ANTIMICROBIAL RESISTANCE

Altered expression of oligopeptide-binding protein (OppA) and aminoglycoside resistance in laboratory and clinical *Escherichia coli* strains

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Oligopeptide-binding protein (OppA) is the periplasmic component of the major oligopeptide transport system of enteric bacteria. Genetic and biochemical evidence suggests that OppA plays a role in the uptake of aminoglycoside antibiotics in *Escherichia coli* K-12. Forty-six (82%) of 56 aminoglycoside-resistant mutants of *E. coli* K-12 selected *in vitro* had reduced or undetectable OppA levels, as compared with their parent strain. Moreover, nine (36%) of 25 aminoglycoside-resistant clinical isolates of *E. coli* expressed reduced or undetectable levels of OppA. No decrease in OppA expression was observed among aminoglycoside-sensitive *E. coli* strains from patients. Twenty-three (42%) of 56 aminoglycoside-resistant mutants of *E. coli* K-12 and six (24%) of 25 clinical isolates also were deficient for expression of ornithine or arginine decarboxylases, or both, and these deficiencies might negatively affect OppA expression by reducing polyamine synthesis. These results support the view that reduced OppA expression is associated with aminoglycoside resistance in *E. coli* strains.

Introduction

Resistance to aminoglycoside antibiotics can entail diverse mechanisms including target modification, production of inactivating enzymes and reduced uptake [1, 2]. Reduced aminoglycoside uptake can confer clinical resistance in Enterobacteriaceae and other gram-negative pathogens [3–5]. In some cases decreased uptake may reflect loss of respiratory chain components, but this mechanism reduces the bacterial growth rates of the cells and only confers low-level resistance, meaning that such isolates pose little clinical threat [2]. Other cell envelope changes may allow the emergence of substantial aminoglycoside resistance in pathogenic *Escherichia coli*. Recent evidence indicates that oligopeptide-binding protein (OppA), the periplasmic component of a major oligopeptide permease system, may act as carrier for aminoglycoside antibiotics in *E. coli* K-12 [6], and reduced OppA expression seems to confer resistance to several aminoglycosides in this strain [7–10]. However, the clinical relevance of such resistance remains

unknown. This study investigated the possibility that altered expression of OppA might be involved in the aminoglycoside resistance among laboratory and clinical *E. coli* strains.

Materials and methods

Bacterial strains and growth conditions

E. coli K-12 strain J53 was used for selection of aminoglycoside-resistant mutants [11]. Fifty-six mutant colonies were selected by plating overnight growth on to L2 agar medium (tryptone 2%, yeast extract 2%, NaCl 1%, agar 1.5%, 448.6 mOsm) containing kanamycin 20 mg/L, as described previously [8]. Under such conditions, the frequency of kanamycin-resistant colonies ranged from 10^{-5} to 10^{-6} . Twenty-five aminoglycoside-resistant *E. coli* isolates from patients with urinary tract infection (13), diarrhoea (4), sepsis (4) or infected wounds (4) were supplied by Dr L. Moreira, Fortaleza City Hospital (Ceará State, Brazil). These isolates displayed high-level resistance (MIC at least 20-fold higher than for *E. coli* K-12 strain J53) and expressed aminoglycoside-modifying enzymes (unpublished observation). A set of 16 aminoglycoside-sensitive enteropathogenic *E. coli* (EPEC) isolates belonging to serogroup O55 were from patients with

diarrhoea in São Paulo City, Brazil and were kindly supplied by Dr L. R. Trabulsi (Instituto Butantan, São Paulo).

All isolates were grown overnight at 37°C in L2 medium before evaluation of expression of OppA. Möeller decarboxylase broth was used for the detection of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), as described previously [12]. Minimum inhibitory concentrations (MICs) were determined on L2 and Mueller Hinton (Difco, Detroit, USA) agars. Growth rates were measured in L2 broth at 37°C under aeration. The ability to grow on a single carbon source was evaluated in minimal medium [13] with succinate 1% added as the sole carbon and energy source.

OppA expression in periplasmic and whole-cell extracts

Periplasmic proteins were isolated from overnight cells after gentle treatment with chloroform, then concentrated with acetone and suspended in electrophoresis sample buffer [14]. Whole-cell extracts were prepared from 1-ml volumes of overnight cultures diluted to an OD₆₀₀ of 0.65. The cells were suspended in 100 µl of electrophoresis sample buffer and, after boiling for 5 min, one-tenth of each sample was subjected to SDS-PAGE [15]. Strains expressing ≤30% of the parental OppA levels were considered deficient for this protein.

SDS-PAGE and immunoblotting

Proteins were sorted in acrylamide 9% gels (C = 5%) at 100 V for 2 h by the SDS-PAGE procedures described previously [15]. Electroblothing to nitrocellulose membranes was performed in a model EPS 500/400 Semidry Electro-blotter Unit (Pharmacia, Uppsala, Sweden). The membranes were blocked with phosphate-buffered saline (PBS) containing BSA 1% and Tween 20 0.05% [10]. Monospecific polyclonal rabbit anti-OppA serum (kindly supplied by Dr K. Igarashi, Chiba University, Japan) was used at a final dilution of 1 in 2000 in PBS-Tween 20 0.5% (PBST) and incubated for 1 h at room temperature. After washing, the membranes were incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma) for 1 h. Reactive protein bands were visualised by chemiluminescence with the Super Signal Kit reagents (Pierce, Rockford, IL, USA). Samples analysed in Western blots also were electrophoresed and stained with silver to confirm that equal loads were applied to each slot [8, 10].

Detection of ODC and ADC activities

The presence of ODC was detected by growing one colony of each strain in Möeller decarboxylase broth supplemented with ornithine 1% and layered with mineral oil [12]. The same procedure was followed to detect ADC except that arginine 1% replaced ornithine

in the culture medium. After overnight incubation at 37°C, a brown/purple colour was taken to indicate enzyme activity, whereas enzyme deficiency resulted in yellow, i.e., unchanged medium colour.

Growth curves and MIC determination

MICs of kanamycin (Km), neomycin (Nm), streptomycin (Sm), gentamicin (Gm) and tobramycin (Tm) were determined on Mueller Hinton and L2 agar plates with inocula containing *c.* 10⁴ cfu. Results were read after overnight incubation at 37°C. Resistance was defined as an MIC at least four times that for the parental strain. Growth curves were measured at 37°C in L2 medium seeded with *c.* 10⁶ cfu from overnight cultures. Optical densities (ODs) were determined in a Hitachi spectrophotometer (model U-2000) at 600 nm.

Determination of outer-membrane protein and lipopolysaccharide profiles

Cell envelopes were isolated after ultrasonic disruption of cells, harvested by centrifugation and submitted to differential solubilisation with Sarkosyl 2%, as described previously [16]. LPS was obtained by proteinase K digestion of cell envelopes, as described by Darveau and Hancock [17], electrophoresed in polyacrylamide 12% gels and visualised by silver staining [18].

Results

Expression of OppA and aminoglycoside resistance in E. coli K-12

Fifty-six spontaneous kanamycin-resistant colonies of *E. coli* K-12 strain J53 were chosen from among those selected on L2 agar plates containing kanamycin 20 mg/L. Western blot analysis showed that 46 (82%) of these mutants had reduced OppA levels, as determined with both periplasmic fractions and whole-cell extracts (Fig. 1). In contrast, 25 colonies of *E. coli* K-12 strain J53 harvested from L2 agar without antibiotics all had parental levels of OppA. The kanamycin-resistant variants were cross-resistant to other aminoglycosides tested (neomycin, streptomycin, gentamicin and tobramycin) as compared with the parent strain (Table 1). Resistant mutants did not revert to the sensitive phenotype after five subcultures on antibiotic-free media. In view of their cross-resistance, these mutants were considered to owe their behaviour to reduced uptake of the antibiotics tested. None of the kanamycin-selected mutants had significant changes in the outer-membrane protein and lipopolysaccharide (rough) profiles compared with the parent strain (not shown).

The intracellular polyamine pool can affect post-transcriptional OppA expression in *E. coli* K-12 [19]. Therefore, expression of the two major polyamine synthesising enzymes, ODC and ADC, was examined

Fig. 1. Immunodetection of OppA in laboratory mutants of *E. coli* K-12 strain J53 (a) and clinical *E. coli* isolates (b). (a) Lane 1, parent *E. coli* K-12 strain J53; 2–8, aminoglycoside-resistant OppA[−] *E. coli* K-12 strain J53 mutants. (b) Clinical *E. coli* strains: lane 1, *E. coli* K-12 strain J53 as a control; 2–4, aminoglycoside-sensitive OppA⁺ *E. coli* O55 strains; 5–8, aminoglycoside-resistant OppA[−] *E. coli* strains.

Table 1. Aminoglycoside resistance, OppA expression and polyamine synthesising enzymes among derivatives of *E. coli* K-12 J53

Number tested	Aminoglycoside resistance pattern*	Expression of OppA [†]	ODC/ADC [‡] (n)
46	Km, Nm, Sm, Gm, Tm	−	+/+ (24) −/+ (12) +/- (5) −/- (5)
10	Km, Nm, Sm, Gm, Tm	+	+/+ (9) −/+ (1)
25 (controls) [§]	−	+	+/+ (25)

Km, kanamycin; Nm, neomycin; Sm, streptomycin; Tm, tobramycin.

*Isolates were considered resistant if the MICs (minimum values of 10 mg/L for Km, Nm or Sm and 2.5 mg/L for Gm or Tm) were at least four-fold above those for the parent strain on L2 agar medium.

[†]Determined by Western blots of periplasmic and whole cell extracts: +, 100–70% of OppA level in the *E. coli* K-12 strain J53; −, 0–30% of the level in *E. coli* K-12 strain J53.

[‡]Determined by colour reaction in Möeller decarboxylase broth; numbers in parentheses indicate the number of strains with the same enzymic (ODC/ADC) profile.

[§]Colonies of *E. coli* K-12 strain J53 from L2 agar without antibiotics.

for aminoglycoside-selected mutants. Twenty-four (52%) of the mutants expressing reduced OppA levels retained ODC and ADC activities, whereas 12 (26%) were deficient for ODC, five (11%) for ADC and five (11%) for both enzymes (Table 1). Nine of 10 aminoglycoside-resistant mutants expressing OppA at levels similar to the parent strain expressed both ODC and ADH. Twenty-five colonies of the parent strain harvested from L2 agar expressed normal OppA levels and had both ODC and ADC activities (Table 1).

In an antibiotic-free medium, most OppA[−] aminoglycoside-resistant mutants had slightly slower exponential growth rates than the parent strain, but ultimately grew to similar optical densities to the parent strain (Fig. 2). Addition of kanamycin did not further decrease the growth rate of the aminoglycoside-resistant mutants with reduced OppA levels (Fig. 2). The aminoglycoside-selected mutants could grow in minimal medium containing succinate as the sole carbon energy source, suggesting that they did not

harbour defects in oxidative respiratory chain components (not shown).

Expression of OppA and aminoglycoside resistance among clinical E. coli strains

Nine (36%) of 25 aminoglycoside-resistant *E. coli* strains expressed reduced levels or lacked OppA. These included four of 13 from urine, one of four from faeces, one of four from blood and three of four from wounds (Table 2 and Fig. 1). Only one strain expressing reduced OppA was deficient in ODC whereas five OppA⁺ strains were defective for at least one polyamine-synthesising enzyme (two ODC[−], two ADC[−] and one ADC[−] ODC[−]) (Table 2). All these *E. coli* isolates were cross-resistant to other aminoglycosides in L2 medium and none had decreased growth rates in L2 medium at 37°C as compared with the K-12 strain J53 or other aminoglycoside-sensitive strains (not shown). Sixteen aminoglycoside-sensitive *E. coli* strains of serogroup O55 expressed OppA levels similar

Fig. 2. Growth curves of *E. coli* K-12 strain J53 (closed symbols) and two OppA⁻ aminoglycoside-resistant mutants (open symbols) in L2 broth. **(a)** Resistant mutant R9 (OppA⁻, ODC⁻/ADC⁺); **(b)** resistant mutant R53 (OppA⁻, ODC⁺/ADC⁺). The organisms were cultivated without kanamycin (□) or with kanamycin 10 mg/L (Δ) or 20 mg/L (○). Kanamycin was added at time 0.

to that of *E. coli* J53 and expressed both ODC and ADC (Table 2 and Fig. 1).

Discussion

The uptake of aminoglycoside antibiotics by gram-negative bacterial cells growing aerobically occurs in three consecutive steps. A rapid initial electrostatic binding to the outer surface is followed by a slow uptake termed 'energy-dependent phase I' (EDP I) [2, 20], which gives way to enhanced accumulation as mistranslated proteins, generated by the antibiotic's action on ribosomes, are incorporated into the cytoplasmic membrane, disrupting its barrier function [21, 22]. Recent evidence indicates that the accumulation of aminoglycosides during EDP I in *E. coli* K-12 may include the action of an oligopeptide uptake system carrier, the OppA permease [6, 7, 9]. OppA binds di- to penta-peptides, independent of sequence, and so has many potential ligands, including cell wall peptides, and also peptide-based and aminoglycoside antibiotics [23–25]. The present study found that most (82%) kanamycin-resistant mutants of an *E. coli* K-12 strain selected under conditions of high osmolarity had reduced or undetectable levels of OppA, as based on SDS-PAGE and Western blot analysis. A significant fraction (36%) of aminoglycoside-resistant *E. coli* isolates from infected patients showed reduced expression of OppA. Supporting previous data, which indicated the involvement of OppA in the uptake of aminoglycosides in *E. coli* K-12 [6–10], the present observations indicated that OppA expression affected the susceptibility to aminoglycosides in different *E. coli* strains. Thus, OppA expression may represent a possible source for the emergence of aminoglycoside resistance *via* reduced drug uptake in *E. coli* strains and possibly in other gram-negative pathogens (unpublished observations).

Resistance levels to aminoglycosides have been known to be affected by the osmolarity of the growth medium, independently of OppA expression [10, 25]. However, under conditions of high osmolarity, some *E. coli* K-12 strains yield aminoglycoside-resistant variants at fre-

Table 2. Aminoglycoside resistance, OppA expression and polyamine synthesising enzymes among clinical *E. coli* isolates

Origin of tested strains (n)	Aminoglycoside resistance pattern*	Expression of OppA [†]	ODC/ADC [‡] (n)
Urinary tract (13)	Km, Nm, Sm, Gm, Tm	–	+/+ (3)
		–	–/+ (1)
		+	+/+ (7)
		+	+/- (2)
Diarrhoea (4)	Km, Nm, Sm, Gm, Tm	–	+/+ (1)
		+	–/+ (2)
		+	+/+ (1)
Blood (4)	Km, Nm, Sm, Gm, Tm	–	+/+ (1)
		+	–/- (1)
		+	+/+ (2)
Wounds (4)	Km, Nm, Sm, Gm, Tm	–	+/+ (3)
		+	+/+ (1)
EPEC [§] (16)	–	+	+/+ (16)

*[†]See footnote to Table 1.

[§]EPEC O55 strains isolated from stools of patients with diarrhoea.

quencies two to three orders of magnitude higher than those found in media of low osmolarity [8] (and unpublished observations). This behaviour remains to be elucidated, but a synergic effect between low OppA levels and enhanced osmotic pressure seemingly favours expression of aminoglycoside resistance.

In the present study both *E. coli* K-12 mutants and clinical isolates were found to lack or have deficient expression of OppA. The reduction in the expression of OppA did not impair growth dramatically and so such variants seem unlikely to be easily overgrown under antibiotic-free conditions. This behaviour distinguishes them from aminoglycoside-resistant mutants with mutations affecting oxidative respiratory chain components. It remains to be elucidated if mutations affecting expression or activity of OppA, or both, could arise under non-selective conditions and under selective environments act synergistically with other resistance mechanisms to enhance the resistance levels to aminoglycoside antibiotics.

Polyamines, such as putrescine and spermidine, are polycationic molecules essential for nucleic acid and protein synthesis [26] and can stimulate OppA expression at a post-transcriptional level in *E. coli* [19]. In the present study, half of the *E. coli* K-12 mutants expressing reduced OppA levels were found to be defective for ODC or ADC, or both. This linkage supports previous observations that a reduced polyamine pool may affect OppA levels and aminoglycoside resistance in *E. coli* K-12 [7]. On the other hand, no clear correlation between reduced OppA expression and reduced ODC or ADC activities, or both, could be established among clinical *E. coli* strains with aminoglycoside resistance. Regulation of OppA expression in such strains may differ from that in the laboratory mutants; alternatively, other polyamine synthesising enzymes besides ODC and ADC may have a predominant role in the intracellular accumulation of polyamines. Determination of the polyamine content of OppA⁻ deficient strains may help to elucidate these possibilities.

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