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Isolation of *Escherichia coli* Mutants with an Adenosine Triphosphatase Insensitive to Aurovertin

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Energy-transducing adenosine triphosphatase (ATPase) from *Escherichia coli* is inhibited by aurovertin. Aurovertin-resistant mutants were generated by nitrosoguanidine mutagenesis of *E. coli* AN180, whose growth on a nonfermentable carbon source was blocked by aurovertin. The ATPase activity of cell extracts from 15 different mutants (designated MA1, MA2, MA3, etc.) was found to be at least 20 times less sensitive to aurovertin than that from the parent strain. The aurovertin-resistant mutants did not show cross-resistance towards a number of ATPase inhibitors including azide, dicyclohexylcarbodiimide, quercetin, 7-chloro-4-nitrobenzofurazan, and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. Aurovertin inhibited the energization brought about by addition of ATP to *E. coli* AN180 membrane vesicles; it was without effect on MA1 and MA2 membrane vesicles energized by ATP. The mutation in MA1, like other mutations of the ATPase complex, maps in the *unc* region of the bacterial chromosome.

Aurovertin was first reported by Lardy et al. (26, 27) as a potent inhibitor of oxidative phosphorylation in mitochondria. Later it was shown to inhibit soluble mitochondrial adenosine triphosphatase (ATPase) (34). Aurovertin binding to mitochondrial membrane or to F_1-ATPase is accompanied by enhancement of aurovertin fluorescence (6, 10, 11, 27). Aurovertin also inhibits oxidative phosphorylation in *Rhodospirillum rubrum,* but aurovertin fluorescence is not enhanced (19). Likewise, there is no fluorescence increase when aurovertin is added to chloroplast ATPase and to *Escherichia coli* ATPase (42). Because of the lack of fluorescence response, the interaction of aurovertin with the chloroplast and bacterial ATPase has not been studied in detail. It may be recalled that, in their pioneering work on the isolation of aurovertin, Baldwin et al. (5) found that aurovertin is unable to antagonize the growth, on agar plates, of a number of bacteria including *E. coli.* However, it has been shown more recently that aurovertin inhibits oxidative phosphorylation in *Alcaligenes faecalis* (2) and *Paracoccus denitrificans* (22).

In this paper we show that aurovertin inhibits the growth of *E. coli* on a nonfermentable carbon source at a slightly alkaline pH. The ATPase activity of cell extracts of *E. coli* was found to be highly sensitive to aurovertin. We also describe the isolation of *E. coli* aurovertin-resistant mutants in which the ATPase is no longer sensitive to aurovertin. A brief report (3) has appeared on the isolation of aurovertin-resistant mutants of *Saccharomyces cerevisiae.*

MATERIALS AND METHODS

Preparation of aurovertin D. Aurovertin was extracted from 2-week-old cultures of *Calcarisporium arbusculum* (5). The growth medium and the mycelium were extracted separately, and aurovertin D was purified by preparative thin-layer chromatography on silica gel plates as described by Osselton et al. (32) for aurovertin B.

Aurovertin D was stored in a concentrated ethanolic solution protected from light. It was diluted before use, and its concentration was calculated from a molar absorption coefficient of 42,700 at 367.5 nm (5).

Organisms and growth conditions. *E. coli* strains used in this study are described in Table 1. Cells were cultured at 37°C on a rotary shaker in medium E (9) supplemented with L-arginine (40 μg/ml), thiamine (1 μg/ml), and a carbon source, which was either 0.2% glucose or a mixture of sodium succinate, sodium acetate, and sodium malate (each at a final concentration of 0.4%). When appropriate, streptomycin was added at 110 μg/ml. The final concentration of ethanol resulting from the addition of the ethanolic solution of aurovertin was less than 0.2%. Growth in liquid medium was monitored by following the change in turbidity at 660 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. Agar plates were prepared from the same medium as that described above, supplemented with 1.5% agar.

Selection of aurovertin-resistant mutants. A procedure similar to that described by Fillingame (15) and Friedl et al. (17) was used to select aurovertin-resistant mutants on agar plates. One-milliliter amounts of 9 mM aurovertin in ethanol were spread over the surface of agar plates containing succinate, acetate, and malate as carbon sources; the plates were kept at 37°C until ethanol disappeared completely. Strain AN180 was grown overnight in 5 ml of LB

TABLE 1. *Major properties of the E. coli K-12 strains used in this work*

Strain	Relevant characteristics	Origin or derivation
AN180	*argE3 thi-1* Str^r (wild-type ATPase)	Butlin et al. (7) via CGSC[a]
AN120	*argE3 thi-1 uncA401* Str^r (lacks ATPase activity)	Butlin et al. (7) via CGSC
MA1, MA2, MA3	*argE3 thi-1* Str^r Aur^r (aurovertin-resistant ATPase)	Selected from AN180 after mutagenesis (see text)

[a] CGSC, *Escherichia coli* Genetic Stock Center, Yale University, New Haven, Conn.

medium (30) containing 0.2% glucose. The cells were washed with 5 ml of 0.85% NaCl and resuspended in the same medium. A sample of the suspension was mixed with 3 ml of F-top agar (30) to give a concentration of about 10^8 cells per ml, and the mixture was poured over the agar plates supplemented with aurovertin. A crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was placed in the center, and the plates were incubated at 37°C for 3 days. Of the distinct colonies that were obtained, the largest ones were picked and grown at 37°C in 2 ml of medium supplemented with succinate, acetate, malate, arginine, thiamine, streptomycin, and 0.3 mM aurovertin. Fifteen aurovertin-resistant colonies were further purified by two successive transfers of single colonies onto agar plates supplemented with 0.2 mM aurovertin.

Transduction assays. Phage (P1) transduction was performed as described by Miller (30). A P1 lysate obtained on mutant MA1 was used to infect strain AN120 (*uncA401*). Transductants were selected on agar plates supplemented with succinate, acetate, and malate as carbon sources. Several recombinants capable of growth were selected and further grown in liquid culture in the same medium. The sensitivity of ATPase activity to 20 μM aurovertin was then measured in cell extracts.

Preparation of cell extracts. Crude extracts were prepared by lysozyme-disodium ethylenediaminetetraacetate (EDTA) treatment as described by Hawrot and Kennedy (23). The cells were harvested, washed twice in 0.85% NaCl at room temperature, suspended in an ice-cold medium containing lysozyme (0.1 mg/ml), 25 mM tris(hydroxymethyl)aminomethane (Tris)-sulfate, and 2.5 mM EDTA, and brought to 37°C for 10 min. The suspension was then briefly homogenized with a Potter-type homogenizer, and the lysates were stored at room temperature before being assayed for ATPase activity.

Preparation of membranes and of a crude soluble fraction containing ATPase activity. Membranes were prepared by rupturing the cells in a Sorvall Ribi press. A soluble fraction containing the ATPase activity was obtained by washing the membranes with Tris-EDTA medium by the method of Roisin and Kepes (35, 36) and kept at room temperature before being assayed for ATPase activity.

ATPase assay. The Mg-ATPase activity of cell extracts, which had been preincubated with inhibitors, was assayed at 37°C as described by Butlin et al. (8). The reaction mixture contained 20 mM ATP, 10 mM $MgSO_4$, 1.5 mM EDTA and 100 mM Tris-sulfate (pH 9) in a final volume of 0.5 ml. Addition of 0.4% Triton X-100, which has been reported to increase the membrane permeability of vesicles (43), did not increase the ATPase activity of the lysozyme-EDTA extract. After incubation for 15 min at 37°C, the reaction was terminated with 0.1 ml of 2.5 N perchloric acid, and the amount of phosphate released was measured (16). When samples contained Triton X-100, 0.5% sodium dodecyl sulfate was included in the colorimetric assay of phosphate.

Atebrin fluorescence. Energy-dependent quenching of atebrin fluorescence was measured with a Perkin-Elmer MPF2 fluorimeter (excitation wavelength, 450 nm; emission wavelength, 510 nm) by the method of Gibson et al. (18).

Protein determination. Proteins was determined with the Folin phenol reagent, with bovine serum albumin as a standard (29).

RESULTS

Inhibition of growth by aurovertin. *E. coli* AN180 was sensitive to aurovertin when grown on a nonfermentable carbon source (see Fig. 1). For example, the mean generation times in a medium supplemented with succinate, acetate, and malate were 6 h in the presence of 0.2 mM aurovertin and 10 h in the presence of 0.3 mM aurovertin as compared with 2.5 h for the control. In contrast, the sensitivity to aurovertin was much less when the bacteria were grown on glucose; the mean generation time was 1.6 h in the presence of 0.3 mM aurovertin as compared with 1 h for the control. The inhibitory effect of aurovertin on the growth of *E. coli* AN180 was pH dependent, being greater at alkaline pH than at neutral or slightly acid pH. With respect to that of the control culture, the generation time in the presence of 0.13 mM aurovertin increased 1.9-fold at pH 6.6, 2.5-fold at pH 7.0, and 3.6-fold at pH 7.8. When *P. denitrificans* was tested with succinate as a carbon source, growth was strongly inhibited by aurovertin. The doubling time (2.5 h at 30°C) increased more than sevenfold in the presence of 0.3 mM aurovertin. This sensitivity is in agreement with the report of Harris et al. (22) that aurovertin inhibits oxidative phosphorylation in *P. denitrificans* membranes.

Isolation of aurovertin-resistant mutants. Mutagenesis by nitrosoguanidine on agar plates containing succinate, acetate, and malate as nonfermentable carbon sources and 0.2 mM aurovertin resulted in the selection of aurovertin-resistant mutants. Resistance to aurovertin may arise from different types of mutations, including aurovertin-resistant ATPase, lack of permeability to aurovertin, or development of

scavenging mechanisms. Fifteen aurovertin-resistant mutants were selected and purified. Cell extracts from the aurovertin-resistant cells were prepared, and their ATPase activities were compared with that of the parent strain AN180. All 15 mutants contained an ATPase totally resistant to 20 μM aurovertin. Under the same conditions (same aurovertin concentration and same aurovertin to protein ratio), the ATPase activity from the parent strain was inhibited up to 80%. Three mutants (MA1, MA2, and MA3) were chosen at random for further characterization.

Growth of the MA1 mutant in the presence of aurovertin. The effect of aurovertin on the growth of the MA1 mutant in medium supplemented either with glucose or with succinate, acetate, and malate is shown in Fig. 1.

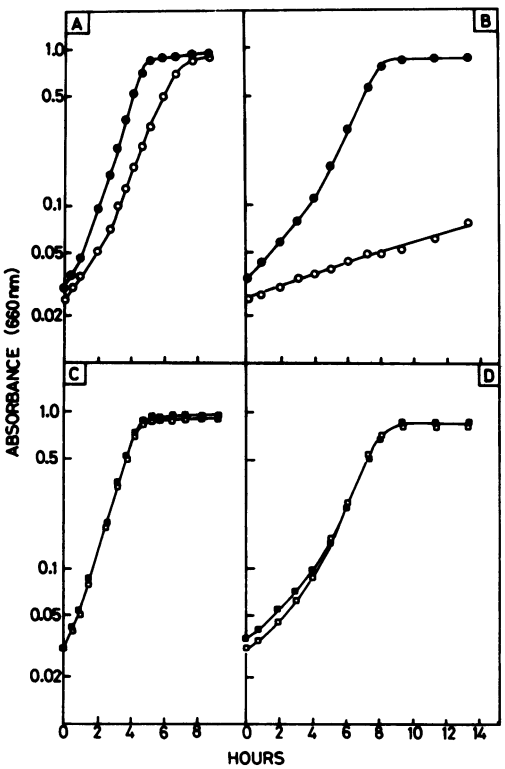

FIG. 1. *Effect of aurovertin on growth of E. coli AN180 and of MA1, an aurovertin-resistant mutant. (A) AN180 cells grown with glucose as a carbon source, without (●) and with (○) 0.3 mM aurovertin. (B) AN180 cells grown with succinate, acetate, and malate as carbon sources, without (●) and with (○) 0.3 mM aurovertin. (C) MA1 cells grown with glucose as a carbon source, without (■) and with (□) 0.3 mM aurovertin. (D) MA1 cells grown with succinate, acetate, and malate as carbon sources, without (■) and with (□) 0.3 mM aurovertin.*

Aurovertin had no effect on the rate and extent of growth in a liquid medium whatever the nature of the carbon source.

Aurovertin-resistant ATPase in the MA1 and MA2 mutants. No inhibition by aurovertin, even at concentrations as high as 20 μM, was found in cell extracts of mutants MA1 and MA2 (Fig. 2). By comparison, the ATPase activity in an extract of the parent strain AN180 was highly sensitive to aurovertin; half-inhibition was obtained with 1 μM aurovertin irrespective of whether the bacteria were grown on glucose or on succinate-acetate-malate.

We investigated whether the aurovertin resistance of mutants MA1 and MA2 is due to a modified soluble BF_1-ATPase. Crude soluble fractions were prepared from AN180, MA1, and MA2 cells (see Materials and Methods). The ATPase activity of all three extracts was insensitive to 0.2 mM dicyclohexylcarbodiimide (DCCD) but was still inhibited by 10 mM azide (Table 2). Aurovertin at a concentration of 20 μM inhibited the ATPase activity of the parent strain by 83% but had no effect on the ATPase of mutants MA1 and MA2.

Absence of cross-resistance to other ATPase inhibitors in aurovertin-resistant mutants. ATPase of cell extracts from aurovertin-resistant mutants retained sensitivity to a number of ATPase inhibitors including azide, DCCD, 7-chloro-4-nitrobenzofurazan, and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Table 3). Figure 3 shows the inhibitory effect of DCCD on the ATPase activity of mutants MA1 and MA2 and of the parent strain. The slightly lower degree of inhibition found for the aurovertin-resistant mutants cannot be considered as highly significant.

Atebrin fluorescence in membrane vesicles. Atebrin fluorescence was used to monitor energization of *E. coli* membrane vesicles by ATP (18). The ATP-induced quenching of fluorescence in vesicles of AN180 (wild type) was fully reversed by addition of aurovertin (Fig. 4). In contrast, the ATP-induced quenching of fluorescence in MA1 vesicles was no longer reversed by aurovertin, but it could still be reversed by uncouplers. Identical results were obtained with the MA2 mutant.

Mapping of the mutation in MA1 leading to an aurovertin-resistant ATPase. It has been established that all mutations of the ATPase complex map in the *unc* region at 83 min on the genetic map of the *E. coli* K-12 chromosome (4). It was therefore desirable to determine whether the mutation responsible for the aurovertin resistance would also map at the *unc* locus. A phage P1 lysate obtained with strain

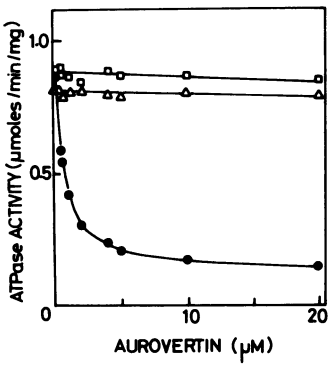

FIG. 2. *Inhibitory effect of aurovertin on ATPase activity in E. coli AN180 and in aurovertin-resistant mutants MA1 and MA2. Cell extracts were prepared from E. coli cells (parent strain [●] and mutants MA1 [□] and MA2 [△]) grown in rich LB medium. Aurovertin was added to the cell extracts in 25 mM Tris-sulfate-2.5 mM EDTA (pH 8.2). The final volume was 0.3 ml and the protein concentration was about 0.3 mg/ml; the ethanol concentration resulting from addition of the ethanol solution of aurovertin was kept constant at 0.3%. After 15 min of preincubation at 37°C, the samples were diluted with 0.2 ml of an ATPase assay mixture to obtain the conditions described in the text. Aurovertin concentrations refer to final concentrations during the ATPase assay.*

TABLE 2. *Effect of aurovertin, DCCD, and sodium azide on solubilized ATPase from E. coli AN180 and mutants MA1 and MA2*[a]

Inhibitor	Inhibition of ATPase activity (%)		
	AN180	MA1	MA2
Aurovertin[b] (20 μM)	83	0	0
DCCD[b] (200 μM)	0	0	0
Sodium azide (10 mM)	87	86	82

[a] Bacterial extracts were preincubated for 10 min at 37°C in a 0.45-ml solution of 0.1 M Tris-sulfate buffer (pH 9.0), 10 mM $MgSO_4$, and inhibitors at the indicated concentrations. The ATPase specific activities were 0.81, 0.51, and 0.56 for the AN180 parent strain and the MA1 and MA2 mutants, respectively.

[b] The final concentration of ethanol was 0.3%.

MA1 was transduced into an ATPase-negative mutant, strain AN120 (*uncA401*) (7). *unc*$^+$ recombinants were selected by growth on succinate-acetate-malate. To spare aurovertin, we avoided the screening of recombinants by replication on aurovertin plates. Instead we grew single colonies in liquid medium with succinate, acetate, and malate as carbon sources and assayed the sensitivity of ATPase to 20 μM aurovertin in cell extracts. Of 43 clones examined, 40 were found to have an ATPase fully resistant to aurovertin. Thus, more than 90% of the *unc*$^+$ recombinants were aurovertin resistant. This strongly suggests that the mutation in MA1 maps in the *unc* region.

DISCUSSION

The fact that aurovertin inhibits growth of *E. coli* on a nonfermentable carbon source more efficiently than on a fermentable carbon source appears to be related to the inhibitory effect of aurovertin on oxidative phosphorylation. This is in agreement with the finding that venturicidin and other inhibitors of oxidative phosphorylation are more inhibitory for growth of *S. cerevisiae* under conditions in which cellular ATP is derived from oxidative phosphorylation rather than from glycolysis (24, 25). It must be noted that the concentration of aurovertin required to inhibit growth is much higher for bacteria like *E. coli* than for the yeast *S. cerevisiae* (3). This may be due to low permeability of bacterial cells to aurovertin, since the sensitivity of *E. coli* ATPase to aurovertin is quite similar to that of the mitochondrial F_1-ATPase.

Fifteen *E. coli* mutants capable of growing on an aurovertin-supplemented medium were selected. Their resistance to aurovertin did not depend on a permeability barrier to aurovertin or on the development of scavenging mechanisms. They all had aurovertin-resistant ATPase.

The frequency of the aurovertin-resistant mutants, characterized by an aurovertin-resistant ATPase, appears to be much higher for *E. coli* than for *S. cerevisiae*. In fact, as recently reported, only 2 of 300 aurovertin-resistant yeast mutants were ATPase mutants; all others were

TABLE 3. *Effect of aurovertin, DCCD, 7-chloro-4-nitrobenzofurazan (Nbf-Cl), quercetin, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), and sodium azide on ATPase activities of membrane-bound ATPase of E. coli AN180 and aurovertin-resistant mutants MA1, MA2, and MA3*

Inhibitor	Inhibition of ATPase activity[a] (%)			
	AN180	MA1	MA2	MA3
Aurovertin[b] (20 μM)	81	6	0	1
DCCD[b] (100 μM)	93	89	85	86
Nbf-Cl[c] (50 μM)	91	89	92	92
Quercetin (0.8 mM)	11.4	9.6	8.6	NT[d]
EEDQ (0.4 mM)	90	92	90.5	NT
Sodium azide (10 mM)	91	92	91	92

[a] The ATPase specific activities were 1.50, 1.09, 1.27, and 1.21 for strains AN180, MA1, MA2, and MA3, respectively.

[b] The final ethanol concentration was 0.3%.

[c] Nbf-Cl inhibition was completely prevented if 0.4 mM dithiothreitol was present in the medium.

[d] NT, Not tested.

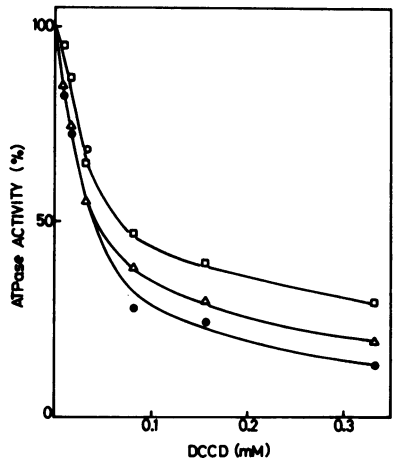

FIG. 3. *Inhibitory effect of DCCD on ATPase activity in cell extracts of E. coli AN180 and in aurovertin mutants MA1 and MA2. Cell extracts were prepared from bacteria grown on rich LB medium (30) and incubated with various concentrations of DCCD. The volume was 0.3 ml, the protein concentration was 0.3 mg/ml, and the final ethanol concentration was 0.3%. After 15 min of preincubation at 37°C, the samples (AN180 [●], MA1 [□], MA2 [△]) were diluted with 0.2 ml of an ATPase assay mixture to obtain the conditions described in the text. The DCCD concentrations refer to final concentrations during the ATPase assay. The ATPase specific activities of AN180, MA1, and MA2 were 0.83, 0.70, and 0.66 μmol/min per mg of protein, respectively.*

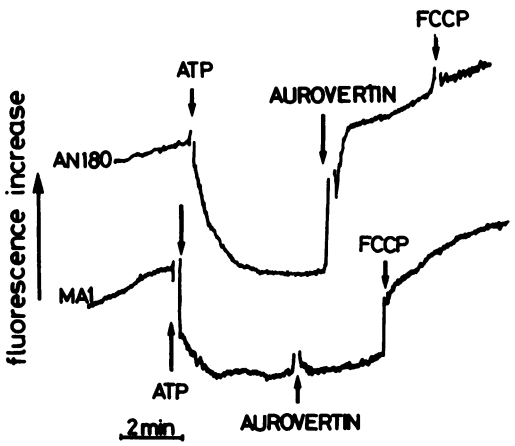

FIG. 4. *ATP-induced fluorescence quenching of atebrin in vesicles from E. coli AN180 and MA1. The reaction medium (2.5 ml) consisted of 10 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES; pH 7), 300 mM KCl, 5 mM $MgCl_2$ and 1.7 mg of particle protein. The assay was carried out at 30°C. The following concentrations were used: atebrin, 4 μM; ATP, 0.8 mM; aurovertin, 20 μM; and carboxyl cyanide-p-trifluoromethoxy-phenylhydrazone (FCCP), 0.4 μM.*

permeability mutants (3). It is interesting that *E. coli* mutants resistant to DCCD are usually permeability mutants (15, 17). However *Streptococcus faecalis* mutants resistant to DCCD were all found to contain a DCCD-resistant ATPase (1).

Three aurovertin-resistant mutants (MA1, MA2, and MA3) were examined in more detail. Their membrane-bound ATPase, although insensitive to aurovertin, was inhibited by DCCD, 7-chloro-4-nitrobenzofurazan, and azide to the same degree as the parent strain. The fact that an aurovertin-resistant ATPase remains sensitive to 7-chloro-4-nitrobenzofurazan is noteworthy in view of the fact that a 7-chloro-4-nitrobenzofurazan-modified mitochondrial F_1-ATPase still interacts with aurovertin indistinguishably from native enzyme (14).

The increase in fluorescence of aurovertin when bound to ATPase has often been correlated with the inhibition of the ATPase activity by aurovertin. Conversely, no increase in fluorescence was found when aurovertin was added to a mitochondrial ATPase preparation from an aurovertin-resistant mutant of *S. cerevisiae*. In the case of *E. coli*, although aurovertin was an efficient inhibitor of ATPase, no fluorescence enhancement was obtained (42).

Aurovertin-resistant mutants described in this paper have an ATPase that is normally energy coupled, as shown by aerobic growth on succinate-acetate-malate and by the response of atebrin fluorescence to energization, but that is no longer inhibited by aurovertin. Although occurring at the *unc* locus, the mutation is phenotypically distinct from other ATPase mutations characterized by uncoupling (*unc*) or by a decreased sensitivity to DCCD. It is not unlikely that the mutated ATPase subunit in aurovertin-resistant mutants is identical to that in some other *unc* mutants.

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