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A Novel Method for Isolating Chlorate-resistant Mutants of *Escherichia coli* K12 by Anaerobic Selection on a Lactate plus Fumarate Medium

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INTRODUCTION

Under anaerobic conditions *Escherichia coli* is unable to grow in minimal medium with non-fermentable substrates unless an electron acceptor such as nitrate or fumarate is provided.

Mutants defective in the nitrate reductase system have been isolated by several methods. Direct selection for chlorate resistance has been used because the anaerobic sensitivity of *E. coli* to chlorate depends on the presence of a functional nitrate reductase which reduces chlorate to chlorite with lethal consequences (Piechaud *et al.*, 1967). Mutants defective in nitrate respiration have been isolated by an indirect selection based on their failure to grow anaerobically on an amino acid-enriched lactate plus nitrate medium (Venables & Guest, 1968). They have also been isolated by screening colonies directly for their inability to reduce nitrate to nitrite with formate as the electron donor (Ruiz-Herrera, Showe & DeMoss, 1969). Some seven distinct classes of mutants deficient in the reduction of nitrate have been defined. They have all been designated *chl* mutants (*chlA–F*) although some are chlorate-sensitive and others show different degrees of resistance. Furthermore, the selections based either on chlorate resistance or on the inability to reduce nitrate yield entirely different distributions of mutant types (Glaser & DeMoss, 1972).

Failure to grow anaerobically on a glycerol plus fumarate medium has been used to isolate *E. coli* mutants (*frd*) deficient in fumarate reductase (Spencer & Guest, 1973). This communication reports how attempts to use an analogous lactate plus fumarate medium for isolating *frd* mutants led to the discovery that selection for the ability to grow on this medium provides a new method for the direct selection for chlorate-resistant mutants (*chl*).

METHODS

Organisms and media. Escherichia coli K12 strain WGAS, a spontaneous streptomycin-resistant derivative of WGA (W3110 *gal trpA*, F^-) was used as the parental strain for the isolation of mutants. The basal minimal medium contained (per litre): KH_2PO_4, 5·44 g; K_2HPO_4, 10·49 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4.7H_2O$, 0·05 g; $MnSO_4.4H_2O$, 5 mg; $FeSO_4.7H_2O$, 0·125 mg; $CaCl_2$, 0·5 mg. The substrates glycerol, sodium fumarate, potassium lactate and potassium nitrate were added as required, each at a final concentration of 0·04 M. The media were enriched with Casamino acids (Difco, vitamin-free; 0·5 g l^{-1}) supplemented with L-tryptophan (30 mg l^{-1}) and solidified with 1·5 % (w/v) agar (Difco). Anaerobic incubations were carried out under H_2–CO_2 (95:5, v/v). Gas production was tested according to Guest (1969). Bacterial stocks were maintained on L-agar (Lennox, 1955).

Genetic methods. Conjugations were performed by cross-streak tests on solid media. Donor strains carrying the following F′ factors: F126, F133, F152 and F450 (Low, 1972) in auxotrophic *recA* hosts, were grown to exponential phase in L-broth and streaked at right-angles across streaks of twice-washed stationary-phase cultures of the LF^+-recipient strain on a lactate plus nitrate selective medium. The plates were incubated aerobically for 30 min and then anaerobically for 3 days at 37 °C before scoring. Counter-selection against the donor strains was accomplished nutritionally by replacing the Casamino acids with a defined amino acid mixture based on the composition of casein but lacking the amino acids required by the donor strains.

Transductions were performed with phage P1 lysates of the wild-type strain *E. coli* W3110 as the donor, according to the methods of Spencer & Guest (1973). Transductants were selected anaerobically on lactate plus nitrate medium, purified on the same medium, and scored for the inheritance of the Gal^+ and Trp^+ markers of the donor by replica-plating on to galactose and glucose minimal media (Venables & Guest, 1968).

RESULTS AND DISCUSSION

All the strains of *E. coli* K12 tested grew slowly on glycerol plus fumarate medium (GF) and extremely slowly on lactate plus fumarate medium (LF). However, derivatives which grew more rapidly (GF^+ and LF^+) were readily isolated as larger colonies which appeared after anaerobic incubation on the corresponding solid media. The biochemical basis for this adaptation is not understood although the results presented here indicate that changes in the pathways of electron transport, particularly to nitrate, may be involved.

Colonies of *E. coli* WGAS growing on L-agar were picked and streaked directly on to GF medium or LF medium. The larger GF^+ colonies appeared against a background of smaller colonies within 3 days whereas discrete LF^+ colonies appeared within 7 days at 37 °C under H_2–CO_2. Approximately 50 isolates of each type were purified on the same media and both GF^+ and LF^+ derivatives grew well anaerobically, the colonies being visible at 24 h.

Further tests (Table 1) showed that all the LF^+ strains grew equally well on GF and LF medium but they were unable to grow anaerobically on glycerol plus nitrate (GN) or lactate plus nitrate (LN) media. By contrast, the GF^+ derivatives retained the parental ability to use nitrate as the terminal electron acceptor but they were not adapted for growth on LF medium (Table 1). Thus, adaptation for growth on LF (but not GF) medium is accompanied by a defect in nitrate respiration.

When GF^+ derivatives were subjected to a further selection on LF medium, the sequentially-adapted GF^+/LF^+ derivatives gained the LF^+ phenotype without losing the ability to grow on GN and LN media (Table 1). Thus it appears that preliminary selection for the GF^+ phenotype permits the isolation of LF^+ strains without concomitant loss of the capacity to reduce nitrate.

It was also noted that the GF^+, LF^+ and GF^+/LF^+ derivatives were capable of growing on the amino acid-enriched medium under H_2–CO_2 with fumarate alone. This growth was consistently slower and less prolific than that observed when glycerol or lactate was also present. It is presumed that the growth is supported by energy derived from the fermentation of fumarate or fumarate plus hydrogen. Such fermentations have been observed previously in manometric studies with washed suspensions of *E. coli* (Krebs, 1937).

Tests for chlorate resistance indicated that for the parental strain, the GF^+ and GF^+/LF^+ derivatives were all sensitive to chlorate whereas the LF^+ derivatives were all chlorate-resistant (Table 1). The chlorate phenotypes were therefore entirely consistent with the

Table 1. *Growth characteristics of strains of E. coli*

Washed suspensions of the strains were streaked on the media indicated and growth scored after 72 h at 37 °C under an atmosphere of H_2–CO_2 (95:5, v/v).

	Medium				
Strain	GF	LF	GN	LN	Chlorate
WGAS	tr	−	++	+	−
WGAS LF^+	+++	+++	tr	−	+++
WGAS GF^+	+++	−	+++	++	−
WGAS GF^+/LF^+	+++	+++	++	++	−
WGAS Chl^R	+++	+++	−	−	+++

−, No growth; tr, trace of growth; +, ++ and +++, increasing degrees of growth.

growth patterns observed on GN and LN media. The LF^+ derivatives also failed to produce gas during anaerobic growth in L-broth plus extra glucose (1%, w/v), indicating that they closely resemble *chl* mutants of the pleiotropic types (e.g. *chlA*, *B*, *D* and *E*).

Twenty independently-derived chlorate-resistant mutants of *E. coli* strain WGAS were isolated by direct selection anaerobically on nutrient agar (Oxoid) containing glucose (0·2 %) plus potassium chlorate (0·1 %). All these Chl^R mutants grew rapidly on both GF and LF media without prior adaptation; they failed to grow on GN and LN media (Table 1) and were all judged to be of the pleiotropic types by their failure to produce gas. The behaviour of the Chl^R mutants was therefore identical to that of the LF^+ derivatives.

The approximate locations of the mutations in the LF^+ strains were determined by conjugation with several F′ strains and by transduction with phage P1 to test for linkage with *trpA* and *gal*. The F′ strains were chosen for their ability to transfer one or more of the *chl* regions: F133 (*chlB*), F450 (*chlA* and *D*), F126 (*chlA*, *C*, *D* and *E*) and F152 (part of the *chlD* region). The selection was for conjugants which grew anaerobically on LN medium, and positive results were obtained for all of the LF^+ mutants when crossed with at least one of the F′ donors. As a consequence, representatives of the *chlB*, *chlE* and *chlA* or *D* types were detected. Using the same selection in transductions with the prototrophic strain W3110 as donor, none of the LF^+ mutants resembled *chlC* mutants by being cotransducible with *trpA*. However, two classes of LF^+ mutant were recognized by their cotransduction frequencies with *gal* and these corresponded closely to the values observed with *chlA* and *chlE* mutants (Venables & Guest, 1968). Consequently, LF^+ derivatives possessing the phenotypic and genetic characteristics of *chlA*, *chlB* and *chlE* mutants were identified.

Selection for the ability to grow anaerobically on lactate plus fumarate provides a novel method for the direct selection of mutants defective in nitrate respiration. The biochemical basis for this selection is not understood, although it is clear from the growth patterns of the LF^+ and GF^+/LF^+ derivatives that there are at least two mechanisms for attaining the LF^+ phenotype. Direct selection for LF^+ favours the isolation of pleiotropic *chl* mutants defective in the nitrate reductase system. By contrast, prior selection for the GF^+ phenotype seems to evoke a different response, such that the ability to grow on lactate with fumarate as the electron acceptor is not coupled to the elimination of electron transport to nitrate.

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