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Role of the culture medium in porin expression and piperacillin-tazobactam susceptibility in *Escherichia coli*

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The continuing emergence of the multidrug resistance phenotype in Gram-negative bacteria makes the development of rapid susceptibility tests mandatory. To achieve this goal, proprietary specific media for bacterial growth can be used but may have some adverse effects. In this study, we dissected the role of media on porin, efflux pump and β-lactamase expression. Depending on the medium used, we observed a change in piperacillin-tazobactam susceptibility for some isolates, such as increases in MIC values. No significant alteration in efflux activity or in β-lactamase production was detected after changing the incubation medium. The ratio of piperacillinase:nitrocefinase showed no specific alteration, indicating that the various media did not affect significantly the relative enzymic affinity for the substrates. In contrast, osmotic variation was able to modulate both porin expression and OmpC : OmpF balance, thus modulating the antibiotic uptake. This study suggests that porin expression may be impacted by a susceptibility testing medium, which may modify the antibiotic diffusion into the bacteria, thus affecting MIC results.

INTRODUCTION

The number of infections due to multidrug-resistant Gram-negative bacteria is increasing rapidly, and outbreaks due to these strains have been reported in hospital wards including intensive care units (Grundmann *et al.*, 2011; Khan *et al.*, 2012; Cohen, 2013). Studies on hospital-acquired infections have also claimed the involvement of multidrug-resistant bacteria, and the recent emergence of carbapenem resistance has amplified the phenomenon (Khan *et al.*, 2012; Theuretzbacher, 2012; Cohen, 2013). Consequently, an early diagnosis of resistant clinical isolates is key information for the clinician to select an appropriate treatment (Jorgensen & Ferraro, 2009; Orenga *et al.*, 2009; Jenkins & Schuetz, 2012; Bhattacharya, 2013; Laxminarayan *et al.*, 2013). Today, it is well recognized that the first step of antibiotic use is a key action that can govern the fate of the infection by selecting or rendering the bacteria more resistant via their adaptive response and resistosome (Schuldiner, 2009; Bhattacharya, 2013).

Reducing the time required for microbial identification and susceptibility tests represents a challenge and needs continuous improvement of methods, selected media and interpretation of raw data (Jorgensen & Ferraro, 2009; Jenkins & Schuetz, 2012; Bhattacharya, 2013). This detection is often carried out by automated systems that are based on early growth detection in an appropriate antibiotic containing medium that may differ in nutrients from the Mueller–Hinton (MH) reference medium (Livermore *et al.*, 2002; Lee *et al.*, 2008; Pitout *et al.*, 2008; Jorgensen & Ferraro, 2009; Orenga *et al.*, 2009). Corresponding to the various methods, the medium used for bacterial growth can play an important role by modulating the permeability of the bacterial membrane, e.g. the expression and balance of porins. These outer-membrane proteins allow permeation through the outer membrane for β-lactams and fluoroquinolones, which use their hydrophilic channels to penetrate the cell (Pagès *et al.*, 2008; Davin-Regli *et al.*, 2008; Martínez & Rojo, 2011; Acar & Moulin, 2012; Fernández & Hancock, 2012).

In this study, the effect of two culture media, the MH reference medium for susceptibility testing and the GN1 medium used for *Enterobacteriaceae* by an automated system, was investigated with respect to antibiotic susceptibility, β-lactamase production, porin level expression, porin type and possible involvement of efflux pump expression.

Abbreviations: BCA, bicinchoninic acid; PAβN, phenylalanine arginine β-naphthylamide.

METHODS

Bacterial strains, growth conditions and media. The bacterial strains used in this study are listed in Table 1. Bacteria were grown

Table 1. Strains and plasmids used in this study

E. coli isolates were classified in four groups related to their piperacillin-tazobactam susceptibility determined according to the method and medium used: reference method (broth microdilution in MH medium) versus GN1 medium, respectively, e.g. S/R for susceptible/resistant for MH versus GN1.

Strain or plasmid	Description	Reference
Clinical strains		
1103137	Phenotype group 1: susceptible/resistant (S/R)	This study
1103138		
1103143		
1103144		
1103145		
1103132	Phenotype group 2: susceptible/susceptible (S/S)	This study
1103134		
1103135		
1103139		
1103146		
1103140	Phenotype group 3: resistant/resistant (R/R)	This study
1103148		
1103150		
1103141	Phenotype group 4: resistant/susceptible (R/S)	This study
1103149		
Laboratory strains		
MH225	MC4100 Φ(*ompC–lacZ* $^+$)10-25	Liu & Ferenci (2001)
MH513	MC4100 *araD* $^+$ Φ(*ompF′–lacZ* $^+$)16-13	
JM109	*E. coli*	Dupont *et al.* (2007)
MC1061	*E. coli* WT	
Plasmids		
pMD08	pFus2K with the *ompX–lacZ* fusion	Dupont *et al.* (2007)
pCeJa01	pFus2K with the *ompF–lacZ* transcription fusion	
pCeJa02	pFus2K with the *ompF–lacZ* translation fusion	

routinely at 37 °C on Luria–Bertani (LB) agar supplemented with kanamycin (50 mg l^{-1}) for the strains MH225, MH513 and JM109 pMD08. The genetic constructs *ompC–lacZ*, *ompF–lacZ* and *ompX–lacZ* have been described previously (Liu & Ferenci, 2001; Dupont *et al.*, 2007). Two culture media, MH broth and GN1 broth (one of the media used by the VITEK2 automated system) were compared. Clinical isolates of *Escherichia coli* from the bioMérieux collection were classified into four groups based on their piperacillin-tazobactam susceptibility phenotype according to the method and medium used: reference method (broth microdilution in MH medium) versus GN1 medium, respectively (e.g. S/R for susceptible/resistant to describe their resulting antibiotic susceptibility phenotype; Table 1). In parallel, nutrient broth (NB) supplemented or not with 20% sorbitol (NBS) was used as a control (Bornet *et al.*, 2004; Dupont *et al.*, 2007). The osmolarity of the different media was determined using an osmometer apparatus according to the manufacturer's instructions (Gonotec): LB=424(±12) mOsmol kg^{-1}, MH=312(±4) mOsmol kg^{-1}, NB=46(±3) mOsmol kg^{-1}, NBS=1290(±23) mOsmol kg^{-1} and GN1=476(±9) mOsmol kg^{-1}.

Antibiotic susceptibility assays. Clinical *E. coli* strains were grown in the different media at 37 °C for 18–24 h with shaking. Susceptibility to imipenem (US Pharmacopeia), piperacillin sodium salt (MP Biomedicals), ticarcillin (GlaxoSmithKline), cloxacillin (Sigma-Aldrich), chloramphenicol (Sigma-Aldrich), nalidixic acid (Sigma-Aldrich) and cefoxitin (Molcan Corp.) was determined by the broth microdilution method as described previously (Burchak *et al.*, 2011). MICs were determined with an inoculum of 10^6 c.f.u. in 200 µl broth containing twofold serial dilutions of each antibiotic. The efflux modulator phenylalanine arginine β-naphthylamide (PAβN) (5 or 20 mg l^{-1}; Sigma-Aldrich) and/or the β-lactamase inhibitors tazobactam (Molekula) and clavulanate (GlaxoSmithKline) (4 mg l^{-1}) were added when required. Experiments were performed in triplicate for each antibiotic, each strain and each condition. Isolates were classified as susceptible, intermediately susceptible or resistant to the antibiotics tested according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2013). The MICs results were scored after 18 h at 37 °C.

β-Lactamase assays. The β-lactamase activity of clinical strains in GN1 and MH medium was monitored using nitrocefin or piperacillin as the respective substrate. Samples of exponential-phase bacteria at 6 UDO ml^{-1} (where UDO corresponds to units of optical density of 1 $ml^{(1}$ at 600 nm) were pelleted and sonicated in 6 ml Milli-Q water. Assays were performed on the sonicated supernatants. First, the enzymic activity corresponding to the increase in hydrolysis product of chromogenic β-lactam nitrocefin (Oxoid) was measured at 486 nm for 2 h at 37 °C in the presence of 50 mg nitrocefin l^{-1} and 10 µl sonicated extract in phosphate buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4) (O'Callaghan *et al.*, 1972). Second, piperacillin hydrolysis was measured at 235 nm for 10 min at 37 °C in the presence of 0.2 mM piperacillin and 100 µl sonicated extract in 0.1 M phosphate buffer complemented with 0.01 µM $ZnCl_2$ (Babini *et al.*, 2003). Specific activity was defined as UDO min^{-1} (mg protein)$^{-1}$, with protein measured by the bicinchoninic acid (BCA) method (Burgess & Deutscher, 2009).

β-Galactosidase assays. The effect of medium on *ompF*/*ompC*/*ompX* expression was investigated using *E. coli* MH513, MH225 and JM109 pMD08 strains, respectively (Table 1). In addition, the effect on *ompF* expression was investigated using the pCeJa01 and pCeJa02 plasmids in the parental strain MC1061 (Table 1). Strains harbouring *lacZ* fusion constructs were grown overnight in MH broth. These pre-inocula were diluted to 0.1 UDO ml^{-1} in 20 ml each medium. Samples of 1 ml were harvested at various times. Strains were then grown overnight in each medium and 1 ml samples were harvested. The β-galactosidase activity was assayed as described by Miller (1972).

Briefly, samples were centrifuged for 10 min at 3007 ***g*** and 4 °C, and pellets were lysed with 500 µl Milli-Q water, 25 µl 0.1% SDS and 25 µl chloroform and incubated for 10 min at 37 °C with shaking. β-Galactosidase activity was measured in 100 µl lysate diluted in 900 µl Z buffer (composition per 100 ml: β-mercaptoethanol, 0.27 ml; Na_2HPO_4 $7H_2O$, 1.61 g; NaH_2PO_4 $7H_2O$, 0.55 g; KCl, 0.075 g; $MgSO_4$ $7H_2O$, 0.0246 g) in the presence of 100 µl ONPG (4 mg ml^{-1}) with incubation in a 37 °C bath. The reaction was stopped with 500 µl 1 M Na_2CO_3 and the absorbance at 420 nm (A_{420}) was measured after centrifugation as described previously (Dupont *et al.*, 2007). Curves were plotted as E[UA] (enzymatic activity expressed as arbitrary units) =UDO min^{-1} (mg protein)$^{-1}$, with protein measured by the BCA method (Burgess & Deutscher, 2009).

SDS-PAGE analyses and immunodetection. Samples at 1 UDO ml^{-1} of exponential-phase bacteria in tested medium were pelleted and solubilized in 200 µl SDS-PAGE loading buffer and boiled at 96 °C. Bacterial proteins were analysed by SDS-PAGE with 10% acrylamide supplemented with or without 4 M urea. Gels were stained with Coomassie Brilliant Blue R-250 to standardize protein samples (Dupont *et al.*, 2007). For Western blots, proteins were electro-transferred onto nitrocellulose membranes (Schleicher & Schuell) in transfer buffer [20 mM Tris/HCl, 150 mM glycine, 20% 2-propanol, 0.05% SDS]. An initial blocking step was performed overnight at 4 °C with Tris-buffered sodium [50 mM Tris/HCl (pH 8), 150 mM NaCl] containing skimmed milk powder (4%). The nitrocellulose membranes were then incubated in Tris-buffered sodium containing skimmed milk powder (4%) and Triton X-100 (0.2%) for 1.5 h at room temperature in the presence of polyclonal antibodies directed against denatured OmpC or OmpF (Dupont *et al.*, 2007). The detection of antigen–antibody complexes was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch).

RESULTS

Antibiotic susceptibility determination

The MICs of the clinical strains as determined by broth microdilution are shown in Table 2. Four strains were selected to illustrate each group. The addition of tazobactam and clavulanate increased piperacillin and ticarcillin susceptibility for strains 1103137, 1103140 and 1103141. MIC values for these combinations varied when GN1 and MH were used. Without β-lactamase inhibitors, piperacillin and ticarcillin MICs were unchanged in both media. For the other antibiotics, MIC values were similar whichever medium was used, except for cefoxitin in strain 1103141 and cloxacillin and nalidixic acid in strain 1103140.

Effect of efflux pump blocker

The impact of the different media on efflux pumps was tested by the addition of a well-described efflux pump blocker, PAβN (Pagès *et al.*, 2009, 2011). The results in Table 3 showed that PAβN decreased the resistance level to nalidixic acid and cloxacillin. We also note that the cloxacillin/PAβN combination was more efficient in GN1 medium for strain 1103140.

The MIC values of the nalidixic acid/PAβN combinations varied between the different media. However, no significant changes in piperacillin and ticarcillin susceptibility

Table 2. MICs of various antibiotics tested alone and with β-lactamase inhibitor against *E. coli* clinical strains

Values are means of three independent assays listed in mg l^{-1}.

Antibiotic*	MIC (mg l^{-1})							
	Strain 1103137 (S/R)		Strain 1103146 (S/S)		Strain 1103140 (R/R)		Strain 1103141 (R/S)	
	GN1	MH	GN1	MH	GN1	MH	GN1	MH
CHL	4	4	8	8	4	2	4	4
PIP	32	32	2	2	>1024	>1024	512	1024
TZP†	8	8	2	2	16	>1024	4	64
FOX	8	8	8	4	4	4	8	16
TIC	1024	1024	4	4	>1024	>1024	>1024	>1024
TIM‡	128	64	2	4	>256	>1024	128	256
CLX	>1024	>1024	256	512	128	1024	512	1024
IMP	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
NAL	>1024	>1024	>1024	>1024	512	256	>1024	>1024

*CHL, chloramphenicol; PIP, piperacillin; TZP, piperacillin + tazobactam; FOX, cefoxitin; TIC, ticarcillin; TIM, ticarcillin + clavulanic acid; CLX, cloxacillin; IMP, imipenem; NAL, nalidixic acid.

†Concentration of 4 mg tazobactam l^{-1}.

‡Concentration of 4 mg clavulanic acid l^{-1}.

Table 3. MICs of various antibiotics tested alone and with efflux inhibitor PAβN against *E. coli* clinical strains

Values are means of three independent assays listed in mg l^{-1}.

Antibiotic*	MIC (mg l^{-1})									
	Strain 1103137 (S/R)		Strain 1103146 (S/S)		Strain 1103140 (R/R)		Strain 1103141 (R/S)		Strain 113138 (S/R)	
	GN1	MH	GN1	MH	GN1	MH	GN1	MH	GN1	MH
PIP	32	32	2	2	>1024	>1024	512	1024	1024	1024
PIP + PAβN†	32	32	2	2	>1024	256	256	512	1024	1024
CLX	>1024	>1024	256	512	128	1024	512	1024	512	512
CLX + PAβN†	512	256	64	64	16	256	128	256	128	128
TIC	1024	1024	4	4	>1024	>1024	>1024	>1024	>1024	>1024
TIC + PAβN†	512	512	4	4	>1024	>1024	>1024	>1024	>1024	>1024
NAL	>1024	>1024	>1024	>1024	512	256	>1024	>1024	4	4
NAL + PAβN†	512	128	512	128	256	64	256	128	0.5	0.5

*PIP, piperacillin; CLX, cloxacillin; TIC, ticarcillin; NAL, nalidixic acid.
†20 mg PAβN l^{-1}.

were observed in the presence of PAβN, except in strain 1103140 with piperacillin in MH broth.

Different antibiotic combinations were examined in MH broth in an attempt to saturate the pumps and to inhibit β-lactamase activities at the same time. Cloxacillin, described previously as a good efflux substrate, can decrease the β-lactam efflux (Pagès *et al.*, 2009). Cloxacillin was added at a subinhibitory concentration (0.2 MIC) during MIC determination. No significant effect of cloxacillin, PAβN or cloxacillin/PAβN was detected when they were combined with piperacillin or ticarcillin (Table 4). When tazobactam was combined with piperacillin (Table 4), we observed a modest effect (twofold decrease) of cloxacillin/PAβN on strain 1103146. For the ticarcillin/clavulanate combination (Table 4), a twofold decrease in MIC was observed when PAβN and cloxacillin/PAβN were combined for strain 1103141. Strain 1103138 demonstrated a twofold decrease in MIC in the presence of PAβN and a threefold decrease in the presence of cloxacillin/PAβN.

β-Lactamase activity in the various media

The activity corresponding to nitrocefin hydrolysis (Table 5) was measured in the two different media for six selected strains (two S/R, one S/S, one R/R and two R/S). For three strains (1103138,1103140 and 1103149), the levels of nitrocefinase activity were similar in GN1 and MH. However, the level was higher in MH than in GN1 medium for 1103145 and 1103149, and there was no activity detected for 1103146.

On the second run, hydrolysis of piperacillin (Table 5) was followed with the same samples in same conditions. The activity was similar in GN1 and MH for strains 1103138 and 1103140, but it was variable for strains 1103141 and 1103145. For 1103141, piperacillin hydrolysis was greater in GN1 than in MH medium and the opposite was observed for 1103145. There was no activity detected for 1103146 or 1103141 in MH medium, and activity was very low in GN1 medium for 1103149.

To compare the respective activities obtained with the two media, the ratio of piperacillinase:nitrocefinase activity was calculated for each medium (Table 5). Values obtained in the two conditions exhibited less than 1% difference in the calculated ratio.

Porin expression in the various media

The expression of OmpF and OmpC can be modulated by the culture conditions. As described previously (Liu & Ferenci, 2001; Bornet *et al.*, 2004; Dupont *et al.*, 2007), medium osmolarity influences the OmpF:OmpC balance due to bacterial osmotic two-component system (TCS) regulators (Masi & Pagès, 2013). Under low osmolarity, OmpF is preferred to OmpC and under high osmolarity the opposite occurs. The porin contents of four selected strains were checked in GN1, MH, NB and NBS media (Fig. 1) with specific antisera prepared against OmpF and OmpC, respectively (Dupont *et al.*, 2007). In the low-osmotic medium (NB), two products were detected in strains 1103145, 1103139 and 1103143. When the growth was carried out with the high-osmotic medium (NBS), the lower band disappeared. By analogy to previous studies, we inferred that the upper band was OmpC while the lower band corresponded to OmpF (Bornet *et al.*, 2004). In strains 1103140 and 1103148, a doublet exhibiting a very similar-sized product to OmpC migration was detected in all of the different media and no band exhibiting OmpF migration characteristics was observed. Regarding strain 1103141, only one

Table 4. MICs of piperacillin and ticarcillin with and without β-lactamase inhibitor alone or in combination with a sub-inhibitory concentration of cloxacillin and with efflux inhibitor PAβN in MH broth

Values are means of three independent assays, listed in mg l^{-1}. PIP, piperacillin; TZP, piperacillin + 4 mg tazobactam l^{-1}; TIC, ticarcillin; TIM, ticarcillin + 4 mg clavulanic acid l^{-1}; CLX, cloxacillin.

Strain	MIC (mg l^{-1})							
	PIP				TZP			
	0	+CLX*	+PAβN†	+PAβN+CLX‡	0	+CLX*	+PAβN†	+PAβN+CLX‡
1103137 (S/R)	32	32	32	64	8	8	4	16
1103138 (S/R)	1024	512	1024	1024	2	8	1	4
1103146 (S/S)	2	1	2	1	2	1	1	0.5
1103140 (R/R)	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
1103141 (R/S)	1024	512	512	1024	64	256	64	256
	TIC				TIM			
	0	+CLX*	+PAβN†	+PAβN+CLX‡	0	+CLX*	+PAβN†	+PAβN+CLX‡
1103137 (S/R)	1024	1024	512	1024	64	64	32	32
1103138 (S/R)	>1024	>1024	>1024	>1024	64	64	16	8
1103146 (S/S)	4	2	4	2	4	2	1	2
1103140 (R/R)	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
1103141 (R/S)	>1024	>1024	>1024	>1024	256	256	64	64

*0.2 MIC cloxacillin.
†20 mg l^{-1} PAβN.
‡0.2 MIC cloxacillin in the presence of 20 mg PAβN l^{-1}.

Table 5. Nitrocefinase and piperacillinase activity in the tested media

Values are listed in UDO min^{-1} (mg protein)$^{-1}$ and are means of three independent assays.

Strain	Nitrocefinimase activity		Piperacillinase activity		Ratio piperacillinase: nitrocefinase (%)	
	GN1	MH	GN1	MH	GN1	MH
1103138 (S/R)	55.4	57.4	4.1	3.8	7.4	6.6
1103145 (S/R)	9.5	28.9	0.7	2.2	7.7	7.5
1103146 (S/S)	0	0	0	0	0	0
1103140 (R/R)	9.5	9.2	0.5	0.5	5.7	5.2
1103141 (R/S)	16.5	14.2	1.1	0.7	6.9	5.2
1103149 (R/S)	6.3	12.6	0.1	0	1.6	0

signal was obtained, whichever medium was used, with migration corresponding to OmpC porin, suggesting that 1103141 is an $OmpF^{-}$ strain.

The analyses were also carried out with SDS/urea-PAGE and the results confirm porin detection after SDS-PAGE.

Interestingly, the upper band (OmpC) seemed to be over-expressed in GN1 and NBS media compared with MH and NB media, whereas the lower band (OmpF) was expressed more in NB and MH versus GN1 and NBS media for strains 1103143 and 1103145, and also for strain 1103139 (S/S phenotype). The strains 1103140 and 1103148 presented one doublet [upper] that was more intense in MH compared with other media. Finally, strain 1103141 exhibited a different behaviour with only one band (upper), which seemed not to be modulated by the medium used.

Quantification of OmpC/OmpF expression in the various media

To investigate the role of medium in OmpC and OmpF expression, *E. coli* strains MH 225 and MH 513 (Liu & Ferenci, 2001) containing *ompC–lacZ* and *ompF–lacZ* genomic fusions, respectively, were used. β-Galactosidase activity was followed for 4 h after transfer in the different media.

OmpC was highly expressed in GN1 medium compared with the level observed in MH medium (Fig. 2a). In contrast, OmpF was expressed more in MH compared with GN1 medium (Fig. 2b). Concerning OmpF, additional investigations were performed by using multi-copy plasmids encoding *ompF–lacZ* transcription fusion and *ompF–lacZ* translation fusion (Dupont *et al.*, 2007). In both cases, the expression of OmpF was more intense in MH compared with GN1 medium (Fig. 2c, d). Finally, as reported previously (Dupont *et al.*, 2004), OmpX expression was controlled by environmental osmolarity, and the expression of OmpX was as important in GN1 as in MH medium (Fig. 2e).

To complete these investigations, β-galactosidase units were measured on overnight cultures with the same constructions. As shown in Fig. 3(a), OmpC expression was higher in GN1 than in MH medium, while OmpF was expressed more highly in MH than in GN1 medium, as was OmpX (Fig. 3b). The expression of OmpF–LacZ transcription and translation constructions was similar and the level was higher in MH compared with GN1 medium. Finally, the β-galactosidase activity of these constructions was assayed in NB and NBS media (Fig. 3c) and, as expected, OmpC was more highly expressed in NBS medium, while OmpF and OmpX were expressed more in NB medium.

OmpC : OmpF balance and bacterial susceptibility

In order to determine whether the variations observed in porin expression could modulate piperacillin-tazobactam

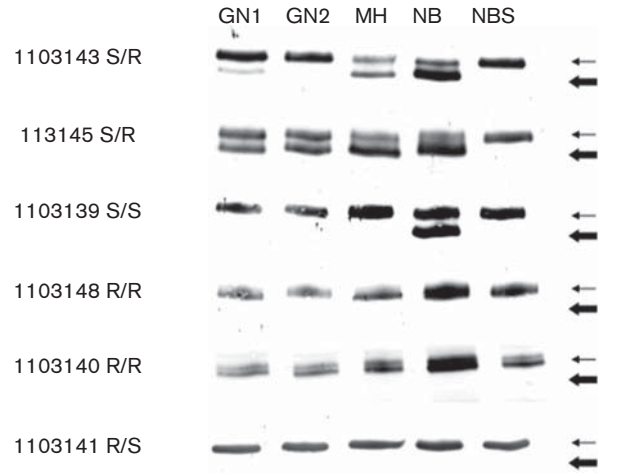

Fig. 1. Immunodetection of porins in *E. coli* clinical strains growing in various media. Total proteins were resolved by 10 % SDS-PAGE. The proteins were electro-transferred to nitrocellulose membrane and immunodetected with polyclonal antibody directed against denatured OmpF porin. Only the relevant part of the blot is shown; thin and thick arrows indicate OmpC and OmpF migration, respectively.

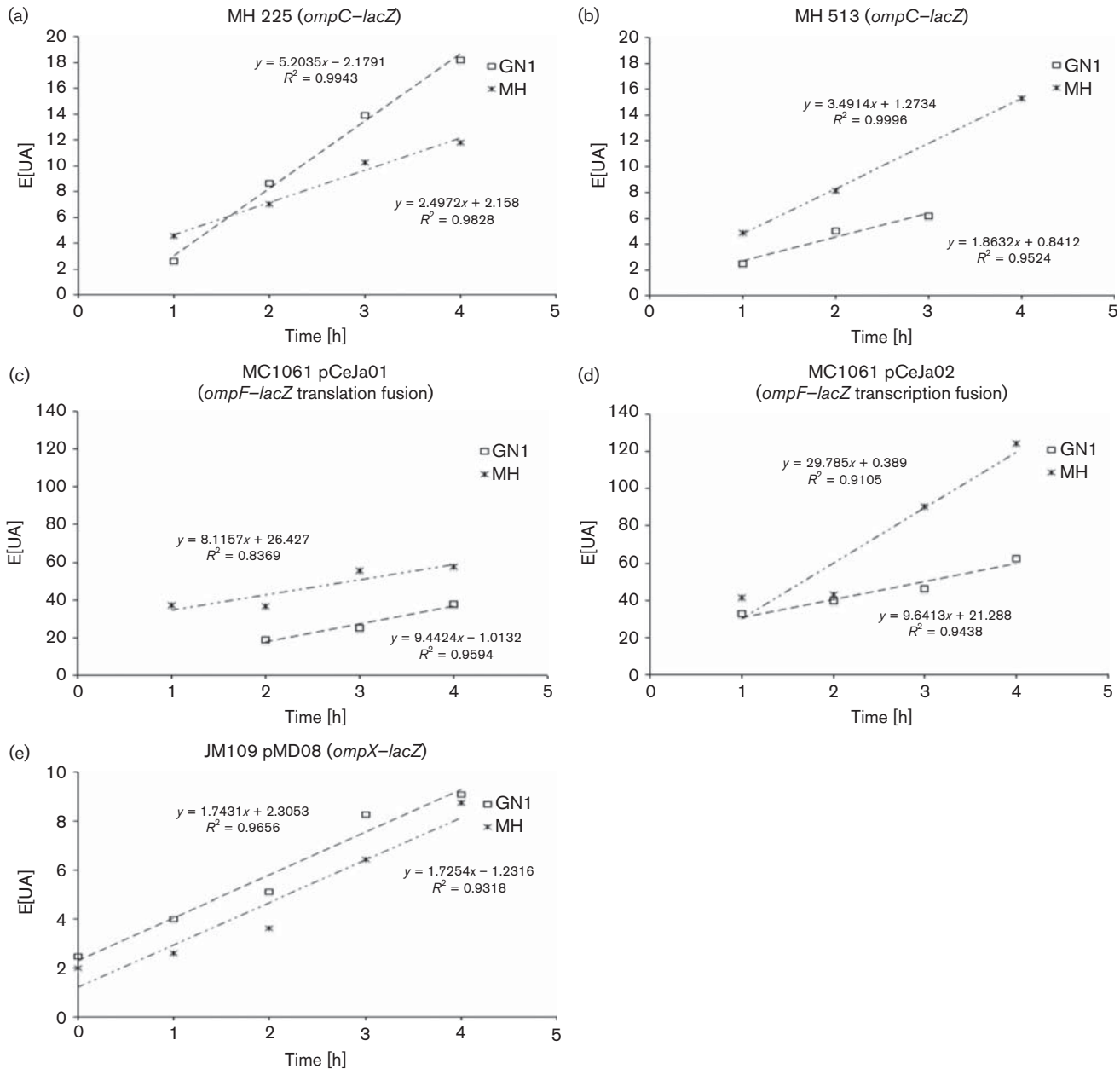

Fig. 2. Expression of *ompC–lacZ*, *ompF–lacZ* and *ompX–lacZ* fusions in GN1 and MH media. Three independent experiments were carried out for each fusion in each condition. Curves were plotted as E[UA] = UDO min^{-1} (mg protein)$^{-1}$, with protein measured by the BCA method (Burgess & Deutscher, 2009).

activity, the MICs were determined in NB and NBS media, respectively (Table 6).

As reported previously (Bornet *et al.*, 2004), changes in medium osmolarity had no significant effect on imipenem MIC except for strain 1103146, while the susceptibility to cefepime and ceftriaxone was altered by osmolarity increase except for strain 1103140. Concerning piperacillin, sorbitol addition increased MIC from one to two dilutions. The effect of osmolarity on tazobactam varied depending on the strain tested. For strains 1103138, 1103140, 1103141, 1103149 and 1103146, the presence of high osmolarity induced a decrease in MIC. In contrast, there was a small increase in MIC for strain 1103143 and no change for strain 1103145. Interestingly, high osmolarity induced an increase (two- to threefold) in the piperacillin-tazobactam MIC for each strain except for strains 1103140 and 1103141.

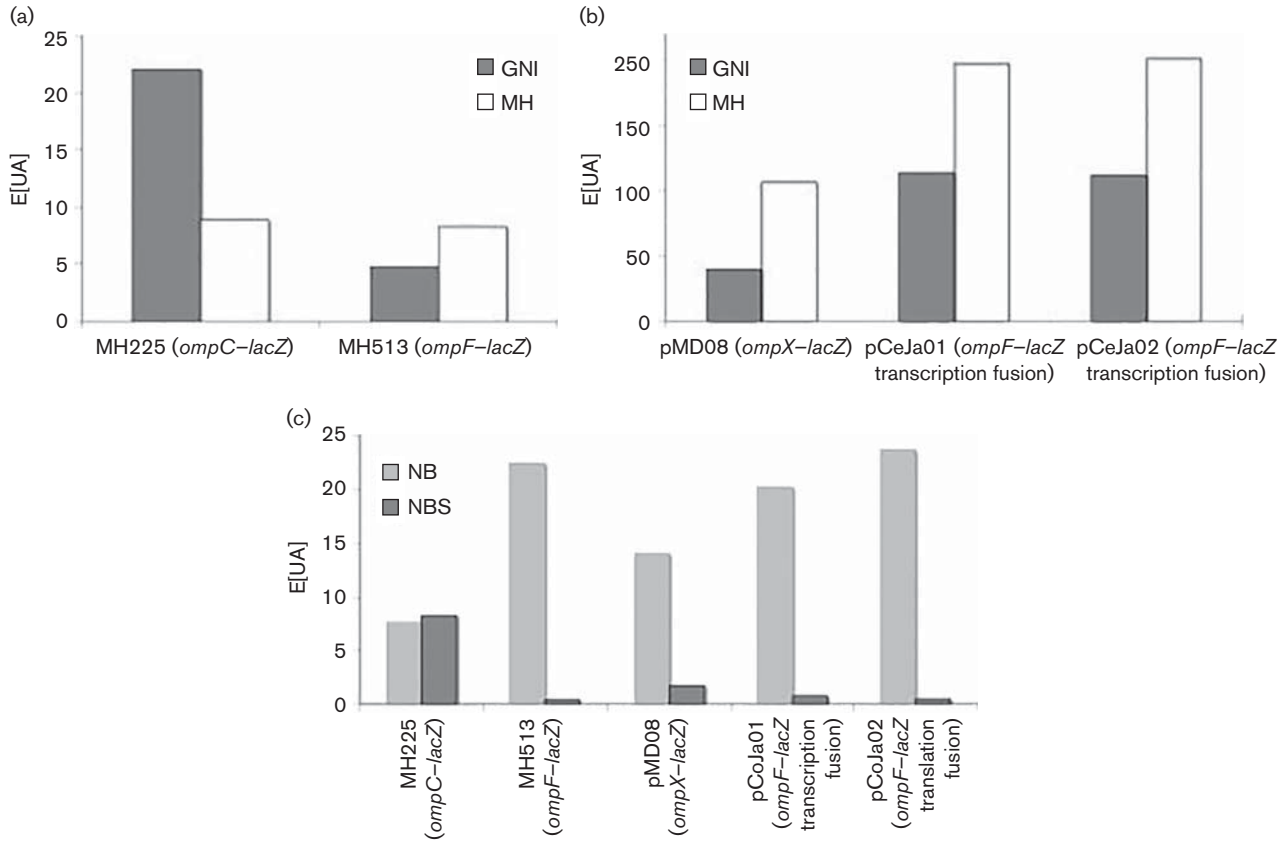

Fig. 3. Overnight β-galactosidase activity in various media. Values are means of three independent assays. Curves were plotted as E[UA] = UDO min^{-1} (mg protein)$^{-1}$, with protein measured by the BCA method (Burgess & Deutscher, 2009).

DISCUSSION

In this study, we aimed to define a possible correlation between the medium used and its effect on the bacterial physiology involved in antibiotic susceptibility. Measurement of nitrocefinase and piperacillinase activity in strains growing in various media indicated no significant variation, except for strain 1103145. When the ratio of piperacillinase:nitrocefinase was compared, no difference was observed, indicating that the medium shift had not significantly changed the relative expression of hydrolytic activities. Regarding efflux involvement, using PAβN susceptibility as a reporter of efflux pump activity (Pagès *et al.*, 2009; Nikaido & Pagès, 2012), no significant modulation of antibiotic susceptibility was noted in the presence of the blocker. Consequently, a potential efflux contribution to penicillin (piperacillin, ticarcillin) activity was not detected in the strains in this assay.

The main effect of growth medium on MIC appeared to be based on porin expression. Two methods were used: immunodetection by specific antibodies and quantification of porin expression using gene reporter fusions. Both approaches clearly demonstrated that, depending on the medium used, there were changes in the level of porin expression and the type of the porin produced. Moreover, some strains such as 1103139 expressed only OmpC in GN1, MH and NBS media, suggesting an alteration in the porin regulation cascade compared with the other strains. These changes contributed to the modification of piperacillin-tazobactam susceptibility reported here: a natural balance of porin type, OmpC versus OmpF, via the TCS EnvZ–OmpR associated with *micF* RNA (Liu & Ferenci, 2001; Delihas, 2012), as well as additional mutations in other regulators that independently or jointly enhance the production of a porin exhibiting a restricted channel such as OmpC.

From our investigations focused on the four phenotypic groups, S/S, S/R, R/S and R/R, it was clear that the results obtained with strain 1103140 (R/R) – enzymatic production associated with the production of only OmpC and a less efficient porin channel – explained the resistant phenotype preserved, regardless of the medium used for susceptibility determination.

For the S/S group, strain 1103146 exhibited neither significant piperacillinase nor nitrocefinase activity. In addition, two representative strains, 1103146 and 1103139, produced

Table 6. Influence of medium osmolarity on MICs of different antibiotics tested alone or with β-lactamase inhibitor against *E. coli* clinical strains

Values are means of three independent assays listed in mg l^{-1}.

Antibiotic*	MIC (mg l^{-1})													
	Strain 1103138 (S/R)		Strain 1103143 (S/R)		Strain 1103145 (S/R)		Strain 1103140 (R/R)		Strain 1103141 (R/S)		Strain 1103149 (R/S)		Strain 1103146 (S/S)	
	NB	NBS	NB	NBS	NB	NBS	NB	NBS	NB	NBS	NB	NBS	NB	NBS
PIP	256	512	256	1024	256	1024	384	192	768	768	96	192	2	6
TZB	192	64	128	192	128	128	256	96	192	96	128	96	192	96
TZP†	96	384	128	768	96	768	256	192	512	384	48	128	2	6
IMP	0.03	0.015	0.03	0.03	0.015	0.015	0.015	<0.015	0.03	0.015	0.03	0.015	0.03	0.0075
FEP	0.125	2	0.125	2	0.06	1	1	1	0.25	2	1	2	0.06	0.5
CRO	0.125	1	0.06	1	0.06	0.5	0.25	0.5	0.125	1	0.125	0.5	0.06	0.5

*PIP, piperacillin; TZB, tazobactam; TZP, piperacillin + tazobactam; IMP, imipenem; FEP, cefepime; CRO, ceftriaxone.
†Concentration of 4 mg tazobactam l^{-1}.

the two porins, OmpC and OmpF, that were osmotically regulated depending on the selected medium. These findings support the stability of the piperacillin-tazobactam-susceptible phenotype observed here.

Regarding the S/R group, the key results concerned OmpC/OmpF regulation in strains 1103138, 1103143 and 1103145. A strong downregulation of OmpF was observed in GN1 and NBS media, further confirmed by the findings from the gene fusion reporter. Consequently, we hypothesize that the medium components play a role in the shift of susceptibility reported for these strains by modulating membrane permeability.

Porin–β-galactosidase gene fusions have been used to quantify the effect of a medium shift on the expression of porins. It was clear that NB, GN1 and MH media favoured porin expression, especially OmpF in NB medium. OmpX is a small, outer-membrane protein and its overexpression represses porin expression. Interestingly, during the medium shift, we observed the same regulation behaviour for porin and OmpX (Dupont *et al.*, 2004; 2007). This finding suggests a more complex regulator cascade in addition to the TCS such as OmpR–EnvZ (De la Cruz & Calva, 2010; Fernández & Hancock, 2012; Masi & Pagès, 2013) in the studied strains. Moreover, we obtained different porin profiles, including porin absence or changes in porin level, with the various isolates treated under the various conditions. Modification of the medium osmolarity, with NBS and GN1 media, induced an imbalance of porin expression (OmpC versus OmpF), which resulted in a decrease in piperacillin-tazobactam susceptibility with the less efficient OmpC porin. This may also explain the emergence of variants exhibiting low-level resistance within the bacterial population.

It will be interesting to test other Enterobacteriaceae to check whether the response identified here for *E. coli* is also present in *Enterobacter*, *Klebsiella* and *Salmonella* isolates, for example. A similar response could support a substantial review of the various media recommendations for susceptibility determination to define the parameter 'bacterial permeability': the medium can generate certain osmotic effects, depending on the strain tested, on drug diffusion by altering the normal uptake pathway. In addition, this work showed that modification of membrane physiology by the test medium suggests the development of an assay allowing the determination of the permeability status in clinical isolates.

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