JOURNAL OF BACTERIOLOGY, July 1972, p. 287-289
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Vol. 111, No. 1
Printed in U.S.A.

Use of Neomycin in the Isolation of Mutants Blocked in Energy Conservation in *Escherichia coli*

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Received for publication 22 November 1971

A neomycin-resistant mutant of *Escherichia coli* K-12 unable to grow on Krebs cycle intermediates has been isolated. The mutant retained its respiratory capacity, but lacked membrane Mg^{2+}-Ca^{2+}-stimulated adenosine triphosphatase activity (EC 3.6.1.3).

Selection for neomycin resistance has been used in the isolation of hemin-deficient mutants of *Escherichia coli* K-12 (13) and *Salmonella typhimurium* (12). It was postulated that respiratory-deficient cells incorporated less neomycin than normal cells (12). One explanation for this may be the inability of these mutants to generate sufficient energy to concentrate the antibiotic. It appeared possible, therefore, to employ neomycin in the positive selection of mutants rendered resistant by virtue of a defect in the ability to couple respiration to the synthesis of adenosine triphosphate (ATP). This report describes the isolation and partial characterization of one such neomycin-resistant mutant of *E. coli* K-12.

Strain A428 (F^-, Pro^-, Lac_1^-, $T6^R$, Gal_2^-, Ara^-, His^-, Xyl^-, Man^-, B_1^-, Str^R) was used as a parent organism and grown in nutrient broth to a cell density of 1.5×10^9 bacteria/ml. The cell suspension was diluted 10-fold into Davis minimal medium (4) and 4-ml portions were irradiated in petri dishes with a Phillips germicidal lamp (30 w) at a distance of 57.5 cm for 90 sec. Under these conditions the viable count was reduced about 1,000-fold. After irradiation, cells were diluted into nutrient broth containing glucose (0.5%) and yeast extract (0.5%) and incubated for 2 hr at 37 C. Samples containing between 10^4 and 10^6 cells were passed through membrane filters (Sartorius; pore size, 0.45 μm; diameter, 50 mm). The filters were washed with saline and placed on agar plates containing supplemented Davis medium (see legend to Fig. 1), glucose (0.5%), and neomycin sulfate (50 μg/ml, Sigma Chemical Co.). The plates were incubated for 3 days at 37 C, and small colonies were picked and transferred to minimal plates supplemented as above but without neomycin, and incubated at 37 C. They were then replica-plated onto supplemented minimal plates containing either succinate, malate, α-ketoglutarate, or glucose (all at 0.5%) as sole source of carbon. Out of 500 neomycin-resistant colonies examined, 14 were able to grow on glucose but were unable to utilize these Krebs cycle intermediates as sole sources of carbon and energy. Respiration was then examined in washed whole cells of these 14 mutants by using either glucose or the tricarboxylic acid cycle intermediates as substrates. A mutant blocked in coupled ATP synthesis would be expected to grow on glucose but not on Krebs cycle intermediates, yet it should retain its ability to oxidize these compounds. Three mutants were found which respired on each of the substrates to the same extent as the parent. One of these, N_{144}, was retained for further study.

A mutant unable to couple respiration to the synthesis of ATP might be expected to exhibit much lower growth yield under aerobic conditions than the corresponding parent strain in the presence of limiting concentrations of a carbon source (1-3, 8). A comparison of the growth yields of mutant N_{144} with those of the parent A428 on limiting glucose concentrations is illustrated in Fig. 1. Under aerobic conditions the growth yield of the mutant was much lower than that of the parent. Anaerobically, the yield of mutant and parent differed slightly. Nitrate has been shown to serve as a terminal electron acceptor in place of oxygen under anaerobic conditions, and its reduction is coupled to the synthesis of ATP (11). It is of interest that, whereas the anaerobic growth

yield of the parent was stimulated by the presence of nitrate, no effect of nitrate was observed in the case of the mutant (Fig. 1).

Butlin et al. (2) recently described a mutant of *E. coli* K-12, *uncA*, with similar growth characteristics (although the effect of nitrate was not reported). Evidence from cell-free studies was presented indicating that *uncA* was defective in oxidative phosphorylation and that this defect was due to an impaired Mg^{2+}-Ca^{2+}-stimulated adenosine triphosphatase (EC 3.6.1.3). The Mg^{2+}-Ca^{2+}-stimulated adenosine triphosphatase activity was therefore examined in preparations of A428 and N_{144} (Table 1). Membrane ghosts were prepared, and the adenosine triphosphatase was assayed by the procedure of Evans (5). Virtually no membrane-bound adenosine triphosphatase activity could be detected in the mutant. Evans has shown that treatment of *E. coli* membrane ghosts with sodium dodecyl sulfate (SDS) results in the solubilization and stimulation of cation-dependent adenosine triphosphatase (6). The data in Table 1 demonstrate that the adenosine triphosphatase activity of the parent was stimulated about threefold after solubilization with SDS. In contrast, even after SDS treatment, there was only slightly detectable adenosine triphosphatase activity in the mutant preparation. Mixing of membrane ghosts or solubilized preparations from N_{144} with corresponding preparations from A428 had no effect on the adenosine triphosphatase activity of the parent, suggesting that the defect is not due to the presence of a diffusible inhibitor. The mixing experiments do not rule out the possibility that the absence of activity in N_{144} is due to the presence of a tightly bound inhibitor.

TABLE 1. *Membrane-bound and solubilized Mg^{2+}-Ca^{2+}-activated adenosine triphosphatase activity in strains A428 and N_{144}*[a]

Addition	Specific activity[b]			
	Membrane-bound		Solubilized	
	A428	N_{144}	A428	N_{144}
None	5.4	0	19.0	1.0
Mg^{2+}	29.4	0	66.0	3.6
Ca^{2+}	18.1	0	74.4	0.6

[a] Bacteria were grown to late exponential phase on supplemented Davis minimal medium (see legend to Fig. 1) containing 0.5% glucose. Membrane ghosts were prepared as described by Evans (5).

[b] The enzyme was assayed and solubilized with sodium dodecyl sulfate according to the procedures of Evans (5, 6). Magnesium chloride and calcium chloride were added where indicated at 2 mM and 1 mM, respectively. Protein was determined by the method of Lowry et al. (9) with bovine serum albumin (Sigma Chemical Co.) as a standard. Specific activity is recorded as micromoles of orthophosphate (7) released per milligram of protein per hour.

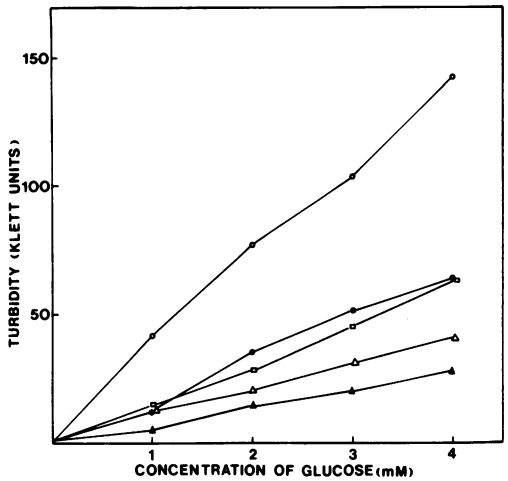

FIG. 1. *Growth yields of strains A428 and N_{144} grown on limiting concentrations of glucose. The measurement of aerobic growth yields was carried out as described by Cox et al. (3) except that Davis minimal medium (4) supplemented with citrate, histidine (50 μg/ml), proline (50 μg/ml), and vitamin B_1 (1 μg/ml) was used. Anaerobic experiments were performed in glass-stoppered Thunberg tubes, especially adapted to fit the Klett-Summerson colorimeter, filled to capacity with supplemented Davis minimal medium and 25 mM $NaHCO_3$. Potassium nitrate (0.1%) was added where indicated. Maximum turbidity readings were recorded when growth of the cultures had ceased. Strain A428: aerobic (○), anaerobic (△), anaerobic + nitrate (□). Strain N_{144}: aerobic (●), anaerobic or anaerobic + nitrate (▲).*

The defect in N_{144} appeared to be due to a single-point mutation since the mutant reverted to growth on Krebs cycle intermediates with a frequency of 1 in 10^8. In addition, N_{144} could be transduced to growth on tricarboxylic acid cycle intermediates (10), and no differences in growth or adenosine triphosphatase activity could be observed between a transductant and A428. Butlin et al. (2) reported that *uncA* was cotransducible with *ilv* but not with *metE* and was located at 73.5 min on the *E. coli* chromosome. Similarly, the mutation in N_{144} was cotransducible with *ilv* at a frequency of 17%; no cotransduction was found with *metE*. The defect in N_{144} may thus be due to a mutation in the *uncA* gene or in a gene close to it.

We thank Eliora Z. Ron for the strain A428 and for her critical reading of the manuscript. We also thank Eugene Rosenberg and Joseph Neumann for their helpful suggestions and criticisms, and Tamar Tuito and Vanda Freudis for their expert technical assistance.

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