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Use of *chlC-lac* Fusions to Determine Regulation of Gene *chlC* in *Escherichia coli* K-12

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Gene fusions between the *lac* structural genes and the *chlC* locus were isolated, and the regulation of *lac* gene expression was studied. The fused *lac* genes were induced by nitrate anaerobically and repressed by the presence of oxygen.

The membrane-bound, proton-translocating, anaerobic formate-dependent nitrate reductase activity of *Escherichia coli* has been studied extensively both biochemically and genetically (13). Nitrate reductase (EC 1.7.99.4) as isolated from the cytoplasmic membrane is a molybdenum, iron-sulfur protein composed of two nonidentical subunits designated α and β; a third polypeptide, the γ-subunit or cytochrome $b_{556}^{NO_3^-}$, is required for functional ubiquinol-dependent nitrate reductase activity. A structural gene for nitrate reductase, designated *chlC*, has been identified from analysis of membrane proteins of a *chlC* mutant using specific antisera (18) and from the isolation of temperature-sensitive *chlC* mutants (9). It is thought that the *chlC* gene codes for the α-subunit of nitrate reductase (18), and mutants with a specific defect in their ability to synthesize the β-subunit have not yet been described. In addition it is known that conversion of the apopolypeptide product of the *chlC* gene into a functional holoenzyme requires the expression of at least three other genes, designated *chlA*, *chlB*, and *chlD*, which are apparently required for the synthesis and/or insertion of the molybdenum and iron-sulfur centers into both nitrate reductase and formate dehydrogenase (EC 1.2.1.2). The structural gene for the γ-subunit (cytochrome $b_{556}^{NO_3^-}$) has been tentatively ascribed to the *chlE* gene (18). There appears to be no genetic linkage between any of the *chl* genes so far described.

The synthesis of nitrate reductase is induced by growth anaerobically in the presence of nitrate and repressed by growth in the presence of oxygen (13). Formate dehydrogenase, however, is synthesized by *Escherichia coli* under both aerobic and anaerobic growth conditions. Therefore, the factors regulating the expression of the *chlC* and *chlE* genes must be different from those regulating the *chlA*, *chlB*, and *chlD* genes. Since the product of the *chlC* gene has no known enzymatically assayable activity (in the absence of expression of the *chlA*, *chlB*, and *chlD* genes), we have employed the technique of operon fusion described by Casadaban (5) in our analyses of the control mechanism(s) regulating the specific expression of the *chlC* gene. The basis of this technique is to transpose the *lac* structural genes so that they are placed under the control of the particular regulatory genes under study. We describe here a new procedure for the specific identification of *chlC* mutants and the isolation of gene fusion strains in which β-galactosidase (EC 3.2.1.23) synthesis and growth on lactose only occurs under anaerobic conditions in the presence of nitrate, implying fusion of the *lac* structural genes to the promoter/operator region regulating the *chlC* locus.

MATERIALS AND METHODS

Chemicals. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine and *o*-nitrophenol-β-D-galactopyranoside were obtained from Sigma Chemical Co., St. Louis, Mo. Benzamidine was from Aldrich Chemical Co., Gillingham, England. 2,3,5-Triphenyltetrazolium chloride (TTC) and all other reagents were from British Drug Houses, Poole, England.

Media. All solidified media contained 1.5% (wt/vol) agar. L-broth (16) was used routinely with the exceptions (where indicated) of: tryptone broth (supplemented with 0.2% [wt/vol] maltose [23]), tryptone agar, and tryptone top agar (11). The solid minimal medium used was medium E of Vogel and Bonner (25), supplemented with glucose 0.2% (wt/vol), amino acids (40 μg/ml), thiamine (4 μg/ml), and other required growth factors. KNO_3 (0.1 M) and potassium fumarate (0.05 M) were added where indicated. Lactose MacConkey plates were prepared as described by Miller (20). TTC was added at a concentration of 50 μg/ml to glucose-nitrate minimal plates.

Cells used for enzyme assays were grown in liquid minimal Cohen and Rickenberg medium (8) supplemented as described for medium E.

Bacterial and phage strains. All the bacterial strains used were derived from *E. coli* K-12. Bacterial and phage strains are listed in Table 1.

Transduction. Transductions with the generalized transducing phage P1 were carried out according to Miller (20).

Preparation of phage lysates. Mu*cts* lysates were prepared by temperature induction of the lysogenic strain KMBL1614 and Mu*c* lysates by lytic growth on strain MC4100, as described by Bukhari and Ljungquist (4). Lambda lysates were prepared by lytic growth on strain MC4100. Once lysis had occurred, chloroform was added (2%, vol/vol), and the lysate was shaken for 10 min and centrifuged to remove cell debris.

Mu*cts* lysogeny. Independent Mu*cts* lysogens were isolated by the following two methods. First, drops of a Mu*cts* lysate were spotted onto a lawn of strain 4100T on L-agar plates supplemented with 1 mM $CaCl_2$ and 2.5 mM $MgSO_4$ and incubated overnight at 30°C (4). Second, infection with Mu*cts* was carried out in liquid culture as described by Smith and Umbarger (22). An L-broth culture of strain 4100T was suspended in $MgSO_4$ (5 mM) plus $CaCl_2$ (5 mM), and Mu*cts* were absorbed at multiplicities of infection of 1 and 0.1 for 15 min at 30°C. The cells were suspended in L-broth and grown overnight at 30°C to allow segregation of the Mu-induced mutants. Mu*c* 25 was sometimes added at a multiplicity of infection of 1 to kill any nonlysogens.

Isolation of *chlC* mutants. Point mutations within the *chlC* locus were induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutangenesis as described by Miller (20). Cells were then streaked onto glucose-nitrate minimal plates containing TTC and incubated at 37°C anaerobically for 3 days. Small, dark-red colonies were picked for further analysis (see Results).

This identification procedure was used in the present study to isolate Mu insertion mutations specifically in the *chlC* gene. Survivors of Mu*cts* infection were streaked onto glucose-nitrate minimal plates containing TTC and incubated anaerobically at 30°C for 6 days. Small, dark-red colonies were picked and tested for nitrate reductase and formate dehydrogenase activities (3, 19). To verify that these *chlC* mutants were single lysogens for phage Mu*cts*, they were transduced to *chlC*$^+$ with phage P1 using anaerobic growth on lactate-nitrate plates as the selection for the Chl$^+$ phenotype (24). Single Mu*cts* insertions into the *chlC* locus thereby became heat resistant at 42°C due to the loss of the temperature-inducible Mu prophage.

Lambda lysogeny. λp1(209) and λp123(209) lysogens of independent *chlC*::Mu*cts* mutants were isolated by the method of Casadaban (5). A drop of the lambda lysate was placed on a lawn of the mutant on a tryptone plate. After incubation overnight at 30°C, cells from the center of the turbid plaque were purified by single-colony isolation on tryptone agar plates seeded with 10^8 λ*cIh80del9* phage to kill any nonlysogens. The lysogens were then tested for the stable integration of the λ phage by the method of Eckhardt (10).

Isolation of *chlC-lac* fusion strains. An overnight L-broth culture of the λ-Mu lysogen strain AF15 was centrifuged and suspended in 0.9% (wt/vol) NaCl. Up to 10^{10} cells were spread onto a lactose-nitrate minimal plate (modified by the omission of citrate), incubated anaerobically at 42°C overnight, and then grown for an additional 5 days at 37°C. Colonies were

TABLE 1. *E. coli K-12 and phage strains*

Strain	Description	Source or derivation
E. coli		
4100T	F$^-$ Δ*lacU169 trp*[a]	Spontaneous *trp*$^-$ derivative of strain MC4100[b]
AF15	F$^-$ Δ*lacU169 trp chlC*::Mu*cts*:: λp1(209)[a]	This work
AF16	F$^-$ Δ*lacU169 trp chlC*::λp1(209)[a]	This work
EMG29	F$^-$ *pro trp his lac str*^{r}	D. Old
KMBL1614	Mu*cts*[c]	P. van der Putte
MC4100	F$^-$ Δ*lacU169*[a]	R. E. Loughlin
Phage		
Muc25		H. Tabor
λp1(209)	*lacA?YZO'*-Δ*W209-trp'AB'* ::(+Mu')	R. E. Loughlin
λp123(209)	*lacA?YZO'*-Δ*W209-trp'ABCDE?'*:: (−Mu')	R. E. Loughlin
λ*cIh80del9*		Cold Spring Harbor

[a] Other markers: *araD139 rpsL thi.*
[b] Obtained by a double penicillin-enrichment procedure carried out as described by Miller (20).
[c] Other markers not known.

patched onto both lactose MacConkey plates, incubated aerobically (repressing conditions for nitrate reductase), and lactose-nitrate MacConkey plates, incubated anaerobically (derepressing conditions). Those colonies that gave a Lac$^-$ phenotype aerobically and a Lac$^+$ phenotype anaerobically in the presence of nitrate were chosen for further analysis.

Growth and disruption of bacteria. Cultures used for enzyme assays were grown aerobically and anaerobically as described previously (14). Cells were disrupted with an MSE 150 W ultrasonic disintegrater fitted with a microtip, and the resulting crude extracts were used for enzyme assays. For nitrate reductase assays the crude extracts contained benzamidine (5 mM) to inhibit protease activity.

Enzyme assays. Nitrate reductase was assayed spectrophotometrically using reduced benzyl viologen as electron donor, and formate dehydrogense was assayed using 2,6-dichlorophenolindophenol as electron acceptor with phenazine methosulfate as mediator, as described previously (14). β-Galactosidase was assayed by measuring the initial rate of *o*-nitrophenol-β-D-galactopyranoside hydrolysis at 420 nm with a Cecil spectrophotometer. For β-galactosidase assays, cuvettes contained (in 3.0 ml): Z buffer (20) adjusted to pH 8.0, *o*-nitrophenol-β-D-galactopyranoside (2.2 mM), and 0.1 ml of crude extract. Protein was determined by the method of Lowry et al. (17).

RESULTS

Isolation and mapping of *chlC* mutants. During anaerobic growth on glucose-nitrate minimal plates containing TTC, wild-type and *chlA* and *chlB* mutants of *E. coli* give white colonies, whereas *chlC* mutants yield red colonies. The biochemical reason for this experimental observation is not fully understood, but our working hypothesis is that the reduction of TTC to the corresponding red formazan derivative is catalyzed by a cytochrome oxidase, possibly cytochrome *d*, and that the activity of this enzyme is inhibited in wild-type strains by nitrite (which accumulates in the growth plates due to the action of nitrate reductase) but not in *chlC* mutants (which cannot reduce nitrate to nitrite). In addition, the production of the red formazan derivative is known to be inhibited at low pH (1). Since *chlA* and *chlB* mutants produce and accumulate formic acid during growth on glucose anaerobically due to their inability to synthesize a functional formate dehydrogenase, these mutants should not be able to produce the formazan and would therefore be expected to have a phenotype superficially similar to the wild type under these conditions.

Of 60 red colonies of strain EMG29 picked from glucose-nitrate minimal plates containing TTC and incubated anaerobically after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, all lacked nitrate reductase activity but retained formate dehydrogenase activity as assayed by dye overlay techniques (3, 19), and all showed between 40 and 50% linkage with *trp* estimated by P1 transduction when *trp* was used as the selected marker (Y. A. Begg, unpublished data). This cotransduction frequency indicates that the mutants are of the *chlC* type previously described by Guest (12). Previous linkage data of *chlC* with *trp* (12) indicate that a linkage value between 55 and 95% is obtained when *chlC* is used as the selected marker, but this value is considered unreliable due to interference from the nonselected marker (12).

chlC::Mu*cts* mutants were isolated and tested for single Mu lysogeny as described in Materials and Methods. The frequency of Mu lysogens that possessed prophages not linked to the inactivated gene varied considerably in different experiments. Four independent *chlC*::Mu*cts* single lysogens were isolated which lacked nitrate reductase activity but possessed formate dehydrogenase activity as determined by dye overlay tests. The linkage of the *chl* mutation in these mutants with the *trp* operon was tested by P1 transduction using *chl* as the selected marker, since the presence of the Mu insert into the *chl* locus would be expected to reduce the cotransduction frequency if *trp* was used as the selected marker. Of 168 Chl^+ colonies, 87 were also found to be Trp^+, indicating that the *chl* mutation in these mutants and the *trp* locus are 48% cotransducible.

Isolation of *chlC-lac* fusion strains. The *lac* structural genes were transposed near the promoter of the *chlC* genes by lysogenizing the four strains containing Mu*cts* insertions in *chlC* with the λ-Mu hybrid phages, λp1(209) and λp123(209). Lysogenization will occur preferentially via recombination between the homologous region of Mu DNA, since the λ-Mu hybrid phages carry no λ attachment site and the host chromosome is deleted for *lac*. The Mu phage can insert in the *chlC*::Mu*cts* mutants in one of two directions with respect to the *chlC* promoter, but, provided that both λ-Mu hybrid phages are used, each carrying a fragment of Mu DNA in a different orientation, the *lac* genes carried by one of the phages will be placed in the same direction for transcription as the *chlC* gene. Deletions that remove the temperature-inducible Mu prophage and generate fusion were obtained by selecting for a Lac^+ phenotype anaerobically on lactose-nitrate minimal plates.

Of the four original *chlC*::Mu*cts* mutants lysogenic for λp1(209), one yielded fusions at a frequency of 5×10^8, 60% of which were under the control of the *chlC* promoter, based on the following assumption. Since the regulation of any of the individual *chl* genes is not known, we assumed that expression of the *chlC* gene would be governed by the same factors that affect the expression of nitrate reductase activity. Hence we selected for the Lac^+ phenotype and determined whether this was dependent upon the presence of nitrate together with anaerobic growth conditions (Table 2).

Enzyme assays. We examined in detail one of the potential fusion strains for inducibility of β-galactosidase activity by nitrate under both aerobic and anerobic growth conditions. The results shown in Table 2 indicate that the synthesis of β-galactosidase activity and the Lac^+ phenotype in fusion strain AF16 appear to be under the same control as that regulating the synthesis of nitrate reductase activity in the parental strain 4100T.

Recently a mutation has been described, *ana*, mapping near 26 min on the *E. coli* linkage map, whose phenotype is the inability to grow anaerobically on glucose unless the growth medium is supplemented with either nitrate or fumarate as terminal electron acceptor (6). The mutation has not been characterized biochemically, but, to exclude the possibility that in the construction of these fusion strains we had inadvertently affected *ana* gene function, β-galactosidase activity was assayed after anaerobic growth on glucose in the presence of fumarate. The results shown in Table 2 exclude this possibility, since β-galactosidase activity in strain AF16 was not induced by fumarate under these conditions, and

TABLE 2. *Properties of chlC-lac fusion strain AF16 and parent strain 4100T*

Strain	Growth conditions[a]	Lactose phenotype[b]	Enzyme sp act[c]		
			β-Galactosidase[d]	Nitrate reductase[e]	Formate dehydrogenase[f]
4100T	$+O_2$	Lac⁻	<5	<5	ND[g]
	$+O_2$, $+NO_3^-$	Lac⁻	<5	<5	ND
	$-O_2$	Lac⁻	<5	<5	ND
	$-O_2$, $+NO_3^-$	Lac⁻	<5	100	45
AF16	$+O_2$	Lac⁻	<5	<5	ND
	$+O_2$, $+NO_3^-$	Lac⁻	<5	<5	ND
	$-O_2$	Lac⁻	<5	<5	ND
	$-O_2$, $+NO_3^-$	Lac⁺	100	<5	100
	$-O_2$, + fumarate	ND	<5	ND	ND

[a] Cells were grown with glucose as the carbon source as described in the text.
[b] Determined by growth on lactose minimal agar.
[c] Enzyme assays were carried out as described in the text.
[d] A specific activity of 100 corresponds to 0.23 μmol of o-nitrophenol produced per mg of protein per min.
[e] A specific activity of 100 corresponds to 0.72 μmol of reduced benzyl viologen oxidized per mg of protein per min.
[f] A specific activity of 100 corresponds to 0.6 μmol of 2,6-dichlorophenolindophenol reduced per mg of protein per min.
[g] ND, Not determined.

indeed this strain is capable of anaerobic growth on glucose in the absence of a terminal electron acceptor.

DISCUSSION

We have used the technique of gene fusion described by Casadaban (5) to study the regulation of the *chlC* gene in *E. coli*. The results obtained indicate that the expression of the *chlC* locus is regulated by nitrate and oxygen in the following manner. Nitrate and anaerobic growth conditions are both required for induction of the *chlC* locus, whereas the presence of oxygen results in repression.

The TTC method described for the specific isolation of *chlC* mutants yields mutants which lack nitrate reductase, possess formate dehydrogenase activity, and display between 40 and 50% cotransduction between their *chl* locus and the *trp* operon when *trp* is used as the selected marker.

Evidence that fusion strain AF16 described here has the *lac* structural genes fused to the *chlC* promoter is as follows. First, the four *chlC* ::Mu*cts* mutants studied were isolated using the TTC procedure and are cotransducible with *trp*. Second, fusion strain AF16 lacks benzyl viologen-dependent nitrate reductase activity but retains formate dehydrogenase activity when assayed spectrophotometrically. Third, β-galactosidase activity in strain AF16 is regulated by the same effectors that regulate nitrate reductase activity in the wild type.

We have excluded the possibility that the *lac* structural genes in fusion strain AF16 are fused to the *ana* gene promoter. In addition, a mutation has been described, designated variously *nirA* (21), *fnr* (15), or *nirR* (7), which maps at approximately 29 min on the *E. coli* linkage map (2). Such mutants are pleiotropic and are unable to grow on a range of anaerobic electron acceptors including fumarate, nitrite, and nitrate. Our data for the cotransduction frequency of the original Mu-induced mutations with *trp*, together with the nutritional requirements of fusion strain AF16, exclude the possibility that a large deletion has arisen placing the *lac* structural genes under the control of the promoter regulating this locus.

Thus we have isolated a strain which has the *lac* structural genes regulated by conditions identical to those that regulate the synthesis of nitrate reductase; further work with this strain should assist our study of this enzyme complex. In an attempt to isolate regulatory mutants for nitrate reductase we have isolated Lac⁺ revertants of the fusion strain which grow under conditions that normally repress nitrate reductase synthesis. The Casadaban technique also facilitates the isolation of specialized transducing lambda phages carrying the *chlC-lac* fusion which would make possible the DNA sequencing of the *chlC* promoter/operator region and the investigation of the in vitro regulation of β-galactosidase synthesis (26) under the control of the *chlC* promoter. In addition, if the *chlC* gene is a structural gene for nitrate reductase, characterization of the polar *chlC*::Mu*cts* mutations should allow the unequivocal identification of the structural subunit(s) coded by this locus.

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