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Influence on *Enterobacter cloacae* Metabolism, Cell-Surface Hydrophobicity and Motility of Suprainhibitory Concentrations of Carbapenems

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ABSTRACT. The impact of postantibiotic effect (PAE) of carbapenems (imipenem, meropenem) on the metabolism (biosynthesis of macromolecules, respiration), cell-surface hydrophobicity and motility of a clinical isolate of *Enterobacter cloacae* was examined. The metabolism was evaluated after 16 h and after 1 d of cultivation using 2× and 4× minimum inhibitory concentrations (MIC) of both antibiotics for the induction of PAE. Imipenem at 4 × MIC did not induce PAE. After a 16-h cultivation (in the postantibiotic phase of both carbapenems), inhibition of nucleosynthesis and protein synthesis was found; after a 1-d cultivation, during regrowth stimulation of mainly ^{14}C-leucine incorporation was found. The presence of the exogenous intermediates of citrate cycle, *viz.* 2-oxoglutarate, increased the respiratory activity of the cells. The cell-surface hydrophobicity (evaluated by three methods – bacterial adhesion to hydrocarbon, nitrocellulose-filter test and salt-aggregation test) decreased after PAE of both carbapenems; meropenem was more effective. Motility (an important virulence factor) was inhibited in the postantibiotic phase of both carbapenems; the 4 × MIC caused a higher inhibition.

Carbapenems are broad-spectrum agents with excellent *in vitro* activity against Gram-positive and Gram-negative bacteria including strictly anaerobic bacteria. Imipenem and meropenem, which are registered on the market, are highly resistant to hydrolysis by β-lactamases (with a few exceptions – the β-lactamases of *Stenotrophomonas maltophilia* and of some *Aeromonas* strains; Labia *et al.* 1986; Jones *et al.* 1989; Moellering *et al.* 1989). The carbapenems have been shown to exhibit a postantibiotic effect (PAE) against Gram-positive bacteria and also against some Gram-negative strains (Bustamente *et al.* 1984; Nadler *et al.* 1989; Nadler and Sheikh 1993; Odenholt-Tornqvist 1993; Hanberger *et al.* 1995; Odenholt *et al.* 1997; Majtánová and Majtán 1998*a*; Hoštacká 1999, 2000). Imipenem is less stable than meropenem against human dehydropeptidase and must therefore be administered with cilastatin; both drugs have half-lives of approximately 1 h (Buckley *et al.* 1992). Except for experiments for induction of PAE with carbapenems *in vitro*, *in vivo* PAE were also published with *Pseudomonas aeruginosa* in the neutropenic mouse mode (Tanio and Fukasawa 1992).

During the postantibiotic phase and after PAE specific physiological characteristics of bacteria may be affected, including the virulence factors (Guan and Burnham 1992; Majtán and Hoštacká 1996; Hybenová and Majtán 1997; Majtánová and Majtán 1998*b*; Latrache *et al.* 2000), postantibiotic leukocyte enhancement (Pruul *et al.* 1988; Horgen *et al.* 1998), morphological changes (*e.g.*, filamentation; Guan and Burnham 1992; Barmada *et al.* 1993; Gottfredsson *et al.* 1993) and increased or decreased susceptibility to antibiotic re-exposure (MacKenzie and Gould 1993).

Here we ascertained the PAE of imipenem and meropenem on clinically important *Enterobacter cloacae* and evaluated the effect of pharmacodynamic parameters on its metabolism, cell-surface hydrophobicity and motility after regrowth.

MATERIALS AND METHODS

Bacterial strain. Enterobacter cloacae strain 17/97 was isolated from a patient suffering from nosocomial infection. The actual minimum inhibitory concentrations (MIC) of imipenem and meropenem against this strain were 6.25 and 0.78 mg/L, respectively.

Antibiotics. Imipenem (*Merck Sharp & Dohme*, Germany) and meropenem (*Zeneca Pharmaceuticals*, UK) were used.

Growth medium. Mueller–Hinton broth (*Difco Laboratories*, USA) supplemented with 50 mg/L of Ca^{2+} and 25 mg/L of Mg^{2+} was used for MIC determination and in the PAE experiments.

MIC determination. MICs of both carbapenems were determined using the macrodilution broth method: to 6.7 mL of culture medium in an L-shaped test tube 0.2 mL of bacterial suspension (A_{600} = 0.5) and 0.1 mL of the tested antibiotic were added. Growth of the bacterial suspension was estimated spectro-

photometrically (A_{600}). The lowest dilution of the antibiotic which inhibited bacterial growth was considered as the MIC.

PAE determination. A bacterial suspension in the exponential phase of growth (A_{600} = 0.3) (Zhanel *et al.* 1992) diluted 1 : 10 was exposed to the antibiotic at the 2× or 4 × MIC for 30 min (postantibiotic phase); control cultures were left untreated. After 30 min, the exposed bacterial suspension was diluted 1 : 100 (in order to eliminate the effect of the antibiotic). Three or four different dilutions of the control cultures were made (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) to obtain an inoculum as close as possible to the inoculum of the treated culture on the basis of colony-forming units. Controls as well as treated samples were then incubated (37 °C) and regrowth of bacteria (A_{600}) was followed after 16 h and 1 d. Viable counts in the bacterial suspension before antibiotic exposure and after postantibiotic phase were determined. PAE was defined as the difference between the time required for exposed samples and the corresponding unexposed cultures to grow to a chosen point on the absorbance curve (A_{50}; defined as 50 % of the maximum absorbance of the control culture; Löwdin *et al.* 1993).

Incorporation of ^{14}C-precursors. Bacterial suspensions after treatment with 2× and 4 × MIC of meropenem and 4 × MIC of imipenem were centrifuged (10 000 ***g***, 4 °C, 30 min), cell pellets were washed twice in Tris-buffer (pH 7.4) and concentrated to A_{600} = 0.2. One mL of the suspension was pipetted into tubes and 10 μL of ^{14}C-precursors were added (stock solutions: 8-^{14}C-adenine, 154 MBq per 10 mg/L; L-^{14}C-*U*-leucine, 61.7 MBq per 80 mg/L; both from the *Institute for Research, Production and Application of Radioisotopes*, Prague, Czechia); the incubation lasted for 6 min. The tubes were transferred to an ice bath and the content was precipitated with 1 mL of 10 % ice-cold trichloroacetic acid (TCA). The precipitate was washed with 10 mL 2.5 % TCA and 10 mL solutions of nonradioactive precursors (200 mg/L) were added. After filtration (membrane filters 0.45 μm; *Sartorius*) and radioactivity of precipitates was measured with scintillation counter Rack-Beta (*LKB*).

Respiration was determined polarographically with a Clark-type oxygen electrode (*Gilson Medical Electronics*, USA). The substrates added for exogenous respiration were glucose, 2-oxoglutarate and acetate (all 0.2 mol/L). The bacterial suspensions after treatment with 2× and 4 × MIC of meropenem and 4 × MIC of imipenem were centrifuged (10 000 ***g***, 4 °C, 30 min), cell pellets were washed twice in Tris-buffer (pH 7.4) and concentrated to a concentration corresponding to 46 mg/L dry mass. Tris-buffer (1.5 mL) was pipetted into the respiration vessel; after the system reached 37 °C, the cell suspensions of controls and cells after PAE were added under constant stirring. Oxygen consumption was monitored for approximately 10 min and the linear part of the oxygen-consumption curve was used to calculate the oxygen-consumption rates. The respiratory rate was expressed as nmol/min of oxygen consumed per mg dry cell mass.

After cultivation for 16 h and 1 d (for PAE induction), bacterial hydrophobicity was assayed using three techniques – bacterial adhesion to hydrocarbon (BAH), the salt aggregation test (SAT), and nitrocellulose-filter test (NCF).

The *BAH (xylene)* test was done according to Rosenberg *et al.* (1980). Bacteria were harvested, washed twice, and resuspended in phosphate–urea–magnesium (PUM) buffer (in g/L distilled water: K_2HPO_4 16.9, KH_2PO_4 7.3, urea 1.8, $MgSO_4 \cdot 7H_2O$ 0.2; pH 7.0). The cells were resuspended in the PUM buffer to A_{400} = 1.0 in a total volume of 10 mL. Xylene (1 mL) was added to a test tube containing 4 mL of bacterial suspension and the tubes were shaken for 1 min on a vortex mixer and then left for 30 min at room temperature. After the samples were separated into two layers, the aqueous layer was removed and the A_{400} was estimated. Results were expressed as the ratio (in %) of absorbance of the aqueous layer after xylene addition with reference to the cell suspension without xylene; the strain was considered hydrophobic when the adsorption to xylene was greater than 35 %.

NCF test. Adherence of bacteria to nitrocellulose filters was assessed according to Lachica and Zink (1984). Four-mL aliquots of bacterial suspensions after PAE with A_{600} = 1.0 in saline solution were passed dropwise through an 8.0-μm pore-size nitrocellulose filter (*Sartorius AG*, Germany) and the absorbance of the resultant filtrate was compared with that of the initial bacterial suspension (control). The NCF (in %) was defined as $[(A_{600,\ \text{control}} - A_{600,\ \text{NCF treated}}) / A_{600,\ \text{control}}] \times 100$; the strain was considered hydrophobic when the binding efficiency was greater than 50 %.

SAT was performed according to Blanco *et al.* (1990) with ammonium sulfate solutions (0.2, 0.4, 0.6, 0.8, 1.0, 1.4, 2.0 mol/L) in 0.2 mol/L phosphate buffer (pH 6.8). One drop of a bacterial cell suspension after PAE and nontreated bacteria and one drop of an ammonium sulfate solution were mixed on a glass slide and the results were scored after 2 min. The strain was considered hydrophobic when it aggregated in ammonium sulfate concentrations lower than 1.4 mol/L.

Assay for motility. The strain was grown after PAE at 37 °C for 1 d, harvested by centrifugation and resuspended in phosphate-buffered saline (PBS, pH 7.2) to A_{400} = 1.0. Five-μL aliquots were inoculated on the agar surface of the semisolid swarming medium (in %: tryptone 1, NaCl 0.5, agar 0.25, dissolved in

distilled water; pH 7.1) (Braga *et al.* 1995). The plates were incubated at 37 °C; diameters of the swarming zone were measured at regular time intervals and growth was expressed as the mean from 3 experiments.

RESULTS AND DISCUSSION

Induction of PAE. After exposure to imipenem and meropenem at suprainhibitory concentrations, the 2 × MIC of both carbapenems induced nearly identical PAE (0.6 h for imipenem and 0.5 h for meropenem). The 4 × MIC of imipenem totally suppressed bacterial regrowth and the same concentration of meropenem induced the PAE for 11 h (Table I). In contrast to our results, similar PAE (11 h after affecting 4 × MIC of meropenem) was not found by other authors (cf. Nadler and Sheikh 1993; Hanberger *et al.* 1995). Why the carbapenems produce PAE against some Gram-negative bacteria is not clear; consequence of the ability of these agents to penetrate through the outer cell envelope and their high affinity for specific penicillin-binding protein (PBP) targets has been considered (Moellering *et al.* 1989). The above antibiotics produce a longer PAE than other β-lactams because they bind preferentially to the PBP 2 of some species whereas most β-lactams do not. It has been suggested that binding to PBP 2 itself produces the PAE (Gould *et al.* 1989). However, Majcherczyk and Livermore (1990) demonstrated that imipenem was still able to produce PAE in mutants with thermally inactivated PBP 2; hence the protein cannot be responsible for the PAE phenomenon. At this concentration the PAE for 0 h is remarkable due to 4 × MIC of imipenem against *E. cloacae.*

Table I. Effect of PAE of carbapenems[a] on the incorporation (%) of the ^{14}C precursors in *E. cloacae*

Antibiotic	Induction of postantibiotic phase	PAE h	^{14}C-Adenine		^{14}C-Leucine	
			16	24	16	24
Imipenem	0		100	100	100	100
	2 × MIC	0.6	74	83	78	102
	4 × MIC		_[b]	_[b]	_[b]	_[b]
Meropenem	0		100	100	100	100
	2 × MIC	0.5	49	112	38	112
	4 × MIC	11.0	52	92	77	118

[a]Regrowth after 16 h and 1 d of cultivation.
[b]Not determined; at this concentration no regrowth of *E. cloacae* during additional 1-d cultivation was observed.

Macromolecule biosynthesis. The effect on macromolecule synthesis (kinetics of DNA, RNA and protein synthesis inhibition) after affecting the PAE of carbapenems was demonstrated. The cells after 16-h cultivation were more susceptible to the PAE effect than those after 1-d cultivation (Table I). After 16-h cultivation, higher inhibition of the incorporation of both ^{14}C precursors (particularly at 2 × MIC of meropenem) was determined. After a 1-d incubation, no inhibition was found with the exception of incorporation of ^{14}C-adenine (2 × MIC of imipenem; 83 %).

The concentration of cells in both time intervals was adjusted to the same value but the metabolic state of cells was different. After 16-h cultivation, when the bacteria were in the exponential phase of growth and after 1-d cultivation, which represented the maximum stationary phase and similarly at the end of cultivation, these differences indicate that the cells during regrowth did not complete the synthesis of enzymes needed for cell repair. Bacteria after the PAE require a longer time for complete metabolic facilities. Similar but milder inhibitory effects were found with imipenem.

The 2 × MIC of meropenem after 1-d cultivation showed a stimulation of incorporation of ^{14}C-adenine; we observed a similar effect previously with *P. aeruginosa* (Majtán and Majtánová 1998). After exposure of *E. coli* and *P. aeruginosa* to imipenem, Gottfredsson *et al.* (1995) obtained similar results which probably represent continual DNA replication and inhibition in bacterial septation and multiplication at the same time.

More interesting is the stimulation of ^{14}C-leucine incorporation after treatment with both 2 × MIC and 4 × MIC of meropenem for 1 d. Guan *et al.* (1992) demonstrated also an increase in the rates of protein synthesis in *E. coli* during and/or after a 1-h treatment with enoxacin. On the other hand, we previously

described inhibition of protein synthesis in *P. aeruginosa* after treatment with both 2 × MIC and 4 × MIC of meropenem and after 4 × MIC of imipenem (Majtán and Majtánová 1998).

These results suggest that biosynthetic processes are influenced differently, depending on the antibiotic, on the postantibiotic phase and also on the maturity of the bacterial cells.

Endogenous and exogenous respiration. The presence of energy sources in suspensions after a 16-h as well as after a 1-d incubation stimulated respiration (Table II). Unambiguously, the highest stimulation was induced by 2-oxoglutarate in all the bacterial suspensions tested (about 10–20 fold) and always higher after a 1-d cultivation. The 2 × MIC of imipenem and 4 × MIC of meropenem caused a higher inhibition in the presence of all substrates, compared to endogenous respiration; its values were, however, higher after 2 × MIC of meropenem (except the acetate).

Table II. Effect of PAE of carbapenems[a] on endogenous and exogenous respiration (nmol/min O_2 per mg dry mass) of *E. cloacae*

Antibiotic	Induction of postantibiotic phase	Substrate	16 h	1 d
–	0	endogenous	9.9	5.9
		glucose	81	29.7
		2-oxoglutarate	103	198
		acetate	55	89
Imipenem	2 × MIC	endogenous	9.9	5.9
		glucose	50	21.7
		2-oxoglutarate	69	79
		acetate	29.7	39.6
Meropenem	2 × MIC	endogenous	9.9	5.9
		glucose	45.5	13.8
		2-oxoglutarate	59	79
		acetate	21.7	17.8
	4 × MIC	endogenous	9.9	5.9
		glucose	9.9	25.7
		2-oxoglutarate	25.7	119
		acetate	10.9	31.6

[a]Regrowth after 16 h and 1 d of cultivation.

In our previous work (Majtán and Majtánová 1998) the endogenous respiration of *P. aeruginosa* cells after induction of the PAE by ciprofloxacin, pefloxacin, amikacin, imipenem and meropenem at 2× and 4 × MIC was inhibited, mainly after the effect of 4 × MIC of quinolones. The presence of endogenous substrate intermediates of citrate cycle, *viz.* 2-oxoglutarate, increased the respiratory activity of *E. cloacae* cells. The differences in their influence cannot only be due to their degradation and subsequent entry into the respiratory chain but also due to the rate of their transport into the cell. We assume that the stimulatory effect of 2-oxoglutarate could represent its direct connection with the respiratory chain.

Cell-surface hydrophobicity. Different effects of changing the pharmacodynamic parameters have been observed on the virulence factors of *E. coli*, *P. aeruginosa*, *S. typhimurium* and *E. cloacae* (Guan and Burnham 1992; Majtán and Hoštacká 1996; Hybenová and Majtán 1997; Majtánová and Majtán 1998*a*,*b*; Latrache *et al.* 2000). The cell-surface hydrophobicity, as a modulator of bacterial adhesion, is generally considered to be one of the most important virulence factors in the bacterial infection.

Adhesion to xylene was inhibited more after 2 × MIC and 4 × MIC of meropenem in both time intervals (Table III). Imipenem did not influence the adhesion to xylene at 2 × MIC (inhibition only 4.7 %) after a 1-d cultivation. Similar effects but at lower intensity were found also in the NCF test. After treatment with imipenem, the SAT values decreased as could also be indicated by the increase of hydrophobicity; in contrast, meropenem decreased the hydrophobicity to 70 %.

We found a general lack of correlation after application of these methods. The decrease of adhesion of *E. cloacae* to xylene after the PAE of carbapenems was similar to those we found earlier in this strain after PAE of ciprofloxacin, pefloxacin, netilmicin and tobramycin (Majtánová and Majtán 1998b). This suggests that the inhibition of adhesion is the result of strain susceptibility and of the pleiotropic effect of antibiotics, including cell-wall damage and/or cell-surface-structure modifications (where glycosidic-bound side-chains of the lipopolysaccharide play an important role).

Table III. Effect of PAE of carbapenems on cell-surface hydrophobicity[a] of *E. cloacae* during regrowth after 16-h and 1-d cultivation

Antibiotic	Induction of PA phase	PAE h	BAH[b]	NCF[b]	SAT[c]
Regrowth after 16 h					
Imipenem	0		81 ± 0.7 (100)	93 ± 0.5 (100)	1.0
	2 × MIC	0.6	76 ± 0.8 (94)	89 ± 0.1 (96)	0.8
Meropenem	0	–	93 ± 1.5 (100)	99 ± 0.3 (100)	1.0
	2 × MIC	0.5	28 ± 0.7 (30)	79 ± 0.1 (80)	1.4
	4 × MIC	11.0	58 ± 0.4 (62)	91 ± 0.2 (92)	1.4
Regrowth after 1 d					
Imipenem	0	–	82 ± 0.1 (100)	98 ± 0.1 (100)	1.0
	2 × MIC	0.6	77 ± 1.0 (94)	93 ± 0.2 (95)	0.8
Meropenem	0	–	78 ± 1.5 (100)	66 ± 2.6 (100)	1.0
	2 × MIC	0.5	28 ± 0.4 (36)	63 ± 1.1 (96)	1.0
	4 × MIC	11.0	10 ± 0.8 (13)	38 ± 0.2 (58)	1.4

[a]BAH – test of bacterial adhesion to hydrocarbon (xylene), NCF – nitrocellulose-filter test, SAT – salt aggregation test; for further details *see Materials and Methods*.
[b]In %, mean ±SD; in parentheses percentage of controls without antibiotics.
[c]Concentration of $(NH_4)_2SO_4$ at which aggregation was observed; in mol/L.

Motility is an important virulence factor which contributes substantially to the invasive capabilities of bacteria (Craven and Montie 1981) and its inhibition reduces the ability of forming new colonies and spreading the infection. Significant reduction of these bacterial properties after treatment with a subinhibitory concentration of amikacin and gentamicin on *Proteus* strains (Tawfik *et al.* 1997) as well as of brodimoprim and trimetoprim on *E. coli* (Braga *et al.* 1995) were described. According to our knowledge, such data lack do not hold after influencing bacteria with suprainhibitory antibiotic concentrations (PAE).

A significant reduction of the swarming motility of *E. cloacae* (determined after PAE of meropenem and imipenem in stationary-phase cells after an additional 6-h cultivation on semisolid agar) was found; this reduction was similar with both carbapenems (Table IV). The diameters of the extending zones increased

Table IV. Swarming zones (diameter, mm; mean ±SD) at different time periods (0–6 h) of additional cultivation in *E. cloacae* after 1-d postantibiotic effects of carbapenems[a]

Concentration	0		2		4		6	
	I	M	I	M	I	M	I	M
Control	2.5 ± 0.8	2.5 ± 0.8	5.0 ± 0	4.5 ± 0.8	24.0 ± 0	16.5 ± 0.8	32.0 ± 1.7	35.0 ± 3.5
2 × MIC	2.3 ± 0.4	3.0 ± 0	5.0 ± 0	3.5 ± 0.8	22.5 ± 0.8	12.5 ± 0.8	25.5 ± 0.8	30.0 ± 8.8
4 × MIC	–	2.3 ± 0.4	–	5.0 ± 0	–	15.5 ± 0.8	–	28.5 ± 0.8

[a]I – imipenem, M – meropenem.

with longer time of cultivation. Such a reduction in motility may be due to the absence of flagella (Molinari *et al.* 1992) in which protein synthesis (flagellin) was inhibited during the bacterial growth in the presence of these antibiotics.

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