J. Biochem. 120, 301-305 (1996)

Open Reading Frame 3 of the Barotolerant Bacterium Strain DSS12 Is Complementary with *cydD* in *Escherichia coli*: *cydD* Functions Are Required for Cell Stability at High Pressure[1]

Chiaki Kato,*,[2] Hideyuki Tamegai,* Akihiko Ikegami,† Ron Usami,† and Koki Horikoshi*,†

The DEEP STAR group, Japan Marine Science and Technology Center, 2-15 Natsushima-cho, Yokosuka, Kanagawa 237; and †Department of Applied Chemistry, Faculty of Engineering, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350

Received for publication, February 16, 1996

***Escherichia coli* strain JD518, a *cydD*-deficient mutant, displayed temperature-sensitive and pressure-sensitive growth. The defective *cydD* gene in this strain was complemented by open reading frame 3 (ORF3), previously identified in DNA from a barotolerant bacterium, strain DSS12, allowing growth of the *cydD* mutant under high temperature and high pressure conditions. Spectrophotometrical analysis indicated that the cytochrome *bd* complex which is assembled by the CydD protein was expressed in *E. coli* strain JD518 carrying the ORF3 gene at the same level as occurred in the wild-type strain. Our results indicate that the *cydD* gene functions are required for cell stability under the condition of high pressure stress in bacteria.**

Key words: barotolerant bacterium, *cydD*, *Escherichia coli*, high pressure, respiratory system.

We have isolated several deep-sea barophilic and barotolerant bacteria to investigate how these bacteria have adapted to life in an environment characterized by conditions of extremely high pressure (*1, 2*). A pressure-regulated promoter region from the barophilic bacterium strain DB6705, isolated in our laboratory, was cloned and analyzed, and this promoter was shown to function under elevated hydrostatic pressures in both the barophilic strain and *Escherichia coli* (*3*). A pressure-regulated operon was found downstream of this promoter region in DNA from the same strain, and expression of the genes in this operon was controlled by elevated pressure at the level of transcription (*4*). Interestingly, the cloned pressure-regulated promoter region is one of the common sequences found in the DNA of deep-sea, high-pressure-adapted bacteria isolated in our laboratory (*3, 5*). Thus, this DNA region might have important functions required for survival of deep-sea-adapted microorganisms in a high-pressure environment. Another operon downstream of the pressure-regulated operon in the barotolerant strain DSS12 was found to consist of 2 open reading frames, ORF3 and ORF4, which were homologous to genes in the *cydD-cydC* operon of *E. coli*. Expression of this operon was enhanced under elevated pressure at the level of transcription (*6*). In *E. coli*, CydD is required for assembly of the cytochrome *bd* complex, which is one of the components in the respiratory system of bacteria. The *cydD* gene product is also a necessary component of the bacterial respiratory system (*7-9*).

In this paper, we demonstrate that a *cydD*-deficient mutant of *E. coli* is not only temperature-sensitive, but also pressure-sensitive, and that the ORF3 of strain DSS12 is complementary to *cydD*. Our findings indicate that *cydD* may function against physical stress caused by extremes of temperature, pressure, *etc.*, allowing survival in an extreme environment. This is the first report to indicate that the bacterial respiratory system may have an important role in the mechanism of survival in high-pressure environments.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Study—*E. coli* strain B178 (wild type) and *cydD*-deficient mutant strain JD518 were kindly provided by Dr. J. M. Delaney (*10*). The recombinant plasmid, pAI-16 (Fig. 1), which contains the ORF3 sequence region of the barotolerant strain DSS12 (*6*), was used in complementation studies concerning *cydD*. Recombinant DNA work was carried out as described by Sambrook *et al.* (*11*). LB agar medium [1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 1% (w/v) NaCl, and 2% agar] containing ampicillin (50 μg/ml) was used to select transformants. *E. coli* strains were cultivated under atmospheric pressure and under high pressure as described previously (*12*).

Preparing the Membrane Fractions of Bacterial Cells—Each strain of *E. coli* was grown in 500 ml of medium at 30°C for 18 h. After cultivation, the cells were collected by centrifugation (10,000 × *g*, 10 min), and resuspended in 50 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 0.3 M NaCl. Cells were disrupted with a sonic oscillator (20 kHz, 200 W, Tomy, Model UR-20P, Tokyo) for a total period of 30 min, and centrifuged (10,000 × *g*, 10 min) to remove

[1] The nucleotide sequence data reported in this paper will appear in DDBJ/EMBL/GenBank DNA databases with the accession number D83386.

[2] To whom correspondence should be addressed. Tel: +81-468-67-5555, Fax: +81-468-66-6364, E-mail: katoc@jamstec.go.jp

unbroken cells. The cell-free extract thus obtained was centrifuged at 143,000×*g* for 1 h (Beckman ultracentrifuge, Model XL-80, Paloalto, CA, USA). The resulting pellets were suspended in 30 ml of 10 mM Tris-HCl buffer, pH 8.0. This suspension was used as the membrane fraction.

Physical and Chemical Measurements—Spectrophotometrical analyses were performed with a Beckman Model DU-7500 spectrophotometer with 1-cm light path cuvettes. The protein concentrations were estimated by the method of Lowry *et al.* (*13*) with slight modifications (*14*). The contents of cytochrome *bd* were calculated with $\Delta\varepsilon$ of 18.8 $cm^{-1} \cdot mM^{-1}$ at the wavelength pair 628-649 nm of the reduced *minus* oxidized difference spectra (*15*).

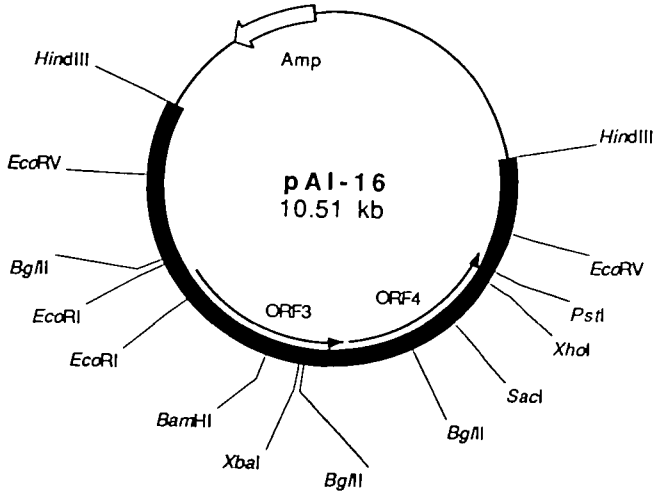

Fig. 1. **Restriction map of the recombinant plasmid, pAI-16.** The thin lines represent the pMW118 DNA (Nippon Gene, Toyama). The white arrow shows the region of the antibiotic-resistance gene. Amp, ampicillin-resistance gene. The thick line represents DNA from strain DSS12 (the nucleotide sequence data will appear in DDBJ/EMBL/GenBank DNA databases with the accession number D83386).

RESULTS AND DISCUSSION

Growth Study—The plasmid pAI-16 was introduced into *E. coli* strain JD518, and transformants were obtained on LB agar plates containing 50 μg/ml ampicillin. *E. coli* strains, B178, JD518, and a transformant of JD518 [JD518 (pAI-16)], were cultivated at 37°C under atmospheric pressure (0.1 MPa) with shaking, and at 30°C under a pressure of 0.1 or 30 MPa with oxygenated Fluorinert in the pressure vessels, without shaking. The cell growth profiles are shown in Figs. 2 and 3. Strain JD518, the *cydD*-deficient mutant, was unable to grow at 37°C, whereas the wild-type strain B178 and the transformant strain JD518 (pAI-16) were able to grow and had similar growth profiles (Fig. 2). Delaney *et al.* (*10*) have reported that strain JD518 is a temperature-sensitive mutant, so this result indicates that the ORF3 carried on plasmid pAI-16 complements *cydD*. When cultured at 30°C under a pressure of 0.1 MPa the growth profiles of all three strains were

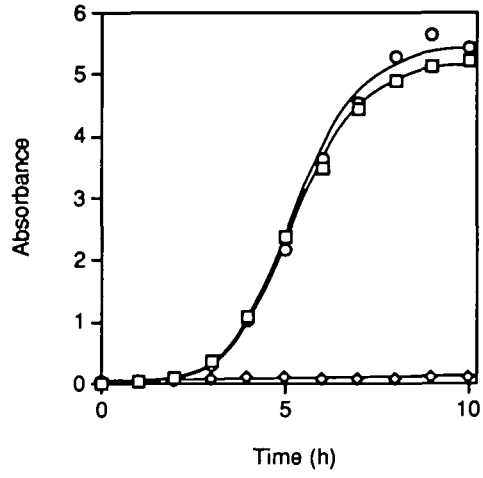

Fig. 2. **Growth profiles of the *E. coli* strains, B178 (□), JD518 (◇), and JD518 carrying the plasmid pAI-16 (○), grown at 37°C in LB broth with shaking (0.1 MPa).** The absorbance of the culture was determined at 660 nm.

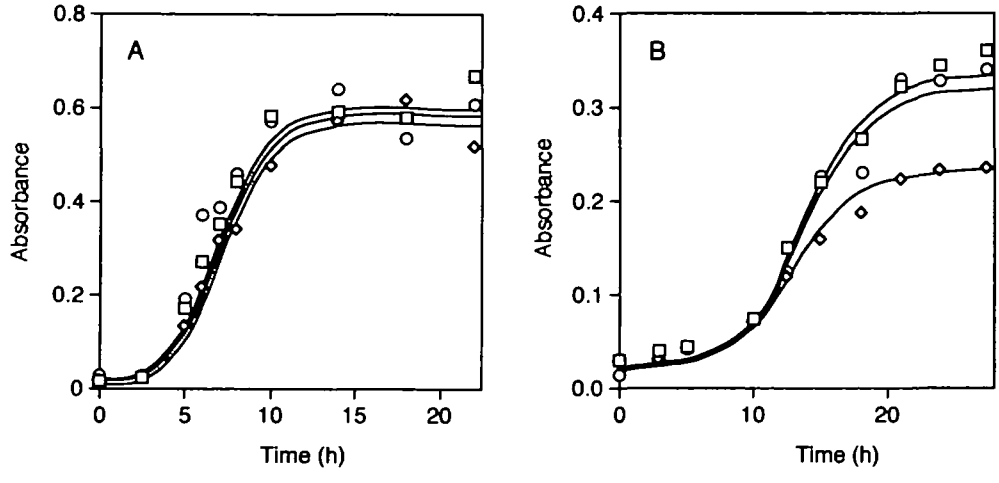

Fig. 3. **Growth profiles of the *E. coli* strains, B178 (□), JD518 (◇), and JD518 carrying the plasmid pAI-16 (○), grown at 30°C in LB broth with oxygenated Fluorinert in the pressure vessels.** The absorbance of the culture was determined at 660 nm. A, cells grown under 0.1 MPa; B, cells grown under 30 MPa.

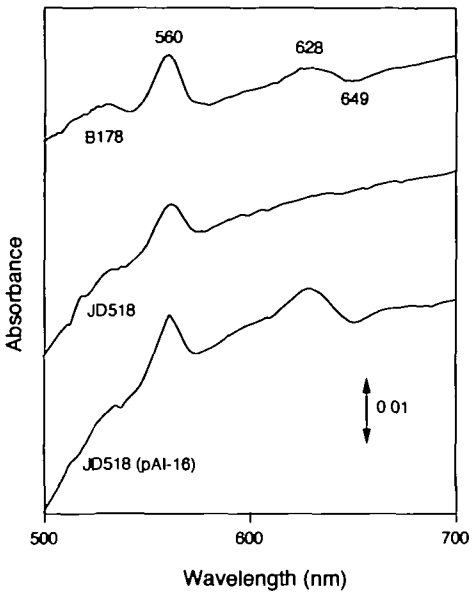

Fig. 4. **Reduced *minus* oxidized difference spectra of the membrane fractions from each strain of *E. coli*.** The protein concentration in each fraction was 1.29 mg/ml. The reduced form was prepared by adding a small amount of $Na_2S_2O_4$. The contents of cytochrome *bd* from the wild type strain B178 and the transformant strain JD518 (pAI-16) were 0.0868 and 0.202 nmol/mg of protein, respectively.

```
DSS12 ORF3   1' MDKSLEKQLTSWLRLQKKACGFYLNLTVLFGLLTGLSLIIQAYLLSTILHGVIILELDKS
                *.** .*.**.**. *.  .  .**.. *.*...*. .* **.... **. .*. .. ..
E.coli CydD  1* MNKSRQKELTRWLKQQSVISQRWLNISRLLGFVSGILIIAQAWFMARILQHMIMENIPRE

            61' HYTHEFILLLVLIPIRALLAFARERACFESGKRLRLQIRSAVLDKLTELGPAFIKGKPVG
                  *.**.. . .** ... ***. ...*...*..**..***.* . ***.*.***.*
            61* ALLLPFTLLVLTFVLRAWVVWLRERVGYHAGQHIRFAIRRQVLDRLQQAGPAWIQGKPAG

           121' SWASIVLEQVEDLHDFYARYLPQIILAGFIPLTILIVVFPLNWAAGLILLATAPLIPMFM
                ***..****..*.**.*******. **  .** *....** ****.****.******.**
           121* SWATLVLEQIDDMHDYYARYLPQMALAVSVPLLIVVAIFPSNWAAALILLGTAPLIPLFM

           181' ILVGMGAADANRKNFSALAKLSGHFMDRLKGLPTLKLFNRGDAEVKEIETASEEFRERTM
                ***********.** ***.*****.***.*. **..*.**.**...* .***.**.***
           181* ALVGMGAADANRRNFLALARLSGHFLDRLRGMETLRIFGRGEAEIESIRSASEDFRQRTM

           241' SVLRMAFLSSAVLEFFSAVSIAVLAVYFGFSYLGHLNFGDYNGGVSLFIGLFVLMLAPEF
                .***.*****..****...***..**********.*.**.*..**.*  *...*.*****
           241* EVLRLAFLSSGILEFFTSLSIALVAVYFGFSYLGELDFGHYDTGVTLAAGFLALILAPEF

           301' YQPLRDMGTHYHAKAQAIGAAEELMALLEYRVESNSVESKSGGNEIKHMAVNDKAIDWKA
                .*****.** *******.***..*....* ..    .....*..*   .*        ..
           301* FQPLRDLGTFYHAKAQAVGAADSLKTFMETPL----AHPQRGEAE---LA-------STD

           361' GVEIEARDLKVLSHDGATLVGPVSFHLSPGQHVAIVGPSGSGKTSLLNAMLGFLPYEGSL
                *.*** .* . *..* **.**..* *..**....**.*****.*****. ***.*.***
           347* PVTIEAEELFITSPEGKTLAGPLNFTLPAGQRAVLVGRSGSGKSSLLNALSGFLSYQGSL
                                                   ATP_A

           421' KVNGLELSDLDMTLWRRHLVWLAQDPQLFHGTVRDNVAMADVAMGDEAIKLLLEKATILE
                ..**.**.**. . **.**  ..*.***  .*.**** .*  . .....  *..* . *
           407* RINGIELRDLSPESWRKHLSSVGQNPQLPAATLRDNVLLARPDASEQELQAALDNAWVSE

           481' CVEQQSLGLSHPMGEQMAGLSVGQAQRLALARAGGQDPHLPLLDDPTASLDSHSEQAVLS
                .  . *.. *.*.* * ********.*.*** .  *.***.*.****.**** *..
           467* FLPLLPQGVDTPVGDQAARLSVGQAQRVAVARALLNPCSLLLLDEPAASLDAHSEQRVME
                                                   ATP_B

           541' TLWQAMDKTSCLMVTHRLDQLHQMDTILVLDAGAIVQRGSFEDLNRAEGLF-KLMQEDET
                .* .*  . . *****.*..* . *.* *...* *...*....*. *.* * .*... ..
           527* ALNAASLRQTTLMVTHQLEDLADWDVIWVMQDGRIIEQGRYAELSVAGGPFATLLAHRQE

           600' DVEAQANKESNS
                ..
           587* EI
```

Fig. 5. **Alignment of the DSS12 ORF3 and *E. coli* CydD protein sequences using the GENETYX-MAC program (ver. 8.0, Software, Tokyo).** Identical residues, (*); similar residues, (.). The boxed sequences indicate the ATP_A and ATP_B-binding sites, respectively.

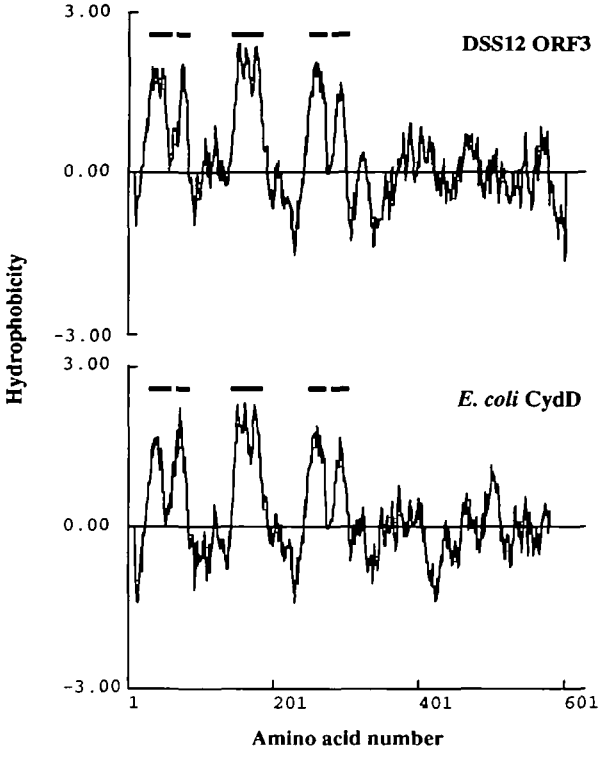

Fig. 6. **Predicted hydrophobicity plots of the polypeptides of the DSS12 ORF3 and *E. coli* CydD using the parameter of Kyte & Doolittle in the GENETYX-MAC program (ver. 8.0, Software).** The hydropathy indices were calculated with a window of 19 amino acids. The horizontal bars indicate the putative membrane-spanning regions.

very similar (Fig. 3A); however, when cultured at 30°C under a pressure of 30 MPa the growth profile of the *cydD* mutant, strain JD518, differed from the others (Fig. 3B). Strain JD518 was pressure-sensitive. In the case of this strain, the maximum absorbance of the culture at 660 nm was around 0.2, whereas that of strains B178 and JD518 (pAI-16) was around 0.35. These results suggest that the ORF3 of the barotolerant strain DSS12 functions as *cydD*, and apparently this gene product is very important for growth of the cells under conditions of high temperature and high pressure.

Spectrophotometric Analyses of the Membrane Fractions—Reduced *minus* oxidized difference spectra of the membrane fractions isolated from each strain of *E. coli* are shown in Fig. 4. In the case of the membranes of the wild-type strain B178, an absorption peak at 628 nm and a valley at 649 nm typical of cytochrome *bd* (*15*) were clearly observed, but these were not apparent in the case of the mutant strain JD518. On the other hand, the transformant strain JD518 (pAI-16) expressed cytochrome *bd* as shown in Fig. 4.

Similarity of the Amino Acid Sequences between the DSS12 ORF3 and E. coli CydD—The amino acid sequences of the DSS12 ORF3 and the *E. coli* CydD proteins have 83% similarity and 49% identity (Fig. 5). However the hydropathy profiles of the two proteins (Fig. 6) indicate both that they are membrane proteins and that they are very similar. The N-terminal half of each protein sequence contains stretches of hydrophobic amino acids corresponding to six transmembrane helices. The C-terminal half of each protein sequence is hydrophilic and contains an ATP-binding site (Fig. 5) comprising the Walker A (ATP_A) and B (ATP_B) motifs (*16*). The ORF3 protein therefore appears to be subunits of an ABC membrane transporter (*17*) containing both a membrane domain and an ATP-binding domain.

The ORF3 from the barotolerant strain DSS12 complemented the *cydD* gene of *E. coli*, indicating that it is homologous to *cydD* in terms of physiological function. Further, ORF3 (*cydD*) seems to function to allow cell growth under high pressure, as shown in Fig. 3B. The *cydD* gene is known to be necessary for expression of the components of respiratory systems (*7-9*). Considering these results, it seems possible that regulation of the respiratory system in strain DSS12 is responsive to high hydrostatic pressure, and the respiratory system appears to play a significant role in cell growth under high pressure.

This is the first report to demonstrate that the bacterial respiratory system may have an important role for survival in high-pressure environments.

We are very grateful to Dr. J.M. Delaney for providing *E. coli* strains, B178 and JD518, for this research. We thank Dr. W.R. Bellamy for assistance in editing the manuscript.

REFERENCES

1. Kato, C., Sato, T., and Horikoshi, K. (1995) Isolation and properties of barophilic and barotolerant bacteria from deep-sea mud samples. *Biodiv. Conserv.* **4**, 1-9
2. Kato, C., Inoue, A., and Horikoshi, K. (1996) Isolating and characterizing deep-sea marine microorganisms. *Trends Biotechnol.* **14**, 6-12
3. Kato, C., Smorawinska, M., Sato, T., and Horikoshi, K. (1995) Cloning and expression in *Escherichia coli* of a pressure-regulated promoter region from a barophilic bacterium, strain DB6705. *J. Mar. Biotechnol.* **2**, 125-129
4. Kato, C., Smorawinska, M., Sato, T., and Horikoshi, K. (1996) Analysis of a pressure-regulated operon from the barophilic bacterium strain DB6705. *Biosci. Biotech. Biochem.* **60**, 166-168
5. Kato, C., Masui, N., and Horikoshi, K. (1996) Properties of obligately barophilic bacteria isolated from a sample of deep-sea sediment from the Izu-Bonin trench. *J. Mar. Biotechnol.* (in press)
6. Kato, C., Ikegami, A., Smorawinska, M., Usami, R., and Horikoshi, K. (1996) Structure of genes in a pressure-regulated operon and adjacent regions from a barotolerant bacterium strain DSS12. *J. Mar. Biotechnol.* (in press)
7. Poole, R.K., Williams, H.D., Downie, A., and Gibson, F. (1989) Mutations affecting the cytochrome *d* containing oxidase complex of *Escherichia coli* K12: Identification and mapping of a fourth locus, *cydD*. *J. Gen. Microbiol.* **135**, 1865-1874
8. Bebbington, K.J. and Williams, H.D. (1993) Investigation of the role of *cydD* gene product in production of a functional cytochrome *d* oxidase in *Escherichia coli*. *FEMS Microbiol. Lett.* **112**, 19-24
9. Poole, R.K., Gibson, F., and Wu, G. (1994) The *cydD* gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome *c* and of cytochrome *bd* in *Escherichia coli*. *FEMS Microbiol. Lett.* **117**, 217-224
10. Delaney, J.M., Wall, D., and Georgopoulos, C. (1993) Molecular characterization of the *Escherichia coli htr*D gene: Cloning, sequence, regulation, and involvement with cytochrome *d* oxidase. *J. Bacteriol.* **175**, 166-175
11. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

12. Kato, C., Sato, T., Smorawinska, M., and Horikoshi, K. (1994) High pressure conditions stimulate expression of chloramphenicol acetyltransferase regulated by the *lac* promoter in *Escherichia coli*. *FEMS Microbiol. Lett.* **122**, 91-96
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
14. Dulley, J.R. and Grieve, P.A. (1975) A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal. Biochem.* **64**, 136-141
15. Kita, K., Konishi, K., and Anraku, Y. (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. II. Purification and properties of cytochrome b_{558}-d complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. *J. Biol. Chem.* **259**, 3375-3381
16. Walker, J.E., Saraste, M., Runswich, M.J., and Gray, N.J. (1982) Distantly related sequences in the alpha-subunits and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945-951
17. Higgins, C.F. (1992) ABC transporters: From microorganisms to man. *Annu. Rev. Cell Biol.* **8**, 67-113