

## Identification of an 82,000-dalton Protein Responsible for $K^+/H^+$ Antiport in Rat Liver Mitochondria\*

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**Under highly selective conditions, pretreatment of rat liver mitochondria with *N,N'*-dicyclohexylcarbodiimide (DCCD) results in 95% inhibition of  $K^+/H^+$  antiport. This inhibition is irreversible.**

**The  $K^+/H^+$  antiporter is reversibly inhibited by physiological ( $Mg^{2+}$  and  $H^+$ ) and pharmacological (quinine and propranolol) inhibitors. Each of these inhibitors protects the  $K^+/H^+$  antiporter against inhibition by DCCD.**

**DCCD is without effect on  $Na^+/H^+$  antiport under all conditions tested, confirming our contention that rat liver mitochondria possess two different alkali cation/proton antiporters (Nakashima, R. A., and Garlid, K. D. (1982) *J. Biol. Chem.* 257, 9252-9254).**

**The selective nature of irreversible inhibition by DCCD has enabled us to label and identify the protein responsible for  $K^+/H^+$  antiport. This protein, the first cation antiporter so identified, migrates on sodium dodecyl sulfate-polyacrylamide gels with a molecular weight of 82,000.**

The best clues to the mysteries of a particular transport process are often provided by inhibitors. The lack of specific inhibitors of  $Na^+$  and  $K^+$  transport in mitochondria has long impeded progress toward characterization of cation transport in these organelles (1). With respect to the  $K^+/H^+$  antiporter, this state of affairs is now improving. Thus, the finding that matrix  $Mg^{2+}$  inhibits the  $K^+/H^+$  antiporter (2) has led to valuable insights into the mechanism by which this porter regulates mitochondrial volume *in vivo* (2-7). Inhibition by protons (8), presumably due to protonation of the  $Mg^{2+}$  site, has provided an explanation for the marked difference in pH profiles for  $K^+/H^+$  versus  $Na^+/H^+$  antiport (9). The observation that quinine (9) and other hydrophobic amines (10) inhibit  $K^+/H^+$ , but not  $Na^+/H^+$ , antiport has permitted us to conclude that mitochondria possess two distinct alkali cation/proton antiporters with very different cation specificities and physiological functions (9). Each of these interactions is rap-

idly reversible; therefore, we have not been able to use these inhibitors to label and identify the integral membrane protein presumed to be responsible for transport.

We now report that the  $K^+/H^+$  antiporter is irreversibly inhibited by DCCD<sup>1</sup> and that DCCD inhibition is prevented by the reversible physiological ( $Mg^{2+}$  and  $H^+$ ) and pharmacological (quinine and propranolol) inhibitors of the antiporter. The highly selective nature of DCCD inhibition has enabled us to label and identify the protein responsible for  $K^+/H^+$  antiport in intact mitochondria. To our knowledge, this is the first assignment of alkali cation/proton antiport function to a particular membrane polypeptide.

### EXPERIMENTAL PROCEDURES

**Preparation of Mitochondria**—Rat liver mitochondria were isolated as previously described (2). Stock suspensions containing 50 mg/ml of protein were stored in 0.25 M sucrose at 0 °C and used within 4 h of preparation.

**Transport Studies**—To assay the effects of DCCD on  $K^+/H^+$  antiport, we followed mitochondrial volume changes in potassium acetate medium, in which the rate of uptake of potassium acetate and water depends on the rate of electroneutral  $K^+/H^+$  antiport (9). The points in Figs. 1-3 are values of inverse absorbance, which is linearly related to matrix volume (11), printed out at 0.6-s intervals after averaging 100 samples taken at 3-ms intervals. The apparatus consists of a Brinkmann PC 700 colorimeter with a 520-nm filter and a fiberoptic probe. The probe is immersed in 10 ml of assay medium, maintained at 25 °C, to which 1 mg of mitochondrial protein is added. The analog signal, linear with transmittance, is fed through a Cyborg 91A analog/digital converter to an Apple IIe computer, where smoothing, conversion to inverse absorbance, storage, and real-time plotting are carried out. The assay medium for Figs. 1-3 contains 55 mM  $K^+$  acetate, 5 mM TES (pH 7.8), 0.1 mM EDTA, 0.1 mM EGTA, and 1  $\mu$ g/mg of rotenone. This assay for  $K^+/H^+$  antiport yields reproducible results and is sufficiently sensitive for rapid transport processes.

**SDS-Polyacrylamide Gel Electrophoresis of [<sup>14</sup>C]DCCD-labeled Mitochondrial Proteins**—Following preincubation of mitochondria (1 mg/ml) with [<sup>14</sup>C]DCCD, 0.1 ml of suspension was added to 0.9 ml of ice-cold acetone (12). The precipitated protein was solubilized with SDS and subjected to SDS-PAGE on 7.5 and 15% gels by the method of Laemmli (13). Fluorographs were prepared by the method of Bonner and Laskey (14). [<sup>14</sup>C]DCCD was obtained from Research Products International with a stated specific activity of 54 mCi/mmol. Bio-Rad standards were used for the estimates of molecular weight.

### RESULTS

**Effects of DCCD on  $K^+/H^+$  and  $Na^+/H^+$  Antiport**—Fresh mitochondria exhibit little tendency to swell in potassium acetate, because the  $K^+/H^+$  antiporter is fully inhibited by endogenous  $Mg^{2+}$  (2, 5, 6). Upon addition of EDTA and the divalent cation ionophore, A23187, the carrier is released from inhibition and the mitochondria swell, as reflected in the trace labeled -DCCD in Fig. 1. When the same assay is carried out on mitochondria preincubated with DCCD (50 nmol/mg) for 45 min, there is no inhibition of  $K^+/H^+$  antiport (Fig. 1, +DCCD). The failure of DCCD to inhibit  $K^+/H^+$  antiport when preincubated with *normal*,  $Mg^{2+}$ -containing mitochondria is consistent with the findings of Jung *et al.* (15).

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<sup>1</sup> The abbreviations used are: DCCD, *N,N'*-dicyclohexylcarbodiimide; TES, *N*-tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

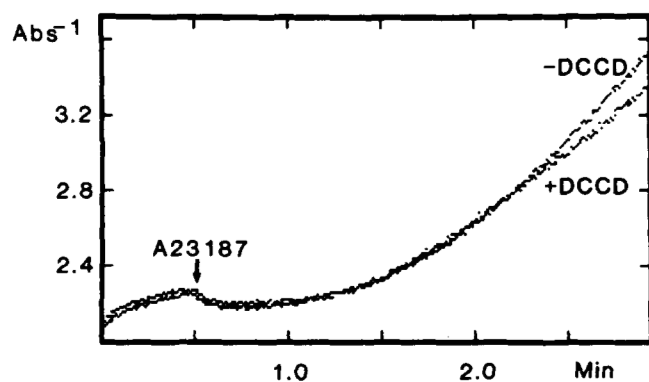


FIG. 1. DCCD does not inhibit the  $K^+/H^+$  antiporter in normal  $Mg^{2+}$ -containing mitochondria. Normal mitochondria (10 mg/ml) were preincubated at  $0^\circ C$  for 45 min in the presence (+DCCD) or absence (-DCCD) of DCCD (50 nmol/mg). The preincubation medium (0.27 osmolal) contained 210 mM sucrose and potassium salts of TES (24.6 mM, pH 7.8) and EGTA (50  $\mu M$ ) at  $0^\circ C$ . 0.1 ml was transferred to the assay medium, and A23187 (1 nmol/mg) was added after 30 s. See "Experimental Procedures" for details of the assay.

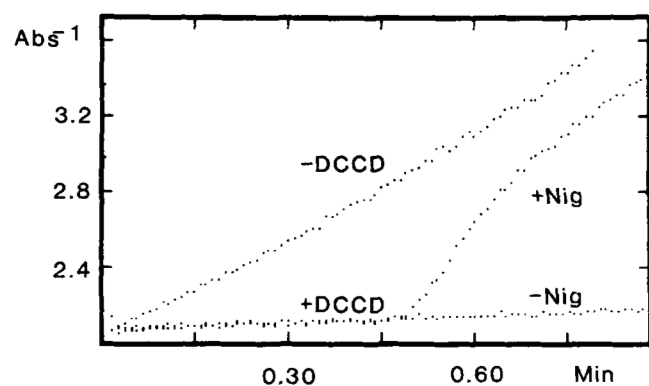


FIG. 2. DCCD inhibits the  $K^+/H^+$  antiporter in  $Mg^{2+}$ -depleted mitochondria. Mitochondria (10 mg/ml) were first preincubated at  $25^\circ C$  in a medium containing A23187 and EDTA, resulting in rapid depletion of matrix  $Mg^{2+}$  from 38 nmol/mg to 1 nmol/mg (6, 42). After 2 min, they were cooled to  $0^\circ C$  and preincubated for 45 min in the presence (+DCCD) or absence (-DCCD) of DCCD (50 nmol/mg). In one assay of DCCD pretreated mitochondria, nigericin (0.1 nmol/mg) was added at 0.5 min (+Nig). The preincubation medium (0.11 osmolal) contained 50 mM sucrose and potassium salts of TES (18.8 mM, pH 7.8) and EDTA (4.4 mM) plus rotenone (1  $\mu g$ /mg) and A23187 (1 nmol/mg). 0.1 ml was transferred to the assay medium. See "Experimental Procedures" for details of the assay.

Remarkably,  $K^+/H^+$  antiport is inhibited by 95% by DCCD when  $Mg^{2+}$  is removed from the matrix before exposure to DCCD (Fig. 2). This inhibition is not relieved by three washes in 0.25 M sucrose containing 3 mg/ml of bovine serum albumin (not shown), and we conclude that the inhibition by DCCD is irreversible. This indicates that DCCD binds covalently to a site on the  $K^+/H^+$  antiporter, but only in the absence of  $Mg^{2+}$ . The response to nigericin (See Fig. 2) demonstrates that the DCCD-treated preparation responds normally to an exogenous  $K^+/H^+$  antiporter.

Under conditions identical to those described for Fig. 2, DCCD inhibition of  $K^+/H^+$  antiport is found to depend strongly on the pH of the preincubation medium. For example, preincubation with DCCD at pH 6.8, rather than 7.8 (as in Fig. 2), results in less than 10% inhibition of  $K^+/H^+$  antiport (data not shown). Thus, protons and magnesium, the physiological inhibitors of the  $K^+/H^+$  antiporter (2, 8), both protect this protein against inhibition by DCCD.

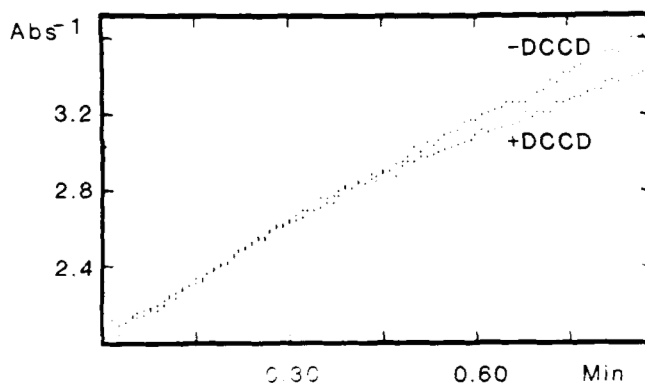


FIG. 3. Quinine protects the  $K^+/H^+$  antiporter from DCCD inhibition.  $Mg^{2+}$ -depleted mitochondria (10 mg/ml) were preincubated with 0.5 mM quinine in the presence (+DCCD) or absence (-DCCD) of DCCD (50 nmol/mg). Except for the presence of quinine, the preincubation medium was identical to that described in the legend to Fig. 2. See "Experimental Procedures" for details of the assay.

The reversible pharmacological inhibitors of the  $K^+/H^+$  antiporter (9, 10) also protect against inhibition by DCCD. In the experiments reported in Fig. 3,  $Mg^{2+}$  depleted mitochondria were incubated with 0.5 mM quinine at pH 7.8 prior to the assay. This concentration of quinine is about 30 times the dose required to inhibit antiport by 50% at pH 7.8 (10). The quinine is diluted 101-fold when the suspension is transferred to the assay medium, and the lack of inhibition in the control trace (Fig. 3, -DCCD) illustrates the rapid reversibility of quinine inhibition. It can be seen that quinine provides nearly complete protection against DCCD inhibition (Fig. 3, +DCCD). The same result is obtained with 0.5 mM propranolol, another reversible inhibitor of  $K^+/H^+$  antiport (10) (data not shown).

We have carried out numerous experiments attempting to inhibit  $Na^+/H^+$  antiport with DCCD. We have varied anions, cations, pH, temperature, and osmotic strength of the preincubation medium, and all of these attempts have been unsuccessful. This lack of effect of DCCD on  $Na^+/H^+$  antiport is in agreement with, and extends, the results of a previous study (15).

**Labeling the  $K^+/H^+$  Antiporter with [ $^{14}C$ ]DCCD**—These findings immediately suggested the possibility that the  $K^+/H^+$  antiporter protein could be labeled with [ $^{14}C$ ]DCCD and identified by SDS-PAGE. The fluorographs in Fig. 4, lanes 1-3, are the results of experiments in which the conditions of exposure to [ $^{14}C$ ]DCCD are identical to the conditions of DCCD treatment used in the kinetic assays of Figs. 1-3. Accordingly, we expect to see labeling of the antiporter protein in  $Mg^{2+}$ -depleted mitochondria (lane 2), but not in normal mitochondria (lane 1) or in  $Mg^{2+}$ -depleted mitochondria protected by quinine (lane 3). The 82,000-dalton band which appears in lane 2 is the only labeled band which fulfills these criteria (Fig. 4).<sup>2</sup> In accordance with the pH dependence of DCCD inhibition, the 82,000-dalton band also does not appear when  $Mg^{2+}$  depleted mitochondria are incubated with [ $^{14}C$ ]DCCD at pH 6.8 (not shown).

Coomassie blue staining of gels reveals a distinct band which migrates with an apparent molecular weight of 82,000. Neither the position nor the intensity of this band is affected

<sup>2</sup> Fluorographs of 15% gels (not shown) have enabled us to study [ $^{14}C$ ]DCCD-labeling of low molecular weight polypeptides which are not visualized in 7.5% gels (Fig. 4). Applying the same criteria to these bands, we find no candidates for the  $K^+/H^+$  antiporter in this region.

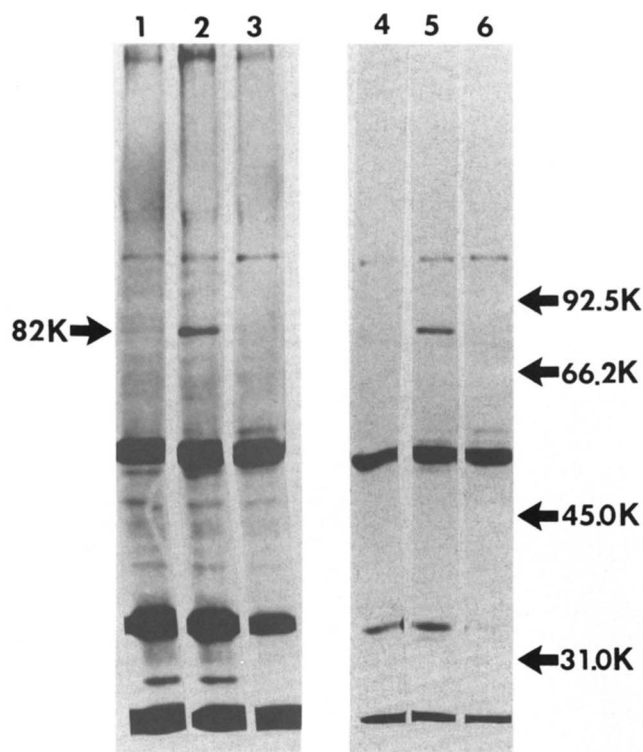


FIG. 4. Identification of the  $K^+/H^+$  antiporter on fluorographs prepared from [ $^{14}C$ ]DCCD-labeled mitochondria. Fluorographs were obtained following SDS-PAGE using 7.5% gels. The conditions of [ $^{14}C$ ]DCCD treatment corresponded closely to the conditions of the assays in Figs. 1–3. Mitochondria (1 mg/ml) were incubated for 45 min at 0 °C with 100 nmol/mg of [ $^{14}C$ ]DCCD. Lanes 1–3, aliquots of stock mitochondria were incubated with [ $^{14}C$ ]DCCD in the labeling media described below. Lanes 4–6, mitochondria were first pretreated with cold DCCD (50 nmol/mg) for 45 min in a 0.27 osmolal medium identical to the preincubation medium described in the legend to Fig. 1. The cold DCCD was removed by washing the mitochondria three times with 0.1 mM  $K^+$  EGTA, 5 mM  $K^+$  TES (pH 6.7), 240 mM sucrose, and 3 mg/ml of bovine serum albumin. Following the washes, mitochondria were suspended in 0.25 M sucrose and aliquots were incubated with [ $^{14}C$ ]DCCD in the labeling media described as follows. Lanes 1 and 4 ( $Mg^{2+}$ -containing mitochondria), the composition of the labeling medium was identical to the preincubation medium described in the legend to Fig. 1. Lanes 2 and 5 ( $Mg^{2+}$ -depleted mitochondria), the composition of the labeling medium was identical to the preincubation medium described in the legend to Fig. 2. Lanes 3 and 6 ( $Mg^{2+}$ -depleted mitochondria with quinine), the composition of the labeling medium was identical to the preincubation medium described in the legend to Fig. 3. The observed mean and standard deviation for the molecular weight of the putative  $K^+/H^+$  antiporter was found to be  $82,400 \pm 2,700$  ( $n = 11$ ).

by any of the factors which affect [ $^{14}C$ ]DCCD labeling of the bands.

We attempted next to reduce the  $^{14}C$  labeling of non-antiporter proteins by pretreating normal,  $Mg^{2+}$ -containing mitochondria with unlabeled DCCD, then washing with bovine serum albumin to remove unreacted DCCD. Subsequent exposure of these mitochondria to [ $^{14}C$ ]DCCD results in fewer labeled bands, as expected (Fig. 4, lane 4). When [ $^{14}C$ ]DCCD is added after  $Mg^{2+}$  depletion, the label is again concentrated in the 82,000-dalton band (Fig. 4, lane 5) but does not appear following co-treatment with quinine and DCCD (Fig. 4, lane 6).

The reversibility of DCCD binding was examined using SDS-PAGE, as follows.  $Mg^{2+}$ -depleted mitochondria were reacted with unlabeled DCCD, then washed with bovine serum albumin. After exposure of these mitochondria to [ $^{14}C$ ]

DCCD under the conditions of Fig. 4, lane 5, the 82,000-dalton protein was not labeled (not shown). Thus, prior exposure to DCCD under conditions favorable for DCCD inhibition and binding prevents subsequent binding of [ $^{14}C$ ]DCCD to the 82,000-dalton protein, indicating that the binding is irreversible.

#### DISCUSSION

DCCD is gaining increasing recognition as a valuable probe of ion transport proteins. In mitochondria, DCCD has been reported to inhibit proton translocation through the  $F_0$  component of the ATPase (16, 17) through cytochrome *c* oxidase (18, 19), through transhydrogenase (20, 21), and through the cytochrome *b-c*<sub>1</sub> complex (22, 23). DCCD has also been shown to inhibit anion transport in liver (24, 25), heart (26), and brown adipose tissue mitochondria (27) and to inhibit respiration-dependent  $K^+$  transport in liver (28) and heart (15) mitochondria. Many mitochondrial proteins have been visualized by SDS-PAGE with the aid of [ $^{14}C$ ]DCCD (12, 18–21, 26, 27, 29–34) (see Fig. 4), but none has previously been reported with a molecular weight of 82,000.

The kinetic assays of Figs. 1–3 reveal that DCCD is a potent inhibitor of the  $K^+/H^+$  antiporter provided that the antiporter is in an active configuration.<sup>3</sup> The finding that DCCD can neither inhibit nor bind to the 82,000-dalton  $K^+/H^+$  antiporter in the presence of physiological ( $Mg^{2+}$  and  $H^+$ ) or pharmacological (quinine and propranolol) inhibitors is consistent with our proposals that these factors interact directly with inhibitory sites on the antiporter protein (2–6, 8, 9). The dramatic effect of  $Mg^{2+}$  depletion on DCCD inhibition and binding (Figs. 2 and 4) strongly supports our contention that it is *matrix*  $Mg^{2+}$  which inhibits the  $K^+/H^+$  antiporter (2–6). Thus, DCCD is unable to inhibit (Fig. 1) or label (Fig. 4, lane 1) normal,  $Mg^{2+}$ -containing mitochondria despite the absence of  $Mg^{2+}$  from the external medium. Protection by *matrix*  $Mg^{2+}$  against binding of DCCD to the antiporter protein best explains why Jung *et al.* (15) failed to observe DCCD inhibition of  $K^+/H^+$  antiport.

We agree with Jung *et al.* (15) that DCCD does not inhibit  $Na^+/H^+$  antiport. The differential effects of DCCD on  $Na^+/H^+$  versus  $K^+/H^+$  antiport confirm our proposal, based on quinine inhibition studies, that rat liver mitochondria possess two independent alkali cation/proton antiporters (see Ref. 9 for a description of their properties and possible physiological roles).

In all studies we have carried out, we have observed a one to one correspondence between DCCD inhibition of  $K^+/H^+$  antiport, assayed as in Figs. 1–3, and [ $^{14}C$ ]DCCD labeling of the 82,000-dalton protein. This constitutes strong evidence for identifying this protein with the  $K^+/H^+$  antiporter and permits preliminary speculation on the nature of the reversible and irreversible inhibitory sites on this transport protein. We envisage three distinct reactive sites on the antiporter protein. 1) *Transport sites* bind  $K^+$  and  $H^+$  prior to obligatorily coupled, electroneutral  $K^+/H^+$  antiport (2–4). We find that  $K^+$  does not affect the kinetics of DCCD inhibition,<sup>3</sup> suggesting little influence of the cation transport sites on DCCD binding. 2) A *hydrophilic regulatory site* is localized on the side of the  $K^+/H^+$  antiporter exposed to the matrix (3, 4). This site binds  $Mg^{2+}$  (2) and can be protonated when free of

<sup>3</sup> It is of interest that DCCD inhibition does not require the presence of a transportable cation. DCCD inhibits  $K^+/H^+$  antiport and labels the 82,000-dalton protein in preparations in which the nontransported tetraethylammonium cation is substituted for matrix  $K^+$  and  $Mg^{2+}$  (W. H. Martin, A. D. Beavis, and K. D. Garlid, unpublished observations).

$Mg^{2+}$  (8). The result in either case is inhibition of antiport. Protection against DCCD by  $Mg^{2+}$  and  $H^+$  acting at a hydrophilic site is interesting, since DCCD most likely reacts in a hydrophobic environment (35–37). This may indicate that the binding of  $Mg^{2+}$  or protons induces a conformational change in the antiporter which prevents the DCCD-binding site from entering the hydrophobic domain of the membrane or, equivalently, which protects this site from access by DCCD. 3) A hydrophobic reactive site interacts with quinine and other hydrophobic amines (9, 10), resulting in inhibition of antiport. Protection against DCCD by hydrophobic amines suggests that these agents compete with DCCD for the hydrophobic site. We are currently studying the kinetics of DCCD inhibition of  $K^+/H^+$  antiport in an attempt to shed further light on the nature of these interactions.

The existence of cation/proton antiporters in energy transducing systems was deduced by Mitchell (38) long before there was experimental evidence to support such a mechanism. The physiological necessity for such antiporters derives from the fact that chemiosmotic mitochondria respiring *in vivo* are infinite sinks for alkali cations crossing the inner membrane electrophoretically. Consideration of the osmotic consequences of net potassium transport led Garlid (2) to conclude that the  $K^+/H^+$  antiporter must be precisely regulated in order for mitochondria to maintain constant volume. The  $Mg^{2+}$  carrier brake hypothesis (2), for which there is now considerable evidence (2–9), provides this regulation and emphasizes the central, homeostatic role which the  $K^+/H^+$  antiporter plays in energy transduction.

To our knowledge, the present report is the first to identify a specific membrane protein responsible for alkali cation/proton antiport. This identification constitutes a practical assay for the  $K^+/H^+$  antiporter which can be used to monitor purification and reconstitution procedures. This assay may also be useful for identifying  $K^+/H^+$  antiporters in other systems, such as the red blood cell (39) and *Escherichia coli* (40, 41). Such studies are currently underway in our laboratory.

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