

SPONTANEOUS KANAMYCIN-RESISTANT *ESCHERICHIA COLI* MUTANT WITH ALTERED PERIPLASMIC OLIGOPEPTIDE PERMEASE PROTEIN (OPPA) AND IMPERMEABILITY TO AMINOGLYCOSIDES

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SHORT COMMUNICATION

ABSTRACT

A spontaneous kanamycin-resistant *Escherichia coli* mutant, showing cross resistance to five other aminoglycosides and absence of the OppA protein was isolated. [³H]-dihydrostreptomycin uptake is reduced in this mutant, implying that the oligopeptide transport system is involved in accumulation of aminoglycosides, although apparently not related with aminoglycoside permeability alteration due to bacterial adaptation to osmotic changes.

Key words: aminoglycosides-resistance, *Escherichia coli*, OppA.

A new class of mutants that show resistance to several aminoglycoside antibiotics has been selected by exposing *Escherichia coli* and other Gram-negative bacteria to kanamycin in high osmolarity media (12, 13). Kashiwagi *et al.* (6) suggested that the oligopeptide carrier system was involved with slow accumulation phase (EDP-I, ref. 3) of those antibiotics because the introduction of additional copies of the *oppA* gene increases *Escherichia coli* susceptibility to aminoglycosides. In fact, some *E. coli* kanamycin resistant mutants show decrease or deficiency of the oligopeptide permease OppA, a periplasmic protein, as found by SDS-PAGE and Western Blotting with the specific antibody (15). Those mutants revert to the kanamycin sensitive

phenotype when transformed with the wild-type *oppA* gene. Two distinct mutants were detected: one carries a nonsense mutation that abolishes translation of OppA and the other one shows a reduction of about one third of the OppA protein translation, although transcriptional levels are about normal (K. Kashiwagi, personal communication). The reduction of OppA synthesis was correlated with reduction of ornithine synthesis and subsequently of polyamine contents, which is important for stimulation of OppA synthesis at the translational level (5, 7). We show here that an *E. coli* mutant lacking OppA is resistant at the same time to different aminoglycosides due to a reduced accumulation of the drug, thus confirming the participation of the oligopeptide transport system

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in the uptake of several aminoglycosides. We also show that, even lacking OppA, this mutant still responds to the osmolarity effect.

Strain and culturing conditions. The *Escherichia coli* strain J53 *proA met* (4) was used. All media components were from Difco Labs. and hydrated with distilled water. Media used: Nutrient Broth (NB) and Nutrient Agar (NA), low osmolarity medium; and high osmolarity L2 medium (2% tryptone, 1% yeast extract, 1% NaCl and 2% bacto-agar when required). Media osmolarity was measured in an Osmette-Precision Systems Inc. osmometer.

Susceptibility and resistance evaluation. Bacterial overnight cultures were used after 1000 fold dilution to inoculate plates with increasing concentration of antibiotic. After incubation for 24 hours at 37°C, the minimal inhibitory concentration or the level of resistance were estimated from the resulting growth. The following antibiotics were used: amikacin and kanamycin from Bristol Laboratories, streptomycin (Squibb & Sons Ltd.) gentamicin (Schering Corp.), neomycin (Lafi Lab.), tobramycin (Eli Lilly & Co.), dihydrostreptomycin (Fontoura) and [³H]-dihydrostreptomycin (Amersham).

[³H]-dihydrostreptomycin uptake measurement. Exponentially growing cells (OD₆₂₀=0.5) were diluted 10 times in the same medium used for growth plus 4,000cpm/μg of dihydrostreptomycin (at a final concentration of 50μg/ml), and incubated at 37°C for 30 minutes. Duplicated 1ml samples were taken at 0, 5, 10, 15, 20 and 30 minutes after culture dilution and kept on ice until filtration on Millipore 0.22μm teflon coated filters (GYWP01300), previously saturated with 1ml of culture medium plus 1mg of streptomycin. After sample filtration, filters were washed with 2ml of culture medium and 3ml of 3% (w/v) NaCl and dried at room temperature. Radioactivity was measured using scintillation liquid in a Beckman LS 5000TD scintillation counter. Dihydrostreptomycin uptake was calculated by subtracting background radioactivity and dividing by cpm value obtained by measuring 1ng of dihydrostreptomycin.

Periplasmic proteins extraction (Ames *et al.*, 1984, modified). Cells from 3ml of overnight culture were collected by centrifugation at 3,000rpm for 10 minutes at room temperature, resuspended on residual medium, mixed with 40μl of chloroform and incubated for 25 minutes at room temperature. 200μl of 10mM Tris-HCL pH 8.0 was added and the

suspension was centrifuged at 3,000rpm for 25 minutes at 4°C. Supernatant was carefully recovered (periplasmic proteins fraction). Protein concentration was measured as in Lowry *et al.*, 1951.

SDS-PAGE and Immunoblotting. 11% SDS-PAGE was performed as in Lugtenberg *et al* (1975) with modifications: running gel was made by mixing 6.25ml of 44% acrilamide/0.8% bis-acrilamide, 0.5ml of 10% SDS, 0.63ml of 1% ammonium persulfate, 12ml of 0.75M Tris-HCl pH8.8 and 5.12ml of deionized water. Stacking gel was made by mixing 0.5ml of 44% acrilamide/0.8% bis-acrilamide, 50μl of 10% SDS, 0.12ml of 1% ammonium persulfate, 2.5ml of 0.25M Tris-HCl pH6.8 and 1.83ml of deionized water. Polymerization was started by addition of TEMED to 0.02%. After running the gel, proteins were silver stained as in Morrissey (1981) or transferred to nitrocellulose filter in a MultiphorII apparatus (Pharmacia). Western blotting was performed as described in Maniatis *et al.* (1989) using specific anti-OppA rabbit serum kindly offered by D. D. Santos (Centro de Biotecnologia, UFRGS) diluted 1: 1,000, anti-rabbit IgG peroxidase conjugated diluted 1: 2,500 and diaminobenzidine as chromogenic substrate.

When the aminoglycoside sensitive *Escherichia coli* J53 is plated on the high osmolarity medium L2 (448.6 mOsm) containing 20μg/ml of kanamycin, resistant colonies arise at frequencies around 5x10⁻⁶. One high level kanamycin-resistant clone (A31) was chosen to have its permeability to aminoglycosides examined, in comparison with the original sensitive strain J53. This clone is resistant to 200μg/ml of kanamycin, streptomycin, tobramycin, gentamicin, amikacin and neomycin when L2 medium is used, and lacks a periplasmic protein (8) that was identified as OppA by Western Blotting (Fig. 1). Given that previous data pointed out to the importance of the medium osmolarity on aminoglycoside susceptibility (8, 9), we evaluated the [³H]-dihydrostreptomycin uptake on NB (63.1 mOsm) and NB plus 0.5M sorbitol (531.1 mOsm). The resistant mutant showed remarkably reduced antibiotic accumulation (Fig. 2) even in low osmolarity conditions, where the susceptibility is higher (13), thus confirming the mutant impermeability hypothesis as the resistance mechanism. The osmolarity effect on bacterial susceptibility to aminoglycosides has been shown to be present on sensitive bacteria (14) as well as on resistant strains (16), even if the resistance is not related with OppA alteration, but is due to other

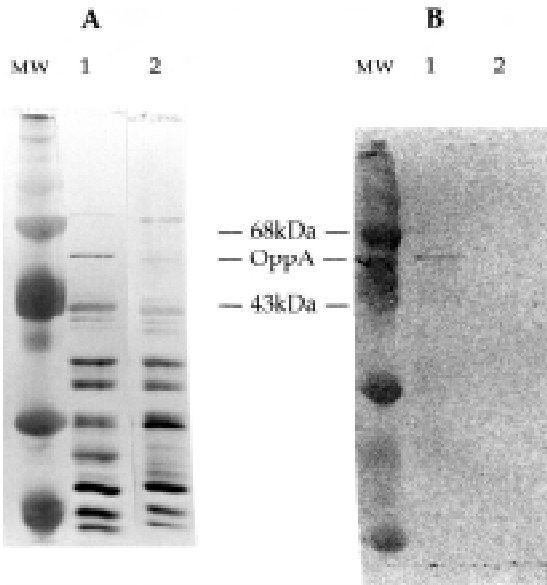


Figure 1. OppA absence on kanamycin resistant mutant. (A) shows a 11% SDS-PAGE of periplasmic proteins and (B) shows a Western blot using anti-OppA, from the aminoglycoside sensitive strain J53 (lanes 1) and the resistant clone A31 (lanes 2).

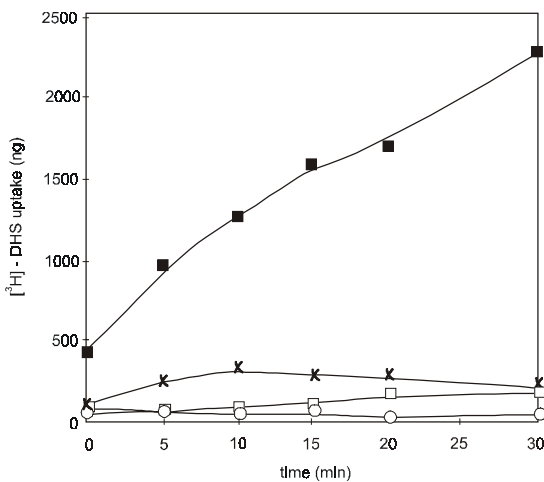


Figure 2. [³H]-dihydrostreptomycin ([³H]-DHS) uptake by aminoglycoside resistant A31 cells in low osmolarity NB (x) and high osmolarity NB + 0.5M sorbitol (o); and by sensitive J53 cells in NB (filled squares) and NB + 0.5M sorbitol (open squares).

mechanisms such as enzymatic inactivation of the drug (1). Our results show that the high osmolarity protective effect is indeed due to a reduction of the bacterial permeability to aminoglycosides. This may be a side effect of a general reduction of permeability for high osmolarity adaptation and rather than a

specific one for aminoglycosides. On the other hand, the fact that a spontaneous aminoglycoside resistant mutant, lacking OppA, has reduced permeability to these antibiotics, confirming that the oligopeptide carrier system plays a role on aminoglycoside transport, may represent an important information for antimicrobial therapy, since mutants lacking OppA have recently been isolated from clinical samples (1).

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RESUMO

Mutante espontâneo de *Escherichia coli* resistente à canamicina com expressão de oligopeptídeo permease periplasmática (OppA) alterada e impermeabilidade aos aminoglicosídeos

Um mutante espontâneo de *Escherichia coli* foi selecionado com canamicina e mostrou resistência cruzada a cinco outros aminoglicosídeos e ausência da proteína OppA. A incorporação de diidroestreptomicina tritíada mostrou-se reduzida nesse mutante, implicando que o sistema de transporte de oligopeptídeos está envolvido na acumulação de aminoglicosídeos, embora aparentemente não esteja relacionado com a alteração de permeabilidade aos aminoglicosídeos decorrente da adaptação bacteriana a mudanças osmóticas.

Palavras-chave: aminoglicosídeos, *Escherichia coli*, OppA.

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