ACCLIMATIZATION RESISTANCE OF A *Pseudomonas aeruginosa* PROTOTYPE STRAIN TO IMIPENEM

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A prototype sensitive strain of *Pseudomonas aeruginosa* (IR^s) was acclimatized *in vitro* to imipenem by serial transfers in broth containing increasing concentrations of the antibiotic up to 32 μg/ml. Serial subculture of the resistant progeny ($IR1^r$) in antibiotic-free solid media resulted in a "revertant" progeny ($IR2^r$) that retained resistance to imipenem. Acclimatization resistance to imipenem and the attempted reversion procedure affected colony morphology, growth rate, pigment production, growth at 42°C and glucose oxidation. SDS-PAGE analysis of the outer membrane proteins revealed in strain $IR1^r$ a complete loss of 6 proteins that were present in IR^s (85 kdaltons, 46 kdaltons or porin D1, 45.5 kdaltons or porin D2, 43 kdaltons or porin E, 21 kdaltons or protein H1 and 20.5 kdaltons or lipoprotein H2) and in strain $IR2^r$ a complete loss of 3 proteins (46 kdaltons, 43 kdaltons, 20.5 kdaltons), while three others were found only in trace amounts (75 kdaltons, 45.5 kdaltons and 21 kdaltons). In the outer membranes of strains $IR1^r$ and $IR2^r$ an acquisition of a 56-kdalton protein was noted. Lipopolysaccharide chemical analysis revealed a marked, partially reversible increase in 2-keto-3-deoxyoctonate, total hexose and heptose constituents; readily extractable lipid chemical analysis and TLC, revealed a marked, partially reversible, increase in the phospholipid content of the outer membrane. Acclimatization resistance to imipenem was accompanied by cross-resistance to gentamicin, by partially reversible cross-resistance to moxalactam, carbenicillin and ticarcillin and by fully reversible cross-resistance to aztreonam. Sensitivity to azlocillin and polymyxin B remained unaltered. To explain this type of resistance to imipenem, an irreversible, non-inducible "loss" mutation mechanism, working in concert with a partially reversible mechanism, is proposed.

Recent investigations with clinical or *in vitro* selected imipenem-resistant *Pseudomonas aeruginosa* strains have shown that all resistant progeny lacked an outer membrane protein (OMP) of the D family[1~8] (nomenclature according to HANCOCK and CAREY[9]). This family consists of two proteins, D1 (46 kdaltons) and D2 (45.5 kdaltons)[5,8]. Initially the absent protein was believed to be D1, the glucose-inducible protein of the outer membrane (OM) with a known porin activity[1,2]. Recently, the missing protein is considered to be D2 because it has been shown that this protein also has porin activity[5,8] and catalyzes facilitated diffusion of carbapenems and penems through the OM of *P. aeruginosa* ("imipenem channel")[7].

In studying the mechanisms of resistance to imipenem in *P. aeruginosa*, a prototype sensitive strain (MIC 0.5 μg/ml) was acclimatized to a high concentration of imipenem with serial passages in broth containing increasing concentrations of the antibiotic up to 32 μg/ml. To revert the resistant progeny and observe if this type of *in vitro* acquired resistance was an adaptational procedure, serial subculture in antibiotic-free solid media was performed. Adaptational resistance in *P. aeruginosa* has been described for penicillins, tetracyclines, chloramphenicol, aminoglycosides and polymyxin B[10,11]. The use of a prototype strain instead of a clinical one has the advantage of a clonal population with defined properties; thus cells

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that manage to acclimatize by step mutations to the high concentration of the antibiotic, and then revert, have to vary from the parent only in those respects stressed by imipenem. Furthermore, in the case of imipenem, acclimatization overwhelms random selection because the antibiotic is strongly bactericidal and does not favor spontaneously resistant mutants[12].

Materials and Methods

Strains

The parental strain *P. aeruginosa* IR^s-PAC (smooth) was kindly provided by Dr. P. MEADOW, Department of Biochemistry, University College, London. IR^s strain used for further analysis was grown in basal medium broth 1 (BMB1, GILLELAND *et al.*[13]), Mg^{2+}-deficient and supplemented with 1% glucose (the cells were grown aerobically with vigorous shaking at 37°C for 18 hours).

Growth Media

Mueller-Hinton Broth (MHB; Difco) was used for acclimatization and MIC determinations. The following four solid media were independently used for reversion: Basal medium 1 agar (BMA1, GILLELAND *et al.*[13]), Mg^{2+}-deficient (0.6 mg/liter) or Mg^{2+}-sufficient (60 mg/liter), supplemented with glucose 1% as carbon source; Basal medium 2 agar (BMA2, GILLELAND *et al.*[13]), Mg^{2+}-sufficient (60 mg/liter), supplemented with glucose (1%) and Tryptic Soy Agar (Difco). Tryptic Soy Broth (Difco) was used for growth rate determinations and subculture at 42°C. O-F basal medium (HUGH and LEIFSON[14]) was used to determine sugar (glucose, lactose, sucrose and xylose) oxidation. Bacto Pseudomonas agar P and F (Difco) were used to ehance pyocyanin and fluorescent pigment production.

Antimicrobial Agents

Drug solutions for MIC determinations and hydrolysis studies were prepared from standard laboratory powders according to the manufacturers instructions. These were moxalactam (Eli Lilly and Company, U.S.A.), carbenicillin (Sigma Chemical Company, England), ticarcillin (Sigma), azlocillin (Bayer AG, Leverkusen-Bayerwerk, W. Germany), gentamicin (Sigma), aztreonam (The Squibb Institute for Medical Research, Princeton, NJ) and polymyxin B (Sigma). Imipenem powder was kindly provided by Merck, Sharp & Dohme (U.S.A). Solutions of each drug were prepared (weight corrected for potency) on the day of use.

Acclimatization Procedure

The resistant progeny IR1^r was produced from the wild-type strain by repeated daily passage in MHB containing increasing levels of imipenem up to 32 μg/ml (the cells were grown aerobically with vigorous shaking at 37°C for 18 hours). For each successive step, the MIC of the intermediate strain was thoroughly tested in order to avoid a spontaneous mutant with a MIC above the levels of acclimatization. No such mutant was observed. IR1^r strain used for further analysis was grown in BMB1, Mg^{2+}-deficient, supplemented with 1% glucose and 32 μg/ml imipenem (the cells were grown aerobically with vigorous shaking at 37°C for 18 hours).

Reversion Procedure

The "revertant" progeny IR2^r was produced from IR1^r strain by repeated daily subcultures (aerobically, 18 hours, 37°C), independently performed in the four antibiotic-free solid media described above. 30 subcultures were made in Tryptic Soy Agar and in either BMA1 (Mg^{2+}-sufficient) or BMA2, supplemented with glucose in order to induce the production of OMP D1[15]. The same number of subcultures were made in BMA1, Mg^{2+}-deficient, in order to induce the production of OMP H1[11]. Despite the 4 media used, IR2^r strain remained resistant to imipenem (MIC 32 μg/ml). IR2^r strain used for further analysis was grown in BMB1, Mg^{2+}-deficient, supplemented with 1% glucose and 16 μg/ml imipenem (the cells were grown aerobically with vigorous shaking at 37°C for 18 hours).

β-Lactamase Assays

Enzyme reaction rates in whole cell preparations and in the cell-free supernatants were quantified

using the iodometric method of NOVICK[16] with cephaloridine (Eli Lilly) as the substrate. The strains were grown until mid-exponential growth phase in MHB supplemented with the corresponding subinhibitory concentrations of imipenem. Bacteria were harvested by centrifugation in a Sorvall SS34 rotor and $1,500 \times g$ for 20 minutes. They were washed twice with 50 mM potassium phosphate buffer (pH 7.0), supplemented with 5 mM $MgCl_2$, and were resuspended in a volume of the same buffer, so as to match the turbidity of a McFarland 4 standard. This suspension was used as the intact-cell enzyme source. An equal quantity of bacterial suspension was lysed by addition of 10 mM EDTA, 20% sucrose (final concentrations) and lysozyme (1 mg/ml) to provide the released-enzyme preparation. Enzyme reaction rates were compared for the intact-cell and released-enzyme preparations over a range of five cephaloridine concentrations (500 to 7.81 μg/ml). To convert rates of decolorization of the starch-iodine complex to enzyme reaction rates, the absorbencies were compared with those of completely hydrolyzed cephaloridine preparations. Similarly, to determine hydrolysis of selected β-lactams by the inducible enzyme of the strains, enzyme activity was measured against concentrations (250 μg/ml) of imipenem, moxalactam, carbenicillin, ticarcillin, azlocillin and aztreonam. To measure the extent of leakage of enzyme into the medium, the rate of β-lactam hydrolysis was measured with supernatants obtained by centrifugation of the intact-cell suspensions and the corresponding intact-cell rates were corrected for these leakage rates.

Outer Membrane Preparations

OMs were prepared from overnight cultures in the media described above by the method of MÜHLRADT and GOLECKI[17], as modified by IRVIN *et al.*[18]. Final protein concentration was 5 mg/ml.

PAGE Procedures

For estimation of OMPs the discontinuous SDS-PAGE system described by LUGTENBERG *et al.*[19] as modified by HANCOCK and CAREY[9] for solubilization of heat modifiable proteins was used utilizing resolving gels of 14% acrylamide. The gels were stained with Coomasie brilliant blue.

Isolation of Lipopolysaccharide (LPS)

LPS was extracted from whole cell preparations by the phenol extraction technique of WESTPHAL and JANN[20].

Isolation of Readily Extractable Lipid (REL) Fractions

The REL fraction was obtained from whole cell preparations by the chloroform - methanol (2:1) extraction method of FOLCH *et al.*[21].

TLC Procedures

Polar lipids in the REL fractions were separated by TLC in a multisolvent system described by CONRAD and GILLELAND[22] and spotted on commercial Silica gel 60 plates (Merck). They were visualized by spraying the developed plates with 50% H_2SO_4 saturated with potassium dichromate and heating at 155°C for 45 minutes.

Chemical Analyses

Total protein was estimated by the Bicinchoninic acid method (Bicinchoninic acid Protein Assay Kit, Sigma). 2-Keto-3-deoxyoctonate (KDO) was estimated in samples after hydrolysis at 100°C for 20 minutes in 0.04 N H_2SO_4 by the method of OSBORN[23], and ELLWOOD[24]. Total hexose and heptose were estimated in LPS samples by the method of DISCHE (1953) as modified by WRIGHT and REBERS[25]. As standards D-glucose and D-glucoheptose (Sigma) were respectively used. Total lipids were quantitated in REL fractions by the method of ZOLLNER and KIRSCH (1962) (Total Lipids Kit, Boehringer Mannheim). Phospholipid phosphorus was quantitated in REL fractions by the molybdate/vanadate reaction method (Test Combination Phosphorus, Phospholipids, Boehringer Mannheim).

Results

Serial transfers of a *P. aeruginosa* prototype strain in MHB containing increasing concentractions of imipenem resulted in a progeny ($IR1^r$) growing in the presence of 32 μg/ml. Thirty independent serial

transfers of IR1^r in four imipenem-free solid media resulted in the recovery of a strain (IR2^r) that remained resistant to the antibiotic. Acclimatization to imipenem affected colony morphology, growth rate, pigment production, growth at 42°C and glucose oxidation. Some of these properties reverted in strain IR2^r, but others remained unchanged. Subculture (aerobically, 18 hours, 37°C) on Tryptic Soy Agar revealed significant differences in colonial morphology. The colonies of IR^s were large, wrinkled, irregular, mucoid and pigmented; the colonies of IR1^r were small, smooth with well-defined edges, much less mucoid and did not produce pigment; the colonies of IR2^r had a medium size, were somewhat wrinkled, mostly irregular, mucoid and pigmented (data not shown). Subculture (aerobically with vigorous shaking at 37°C) in Tryptic Soy Broth free of antibiotic revealed a significant decrease in the growth rate of IR1^r strain, but the growth rate of IR2^r strain was almost similar to that of IR^s (corresponding generation times determined on the basis of the rate of increase in optical density in the medium: IR^s = 45 minutes, IR1^r = 130 minutes, IR2^r = 52 minutes). Subculture in Tryptic Soy Broth at 42°C (aerobically, vigorous shaking) revealed that IR1^r was unable to grow, but this property was restored in IR2^r as well. Finally, glucose oxidation was observed in both progeny strains after 48 hours, while the rest of the biochemical properties did not change (data not shown).

Table 1 shows the relative resistances of the isolates to selected antibiotics. IR^s was susceptible to all the antibiotics tested. Besides imipenem, the acclimatized strain IR1^r became resistant to moxalactam, carbenicillin, ticarcillin and gentamicin, exhibited an intermediate state of susceptibility to aztreonam and remained sensitive to azlocillin and polymyxin B. The progeny strain IR2^r was resistant to imipenem (MIC 32 μg/ml) and gentamicin, sensitive to aztreonam, azlocillin and polymyxin B and exhibited an intermediate state of susceptibility to moxalactam, carbenicillin and ticarcillin.

β-Lactamase Induction

It is known that imipenem is a potential inducer and a potential inhibitor of the chromosomal type Id enzyme[13,26,27]. After induction with the corresponding subinhibitory amount of imipenem (IR^s = 0.25 μg/ml, IR1^r = 32 μg/ml, IR2^r = 16 μg/ml), the β-lactamase produced by strains IR^s, IR1^r and IR2^r (Table 2) did not hydrolyze imipenem nor the other β-lactams tested. Strains IR1^r and IR2^r were not stably-derepressed mutants since only minimum hydrolysis of cephaloridine was observed in released enzyme-preparations of cells grown in the absence of imipenem (Table 2). Antagonistic drug assays revealed that, in the presence of corresponding subinhibitory concentrations of imipenem, the MICs of moxalactam, carbenicillin and ticarcillin showed a 2-fold increase in strains IR^s and IR2^r and a 4-fold increase in strain

Table 1. MICs of selected antibiotics for the three strains.

Antibiotics	MIC (μg/ml)		
	IR^s	IR1^r	IR2^r
Imipenem	0.5	64	32
Moxalactam	32	128	64
Carbenicillin	64	256	128
Ticarcillin	32	256	64
Azlocillin	4	4	4
Aztreonam	6.25	12.5	6.25
Gentamicin	2	4	4
Polymyxin B	25 U/ml	25 U/ml	25 U/ml

Table 2. β-Lactamase activity of cell extracts expressed as hydrolysis rate (μg/ml) of cephaloridine (250 μg/ml).

Strain	Hydrolysis rate (μg/ml)	
	Uninduced	Imipenem induced
IR^s	0.105	2.08
IR1^r	0.112	2.08
IR2^r	0.110	2.08

For β-lactamase induction, the strains were grown in MHB supplemented with the corresponding subinhibitory concentrations of imipenem.

Table 3. Rate ratios of cephaloridine hydrolysis for intact cells against lysed cells for each strain.

Strains	Rate ratios at cephaloridine conc (μg/ml)				
	500	250	31.25	15.62	7.81
IR^s	0.22	0.43	0.80	1.400	2.000
IR1^r	0.20	0.20	0.16	0.036	0.004
IR2^r	0.20	0.20	0.36	0.065	0.008

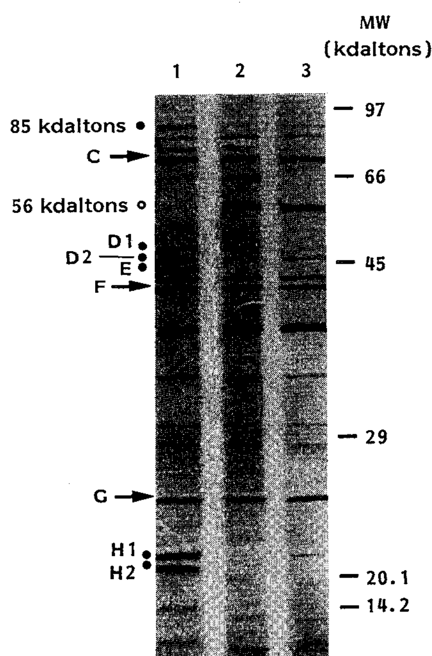
The strains were grown in MHB supplemented with the corresponding subinhibitory concentrations of imipenem.

IR1^r. The MIC of aztreonam showed an 1-fold increase in strains IR^s and IR2^r and 2-fold increase in strain IR1^r. In contrast, the MIC of azlocillin remained unaltered in all strains (data not shown). Moreover, the use of corresponding subinhibitory concentrations of cefoxitin as an inducer had no effect on the MICs of the strains to imipenem (data not shown).

Permeability of the OM

The permeability barrier to cephaloridine was determined for all strains based on the work of ZIMMERMAN and ROSSELET[28] on the role of the OM as a permeability barrier to β-lactams. The rates of cephaloridine hydrolysis by intact cells and their lysates for each strain were compared. Table 3 shows ratios of the enzyme hydrolysis rates for intact *versus* lysed cells at different caphaloridine concentrations. The trend of the ratios to approach zero at low substrate concentrations reflects the permeability barrier. This barrier was evident in both IR1^r and IR2^r strains. Furthermore, the distinction of the rate ratios for strains IR^s, IR1^r and IR2^r at each cephaloridine concentration revealed that an increased permeability barrier in the resistant strains was the most probable reason for the increased resistance to imipenem.

Fig. 1. SDS-PAGE analysis of outer membrane proteins.



Lane 1: IR^s, lane 2: IR1^r, lane 3: IR2^r. MW markers are indicated at right: phosphorylase b (97,000), bovine albumin (66,000), egg albumin (45,000), carbonic anhydrase (29,000), trypsin inhibitor (20,100) and α-lactalbumin (14,200). Porin C and OMPs F and G are indicated at left with arrows; the presence of a 56-kdalton protein in the OMs of strains IR1^r and IR2^r is indicated by an open circle; porins D1, D2, E and OMPs 85 kdaltons, H1 and H2 are indicated by closed circles. The gel was stained with Coomasie brilliant blue following electrophoresis.

Analysis of OMPs

SDS-PAGE gels of OMPs revealed that there were distinct differences among the strains (Fig. 1). Among others, strain IR1^r lacked 5 OMPs that were present in the wild-type strain. These were the proteins of 46 kdaltons, 45.5 kdaltons, 43 kdaltons, 21 kdaltons and 20.5 kdaltons. As compared with IR^s and IR1^r, strain IR2^r lacked 3 OMPs, those of 46 kdaltons, 43 kdaltons and 20.5 kdaltons; two other OMPs were found in trace amounts (45.5 kdaltons and 21 kdaltons). According to the nomenclature of HANCOCK and CAREY[9], the proteins of 46 kdaltons, 45.5 kdaltons and 43 kdaltons correspond to porins D1, D2 and E, and proteins of 21 kdaltons and 20.5 kdaltons correspond to OMPs H1 and H2, respectively. Furthermore, a 56-kdalton protein, not seen in the OM of strain IR^s, was present in the OMs of both resistant strains;

Table 4. LPS composition of strains.

	Strains (% dry weight)		
	IR^s	IR1^r	IR2^r
KDO ($\times 10^{-3}$)	1.7±0.2	13.0±0.4	3.0±0.3
Hexose ($\times 10^{-2}$)	52±10	116±12	54±15
Heptose ($\times 10^{-2}$)	13±2.5	30±3.0	25±3.5

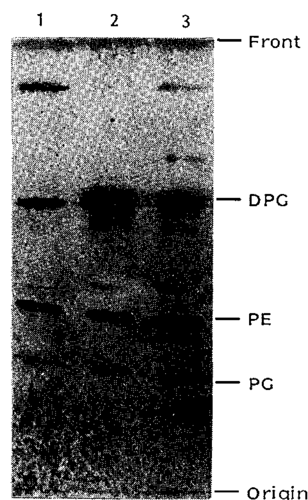
Mean values±SD of six independent determinations.

Table 5. REL composition of strains.

	Strains (% dry weight)		
	IR^s	IR1^r	IR2^r
Total lipids	3.8±0.4	7.8±0.6	7.2±0.4
Phospholipid phosphorus ($\times 10^{-3}$)	16.5±2.0	53.3±2.8	42.4±2.5
Phospholipids	24.5±1.0	92.0±2.3	69.8±1.8

Mean values±SD of six independent determinations.

Fig. 2. TLC analysis of REL fractions.



Lane 1: IR^s, lane 2: IR1^r, lane 3: IR2^r. Phospholipid standards are indicated at right: DPG, PE and phosphatidylglycerol (PG). Lipids were visualized after development of the TLC with sulfuric acid/potassium dichromate reagent. Other lipids revealed in the TLC were not identified.

a 66-kdalton protein was present only in the OM of IR1^r; an 85-kdalton protein, present in both the OMs of IR^s and IR2^r, had disappeared from the OM of IR1^r; and a 75-kdalton protein, present in both the OMs of IR^s and IR1^r, was found in trace amounts in the OM of IR2^r. Finally, it is noteworthy that porin C (70 kdaltons) and OMPs F (39 kdaltons) and G (25 kdaltons) were present in the OMs of all strains.

LPS Analysis

Table 4 shows the LPS composition of the strains. As compared with strain IR^s, the resistant progeny IR1^r showed a highly significant increase of all LPS components especially of the KDO (673%) and heptose or inside core constituents (130%). The phenomenon was partially reversible as shown by IR2^r's LPS composition, where the amount of total hexose reverted to that of the wild-type strain. The quantitative distinctions were not accompanied by any qualitative distinctions in the core or side chain saccharides (data not shown).

REL's Analysis

Table 5 shows the comparative composition of the REL fractions of the strains. The quantitative analysis is accompanied by the TLC (Fig. 2). Both approaches reveal a highly significant increase in the amount of total lipids (105%) and phospholipids (67.5%), especially diphosphatidylglycerol (DPG) and phosphatidylethanolamine (PE), observed in strain IR1^r. The phenomenon was partially reversible as shown by IR2^r's REL composition and TLC.

Discussion

The results show that it is possible to acclimatize a strain of *P. aeruginosa* to imipenem *in vitro*. However, the resistant progeny did not revert although 30 antibiotic-free subcultures were performed, and media that are known to induce the production of certain OMPs were used. *In vivo* resistant strains also

failed to revert[2]. Thus the mechanism involved in resistance to imipenem is not adaptation. An irreversible, non-inducible "loss" mutation mechanism working in synergy with a partially reversible mechanism is proposed to explain this type of resistance to imipenem.

In Gram-negative bacteria, impaired permeability of the OM has been reported to broaden the spectrum and convey a high level of resistance to β-lactams[3,6,29,30,31]. The SDS-PAGE findings confirm previous observations that imipenem resistance in *P. aeruginosa* is primarily associated with characteristic alterations of the OM porins[1~3,5~8]. The resistant strain IR1^r lacked 3 porins (D1, D2 and E) of the OM and the resistant strain IR2^r lacked two porins (D1 and E) and exhibited trace amounts of porin D2. The lack of porin E is unique among clinical or *in vitro* imipenem-resistant *P. aeruginosa* strains analyzed to date. Previous work has shown[32] that imipenem, because of its compact molecular structure and zwitterionic behavior, may transverse the OM of *P. aeruginosa* through nonspecific porin channels (D1 and E[7,8]), but it also utilizes some saturable and therefore specific channels (D2)[5,7,8]. The trace amounts of porin D2 produced in the OM of strain IR2^r possibly, but not exclusively, account for the reduction of its MIC to 32 μg/ml in comparison with the 64 μg/ml of IR1^r strain. Thus the resistance of strains IR1^r and IR2^r to imipenem may be related to the absence of both the non-specific (D1 and E) and specific (D2) pores from their OMs. Moreover, this absence rationally accounts for the increased permeability barrier observed with the β-lactamase assays in IR1^r and IR2^r strains[30,33,34].

Subcultures of IR1^r strain on media containing only glucose as carbon source did not succeed in inducing normal production of porin D1 nor sensitivity to imipenem. The D1 porin of *P. aeruginosa* is induced by glucose and facilitates specifically the diffusion of glucose[10]. When D1 is absent or not normally functioning, the uptake of glucose is retarded[10] and consequently its oxidation by the cell should be retarded. Thus, it is plausible that the absence of D1 from the OM of strains IR1^r and IR2^r accounted for the observed retardation of glucose oxidation by these strains.

Lipoprotein H2 was one of the proteins totally missing from the OMs of both resistant strains and OMP H1 was missing from the OM of IR1^r and found in trace amounts in the OM of IR2^r; these findings are unique among clinical or *in vitro* imipenem-resistant *P. aeruginosa* strains analyzed to date. The actual role of lipoprotein H2 still remains obscure. Induction of H1 protein as well as reduced amounts of phospholipids, have been associated with decreased susceptibility to polymyxin B and gentamicin[10,11]. Subcultures of IR1^r strain on BMA1 Mg^{2+}-deficient did not induce normal production of protein H1[11,13]. The unaltered susceptibility to polymyxin B is compatible with the absence of protein H1 and the increased amounts of phospholipids observed in strains IR1^r and IR2^r. On the other hand, since "permeability resistance" to aminoglycosides has been described for strains of *P. aeruginosa*[10], the impaired permeability observed in the OMs of strains IR1^r and IR2^r is the most likely plausible cause for the cross-resistance observed between imipenem and gentamicin. In Gram-negative bacteria and in respect of β-lactams, for slow uptake to result in resistance, a secondary defense mechanism (clearance mechanism) must work in synergy to prevent the periplasmic drug concentration from rising above the toxic threshold for intolerable penicillin-binding proteins (PBP) activation[12,29,30,35]. This secondary defense mechanism entails slow hydrolysis or nonhydrolytic binding by β-lactamases and PBPs and, perhaps, transport across the cytoplasmic membrane[12,29,30,35,36]. Imipenem has the ability to overcome the clearance mechanism[12,27,29] (shown by the inability of cefoxitin to antagonize imipenem), but the synergy of both the increased permeability barrier and the clearance mechanism seems to be the most probable overall mechanism responsible for the cross-resistance observed between imipenem and moxalactam, carbenicillin and ticarcillin in strains IR1^r and IR2^r.

The discrepancy observed in cross-resistance between imipenem and azlocillin compared with other β-lactams and the fully reversible susceptibility to aztreonam are two additional aspects that have to be evaluated. A similar non-antagonistic pattern between imipenem and azlocillin is observed elsewhere[37,38]. Previous work with *P. aeruginosa* mutants has shown[5] that monoanionic β-lactams diffuse mainly through porin C, which has a slight anion selectivity, and much less efficiently through D2 and E and that the penetration of monobasic penams seems to be enhanced through OMs with increased selectivity for hydrophobic molecules[39], where the existence of nonporin pathways has been strongly implicated[30,40]. Thus in respect of the resistant strains IR1^r and IR2^r it is possible that azlocillin transverses their OMs mainly through porin C and that the hydrophobic shift observed in these OMs (with the significant decrease of hydrophilic proteins and the increase of the total amount of LPS and lipids[34]) has favored azlocillin

to dibasic penams possibly through a nonporin pathway. Aztreonam, on the other hand, seems to diffuse mainly through porin C, less efficiently through E and poorly through D[25]. Moreover, aztreonam is a good substrate for the Id β-lactamase[27,35]. Under imipenem induction of the β-lactamase, the MIC of aztreonam in strains IR^s and $IR2^r$ revealed an 1-fold increase and that in strain $IR1^r$ a 2-fold increase. Thus there seems to be an increased permeability barrier for aztreonam in strain $IR1^r$ that works in synergy with the clearance mechanism. The trace amounts of protein D2 produced in the OM of strain $IR2^r$ possibly, but not exclusively, may account for the revert of the susceptibility to aztreonam to the levels of the wild-type strain.

The changes observed in LPS and lipid composition of the resistant strains are consistent with other findings; LPS and lipids—particularly phospholipids—increase presumably to cover gaps due to protein loss from the OM[5,33]. The same phenomenon was observed in protein F-deficient mutants of *P. aeruginosa*[41]. Thus it seems that the porin (D1, D2 and E) and nonporin (H1, H2, 85 kdaltons and 75 kdaltons) proteins that were totally or partially missing from the OMs of the resistant strains not only functioned as channels permitting the passage of hydrophilic molecules, but contributed to an unknown extent as cellular structural components as well[41]. Moreover, when some of these proteins are partially restored, as seen in strain $IR2^r$, the amount of LPS and phospholipids trends to revert to the levels of the wild-type strain. Here the presence of the 56-kdalton protein in the OM of the resistant strains $IR1^r$ and $IR2^r$ must be highlighted. This protein is overproduced in multiresistant strains of *P. aeruginosa*, interacts with either LPS or other membrane proteins and affects the permeation of β-lactams (excluding imipenem), aminoglycosides, chloramphenicol and quinolones into the cell[31,42].

In conclusion, the following mechanism is proposed as the most plausible one to explain the *in vitro* acclimatization resistance of *P. aeruginosa* to imipenem. In the sensitive strain, the zwitterionic, compact molecules of imipenem diffuse rapidly through all OM porins, but particularly through porin D2. Antibiotic stress leads to a consecutive selection of mutants, probably *via* a "loss" mutation procedure, with an altered OM structure and a prominent permeability barrier due to quantitative and/or qualitative alterations of basic porins such as D1, D2 and E and OMPs such as H1, H2 and 85 kdaltons. Increased amounts of LPS, phospholipids and of a 56-kdalton protein are produced to cover the gaps and restore the integrity of the OM. Overall, a more hydrophobic and much less permeable OM results. Some of the changes stressed by imipenem are partially reversible, but most of them are non-inducible and irreversible. The above processes result in the emergence of a stably imipenem-resistant *P. aeruginosa* isolate that reveals significant cross-resistance to other antipseudomonal β-lactams—excluding azlocillin and possibly aztreonam—and to gentamicin as well.

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