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Genes, Enzymes and Membrane Proteins of the Nitrate Respiration System of *Escherichia coli*

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Summary. A method was devised to isolate mutants carrying deletions through several genetic loci (*chlD*$^+$ and *chlA*$^+$) which are involved in the membrane-bound nitrate respiratory complex of *Escherichia coli*. Specific λ transducing phages were used to reintroduce these genes. Comparisons of membrane fractions from these transduced strains showed five membrane proteins that are necessary for the formation of an active nitrate respiration system. Two particular bacterial genes (*chlD*$^+$ and *chlA*$^+$) were shown to control these five membrane proteins. Three of the proteins specified by *chlA*$^+$, appear to be constitutively controlled and always present in the membrane of *E. coli* irrespective of growth conditions, while the other two proteins, specified by *chlD*$^+$, appear to be induced by *anaerobic* growth in the presence of nitrate.

Escherichia coli is a facultative *anaerobe* and can grow in the presence or absence of oxygen. Under *anaerobic* conditions, cell growth is markedly reduced [11, 12]. However, because nitrate can be used as a terminal electron acceptor, the addition of nitrate to the medium enables growth at rates comparable to *aerobic* conditions [4, 5, 21, 31, 37, 39, 40, 41]. This membrane-bound system of electron transport is induced by nitrate and repressed by the presence of oxygen [3, 21, 37] and is summarized in Fig. 1.

The nitrate respiratory complex of enzymes also reduces chlorate to chlorite with lethal consequences for the cell. Thus, mutants lacking nitrate reductase have been isolated because of resistance to chlorate and by a failure to use nitrate as a terminal electron acceptor during *anaerobic* growth [1, 7, 13, 21, 22, 23, 24, 25, 32, 33, 39, 40, 41]. Many of the mutants exhibit a pleiotropic phenotype and have lost in addition to the nitrate reductase activity, the formate hydrogenlyase system and an independent chlorate reductase activity [3, 13, 22, 31, 32, 40, 41]. Genetic analysis of

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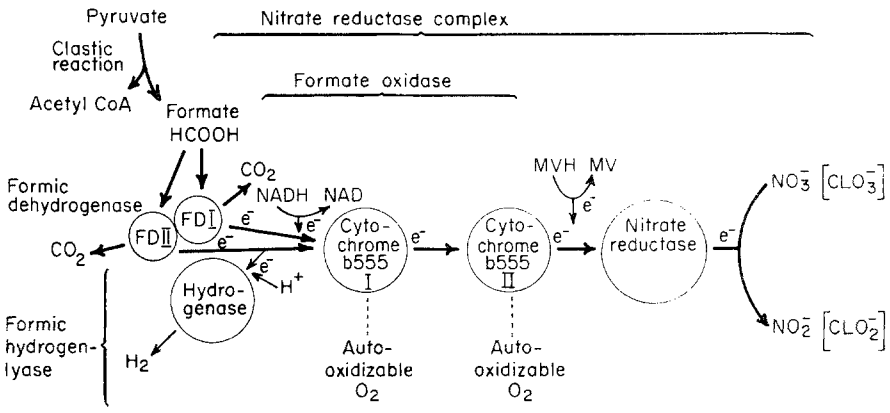

Fig. 1. The nitrate respiratory complex of enzymes of *E. coli* modified after Gray and co-workers [11, 12] and Ruiz-Herrera and DeMoss [31]. It consists of a membrane-bound nitrate reductase, cytochrome *b*1, formate dehydrogenase (FDI), closely associated formate hydrogenlyase complex containing a distinct formate dehydrogenase (FDII) and a hydrogenase

these mutants has shown that at least five different loci affect nitrate reductase activity in *E. coli* [7, 13, 22, 25]. Three of the loci (*chlD*$^+$, *chlA*$^+$ and *chlE*$^+$) specifying components of this complex of respiratory enzymes have been located on the bacterial chromosome near the attachment site of the bacteriophage lambda [1, 2, 20, 22, 23, 24, 27, 29, 36]. In this paper, we wish to describe the interaction of the genes *chlD*$^+$ and *chlA*$^+$ and their effect on membrane structure and function.

Materials and Methods

A. Media

Nutrient broth, minimal medium, phage adsorption buffers and eosine methylene blue (EMB) plates, supplemented with galactose as required, have been described previously [6, 10, 14, 26, 28]. Nitrate medium was prepared by adding sodium nitrate at a final concentration of 5 mg/liter to nutrient broth.

B. Bacterial and Phage Strains

The genotypes of the *E. coli* strains used are shown in Fig. 2. Strain KA 56 contains a mutation in the galactose operon (*gal*) of *E. coli* (gene for epimerase enzyme) which makes it sensitive to the addition of the sugar galactose. The deletion mutant KB5 was derived from strain KA56 [20]. The partially reconstructed strain KB5-1 was derived from KB5 by phage P1 transduction. A *bio*$^+$ (biotin) transductant (KB5-1) was selected and shown to contain the bacterial genes *chlA*$^+$, *uvrB*$^+$, *bio*$^+$, *att*λ and *mdr*$^+$, while

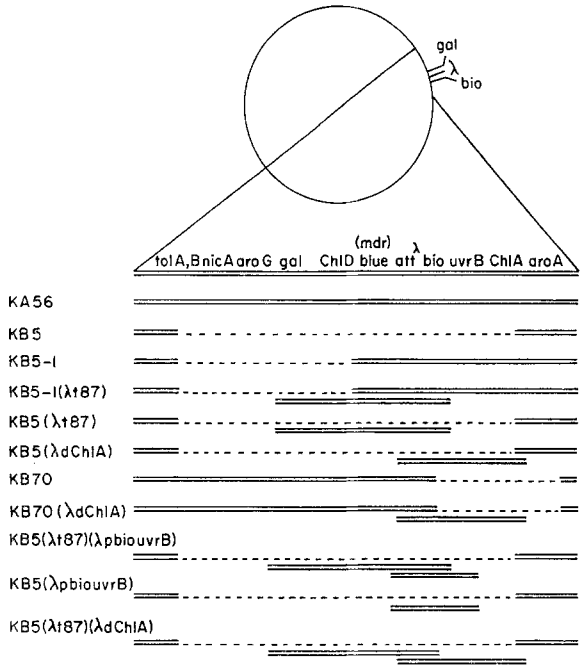

Fig. 2. Genotypes of the series of deletion and constructed strains used in this investigation. The broken line indicates the region deleted from the *E. coli* chromosome. The double lines indicate the bacterial chromosome and transducing phage genome of the constructed bacterial strains. The different λ transductant derivatives of strains KB5, KB5-1 and KB70 are shown with the particular transducing phage indicated in parentheses after each strain. Gene symbols used are *tolA, B* (tolerance to colicins); *nicA* (nicotinic acid requirement); *aroG* (aromatic amino acid synthesis); *gal* (galactose utilization); *chlD* (chlorate resistance, nitrate-chlorate reductase); *mdr* (multi-drug resistance); *blue* (6-phosphogluconolactonase); *att*λ (attachment site for prophage λ); *bio* (biotin synthesis); *uvrB* (ultraviolet radiation repair); *chlA* (chlorate resistance, pleiotrophic mutations affecting nitrate-chlorate reductase and hydrogenlyase activity); and *aroA* (aromatic amino acid synthesis)

still missing the genetic information between the *tolA, B* and *chlD* loci. Strain KB70 was isolated from KA56 as a chlorate-resistant mutant after the method of Adhya, Cleary and Campbell [1]. Strains KB5, KB5-1 and KB70 were transduced with λ phages carrying specific, well-defined genes. Transductant colonies were isolated from plates of minimal medium supplemented with glucose and nicotinic acid (selection for *bio*$^+$ colonies) or with galactose, nicotinic acid and biotin (selection for *gal*$^+$ colonies) and tested for the presence or absence of various bacterial loci. The different λ transductant derivatives of strains KB5, KB5-1 and KB70 are shown in Fig. 2 with the particular transducing phage indicated in brackets after each strain. These reconstructed strains were examined for their ability to maintain their λ transducing particle [26], and were found to be sufficiently stable for both physiological and biochemical analysis.

The λ transducing phages have been described elsewhere, λ*t*87 [26]; λ*dbio uvrB chlA* (hereafter called λ*dchlA*) [22]; λ*pbio uvrB* [10].

C. Preparation of Aerobically and Anaerobically Grown Cell Suspensions

Aerobically Grown Suspensions. These suspensions were prepared by growing cells overnight at 37 °C with vigorous shaking in nutrient broth [14]. These cells were then diluted into fresh prewarmed medium and regrown in the same manner until late exponential phase of growth before being harvested, washed and disrupted as described below. The level of nitrate reductase activity for both mid and late exponentially grown cells was measured to establish whether cell suspensions of late exponential phase were representative of *aerobically* grown cells. It was concluded that the procedures of vigorous shaking to provide aeration and a regular check on the optical density of the growing culture enabled the harvesting of large volumes of *aerobically* grown cells.

Anaerobically Grown Suspensions. The cells were grown for 18 to 20 hr at 37 °C in stationary Erlenmeyer flasks which were three-fourths filled with growth medium and flushed with nitrogen gas before sealing [13]. It should be noted that mutants which lack the nitrate reduction system can still grow *anaerobically* but at a greatly reduced rate.

D. Enzymatic Analysis and Preparation of Particulate Material

Cells were harvested and washed in a Tris buffer and either used as whole cell suspensions or disrupted by a single passage through an Aminco French pressure cell at 20,000 psi. The disrupted cell homogenate was then contrifuged at $3{,}000 \times g$ for 20 min to remove whole cells. The supernatant was centrifuged at $37{,}000 \times g$ for 60 min. The pellet was resuspended in 0.1 M Tris-HCl buffer (pH 8.1) containing 0.1 M $MgCl_2$ and washed 3 times. The final membrane pellet was either resuspended in buffer to be used for the analysis of different enzymes or prepared for polyacrylamide gel electrophoresis. Before carrying out the enzyme assays the amount of protein was measured and the various enzyme suspensions from the different mutants were diluted to equivalent concentration of protein.

Formic Dehydrogenase (FDI). The enzyme was measured by a colorimetric method described by Ruiz-Herrera, Showe and DeMoss [32]. The results were expressed as the change in optical density units at 600 nm per minute per milligram of protein. A second method followed the reduction of methylene blue in Thunberg tubes flushed with nitrogen gas [31].

Enzyme activities of *aerobic* cells grown with vigorous shaking in complete medium without nitrate addition gave values around 0.230 A_{600} units per min per mg of protein for the parent strain. The results of the enzyme assay were calculated as a per cent of the specific activity of the parent strain and indicated as either present (+), absent (−) or as a percentage where some activity was detected.

Formic Dehydrogenase (FDII). The FDII enzyme was measured with the electron acceptor benzyl viologen using Thunberg tubes and the procedure of Ruiz-Herrera and DeMoss [31]. The same amount of protein extract was used in comparative experiments between parent and mutant strains. Where some partial activity was observed in *chlD*$^-$ *chlA*$^+$ cells, the intensity of the developed color was measured and the results expressed as a percentage of the parent strains activity.

Formic Hydrogenlyase System. This enzyme system was observed in tubes containing nutrient broth plus glucose, indicator dye and small inverted Durham tubes [13]. A positive result is indicated by the medium turning yellow (acid production) and the presence of a gas bubble in the Durham tube. The method can be quantitated by measuring the height of the bubble in the Durham tube.

Formic: Nitrate Oxidoreductase. The reaction was measured by the method of Ruiz-Herrera, Showe and DeMoss [32] using either sodium formate or reduced methyl viologen as the electron donor. The specific activity was expressed as μmoles of nitrite formed per min per mg of protein.

Protein Determination. Protein was determined by the procedure of Lowry, Rosebrough, Farr and Randall [17].

E. Extraction of Lipid from Cell Membrane

Preparations of membrane proteins with lipids removed were made as follows. Lipid from the final membrane pellet (prepared as described in section D above) was extracted with 10 ml of ether/ethanol (1:3) solvent for 15 to 18 hr. The extracted membrane was centrifuged at 37,000×*g* for 60 min. The organic solvent was decanted and the membrane pellet was solubilized with phenol/acetic acid/water (2:1:1 w/v/v), centrifuged at 37,000×*g* for 60 min, and the supernatant was the solubilized membrane protein.

F. Preparation of Material for Polyacrylamide Gel Electrophoresis

For electrophoresis on acetic acid-urea gels, lipids were removed from the pelleted particulate fractions as described above. For analysis on SDS gels, the particulate pellets were resuspended in 0.1 ml of dissolving buffer containing 0.6 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.5 M urea and 1% 2-mercaptoethanol solution. The membrane pellets were left to solubilize overnight in the cold. To further dissociate the protein, the membrane fraction was boiled for 3 min and the amount of protein determined. The amount of protein layered on to each SDS gel was between 150 and 200 μg.

G. Polyacrylamide Gel Electrophoresis

Acetic acid-urea gels contained 5% (w/v) acrylamide, 0.1% *bis*-acrylamide in 25% (v/v) acetic acid and 2.5 M urea. The gels were run in 10% (v/v) acetic acid, 1 M urea at 3.3 ma/tube for 4 hr. The protein in the gel was fixed in 12.5% trichloroacetic acid (TCA) and stained with Amido Black 10B [30].

Sodium lauryl sulfate-acrylamide (SDS) gel electrophoresis was performed in a discontinuous gel system. Gels of 10 cm were prepared in glass tubes with a diameter of 0.6 cm. The stacking gel of 3% acrylamide and 2 cm in length contained 0.08% *bis*-acrylamide and 0.1% SDS in 0.125 M Tris buffer (pH 6.8) and contained TEMED and ammonium persulfate at the same concentration as the separating gel. The separating gel had an acrylamide concentration of 7% and *bis*-acrylamide concentration of 0.02% with 0.1% SDS in 0.375 M Tris buffer (pH 8.8). TEMED and ammonium persulfate were added to a concentration of 0.025% for initiating and catalyzing the polymerization reaction. The gels were run in a buffer containing 0.15% Tris, 7.2% glycine and 0.1% SDS. The amount of membrane sample applied directly to the top of each gel was 150 to 200 μg protein. One of the gels was kept for the electrophoresis of a 0.2-ml sample of a 1% solution of bromo phenol blue dye. The gels were run at 2.5 ma/gel until the complete passage of the dye in the indicator gel. The gels were fixed and stained for 36 hr in 12.5% TCA containing 0.1% Coomassie brilliant blue [34]. The gels were destained by repeated washings of 7% acetic acid in a 37 °C room.

Results

Deletion Mutants and Reconstructed Strains

Strain KA56 is killed by both galactose and a wide variety of bacterial colicins (protein antibiotics liberated by certain bacterial strains) [19]. Mutations to tolerance to the killing action of a group of these colicins occur at a site (*tolA, B*) on the bacterial chromosome near the galactose operon of *E. coli* [38]. A double selection procedure was devised using EMB plates containing both galactose and a group of colicins, to isolate from strain KA56 a whole series of deletion mutants which were resistant to the killing effects of both these agents [20, 27]. This method enables the isolation of mutants carrying deletions both into and through the nitrate reductase loci *chlD* and *chlA* without involving the use of the nitrate reductase complex as part of the selection system (Fig. 2). One such mutant strain KB5 has lost the bacterial genes located in the region of the attachment site of phage λ due to a deletion of the chromosome between the genes *tolA, B* and *chlA* (Fig. 2; refs. [20, 27]). This strain was used as the recipient for a series of transduction experiments using either phage P1 or different λ transducing phages. Strain KB5-1 ($chlD^-$ $chlA^+$) was constructed from mutant KB5 ($chlD^-$ $chlA^-$) by phage P1 transduction. Selection on different media (*see* Materials and Methods) enabled bacterial strains KB5 and KB5-1 to be transduced with λ phages which carry specific genes. These genes are located on either side of the attachment site used by phage λ to integrate into the *E. coli* chromosome (Fig. 2). Thus it was possible to reintroduce the $chlD^+$ or $chlA^+$ loci or both by the addition of specific λ transducing phages.

Enzymic Activity of the Nitrate Respiratory Complex

A comparison of the enzymic activities of the nitrate respiratory complex from either whole cells or membrane preparations confirmed the pleiotropic phenotype found with chlorate mutants of the *chlA* locus, where a complete loss occurs of many of the activities normally associated with the complex (Table 1). An exception appears to be strains of the $chlD^-$ $chlA^+$ genotype (strains KB5-1 and KB5 ($\lambda dchlA$)) which still have some formic dehydrogenase activity (both FDI and FDII enzymes) and partial formic oxidase activity. However, after *anaerobic* growth in the presence of nitrate, no formic dehydrogenase activity could be detected (Table 2).

A third bacterial gene which is involved in the nitrate respiration system and located on the bacterial chromsome near both the genes *chlA* and *att* λ is the chlorate resistance locus *chlE* [38, 39]. As part of the characterization

Table 1. Nitrate respiratory complex in *aerobically* grown cultures

Strain genetic characteristics	Formate hydrogenlyase		Nitrate reductase system			Formate oxidase
	Acid and gas production	FDII	FDI	Cytochrome *b*1 complex	Nitrate reductase	
chlD$^+$ *chlA*$^+$	AG	100	100	+	100	100
chlD$^-$ *chlA*$^-$	A	0	0	+	0	0
chlD$^-$ *chlA*$^+$	A	~5	~20	+	0	~10
chlD$^+$ *chlA*$^-$	A	0	0	+	0	0

The enzyme results in Tables 1, 2 and 3 are shown as the detected per cent of wild-type activity (*see* Materials and Methods). The different strain genotypes represent the various strains shown in Fig. 2 which were used in Tables 1 through 4 and Figs. 3 and 4.

Table 2. Nitrate respiratory complex in cultures grown *anaerobically* in the presence of nitrate

Strain genetic characteristics	Formate hydrogenlyase		Nitrate reductase system		
	Acid and gas production	FDII	FDI	Cytochrome *b*1 complex	Nitrate reductase
chlD$^+$ *chlA*$^+$	AG	100	100	+	100
chlD$^-$ *chlA*$^-$	A	0	0	+	0
chlD$^-$ *chlA*$^+$	A	0	0	+	0
chlD$^+$ *chlA*$^-$	A	0	0	+	0

Table 3. Effect of gene *chlA*$^+$ in a *chlA*$^-$ *chlE*$^-$ deletion mutant

Bacterial strain	Relevant strain genotype	Formic hydrogenlyase		Nitrate reductase system	
		Acid and gas production	FDII	FDI	Nitrate reductase
KA56	*chlA*$^+$ *chlE*$^+$	AG	100	100	100
KB70	*chlA*$^-$ *chlE*$^-$	A	0	0	0
KB70 (λ*dchlA*)	*chlA*$^+$ *chlE*$^-$	A	100	100	0

The various strains were grown to late exponential phase before harvesting for enzyme analysis.

of the interaction between the *chlE* gene and the *chlA* locus, the *chlA*$^+$ gene was reintroduced into a *chlA*$^-$ *chlE*$^-$ deletion mutant (KB70) by λ phage transduction (Fig. 2). When the enzyme properties of this reconstructed

strain KB70 (*λdchlA*) were examined, it was found that its formic dehydrogenase activities (FDI and FDII) were similar to parent strain KA56 (Table 3). However, the transduced strain KB70 (*λdchlA*) (still *chlE*$^-$) was unable to utilize its hydrogenlyase system under *anaerobic* growth conditions (Table 3). Examination of the cell inoculum used in these tests [29] confirmed that the transducing phage was still present during each growth experiment. The results therefore, suggest that the *chlE*$^+$ locus influences preferentially the hydrogenase enzyme activity rather than the FDII enzyme activity of this complex.

Loss of Membrane Components

An attempt was made to discover which bacterial genes controlled which proteins of the nitrate respiration complex. Membrane fractions were prepared from *aerobically* grown cultures and after lipid extraction, the solubilized membrane proteins (*see* Materials and Methods) were analyzed on acetic acid-urea polyacrylamide gels after the method of Rottem and Razin [30]. A comparison of membrane fractions shows that the *chlA*$^+$ locus (rather than *chlD*$^+$) controls either directly or indirectly three protein components (Fig. 3). Similar results were obtained by comparison of membrane fractions of *aerobically* grown cells on SDS polyacrylamide gels (Table 4; *see* Fig. 4 for typical band pattern). However, when membrane preparations of *anaerobically* grown cells were compared, two proteins were found to be controlled by the *chlD*$^+$ locus (Table 4). Thus *chlA*$^+$ is phenotypically expressed under both *aerobic* and *anaerobic* conditions but *chlD*$^+$

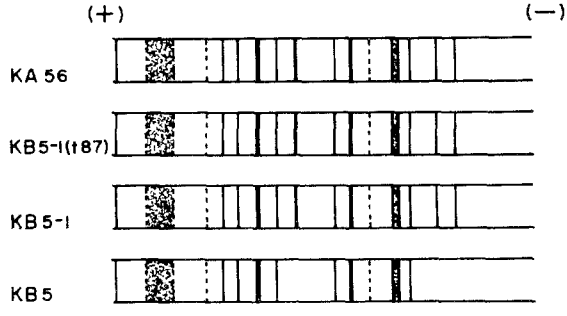

Fig. 3. Analysis of membrane protein components (after lipid removal) by electrophoresis using acetic acid-urea polyacrylamide gels. Strains used; parent KA56, deletion mutant KB5 (*chlD*$^-$ *chlA*$^-$) constructed strains KB5-1 (*chlD*$^-$ *chlA*$^+$), and KB5-1 (*t*87) (*chlD*$^+$ *chlA*$^+$). Strains also analyzed were KB5 (*λpbio uvrB*), KB5 (*λdchlA*), and KB5 (*λt*87) (*λdchlA*). Strains KB5 (*λdchlA*) and KB5 (*λt*87) (*λdchlA*) had a gel profile the same as KB5-1 while KB5 (*λpbio uvrB*) was the same as KB5

Table 4. Presence of nitrate respiratory components as a function of strain genotype

Condition	Protein band no.	Genotype			
		chlD$^+$ *chlA*$^+$	*chlD*$^-$ *chlA*$^+$	*chlD*$^+$ *chlA*$^-$	*chlD*$^-$ *chlA*$^-$
A	1	−	−	−	−
	2	−	−	−	−
	3	+	+	−	−
	4	+	+	−	−
	5	+	+	−	−
B	1	+	−	+	−
	2	+	−	+	−
	3	+	+	−	−
	4	+	+	−	−
	5	+	+	−	−

Analysis of membrane proteins prepared from (*A*) *aerobically* grown cultures (with and without nitrate), and (*B*) *anaerobically* grown cultures in the presence of nitrate, using SDS gel electrophoresis as described in Materials and Methods. Refer to Fig. 4 for typical gel profiles. The sign (+) indicates presence and (−) indicates absence of a particular protein band on the gels.

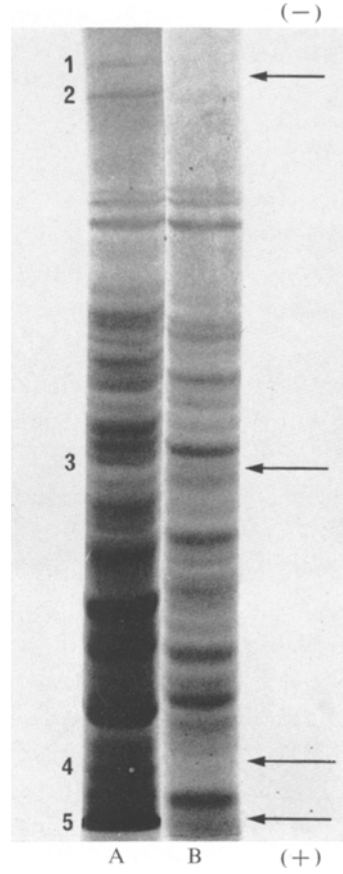

Fig. 4. Analysis of membrane proteins from cells grown *anaerobically* in the presence of nitrate using SDS gel electrophoresis as described in Materials and Methods; gel *A* (parent strain KA 56) and gel *B* (deletion mutant KB 5 (*chlA*$^-$ *chlD*$^-$). The gels show the typical band pattern obtained with this procedure. Similar gels were used for the results recorded in Table 4

requires *anaerobic* conditions for expression. At present, using the same methods and membrane preparations, it has not been possible to detect membrane components specified by the *chlE*$^+$ locus.

Discussion

From the analysis of membrane preparations from strains used in this investigation, it is argued that the genetic locus *chlA*$^+$ controls, either directly or indirectly, at least three different membrane proteins. For these components to be under the control of genes other than the *chlA*$^+$ locus, would mean that the proposed locus would have to be closely linked with *chlA* and located between genes *uvrB* and *chlA* on the chromosome. Against this possibility is: (a) the physical limit on how much information can be carried by the particular *λdchlA* transducing phages that were used [15]; (b) a preliminary investigation of point mutants in the *chlA* locus show a loss of bands similar to *chlA* deletion strains; and (c) recent genetic analysis indicates that there are at least two complementation groups for the *chlA*$^+$ locus in *E. coli* (W. A. Venables, *personal communication*; Guest & Rolfe, *unpublished*).

Genetic control of two membrane components, only observed in membrane preparations from cultures grown *anaerobically* was attributed to the *chlD*$^+$ locus. The control of the observed bands must be located between the bacterial genes *blue* and *gal* on the chromosome as strains carrying a deletion of the *blue* locus still have a normal nitrate respiration system (Onodera & Rolfe, *unpublished*). This would locate the genetic control in the region of the *chlD*$^+$ locus. In addition, genetic studies indicate that *chlD*$^+$ contains at least two complementation groups (W. A. Venables, *personal communication*).

MacGregor and Schnaitman [18] recently, have described electrophoretic gel profiles which show four components in the cytoplasmic membrane which influence the nitrate respiration system. One of the protein bands was identified as the nitrate reductase enzyme which is present in *chlA*$^-$ mutants in a nonfunctional form but absent in *chlC*$^-$, *chlD*$^-$ and *chlE*$^-$ mutants [18]. Presumably, one of the stainable bands observed in this investigation could represent the nitrate reductase enzyme.

The synthesis of the nitrate reductase of *E. coli* is controlled by two principle growth conditions: (a) the presence of nitrate, which acts to induce, and (b) aeration of the medium which serves to repress [37]. The observation that the components of the nitrate respiratory complex may be differentially affected in *anaerobiosis* may be an important step to an understanding of

the nature of the regulatory control of this system in *E. coli*. Apparently, the gene products under the control of the *chlA*$^+$ locus are *constitutive* proteins and form part of the membrane irrespective of the presence of nitrate and the aeration of the growth medium. In constrast, the presence of membrane proteins controlled by the *chlD*$^+$ locus are apparently subject to the levels of oxygen and nitrate in the growth medium and are probably *inducible* proteins of the nitrate respiratory complex. However, although it has been impossible to detect gene products from the *chlD*$^+$ locus in membrane preparations from *aerobically* grown cultures, some contribution from this locus to membrane organization seems necessary to obtain complete formic dehydrogenase (FD I) activity. Recently, evidence has been presented on the effect of molydbate and selinite on formate and nitrate metabolism in *E. coli* with particular reference to the restoration of nitrate reductase activity in *chlD*$^-$ mutants [9, 16]. It was concluded that the *chlD*$^+$ locus might be responsible for processing molybdate in a form which is capable of activating nitrate reductase and the formate hydrogenlyase system and play a role in the regulation of the synthesis of the components of both enzyme systems [9]. It is well documented that changes occur in the activities of oxidative enzymes in cells changed from *aerobic* to *anaerobic* growth conditions [11, 12], although only minor alterations in the major membrane proteins were observed during such growth changes [34, 35]. Experiments are in progress to determine the relationship between this differential contribution of membrane components by the various genetic loci (especially *chlD*$^+$ and *chlA*$^+$) in response to different growth conditions and the regulation of the nitrate respiratory complex.

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