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Role of the *chlC* Gene in Formation of the Formate-Nitrate Reductase Pathway in *Escherichia coli*

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Five temperature-sensitive *chlC* mutants were isolated from *Escherichia coli* by the technique of localized mutagenesis. All of the mutants produced severely reduced levels of both nitrate reductase and formate dehydrogenase when grown at 43°C. In three of the mutants, the nitrate reductase activity produced at the permissive temperature was shown to be thermolabile compared with the activity produced by the parent wild-type strain, both in membrane preparations and in preparations released from the membrane by deoxycholate. In each case, formate dehydrogenase activity was similar to the wild-type activity in its stability to heat. It is concluded that the *chlC* gene codes for at least one of the polypeptide chains of nitrate reductase and that the *chlC* mutations affect indirectly the formation of formate dehydrogenase.

The formation of the formate-nitrate reductase system, which includes a specific formate dehydrogenase, cytochrome b_1, and nitrate reductase (14, 15), is under the control of at least seven genetic loci in *Escherichia coli* (1, 4, 5, 12, 13, 19). Mutants defective in the formation of this membrane-bound, multienzymatic pathway have been selected by several different procedures and have been variously designated *chl* (12, 13), *nar* (5, 19), and NR^- (14) mutants. Because most of the mutants are resistant to chlorate, they are now generally designated *chl* mutants.

Four of the *chl* genes (*A, B, D,* and *E*) are pleiotropic; mutations at these loci lead to the loss of both formate dehydrogenase and nitrate reductase as well as another anaerobic electron transport pathway, the formate hydrogenlyase system (4, 13). Except for *chlD*, which affects molybdenum uptake or processing (3, 18), the functions of these genes are unknown.

It has generally been concluded that the *chlC* locus is the structural gene for nitrate reductase because mutations at this locus result in the loss of nitrate reductase without affecting the formation of the formate hydrogenlyase system (4, 5, 12, 13). However, the *chlC* mutations also lead to a 10 to 90% decrease in the specific activity of formate dehydrogenase (4, 5), creating some doubt about the assignment of this locus as the structural gene of nitrate reductase.

For two of the remaining genes, *chlF* and *chlG*, only a few mutants have been isolated, and these are poorly characterized (4). *chlF* mutants were indistinguishable from *chlC* mutants by conjugative mapping but were separated as a distinct genetic locus on the basis of frequency of cotransduction with *trp* (4). The mutations designated as *chlF* were cotransduced with *trp* at a frequency of less than 10%, whereas *chlC* mutations exhibited frequencies of 40 to 60%. Because formate dehydrogenase was depressed more severely than nitrate reductase in the *chlF* mutants, it was suggested that *chlF* may be a structural gene for formate dehydrogenase. However, no compelling biochemical or genetic evidence has been obtained to support this idea, and the role of the *chlF* gene remains unknown.

To understand the role of the various *chl* genes in the expression of the nitrate reductase system, it is essential that the structural genes of the various polypeptide components be established. In the present study, several temperature-sensitive *chlC* mutants have been isolated and studied in an attempt to define the role of this gene in the formation of the formate-nitrate reductase system.

MATERIALS AND METHODS

The wild-type strain *E. coli* K-12 strain PK27 (4) and the mutant strains were maintained on L agar (7) and grown on L broth (7) or minimal medium (20) supplemented as indicated.

Phage P1 transduction was carried out employing the general condition described by Lennox (7) except that *E. coli Δtrp ins*, obtained from E. J. Murgola, was used as the indicator strain for titrating the phage.

Mutant *trpDL1* is a tryptophan auxotroph derived from strain PK27 by P1 transduction from *E. coli* C9830, a *trpC* mutant obtained from C. Yanofsky. The

temperature-sensitive mutants TS4, TS5, TS6, TS8, and TS9 were isolated by employing the localized mutagenesis technique of Hong and Ames (6) as modified by Murgola and Yanofsky (11). Phage P1, propagated on wild-type strain PK27, was mutagenized with hydroxylamine and used to transduce strain *trpDL1* to prototrophy. The prototrophic transductants were selected on minimal medium supplemented with 0.2% Casamino Acids and stabbed into three L-agar plates. Plates were incubated at 25, 37, and 43°C until appropriate growth occurred, and the plates from 25 and 43°C were scored for formate-nitrate reductase activity by the agar overlay technique of Glaser and DeMoss (4). The strains that exhibited little formate-nitrate reductase activity at 43°C relative to strain PK27 but exhibited approximately equal levels at 25°C were isolated from the corresponding 37°C plates by restreaking on L agar. Only those strains that produced growth zones equivalent to the wild type were selected for restreaking, and the isolates were finally retested for temperature-sensitive, formate-nitrate reductase activity by the overlay technique.

Strain TS9A was isolated after P1-mediated transduction of the temperature-sensitive phenotype from mutant TS9 into strain PK27. The temperature-sensitive transductants were selected by plating on L agar supplemented with 0.2% potassium chlorate and incubating at 43°C. The resulting chlorate-resistant colonies were picked and scored for the temperature-sensitive, formate-nitrate reductase phenotype as above. Strain TS9A was isolated from one such colony by restreaking on L agar and retesting by the overlay technique.

For the assay of activities, strains were grown on L broth supplemented with 1% potassium nitrate sparged with a mixture of 95% N_2 and 5% CO_2. Cultures were harvested at 80 to 100 Klett units (no. 54 filter); the cells were washed once with 0.05 M potassium phosphate (pH 7.0) and resuspended in the same buffer for assay or for preparation of extracts.

Extracts were prepared from 10% cell suspensions (wet wt/vol) by passing through a French press and centrifuging the crude extract at 3,000 × *g* for 15 min to remove whole cells. Ammonium sulfate (23 g/100 ml) was added to the supernatant, and the mixture was stirred for 1 h at 4°C. The membrane fraction was sedimented by centrifugation at 39,000 × *g* for 45 min and finally suspended in 0.05 M potassium phosphate (pH 7.0) for assay. Nitrate reductase was released from the membrane fraction by addition of deoxycholate (1 mg/mg of protein) and ammonium sulfate (0.30 saturation) as described by Scott and DeMoss (16). The supernatant remaining after removal of the residual membrane fraction by centrifugation was used for assay without further purification.

Formate dehydrogenase (15) and nitrate reductase (17) activities were assayed in both whole-cell suspensions and cell extracts as previously described and are expressed in units of micromoles per minute.

For the assessment of temperature stability of enzyme activities, extracts (0.3 to 2.0 mg of protein per ml) were incubated at the temperature indicated; samples were withdrawn at the indicated intervals and immediately assayed at 37°C.

Protein was determined by the Lowry procedure (8), and specific activity was expressed as units per milligram of protein.

RESULTS

A series of putative temperature-sensitive *chlC* mutants were isolated by employing the localized mutagenesis technique (6). With the agar overlay procedure of Glaser and DeMoss (4), mutants were selected that exhibited considerably less formate-nitrate reductase activity after growth at 43°C than at 25°C. This was in contrast to the parental strain, PK27, which exhibited considerably more activity after growth at 43°C than at 25°C. To eliminate possible indirect growth effects, only those mutants that produced larger zones of growth at 43°C than at 25°C were selected for further study.

To establish that the temperature-sensitive mutants were *chlC* mutants, the frequency of cotransduction of the temperature-sensitive phenotype with *trp* was determined with five of the isolates. Of the known *chl* genes, only *chlC* and *chlF* are cotransduced with *trp*, and only *chlC* is cotransduced at a high frequency with *trp* (4). Phage P1 was propagated on mutant strains TS4, TS5, TS6, TS8, and TS9 and used to transduce with strain *trpDL1* to auxotrophy. For all five mutants, cotransduction frequencies of 37 to 46% were obtained for the temperature-sensitive phenotype with the *trp* marker, verifying that all five are *chlC* mutants.

Because reduced formate-nitrate reductase activity can result from reduced levels of either formate dehydrogenase or nitrate reductase activity, the temperature-sensitive mutants were assessed for their ability to produce these activities at the restrictive and permissive temperatures (Table 1). The specific activity of nitrate reductase in the wild-type strain PK27 was about twofold higher in cells grown at 43°C than in those grown at 25°C. Compared with the wild-type, the temperature-sensitive strains produced reduced levels of nitrate reductase activity at both 25 and 43°C. However, the specific activity of nitrate reductase was reduced 30- to 50-fold in cells grown at 43°C and reduced only 4-fold in those at 25°C, resulting in each case in a lower specific activity in 43°C cells than in 25°C cells. The mutants were also affected in their ability to produce active formate dehydrogenase. The specific activity of this enzyme was reduced at both temperatures, relative to the wild type, but the relative levels at 25 and 43°C were different in different mutants. In mutants TS8 and TS9, the specific activity of formate dehydrogenase was severalfold lower in cells grown at 43°C compared with those grown at 25°C. The specific activity of formate dehydro-

genase in mutant TS4 was severalfold higher in cells grown at 43°C compared with those grown at 25°C, in contrast to the pattern observed for nitrate reductase. These results demonstrated that temperature-sensitive *chlC* mutations affect the formation of both formate dehydrogenase and nitrate reductase activities. These data are in agreement with the earlier demonstration by Guest (5) and by Glaser and DeMoss (4) that many *chlC* mutations lead to a reduction in formate dehydrogenase activity, and they further emphasize the ambiguity of the assignment of *chlC* as the structural gene for nitrate reductase.

To eliminate any possible differences in the genetic background of the mutants and the wild-type strain, one of the temperature-sensitive lesions was transferred into PK27 without tryptophan selection by the procedure outlined above. The resulting strain, TS9A, produced enzyme levels similar to its temperature-sensitive parent, TS9, at 25 and 43°C. This rules out the possible involvement of other unlinked genetic factors that might have been present in the recipient strain in which the mutants were originally isolated.

To assess what direct effects these mutations have on the structures of the enzymes, the relative heat stabilities of formate dehydrogenase and nitrate reductase were examined in the membrane fraction of cells grown at the permissive temperature (Fig. 1). The wild-type strain PK27 and mutants TS4, TS9, and TS9A were grown at 25°C, and membrane fractions were

TABLE 1. *Effect of growth temperature on activities of the formate-nitrate reductase*

Strain	Growth temp (°C)	Formate dehydrogenase (U/mg of protein)[a]	Nitrate reductase (U/mg of protein)[a]
PK27	25	0.29	1.2
	43	0.79	2.7
TS9	25	0.045	0.33
	43	0.013	0.051
TS8	25	0.049	0.26
	43	0.012	0.055
TS4	25	0.046	0.28
	43	0.084	0.047
TS5	25		0.30
	43		0.089
TS6	25		0.23
	43		0.098

[a] Determined in whole cell suspension at 37°C as described in the text.

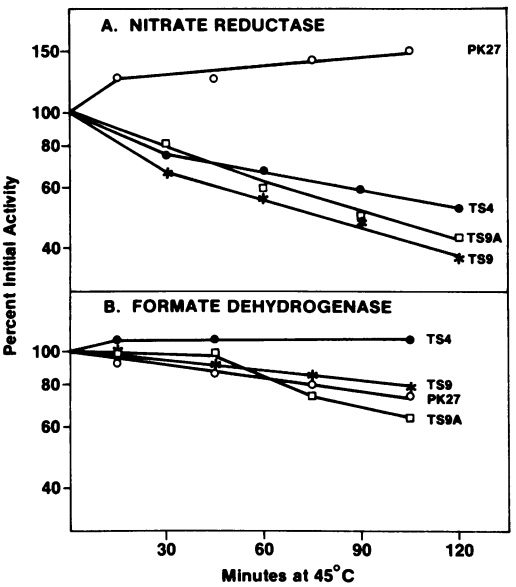

FIG. 1. *Stability of activities in membrane fractions at 45°C. The fractions were incubated and assayed as described in the text.*

prepared by differential centrifugation after disruption in a French press. In the preparation from wild type, nitrate reductase activity was quite stable at 45°C and actually increased about 50% over the incubation period. It has been shown previously that nitrate reductase can be released from the membrane by heating at 60°C and that a significant activation occurred during that release (9, 10). In the membrane preparation from each mutant, nitrate reductase activity was relatively unstable at 45°C, with inactivation occurring with a half-time of 80 to 120 min. In contrast, formate dehydrogenase in the membrane preparations from the temperature-sensitive mutants was as stable as, or more stable than, the activity in the membrane fraction from the wild type.

These results established that mutants TS4, TS9, and TS9A produce a temperature-sensitive form of membrane-bound nitrate reductase. It remained possible that the alteration of some other component that is associated with nitrate reductase in the membrane was leading to the temperature-sensitive state. To eliminate this possibility, nitrate reductase was released from membrane preparations of the wild type and of mutant TS9A by treatment with deoxycholate and ammonium sulfate as described above. This procedure has been used to release nitrate reductase from the membrane in a form that can be purified to homogeneity free of other membrane components (2). The solubilized nitrate reductase from the mutant was markedly tem-

perature sensitive relative to that from the wild type (Fig. 2).

DISCUSSION

The results presented here establish that the *chlC* gene codes for at least part of the polypeptide structure of nitrate reductase, because mutations at this locus result in the formation of a temperature-sensitive enzyme. Nitrate reductase is composed of two distinct polypeptide chains (9, 10), and it is not possible to determine which of the polypeptides is rendered temperature sensitive in the mutants studied because conditions have not been established for dissociation and reconstitution of the enzyme. Because no other genetic loci are known to specifically affect nitrate reductase activity, it remains a possibility that the *chlC* gene codes for both polypeptide chains of the enzyme.

Mutations at the *chlC* gene also result in reduced levels of formate dehydrogenase activity, although the heat stability properties of this enzyme are not affected by the temperature-sensitive mutations. There are several possible explanations for the reduction in formate dehydrogenase levels in *chlC* mutants. This enzyme may be associated with nitrate reductase in the membrane, and its synthesis or turnover may be affected by the presence of stable nitrate reductase. Alternatively, the formation of formate dehydrogenase may be regulated directly or indirectly by the activity of nitrate reductase. Another possibility suggested by Guest (5) is that formate dehydrogenase (or at least one of its peptides) is encoded by an adjacent gene that is part of an operon including the *chlC* gene and that *chlC* mutations exert polar effects on its expression. The latter possibility is attractive because several mutants (*chlF*) that appeared to be most severely affected in their formate dehydrogenase activity were found to map near the *chlC* locus.

It has not been possible to carry out reversion studies to establish that both the temperature-sensitive phenotype and the reduced level of formate dehydrogenase are the result of a single mutation. Revertants to wild type are apparently too rare to detect with the nonselective procedure of Glaser and DeMoss (4), and the mutants grow too well at the nonpermissive temperature on the selective lactate-nitrate medium of Venables and Guest (19) to permit the isolation of revertants by that procedure. Although it has not been proven by reversion studies that both characteristics are the result of a single mutation in the *chlC* gene, the results strongly suggest that this is the case. Both were characteristics of the three temperature-sensitive mutants that were examined in detail, and both were transferred at high frequency with *trp* during phage P1 transduction.

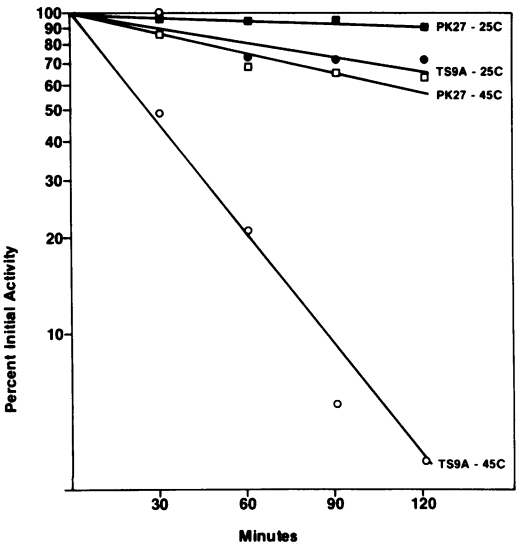

FIG. 2. *Stability at 25 and 45°C of nitrate reductase released from membrane fractions by deoxycholate treatment. Nitrate reductase was released, and the samples were incubated and assayed as described in the text.*

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