (169; see above) was consistent with this idea. Thus, that many chlorate-resistant mutants lacked both of these enzyme activities suggested that some *chl* mutants were defective in "structural protein."

In support of this idea, Azoulay et al. were able to reconstitute nitrate reductase activity by mixing cell-free, "soluble" extracts of different *chl* mutants; the reconstituted activity was in a newly formed "particulate" fraction. They concluded that some *chl* mutants were defective in structural protein components of the formate dehydrogenase-nitrate reductase "particle" (18, 19). This result suggested that *chl* mutants would be ideal for studying the role of "structural protein" in respiratory chains (19, 257, 342, 372; but see also references 330 and 332).

The view of membrane structure continued to change. The concept that quinones form a diffusible pool to carry electrons between the enzymes and cytochromes of respiratory chains implied that individual components of a given respiratory chain need not be rigidly associated with each other (228). Soon thereafter, Singer and Nicholson's fluid mosaic model of membrane structure put an end to the structural protein hypothesis (356). Meanwhile, Nason and colleagues found that active assimilatory nitrate reductase could be reconstituted by mixing extracts of an appropriate *Neurospora crassa* mutant with various sources of Mo cofactor (282). Analogous experiments with *E. coli* suggested that the in vitro reconstitution of *chl* mutant extracts similarly involved incorporation of Mo cofactor into nitrate reductase apoenzyme (258, 259). Thus, at least some of the pleiotropic properties of *chl* mutations were recognized as being defects in Mo metabolism, but some time elapsed before the generality of this hypothesis was fully accepted (83, 91, 127, 251, 271, 368).

Nitrate reductase remains an attractive system for studying respiration. A variety of specific mutants is now available. Artificial electron donors, especially viologen dyes, provide powerful probes for understanding enzyme action and organization (197, 276). The subunit structure of the enzyme is being explored, detailed studies on the structural genes and protein structure are under way, and the genetic basis for regulation of its synthesis is understood in principle.

NITRATE RESPIRATION AND FACULTATIVE METABOLISM

Nitrate respiration occupies a central position in facultative metabolism of enterics and is a key regulation point for a variety of energy-generating pathways. I provide here a selective overview of facultative metabolism, with emphasis on points germaine to the role played by nitrate respiration.

Aerobic Respiration

This section concentrates on new information on *E. coli* aerobic cytochrome oxidases that has appeared since a recent review (164).

Enteric respiratory chains can use a variety of substrates. Any of several membrane-bound dehydrogenases (e.g., NADH, lactate, succinate, formate, or glycerol-3-phosphate dehydrogenases) can donate electrons to a common quinone pool. The predominant quinone, ubiquinone-8, functions in aerobic respiration; menaquinone-8 functions in anaerobic respiratory chains. The quinone pool, in turn, donates electrons directly to terminal reductases, the cytochrome *o* and *d* complexes (14). Thus, the respiratory chains are branched on both sides of quinone, allowing for great flexibility for using oxidants and reductants. Any given electron transport chain has a rather simple structure: dehydrogenase-quinone-reductase (46, 216).

Biochemical analysis of cytochrome oxidases. *E. coli* contains two terminal dioxygen reductase (cytochrome oxidase) complexes, both of which are now well studied (reviewed in reference 13). The first, termed cytochrome *o* complex, consists of at least two (208) and probably four (224, 264, 265) polypeptide subunits. Cytochrome *o* generates two spectral signals, termed cytochromes b_{555} and b_{562}, that may represent a split α-band generated by a single cytochrome species (243). Cytochrome *o* has been reconstituted into proteoliposomes, where it oxidizes ubiquinol-1 to generate PMF (154, 207, 264, 265). This result indicates that cytochrome *o* is a coupling site for energy conservation. Indeed, complete proton-translocating electron transport chains (pyruvate-dioxygen and lactate-dioxygen oxidoreductases) have been reconstituted in proteoliposomes from purified pyruvate oxidase or lactate dehydrogenase, ubiquinone-8, and cytochrome *o* (46, 263).

In the second terminal dioxygen reductase, the cytochrome *d* complex, only two polypeptide subunits have been detected (209, 273). However, potentiometric analysis of the purified complex indicates the presence of three heme signals, cytochromes b_{558}, b_{595}, and *d* (217, 244). Subunit I of the complex corresponds to cytochrome b_{558} (136, 137) and contains the site for ubiquinol oxidation (225, 434). The cytochrome *d* complex has been reconstituted in proteoliposomes, where it oxidizes ubiquinol-1 to generate PMF (209, 274, 434). Thus, the cytochrome *d* complex is also a coupling site for energy conservation. Pyruvate-dioxygen oxidoreductase has also been reconstituted in proteoliposomes with cytochrome *d* complex as the terminal oxidase (216).

Both cytochrome *o* and *d* complexes may have similar mechanisms for proton translocation (46, 265, 274). It is throught that the dioxygen reductase site faces the cytoplasm, whereas the ubiquinol oxidase site faces the periplasm. Thus, release of two protons in the periplasm (ubiquinol-8 → ubiquinone-8 + $2H^+$ + $2e^-$) is coupled to the

consumption of two protons in the cytoplasm ($2H^+ + 2e^- + 1/2\ O_2 \rightarrow H_2O$). An identical mechanism was proposed for nitrate reductase (197; see "Nitrate Reductase Structure and Mechanism" section). If this hypothesis is correct, it explains the mechanism of proton translocation as originally proposed by Mitchell (discussed in reference 265).

Abundance of the two terminal oxidase complexes is regulated by the availability of oxygen. The cytochrome *o* complex predominates when cells are grown with vigorous aeration, while the cytochrome *d* complex predominates under conditions of limited aeration or anaerobiosis (164, 208, 209, 222, 224). Immunochemical analysis suggests that synthesis of cytochrome *o* is repressed, and synthesis of cytochrome *d* is induced, upon a shift from high to low aeration (222, 224). However, oxygen appears not to be the direct signal for regulation, because a mutant (*cyd*) lacking cytochrome *d* synthesizes cytochrome *o* even at low levels of oxygen (224).

Another cytochrome, b_{556}, is present in the membranes of aerobically grown cells. This cytochrome donates electrons to ubiquinone and thus was thought to be required for both the cytochrome *o* and *d* branches of aerobic respiration (207–209). Others argue that quinone is located exclusively on the dehydrogenase side of aerobic respiratory chains (14). Indeed, pyruvate-dioxygen oxidoreductase has been reconstituted in proteoliposomes with either cytochrome *o* or *d*, indicating that, at least in vitro, cytochrome b_{556} is not an obligatory component of these respiratory chains (46, 216). Further, cytochrome b_{556} levels are not regulated by the availability of oxygen (222). This paradox has apparently been resolved with the identification of cytochrome b_{556} as a subunit of the succinate dehydrogenase complex (278).

Finally, cytochrome b_{561} also donates electrons to ubiquinone, but its role in aerobic respiration is not yet clear (277).

Spectroscopic studies indicate that the aerobic cytochrome complements of *S. typhimurium* and *P. mirabilis* are similar to those of *E. coli* (232, 406, 408).

Genetic analysis of cytochrome oxidases. *E. coli* mutants defective in cytochrome *d* have been isolated by screening for strains that either lack *N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidase activity (135) or are hypersensitive to azide (136). Two distinct classes of mutants have been found. *cydA* mutants lack both subunits and all three spectroscopic signals (cytochromes b_{556}, b_{595}, and *d*) of cytochrome *d*. *cydB* mutants only lack subunit II and the signals of cytochromes b_{595} and *d* (136). *cydA* and *cydB* are tightly linked and are the structural genes for the cytochrome *d* complex (136). These mutants have proved invaluable in characterizing both of the aerobic cytochrome oxidase complexes (14, 223, 243, 264, 265). More recent screens have revealed an unlinked gene, *cydC*, that may be involved in heme *d* synthesis (107).

Mutants defective in cytochrome *o* have also been identified. In this case, a *cyd* strain was mutagenized, and mutants incapable of aerobic respiration were isolated after ampicillin enrichment (15). One mutant (*cyo*) was shown by spectroscopic and immunological characterization to lack specifically cytochrome *o*. Both *cyd* and *cyo* single mutants have been characterized for oxygen uptake and growth rate on a variety of carbon sources. These studies suggest that, at least in the laboratory, either cytochrome oxidase complex is dispensable for normal aerobic growth (15, 136).

Anaerobic Metabolism

During aerobic growth, NADH is reoxidized via the respiratory chain. In the absence of a terminal electron acceptor, NAD^+ must be regenerated by other means. This requirement leads to dramatic changes in the pathways of glucose catabolism and the tricarboxylic acid (TCA) cycle.

Glucose catabolism. Studies on continuous cultures of *E. coli* indicate that aerobic cultures catabolize glucose predominantly through the pentose phosphate pathway, while anaerobic cultures make greater use of the Embden-Meyerhof pathway (395). However, key enzymes of both pathways are increased as the oxygen supply is decreased. Measurements of steady-state protein levels confirm this pattern: levels of phosphofructokinase, enolase, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate kinase all increase upon a shift to anaerobiosis (358).

A more dramatic effect of anaerobiosis is on the enzymes for pyruvate metabolism, to regenerate NAD^+ at a rate sufficient to meet the needs of glycolysis. One pathway by which this occurs consists of a single enzyme, lactate dehydrogenase, which oxidizes NADH and converts pyruvate to lactate (427; Fig. 1).

Anaerobiosis also inhibits the formation of pyruvate dehydrogenase, which produces NADH (359, 360, 362). The role of pyruvate dehydrogenase is played by pyruvate-formate lyase (NAD independent), which produces acetyl-coenzyme A plus formate (304, 358; Fig. 1). Formate is in turn converted to carbon dioxide and hydrogen by formate-hydrogen lyase. This enzyme complex consists of formate dehydrogenase, unidentified carriers, and a hydrogenase (307; Fig. 1); it is responsible for the vigorous formation of gas during mixed-acid fermentation.

Meanwhile, metabolism of acetyl-coenzyme A occurs by a variety of reactions, some of which are species restricted. First, phosphotransacetylase and acetate kinase combine to generate ATP, producing acetate as the end product (41, 42, 301, 427; Fig. 1). These enzymes are also present in aerobic cultures, where they probably mediate aerobic assimilation of acetate.

Acetyl-coenzyme A is also metabolized by acetaldehyde dehydrogenase and alcohol dehydrogenase, which regenerate two molecules of NAD^+ in forming ethanol (Fig. 1). *E. coli* mutations affecting alcohol dehydrogenase have been mapped to the *adhE* locus (245, 426). A mutant lacking both alcohol and acetaldehyde dehydrogenase activities (termed *ana*) is unable to grow on glucose in the absence of an exogenous electron acceptor, thus demonstrating that this pathway is essential for regenerating NAD^+ during fermentative growth (301). However, a strain that also lacks pyruvate-formate lyase (*ana pfl* double mutant) can grow anaerobically on glucose (plus acetate, to satisfy anabolic reactions). In this case, regeneration of NAD^+ by reduction of pyruvate to lactic acid is sufficient to provide the fermentation balance (301).

E. coli also expresses an alcohol dehydrogenase during aerobic growth; this activity is not affected in *adhE* mutants and thus represents an isoenzyme (426).

Together, the above pathways constitute the mixed-acid fermentation, which is characteristic of most enteric bacteria (Fig. 1). However, some species, including *K. pneumoniae*, also perform the 2,3-butanediol fermentation. In this pathway, catabolic α-acetolactate synthase, α-acetolactate decarboxylase, and 2,3-butanediol dehydrogenase catalyze the conversion of pyruvate to 2,3-butanediol, regenerating a single NAD^+ in the process (42, 427).

Studies with anaerobic, pH-controlled continuous cultures of *K. pneumoniae* NCTC 418 have provided a more refined picture of this organism's fermentative pathways and their regulation (390, 391). The only fermentation products in

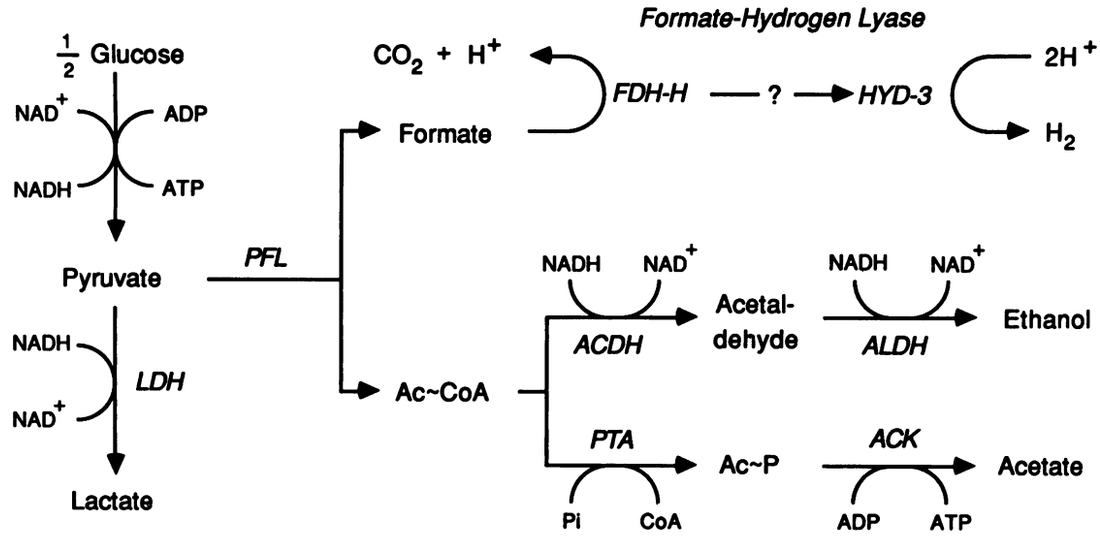

FIG. 1. Mixed-acid fermentation. Enzymes are shown in italics: LDH, lactate dehydrogenase; PFL, pyruvate-formate lyase; FDH-H, formate dehydrogenase-H; HYD-3, hydrogenase isoenzyme 3; ACDH, acetaldehyde dehydrogenase; ALDH, alcohol dehydrogenase; PTA, phosphotransacetylase; ACK, acetate kinase; CoA, coenzyme A; ADP, adenosine 5'-diphosphate. Redrawn from reference 123.

glucose-limited cultures, over a range of dilution rates, are acetate, ethanol, and formate plus carbon dioxide. Acetate formation is energetically the most favorable fermentation pathway, producing 1 mol of ATP per mol of acetate formed; concomitant formation of ethanol is necessary to regenerate NAD^+ (Fig. 1). However, in glucose-sufficient cultures (limited for phosphate, ammonia, or other nutrients), other fermentation products (2,3-butanediol, succinate, and lactate) are also produced. It is argued that these additional fermentation pathways allow for modulation of ATP synthesis relative to glucose uptake (391). Indeed, when glucose-limited cultures are suddenly pulsed with excess glucose, lactate production begins immediately. This is attributed to operation of the methylglyoxal bypass, allowing the rapid catabolism of glucose without unduly straining the immediate capacity for anabolism (390). Thus, the regulation of fermentation pathways is quite complex, not only in terms of enzyme synthesis, but also with respect to metabolic flux through the various pathways.

Nitrate respiration and fermentation. Nitrate reduction coupled to NADH oxidation generates PMF (see "Physiology" section). Thus, the availability of nitrate renders most of the fermentation pathways discussed above superfluous. Measurements of fermentation balances of *K. pneumoniae* and *E. coli* show similar effects of nitrate; ethanol formation is suppressed, with a concomitant increase in acetate formation (89, 101). (Acetate is excreted because the TCA cycle is incomplete in anaerobic cultures; see below.) Nitrate apparently affects the synthesis rather than the activity of anaerobic alcohol dehydrogenase; some regulatory mutants that overproduce alcohol dehydrogenase retain significant levels of this activity in the presence of nitrate (60; see also reference 267).

Nitrate reduction can also be coupled to formate oxidation, again with the production of PMF, and the activity of the fermentation pathway for metabolizing formate, formate-hydrogen lyase, is also greatly decreased by nitrate (423; see next section).

TCA cycle. Enteric bacteria, cultured aerobically, contain an intact TCA cycle (Fig. 2). In anaerobic cultures, the TCA cycle undergoes changes that act to reduce NADH production. The level of NADH dehydrogenase is also greatly decreased (395). The genetics and regulation of TCA enzyme synthesis have recently been reviewed (363).

Activities of the initial enzymes of the TCA cycle, citrate synthase, aconitase, and isocitrate dehydrogenase, decrease during anaerobic growth (102, 132, 359, 360, 362). This portion of the TCA cycle is necessary to generate α-ketoglutarate for biosynthetic purposes (Fig. 2).

α-Ketoglutarate dehydrogenase is severely repressed by anaerobiosis, preventing succinate formation from α-ketoglutarate (9, 204, 359, 360, 395). Instead, the so-called reductive branch of the TCA cycle, consisting of oxaloacetate-glutamate transaminase, aspartase, and fumarate reductase, functions to provide succinate for biosyntheses (73, 165; Fig. 2). (Oxaloacetate is generated by carboxylation of pyruvate.) Fumarate reductase is a respiratory enzyme, so the reductive branch also generates PMF; enteric bacteria will grow anaerobically on nonfermentable substrates if exogenous fumarate is present as an electron acceptor. Succinate is also formed anaerobically via isocitrate lyase (76). Meanwhile, the normal enzymes of this portion of the TCA cycle (malate dehydrogenase, succinate dehydrogenase, and probably fumarase) are repressed during anaerobic growth (361, 385, 395). Thus, the four TCA cycle enzymes involved in NADH formation (isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase) are absent or greatly reduced during anaerobic growth (Fig. 2).

Keevil and colleagues (203, 204), studying *C. freundii*, found that α-ketoglutarate dehydrogenase synthesis is exquisitely sensitive to even traces of oxygen. Enzyme activity was reduced 100-fold when the redox potential of the medium was reduced from −255 to −275 mV. Several other TCA cycle and fermentation enzymes showed little change in activity. These and other observations led them to conclude that the potential TCA cycle is regulated by induction of α-ketoglutarate dehydrogenase synthesis by oxygen and glutamate and by catabolite repression of succinate dehydrogenase (202, 203).

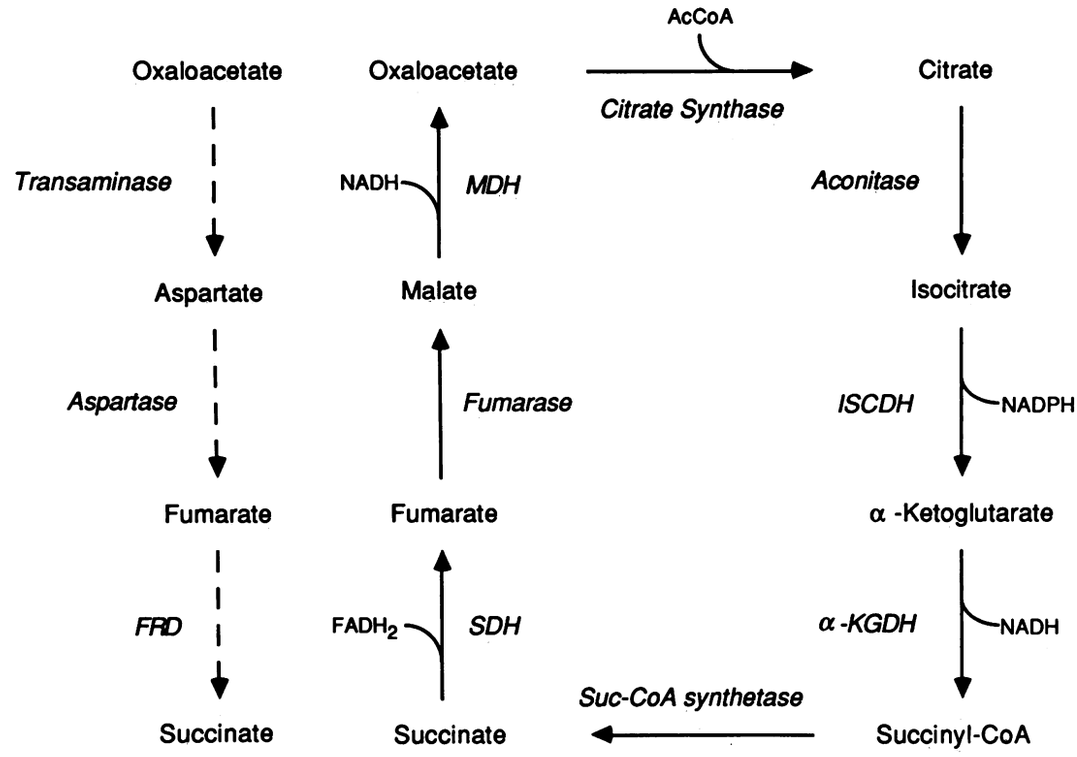

FIG. 2. TCA cycle. This figure is drawn to emphasize those reactions that produce reduced nucleotide cofactors, and therefore it does not show the complete reaction for each step. The "reductive branch" for anaerobic synthesis of succinate is shown with broken lines. Enzymes are shown in italics: ISCDH, isocitrate dehydrogenase; α-KDH, α-ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; MDH, malate dehydrogenase; FRD, fumarate reductase; CoA, coenzyme A; FADH, reduced flavin adenine dinucleotide. Redrawn from references 123 and 165.

Nitrate respiration and the TCA cycle. In contrast to its effects on fermentation pathways, nitrate apparently has little effect on the TCA cycle in glucose-grown cultures, in which most of the enzymes show their typical anaerobic levels (423). Two enzymes, the activities of which are greatly reduced by nitrate, are aconitase and fumarase, but these activities are inhibited in vitro by the nitrite that accumulates from nitrate reduction (425), implying that the synthesis of these enzymes is not depressed by nitrate (430). Cultures growing on lactate plus nitrate may have an intact TCA cycle (432). However, more recent studies of the TCA cycle have not yet addressed the possible regulatory role of nitrate (363), so these conclusions await confirmation.

Anaerobic respiration. The great flexibility of enteric respiratory chains is extended by anaerobic respiration. A variety of compounds serve as electron donors for both aerobic and anaerobic respiration, including lactate, glycerol-3-phosphate, NADH, and formate. Oxidation of these compounds is catalyzed by membrane-bound dehydrogenases that participate in both aerobic and anaerobic respiration. The glycerol-3-phosphate dehydrogenases are exceptions; genetically distinct aerobic and anaerobic enzymes are formed (reviewed in reference 164). Many compounds serve as electron acceptors in anaerobic respiration: nitrate, nitrite, fumarate, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide, tetrahydrothiophene-1-oxide, and tetrathionate. Consideration of these respiratory pathways is beyond the scope of this article, and I defer to others for detailed information (29, 269; reviewed in references 22, 24, 71, and 164).

In general, activities of anaerobic respiratory enzymes are depressed by nitrate. Fumarate reductase enzyme activity in *E. coli* and *P. mirabilis* is depressed by nitrate (400, 423), reflecting an inhibition of fumarate reductase (*frd*) gene expression (171–174, 192, 193). Likewise, the activities of TMAO reductase in *E. coli* and *S. typhimurium* (24) and tetrathionate reductase in *S. typhimurium* and *P. mirabilis* (22, 49, 82) are depressed by anaerobic growth in the presence of nitrate.

Thus, nitrate-respiring cells resemble aerobic cells in that they regenerate NAD^+ through a respiratory chain and they contain low levels of alcohol dehydrogenase and formate-hydrogen lyase, but, like anaerobic cells, they have an incomplete TCA cycle and excrete acetate. These cells are also uninducible for other anaerobic respiratory pathways. This intermediate state is also reflected by molar growth yields of aerobic, anaerobic, and nitrate-respiring cultures (see "Physiology" section). Thus, it is not surprising that much of the regulation of facultative metabolism centers around nitrate respiration, as detailed below.

FORMATE/HYDROGEN METABOLISM

The metabolism of formate and hydrogen is sufficiently intertwined with nitrate respiration to warrant detailed treatment. Formate is produced during anaerobic growth by pyruvate-formate lyase (Fig. 1). Further metabolism of formate by enteric bacteria occurs by two distinct routes. First, it can undergo oxidation coupled to electron transport and thereby generate PMF. Second, it can undergo dismutation

via formate-hydrogen lyase, yielding carbon dioxide plus hydrogen (Fig. 1). This second pathway is generally assumed to minimize acidification and to dispose of excess reducing equivalents (130).

Formate Dehydrogenase

Most early work on formate dehydrogenase was done when formate-hydrogen lyase was considered to be a single enzyme. These early and often conflicting studies are reviewed by Gest (108). The modern era of formate dehydrogenase research began with the work of Gest and Peck (109, 307). They screened a large collection of "*coli-aerogenes*" bacteria and identified six anaerogenic variants (isolates that do not produce gas from glucose). One isolate apparently lacked pyruvate-formate lyase and thus could not produce formate. The other five were specifically deficient in formate-hydrogen lyase and proved invaluable in elucidating its various components.

Perhaps the most important result of this work was the recognition of two distinct forms of formate dehydrogenase, as defined by enzyme assay. One form is particularly active with methylene blue as an artificial electron acceptor. The apparently identical form is also active with phenazine methosulfate plus dichlorophenolindolphenol (55, 97, 109), and this form of formate dehydrogenase is found predominately in oxygen- or nitrate-respiring cells (97, 328). A second form is active with methyl viologen or benzyl viologen as electron acceptor (109), and is found at highest levels in nonrespiring cells, particularly at low pH (131, 328).

Gest and Peck, cautious in their interpretation, suggested that these distinct formate dehydrogenase activities could represent either two separate enzymes or a common "formate-activating enzyme" coupled to different intermediate electron carriers, each specific for a given artificial electron acceptor. The former hypothesis obviates the need to postulate additional carriers (109, 307). Gray and Gest emphasized the likelihood of distinct formate dehydrogenases (130), but many authors continued to focus on the "one enzyme, many carriers" hypothesis. Current evidence overwhelmingly supports the existence of two distinct formate dehydrogenase isoenzymes, the evidence for which is summarized in this section. For simplicity, I refer to the methylene blue-linked activity found in nitrate-respiring cells as formate dehydrogenase-N and the viologen dye-linked activity found associated with formate-hydrogen lyase as formate dehydrogenase-H.

Gest and Peck (109, 307) distinguished three biochemical classes among their formate-hydrogen lyase-deficient anaerogenic variants. One class lacks both of the formate dehydrogenase activities, the second specifically lacks hydrogenase, and the third has very low levels of formate dehydrogenase-H. Formate-hydrogen lyase activity could be reconstituted in vitro by mixing extracts of the first and second classes (providing hydrogenase and formate dehydrogenases-H plus -N, respectively) and the second and third classes (providing formate dehydrogenase-H and hydrogenase plus formate dehydrogenase-N, respectively), but not with the first and third classes (no source of formate dehydrogenase-H). These results indicated that formate-hydrogen lyase is composed of two enzymes, formate dehydrogenase-H and hydrogenase, and that formate dehydrogenase-N does not participate in the formate-hydrogen lyase reaction.

Gest and Peck also reconstituted the formate-hydrogen lyase reaction by mixing an extract of the hydrogenase-deficient variant with a preparation of clostridial hydrogenase. This reconstitution was dependent upon added methyl viologen as an intermediate electron carrier. The physiological electron carrier in formate-hydrogen lyase remains to be identified. Circumstantial evidence had implicated cytochrome c_{552} as a component of formate-hydrogen lyase (69, 130, 131), but more recent studies suggest that cytochrome c_{552} is involved in nitrite reduction (see section, "Nitrite Metabolism"). Others found that quinones are not required for formate-hydrogen lyase activity (418).

Peck and Gest also characterized certain biochemical parameters of the two formate dehydrogenases, and their conclusions have withstood the test of time (307). They found that formate dehydrogenase-N is present in cells grown with high aeration; later it was found to be abundant in nitrate-respiring cells as well (75, 97, 115, 328). Likewise, formate dehydrogenase-H, hydrogenase, and thereby formate-hydrogen lyase are only present in cells grown in the absence of exogenous electron acceptors (75, 108, 115, 131, 307, 328, 339). The two formate dehydrogenases respond differently to pH and inhibitors (115, 307). They also found that the subcellular localization of the enzymes differed; formate dehydrogenase-N was associated with the membrane fraction (75, 97, 115), whereas the bulk of formate dehydrogenase-H activity was in the soluble fraction.

The subcellular location of formate dehydrogenase-H has been problematical: many have found this activity associated with both the soluble and particulate fractions (115, 307), although others have detected it exclusively in the soluble (131) or the membrane (329) fraction. This last study was done with a preparation of lysed spheroplasts; most other studies used harsher methods (such as a French pressure cell) to disrupt cells. Perhaps formate dehydrogenase-H is a loosly associated peripheral membrane enzyme.

A different simple explanation may be found in the fact that purified formate dehydrogenase-N shows a low but significant activity with viologen dyes as electron acceptors (97). Thus, the specificity of formate dehydrogenase-N, at least, is not absolute, and this low activity with viologen may contribute to the membrane-bound activity ascribed to formate dehydrogenase-H (25). Indeed, the third class of anaerogenic variant studied by Gest and Peck (see above) retains a low level of benzyl viologen-formate dehydrogenase activity (109). Further, a strain carrying an insertion mutation in the structural gene for formate dehydrogenase-H (*fdhF*) is devoid of benzyl viologen-formate dehydrogenase activity in nonrespiring cells, but expresses a normally low level of this activity in nitrate-respiring cells (306). Therefore, formate dehydrogenase-H may be a soluble (or peripheral membrane) protein, and the "membrane-bound" form may represent the weak viologen activity of formate dehydrogenase-N.

Biochemistry of formate dehydrogenase-H. Both formate dehydrogenase enzymes require Fe, Mo, and selenium (Se) for activity (236, 314). Numerous claims that formate-hydrogen lyase activity requires complex medium for formation (108, 131) resulted from trace amounts of Se and Mo in the components of the medium (236, 314). The Mo in formate dehydrogenase occurs in Mo cofactor in a form similar or identical to that found in nitrate reductase (see "Molybdenum Cofactor" section).

Selenite and selenocysteine are equally effective supplements of culture medium that support high formate dehydrogenase activity (95, 353). Se is present in active formate dehydrogenase as selenocysteine, and it is likely that specific enzymes catalyze the synthesis of selenocysteine (366). The structural gene for the 80-kilodalton (kDa) subunit of formate

TABLE 1. Proposed functions of *fdh* genes[a]

Gene		Proposed function
E. coli	*S. typhimurium*	
fdhA	*fdhA*	Required for formate dehydrogenase-H and -N; possibly involved in Se metabolism or uptake
fdhB[b]	*fdhB*[b]	Required for formate dehydrogenase-H and -N; possibly involved in Se metabolism or uptake
fdhC	*fdhC*	Required for formate dehydrogenase-H and -N; possibly involved in Se metabolism or uptake
fdhD	—[c]	Required for formate dehydrogenase-H and -N; defect unknown
fdhE	*fdn*	Required for formate dehydrogenase-N; possibly a structural gene
fdhF	*fhl*[d]	Structural gene for 80-kDa selenopolypeptide of formate dehydrogenase-H

[a] See text for references.
[b] It is not known whether these designations represent homologous genes in *E. coli* and *S. typhimurium*.
[c] Not reported in *S. typhimurium*.
[d] Provisional assignment based on phenotype and map position similarities to *fdhF* of *E. coli*.

dehydrogenase-H (*fdhF*) contains a UGA codon at the position of selenocysteine, indicating that selenocysteine is incorporated via a novel cotranslational mechanism (437, 438). The mechanism of Se incorporation into formate dehydrogenase-N is unknown.

Formate dehydrogenase-H has so far been refractory to purification (75, 115, 314, 328), so its biochemical characterization is not yet possible. However, Cox et al. labeled growing cultures with ^{75}Se and found that two major species constitute the bulk of selenopeptides in *E. coli* (75). One, of approximately 110 kDa, is formed under conditions favoring high formate dehydrogenase-N activity and presumably is identical to the 110-kDa subunit of formate dehydrogenase-N. The other, of 80 kDa, is detected in concert with formate dehydrogenase-H activity. This 80-kDa selenopolypeptide is encoded by *fdhF* (305, 306). The predicted amino acid sequence of the *fdhF* gene product shows substantial sequence similarity to that of the 80-kDa subunit of *Methanobacterium formicicum* formate dehydrogenase (352, 437, 438). However, unlike the *M. formicicum* enzyme (352), there is no genetic (or biochemical) evidence for a second polypeptide subunit of formate dehydrogenase-H (437).

Biochemistry of formate dehydrogenase-N. Formate dehydrogenase-N was purified by Enoch and Lester (97). The enzyme contains three subunits of 110, 32, and 20 kDa. The 110-kDa subunit contains Se and probably the active site (75, 97). Formate dehydrogenase-N copurifies with nitrate reductase, suggesting that the two enzymes may form a specific complex (52, 97, 169).

Most preparations of formate dehydrogenase-N contain associated cytochrome *b* (169, 241, 344). Ruiz-Herrera and DeMoss distinguished two distinct forms of cytochrome *b* in nitrate-respiring cells, one associated with formate dehydrogenase-N and the other associated with nitrate reductase (330; see "Physiology" section). It is presumed that the 20-kDa subunit of formate dehydrogenase-N comprises the specific cytochrome (97, 344).

Formate dehydrogenase-N spans the cytoplasmic membrane (125) and, consistent with its role in energy conservation, translocates protons to generate PMF (105, 194; reviewed in reference 164). A formate-oxygen oxidoreductase activity (formate oxidase) is also detected in nitrate-respiring cells, so formate dehydrogenase-N probably couples to the aerobic respiratory chain (328). Likewise, formate dehydrogenase-N has been implicated in formate-nitrite oxidoreductase (see section, "Nitrite Metabolism").

Genetics of Formate Dehydrogenase Formation

Current designations and functional assignments for formate dehydrogenase genes are summarized in Table 1.

***fdhA*.** Mutants lacking formate dehydrogenase-N and -H activities, but retaining hydrogenase, have been isolated in both *S. typhimurium* (23, 55, 300) and *E. coli* (127; B. A. Haddock and M.-A. Mandrand-Berthelot, Biochem. Soc. Trans. **10**:478, 1982). Transductional crosses have revealed in both organisms a gene order of *xyl-fdhA-mtl*, indicating that these mutations affect homologous genes in the two organisms (23, 298; Haddock and Mandrand-Berthelot, Biochem. Soc. Trans. **10**:478, 1982). Immunochemical analysis indicates that *E. coli fdhA* mutants lack formate dehydrogenase-N protein (127), but other evidence suggests that apoformate dehydrogenase-N, when it is unable to complex with Mo cofactor, fails to accumulate (113, 393). Given that formate dehydrogenase-N and formate dehydrogenase-H are distinct enzymes, it is unlikely that *fdhA* is a structural gene for a formate dehydrogenase-N subunit. Rather, the simplest hypothesis is that *fdhA* is required for Se metabolism and thus pleiotropically affects both formate dehydrogenases (115). Analogous hypotheses explain the pleiotropic effects of *cysG* (siroheme) and *chl* (Mo cofactor) mutations (see "Nitrite Metabolism" and "Molybdenum Cofactor" sections).

***fdhB*.** *S. typhimurium* mutations designated *fdhB*, although not precisely mapped, may be located in the general region of *aroA* (23, 57). *fdhB* mutants are devoid of formate dehydrogenase activity but retain hydrogenase. The *fdhB* designation has also been used to designate *E. coli* mutations that reportedly map between *his* and *trp* (262; Haddock and Mandrand-Berthelot, Biochem. Soc. Trans. **10**:478, 1982). It is not known whether "*fdhB*" designates homologous genes in the two organisms.

***fdhC*.** *S. typhimurium* (23) and *E. coli* (Haddock and Mandrand-Berthelot, Biochem. Soc. Trans. **10**:478, 1982), mutations designated *fdhC* are linked to *fdhA*. The gene order in *E. coli* is *xyl-lct-fdhA-mtl-pyrE-fdhC* (Haddock and Mandrand-Berthelot, Biochem. Soc. Trans. **10**:478, 1982). Again, *fdhC* mutants lack both formate dehydrogenase-H and formate dehydrogenase-N activities.

***fdhF*.** Böck and colleagues have identified a Mu dl insertion mutant that specifically lacks formate dehydrogenase-H activity; it retains formate dehydrogenase-N, hydrogenase, and the wild-type low level of benzyl viologen-formate dehydrogenase found in nitrate-respiring cells (306; see above). Genetic mapping indicates an order of *fdhF-mel-frd* (305). This mutant was isolated from a collection of Mu dl insertion mutants in which β-galactosidase synthesis is induced by anaerobiosis. *fdhF* is the structural gene for the formate dehydrogenase-H 80-kDa selenopolypeptide (75, 305, 438; see above). Analogous Mu dl insertion mutants of *S. typhimurium* were isolated on a medium that differentiates

TABLE 2. Proposed functions of hydrogenase isoenzymes in *E. coli* and *S. typhimurium*[a]

Isoenzyme	Proposed function
1	Hydrogen uptake during fermentation; possible role in recycling reducing equivalents
2	Hydrogen uptake during anaerobic respiration; required for use of hydrogen as an electron donor
3	Hydrogen evolution during fermentation; hydrogenase component of formate-hydrogen lyase

[a] From references 20, 178, 179, 235, 335, 339, and 341.

formate-hydrogen lyase mutants (25). The mutations, designated *fhl*, are also linked to *mel* and probably affect the *Salmonella* gene homologous to *fdhF*. These *fhl* mutants lack formate dehydrogenase-H but retain hydrogenase; formate dehydrogenase-N activities were not reported (25).

fdn. Barrett and colleagues developed a differential medium, MacConkey nitrate, to isolate mutants lacking formate dehydrogenase (23). This medium contains glycerol, nitrate, formate, and trace amounts of glucose. Colonies of *fdh* mutants, which cannot metabolize formate, turn red on this medium after anaerobic incubation. Wild-type strains form white colonies and mutants lacking nitrate reductase show various distinguishable phenotypes (23, 368). This medium distinguished a new class of *S. typhimurium* mutants, *fdn*, which lack formate dehydrogenase-N and retain formate dehydrogenase-H. Genetic mapping shows that *fdn* mutations are linked in the sequence *chlB-fdn-rha-metB*. The transduction data were interpreted to indicate the existence of at least two distinct *fdn* genes (26). Biochemical analysis suggests that these mutants retain formate dehydrogenase-N enzyme, but lack the associated cytochrome *b* (26).

Analogous mutations in *E. coli*, termed *fdhE*, are reported to map between *chlB* and *rha* (Haddock and Mandrand-Berthelot, Biochem. Soc. Trans. **10**:478, 1982). Other mutations in this region, termed *fdhD*, are reported to lack both formate dehydrogenase activities (Haddock and Mandrand-Berthelot, Biochem. Soc. Trans. **10**:478, 1982).

Hydrogenase

Hydrogenase serves two roles in enteric bacteria; that of a terminal component in formate-hydrogen lyase is discussed above. Enteric bacteria can also couple hydrogen oxidation, via hydrogenase, to anaerobic respiration (reviewed in reference 164); this activity is termed uptake hydrogenase (Hup). Recent work with both *E. coli* and *S. typhimurium* has defined three distinct membrane-bound hydrogenase isoenzymes (20, 339, 341) (Table 2), which are involved in hydrogen uptake during fermentation, hydrogen uptake for respiration, and formate-hydrogen lyase, respectively. "Soluble" hydrogenase activity is attributed to contamination by hydrogenase released from membranes during cell fractionation (20).

Hydrogenase 1 is an uptake hydrogenase active during fermentation. It has been defined in both *E. coli* K-12 and *S. typhimurium* LT2 on the basis of immunochemical studies (20, 339, 341). This enzyme contains Ni, and has an apparent molecular mass of 64 kDa (20). Active hydrogenase 1 has been purified from *E. coli* (340). Three prototrophic strains of *S. typhimurium* [LT2(Z), LT2(A) and LT7] were found to differ in hydrogenase isoenzyme content. LT2(A) lacks isoenzyme 2, and LT7 lacks isoenzymes 1 and 2. Comparing these strains reveals that hydrogenase 1 catalyzes hydrogen uptake and suggests that it may function to recycle reducing equivalents from hydrogen during fermentation, presumably by using endogenous fumarate as an electron acceptor (341). Consistent with this idea is the fact that isoenzyme 1 activity is induced by growth with formate (a fermentation product), but not with hydrogen or glycerol (nonfermentable substrates) (339, 341).

Hydrogenase 2 is "respiratory" hydrogenase; it allows cells to use hydrogen as an electron donor for anaerobic respiration (195). Hydrogenase 2 has been defined in both *E. coli* K-12 and *S. typhimurium* LT2 on the basis of immunochemical studies (20, 339, 341). This enzyme also contains Ni and exists in two forms as revealed by nondissociating gel electrophoresis (20). An active soluble fragment of hydrogenase 2 has been purified from *E. coli* (21). Isoenzyme 2 activity is induced by growth on nonfermentable substrates (339, 341).

Hydrogenase 3 is probably the hydrogenase component of formate-hydrogen lyase (330, 341). It has not been detected as a physical entity, but its presence in both *E. coli* and *S. typhimurium* is inferred from the fact that substantial hydrogenase activity is refractory to immune precipitation with serum raised against isoenzymes 1 and 2 (339, 341). The hydrogenase 3 content is enhanced after growth with formate, and it is not formed after growth on nonfermentable substrates (339, 341). *S. typhimurium* LT7, which lacks isoenzymes 1 and 2, retains formate-hydrogen lyase activity (341).

Genetics of Hydrogenase

The first-studied mutants deficient in formate-hydrogen lyase were the "*coli-aerogenes*" anaerogenic variants of Gest and Peck, but these strains are not suitable for genetic analysis. Early schemes for isolating hydrogenase (*hyd*) mutants used screens for clones unable to reduce benzyl viologen during anaerobic growth (55). Oxidized benzyl viologen is colorless, while the reduced form is purple, so *hyd* colonies are white on media containing the dye. This procedure yields pleiotropic mutants (*chl*; chlorate resistant) that are affected in Mo cofactor synthesis (see "Molybdenum Cofactor" section). However, *chl*$^+$ strains that lack formate dehydrogenase and hydrogenase activities have also been found. Recently, Shanmugam and colleagues (235) devised a positive selection for *hyd* mutants, taking advantage of the fact that reduced benzyl viologen produces lethal amounts of superoxide radical. *hyd* mutants survive exposure to oxygen after anaerobic growth with hydrogen and benzyl viologen. Other methods, using MacConkey indicator media, have also been used to isolate *hyd* mutants (25, 428).

hyd. Early studies (55, 299) identified hydrogenase-deficient (*hyd*) mutants of *S. typhimurium* and *E. coli*. Genetic mapping of the *E. coli hyd* mutations gave results consistent with a gene order of *cysC-hyd-srl-recA-nalB* (126, 299, 435). The *S. typhimurium hyd* mutations map at the analogous chromosomal location, but transductional crosses to establish a more precise gene order were unsuccessful (299). More recently, Barrett and colleagues isolated Mu d1 insertion mutants of *S. typhimurium* on a medium that differentiates mutants lacking formate-hydrogen lyase (25). These mutations are linked to *srl* and are thus presumed to affect *hyd*.

The low formate dehydrogenase-H activities of these *hyd* mutants, coupled with in vitro reconstitution studies, led Chippaux and colleagues to conclude that hydrogenase is

required for electron transfer from formate to benzyl viologen (57). They proposed a modified form of the Peck and Gest "one enzyme, two carriers" hypothesis (307; see above) in which formate dehydrogenase can donate electrons directly to phenazine methosulfate (and methylene blue), while the entire formate-hydrogen lyase is required for transfer to viologen (57).

Other studies are inconsistent with this proposal. The "*coli-aerogenes*" anaerogenic variant WR3 (109) is devoid of hydrogenase, yet it retains a high level of formate dehydrogenase-H. More recently, two *E. coli* mutants with very low hydrogenase activity but wild-type levels of formate dehydrogenase-H were isolated. These mutants were identified in a screen for Mu dl insertion mutants in which β-galactosidase synthesis is induced by anaerobiosis. The mutations are linked to *cysC* and presumed to affect *hyd* (306). The existence of these mutants argues against a role for hydrogenase in transferring electrons from formate to benzyl viologen. Further, the reconstituted formate-hydrogen lyase consisting of "*coli-aerogenes*" formate dehydrogenase-H, methyl viologen, and clostridial hydrogenase (307) suggests that formate dehydrogenase-H directly donates electrons to viologen.

Nonetheless, many *hyd* mutants do have reduced levels of formate dehydrogenase-H activity (235, 419). Although this may reflect the functional organization of formate-hydrogen lyase (perhaps formate dehydrogenase-H is unstable in the absence of hydrogenase; [419]), it is also likely that at least some of these mutations affect *hyd*-linked genes required for formate dehydrogenase-H gene expression (335, 419, 429; see below).

Yerkes and colleagues identified five Mu dl insertion mutants in a screen for mutants with defective electron transfer to benzyl viologen in a 15% hydrogen atmosphere (435). These strains retain uptake hydrogenase activity and reportedly retain formate dehydrogenase; however, a distinction between different forms of formate dehydrogenase was not made. Physical mapping shows that the Mu dl insertions span a region of approximately 5 kilobases. These mutations were named *ant* (anaerobic electron transport) because they were thought to be distinct from *hyd*; however, genetic mapping reveals an order of *cysC-mutS-ant-srl-recA-nalB* (435). Therefore, *ant* and *hyd* are tightly linked, but apparently not identical (335); that the *ant* insertion mutations span 5 kilobases indicates that this region contains more than one gene (435).

hydAB. More recent analyses of the *hyd* region have begun to address this genetic complexity. Shanmugam and colleagues (235, 335) isolated a large collection of *hyd* mutants, using the benzyl viologen-mediated superoxide selection. Genetic mapping and complementation analyses with cloned DNA fragments indicate that these mutations define two linked, yet distinct regions termed *hydA* and *hydB*. Additional complementation studies with subclones indicates that *hydB* may include genes required for formate dehydrogenase-H (*fdv*) and formate-hydrogen lyase (*fhl*) activities (335). Alterations of *fdv* would provide an explanation for the lowered formate dehydrogenase-H activity observed in many *hyd* mutants. Indeed, *hydB12* (335) and a newly isolated mutation thought to affect *fdv* (429) both eliminate expression of a *fdhF-lacZ* operon fusion (429).

Examination of other previously isolated *hyd* mutants (126) indicates that they affect the *hydB* region (419). Hydrogenase activity in one of these mutants is phenotypically suppressed by growth in 0.6 mM Ni. Complementation studies suggest that at least three genes are present at *hydB*: two required for hydrogenase activity and one required for formate dehydrogenase-H activity (419).

Recently, the mutation *hyd-17*::Mu dl (306) has been shown to specifically lack hydrogenase isoenzyme 3 (30), suggesting that it may define the structural gene for that enzyme.

Obviously, much work remains to define the roles played by different *hyd* genes in encoding hydrogenase structural genes, regulatory proteins, or enzymes associated with nickel metabolism.

hydCD. Three Mu dl insertion mutations affecting hydrogenase activity were mapped to the 77-min region of the *E. coli* genetic map, distinct from the *hydAB* region (426). The hydrogenase-deficient phenotype conferred by two of these mutations, termed *hydC*::Mu dl, is phenotypically suppressed by growth in 0.5 mM Ni. β-Galactosidase activity in these strains is also completely repressed by growth in Ni. These results suggest that *hydC* is required for Ni transport (428). Hydrogenase and β-galactosidase activities in the third mutant, *hydD*, are insensitive to Ni. β-Galactosidase in all three strains is induced by anaerobiosis (428).

hup. Three mutants isolated by Shanmugam and colleagues (235) lack hydrogenase activity as assayed by hydrogen-dependent benzyl viologen reduction and fumarate-mediated hydrogen uptake, but retain substantial tritium exchange activity. Thus, these mutants specifically affect hydrogen uptake and are designated *hup*. Genetic mapping located the *hup* mutations to the 65-min region of the *E. coli* chromosome (235). The relationship between *hup* and specific hydrogenase isoenzymes remains to be determined.

Regulation of Formate-Hydrogen Lyase Synthesis

The effects of growth conditions on production of formate-hydrogen lyase have been amply documented. This activity is not formed in oxygen- or nitrate-respiring cultures (131, 328, 339) and is preferentially synthesized in cultures growing at relatively low pH (e.g., pH 6.2 [131]). Both components of formate-hydrogen lyase, formate dehydrogenase-H and hydrogenase-3, show the same patterns of regulation in *E. coli*, *S. typhimurium*, and *K. pneumoniae* (131, 161, 307, 328, 339, 340). Formate-hydrogen lyase activity is also increased by growth in formate-containing medium (328, 339, 340). In contrast, formate dehydrogenase-N synthesis is high in nitrate-respiring (75, 115, 169, 328) or oxygen-respiring (131, 307, 328) cultures, and its activity is low in cultures grown in the absence of an external electron acceptor (75, 115, 328).

The isolation and characterization of *fdhF-lacZ* operon fusions has confirmed that the regulation of formate dehydrogenase-H synthesis described above occurs at the level of transcription. Specifically, *fdhF* transcription in *E. coli* and *S. typhimurium* (where the gene is designated *fhl*; Table 2) is inhibited by oxygen and nitrate and is induced by formate. Formate also partially relieves the inhibition by nitrate (25, 30, 306, 429). That formate is a specific inducer is shown by studies with pyruvate-formate lyase (*pfl*) mutants, in which *fdhF* expression requires added formate (30, 429).

Wu and Mandrand-Berthelot (429) have studied the effect of two *hyd* region mutations, *hydB12* (335) and a mutation thought to affect *fdv*, on the expression of an *fdhF* operon fusion. Both mutations essentially eliminate *fdhF* expression. Thus, the *hyd* region contains at least one regulatory gene for *fdhF* expression (235, 335), consistent with observations that many *hyd* mutants are also deficient in formate dehydrogenase-H (see above).

Similar patterns of regulation affect *hyd* transcription in *E. coli* and *S. typhimurium* (25, 30, 306) and *hydC* and *ant* transcription in *E. coli* (428, 435). Again, in a *pfl* mutant, expression of *hyd-17*::Mu dl is dependent on added formate (30). Studies of *hyd* gene regulation will be more easily interpreted as specific functions are identified for each of these genes.

The role of nitrate in regulating formate-hydrogen lyase gene transcription is considered below (see "Nitrate Regulation of Anaerobic Enzyme Synthesis" section).

NITRITE METABOLISM

The ultimate fate of nitrite, the reduction product of nitrate, is determined by the type of nitrite reductase expressed by a given organism. Denitrifying nitrite reductase sends nitrite down a path through several gaseous intermediates to dinitrogen, in a process termed denitrification. Each step of denitrification is coupled to energy conservation, and thus this pathway represents a cascade of anaerobic respirations (reviewed in reference 303). However, enteric bacteria are not denitrifiers, and the low level of denitrifying nitrite reductase (nitrous oxide-producing) activity detectable in some strains is attributable to nitrate reductase itself (357).

The other fate of nitrite is its conversion to ammonia by a six-electron transfer. In enteric bacteria, this conversion can serve any of three physiological roles: nitrogen assimilation, disposal of reducing equivalents during anaerobic growth, and generation of a membrane potential during anaerobic growth. Each of these processes is considered here in turn.

Nitrate and Nitrite Assimilation

K. pneumoniae organisms grow well aerobically with nitrate or nitrite as the sole source of nitrogen (280). The assimilatory nitrate and nitrite reductases from *K. pneumoniae* have not been well characterized, but by analogy with other organisms, it is probable that these are soluble, single-subunit, reduced flavodoxin- or NADH-dependent enzymes (140, 141, 409). Synthesis of assimilatory nitrate reductase activity is inhibited by ammonia, but insensitive to aerobiosis; this contrasts with the respiratory activity, whose synthesis is insensitive to ammonia but prevented by aerobiosis (404). Mutants defective only in nitrate and nitrite assimilation have been isolated (338, 371, 372, 380), but they have not been extensively characterized. It was tentatively concluded that nitrate assimilation and respiration are performed by the same enzyme. Such an enzyme would show differential membrane association and interaction with electron donors depending on growth conditions (404). In *Pseudomonas aeruginosa*, the respiratory and assimilatory nitrate and nitrite reductases are genetically distinct (184). Recent genetic studies indicate that *K. pneumoniae* also encodes distinct forms of these enzymes (V. Stewart, B. M. Cali, and J. M. Micca, unpublished observations).

Kinetic studies on nitrate uptake in aerobically grown *K. pneumoniae* M5al, using $^{13}NO_3^-$, have revealed two distinct uptake pathways (394). The high-affinity pathway has a K_m for nitrate of about 5 μM, and the K_m for the low-affinity pathway is about 4 mM. Nitrate uptake is induced by growth with nitrate or nitrite and is both repressed and inhibited by ammonium. The high-affinity pathway almost certainly represents a specific active transport system, while the nature of the low-affinity pathway is unknown (394).

Early reports indicated the presence of an apparently soluble NADH-nitrate reductase activity in "aerobically" cultured *E. coli* strains B (286) and Yamagutchi (170). These results implied that *E. coli* is capable of aerobic nitrate assimilation (reviewed in reference 281). However, immunological characterization showed that the "aerobic assimilatory" and the respiratory forms of nitrate reductase are identical (279). Special precautions are required to ensure that cultures are fully aerobic, and it is likely that these early studies (170, 286) were performed with oxygen-limited cultures. No modern reports of soluble assimilatory nitrate reductase in *E. coli* have been published, and several *E. coli* strains tested failed to grow aerobically with nitrate or nitrite as sole nitrogen source (215). Thus, it is highly unlikely that *Escherichiae* are capable of aerobic nitrate assimilation. Likewise, *S. typhimurium* LT2 also fails to grow aerobically with nitrate or nitrite as sole nitrogen source (143).

NADH-Nitrite Reductase in *E. coli*

The major nitrite reductase activity in *E. coli* is contributed by NADH-nitrite reductase (205). This soluble enzyme is composed of a single polypeptide species of 88 kDa (72). It resembles the assimilatory nitrite reductase from *N. crassa* in its cofactors (flavin adenine dinucleotide, sulfhydryl, Fe-S clusters, and siroheme) and its presumed reaction mechanism (92, 175, 176).

Induction of NADH-nitrite reductase activity is not controlled by the availability of ammonia, suggesting that the enzyme does not serve an assimilatory function (66, 283). Of course, ammonia from any source can be assimilated, so *E. coli* will grow with nitrite (or nitrate; but see below) as the sole nitrogen source under anaerobic conditions (66). However, Cole and Brown argue that the true function of NADH-nitrite reductase activity is to use ammonia generation as an "electron sink" during anaerobic growth (65; see also reference 205). Nitrite reduction consumes six electrons per molecule of nitrite, so it can serve as a significant means of reoxidizing NADH in the absence of other electron acceptors.

Evidence for this hypothesis comes from studies on fermentation balances. In the absence of nitrite, ethanol and acetate are formed in roughly equimolar amounts. However, in the presence of nitrite little ethanol is produced, concomitant with an increased amount of acetate. Large quantities of ammonia are excreted into the medium, even when nitrite is the sole nitrogen source (64). These results indicate that nitrite reduction supercedes the need for regenerating NAD^+ through ethanol formation, and thus most of the acetyl-coenzyme A is used for ATP generation instead (65). Paradoxically, however, nitrite-grown cultures have the same anaerobic growth yield as ammonia-grown cultures; it is suggested that nitrite transport may consume the extra energy produced by the increased formation of acetate (65).

NADH-nitrite reductase and NADPH-sulfite reductase require a common cofactor, siroheme, for enzymatic activity. Thus, *cysG* mutants, defective in siroheme production, lack both activities (67). Recent work has defined a gene, *nirB*, that is closely linked to *cysG*; *nirB* mutants lack only NADH-nitrite reductase activity (248). The genes *cysG* and *nirB* may form an operon, although complementation studies indicate that the genes are expressed independently under some conditions (247). Comparison of the partial DNA and amino acid sequences confirms that *nirB* is the structural gene for nitrite reductase (181).

Formate-Nitrite Reductase in *E. coli*

A minor nitrite reductase activity coupled to formate oxidation has been detected in many strains of *E. coli*; this

activity usually accounts for approximately 25% of the total detectable nitrite reductase activity (1, 248). However, this activity was not found in *S. typhimurium* or *K. pneumoniae* (1). Lactate also serves as an efficient electron donor for nitrite reduction, presumably to the same enzyme (2). Ethanol and pyruvate also serve as electron donors for nitrite reduction, but this probably reflects their metabolism to generate NADH and formate (317).

Nitrate inhibits formate-nitrite reductase activity by 75%, but in cell suspensions both nitrate and nitrite are reduced simultaneously by formate. It was proposed that the same formate dehydrogenase enzyme (i.e., formate dehydrogenase-N) is involved in nitrate and nitrite reduction and that nitrate more effectively competes for electrons from this enzyme (1). Formate-dependent nitrite reduction generates PMF during anaerobic growth (276, 316). Thus, it is likely that this pathway is involved in energy conservation and represents another pathway for anaerobic respiration.

A soluble cytochrome species, cytochrome c_{552}, has long been implicated in nitrite reduction (63, 70, 104), although this role was obscured because of other suggestions that it was involved in formate-hydrogen lyase (69, 131; see reference 90). Cytochrome c_{552} is a periplasmic protein that is lost upon cell disruption (63, 70, 104). Cytochrome c_{552} may be the formate-dependent nitrite reductase, although it has been reported that the nitrite reductase activity of cytochrome c_{552} is obligately coupled to NADH oxidation (242). Others argue that NADH is not a physiological electron donor (198). Unfortunately, no mutants specifically defective in cytochrome c_{552} have been isolated (see reference 248), so the role of this cytochrome in anaerobic metabolism is still not certain.

Regulation of Nitrite Reductase Synthesis

Both of the nitrite reductases are induced by anaerobiosis, and induction is insensitive to the presence of ammonia. The highest activities of NADH-nitrite reductase and cytochrome c_{552} are detected in cultures grown with low concentrations (10 mM) of nitrate, and both activities are greatly depressed in cells grown with high concentrations (100 mM) of nitrate (63, 70, 423). Presumably, nitrite produced by nitrate reductase serves to induce nitrite reductase synthesis at low nitrate concentrations, although it is also possible that nitrate is a direct inducer (138, 284). At high concentrations, nitrate suppresses nitrite reductase synthesis (63, 70, 423). Thus, anaerobic *E. coli* cultures grown with high levels of nitrate may be unable to effectively assimilate the resultant nitrite.

One unresolved aspect of nitrite reductase regulation is that it is constitutive in chlorate-resistant (*chl*) mutants (138, 176, 248, 284). *chl* mutants are defective in synthesis of Mo cofactor (see below). The nitrate reductase operon, as measured by *narC-lacZ* operon fusion expression, was also thought to be constitutive in *chl* mutants (297). However, recent work by Iuchi and Lin suggests that the apparent constitutivity is due to trace amounts of nitrate present in complex media (174; see "Regulation of Nitrate Reductase Synthesis" section). It is possible, but untested, that this could also explain the apparent constitutivity of *nirB* expression in *chl* mutants.

Recently, Griffiths and Cole have used *nirB-lacZ* operon fusion strains to examine *nirB* regulation. In chl^+ strains, *nirB* expression is induced about 10-fold by anaerobiosis; this induction requires fnr^+ (see "Regulation of Nitrate Reductase Synthesis" section). Curiously, nitrite, 10 mM nitrate, and 100 mM nitrate are equally effective inducers during anaerobic growth (approximately two- to threefold). The discrepancy between these results and previous work showing that 100 mM nitrate strongly depresses nitrite reductase synthesis (63, 70, 423) has not been addressed (138).

PHYSIOLOGY

Early debates centered around the physiological role of nitrate reduction in *E. coli* (see "Introduction"). That nitrate reduction is a form of anaerobic respiration is now established, and the various electron transport pathways are understood at least in outline; these topics have been reviewed previously (149, 164, 219, 375, 382). This section emphasizes some points of confusion as well as recent results.

Energy Conservation

Molar growth yields. Growth rate measurements provide the simplest indication that nitrate reduction generates energy. Anaerobic cultures of enteric bacteria, typically grown with glucose, have significantly reduced growth rates compared with aerated cultures. Inclusion of nitrate stimulates anaerobic growth, although the rates are still lower than those of aerobic cultures (199, 358, 378, 431). Variations in reported growth rates undoubtedly reflect differences in strains, culture media, and cultivation techniques, but the consistent finding is that nitrate supports growth rates that are intermediate between those of aerobic and anaerobic cultures.

A related demonstration of improved growth comes from analyses of molar growth yields; this topic has been extensively reviewed (375, 382, 392). Again, nitrate-respiring cultures show molar growth yields that are intermediate between those of aerobic and anaerobic cultures.

Early studies on molar growth yields suggested that the amount of ATP required for growth (Y_{ATP}) is a constant value shared by all microorganisms under all culture conditions. It is now recognized that maintenance energy and futile cycles probably play large roles in determining growth yields and that maintenance energy and Y_{ATP} vary with a number of factors, including growth rate, energy source, growth-limiting nutrient, and medium composition (reviewed in references 376, 379, and 392). Therefore, early attempts to calculate the efficiency of oxidative phosphorylation from molar growth yields were not valid. It is interesting to note, however, that these calculations suggested that aerobic respiration and nitrate respiration were equally efficient (151, 152). Subsequent calculations, attempting to allow for the contribution of maintenance, gave a $P/2e^-$ ratio of about 2 for nitrate respiration in *K. pneumoniae* (379).

The intermediate growth yields of nitrate-respiring cultures reflect two aspects of anaerobic metabolism (375). First, the yield is increased over anaerobic cultures, because nitrate respiration is coupled to NADH oxidation. Thus, more ATP is formed not only by respiration, but also by substrate-level phosphorylation coupled to acetate formation. Within the limitations imposed by maintenance, this point was illustrated by comparing the relative molar growth yields of *K. pneumoniae* on glucose and on mannitol (151, 152). In the absence of a terminal electron acceptor, the yield is lower on mannitol than on glucose, perhaps reflecting the additional mole of NADH formed per mole of mannitol, and thus the (presumed) increase in ethanol formation. In the

presence of electron acceptors, however, the yields with mannitol are greater than those with glucose, suggesting that increased oxidative phosphorylation results from the additional NADH.

Second, nitrate-respiring cultures show lower growth yields than aerobic cultures, because acetate oxidation through the TCA cycle is curtailed during nitrate respiration. Physiological evidence to support this conclusion comes from work with a mutant of *K. pneumoniae* that lacks α-ketoglutarate dehydrogenase and is thus unable to oxidize acetate (373). The molar growth yield of this strain, cultured aerobically, was essentially identical to that of the parent strain cultured anaerobically with nitrate, suggesting that acetate oxidation contributes to the aerobic yield. The yields of both strains grown in the absence of electron acceptors were equally low. Obviously, many other factors contribute to the differences in growth yields.

PMF. The central feature of chemiosmosis is the generation of a proton electrochemical potential, or PMF. PMF is generated by proton translocation to the periplasmic side of the cytoplasmic membrane. One may measure proton translocation in spheroplasts with a sensitive pH electrode connected to a recording instrument. A small pulse of oxidant (or reductant) is added to spheroplasts that have been previously incubated anaerobically with reductant (or oxidant). The initial rate of proton extrusion is detected as a spike of decreased pH in the external volume of the suspension. From these data one can estimate the number of protons extruded for each molecule of acceptor that is reduced ($H^+/2e^-$ ratio). These ratios are subject to a number of assumptions, and the potential limitations of this technique for deriving the exact stoichiometries are beyond the scope of this discussion (see references 157, 201, and 287). At the very least, these experiments demonstrate that proton extrusion is coupled to nitrate respiration.

Using this technique, Garland and colleagues (105) found H^+/NO_3^- ratios of approximately 2 for the oxidation of formate, glycerol-3-phosphate, and D-lactate, in spheroplasts of *E. coli* cultured aerobically with nitrate. Malate oxidation supported ratios closer to 4. The same spheroplasts also oxidized these substrates with oxygen as the electron acceptor, and the estimated H^+/O ratios were similar to the H^+/NO_3^- ratios. These results suggest that oxygen and nitrate respiration are energetically equivalent.

An independent method for detecting proton translocation uses the fluorescent dye atebrin, which is distributed across membranes in response to pH gradients. In membrane preparations of *E. coli* cultured aerobically with nitrate, nitrate-dependent quenching of atebrin fluorescence was observed with formate, glycerol-3-phosphate, D-lactate, or NADH as electron donor (150).

Several lines of evidence demonstrate that uptake of lactose and many amino acids is coupled to PMF (reviewed in reference 219). Thus, the finding that solute uptake by anaerobically grown cells is energized by nitrate provides additional evidence that nitrate reduction generates PMF. Extensive studies by Konings, Kaback, and colleagues showed that lactose and amino acid uptake by whole cells and membrane vesicles of *E. coli* can be energized by nitrate respiration (34, 218) and can also be coupled to a nonphysiological substrate of nitrate reductase, chlorate (36). Formate plus nitrate energizes amino acid uptake in vesicles of cells that have been cultured anaerobically either in the presence or absence of nitrate, and glycerol-3-phosphate plus nitrate energizes uptake after anaerobic growth with glycerol (to induce glycerol-3-phosphate dehydrogenase). These same vesicles are capable of amino acid uptake with oxygen as electron acceptor (34).

PMF consists of two components: the pH gradient (ΔpH) and the electrochemical gradient (ΔΨ; reviewed in references 157 and 287). Boonstra and Konings (35) have estimated the contribution of these components to the overall PMF in nitrate-respiring spheroplasts. They found that membrane vesicles of *E. coli* grown anaerobically with nitrate generated a PMF of about −160 mV in the presence of formate plus nitrate; this PMF was composed of a ΔΨ of about −90 mV (measured by triphenyl phosphonium uptake) and a ΔpH of about −75 mV (measured by weak acid distribution). These values were similar to those obtained by adding ascorbate plus phenazine methosulfate (efficient electron donors for the aerobic respiratory chains) to oxygenated vesicles. Unfortunately, measurements of ΔΨ are subject to a number of constraints, and the derived values are often subject to interpretation (201). Nonetheless, these studies further establish that PMF is generated by nitrate respiration and suggest that its potential efficiency in driving oxidative phosphorylation is close to that of aerobic respiration.

Metabolic Pathways for Nitrate Respiration

Electron donors. Current evidence indicates that respiratory chain components are organized in "modules," each of which is capable of interacting with other components within the limits of substrate availability and thermodynamics (reviewed in reference 164). Thus, the various substrate dehydrogenases transfer electrons to a common, diffusible quinone pool, which in turn donates electrons to the electron acceptor reductases. Nitrate reduction by whole cells, spheroplasts, or membrane preparations of *E. coli* can be coupled to the oxidation of formate (34, 70, 96, 105, 150, 169, 330, 388, 389, 418), glycerol-3-phosphate (34, 105, 150, 418), hydrogen (195), D-lactate (105, 150), malate (105, 233; but see reference 70), NADH (70, 150, 388, 418), and succinate (70, 105). Pyruvate is also an in vivo electron donor, probably through its conversion to formate (70); it is not known if pyruvate oxidase will couple to nitrate reductase. *E. coli* has been shown to grow anaerobically with nitrate and either L-lactate (288, 320) or glycerol (103, 270, 320) as the only oxidizable substrate.

Although succinate oxidation is coupled to nitrate reduction in vitro (70, 105), its role in vivo is not clear. *E. coli* K-12 has been grown anaerobically with nitrate, with succinate as the principal carbon source (105, 320). However, the observed levels of succinate-nitrate oxidoreductase in cell extracts are low (70). It is argued that succinate is not a physiologically relevant electron donor for nitrate respiration, because it is not produced during anaerobic growth with nitrate due to repression of both fumarate reductase and α-ketoglutarate dehydrogenase (70). In addition, succinate dehydrogenase levels are low in nitrate-respiring cultures of *E. coli* K-10 (430, 432). However, succinate dehydrogenase activity in the closely related *C. freundii* appears to be slightly induced during anaerobic growth (203).

Malate is not an electron donor for nitrate reduction in cell extracts of *E. coli* (70), but it does support proton translocation in spheroplasts (105, 233). Indeed, the observed H^+/NO_3^- stoichiometries suggested that malate dehydrogenase itself was providing electrons for respiration, in addition to those from the NADH produced by malate oxidation (105, 233). Again, the physiological relevance may be questioned by the fact that malate dehydrogenase is apparently repressed during anaerobic growth of *E. coli* K-10 with

nitrate (430, 432), although the levels in *C. freundii* showed little change between oxygen and nitrate respiration growth conditions (203).

Most physiological studies use glucose as the principal carbon and energy source, so it is relevant to consider possible electron transport pathways to nitrate in glucose-grown cells. Potential electron donors include NADH, formate, and lactate. Most fermentation balances show that little lactate is accumulated by nitrate-respiring cultures (89, 166, 203, 412). D-Lactate dehydrogenase is constitutive, and L-lactate dehydrogenase is induced by lactate in the presence of nitrate (288), so low recovery of lactate could reflect its role as an electron donor.

There is little doubt that NADH is a significant in vivo electron donor for nitrate respiration. Most fermentation balances show that ethanol production is greatly depressed during nitrate respiration, while the levels of acetate are usually doubled (101, 166, 203, 412). The greatly increased growth rates and yields of *K. pneumoniae* during nitrate respiration in mannitol-grown cultures also support this conclusion (152).

The role of formate as a significant electron donor for nitrate respiration has been questioned (375, 382). Many fermentation balances for *K. pneumoniae* (101; and unpublished data cited in reference 375), *E. coli* (166), and *C. freundii* (203) show significant formate accumulation under conditions in which ethanol is nearly undetectable. This might suggest that NADH is the principal electron donor for nitrate respiration (obviating the need to produce ethanol), while formate is a less significant source of electrons. However, other fermentation balances for *E. coli* show little formate under any growth conditions (89, 412). The simple mixed-acid fermentation predicts that the formate concentration, if not metabolized, will equal the sum of the acetate-plus-ethanol concentrations. However, in many fermentation balances the formate concentration is considerably lower than this sum, irrespective of the presence of nitrate (89, 101, 203, 412). This indicates that at least some formate was being metabolized, through either formate-hydrogen lyase or formate dehydrogenase-N.

In contrast to the fermentation balance studies, there are many reports of both a physical (52, 97, 169) and a functional (34, 70, 96, 105, 150, 169, 330, 332, 388, 418) association between formate dehydrogenase-N and nitrate reductase in *E. coli*. Molar growth yield studies suggest that formate oxidation coupled to nitrate reduction will support anaerobic growth of *E. coli* (431). Formate is also the most efficient electron donor for nitrate reduction in cells or extracts of *K. pneumoniae* M5al (338). It thus seems certain that formate is a significant in vivo electron donor for nitrate respiration.

Pinsent (314), and later Lester and DeMoss (236), showed that culture media must be supplemented with Se and Mo for maximum formate dehydrogenase activity; the requirement for Se is particularly stringent. However, most of the fermentation balance studies cited above do not report the deliberate inclusion of Se and Mo in the culture medium. Although amino acid supplements usually provide some Se and Mo as trace contaminants (236, 314), it is possible that the cultures in some of these fermentation studies had reduced levels of formate dehydrogenase activity due to Se limitation.

Quinones. Quinones probably constitute a diffusible pool in the cytoplasmic membrane, mediating electron flow from dehydrogenases to reductases (reviewed in references 164 and 228). Early evidence of a quinone requirement for nitrate respiration in *E. coli* came from the studies of Egami and colleagues (389), who found that 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) inhibited formate-nitrate oxidoreductase activity, but had little effect on reduced methylene blue-nitrate oxidoreductase activity. Coupled formate-nitrate oxidoreductase activity in partially purified preparations was found to be stimulated by a crude lipid fraction or by menadione (169). Subsequently, Itagaki (167) extracted membrane preparations with acetone and found that formate dehydrogenase and nitrate reductase activities, measured with artificial electron carriers, remained at relatively high levels. However, the acetone extraction had nearly abolished the coupled formate-nitrate oxidoreductase activity. He was able to restore coupled activity by adding crude lipids, purified ubiquinone-8, or purified menaquinone. Ubiquinone-8 was more effective than menaquinone, and some phospholipids were also required for maximal reconstitution of activity. These studies were confirmed and extended some years later by Enoch and Lester, working with purified preparations of formate dehydrogenase-N and nitrate reductase (96).

Knook and Planta, studying *K. pneumoniae* S45, found that addition of ubiquinone reconstituted NADH-nitrate reductase activity in pentane-extracted membranes. However, menaquinone was ineffective in reconstitution, and no menaquinone was detected in *K. pneumoniae* S45 under any growth conditions (212).

Kröger and colleagues examined the relative functions of ubiquinone and menaquinone in *Proteus rettgeri* (227). Ubiquinone was found at highest levels in aerated cultures and supported oxidation of NADH, succinate, and formate. Menaquinone predominated in anaerobic cultures and was coupled to NADH and formate oxidation, with fumarate as terminal electron acceptor. The levels of the two quinone species were regulated by the type of electron acceptor present, rather than by the electron donor, indicating that the differential functions of the quinones reflect properties of the terminal reductases (227).

These observations were extended by Wallace and Young (417, 418) with *E. coli*. They constructed isogenic strains specifically defective in the production of ubiquinone (*ubiA*) or menaquinone and demethylmenaquinone (*menA*) or both (*ubiA menA* double mutant). In glucose-defined medium, the *menA* mutation had little effect on aerobic growth rates and yields, while growth of the *ubiA* strain was severely depressed. In the wild-type strain, anaerobic growth with nitrate greatly stimulated menaquinone production. These observations indicate that the primary role for ubiquinone is in aerobic respiration and that for menaquinone is in anaerobic respiration (418).

Wallace and Young also found that NADH and D-lactate oxidation, coupled to either oxygen or nitrate, could only be restored in membranes of the double mutant by addition of ubiquinone; glycerol-3-phosphate oxidation could be coupled to oxygen or nitrate reduction by either ubiquinone or menaquinone. Formate-nitrate reductase was also restored by either ubiquinone or menaquinone (418). This latter observation further indicates that NADH and formate can be used simultaneously and independently as electron donors for nitrate reduction: NADH through ubiquinone and formate through menaquinone.

Glycerol-3-phosphate oxidation by *E. coli* is catalyzed by either of two distinct glycerol-3-phosphate dehydrogenase enzymes (reviewed in references 164 and 240). One enzyme, encoded by *glpD* (343), is required for aerobic growth on glycerol, while the other, encoded by *glpACB* (94), is required for anaerobic growth on glycerol plus fumarate.

Nitrate-respiring cultures contain intermediate levels of both enzymes (103), and either suffices for anaerobic growth on glycerol plus nitrate (270). It is possible that the *glpD* enzyme uses ubiquinone, while the *glpA* enzyme uses menaquinone, but this point apparently has not been tested.

Why do most enteric bacteria synthesize two distinct quinones? Perhaps the relative redox potentials are such that certain respiratory chains work more efficiently with one or the other quinones (418). Alternatively, the two quinone species may be important for regulating the in vivo activity of various respiratory chains, particularly when two different electron acceptors of differing potential are present simultaneously. Finally, it is suggested that different dehydrogenases may recognize different structural features in quinones (417).

Cytochromes. Early biochemical studies of nitrate respiration in *E. coli* implicated a type *b* cytochrome in the electron transport pathway from formate (reviewed in references 337 and 389). Spectroscopic studies revealed a cytochrome *b* that was oxidizable by nitrate; this oxidation was sensitive to inhibition by HOQNO. The early attempts to solubilize and purify nitrate reductase and formate dehydrogenase also revealed at least one cytochrome *b* (162, 169, 241, 388). A cytochrome *b* specific for nitrate respiration was also detected in *K. pneumoniae* S45 (402).

DeMoss and colleagues isolated *E. coli* mutants defective in nitrate respiration and found that many also had lowered levels of cytochrome *b* after anaerobic growth with nitrate (330). The cytochrome *b* spectra were similar for both wild-type and mutant cells cultured aerobically. Thus, the aerobic and anaerobic cytochrome *b* species are genetically distinct (332).

Low-temperature (77 K) difference spectroscopy revealed only a single α-band absorption maximum at 555 nm (332). However, kinetic studies provided evidence for two distinct *b* cytochromes in the formate-to-nitrate respiratory chain (332). Cytochrome b_{555} was fully reduced by formate and was oxidized by nitrate with biphasic kinetics. Ascorbate reduced only a portion of the cytochrome b_{555}, and nitrate reduced this portion with monophasic kinetics. HOQNO inhibited the second biphasic step in either nitrate oxidation or formate reduction of cytochrome b_{555}. Thus, two distinct cytochromes participate in the formate-to-nitrate respiratory chain, with quinone serving as an intermediate electron carrier. The postulated electron transport chain was diagrammed in schematic fashion (332): formate dehydrogenase → cytochrome *b* → quinone → cytochrome *b* → nitrate reductase.

Subsequent studies have extended this proposal. Similar kinetics for oxidation and reduction of cytochromes *b* in nitrate-grown cells have been reported (333). Again, HOQNO inhibition studies provided evidence for two cytochromes b_{556}. One of these cytochromes, presumably the formate-dehydrogenase-specific cytochrome b_{556}, was inferred to be very electronegative.

HOQNO inhibition studies with *P. mirabilis* (408) have suggested the presence of a proton motive Q-cycle (reviewed in reference 157), so this linear representation of the electron transport chain may be an oversimplification.

Several cytochromes *b* have absorption maxima, measured by difference spectroscopy, of approximately 556 nm. Redox titration can help to resolve these cytochromes by providing an estimate of their individual midpoint potentials. Several studies have demonstrated that nitrate-grown cultures of both *E. coli* and *P. mirabilis* form increased amounts of cytochromes b_{556}, with midpoint potentials of approximately 10 to 25 and 120 to 150 mV (146, 147, 406, 407).

Redox titrations of partially purified nitrate reductase were interpreted by Hackett and Bragg to indicate the presence of two cytochromes b_{556}, with midpoint potentials of approximately 10 and 125 mV (146). Further, membranes of *narI* mutants, thought to specifically lack the nitrate reductase-associated cytochrome *b*, showed no evidence for either the 10- or the 125-mV cytochromes (148). These studies indicate that two cytochromes b_{556} may be associated with nitrate reductase.

Hackett and Bragg also detected a cytochrome b_{556} with a midpoint potential of approximately −110 mV (147, 148). This cytochrome is absent in *fdhA* mutants (lacking formate dehydrogenase) and is overproduced in *nar* mutants. This low-potential cytochrome is probably the formate dehydrogenase-associated cytochrome b_{556} identified by kinetic studies (see above).

Van Wielink and colleagues (406, 407) used both potentiometry and spectrum deconvolution to examine the cytochrome content in *E. coli* and *P. mirabilis* and observed that both organisms had similar cytochrome contents under similar growth conditions. Nitrate-grown cells contained two cytochromes b_{556}, with midpoint potentials of approximately 25 and 150 mV. These values compare well with those (10 and 125 mV) reported by Hackett and Bragg. Cultures grown in the absence of nitrate formed a cytochrome b_{556} with a midpoint potential of approximately −115 mV, but this species was not detected in nitrate-grown cells. Thus, in these studies, the possible relationship between this low-potential cytochrome and the formate dehydrogenase-associated cytochrome b_{556} is unclear. However, the consistent detection of both high- and intermediate-potential cytochromes b_{556} in nitrate-grown cells is striking.

Cytochrome-containing formate dehydrogenase and nitrate reductase have each been purified to homogeneity (e.g., references 52, 96, 97, 250, and 275). The cytochrome *b* subunits for each enzyme are distinct. The spectral properties of these cytochromes are consistent with those detected in crude cell extracts, although low-temperature difference spectroscopy and redox titration have not been performed with the homogeneous enzymes, so direct comparison is not possible.

Enoch and Lester reconstituted purified formate dehydrogenase and nitrate reductase into liposomes, forming an intact respiratory chain (96). The kinetic properties of this reconstituted chain are similar to those observed in whole cells by Ruiz-Herrera and DeMoss (330). Furthermore, at least the nitrate reductase-specific cytochrome is required for respiratory chain activity in liposomes. This study elegantly demonstrated the relatively simple constitution of the formate-nitrate respiratory chain. However, it leaves open the question of whether two cytochromes b_{556}, of intermediate and high potential, are associated with nitrate reductase. It is conceivable that the liposome-reconstituted system does not require both cytochromes. Perhaps the polypeptides for the two cytochromes comigrate on sodium dodecyl sulfate gels, or perhaps both of the distinct redox components are contributed by a single polypeptide species. DNA sequence analysis of the nitrate reductase structural gene (*nar*) operon will help to resolve this question.

NITRATE REDUCTASE STRUCTURE AND MECHANISM

Pichinoty and Piéchaud (311) distinguished two types of nitrate reductases in bacteria, based on response to inhibi-

tors and substrates. Enzyme A is respiratory nitrate reductase; it uses chlorate as a substrate and is inhibited by azide. Enzyme B is inhibited by chlorate. It is thought that enzyme B corresponds to assimilatory nitrate reductase, but it is also possible that, at least in enteric bacteria, it is a respiratory molybdo-enzyme with a gratuitous nitrate reductase activity (e.g., reference 27); "enzyme B" may actually consist of multiple molybdo-enzymes. A third enzyme, chlorate reductase C, is repressed by nitrate; it has no detectable nitrate reductase activity and is insensitive to azide. All three enzymes undoubtedly contain Mo cofactor.

Purification and Properties

Egami, Sato, and colleagues made early attempts to purify nitrate reductase from *E. coli* (see "Introduction"). Although they characterized it as a large Fe-Mo enzyme with associated cytochrome *b*, the technology for purifying and characterizing membrane-bound enzymes did not allow detailed studies. By 1968, Showe and DeMoss (351) were able to demonstrate that *E. coli* nitrate reductase is localized exclusively in the particulate fraction, "bound to a large substructure that is subject to fragmentation during cell disruption procedures."

In the mid-1970s, nitrate reductase was solubilized and purified by a number of groups. The enzyme can be solubilized by incubating envelope fractions at alkaline pH and 60°C (5, 246, 261, 388) or by dissolving the membrane with Triton X-100 (61, 250) or deoxycholate (97, 275, 403). In each case, the purified enzyme contains at least two subunits: α (or A), with a molecular mass of approximately 150 kDa, and β (or B), with a molecular mass of approximately 60 kDa. These two subunits are present in a 1:1 ratio.

Various protease inhibitors reduce the amount of enzyme released by alkaline heat treatment (249). MacGregor and colleagues have detected a protease activity in the outer membrane that may be responsible for this solubilization. Perhaps the effect of heat is to convert the cytoplasmic membrane into a form that is accessible to the protease (254). The β-subunit alterations that accompany this solubilization (254) are described below.

Enoch and Lester (96, 97) purified nitrate reductase after deoxycholate solubilization and found that the enzyme is associated with a third subunit, γ (or C), with a molecular mass of approximately 20 kDa. MacGregor (250) raised antibody to enzyme that had been solubilized by alkaline heat treatment and also found subunit γ associated with enzyme that was solubilized with Triton X-100. This subunit is the nitrate reductase-specific cytochrome b_{556} detected by physiological and spectral studies (52; see preceding section). The α/β/γ ratio in most enzyme preparations is 1:1:2 (52, 250).

Nitrate, chlorate, and bromate are substrates for purified nitrate reductase (100, 275), and a variety of viologen dyes, including methyl viologen and benzyl viologen, act as electron donors (e.g., reference 196). The physiological electron donor, quinol, requires cytochrome-containing enzyme for activity (275; see below). Azide and cyanide are both strong inhibitors; indeed, the affinity of azide is reported to be 1,000-fold greater than that for nitrate (100, 309).

Nitrate reductase has been purified from both *K. pneumoniae* S45 (403) and *P. mirabilis* DG (292). Both enzymes had three subunits with approximate molecular masses of 110, 60, and 55 kDa. In neither case has a specifically associated cytochrome been found (3, 213, 292). More recently, the *K. pneumoniae* enzyme, solubilized by Triton X-100 and analyzed by immune precipitation, was found to contain two subunits of approximately 150 and 60 kDa (3, 421). Perhaps the large subunit was cleaved into species of 110 and 55 kDa during the original purification (382). Subunits α and β of *E. coli* K-12 and *K. pneumoniae* S45 are immunologically cross-reactive (3).

Subunit α from *E. coli*. Subunit α contains 37% hydrophobic residues (53) and has been purified by column chromatography following dissociation of purified enzyme with sodium dodecyl sulfate and guanidinium hydrochloride. The subunit thus purified has lost most of its Mo, is enzymatically inactive, and cannot be immunoprecipitated. All three properties are reconstituted after dialysis against a crude *E. coli* extract, which presumably replaces the Mo (53). Therefore, subunit α contains the active site for nitrate reduction (53).

Subunit β from *E. coli*. Subunit β contains 41% hydrophobic residues (51). All of the Fe and Mo found in native enzyme can be accounted for by subunits α and γ (53), so subunit β is presumed to be devoid of metal. The function of this subunit is unknown, but circumstantial evidence suggests that it is involved in mediating subunit interactions and membrane association (see below).

Subunit γ from *E. coli*. Subunit γ contains 45% hydrophobic residues (52). This subunit is tightly associated with subunits α and β and was purified from the native enzyme only after treatment with sodium dodecyl sulfate followed by electrophoresis. The resulting protein was partially denatured, but was reconstituted with hemin and slow dialysis to remove the sodium dodecyl sulfate. Both renatured subunit γ and purified native nitrate reductase exhibit the spectral properties expected for cytochrome b_{556} (52). Subunit γ aggregates readily, especially after heat treatment, which may explain its variable association with subunits α and β in different purification procedures (52; see below). Trypsin cleaves subunit γ into two peptides of roughly equal size; it is refractory to digestion by other proteases.

Mo. Mo is present in nitrate reductase as Mo cofactor; i.e., it is bound to a small organic moiety, molybdopterin (see next section). Many preparations of nitrate reductase contain substantially less than stoichiometric amounts of Mo (e.g., references 53, 261, and 405), although one study found 0.80 ± 0.05 Mo per αβ monomer (5). It is presumed that low Mo content is due to dissociation during purification and that the native enzyme contains one complete Mo cofactor per αβ monomer.

Electron paramagnetic resonance has been used to examine Mo in nitrate reductase (5, 37, 39, 87, 106, 413, 414). Mo(V) electron paramagnetic resonance signals are consistently detected, and studies with inhibitors and halide complexes suggest that the mechanism of nitrate reduction involves Mo(IV)-Mo(VI) cycling [Mo(IV) and Mo(VI) are electron paramagnetic resonance silent (5, 106)]. The redox potential of the Mo(IV)-Mo(V) couple is 180 mV, while that of Mo(V)-Mo(VI) is 220 mV (413). It is not known whether nitrate reduction involves a one- or two-electron transfer mechanism (414).

Fe. Nitrate reductase has been found to contain as many as 16 molecules each of nonheme Fe and acid-labile sulfide per αβ monomer (5), although others have found only 12 each (53, 246). Electron paramagnetic resonance spectroscopy, magnetic circular dichroism, and Fe-S core extrusion studies indicate that each αβ monomer contains three to four $[4Fe\text{-}4S]^{2+,1+}$ clusters (191). It is suggested that active enzyme also contains a 3Fe cluster, similar to that found in bacterial ferredoxins; it remains possible that this center

results from degradation of a 4Fe-4S cluster during enzyme isolation (191). Fe is also present in subunit γ, of course, in heme.

Subunit Interactions and Assembly

Subunit stoichiometry. The molecular weight of an αβ complex is approximately 200,000. Some cytochrome-free preparations of nitate reductase exhibit molecular weights close to 800,000 (61, 246, 261, 403), indicating that they are αβ tetramers. Other preparations have molecular weights of 200,000 (αβ monomer [61, 246]) or 400,000 (αβ dimer [5, 52]). Deoxycholate converts these oligomeric forms into αβ monomers (5, 403). Lund and DeMoss (246) observed that nitrate reductase (purified after alkaline heat treatment) is a reversibly associating-dissociating system; at low protein concentrations, the enzyme exists as αβ monomers, but it is found primarily as αβ tetramers at high protein concentrations.

MacGregor and colleagues (261) had proposed that native nitrate reductase is an αβ tetramer. The subsequent discoveries of subunit γ (96, 250) and association-dissociation (246) have modified that view. Current evidence suggests that the structure of native nitrate reductase is $\alpha_2\beta_2\gamma_4$ (52, 61).

Role of subunit β. Native subunit β has a molecular mass of approximately 60 kDa. Two other forms of β with approximate molecular masses of 58 and 43 kDa have been observed. In various publications, each of these forms has been named β′. To avoid confusion, I designate these forms as β_{58} and β_{43}, respectively.

DeMoss showed that *E. coli* nitrate reductase subunit β is specifically cleaved by trypsin to form β_{43} (84). Other degraded forms of β found after alkaline heat solubilization (84, 249) are also converted to apparently homogeneous β_{43} by trypsin (84). β_{43}-containing nitrate reductase retains enzyme activity, but no longer behaves as an associating-dissociating system. This implicates at least the 17-kDa portion of β, released by trypsin, in the aggregation of αβ monomers to oligomeric forms. Presumably this β-mediated interaction is important in forming the native ($\alpha_2\beta_2\gamma_4$) holoenzyme (84). *K. pneumoniae* S45 subunit β is also cleaved by trypsin to form β_{43}; however, both subunits of this enzyme are quite sensitive to further degradation by trypsin (3).

β_{58} is often detected in purified preparations of *E. coli* nitrate reductase, and the relative amounts of β and β_{58} are often roughly equivalent (3, 61, 86, 249, 254). β_{58} has not been detected in the enzyme from *K. pneumoniae* S45 (3).

MacGregor and colleagues (254) observed that nitrate reductase, purified from unheated envelope fractions by immunoprecipitation, contains subunits α, β, and γ. Enzyme precipitated from heated envelope contains only subunits α and β_{58}; γ is not detectable. This suggested that removal of a hydrophobic portion of β by proteolysis allows both subunits α and β to be solubilized, presumably by dissociating α and β from γ (254)

DeMoss and colleagues (86) observed conversion of β to β_{58} during purification of deoxycholate-solubilized enzyme, indicating that the heat-activated protease was not responsible for this conversion. The protease inhibitor tosyllysine chloromethyl ketone inhibits further degradation of β_{58}, but does not prevent conversion of β to β_{58}. Partially purified β and β_{58} were both converted to β_{43} by limited trypsin digestion, indicating that the 2-kDa segment lost in β-to-β_{58} conversion is contained within the 17-kDa segment removed by trypsin. The amino terminus of both β and β_{58} is Met, suggesting that the carboxy terminus of β is removed to form both β_{58} and β_{43} (86).

Membrane incorporation. Studies by MacGregor and colleagues have shown that nitrate reductase subunits α and β are synthesized as soluble precursors to membrane-bound nitrate reductase (145, 252, 368). A *hemA* mutant, unable to synthesize heme in the absence of δ-aminolevulinic acid, accumulates subunits α and β in the cytoplasm (252); reduced amounts of α and β are found in the membrane, and the membrane-bound subunits are unstable. The soluble subunits are in a form similar to that released by alkaline heat treatment. The *hemA* mutant grown with δ-aminolevulinic acid accumulates normal membrane-bound nitrate reductase. Double-label experiments showed that, upon addition of δ-aminolevulinic acid to a growing culture, the soluble α and β subunits are incorporated into the cytoplasmic membrane. This indicates that functional subunit γ is required for assembly of stable, native, membrane-bound nitrate reductase (252).

Subsequently, a wild-type strain was pulsed with ^{35}S, and nitrate reductase reductase subunits were immunoprecipitated from both soluble and membrane fractions during a cold chase (145). Both α and β disappeared from the soluble fraction at the same rates that they appeared in the membrane fraction. Furthermore, a mutant specifically lacking subunit γ was shown to accumulate most of the immunoprecipitable nitrate reductase α and β subunits in the cytoplasmic fraction (368). In this case, the soluble β is exclusively in the β_{58} form.

Taken together, these results support the hypothesis that subunit γ is essential for assembly of nitrate reductase into the cytoplasmic membrane. Perhaps subunit β, and particularly the 2-kDa (presumed) carboxy-terminal portion, mediates association of $\alpha_2\beta_2$ with γ_4 to form the holoenzyme. Still, much remains to be learned about the mechanism of membrane incorporation, not only of nitrate reductase but of membrane-bound enzymes in general.

Giordano and colleagues (111), studying in vitro reconstitution of nitrate reductase activity in extracts of *chlA* and *chlB* mutants (see next section), observed that "soluble" β_{58} appeared to be converted to genuine β during reconstitution. Independent studies by MacGregor and McElhaney (256) used pulse-chase experiments to follow the fates of β and β_{58} in vivo. This work indicated that β_{58} was a transient species in the membranes of wild-type cells; immediately after the pulse, a mixture of β and β_{58} was precipitated from the wild-type membrane fraction, whereas only β was observed after a chase. In both a *hemA* and a *chlE* strain, β_{58} was still present in the membrane after the chase. Finally, β_{58} was found in the soluble fraction of all strains tested. The interpretation of these results was that β_{58} is the precursor to membrane-bound β and that, after proper membrane assembly, a posttranslational event converts β_{58} to β. The apparent increase in relative mobility could reflect the addition of a molecule, perhaps a lipid (256).

Subsequently, Chaudhry and MacGregor (51, 52) purified nitrate reductase from Triton X-100-solubilized membranes. They found that further chromatography on Bio-Gel A 1.5 m (Bio-Rad Laboratories) resolved two peaks of nitrate reductase activity. Peak I contains subunits α, β, and γ, whereas peak II contains subunits α and β_{58} only (51). Holoenzyme that is not purified on the Bio-Gel column is gradually converted to the peak II form. This observation provides an explanation not only for the presence of β_{58} in many nitrate reductase preparations, but also for the variable association of subunit γ with purified enzyme.

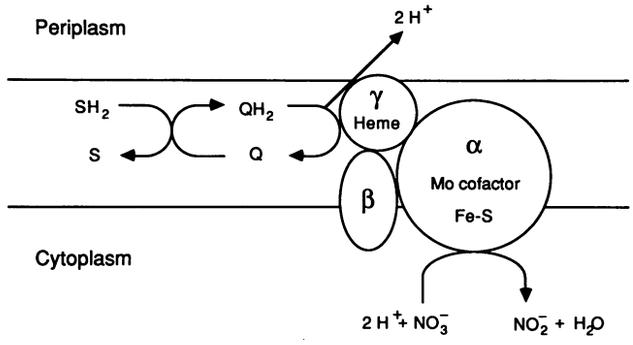

FIG. 3. Schematic representation of nitrate reductase. Reduced substrates (SH_2; e.g., formate, NADH, glycerol-3-phosphate, lactate, etc.) are oxidized by specific dehydrogenases, coupled to reduction of quinol (Q; ubiquinol or menaquinol) to quinone (QH_2). Quinone reduction is in turn coupled to nitrate reduction. The figure is drawn to show the proposed membrane orientation of subunits and mechanism of proton translocation (see text for details); it does not show the likely subunit stoichiometry of $\alpha_2/\beta_2/\gamma_4$.

The peptide maps of β_{58} found in the peak II fraction are identical to those of the β_{58} purified from a *narI* mutant (51). These peptide maps were similar to those for β, which contained a few additional peptides. Thus, it was concluded that the peak II fraction contains a "modifying activity" that converts β_{58} to β. Because it had been concluded previously that β_{58} is a precursor to β (256), this modifying activity was thought to reversibly mediate a β–β_{58} interconversion, possibly through the addition and removal of a small molecule (51). However, these observations do not exclude the possibility that the modifying activity is a protease that converts β to β_{58}. Further work is required to demonstrate that β_{58} is a precursor of β.

At present, then, the simplest hypothesis is that β is the normal component of nitrate reductase, soluble or membrane bound. However, nitrate reductase that is not in the proper association with subunit γ (i.e., soluble $\alpha_2\beta_2$ enzyme, enzyme in *hemA* or *narI* mutants, or enzyme that has been dissociated from subunit γ during purification) is not in the "native" conformation; subunit β in such enzyme is subject to proteolysis to produce β_{58}. Apo-nitrate reductase, lacking Mo cofactor (as in a *chl* mutant) would also be in a non-native conformation. Whether the modifying activity described above is this protease remains to be determined. Regardless, the copurification through several steps of an activity that apparently makes a specific alteration in subunit β is curious indeed. The in vivo role of this activity deserves further attention.

Chemiosmosis

Nitrate reductase is hypothesized to generate PMF through the release of two protons in the periplasm (quinol $\rightarrow$ quinone + $2H^+ + 2e^-$) coupled to the consumption of two protons in the cytoplasm ($NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$). Presumably the electrons pass from a periplasmic site on the enzyme to a distinct cytoplasmic site. Crucial to such a mechanism is the demonstration that, first, the enzyme actually spans the cytoplasmic membrane and, second, the sites for quinol oxidation and nitrate reduction are separate and on the appropriate sides of the membrane (Fig. 3). Nitrate reductase appears to meet both of these conditions.

Membrane topography of nitrate reductase. The transmembrane orientation of nitrate reductase has been demonstrated by a variety of direct and indirect methods, and all are in agreement. Direct methods probe the accessibility of different nitrate reductase subunits to chemical (38, 124, 206, 255, 421) or immunochemical (128, 401, 421) labeling in spheroplasts (native orientation) and everted vesicles. In *E. coli* subunit α can only be labeled in everted vesicles, implying that it is exposed only to the cytoplasmic face. Subunit γ has not been detected in all studies, but two groups, using chemical labeling methods, found that this subunit was only labeled in spheroplasts (38, 255). Likewise, subunit β is difficult to detect, but one study found that it was labeled preferentially from the cytoplasmic face (124). In addition, trypsin was able to cleave subunit β to β_{43} only in everted vesicles (A. Graham, and D. H. Boxer, Biochem. Soc. Trans. **8**:331, 1980).

In *K. pneumoniae* S45, subunit α was also detected only from the cytoplasmic face, while subunit β was not located. Immunofluorescent labeling suggested that at least some nitrate reductase antigens are accessible to the periplasmic face (421). It has recently been reported that this enzyme is covalently bound to peptidoglycan (4), whereas no such observation has been made with the *E. coli* enzyme. Such an association with peptidoglycan, if confirmed, would further support the transmembrane orientation of *K. pneumoniae* nitrate reductase, but its significance with respect to mechanisms of proton translocation remains obscure.

Finally, indirect methods provide further evidence that the catalytic site (and thus, the α subunit; see above) is located on the cytoplasmic face. Nonphysiological electron donors react directly with the active site, bypassing the requirement for cytochrome. Two membrane-impermeable artificial donors, reduced flavin mononucleotide (150) and reduced methyl viologen (196), support nitrate reductase activity only in disrupted cells. In addition, osmotic swelling experiments have been interpreted to show that the nitrate-binding site is on the cytoplasmic side of the membrane (226).

Mechanism of proton translocation. That nitrate respiration generates PMF is established (see section, "Physiology"). Jones and co-workers have elegantly approached the mechanism of proton extrusion in *E. coli* (196, 197). First, they examined the membrane permeability properties of various viologen radicals. Benzyl viologen radical was determined to be membrane permeable, while radicals of methyl viologen (paraquat) and diquat are not. Second, using a *hemA* strain, they studied the requirement of cytochrome for viologen action; diquat requires cytochrome to donate electrons to nitrate reductase in whole cells. Finally, they compared the rates of nitrate reduction with these various dyes in whole and broken cells. All were active in broken cells, but only benzyl viologen and diquat coupled to nitrate reductase in whole cells. Since benzyl viologen is membrane permeable, but methyl viologen is not, the active site must be on the cytoplasmic face of the membrane. Likewise, diquat couples to nitrate reductase only in $hemA^+$ cells, implying that the cytochrome is exposed to the periplasm (196). These results complement the direct labeling studies summarized above and are consistent with the hypothetical mechanism for proton translocation.

Additional studies measured proton consumption with various electron donors (197). With the membrane-permeable benzyl viologen radical as electron donor, proton consumption from the bulk phase is not detected with a pH electrode; this result indicates that the protons consumed at the active site are derived from the cytoplasm. Diquat

radical (an electron donor), reduced phenazine methosulfate (a hydride donor), and ubiquinol-1 (a hydrogen donor) are coupled to nitrate reduction with approximate $H^+/2e^-$ stoichiometries of 0, 1, and 2, respectively. These and other studies support the idea that the observed proton translocation by nitrate reductase actually represents electron translocation, coupled to proton release in the periplasm and proton consumption in the cytoplasm (197).

Independent support for this hypothesis comes from the work of Morpeth and Boxer (275). Kinetic analyses suggest that reduced viologen dyes react with the enzyme by a mechanism different from that of ubiquinol-1; viologens interact directly with the active site for nitrate reduction, whereas ubiquinol-1 acts by a two-site mechanism. Trypsin treatment, producing β_{43}, removes the cytochrome from nitrate reductase. The resulting enzyme has unchanged properties with respect to viologen, but is completely inactive with ubiquinol-1 (275).

Taken together, all of this work supports the idea that nitrate reductase contains two distinct and independent sites: a quinol-oxidizing site on subunit γ, facing the periplasm; and a nitrate-reducing site on subunit α, facing the cytoplasm (Fig. 3). However, many questions remain. For example, there is no information concerning the possible involvement of subunit β in electron transfer between subunits γ and α; perhaps it plays strictly a structural role. Likewise, nitrate must have rapid accessibility to the cytoplasm. One simple hypothesis is that uptake is mediated by a nitrate/nitrite antiporter (197, 226). Nevertheless, these studies with nitrate reductase have provided good evidence to support this mechanism of chemiosmosis.

MOLYBDENUM COFACTOR

Structure and Subcellular Distribution

Mo cofactor consists of an organic moiety, molybdopterin, and a metal, Mo (188). Purification and characterization of Mo cofactor are difficult due to its extreme lability; its biosynthetic pathway remains a mystery. In recent years, Rajagopalan and colleagues have purified and characterized stable, inactive forms of the cofactor. They used the structures of these derivatives to deduce the probable structure for active Mo cofactor (187, 188; reviewed in reference 285). Mo cofactor occurs in an apparently identical form in all molybdo-enzymes with the sole exception of nitrogenase (reviewed in reference 346).

In *E. coli*, soluble Mo cofactor is loosely bound to a carrier protein of approximately 40 kDa and thereby is protected from inactivation by heat and oxygen (12). Mo cofactor is synthesized more or less constitutively in both *E. coli* (11, 271) and *P. mirabilis* (59), although its levels change slightly in response to growth with added molybdate. Mo cofactor is found in both soluble and particulate fractions in both *E. coli* (11, 271) and *P. mirabilis* (59). The soluble Mo cofactor can be assayed directly, but it must be released from the membrane-bound fraction by either heat or detergent treatment (11, 59, 271). Increased Mo cofactor is found in the particulate fraction after anaerobic growth with nitrate, which induces two major molybdo-enzymes (formate dehydrogenase-N and nitrate reductase), but the pool of soluble Mo cofactor remains roughly constant (11, 59, 271).

Effects of tungsten. W (tungsten) interferes with the formation of nitrate reductase and formate dehydrogenase activities (95) and associates with molybdopterin to form an inactive species, W-molybdopterin (12). High concentrations (1 to 10 mM) of tungstate are required for maximum interference, which is reversed by adding 0.1 mM molybdate during growth (95).

Sperl and DeMoss (364) found that *E. coli*, cultured in the presence of 1 mM tungstate, accumulates an inactive form of nitrate reductase. Full enzyme activity is restored in washed, chloramphenicol-treated cells by the addition of as little as 10 μM molybdate. ^{99}Mo associates with nitrate reductase during this reactivation. ^{185}W is not firmly associated with the inactive nitrate reductase: unlike ^{99}Mo, it is released during column chromatography. It is not clear whether W stabilizes an inactive enzyme complex or simply interferes with Mo incorporation (at low molybdate concentrations).

E. coli chlD mutants (see below) also accumulate inactive nitrate reductase when cultured with tungstate (364). In these mutants, 1 mM molybdate is required to fully restore enzyme activity in whole cells (364).

Scott and DeMoss (344) observed that inactive forms of both formate dehydrogenase-N and nitrate reductase accumulate in cells grown with 10 mM tungstate. These inactive species exhibit altered electrophoretic mobilities on nondenaturing polyacrylamide gels. Addition of 1 mM molybdate, in the presence of chloramphenicol, quickly restores active formate dehydrogenase-N and nitrate reductase activities, and after a measurable lag (roughly 30 min) the respiratory chain (formate-nitrate oxidoreductase) becomes functional. The reactivated enzymes exhibit electrophoretic mobilities similar to those of the normal, active enzymes. Glucose is required for the molybdate reactivation, so energy (but not protein synthesis) is necessary.

Reactivation of formate dehydrogenase-N and nitrate reductase activities cannot be demonstrated in cell-free extracts from tungstate-grown cultures (344, 345). ^{99}Mo associates with the inactive enzymes in vitro and restores their electrophoretic mobilities to those of the active enzymes (345). Thus, reactivation does not simply reflect incorporation of Mo into the apoenzymes. The additional factor or step missing in vitro is not known, but it may be related to the requirement for glucose during in vivo reactivation.

In tungstate-grown cells of *P. mirabilis*, molybdate reactivates nitrate reductase, chlorate reductase C, and tetrathionate reductase activities in chloramphenicol-treated cells (291). Reactivation of the latter two enzymes occurred to a lesser extent than that observed for nitrate reductase.

Assays. Mo cofactor is most widely assayed by restoration of nitrate reductase activity to extracts of the *nit-1* mutant of *N. crassa* (282). This mutant lacks molybdopterin (221) and accumulates apo-assimilatory nitrate reductase. Only a high concentration (10 mM) of molybdate restores full activity, so the system can be used as a specific assay for molybdopterin rather than Mo cofactor itself (158, 415). This specificity suggests that Mo cofactor activity, as isolated, largely consists of molybdopterin ("empty cofactor" [11, 271]). The relative amounts of Mo cofactor and molybdopterin help to distinguish certain classes of mutants (271; see below). If special care is taken, the *nit-1* assay provides a quantitative measure of molybdopterin (158, 190).

The *nit-1* mutant also provides a direct (nonenzymatic) assay for molybdopterin (empty cofactor) and W-molybdopterin (12). Monomeric apo-assimilatory nitrate reductase has a sedimentation coefficient of 4.5S, whereas Mo cofactor-containing (dimeric) assimilatory nitrate reductase sediments with a value of 7.9S. Apo-assimilatory nitrate reductase monomers dimerize in the presence of molybdopterin or W-molybdopterin to form the 7.9S species; the 4.5S and 7.9S

TABLE 3. Proposed functions of *chl* and *nar* genes[a]

Locus	Proposed function
chlA	Gene required for molybdenum cofactor synthesis; closely linked to *chlM*
chlB	Locus required for insertion of molybdenum cofactor into apoenzymes; may contain as many as three complementation groups
narC	Structural gene for nitrate reductase subunit α; also designated *narG*
chlD	Locus required for molybdate uptake or processing; possible homology to periplasmic binding protein-dependent transport systems; may contain several complementation groups
chlE	Gene required for molybdenum cofactor synthesis; closely linked to *chlN*
F	Not officially assigned; the original mutant has been lost
chlG	Locus required for molybdenum cofactor activity; no evidence for more than one complementation group
narH	Structural gene for nitrate reductase subunit β
narI	Structural gene for nitrate reductase subunit γ
narJ	Gene of unknown function; located between *narH* and *narI*
narK	Gene of unknown function, linked to *narCHJI*
narL	Positive regulatory gene, linked to *narCHJI*
chlM	Gene required for molybdenum cofactor synthesis; closely linked to *chlA*
chlN	Gene required for molybdenum cofactor synthesis; closely linked to *chlE*
narX	Posible regulatory gene, linked to *narCHJI*

[a] See text for references.

species are resolved on sucrose gradients. Subsequent incubation with molybdate of either form of the 7.9S inactive species (molybdopterin or W-molybdopterin containing) restores nitrate reductase activity (12).

Reverse-phase high-pressure liquid chromatography can be used to measure a fluorescent derivative of Mo cofactor, dephospho-form A (190). Mo cofactor is converted to dephospho-form A by treatment with acidic I_2 and KI at 100°C. This assay is useful for analyzing Mo cofactor biosynthesis (189, 190; see below).

Chlorate-Resistant Mutants

Selection for chlorate resistance has been widely used to obtain mutants (termed *chl*) that are deficient in nitrate reductase activity. Chlorate is a substrate for nitrate reductase, and either chlorite (312) or perhaps chloride (144) is lethal. The method is quick and convenient, and a high spontaneous frequency of *chl* mutations assures success. For many years, most genetic studies of bacterial nitrate respiration used *chl* mutants.

Chlorate-resistant (*cnx*) mutants of the ascomycete *Aspergillus nidulans* cannot use nitrate or hypoxanthine as nitrogen sources (reviewed in reference 74), but they can use nitrite and urea (metabolic products of nitrate and hypoxanthine, respectively), so they are not defective in overall nitrogen assimilation. The recognition that nitrate reductase and xanthine dehydrogenase (but not nitrite reductase or urease) are both molybdo-enzymes showed that the *cnx* mutant phenotype was most likely due to a defect in synthesis of Mo cofactor (reviewed in reference 74).

The analogous hypothesis was not immediately applied to enteric *chl* mutants, for several reasons. First, the fact that *chl* mutants lack formate dehydrogenase, also a respiratory enzyme, implicated these mutants as defective in respiration or membrane structure. This "respiratory particle hypothesis" (18, 19) influenced the thinking of others (e.g., references 257, 342, and 372; but see also references 330 and 332). Second, *E. coli* does not use nitrate or hypoxanthine as nitrogen sources, so the loss of nonrespiratory molybdo-enzymes (as in *A. nidulans*) did not point the way to the analogous hypothesis. In addition, the reported phenotypes of *chl* mutants were often complex (e.g., references 127 and 251), further obviating simple hypotheses of *chl* gene action. Moreover, "detailed biochemical investigations have usually only been performed with a single mutant strain thought typical of a particular *chl* locus" (149). Different laboratories studied spontaneous or mutagen-induced *chl* mutants that were isolated in different genetic backgrounds, complicating the comparison of results. Finally, it is possible that some of these strains also carried *fnr* mutations, contributing to the plethora of reported phenotypes (368). More recently, a collection of *chl* insertion mutations has been isolated and mapped, providing an isogenic set of null mutants (368). These mutants have been used in a number of laboratories for studies of *chl* gene function (28, 30, 83, 148, 163, 174, 185, 271, 324, 336).

Accumulated evidence implicates the *chl* gene products in Mo cofactor synthesis. DeMoss and colleagues observed that high concentrations of Mo phenotypically suppress *chlD* mutants (119, 364). MacGregor and Schnaitman found that certain *E. coli chl* mutants could not provide a source of Mo cofactor for reconstitution of *nit-1* nitrate reductase activity (258, 259). Puig and colleagues, using ^{99}Mo labeling, showed that *chl* mutants do not incorporate Mo into nitrate reductase (91, 129). By the early 1970s, it seemed clear that *chlA*, *chlB*, *and chlD* were involved in Mo cofactor synthesis (259; but see also references 17 and 326). The general role of *chl* gene products in Mo cofactor synthesis is now clear: *chlE and*

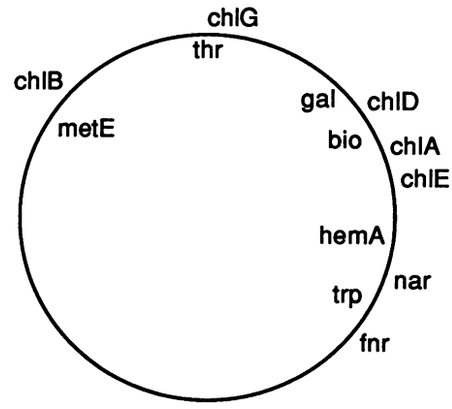

FIG. 4. Approximate map positions of *chl*, *nar*, and *fnr* genes. The *E. coli* genetic map is drawn in the conventional orientation, with coordinate 0/100 at the top.

chlG are not structural genes for nitate reductase subunits (368); extracts of *chlE* mutants behave like those of *chlA* mutants in reconstitution experiments (368); a nonrespiratory enzyme, biotin-D-sulfoxide reductase, is a molybdoenzyme whose activity is absent from *chl* mutants (83); and extracts of *chlA* and *chlE* insertion mutants do not reconstitute *nit-1* nitrate reductase under any conditions (271). Recently, Johnson and Rajagopalan (189, 190) have rigorously demonstrated that genes at *chlA* and *chlE* are involved in Mo cofactor synthesis (see below).

Most mutations in the nitrate reductase structural genes do not confer Chl^r (27, 120, 368; see below). Indeed, chlorate reductase C in *P. mirabilis* is also a molybdo-enzyme (291). Thus, a single mutation confering Chl^r is most likely to affect a common component of all "chlorate reductase" activities. That common component is Mo cofactor.

Nomenclature. The nomenclature of *chl* mutants has been somewhat confusing. Some early chlorate-resistant mutations were first named (*nar* nitrate reductase), before being redesignated as *chl* mutations (410, 411). Later, it was found that the *chlC* region contains several genes involved in nitrate reductase synthesis and regulation (33, 93, 367, 368), so individual genes in this region have been assigned *nar* designations (93, 368). Because of this history, *chl* and *nar* designations are not assigned the same identifying letters (e.g., *chlA*, *chlB*, *narC*, *narH*, etc.). Current gene designations are listed in Table 3, and approximate genetic map positions are shown in Fig. 4. It is desirable that designations for newly discovered *chl* and *nar* genes be cleared with the *E. coli* Genetic Stock Center to help avoid future confusion.

Most *chl* loci contain at least two genes (see below), but rigorous genetic analysis is still lacking in most cases. Detailed molecular genetic studies are sorely needed to identify the number and function of *chl* genes in each locus.

chlAM. Mutations in the *chlA* locus (in the 17-min region of the *E. coli* map) have been mapped in *E. coli* (6, 411), *S. typhimurium* (374), *K. pneumoniae* (381), and *C. freundii* (79). In each case, the order determined by deletion mapping is *bio-uvrB-chlA*.

Genetic complementation studies, using abortive transduction (410) and mini-Mu transduction (190), have revealed that the *E. coli chlA* locus contains at least two complementation groups, while subcloning experiments (324) suggest the presence of three complementation groups. Johnson and Rajagopalan have designated the gene defined by the *chlA1* allele as *chlA* and the gene defined by their complementing mutation as *chlM* (190). Biochemical studies of *chlAM* function are summarized below.

chlB. Mutations in the *chlB* locus (in the 86-min region of the *E. coli* map) have been mapped in *E. coli* (48), *S. typhimurium* (50), and *C. freundii* (79). In each case, *chlB* is linked to *metE*. Abortive transduction experiments (410) suggest that the *E. coli chlB* locus contains at least three complementation groups. Biochemical studies of *chlB* function are summarized below.

It has been reported that *chlB* mutations cause overproduction of nitrate reductase polypeptides (118), although others have not seen this effect (127). Likewise, *chlB* affects the nitrate-specific regulation of *chlD* (272) and *frd* (174) gene expression. These effects, which are not easily interpreted, indicate that the genetics and biochemistry of the *chlB* locus deserves further attention.

chlD. Mutations in the *chlD* locus (in the 17-min region of the *E. coli* map) have been mapped in *E. coli* (6, 411), *S. typhimurium* (374), *K. pneumoniae* (381), and *C. freundii* (79). The overall order of *gal-chlD-bio-uvrB*, determined by deletion mapping, is similar in all of these species, although there are differences; in *E. coli*, *attλ* is between *chlD* and *bio* (6), and in *K. pneumoniae* and *C. freundii*, *hut* is between *chlD* and *bio* (79, 381). Deletion mapping in *S. typhimurium* indicated that the gene order was *gal-bio-chlD-uvrB* (374), but other deletions (8), although not fully characterized, are consistent with the conserved order found in the other three species.

E. coli chlD mutants retain approximately 5% of the wild-type nitrate reductase activity; this phenotypic leakiness has hampered complementation analyses. DeMoss and colleagues (119, 364) discovered that the ChlD$^-$ phenotype is fully suppressed by growth in 1 mM molybdate, which explains the leaky phenotype at normally used (1 μM) molybdate concentrations. They found that a *chlD* mutant accumulated as much total Mo as the wild type after growth with 1 μM molybdate, but that the subcellular distributions differed; most of the Mo in the *chlD* mutant was soluble, while in the wild type the majority of Mo was in the particulate fraction. This and other observations led them to conclude that *chlD* function was not involved in molybdate transport, but rather was required for "processing" of Mo (119). At high molybdate concentrations, this presumptive processing would occur by nonenzymatic means.

Measurements of biotin-D-sulfoxide reductase activity indicate that the phenotypic suppression of *chlD* mutants by molybdate depends on growth conditions (45). *chlD* cultures grown aerobically in rich medium without glucose synthesize high levels of biotin-D-sulfoxide reductase activity, and supplementation with 1 mM molybdate increases that activity only two- to fivefold. In the presence of glucose, the level of activity is more sensitive to molybdate (approximately 10- to 15-fold increase). This suggests that a *chlD*-independent pathway for Mo cofactor synthesis is expressed in the absence of glucose (45). Anaerobic cultures (grown with glucose) have lower levels of biotin-D-sulfoxide reductase activity, which is increased approximately 10-fold by molybdate supplementation. This lower anaerobic level could reflect competition for Mo cofactor with other molybdo-enzymes, such as formate dehydrogenase-H, or it could reflect regulation of biotin-D-sulfoxide reductase synthesis.

Recently, Johann and Hinton (185) have reported the DNA sequence of a clone that complements the *chlD202*:: Mu *cts* mutation. This clone contains one complete open reading frame, termed *chlD*, and the 3′ region of a proximal reading frame, termed *chlJ* by the authors. The predicted protein sequences of ChlD and the sequenced portion of ChlJ are homologous to those of cytoplasmic membrane components of periplasmic binding protein-dependent transport systems (reviewed in reference 10), suggesting that the *chlD* region encodes a molybdate transport system. If analogous to other periplasmic binding protein-dependent transport systems, the *chlD* locus would contain at least four genes, encoding one periplasmic and three membrane proteins (10).

Johann and Hinton also noted the presence of another open reading frame immediately distal to the presumed *chlD* coding region (185). However, the *chlD*::Mu insertion complemented by this plasmid should be strongly polar on the expression of downstream genes. Perhaps the product of the downstream reading frame is not absolutely required for ChlD function, or perhaps overproduction of the presumed *chlD* gene product suppresses the requirement for the downstream gene product.

Unfortunately, this clone has not been mapped to the *chlD*

region (i.e., by Southern blot hybridization) and has not been tested for complementation of other *chlD* mutations (185). The remote possibility exists that it is derived from an unrelated gene system and fortuitously complements the *chlD202*::Mu cts mutation. That *chlD*$^+$ is dispensable when cells are cultured with high molybdate concentrations implies that other transport systems have at least a weak affinity for molybdate.

If the *chlD* locus does encodes components of a periplasmic binding protein-dependent molybdate transport system, then the accumulation of Mo in the soluble fraction of the *chlD* mutant (119) could reflect binding to the periplasmic binding protein without internalization (185). Studies with well-defined mutants are required to test this idea.

Amy and colleagues have studied the regulation of *chlD-lacZ* operon fusions (272). Their work indicates that genes in the *chlD* region are regulated by molybdate and nitrate, but are insensitive to the level of oxygen. These *chlD-lacZ* fusions are efficiently expressed only when cultures are supplemented with <10 nM molybdate; 500 nM molybdate reduces expression by at least 20-fold. Equivalent concentrations of tungstate mimic the effect of molybdate. Nitrate increases the expression of *chlD-lacZ* two- to threefold anaerobically as well as in aerated cultures and also raises the threshold concentration of molybdate required for full *chlD* repression.

Insertion mutations in *chlA*, *chlE*, and *chlG* have no effect on *chlD-lacZ* expression, indicating that Mo cofactor does not regulate *chlD* expression. An insertion in *narL*, which prevents nitrate induction of nitrate reductase gene expression (see section, "Regulation of Nitrate Reductase Synthesis") also abolishes the nitrate induction of *chlD* expression. Finally, insertions in *chlB*, which prevent Mo cofactor incorporation into apo-nitrate reductase or in *narC* and *narI*, which are structural genes for nitrate reductase, cause *chlD-lacZ* to be expressed at the higher (nitrate-induced) level even in the absence of nitrate. These latter effects are not yet understood. None of these mutations affects molybdate-dependent *chlD* expression, so the molybdate-sensitive regulator remains to be identified (272).

K. pneumoniae chlD mutants, termed *mol*, are similar to the corresponding *E. coli* mutants: nitrate reductase activity is phenotypically restored after growth with 1 mM molybdate. These mutants also show decreased Mo accumulation at low molybdate concentrations (163).

chlEN. Mutations in the *chlE* locus (in the 18-min region of the *E. coli* map) have been mapped in *E. coli* (318, 411), *S. typhimurium* (98), and *C. freundii* (79). In each case, the *chlE* region maps just distal to *chlA*.

Genetic complementation studies, using abortive transduction (410), mini-Mu transduction (190), and subcloning (324), have revealed that the *E. coli chlE* locus contains at least two complementation groups. Johnson and Rajagopalan have designated the gene defined by the *chlE5* allele as *chlE* and the gene defined by their complementing mutation as *chlN* (190). Biochemical studies of *chlEN* function are summarized below.

chlF. Glaser and DeMoss (120) isolated two mutants, defective in formate dehydrogenase activity, whose lesions mapped near *chlC* (see next section) in the 27-min region of the *E. coli* map. They designated these mutations *chlF*. Unfortunately, these strains have been lost. One may only speculate that the *chlF* mutations identified one of the regulatory genes in the *chlC* region (see below).

chlG. Mutations in the *chlG* locus (in the 0-min region of the *E. coli* map) have been mapped in *E. coli* (120, 182, 368) and *C. freundii* (79). In each case, the *chlG* locus is linked to *thr*.

The original *E. coli chlG* mutant was isolated in a screen for mutants deficient for formate-dependent nitrate reduction, although it was chlorate resistant (120). Subsequent isolation of *chlG* mutants required special procedures (182). However, in a large-scale search for chlorate-resistant insertion mutants, roughly 4% were *chlG* (368); these are the only gas-producing mutants in this collection. This mutant search used a derivative of the *E. coli* K-12 strain MC4100, which carries the *thr* region of *E. coli* B (47). An independent study reported that the ChlG phenotype in *E. coli* K-12 is dependent on the strain background (44).

chlG mutants, like *chlD* strains, retain detectable activity for molybdo-enzymes, including formate dehydrogenase (120, 182, 368), biotin-D-sulfoxide reductase (83), dimethyl sulfoxide reductase (28), and TMAO reductase (367). Although the growth of *chlG* mutants in 1 mM molybdate leads to some phenotypic suppression as measured by biotin-D-sulfoxide reductase (45, 83) and nitrate reductase (368) activities, wild-type levels of enzyme activity are not restored as in *chlD* mutants. The ChlG and ChlD mutant phenotypes are further differentiated by the *nit-1* assay (271). *chlD* mutants, after growth with a low molybdate concentration, produce low levels of Mo cofactor as detected by *nit-1* reconstitution. However, the relative proportion of Mo cofactor to molybdopterin ("empty cofactor"), distinguished by reconstitution without and with added molybdate in the assay, is roughly equivalent to that of the wild type. *chlG* mutants, after growth with a low molybdate concentration, do not complement *nit-1* extracts in the absence of molybdate, but give full reconstitution in the presence of molybdate (271). Thus, *chlG* mutants synthesize wild-type levels of molybdopterin when grown in the absence of added molybdate, while *chlD* mutants require high molybdate levels to accumulate wild-type levels of molybdopterin. The biochemical bases for these observations are unknown.

Other *chl* genes? Mutations termed *chlG* in *S. typhimurium* (377) and *K. pneumoniae* (381) and *chlH* in *C. freundii* (79) are linked to genes analogous to *nadB* and *purL* (in the 55-min region of the *E. coli* map). In *C. freundii*, this *chl* mutation is complemented by an *E. coli* F′ episome from this region, indicating that the analogous gene is present and functional in *E. coli* as well (79). However, no *E. coli chl* mutations have been located in this region. Perhaps mutations in the analogous *E. coli* gene do not confer Chl^r.

Synthesis and Assembly

Reconstitution in *E. coli* extracts. Azoulay and colleagues discovered that nitrate reductase activity is reconstituted when cell-free extracts of *chlA* and *chlB* mutants are mixed and incubated at 32°C for 2 h (18, 19). Mixing "soluble" fractions (i.e., high-speed centrifugation supernatants) leads to reconstitution of nitrate reductase activity that is associated with a newly formed "particulate" fraction. Their interpretation was that the "membrane particle" was altered in *chl* mutants and that reconstitution reflected assembly of "structural protein" components to form an active respiratory complex (18, 19). This view was subsequently modified to postulate that, in *chl* mutants, a polyenzymatic respiratory complex (of which nitrate reductase is one component) cannot aggregate correctly in the cytoplasmic membrane (17, 326).

An alternate interpretation was provided by the work of MacGregor and Schnaitman. They showed that *E. coli*

extracts complement a *nit-1* extract for reconstitution of *N. crassa* nitrate reductase activity, presumably by providing Mo cofactor. The soluble portion of a *chlB* mutant extract also complements *nit-1*, but the particulate fraction has no complementing activity. The other mutants tested (*chlA*, *-C*, *-D*, and *-E*) have little or no complementing activity (258). These results suggested that the *chlA* locus is required for Mo cofactor synthesis and that the *chlB* gene product mediates the incorporation of Mo cofactor into apo-respiratory nitrate reductase (258). Subsequent work established that the apo-nitrate reductase polypeptides present in the high-speed supernatant are derived from the cytoplasmic membrane during cell breakage and that formation of particulate material during incubation of the extracts is not a consequence of enzyme activity reconstitution, but reflects aggregation of membrane fragments (259). MacGregor and Schnaitman proposed that reconstitution depends on at least three components: apo-nitrate reductase protein; a soluble factor (Mo cofactor) present in *chlB* extracts; and a component, presumably the *chlB* gene product, that inserts Mo cofactor into apo-nitrate reductase (259, 260). The reconstitution system does not provide a model for in vivo assembly of nitrate reductase into the cytoplasmic membrane (260). Work by Amy and colleagues has established that *chlB* mutants synthesize wild-type levels of active Mo cofactor (11, 271).

The presumed *chlB* gene product, termed F_A (association factor), was purified from a *chlA* mutant by assaying for reconstitution of a *chlB* extract (326). F_A is a protein of approximately 35 kDa and is associated with the cytoplasmic membrane (326). A second protein, termed PA, has been purified from the soluble fraction of a *chlB* extract and was presumed to be the product of the *chlA* gene (139). PA isolated from *chlA*, *-D*, *-E*, and *-G* mutants is incompetent for reconstitution of a *chlB* extract (117). It has been suggested that PA is not the *chlA* gene product, but represents a molybdopterin carrier protein (189). Clearly, the gene-protein relationships for the *chl* loci remain to be unambiguously identified.

Formate dehydrogenase-H and TMAO reductase activities are also reconstituted in vitro by either mixing extracts of *chlA* and *chlB* mutants or adding purified F_A or PA to the appropriate mutant extract (118). Both of these reconstituted enzyme activities are only weakly associated with the particulate fraction, again showing that reconstitution does not assay for membrane protein assembly (118). The active species in nitrate reductase and TMAO reductase reconstitution fractionates identically with the component that reconstitutes *nit-1*, further demonstrating that the *E. coli* reconstitution system reflects Mo cofactor incorporation into apoenzymes (336, 353).

An extract from a *chlB* mutant grown with tungstate cannot reconstitute nitrate reductase activity in a *chlA* extract, leading to the postulation that tungstate inhibits the expression of the *chlA* gene (114). However, a tungstate-grown *chlB* mutant would presumably contain W-molybdopterin, which is incompetent to reconstitute enzyme activity. Indeed, nitrate reductase activity in tungstate-grown *chlB* extracts is not reconstituted by purified F_A plus molybdate, but it is reconstituted by purified F_A plus a heat-treated wild-type extract, as a source of Mo cofactor (336).

Biosynthesis of Mo cofactor. Amy found active Mo cofactor in the soluble fractions of both the *chlA1* and *chlE5* mutants, but failed to detect cofactor in the membrane fractions of the same mutants (11). *nit-1* reconstitution with the *chlA* extract was absolutely dependent on added molybdate in the reconstitution mixture, suggesting that the *chlA* defect prevents Mo insertion into molybdopterin (11). Subsequently, Miller and Amy found that *chlA*::Mu *cts* and *chlE*::Mu *cts* mutants synthesize no Mo cofactor that is detectable by *nit-1* reconstitution, irrespective of added molybdate (271). They also reproduced the earlier results with *chlA1* and *chlE5* (271). The genetic and biochemical bases for these observation are unknown, as the Mu insertion mutants that were studied included examples from each of the three *chlA* and two *chlE* complementation groups detected by subcloning experiments (324).

Recently, Johnson and Rajagopalan have described an in vitro assay for detecting Mo cofactor synthesis (189, 190). They found that the *chlA1* mutant, which lacks molybdopterin by both *nit-1* and dephospho-form A assays, contains a protein that synthesizes active Mo cofactor from a low-molecular-weight fraction in the *nit-1* extract (189, 190). (This result suggests that *nit-1* reconstitution by *chlA1* [11] may reflect de novo synthesis of Mo cofactor.) This protein, termed molybdopterin converting factor, has been partially characterized, but it is unstable during purification (189). This work also identified several probable molybdopterin precursors (189) and, using dephospho-form A assays coupled with reconstitution experiments, established the involvement of *chlA*, *chlE*, *chlM*, and *chlN* in molybdopterin biosynthesis (190). Despite these advances, it is clear that a full understanding of molybdopterin and Mo cofactor biosyntheses remains a formidable task.

chlC LOCUS

Isolation of *chlC* Mutants

A minority class of *E. coli chl* mutants (*chlC*) was found to retain substantial formate dehydrogenase activity. Only 1 to 2% of spontaneous Chl^r mutants are *chlC* (48, 120, 142, 319), but this proportion is increased to as much as 20% following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (142). The mutations in these strains map near *trp*, in the 27-min region of the genetic map (142, 319). Transduction studies by Guest (142) demonstrated a genetic order of *purB-hemA-chlC-tyrT-galU-att80-tonB-trp*. Retention of high formate dehydrogenase activity distinguishes *chlC* from all other *chl* mutants; this indicated that *chlC* contains the structural genes for nitrate reductase (142, 319), a fact that has now been demonstrated (see below).

Guest (142) performed an early comprehensive study of *chlC* mutants. He selected 28 independent spontaneous and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced Chl^r *chlC* mutants, which were initially distinguished from pleiotropic *chl* mutants by the fact that they formed gas. Twenty of the mutants produced at least 80% of the wild-type level of gas; other mutants formed as little as 40%. Some of the mutants were partially Chl^s. Biochemical analyses indicated that the primary defect in these mutants was in nitrate reductase activity. Decreased gas production correlated with decreased activity of both formate dehydrogenase activities and was presumed to reflect a secondary consequence of nitrate reductase deficiency. Reversion and mapping studies indicated that most of the strains each carried a single mutation, although the possibility of multiple, closely linked mutations was not ruled out (142).

DeMoss and colleagues developed alternate methods, screening either for colonies that do not produce nitrite or for those with a characteristic phenotype on eosin-methylene blue–nitrate medium, to isolate a number of *chlC*

mutants (120, 332). In three different screens with these procedures, 34 of 65 mutations were linked to *trp* and presumed to affect *chlC*. In contrast, only 1 of 45 mutants selected for chlorate resistance was *chlC*. Indeed, many representatives of the first class retained full or partial sensitivity to chlorate. This suggested that chlorate resistance selected for only the most severe lesions in *chlC* (120). Again, however, most of these mutants were partially defective in formate dehydrogenase activity.

A different isolation procedure takes advantage of the fact that *chlC* mutants, in contrast to the wild type or pleiotropic *chl* mutants, reduce 2,3,5-triphenyltetrazolium chloride during anaerobic growth with nitrate (99). Mu *c*ts and Tn*5* insertions isolated by this screen all map to the *chlC* region, and all such mutants produce at least wild-type levels of formate dehydrogenase activity (93, 99). The Chl phenotype of these mutants has not been reported.

Barrett and Riggs (27) isolated *S. typhimurium chlC* mutants on MacConkey nitrate medium (23), which also differentiates formate dehydrogenase mutants (see section, "Formate/Hydrogen Metabolism"). Three independent *chlC* mutants retained both sensitivity to chlorate and significant nitrate reductase activity. By a variety of criteria, it is likely that this residual nitrate reductase activity is contributed by a distinct enzyme, probably another molybdo-enzyme with an incidental nitrate reductase activity (27).

Nitrate respiration prevents the formation of tetrathionate reductase activity (49, 81). On eosin-methylene blue medium containing nitrate plus tetrathionate, only *chlC* mutants of *S. typhimurium* express tetrathionate reductase activity, which leads to a distinctive color change on this medium (49).

An analogous isolation procedure was applied to *E. coli* (368), using a medium (MacConkey glucose-TMAO [77]) that detects TMAO reductase expression. Wild-type colonies, after anaerobic incubation, accumulate enough trimethylamine from TMAO to counteract the acid produced from glucose and are white on this medium. Inclusion of nitrate prevents TMAO reductase synthesis or activity, so wild-type colonies are red. Pleiotropic *chl* mutants do not synthesize TMAO reductase, a molybdo-enzyme, and are also red. White colonies recovered with this medium carry mutations in the *chlC* locus (368). Tn*10* insertion mutants isolated by this procedure are fully Chl^s and accumulate wild-type levels of gas. These mutants retain a small but detectable level of nitrate reductase activity which, as in *S. typhimurium*, has been attributed to the action of some other molybdo-enzyme(s) (27, 45, 83, 368).

In general, these studies are consistent with the hypothesis that single mutations at *chlC*, including insertions, do not confer a Chl^r phenotype; perhaps two mutations, affecting both a nitrate reductase structural gene and a closely linked regulatory gene, are required for Chl^r. Indeed, one *chlC* allele (*chlC19*) is separable into two distinct, closely linked mutations, either of which fails to confer a Chl^r phenotype (368; V. Stewart, Ph.D. dissertation, University of Virginia, Charlottesville, 1982). Many more mutants need to be characterized to fully test this hypothesis.

Multiple Genes at *chlC*

From the earliest studies, it had been assumed that *chlC* contains at least one structural gene for nitrate reductase. This was formally demonstrated by MacGregor (251), who showed that a *chlC* mutant produces degraded nitrate reductase polypeptides, and by DeMoss (85), who isolated *chlC* mutants with thermolabile nitrate reductase activity.

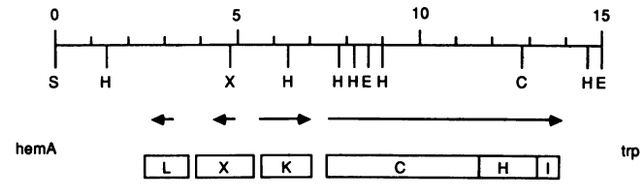

FIG. 5. Physical organization of the *nar* (*chlC*) locus. A partial restriction map for the region is shown; the scale is in kilobase pairs. C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I. The region is oriented with respect to the outside markers *hemA* and *trp* (cf. Fig. 4). Approximate positions of protein coding regions are shown by boxes. *narC*, *-H*, and *-I* are the structural genes for nitrate reductase subunits α, β, and γ, respectively. *narL* encodes a positive activator of *nar* operon expression. The functions of *narK* and *narX*, and whether *narL* and *narX* form a complex operon, are unknown. The direction and extent of transcription are shown by arrows. Compiled from references 93, 268, 327, and 369 and V. Stewart and J. Parales, Jr., unpublished observations.

Bonnefoy-Orth and colleagues (33, 294) isolated *chlC* mutants with a novel phenotype: they retain high levels of viologen-dependent nitrate reductase activity, but lack formate-nitrate oxidoreductase. They termed these mutants, which presumably lack cytochrome b_{556}, *chlI*. They also isolated Mu *c*ts insertion mutants (termed *chlC*) that lack nitrate reductase activity with either formate or viologen as electron donor (33). These results suggested that the structural genes for nitrate reductase subunits α (*chlC*) and γ (*chlI*) are in an operon. Complementation and mapping experiments established a genetic map order of *purB-chlC-chlI-trp* (33).

Analogous Tn*10* insertion mutants were termed *nar*, to distinguish them from the pleiotropic *chl* mutants (368). Immune precipitation established that *narC*::Tn*10* (originally designated *narG*::Tn*10*) mutants fail to accumulate any nitrate reductase subunits, while *narI*::Tn*10* mutants accumulate subunits α and β in the cytoplasm and contained no detectable subunit γ (368). Spectroscopic studies by Hackett and Bragg (148) demonstrated that *narI* mutants specifically lack the cytochrome b_{556} associated with nitrate reductase (e.g., subunit γ). Genetic mapping studies are consistent with the chromosomal locations proposed by Bonnefoy-Orth and colleagues (33).

None of these mutants appear to specifically lack subunit β (368). However, Edwards and colleagues (93), studying Tn*5* insertion mutants, showed that two retain subunit α but produce no subunit β. They designated the gene for subunit β as *narH*.

Taken together, these studies indicate that the structural genes for nitrate reductase subunits α, β, and γ are in an operon of promoter-*narC-narH-narI* and are transcribed counterclockwise with respect to the *E. coli* genetic map (Fig. 5). Formal proof that *narC* is the structural gene for subunit α is provided by comparisons of the 5′ DNA sequence of this gene with the amino-terminal amino acid sequence of purified subunit α (239, 268).

Three additional genes have been found in the *chlC* locus (368, 369; Fig. 5). Mutations in *narL* identify a positive regulatory gene for nitrate reductase gene expression (367, 369; see "Regulation of Nitrate Reductase Synthesis" section). The *narL* gene encodes a protein of 28 kDa (369). A second gene, termed *narX*, has a subtle effect on *nar* operon expression, and its function is unknown. The *narX* gene encodes a protein of 66 kDa (369).

Tn*10* insertion mutations in the third gene, termed *narK*, were identified in strains that form white colonies on Mac-

Conkey nitrate-TMAO medium. These strains synthesize normal amounts of nitrate reductase activity, and nitrate reductase gene expression is also regulated normally. They also accumulate all three nitrate reductase subunits, as determined by immune precipitation. It was suggested that *narK* might encode a regulator for nitrate repression of other anaerobic respiratory enzymes (368). However, a *narK*::Tn*10* mutation has little effect on nitrate repression of fumarate reductase gene expression (173, 193). The *narK* gene is between *narL* and the *narCHI* operon, is transcribed in the same direction as *narCHI*, and is induced by nitrate (369). The function of $narK^+$ remains to be determined.

REGULATION OF NITRATE REDUCTASE ACTIVITY BY OXYGEN

Strickland observed that oxygen inhibits the in vivo activity of nitrate reductase in *E. coli* (370). He concluded that oxygen is a noncompetitive inhibitor of nitrate reductase that acts by withdrawing electrons from the respiratory chain. This observation was extended with the finding that oxygen reversibly inhibits nitrate reductase activity in chloramphenicol-treated suspensions of *K. pneumoniae* LIII-1 (310). However, oxygen has little effect on the viologen-dependent nitrate reductase activity in cell-free extracts (310).

Likewise, nitrate reductase activity in whole cells of *P. mirabilis* PG ceases upon shift to aerobic cultivation (81). The specific activity decreases about twofold in the first hour after the shift and then levels off through the remainder of growth. Nitrate reductase activity in a *hemA* mutant, cultured without δ-aminolevulinic acid, does not decrease after shift to aerobiosis (81). These results indicate that electron transport to oxygen, rather than oxygen per se, is required for oxygen inhibition of nitrate reductase activity.

More recently, John (186) used sensitive electrodes to directly measure oxygen and nitrate uptake in cell suspensions of *E. coli* K-12. Cells were incubated with an electron donor and nitrate to initiate respiration. With succinate as donor, nitrate uptake ceased immediately upon addition of oxygen; as soon as the oxygen was consumed, nitrate respiration resumed at its initial rate. In contrast, with formate as donor, oxygen caused only about a fourfold inhibition of nitrate uptake (186). Formate supported a 10-fold-higher rate of oxygen uptake than succinate. Thus, it was suggested that the residual formate-nitrate uptake in the presence of oxygen represented "spillover" of reducing equivalents from quinone to nitrate reductase (186). Formate dehydrogenase-N can donate electrons to ubiquinone or menaquinone, whereas succinate dehydrogenase only donates to ubiquinone (418; see below). Perhaps the residual formate-dependent nitrate uptake in the presence of oxygen represents electron transport from menaquinone.

Recent work has examined oxygen inhibition of nitrogenase in *K. pneumoniae* KP (121). Nitrogenase is a soluble electron transfer enzyme that is not involved in respiration. Low levels of oxygen (0.37 μM) inhibit nitrogenase activity by 50%, and this inhibition is completely reversible. In the presence of 90 μM cyanide, which inhibits aerobic respiration by 60 to 70%, 2.9 μM oxygen is needed to effect the same 50% inhibition of nitrogenase activity. These and analogous results were interpreted to reflect competition for electrons between the aerobic respiratory chain and nitrogenase.

Competition for electron flow may affect the activity of other anaerobic oxidoreductases as well. For example, in *P. mirabilis* PG, nitrate inhibits thiosulfate reductase activity, but this inhibition is reversed by azide, a nitrate reductase inhibitor that does not affect thiosulfate reductase activity. Both nitrate and thiosulfate reductase activities are inhibited by oxygen (82). Other work with *P. mirabilis* PG has led to the hypothesis that formate-hydrogen lyase activity is regulated by a factor that responds to the redox state of alternate acceptor couples (220). This factor was postulated on the basis of indirect experiments, measuring hydrogen evolution in the presence of various high- and low-potential electron acceptors, and its existence remains unproven.

Recently, Noji and Taniguchi (289) used electrodes to simultaneously measure oxygen and nitrate consumption in *E. coli*, with formate as electron donor. Oxygen inhibited nitrate uptake by two- to threefold. Octyl glucoside largely relieved this inhibition, without affecting oxygen uptake. These and other results were interpreted to reflect control of nitrate transport by oxygen; release of the permeability barrier by octyl glucoside allows nitrate to gain access to the active site of nitrate reductase (289).

Finally, indirect studies, using inhibitors of transcription and translation, indicate that oxygen also blocks a posttranslational step in nitrate reductase biosynthesis or assembly (200, 331). Hackett and MacGregor (145) found that the membrane incorporation of soluble precursor nitrate reductase is blocked immediately by a shift from anaerobic to aerobic growth. The enzyme subunits are not degraded during aeration (145). Perhaps nitrate reductase and other anaerobic respiratory enzymes cannot be incorporated into the membrane under conditions in which they cannot function in electron transport (82).

To summarize, oxygen has been suggested to inhibit nitrate reduction in whole cells by competing for electrons or inhibiting nitrate transport and also to block enzyme assembly. This topic is clearly ripe for further study with modern methods.

REGULATION OF NITRATE REDUCTASE SYNTHESIS

Physiology

Induction of nitrate reductase synthesis by nitrate was demonstrated in "resting suspensions" of *C. freundii* 1433 by Pollock (315) and of *K. pneumoniae* ("*Bacterium lactis-aerogenes*") by Lewis and Hinshelwood (237) in the course of studies on "enzymatic adaptation." Subsequently, Pichinoty and D'Ornano (310), studying washed suspensions of *K. pneumoniae* LIII-1, demonstrated that nitrate induces nitrate reductase synthesis only in anaerobic suspensions. Inhibition by oxygen is reversible: nitrate reductase is induced immediately after an anaerobic shift. Thus, nitrate reductase synthesis is subject to dual control: induction by nitrate and inhibition by oxygen.

Showe and DeMoss (351) studied the kinetics of nitrate reductase synthesis. They measured the differential rate of nitrate reductase synthesis in *E. coli* K-12 during three types of shifts: an aerobic culture was made anaerobic ("derepression"); nitrate was added to an anaerobic culture ("induction"); and an aerobic culture was made anaerobic in the presence of nitrate ("combination"). In each case, the differential rate of synthesis was biphasic: the initial high rate lasted for approximately one-half generation and was then replaced by a lower steady-state rate. Even in the absence of nitrate, there was significant nitrate reductase induction after an anaerobic shift. Similar kinetics have been reported by others (331).

They interpreted these results in terms of two regulatory elements: a "redox repressor," which responds to the

respiratory capacity of the cell, and a "nitrate repressor," which responds directly to nitrate. The biphasic kinetics were explained by this model as reflecting transients in the internal redox potential, rather than as a consequence of nitrate metabolism, because the same pattern of synthesis was observed during anaerobic shift in the absence of nitrate. By this hypothesis, aerobic respiration is immediately halted upon anaerobic shift, and the suddenly lowered redox potential inactivates the redox repressor, causing a mass induction of anaerobic enzymes (including nitrate reductase). As anaerobic metabolism becomes established, the redox potential is raised again, although not to the fully aerobic level. Thus, the redox repressor assumes an intermediate activity, and the rate of anaerobic enzyme synthesis decreases to a steady-state level. In the presence of nitrate, establishment of nitrate respiration raises the redox potential, and thus the differential rate of nitrate reductase synthesis is also decreased as a consequence of the change in the redox repressor.

Support for this idea came from an experiment with a mutant lacking formate dehydrogenase activity and thus unable to form a formate-nitrate oxidoreductase respiratory chain. This mutant did not produce detectable levels of nitrite and thus presumably did not reduce nitrate in vivo. After anaerobic induction with nitrate, nitrate reductase synthesis did not exhibit the biphasic kinetics seen in the wild type. The interpretation was that the failure to establish nitrate respiration meant that the internal redox potential, and thus the hypothetical redox repressor, was unchanged (351). Unfortunately, this mutant has not been characterized further.

Studies with *K. pneumoniae* S45 (404) and *P. mirabilis* PG (81) gave analogous results. *K. pneumoniae*, growing aerobically, was shifted to anaerobic conditions in the presence of nitrate; *P. mirabilis* was subjected to anaerobic shift in the absence of nitrate. In both cases, nitrate reductase was initially synthesized at a high rate which soon assumed a lower steady-state value. Anaerobic cultures of *P. mirabilis* were also induced with either nitrate or azide (81), a reversible competitive inhibitor of nitrate reductase synthesis (309). The nitrate-induced culture exhibited an initial burst of nitrate reductase synthesis followed by a lower steady state, while the azide-treated culture continued to synthesize nitrate reductase at the initial high rate throughout the growth period. Studies with several enteric bacteria showed that nitrate reductase is formed at higher levels after growth with azide than with nitrate (58). Finally, MacGregor (250) studied a mutant (*hemA*) of *E. coli* K-12 that fails to synthesize heme unless provided with δ-aminolevulinic acid. She found that nitrate reductase is overproduced in this mutant after anaerobic growth in the absence of δ-aminolevulinic acid, although she did not examine the kinetics of synthesis during growth. All of these results are consistent with the original model of Showe and DeMoss.

An analogous "overshoot" phenomenon was described by Gorini and Maas in 1957 (122); they studied the synthesis of ornithine transcarbamylase, an enzyme of arginine biosynthesis. They found that shifting a culture from arginine-supplemented to arginine-free medium resulted in an initially high rate of ornithine transcarbamylase synthesis, which was then followed by a lower steady-state rate. An arginine auxotroph, grown in chemostat culture with limiting arginine, exhibited a constant high rate of ornithine transcarbamylase synthesis. The interpretation of these results rests on the fact that formation of endogenous arginine results in repression of ornithine transcarbamylase synthesis. Thus, when a culture is shifted from arginine-supplemented to arginine-free medium, the sudden depletion of arginine results in maximal derepression of the arginine biosynthetic enzymes; subsequent synthesis of arginine then leads to a partial repression of the biosynthetic enzymes. The curves relating enzyme activity to time for both ornithine transcarbamylase (122) and nitrate reductase derepression (81, 404) are strikingly similar.

An alternative explanation for biphasic induction kinetics was developed for the expression of *araC*, the regulator of the arabinose operon in *E. coli* (153). In this case, it is thought that the *araC* gene is autorepressed only with high levels of arabinose. Early in induction, when the intracellular arabinose concentration is low, the *araC* gene is fully expressed; later, as arabinose accumulates, the rate of *araC* expression is reduced to the original preinduction level (153).

The biphasic kinetics of nitrate reductase induction probably do not reflect protein turnover; the enzyme is reportedly quite stable after removal of inducer (416) or addition of oxygen (145).

Thus, the status of the redox control hypothesis to explain the induction of anaerobic respiratory enzymes, including nitrate reductase, is uncertain. Genetic studies (see below) have identified only a single gene, *fnr*, that is required for anaerobic induction of these enzymes; it seems unlikely that the *fnr* gene product responds to a continuum of redox potentials (see below). The nature of the biphasic kinetics of nitrate reductase induction deserves a fresh analysis with modern techniques.

Physiological signal for anaerobic regulation. Molecular oxygen is probably not involved in signalling aerobiosis; mutants unable to form the aerobic respiratory chain are derepressed for nitrate reductase synthesis even in the presence of oxygen. Simoni and Shallenberger (355), studying the energetics of active transport, isolated mutants defective in an unspecified component required for aerobic respiration. These mutants are aerobically sensitive to chlorate, unlike the wild type, implying that nitrate reductase synthesis is derepressed. Subsequently, Giordano and colleagues screened neomycin-resistant mutants for those that were aerobically sensitive to chlorate (116). These mutants lack ubiquinone (16), are defective in aerobic respiration (111, 116), and produce nitrate reductase in the presence of oxygen (112). In addition, Lin and colleagues (229) found that the *glpA* operon (encoding the anaerobic glycerol-3-phosphate dehydrogenase) is aerobically expressed in a *hemA* mutant or in a wild-type strain whose aerobic respiration is inhibited with cyanide.

In contrast, MacGregor and Bishop (253) found that a *hemA* mutant still exhibits proper oxygen regulation of nitrate reductase synthesis. Thus, the correlation between aerobic respiration and oxygen control of nitrate reductase synthesis remains unclear. A systematic genetic and physiological study, using mutants and inhibitors, is needed to reexamine this question.

Wimpenny and colleagues have argued that oxygen control of anaerobic enzyme synthesis reflects the redox potential of the culture medium (422–424). However, this view is contradicted by the existence of mutants that are aerobically derepressed for nitrate reductase synthesis (above). "Redox regulation" more likely reflects physiological parameters which themselves are sensitive to the composition or oxygen saturation of the culture medium.

Various studies have purported to implicate DNA supercoiling (433), oxygen radicals (159), or the lack of certain modified transfer ribonucleic acids (43, 180) as important

TABLE 4. Effects of *fnr* (*oxrA*) mutations on enzyme synthesis and gene expression in *E. coli* and *S. typhimurium*[a]

Enzyme or gene	*E. coli*	*S. typhimurium*	Reference(s)
Enzyme synthesis			
Formate dehydrogenase-N	+	NT	231
Anaerobic glycerol-P dehydrogenase	−	NT	171
Hydrogenase 1	−	−[b]	179, 339
Hydrogenase 2	−	−	179, 339
Fumarate reductase	−	−	179, 231
Nitrate reductase	−	−	179, 231
Nitrite reductase (NADH)	−	NT	284
Nitrite reductase (formate)	−	NT	56
Cytochrome c_{552}	−	NT	56, 284
Dimethyl sulfoxide reductase	−	NT	28
Formate-hydrogen lyase:	+[c]	+[b,c]	179, 339
Formate dehydrogenase-H	+[c]	+[b,c]	179, 339
Hydrogenase 3	+	+[b]	179, 339
Asparaginase II	−	NT	183
Aspartase	−	NT	183
Nitrogenase	+[d]	NT	160
Gene expression			
glpA	−	NT	229
frdABCD	−	NT	173, 193
narCHJI	−	−	54, 99, 238, 230, 367
narK	−	NT	369
nirB	−	NT	138, 181
torA	+	NT	296
fdhF	+[c]	+[b,c]	30, 178, 179, 429
hyd	NT	+[b]	178, 179
hyd-17	+[c]	NT	30
hydC	−	NT	428
pepT	NT	−[b]	178, 383
tppB	NT	+	178
fnr	?	NT	193, 295

[a] +, Efficient expression in an *fnr* background; −, reduced or negligible expression in an *fnr* background. NT, *fnr* effect not tested.
[b] Expression is also dependent on *oxrC*.
[c] After growth with exogenous formate.
[d] *K. pneumoniae nif* genes in an *E. coli* host.

factors in regulating various aspects of anaerobic metabolism. However, alteration of any of these factors has profound consequences on many aspects of cell physiology, and the supposed regulation of anaerobic metabolism may represent indirect effects. The challenge will be to demonstrate that such factors play a direct role in regulating anaerobic gene expression.

Genetics of Anaerobic Regulation

fnr. Lambden and Guest (231) isolated several classes of fumarate reductase-deficient mutants of *E. coli* K-12. One class, which they termed *fnr* (fumarate and nitrate reductases), is also deficient in nitrate reductase and gas production. The *fnr* gene is linked to *pyrF* in the 29-min region of the chromosome. Meanwhile, Cole and Ward (68) had isolated nitrite reductase-deficient mutants termed *nirA*; subsequent work showed that *nirA* is also linked to *pyrF* (284). The Chippaux group (56) also isolated *pyrF*-linked nitrite reductase mutants, which they termed *nirR*. Later, several *fnr* mutations were isolated as spontaneous secondary mutations in *chl* mutants (367). Finally, Strauch and colleagues (383) isolated regulatory mutations (termed *oxrA*) in *S. typhimurium* LT2 that prevent anaerobic synthesis of peptidase T. The *oxrA* gene maps at a position analogous to *fnr* in *E. coli* (383) and is complemented by a plasmid (177) carrying the *E. coli fnr* gene (348). A second putative regulatory gene, *oxrB*, remains to be characterized, although is does not seem to be required for induction of anaerobic respiratory enzymes (383).

Most *fnr*, *nirA*, and *nirR* mutants are defective in nitrate and fumarate reductases, cytochrome c_{552}, and formate-hydrogen lyase activity (56, 284). Newman and Cole (284) were unable to demonstrate complementation between *nirA* and *fnr* mutants. These results reinforce the idea that *fnr*, *nirA*, and *nirR* mutations all identify the same gene, although the possibility that some of these mutations may not be allelic with *fnr* has not been rigorously excluded. Indeed, in her study of the relationship between *fnr* and nitrogenase synthesis, Hill (160) found that *nirA1* is not complemented by a plasmid carrying the cloned *fnr* gene. However, other *nirA* mutations, resulting from Tn*10* insertion, are complemented by essentially the same plasmid (248).

Most of the enzyme systems regulated by *fnr* are involved in anaerobic electron transport and require anaerobiosis for induction (Table 4). Thus, FNR (the *fnr* gene product) is the global regulator for anaerobic induction of respiratory enzyme synthesis. Since *fnr* mutants retain the capacity for anaerobic growth on fermentable carbon sources (Table 4), additional systems are involved in regulation of other anaerobic functions (7, 160, 178).

Both *E. coli* and *S. typhimurium fnr* mutants express decreased levels of hydrogenase and formate-hydrogen lyase activities. Hydrogenase isoenzymes 1 and 2 (thought to be involved in respiration; Table 2) both require fnr^+ for synthesis (179, 339). In contrast, it is now clear that FNR does not directly control formate-hydrogen lyase synthesis; the expression of both *fdhF* and *hyd-17* is *fnr* independent (30, 429). However, exogenous formate is required for wild-type levels of formate-hydrogen lyase synthesis in *fnr* mutants of both *E. coli* and *S. typhimurium* (30, 179, 339, 429). An *E. coli* mutant lacking pyruvate-formate lyase (*pfl*) also lacks formate-hydrogen lyase; exogenous formate restores formate-hydrogen lyase synthesis in *pfl* mutants (30, 339, 429). This indicates that *fnr* mutants are deficient in producing formate, the inducer of formate-hydrogen lyase. It is reported that pyruvate-formate lyase gene expression is *fnr* dependent (unpublished data cited in reference 30).

In addition, total hydrogenase and formate-hydrogen lyase activities are partially restored in an *E. coli fnr* mutant by growth in the presence of 0.5 mM Ni (428). Transcription of the *hydC* locus, which may be required for Ni transport, is dependent on *fnr* (428). Thus, *fnr* may also indirectly control hydrogenase activity via the expression of *hydC*. Whether Ni directly affects hydrogenase synthesis, or simply affects the level of enzyme activity, is unknown; *hyd-17* does not require added Ni for expression (436).

It is established that FNR is a positive regulator of gene expression. Mutations in *fnr* reduce *nar-lacZ* expression to basal levels, indicating that *fnr* is required for nitrate reductase gene transcription (54, 366). *nar* mRNA is greatly reduced in *fnr* mutants (238). Finally, overproduction of the FNR protein results in increased nitrate and fumarate reductase activities and renders their synthesis partially insensitive to oxygen (348). Mutations in *fnr* have been shown to reduce expression of a number of other operon fusions to genes for anaerobic respiratory enzymes (Table 4).

Clearly, *fnr* is not the only anaerobic regulatory gene. A

screen for anaerobically induced operon fusions in *S. typhimurium* revealed several whose induction is independent of *fnr*$^+$ (7). Furthermore, mutations in *oxrC*, in *S. typhimurium*, prevent the anaerobic synthesis of tripeptide permease, peptidase T, formate-hydrogen lyase, and hydrogenase 1 (178, 179). These observations have led to the broad generalization that enzymes synthesized during fermentation require *oxrC* for expression, while respiratory enzyme formation is *fnr* dependent (178, 179). Curiously, *oxrC* mutants are defective in phosphoglucose isomerase, suggesting that a product of glycolysis is involved in signalling anaerobiosis (178). Growth with fructose phenotypically suppresses the effects of *oxrC*, lending support to this idea (178). At least two enzymes, peptidase T and hydrogenase 1, require both *fnr* and *oxrC* for expression (178, 179).

An understanding of anaerobic regulation in all of its aspects remains a distant goal. Much work is needed to characterize fully the anaerobic regulatory genes and signals and the interactions between different regulatory networks.

Mechanism of FNR action. Shaw and Guest have cloned the *E. coli fnr* gene (347), determined its nucleotide sequence (349), and identified its protein product (348). FNR has been purified (398), but it does not bind specifically to the *frdABCD* (fumarate reductase operon) promoter region, due perhaps to the fact that, as purified, FNR is missing eight amino-terminal residues predicted from the DNA sequence (397, 399).

The sequence of FNR, deduced from the DNA sequence, shows regions of similarity with CRP, the cyclic adenosine 3′,5′-monophosphate (cAMP) receptor protein (350). The CRP-cAMP complex is required to activate transcription of many genes that encode enzymes for catabolism and energy metabolism (reviewed in references 78 and 396). One region of similarity between FNR and CRP is in the DNA-binding domain, the familiar "helix-turn-helix" motif. FNR also shares similarity with the CRP nucleotide-binding domain, although residues in CRP that are critical for binding cAMP (266) are not present in the analogous positions of FNR (351). The overall size and organization of the two proteins are quite similar.

These considerations suggested that FNR-mediated activation of gene transcription occurs by a mechanism similar to that of CRP. Strong evidence for that hypothesis comes from the work of Spiro and Guest (365). Certain amino acid residues in the DNA-binding domain of CRP make base-specific contacts in the DNA of the CRP binding site. Spiro and Guest used site-directed mutagenesis to change the amino acid sequence of FNR such that its presumed DNA-binding domain resembles that of CRP. In a Δ*crp* strain containing this engineered *fnr* gene, *lac* operon transcription is activated in response to anaerobiosis. Thus, the engineered FNR presumably binds to the CRP site of the *lac* promoter and promotes efficient transcription initiation by a mechanism similar to that of CRP (365).

The organization of the *narCHJI* (238, 239) and *frdABCD* (192, 193) promoter regions is consistent with a CRP-like mode of FNR transcription activation. The transcription start points have been determined by S1 nuclease analysis. Deletion analysis shows that, at most, sequences to −112 (numbering with respect to the transcription initiation site) are required for anaerobic induction of the *narCHJI* operon (238), while sequences beyond −50 are needed for *frd* transcription (192, 193). The CRP binding site is usually centered at about −60 to −70 (78).

The *narCHJI*, *frd*, and *nirB* (NADH-nitrite reductase) regulatory regions share sequence similarities that may define the FNR binding site (181, 238). Candidate promoter sequences have been identified: for *narCHJI* and *frdABCD*, the −35 regions are similar to the Eσ^{70} promoter consensus, while the −10 regions are less obvious (192, 193, 238, 239); the *nirB* candidate promoter has a recognizable −10 region but no clear −35 region (181). Positively controlled promoters usually have poor matches to the −35 or −10 consensus sequences (155). The complication in comparing potential regulatory sequences for these operons is that, in addition to their positive control by FNR, they are also subject to pathway-specific regulation: the *narCHJI* promoter is activated by nitrate; the *frdABCD* promoter is repressed by nitrate and regulated by fumarate; and the *nirB* promoter is regulated by nitrite. The imposition of these pathway-specific regulatory signals may obscure recognition of FNR binding sites based solely on sequence comparisons (78). Analysis of *cis*-acting mutations that prevent FNR action will be crucial for defining the FNR consensus sequence (78).

cis-acting mutations that affect anaerobic regulation of *narCHJI* (32) and *frdABCD* (172) have been isolated. In both cases, gene expression is less sensitive to aerobiosis and less dependent on FNR, but remains sensitive to regulation by nitrate. Analogous mutations in CRP-dependent operons usually affect the promoter itself, bringing it closer to the consensus sequence, and provide little information about the CRP binding site (322). Indeed, the aerobiosis-insensitive mutations in the *frd* operon [termed *frd*(Oxr)] were interpreted in these terms (172). The phenotype of a mutation affecting the CRP binding site itself is uninducible, rather than constitutive (78). Thus, the hypothesis that the aerobiosis-insensitive mutation in the *narCHJI* operon affects the FNR binding site (32) requires further analysis.

FNR and cAMP. Expression of *fnr-lacZ* operon fusions (193, 295) and measurements of FNR protein levels (397) demonstrate that *fnr* expression is not strongly regulated by anaerobiosis or *fnr*$^+$. Thus, FNR is present during both aerobic and anaerobic growth and must respond to a physiological signal to mediate induction of anaerobic respiratory gene expression. This signal is unknown. If the analogy between CRP and FNR is exact, then a small effector molecule, such as cAMP, would be required for FNR to assume an active conformation. Two observations suggest that this may not be the case. First, mutations in the adenylate cyclase gene (*cya*) confer the same phenotype as *crp* mutations, with the exception that *cya* mutants, unlike *crp* mutants, respond to exogenous cAMP (reviewed in references 78 and 396). However, analogous mutations affecting a second gene required for FNR function have not been reported, although many *fnr* mutations have been isolated in various laboratories. Second, cAMP-independent alleles of *crp* (*crp**) have been isolated, many of which apparently encode CRP proteins that assume active conformations in the absence of cAMP (e.g., references 156 and 420; reviewed in references 78 and 396). An extensive search for analogous *fnr* mutations was unsuccessful (172).

Unden and Guest (398) concluded that cAMP is an effector of FNR function. Subsequently, Unden and Duchene (397) determined that this conclusion is incorrect and was influenced by the poor anaerobic growth of *cya* strains. They were also unable to demonstrate cAMP binding to purified FNR, as measured by equilibrium dialysis (397). In addition, *cya* mutants do not require cAMP for production of nitrite in vivo (302) or synthesis of nitrate reductase (V. Stewart, unpublished data). Expression of an *fnr-lacZ* operon fusion is reported to be stimulated approximately twofold by cAMP

(295; see also reference 365), but it seems unlikely that this effect could account for the several-hundredfold induction of anaerobic respiratory enzyme synthesis. Furthermore, induction of *fnr* expression by cAMP cannot explain how regulation by FNR is specific for anaerobiosis.

The relationship among anaerobic metabolism, catabolite repression, and cAMP concentrations is not fully understood. Anaerobic shock relieves catabolite repression of *lac* operon expression (62, 88) and also increases the rate of cAMP synthesis (234, 397). However, anaerobic shock relief of catabolite repression is also observed in Δ(*cya*) strains that carry *crp** mutations (234). This clouds the conclusion that the increase in cAMP concentration after anaerobic shock is responsible for the relief of *lac* operon catabolite repression in wild-type cells (234; see also reference 396). An additional complication is that adenylate cyclase activity may be regulated by PMF (308), in addition to many other factors (e.g., reference 323).

Nitrate respiration restores *lac* operon catabolite repression to the aerobic level (88, 290). Unfortunately, the effect of nitrate on cAMP concentration has not been reported. Nonetheless, that both nitrate and oxygen respiration enhance catabolite repression to the same extent, while oxygen respiration prevents nitrate reductase expression, further strengthens the idea that gene regulation by anaerobiosis and by catabolite repression results from independent signals.

An alternative hypothesis is that FNR interacts directly with a component of the aerobic respiratory chain (399). For example, during active oxygen respiration, an oxidized form of FNR would be incompetent to stimulate gene transcription; upon anaerobic shift, FNR would become reduced and assume an active conformation. Thus, the functioning respiratory chain, rather than oxygen itself, would serve as the physiological signal for aerobiosis. Such a hypothesis is consistent with the observations that aerobic respiration-deficient mutants are aerobically derepressed for nitrate reductase (see above). In this context, the presence of three cysteine residues near the amino terminus of FNR (349) provides a tantalizing starting point for thinking about direct redox control of FNR conformation or activity (399). Indeed, it is reported that changing Cys-19 to Ser inactivates FNR (unpublished data cited in reference 365).

Genetics of Nitrate Regulation

Nitrate induction of nitrate reductase synthesis. Experiments with *nar-lacZ* operon fusions (54, 99, 367), as well as measurements of *nar* messenger ribonucleic acid synthesis (238), provide formal proof that nitrate acts to induce transcription of the *narCHJI* operon.

Physiological studies, particularly those of Showe and DeMoss (351), indicate that nitrate and aerobiosis are independent regulators of nitrate reductase gene expression. In the absence of nitrate, nitrate reductase is synthesized only when the level of oxygen in the medium falls below 10% saturation. In the presence of nitrate, however, significant nitrate reductase activity is formed at oxygen saturation levels of 40% and below. This suggests that control by nitrate and oxygen are separate, but that the effect of oxygen is "dominant."

Genetic evidence for this conclusion is provided by identification of a regulatory gene, *narL* (367). An insertion mutation, *narL215*::Tn*10*, eliminates induction of nitrate reductase gene expression by nitrate, but has no effect on anaerobic control. Loss of nitrate induction affects both nitrate reductase activity and *narC-lacZ* expression and is complemented in *trans* by a specialized transducing phage. Thus, the *narL* gene product is hypothesized to be a nitrate-responsive positive activator of *narCHJI* operon expression (367). Genetic mapping located *narL* close to the *narCHJI* operon (368). DNA cloning has been confirmed this result and indicates that *narL* is approximately 5 kilobase pairs upstream of the *narCHJI* promoter. The *narK* gene is between *narL* and *narCHJI* (367; Fig. 5).

Li and DeMoss have defined a nitrate-responsive region upstream of the *narCHJI* promoter (238, 239). Deletion analysis shows that sequences between −112 and −215 are required for nitrate induction (238). This result provides independent evidence that *narCHJI* expression is subject to positive control by nitrate and also reveals an interesting example of dual positive control in which the pathway-specific regulator apparently binds DNA sequences upstream of those recognized by the global regulator.

Bonnefoy and colleagues (31) studied the regulation of a chromosomal *nar-lacZ* operon fusion in strains carrying either an F′ *nar* episome or a multicopy *narCHJI* plasmid (pSR95). They found that the operon fusion was poorly inducible by nitrate in these strains. Insertion mutations in the *narCHJI* operon on the multicopy plasmid restored nitrate induction of the chromosomal operon fusion. These results led them to conclude that nitrate reductase gene expression is autoregulated. The physiological relevance of these observations is not obvious. First, nitrate reductase activities were not determined in this study, so the effect on plasmid-borne *nar* gene expression is unknown. DeMoss and colleagues have found that *nar* operon expression from pSR95 is regulated normally by anaerobiosis and nitrate (238, 239, 327). Second, haploid *nar-lacZ* operon fusion strains carry no functional nitrate reductase genes, yet nitrate induction of the operon fusions appears to be an accurate reflection of nitrate reductase gene expression in wild-type strains (54, 99, 367). If functional nitrate reductase acts as a negative regulator of *nar* gene transcription, then the haploid operon fusions should exhibit much greater induction ratios. The proposed autoregulation of nitrate reductase gene expression (31) requires further study.

Molybdate and *nar* operon expression. Sperl and DeMoss (364) noted that *chlD* mutants accumulate fully induced levels of nitrate reductase only when cultured in the presence of 1 mM molybdate. Tungstate-grown cells of the wild type and some *chlD* mutants accumulate an inactive form of nitrate reductase, which can be reactivated in chloramphenicol-treated cells by the addition of molybdate. These and other observations led the authors to conclude that, at normal low concentrations of molybdate, *chlD*$^+$ is required for the accumulation of fully induced levels of nitrate reductase (see section, "Molybdenum Cofactor").

One unresolved dilemma was their finding that two of the five tested *chlD* mutants, including one *chlD* deletion mutant, failed to produce nitrate reductase protein during growth with tungstate, although both strains produced normal levels of nitrate reductase when cultured with molybdate. This result suggested that a different gene in the *chlD* region might be responsible for the accumulation of nitrate reductase protein in tungstate-grown cells. These observations should be reexamined with well-characterized *chlD* mutants, after the genes and products of the *chlD* region are more fully characterized.

Similar results were obtained by Giordano and colleagues (113), who quantitated nitrate reductase protein levels by immunochemical methods. Although *chlD* strains accumulated some nitrate reductase protein in the absence of added

molybdate, higher levels were observed in molybdate- or tungstate-grown cells. Interestingly, a *chlD* deletion strain, different from the one studied by Sperl and DeMoss (364), accumulated high levels of nitrate reductase protein after growth with tungstate. Giordano and colleagues concluded that intracellular molybdate is not absolutely required for nitrate reductase apoprotein synthesis, but that high concentrations of molybdate lead to higher levels of nitrate reductase synthesis.

Pascal and colleagues (297) studied the effects of *chl* mutations on *nar-lacZ* operon fusion expression. In *chlD* strains, the operon fusions are only partially induced by nitrate unless 1 mM molybdate or tungstate is added to the culture medium. Iuchi and Lin (174) confirmed and extended these observations by showing that detectable nitrate induction of *nar-lacZ* expression in a *chlD* strain requires at least 100 nM molybdate in the culture medium, with maximum induction observed at 10 μM molybdate.

These studies indicate that nitrate reductase gene transcription is sensitive to the intracellular levels of molybdate. If molybdate, a trace element, is essentially absent in some of the niches occupied by enteric bacteria, this form of regulation would ensure that cells do not synthesize nitrate reductase under conditions in which it cannot function efficiently. During growth at low extracellular molybdate concentrations, sufficient intracellular molybdate is provided by the *chlD* gene products. The genetic element(s) involved in molybdate-mediated nitrate reductase induction remains to be identified.

Mo cofactor and *nar* operon expression. Pascal and colleagues (297) found that mutations in *chlA*, *chlB*, and *chlE* rendered *nar-lacZ* expression partially constitutive. Different alleles of *chlE* conferred different levels of constitutive expression. This result contradicts the finding that *chlD* mutants have poorly inducible *nar* expression and was interpreted in the context of a complicated model involving repression by molybdopterin (empty cofactor [297]).

Recently, Iuchi and Lin (174) have provided an alternate explanation for these results. They reasoned that *chl* mutants, which are devoid of detectable nitrate reductase activity, would be unable to metabolize trace amounts of nitrate present as a chemical contaminant in phosphate-buffered culture medium. Haploid strains with *nar-lacZ* operon fusions, although defective in the nitrate reductase structural genes, retain measurable nitrate reductase activity contributed by some other molybdo-enzymes (27, 45, 368). Thus, an otherwise wild-type *nar-lacZ* strain can metabolize trace amounts of nitrate present in the medium, whereas *chl* mutants, devoid of molybdo-enzyme activities, cannot; in *chl* mutants, nitrate is a gratuitous inducer. Iuchi and Lin, using a defined, MOPS (morpholinopropanesulfonic acid)-buffered medium, demonstrated that a *chlE* mutation sensitizes the expression of a *nar-lacZ* operon fusion to low levels of nitrate. At 0.1 mM nitrate, the *nar-lacZ* fusion is barely induced in the wild type, but is expressed at nearly full levels in the *chlE* mutant (174).

Pascal and colleagues (300) also concluded that a gene in the *chlE* region is involved in regulation of nitrate reductase and other anaerobic respiratory enzymes. They screened a collection of *chl* insertion mutants for colonies that grew poorly on glycerol plus fumarate. The insertion in one such strain mapped at *chlE*. The original mutant strain had low levels of fumarate reductase and hydrogenase, reminiscent of *fnr*, and undetectable levels of nitrate reductase. They backcrossed the insertion mutation to a *nar-lacZ* operon fusion strain to find that β-galactosidase activity was expressed at high levels in the absence of added nitrate. Unfortunately, they never demonstrated that all of the mutant phenotypes were due to the insertion mutation. It has been found that *fnr* mutations can accumulate in *chl* strains at a significant frequency (368). It is possible that the complex mutant phenotype reported by Pascal and colleagues could be due to an *fnr* mutation, which would reduce fumarate reductase and hydrogenase expression, and the *chlE* mutation, which would abolish nitrate reductase activity. Backcrossing the *chlE* insertion would then render *nar-lacZ* expression hypersensitive to low levels of nitrate.

TABLE 5. Standard redox potentials of selected electron acceptor and donor couples[a]

Couple	E_0' (mV)
O_2/H_2O	+818
NO_3^-/NO_2^-	+433
NO_2^-/NH_4^+	+360[b]
DMSO/DMS[c]	+160
TMAO/TMA[d]	+130
Fumarate/succinate	+33
NAD^+/NADH	−320
H^+/H_2	−432
CO_2/HCO_2^-	−480

[a] Values were extracted from Table 1 in reference 22.
[b] Value quoted in reference 138.
[c] DMS, Dimethyl sulfide.
[d] TMA, Trimethylamine.

NITRATE REGULATION OF ANAEROBIC ENZYME SYNTHESIS

The synthesis of enzyme systems involved in anaerobic respiration (e.g., fumarate reductase) and fermentation (e.g., formate-hydrogen lyase) is prevented by nitrate in all enteric bacteria tested (e.g., references 70, 80, 400, and 423). In general, two types of hypotheses have been proposed to explain this hierarchical control of anaerobic enzyme synthesis. These hypotheses, and the distinctions between them, have been clearly described by Lin and coauthors (171, 229). One idea is that each of the "steps" in the hierarchy (e.g., oxygen respiration, nitrate respiration, fumarate respiration, and fermentation) is mediated by distinct regulators (229, 368). A second idea is that anaerobic regulation responds to redox potential in a continuum (267, 423). In this view, a single regulatory protein could respond to a variety of redox states and differentially affect gene expression accordingly. In the second model, electron acceptor couples with different redox potentials should have different capacities for mediating "redox repression" (Table 5).

The characterization of the *fnr*-dependent regulatory circuit suggests that a continuum of redox potentials does not mediate regulation of anaerobic respiratory enzymes. Synthesis of both nitrate and fumarate reductases is *fnr* dependent, and both are expressed aerobically when FNR is overproduced (348). In contrast, fermentation enzyme synthesis is independent of *fnr* (see above). Thus, at least two distinct pathways are involved in regulation by aerobiosis; it is possible that the effects of nitrate are also mediated by different mechanisms to coordinate with the respective mechanisms of aerobic regulation.

Recent work has examined the regulation of fumarate reductase and formate-hydrogen lyase synthesis. It seems likely that these will become paradigms for understanding hierarchical regulation of enzyme systems involved in respiration (*fnr* dependent) and fermentation (*fnr* independent),

respectively. Although there is much yet to be learned, available data suggest that, at least for fumarate reductase, independent regulatory circuits are involved in hierarchical control.

Fumarate Reductase Gene Expression

Nitrate inhibits the expression of *frd-lacZ* operon fusions in both anaerobic and aerated cultures of *E. coli*; this repression does not require *fnr*$^+$ (173, 193). Iuchi and Lin (173) showed that nitrate repression is abolished by *narL215*::Tn*10*, while control by anaerobiosis is retained. These results provide genetic evidence that anaerobiosis (FNR) and nitrate (NarL) are independent regulators of *frd* expression. Nitrate repression of TMAO reductase formation is also dependent on *narL*$^+$ (173). Other mutations affecting nitrate respiration, including *chlA*, *chlE*, and *nar CHJI*, have little effect on *frd-lacZ* expression (173). Nitrate repression of *frd* expression also requires molybdate, as demonstrated by studies with *chlD* mutants (174).

In addition, Lin and colleagues (171, 172) have isolated and characterized two classes of *cis*-acting mutations that allow aerobic synthesis of fumarate reductase. The first class, *frd* (Con), renders fumarate reductase synthesis insensitive to regulation by aerobiosis, nitrate, fumarate, and *fnr*$^+$. It seems likely that these mutations represent severe promoter alterations or the formation of new, unregulated promoters. The second class, *frd* (Oxr), is more informative: although less dependent on *fnr*$^+$ for anaerobic expression, fumarate reductase expression in these mutants is still induced by fumarate and repressed by nitrate. It is hypothesized that *frd*(Oxr) mutations alter the *frd* promoter so that it does not require FNR for efficient transcription initiation (172). This implies that the *cis*-acting site(s) for control by anaerobiosis is distinct from those required for nitrate repression and fumarate induction (172). These mutations are also inconsistent with the hypothesis (193) that FNR mediates fumarate induction of *frd* operon expression.

Thus, it is likely that aerobiosis and nitrate affect *frd* regulation by independent pathways, rather than through a "redox continuum" which monitors both oxygen and nitrate respiration. Induction of the *nar* operon and repression of the *frd* operon by nitrate share common requirements for both the *narL* gene and molybdate. The simplest hypothesis is that the *narL* gene product is both an activator of *nar* transcription and a repressor of *frd* transcription (173). Further characterization of *narL* and its product is required to test this idea.

Formate-Hydrogen Lyase Gene Expression

Several groups have isolated and characterized operon fusions to *fdhF* (termed *fhl* in *S. typhimurium*) and *hyd*, genes required for formate-hydrogen lyase synthesis (25, 30, 306, 429, 436). *fdhF* is the structural gene for the 80-kDa, Se-containing subunit of formate dehydrogenase (305, 438). One *hyd* fusion, *hyd-17*::Mu d1, abolishes hydrogenase isoenzyme 3, thought to be specifically involved in formate-hydrogen lyase (30). It does not affect hydrogenase isoenzymes 1 and 2. It is not known whether *hyd-17*::Mu d1 defines the structural gene for isoenzyme 3 (30).

Aeration and nitrate inhibit expression of both *fdhF* and *hyd-17*::Mu d1. Formate partially overcomes nitrate repression, but not that by aerobiosis. Birkmann and colleagues suggested that one effect of nitrate may be related to formate metabolism (30). They found that nitrate is a less effective repressor in a mutant lacking formate dehydrogenase-N and thus presumably unable to metabolize formate via formate-nitrate reductase. However, similar results were obtained with an *fnr* mutant, clouding the interpretation; although the *fnr* mutant lacks nitrate reductase, it is also defective in formate production due to reduced pyruvate-formate lyase levels (30, 429).

Some alternate electron acceptors (nitrite and TMAO) also repress *fdhF* transcription (429), while others (fumarate and dimethyl sulfoxide) do not (306, 429). TMAO strongly inhibits *fdhF* expression, while the effect of nitrite is much weaker (429). Thus, there is little correlation between the redox potential of an electron acceptor couple (Table 5) and its ability to mediate redox repression. In contrast to *fdhF*, the expression of *hyd-17*::Mu d1 is significantly reduced by fumarate; the effects of other electron acceptors have not been reported (306).

In *S. typhimurium*, *fdhF* (*fhl*) expression is affected in a medium-dependent manner by *oxrC* (178). Production of formate-hydrogen lyase activity also depends on *oxrC* (179). Conceivably, the repressive effects of alternate electron acceptors could be mediated in part through the *oxrC*-dependent regulatory pathway.

Mutations in *chlA*, *-B*, *-D*, and *-E*, which eliminate Mo cofactor synthesis, have little effect on nitrate repression of *fdhF*. This suggests, first, that nitrate need not be metabolized by nitrate reductase to exert its repressive effect and, second, that Mo cofactor, a component of formate dehydrogenase-H, is not a regulator of *fdhF* (30, 429). Conversely, mutations in *fdhA*, *-B*, and *-C*, which presumably affect Se metabolism, all reduce *fdhF-lacZ* expression, implying that Se is required for *fdhF* transcription initiation (429). Lack of Se would presumably prevent translation of the *fdhF* message (437, 438), so it is important to establish the position of the *lacZ* fusion junction with respect to the UGA codon at position 140 in *fdhF*; premature translation termination could have a polar effect on *lacZ* if the fusion junction is far downstream of the UGA codon (*fdhF* contains 715 codons [438]).

The transcription initiation site for *fdhF* has been determined by S1 nuclease analysis (30). Upstream deletions indicate that sequences between −143 and −184 are required for activation of expression by formate; sites required for oxygen and nitrate control are less well defined but may overlap (30).

Mutants with altered regulation of *hyd-17*::Mu d1 expression were isolated as red colonies on anaerobically incubated MacConkey lactose-nitrate medium (436). Some are pleiotropic Chl^r mutants, but unfortunately these have not been characterized further. A second class of mutants have *cis*-acting lesions that render *hyd-17*::Mu d1 transcription insensitive to nitrate. *hyd-17*::Mu d1 expression in these strains is still repressed by aerobiosis, but, unlike the wild type, formate induces expression in aerated cultures. Thus, these mutants have escaped control by both oxygen and nitrate.

These results were interpreted in terms of a redox continuum model (436). This interpretation will be strengthened by more information. First, the *cis*-acting mutations, although selected to relieve nitrate repression, could be analogous to the *frd*(Con) mutants of Iuchi and Lin (172); it is conceivable that a second class of regulatory mutations similar to *frd*(Oxr) remain to be isolated. Second, the effects of Chl^r mutations should be reevaluated in view of the requirement for *chlD* function in nitrate repression of *frd* transcription (174).

Thus, the hierarchical regulation of *fdhF* and *hyd-17* is still

not well understood and appears to differ at least in detail for the two genes (e.g., in the effect of fumarate). One complication is that, unlike *fnr*-dependent genes, there is no clear candidate for an oxygen-regulatory gene (although *oxrC* may be involved). Thus, one cannot yet determine whether oxygen control is separate from anaerobic respiration control. For *fdhF* expression, the poor correlation between redox potential and repressive effect of alternate electron acceptors appears to argue against a redox continuum model for regulation; whether a similar argument holds for *hyd-17* remains to be seen.

AFTERWORD

We have a rudimentary understanding of central metabolic pathways, but even for such fundamental processes as the TCA cycle the regulation by anaerobiosis and nitrate is poorly understood. The genetics of anaerobic metabolism is in its infancy; for example, the genetics and regulation of hydrogenase isoenzyme synthesis are poorly understood, and the structural genes for such enzymes as TMAO reductase, cytochrome c_{552}, and formate dehydrogenase-N have not been identified. The regulatory circuits for anaerobic gene expression are obviously numerous and complex; much work remains to define these circuits even in outline form. A related challenge is to identify the physiological signals for aerobic and hierarchical regulation.

With respect to nitrate reductase, there are also many significant gaps in our knowledge. The structure of the enzyme is unknown, and we have only a vague understanding of how it spans the cytoplasmic membrane. An attractive hypothesis for the mechanism of proton translocation still awaits direct tests in reconstituted systems. Understanding the structure, genetics, and biosynthesis of Mo cofactor will require an enormous amount of work, particularly from the biochemical side, where the extreme lability of Mo cofactor and its biosynthetic intermediates makes progress difficult.

One sometimes hears statements to the effect that *E. coli* (and by extension all members of the *Enterobacteriaceae*) is so well understood that it has ceased to be an interesting or useful subject for basic research. I hope, at least with respect to nitrate respiration and anaerobic metabolism, that this view will not gain ascendancy. The challenges are numerous, and there are more than enough interesting questions to keep many laboratories occupied for years to come.

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ADDENDUM IN PROOF

A "reductive branch" of the TCA cycle can also be comprised of anaerobically induced malate dehydrogenase and fumarase isoenzymes (J. S. Miles and J. R. Guest, Biochem. Soc. Symp. **54**:45–65, 1987; S. A. Woods and J. R. Guest, FEMS Microbiol. Lett. **48**:219–224, 1987). A new designation, *sel*, has been proposed for pleiotropic *fdh* genes involved in Se metabolism (W. Leinfelder, K. Forchhammer, F. Zinoni, G. Sawers, M.-A. Mandrand-Berthelot, and A. Böck, J. Bacteriol. **170**:540–546, 1988). The *ntrA* gene is required for formate-hydrogen lyase gene expression, and the *fnr* gene is required for formate dehydrogenase-N synthesis (A. Birkmann, R. G. Sawers, and A. Böck, Mol. Gen. Genet. **210**:535–542, 1987). The *S. typhimurium fdn* locus has been resolved into two genes, *fdnB* and *fdnC* (M. T. Paveglio, J. S. Tang, R. E. Unger, and E. L. Barrett, J. Bacteriol. **170**:213–217, 1988). Further characterization of in vitro nitrate reductase activation by Mo cofactor has been reported (G. Giordano, C.-L. Santini, L. Saracino, and C. Jobbi, Biochim Biophys. Acta **914**:220–232, 1987; D. H. Boxer, D. C. Low, J. Pommier, and G. Giordano, J. Bacteriol. **169**:4678–4685, 1987). The *E. coli nar* operon contains a fourth gene, *narJ*, between *narH* and *narI* (E. J. Sodergren and J. A. DeMoss, J. Bacteriol. **170**:1721–1729, 1988). Genetic and biochemical evidence supports the existence of an alternate nitrate reductase (NarZ) in *E. coli* (V. Bonnefoy, J.-F. Burini, G. Giordano, M.-C. Pascal, and M. Chippaux, Mol. Microbiol. **1**:143–150, 1987; C. Jobbi, C.-L. Santini, V. Bonnefoy, and G. Giordano, Eur. J. Biochem. **168**:451–459, 1987). Two-dimensional gel electrophoresis has been used to examine the role of *fnr* in gene regulation (R. G. Sawers, E. Zehelein, and A. Böck, Arch. Microbiol. **149**:240–244, 1988). The *fnr* gene is negatively autoregulated during anaerobic growth, and an interesting consensus sequence for FNR binding has been proposed (S. Spiro and J. R. Guest, J. Gen. Microbiol. **133**:3279–3288, 1987). The *arcA* (*dye*) gene is required for anaerobic repression of many aerobically synthesized enzymes (S. Iuchi and E. C. C. Lin, Proc. Natl. Acad. Sci. USA, in press).

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