Resistance to ceftazidime in *Escherichia coli* associated with AcrR, MarR and PBP3 mutations and overexpression of *sdiA*

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The mechanisms responsible for the increase in ceftazidime MIC in two *Escherichia coli* in vitro selected mutants, Caz/20-1 and Caz/20-2, were studied. OmpF loss and overexpression of *acrB*, *acrD* and *acrF* that were associated with *acrR* and *marR* mutations and *sdiA* overexpression, together with mutations A233T and I332V in *FtsI* (PBP3) resulted in ceftazidime resistance in Caz/20-2, multiplying by 128-fold the ceftazidime MIC in the parental clinical isolate PS/20. Absence of detectable β-lactamase hydrolytic activity in the crude extract of Caz/20-2 was observed, and coincided with Q191K and P209S mutations in AmpC and a nucleotide substitution at −28 in the *ampC* promoter, whereas β-lactamase hydrolytic activity in crude extracts of PS/20 and Caz/20-1 strains was detected. Nevertheless, a fourfold increase in ceftazidime MIC in Caz/20-1 compared with that in PS/20 was due to the increased transcript level of *acrB* derived from *acrR* mutation. The two Caz mutants and PS/20 showed the same mutations in *AmpG* and *ParE*.

**INTRODUCTION**

The emergence and spread of cefotaxime- and ceftazidime-resistant strains among *Escherichia coli* isolates have been frequently described in recent years, with most cases due to some extended-spectrum β-lactamases (ESBLs) of the CTX-M family, which display increased hydrolytic activities against ceftazidime, such as is the case for CTX-M-15 and CTX-M-32 (Oteo et al., 2006). In addition, mutations have been described in AmpC β-lactamases that enhance catalytic efficiency towards oximino-β-lactam substrates (Ahmed & Shimamoto, 2008) or mutations and insertions in the *ampC* promoter/attenuator region, especially those located in the −35 and −10 boxes (Corvec et al., 2003), which have resulted in AmpC chromosomal β-lactamase overexpression. They have all been probed to increase the resistance to cefotaxin and expanded-spectrum cephalosporins (Jacoby, 2009; Tracz et al., 2007). Likewise, other mechanisms of resistance such as increased expression of efflux pumps frequently contribute to the acquisition of resistance to ceftazidime and other antibiotics in *E. coli* (Oteo et al., 2006; Jacoby, 2009). Moreover, the rate of ceftazidime-resistant Enterobacteria has been significantly correlated with daily doses of fluoroquinolones and cephalosporins (Uchida et al., 2010). Other mechanisms such as decreased permeability and *ftsI* (PBP3) mutations can contribute to decreasing the susceptibility to ceftazidime (Jacoby, 2009; Tavío et al., 2010).

Otherwise, in vitro acquisition of multidrug resistance phenotype by ceftazidime or fluoroquinolones in *E. coli* has been previously associated with the quorum-sensing regulator *sdiA* (Tavío et al., 2010). SdiA can upregulate efflux pump transporters such as AcrB and AcrF that increase cefotaxime and ceftazidime MICs (Wei et al., 2001a; Yang et al., 2003; Nishino et al., 2003). Despite the meaning of SdiA in bacterial pathogenesis being known (Lee et al., 2007), its significance in resistance to antibiotics still requires clarification. This suppressor of division inhibition (SdiA) regulates cell division in a cell density-dependent (or quorum-sensing) manner (Wei et al., 2001b). SdiA is homologous to the LuxR family of quorum-sensing transcription factors, and its amplification has a global impact on several functions in bacterial cells, including cell

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**Abbreviations:** ESBL, extended-spectrum beta-lactamase; OD, optical density; OMP, outer-membrane protein; PBP3, penicillin-binding protein 3; QRDR, quinolone resistance-determining region. RT-PCR, reverse transcription of total RNA and PCR of cDNA.

One supplementary figure is available with the online version of this paper.
septation (ftsQAZ cell division genes) (Wei et al., 2001a). In its turn, cell-wall turnover is part of the biochemical events during cell growth and division. In the process of bacterial cell-wall turnover, murein is degraded, resulting in anhydro-muropeptides that are imported into the cytoplasm by AmpG permease, and then muropeptides are broken down to yield tetra- and tripeptides that can re-enter into de novo peptidoglycan synthesis or be secreted into the growth medium. An ATP-binding cassette transporter MppA/OppBCDF (Park et al., 1998; Maqbool et al., 2011) recovers these secreted tri- and tetrapeptides. It has also been suggested that MppA could be involved in the quorum-sensing response (Park et al., 1998).

Therefore, the aim of this study was to characterize ceftazidime resistance determinants and a possible role of sdiA and the genes of its regulon in mutants in vitro selected with ceftazidime.

METHODS

Bacterial strains and drugs. The study was performed using one E. coli clinical isolate, PS/20 strain, as the initial parental strain, and two mutants derived from it through two different and consecutive selective steps that resulted in E. coli mutants Caz/20-1 and Caz/20-2. The parental strain PS/20, which had been isolated from a urine sample from one inpatient at the Hospital Insular of Gran Canaria (Spain), was susceptible to fluoroquinolones and ceftazidime. E. coli AG100 induced by salicylate or paraquat was used as control (Spain), was susceptible to fluoroquinolones and ceftazidime. MICs were determined following CLSI guidelines (CLSI, 2006) with Susceptibility tests and transfer of ceftazidime resistance.

Mutant selection. Spontaneous mutants were consecutively selected on Mueller–Hinton agar plates containing ceftazidime at concentrations 4- and 16-fold times the ceftazidime MIC in the PS/20 strain. Their PCR products were amplified according to GenBank sequence descriptions (Tavío et al., 2010).

Susceptibility tests and transfer of ceftazidime resistance. MICs were determined following CLSI guidelines (CLSI, 2006) with and without the active efflux inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 50 μM, since PS/20 did not grow well with CCCP concentrations higher than 50 μM, as previously described (Tavío et al., 2010). The susceptibility to 2,4-dinitrophenol was also assessed following a previous description (Tavío et al., 2010).

A previously described double-disc synergy test with clavulanic acid was used to identify possible ESBL production in parental strain and Caz/20 mutants (Corvec et al., 2001). Likewise, the CLSI confirmatory test for ESBL production and a boronic acid disc test with or without clavulanic acid were performed as previously described (Song et al., 2007), with a ≥3 mm increase in the inhibition zone diameter of either the cefotaxime/boronic acid disc or the ceftazidime/boronic acid disc in the presence of clavulanic acid considered indicative of an ESBL producer (Song et al., 2007).

Likewise, the possible transfer of ceftazidime resistance by conjugation was studied, using methods described previously (Tavío et al., 2010).

Analysis of outer-membrane proteins (OMPs). The study of OMPs was performed as previously described (Tavío et al., 2010). OMPs were obtained from the pellet after Sarkosyl treatment and separated in an 11% polyacrylamide gel with 6 M urea to achieve a better separation of the major OMPs as previously described (Tavío et al., 2010). Gels were stained using the Imperial Protein Stain kit (Pierce).

β-lactamase hydrolytic activity. β-lactamase activity in sonicated extracts of parental strain and Caz/20 mutants was assayed using 100 μM benzylpenicillin and 100 μM cefaloridine in 0.05 M phosphate buffer (pH 7) at 25 °C, following previous reports (Tavío et al., 2010). In this regard, ceftazidime is generally a poor substrate for AmpC cephalosporinases compared with cefaloridine (Queennan et al., 2007), which, unlike ceftazidime, is frequently used for the determination of hydrolytic activities of chromosomal AmpC β-lactamases (Döi et al., 2004). The activity was expressed as units per milligram of protein, where 1 U represents 1 μmol of substrate hydrolysed min⁻¹ per ml of extract.

Organic solvent tolerance. Tolerance to cyclohexane was measured by a liquid-medium assay, as previously described (Tavío et al., 2010). Cyclohexane tolerance values are reported as the mean determinations from at least three independent measures of optical density (OD) at 660 nm at 3 and 6 h after the addition of cyclohexane. The rate of turbidity (OD at 660 nm) increase of bacterial culture was determined by the formula: Increase in turbidity=OD at 3 and 6 h after cyclohexane addition/OD immediately before cyclohexane addition. The standard deviations for these values were all <10%.

A total increase in turbidity of approximately two to threefold in the first 3–6 h after cyclohexane addition was considered significant following previous descriptions (Asako et al., 1997; Tavío et al., 2010). E. coli AG100 induced by salicylate 5 mM was used as a positive control for tolC overexpression due to its effect inducing increased cyclohexane tolerance. Salicylate 5 mM is a good inducer of marRAB regulon expression and, hence, tolC expression (White et al., 1997; Pompisioello et al., 2001; Tavío et al., 2010) and cyclohexane tolerance in E. coli is TolC dependent (Aono et al., 1998). Cyclohexane tolerance is associated with overexpression of tolC, but it also depends on the concomitant overexpression of two-component efflux pumps exporting organic solvents, such as AcrAB and AcrEF (Aono et al., 1998; Kobayashi et al., 2001).

Analysis of DNA sequences of the acrR, marR, soxR, ftsl, ampG, and ampC genes, ampC promoter-attenuator region and QRDRs of the gyrA, gyrB, parC, and parE genes. The acquisition of possible mutations in the ftsl, ampG, acrR, marR and soxR genes and the QRDR of the gyrA, gyrB, parC and parE genes of PS/20 and the two Caz mutants studied through DNA sequencing of their PCR products were amplified according to GenBank sequence accession no. U00096 and sequenced. Likewise, the ampC and ampC promoter/attenuator regions were amplified in the above three strains according to GenBank sequence accession no. J01611 and sequenced, following previous descriptions (Corvec et al., 2007; Yang et al., 2003).

Reverse transcription of total RNA and PCR of cDNA (RT-PCR). Overnight cultures of the studied strains and AG100 inoculated in Luria–Bertani medium were used for extraction of total RNA, following previous descriptions (Sánchez-Céspedes & Vila, 2007; Tavío et al., 2010). Transcript levels of the acrB, acrD, acrF, tolC, marA, ftsl, mppA and sdiA genes were studied by reverse transcription of total RNA and PCR of cDNA (RT-PCR), using the method and primers previously described (Tavío et al., 2010) with the following bp for the amplified fragments: acrB-336, tolC-398, marA-390,
RESULTS AND DISCUSSION

Different mechanisms have been described as responsible for the acquisition of resistance to ceftazidime in E. coli, in many cases associated with the presence of ESBLs or AmpC β-lactamase hyperproduction concomitantly with active efflux and/or decreased permeability (Martínez-Martínez et al., 2000; Otero et al., 2006; Jacoby, 2009; Oteo et al., 2010). Spontaneous mutants with less susceptibility to ceftazidime were selected from the parental strain PS/20 after two consecutive selective steps with selection frequencies of $10^{-8}$ in the first selective step (Caz/20-1 strain) and $10^{-7}$ in the second selective step (Caz/20-2 strain) when it was used as a concentration 16-fold higher than the MIC of ceftazidime in PS/20. Caz/20-2 exhibited a multidrug resistance phenotype (Tables 2 and 3), including a 128-fold increase in ceftazidime MIC (64 μg ml⁻¹) compared to that in the parental strain PS/20. The mechanisms involved in the ceftazidime resistance phenotype of Caz/20-2 were assessed and compared with those in Caz/20-1 mutant, which was its parental mutant.

The expression of β-lactamases different to chromosomal AmpC was ruled out since the double-disc synergy test between each tested cephalosporin and clavulanic acid did not detect synergy in the parental strain and the two mutants. Likewise, the increases in the inhibition zone diameters of cefotaxime (30 μg) and ceftazidime (30 μg) in the presence of clavulanic acid were all <5 mm in the three studied strains, and in turn, the increases in the inhibition zone diameters of either the cefotaxime/boronic acid or ceftazidime/boronic acid discs in the presence of clavulanic acid were all <3 mm. Furthermore, resistance to ceftazidime was not transferred by conjugation assays to E. coli K12 C600. Resistance to oxyimino-cephalosporins, ceftazidime and cefotaxime, concomitant with susceptibility to both cepedime and imipenem, which are two compounds highly stable to AmpC β-lactamases from E. coli, has been associated with the hyperproduction of chromosomal AmpC β-lactamase and loss of OmpF porin (Martínez-Martínez et al., 2000). The Caz/20-2 mutant indeed showed a resistance profile to β-lactams compatible with that described in E. coli hyperproducing chromosomal β-lactamase.

### Table 1. Oligonucleotides used for RT-PCR and DNA sequence determination

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5′–3′</th>
<th>Reverse primer 5′–3′</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer pairs for RT-PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acrD</td>
<td>GGTGCCCTGCAATGTCGTG</td>
<td>TGGTCAAGATGTCGTGATGC</td>
</tr>
<tr>
<td>acrF</td>
<td>ATCGAAGCAAGATGACAGC</td>
<td>TCTAGATTCAATTACATAC</td>
</tr>
<tr>
<td>mppA</td>
<td>CATTGTCGCCATTTGCAATG</td>
<td>CAGACCAAGCGCTGTGATC</td>
</tr>
<tr>
<td><strong>Primer pairs for sequencing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampC-prt</td>
<td>GATGCTGCTGGGCAATGCTG</td>
<td>GGGCGCAAGAAATGTCGAGGAA</td>
</tr>
<tr>
<td>ampC</td>
<td>GGCCTCTTGTATGAAACAC</td>
<td>GTATAGTGACAGCAAG</td>
</tr>
<tr>
<td>ampG</td>
<td>GAGTTTTCGATCGCCCTGC</td>
<td>GAACCGCTGTGGTTGAG</td>
</tr>
<tr>
<td>ampI</td>
<td>GCCCGGTTATTTCTGTGCCC</td>
<td>CGGAAACAGCATCCATAC</td>
</tr>
<tr>
<td>fisI</td>
<td>GTATGCTGCTGGGCAAGAG</td>
<td>GCCCATGCTGTAGATGTC</td>
</tr>
<tr>
<td>marR</td>
<td>GGTATTCTGCTGGTCTGCT</td>
<td>GCCGTAAATGTTGAG</td>
</tr>
<tr>
<td>soxR</td>
<td>ATCTGATGCTGCTGGTCTG</td>
<td>GGCCGACAAATGTCGAGG</td>
</tr>
</tbody>
</table>

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fisI-380 and sdiA-417. The primers utilized for the amplification of acrF (amplified fragment of 431 bp), acrD (amplified fragment of 380 bp) and mppA (amplified fragment of 554 bp) from cDNA are listed in Table 1. All primers were designed according to GenBank sequence accession no. U00096, as previously reported (Tavío et al., 2010). The data obtained for gene targets were normalized against the reference gene gapA following previous descriptions (Kern et al., 2000; Tavío et al., 2010). The expression level of each of the above-mentioned genes in Caz/20-2, PS/20 and AG100 strains was assessed, although only acrB and sdiA were analysed in the case of Caz/20-1 since marR mutations were not identified in it. The transcript level of soxS was not studied in any of the cases since no mutation other than silent nucleotide substitutions were found in soxR in PS/20, Caz/20-1 and Caz/20-2 strains. Induction of acrB, tolK and marA genes by salicylate 5 mM and acrB by paraquat 0.2 mM were used as positive controls.

At least three different extracts of total RNA were obtained from each studied strain, and RT-PCR was done in triplicate for every RNA extract obtained from each strain and for the different analysed genes. The resulting PCR products (cDNA amplified by each primer pair tested) were separated in 12% polyacrylamide slab gels and detected using a DNA silver staining kit (Amersham Biosciences), as previously described (Tavío et al., 2010). Gel results were analysed using ImageJ analysis software for the measurements of density and pixels of bands to obtain a more accurate measurement of band densities. The accepted standard deviation for these densitometric values for each gene and strain was always <3%.

Changes ≥1.3-fold in the gene expression levels were considered significant, as previously described (Tavío et al., 2010).

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**Journal of Medical Microbiology** 63

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susceptibility of Caz/20-2 mutant and its parental strain, and nucleotide substitutions and mutations in PS/20 and Caz/20 mutants by Martínez-Martínez et al. (2000). Nevertheless, no β-lactamase hydrolytic activity against benzylpenicillin and cefaloridine was detected in the mutant Caz/20-2, whereas low β-lactamase hydrolytic activities were observed in PS20 and Caz/20-1, 0.65 and 0.074 U mg⁻¹ of the total protein in the PS/20 strain and 0.67 and 0.076 U mg⁻¹ of the total protein in Caz/20-1 against 100 mg l⁻¹ of benzylpenicillin and 100 μM cefaloridine, respectively. Furthermore, there were no changes in the nucleotide sequence of the ampC attenuator in Caz/20-1, Caz/20-1 and PS20 strains with respect to wild-type sequence accession no. J01611 previously described (Jaurin, 1982), coinciding with a previous report on non-hyperproducing AmpC E. coli strains (Fernández-Cuenca et al., 2005). Increased transcription of ampC has been more frequently associated with transversions between −35 and −10 boxes such as those at −42 and −32 positions (Caroff et al., 2000) or with C→A transversion at −11 (Tracz et al., 2005), C→T transversions at −1, +58 (Yu et al., 2009) and −88 (Corvec et al., 2003) and A→G transversion at −82 (Yu et al., 2009), also with insertions in the spacer region within −35 and −10 boxes (Tracz et al., 2005; Tracz et al., 2007; Peter-Getzlaff et al., 2011). Nevertheless, G→A transversion at the −28 position within the −35 and −10 boxes, as previously reported in clinical isolates, was not associated with AmpC hyperproduction (Peter-Getzlaff et al., 2011). The same substitution, a G→A transversion at the −28 position located in the spacer region between −35 and −10 boxes, was identified in the Caz/20-2 mutant, and also a C→T transversion at the −73 position in the PS/20 and the two Caz mutants (Table 3), but neither of the two above nucleotide transversions was associated with any AmpC hyperproducing phenotype.

Regarding putative inactivating mutations in AmpC, which could explain the decreased hydrolytic activity of Caz/20-2 crude extract on β-lactams, active site Ser64, as well as Lys67 (Beadle & Shoichet, 2002), Tyr150, Asn152, Lys315 and Ala318 residues, but not Glu272 and His314, have been previously proposed to be important in the catalytic mechanism of class C β-lactamases found in Enterobacteriaceae and Pseudomonas spp. (Dubus et al., 1996; Jacoby, 2009). It has been previously described that substitutions of catalytic residues Ser64, Lys67, Tyr150, Asn152 and Lys315 decreased the activity of the enzyme by 10⁻² to 10⁻⁶-fold compared to AmpC wild-type (Beadle & Shoichet, 2002); nevertheless, residues Glu191 and Pro209, in which mutations were identified in Caz/20-2 mutant (Table 3), have not been described as being among those involved in the catalytic function of AmpC β-lactamases. It must not be overlooked that some mutations modifying the length and charge of the side chain of a certain residue can create electrostatic interactions that slow or decrease the catalytic activity of the AmpC β-lactamase, such as previously described for Ser289 residue (Trépanier et al., 1999), which is not essential in the binding or hydrolytic mechanism of class C β-lactamase from Enterobacter.

Table 2. Antimicrobial agent MICs and the effect of 50 μM carbonyl cyanide m-chlorophenylhydrazone on the susceptibility of Caz/20-2 mutant and its parental strain

<table>
<thead>
<tr>
<th>Drug</th>
<th>PS/20</th>
<th>PS/20 + CCCP</th>
<th>Caz/20-2</th>
<th>Caz/20-2 + CCCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAL</td>
<td>32</td>
<td>16</td>
<td>1024</td>
<td>256</td>
</tr>
<tr>
<td>CEF</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>FOX</td>
<td>1</td>
<td>0.5</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>CTX</td>
<td>0.25</td>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>CPO</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>FEP</td>
<td>0.12</td>
<td>0.12</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>ATM</td>
<td>0.25</td>
<td>0.25</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>IMP</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>CHL</td>
<td>0.5</td>
<td>0.25</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>TET</td>
<td>32</td>
<td>16</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>DNP</td>
<td>0.12</td>
<td>ND</td>
<td>0.06</td>
<td>ND</td>
</tr>
<tr>
<td>MYT-C</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
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</table>

+ CCCP, Antibiotic MIC in the presence of 50 μM carbonyl cyanide m-chlorophenylhydrazone; ATM, aztreonam; CEF, cefalotin; DNP, 2,4-dinitrophenol; FEP, cefpirome; CTX, cefotaxime; FOX, cefoxitin; CPO, cepirome; CHL, chloramphenicol; IMP, imipenem; NAL, nalidixic acid; TET, tetracycline; MYT-C, mitomycin-C.

Table 3. Ceftazidime and norfloxacin MICs (μg ml⁻¹), the effect of 50 μM carbonyl cyanide m-chlorophenylhydrazone on the susceptibility of Caz/20-2 mutant and its parental strain, and nucleotide substitutions and mutations in PS/20 and Caz/20 mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC (μg ml⁻¹)</th>
<th>Mutations</th>
<th>FtsI</th>
<th>AmpC</th>
<th>ampC-pr</th>
<th>AcrR</th>
<th>MarR</th>
<th>ParE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS/20</td>
<td>0.5/0.5</td>
<td>0.5/0.25</td>
<td>−</td>
<td>−</td>
<td>−3 C→T</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Caz/20-1</td>
<td>2/1</td>
<td>2/0.5</td>
<td>−</td>
<td>−</td>
<td>−3 C→T</td>
<td>Q27H</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Caz/20-2</td>
<td>64/8</td>
<td>4/1</td>
<td>A233T I332V</td>
<td>Q191K P209S</td>
<td>−3 C→T</td>
<td>Q27H</td>
<td>c.102_103insT</td>
<td>K443T</td>
</tr>
</tbody>
</table>

CAZ, Ceftazidime; NOR, norfloxacin; +I, antibiotic MIC in the presence of 50 μM carbonyl cyanide m-chlorophenylhydrazone.
cloacae P99. However, when (Trépanier et al., 1999) substituted it by Lys or Arg (polar and positively charged residues), it resulted in decreased catalytic activity (Trépanier et al., 1999). Indeed, substitution of a residue with a small side chain such as serine for a larger amino acid or positively charged could fundamentally change the protein activity (Bett & Russell, 2003). Mutations in AmpC of Caz/20-2 strain were in the amino acids (positions 191 and 209) that are between the catalytic residues Asn152 and Lys315. The amino acid Gln (at position 191), which is a polar or hydrophilic amino acid with a neutral side-chain charge such as Ser (Bett & Russell, 2003), was substituted by serine (Pro209Ser) (Table 3), which is also a small amino acid but which is polar (Betts & Russell, 2003), was substituted by a polar and positively charged residue Ala233Thr, which was only identified in Caz/20-2 mutant P99. However, when (Trépanier et al., 1999) substituted it by Lys or Arg (polar and positively charged residues), it resulted in decreased catalytic activity (Trépanier et al., 1999). Indeed, substitution of a residue with a small side chain such as serine for a larger amino acid or positively charged could fundamentally change the protein activity (Bett & Russell, 2003). Mutations in AmpC of Caz/20-2 strain were in the amino acids (positions 191 and 209) that are between the catalytic residues Asn152 and Lys315. The amino acid Gln (at position 191), which is a polar or hydrophilic amino acid with a neutral side-chain charge such as Ser (Bett & Russell, 2003), was substituted by serine (Pro209Ser) (Table 3), which is also a small amino acid but which is polar (Betts & Russell, 2003). Furthermore, Gln191Lys and Pro209Ser mutations were located within the Ω-loop, which lies from residues 178 to 226, and surrounds the active site of cephalexopinases R1 (Nordmann & Mammeri, 2007). Whatever the explanation of the relationship between the above mutations in AmpC and activity, it must not be overlooked that the simultaneous appearance of the mutations Gln191Lys and Pro209Ser in AmpC of Caz/20-2 was associated with a significant decrease of β-lactamase hydrolytic activity compared with that in PS/20 and Caz/20-1, which did not carry any mutation in AmpC. Nevertheless, the purification of AmpC enzymes of the studied strains and kinetic analysis is necessary as described in Beadle & Shoichet (2002) and Doi et al. (2004) to determine if the difference in protein residues of AmpC β-lactamase in the case of Caz/20-2 strain could confer a lower hydrolysis against ceftazidime, which is far from the objective of the present study: the characterization of resistance to ceftazidime.

Therefore, since β-lactamase hydrolytic activity was not the cause of the resistance to ceftazidime in Caz/20-2, other possible mechanisms were evaluated, such as PBP3 mutations, increased expression of efflux pumps and decreased cell-wall permeability.

Penicillin-binding protein 3 (PBP3; also called FtsI) is a transpeptidase that catalyses cross-linking of the peptidoglycan cell wall in the division septum of E. coli (Georgopapadakou, 1993). The catalytic domain of PBP binds β-lactam antibiotics, which mimics a transpeptidase substrate and serves as a suicide inhibitor by forming a long-lived covalent adduct with the catalytic serine (Wissel & Weiss, 2004). Mutations in PBP3 have been previously described to increase β-lactam resistance in Gram-negative microorganisms such as Haemophilus influenzae, Acinetobacter baumannii and P. aeruginosa (Barbosa et al., 2011; Cayó et al., 2011; Moyá et al., 2012). Furthermore, ceftazidime, cepirome and aztreonam preferentially inhibit PBP3 (FtsI) (Curtis et al., 1979; Maejima et al., 1991). Thus, the two mutations in PBP3, Ile332Val and Ala233Thr, which were only identified in Caz/20-2 mutant (Table 3) and not in PS/20 and Caz/20-1 strains, could decrease the affinity of the protein by ceftazidime increasing its MIC. The mutation Ile332Val was within the catalytic penicillin-binding module Asp237-Val577 (Piette et al., 2004), a highly conserved domain within the transpeptidase superfamily. Interestingly, Ile332Val was near to the previously described PBP3 active site Gly306-Ser-Thr-Val-Lys-Pro311 (Keck et al., 1985). Furthermore, it was previously proposed that activity of the transpeptidase module of PBP3 is regulated by the interaction of its N-terminal non-catalytic module with other cell division proteins, and, in fact, catalytic activity of PBP3 is stimulated by interaction(s) with other division proteins (Eberhardt et al., 2003). Perhaps the mutation Ala233Thr in PBP3 of Caz/20-2 mutant, which is located within the N-terminal non-catalytic module Arg71-Ile236, could abrogate or make more difficult the interaction with the transpeptidase module and/or with other division proteins, as has been previously demonstrated for mutations within the non-catalytic module of PBP3 (Piette et al., 2004); along this line, the mutated residue Ala233Thr is within the highly conserved motif Asn231-Leu-Ala-Leu-Ser-Ile-Asp-Glu-Arg-Leu-Gln241 in n-PBP module (Nguyen-Distete et al., 1998) in PBP3 of E. coli and other Enterobacteria and P. aeruginosa. Further studies investigating possible differences in the affinity of Caz/20-2 mutant’s PBP3 by ceftazidime and other β-lactams, derived from the mutations Ile332Val within the catalytic penicillin-binding module and Ala233Thr within the N-terminal non-catalytic module Arg71-Ile236 are in progress. Likewise, there was no significant difference between the level of expression of ftsI in Caz/20-2 and PS/20 (Table 4, Fig. S1, available in JMM Online).

The condition of AcrD as an aztreonam transporter, and those of AcrB or AcrF as ceftazidime and cefotaxime

Table 4. The fold increase of the expression levels in the studied genes in E. coli PS/20 and Caz/20-2 compared with those in E. coli AG100 strain

<table>
<thead>
<tr>
<th>Genes</th>
<th>Expression ratio over AG100*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS/20</td>
</tr>
<tr>
<td>acrB</td>
<td>0.9</td>
</tr>
<tr>
<td>acrD</td>
<td>1.01</td>
</tr>
<tr>
<td>acrF</td>
<td>2.1</td>
</tr>
<tr>
<td>tolC</td>
<td>0.9</td>
</tr>
<tr>
<td>marA</td>
<td>1.02</td>
</tr>
<tr>
<td>sdiA</td>
<td>4.5</td>
</tr>
<tr>
<td>ftsI</td>
<td>0.8</td>
</tr>
<tr>
<td>mppA</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*The level of expression of genes in the AG100 strain non-induced with salicylate or paraquat was taken as 100.
†AG100 induced with 5 mM salicylate.
‡AG100 induced with 0.2 mM paraquat.
transporters have been previously described (Nishino et al., 2003), and expression of the above efflux pump transporters and possible regulatory genes was analysed. The efflux pump transporters AcrB, AcrD, and AcrF resulted significantly overexpressed in Caz/20-2 the same that SdiA (Table 4, Fig. S1). In turn, the increase in mitomycin-C MIC in Caz/20-2, which was not reversed by CCCP (Table 2), was consistent with increased transcript levels of sdiA as previously described (Tavío et al., 2010). Previous publications found 5.18- to 10.56-fold induction of sdiA expression associated with a 4.9- to 13.93-fold increase in the acrF transcript level (Domka et al., 2006). In other reports, the concurrent overexpression of ramA and marA resulted in 6.6- and 15.1-fold increased transcript levels of acrF and acrB, respectively, whereas a 149.1-fold increase in the transcript level of soxS alone only resulted in a 2.5-fold increase of acrF and 5.5-fold for acrB in Salmonella Typhimurium (Zheng et al., 2009). In the present study, increased transcript levels of both sdiA (65.6-fold) and marA (4.3-fold) in Caz/20-2 were associated with a 6.1-fold increase of acrF expression (transporter of the two-component efflux pump AcrEF) together with acrD (single-component efflux pump) and acrB overexpression (Table 4, Fig. S1). It has previously been demonstrated that when efflux pumps of different structural types combined in the same cell, the observed antibiotic resistance was much higher than that conferred by each of the pumps expressed singly, although simultaneous expression of two multi-component efflux pumps also had an additive effect on antibiotic resistance (Lee et al., 2000). Likewise, it was previously demonstrated that the effect on antibiotic resistance conferred by a multi-component pump was dependent on its level of expression (Lee et al., 2000). Thus, in the present study, an increase of 19.8-fold of acrB, in addition to a 2.9-fold increase in acrF in Caz/20-2 with respect to the expression level of both genes in the parental strain, could multiply the effect of PBP3 mutations reducing the susceptibility to ceftazidime in the Caz/20-2 mutant. In turn, the overexpression of AcrD, AcrB and AcrF transporters could also contribute to increasing the 16-fold aztreonam MIC and fourfold cefotaxime MIC in Caz/20-2. The increased transcript level of acrB in the Caz/20-2 strain was striking, 17.8-fold more than that in AG100 (Table 4, Fig. S1), particularly when compared with previous descriptions in which only frameshift mutation in marR (Keeney et al., 2008) or only acrR mutation (Watanabe & Doukyu, 2012) was detected. Nevertheless, the high expression level of acrB gene in Caz/20-2 is similar to that described in Salmonella typhimurium strains in which simultaneous increased expression oframA and marA was found, 69.1- and 1.9-fold, respectively (Zheng et al., 2009). Likewise, E. coli strains with double mutation in acrR and marR exhibited a higher expression level of AcrB but not TolC, compared with those strains with mutations in only one of the genes, acrR or marR (Watanabe & Doukyu, 2012). In the present study, a mutation in acrC (Gln27His) due to the substitution CAG→CAT was found in Caz/20-2 strain concomitantly with the frameshift mutation in marR that was due to c.102_103insT (Table 3) within the nucleotide sequence of marR. This insertion in marR sequence resulted in Pro35→Ser and generated changes in the following residues, including those lying inside the putative DNA-binding domain of MarR within the region spanning amino acids 61–121, following the description of the DNA-binding domain of MarR by Alekshun et al. (2001). The simultaneous presence of the above two mutations in Caz/20-2 strain (Table 3) explains the higher increase of acrB expression compared to that in Caz/20-1, which carried the same mutation Gln27His in acrR, although without any marR mutation. The above mutation in acrR was just adjacent to the residue Gly28 (G28) in which mutations are described connected to norfloxacin, chloramphenicol and tetracycline resistance (Oteo et al., 2006). Therefore, the moderate increase of acrB expression observed in Caz/20-1, which was 3.6-fold lower than that in Caz/20-2 and 4.3-fold higher than the acrB transcript level in PS/20 (Table 4, Fig. S1), was probably responsible for the increase of ceftazidime MIC in Caz/20-1, 2 μg ml⁻¹, only fourfold higher than that in PS/20, 0.5 μg ml⁻¹. Otherwise, sdiA expression level in the mutant Caz/20-2 did not show a significant difference with respect to that found in PS/20 (4.9-fold increase with respect to that in AG100) (data not shown in Fig. S1).

Likewise, the role of increased active efflux in Caz/20-2 multidrug resistance phenotype was also demonstrated by the increased susceptibility (twofold to 16-fold) not only to ceftazidime but also to quinolones, chloramphenicol, tetracycline and all the tested β-lactams induced by the efflux pump inhibitor CCCP (Tables 2 and 3). Furthermore, increases in turbidity of 3.7- to 4.3-fold at 3 and 6 h after cyclohexane addition were found in Caz/20-2, which meant a 1.9- to 2.8-fold increase in the cyclohexane tolerance with respect to that detected in PS/20 and in the wild-type AG100. The range of increases in turbidity in Caz/20-1 mutant was 2.5- to 2.8-fold, which only meant a 1.6- to 1.8-fold increase with respect to the PS/20 and AG100 strains. Increased cyclohexane tolerance in Caz/20-2 was consistent with tolC overexpression (Table 4, Fig. S1) and this resembled previously reported findings (Tavío et al., 2010). A previous report has demonstrated the synergistic effect of double mutations of marR and acrR improving the solvent tolerance to cyclohexane, compared with that in strains with mutations in only one of the above genes (Watanabe & Doukyu, 2012). The gene acrB, which encodes the transporter of the two-component efflux pump AcrAB, is overexpressed when mutations abrogate the AcrR repressor function on acrAB operon (Webber & Piddock, 2001). Likewise, both tolC and acrB are overexpressed when marR mutations are carried by E. coli strains (Wang et al., 2001; Tavío et al., 2010; Watanabe & Doukyu, 2012) since MarR is a repressor of marRAB operon and therefore also a repressor of marA expression (Alekshun & Levy, 1999). The analysis of OMPs revealed the loss of OmpF only in Caz/20-2 strain, but not in PS/20...
and Caz/20-1, which was concomitant with an increased level of OmpC and the loss of a band migrating between OmpC and OmpF (Fig. 1). The loss of OmpF in Caz/20-2 mutant coincided with the above-mentioned frameshift mutation found in marR (Table 3). The truncated MarR protein probably lost its repressive function on marRAB operon, and this resulted in a marA transcript level in Caz/20-2 mutant 4.3- and 4.2-fold higher than those in AG100 and PS/20, respectively, within the range of previously described marA increased expression in marR mutants (Linde et al., 2000). In turn, the increased MarA level in Caz/20-2 mutant contributed to tolC and acrB overexpression, and was responsible for OmpF loss due to the elevation of micF RNA synthesis and the consequent destabilization of the ompF mRNA, a known effect of MarA overexpression (Aono et al., 1998; Pomposiello et al., 2001). Curiously, the induction of Caz/20-2 with NaCl resulted in increased expression of OmpF and decrease of OmpC (Fig. 1), which means that synthesis of OmpF in Caz/20-2 mutant could be upregulated by NaCl independently of the presence of marR mutations and MarA overexpression. Likewise, Caz/20-2 was twofold more susceptible to 2,4-dinitrophenol than either PS/20 or AG100 (Table 2); this finding coincides with a previous description in other in vitro selected mutants (Tavio et al., 2010), as well as with data obtained in AG100 lon mutant by Nicoloff et al. (2006). Then it is possible to suggest a reduced Lon protease activity that, in addition to the marA gene upregulation due to marR mutation, perhaps could also contribute to increase the level of MarA in the cytoplasm of Caz/20-2 strain by slowing the degradation rate of MarA, since Lon protease degrades MarA and other cellular proteins (Griffith et al., 2004).

Otherwise, no mutations other than silent nucleotide substitutions were found in soxR gene in PS/20, Caz/20-1 and Caz/20-2 strains according to GenBank sequence accession no. U00096.

We previously described the overexpression of sdiA upon in vitro-selection of ceftazidime-resistant E. coli mutants (Tavio et al., 2010). In the present study, the transcript level of the mppA gene showed a significant increase of 3.4- and 9.9-fold in Caz/20-2 compared with its transcript level in PS/20 and AG100 strains, respectively (Table 4, Fig. S1), and the same was observed for transcript levels of sdiA, a 14.5-fold increase compared with that of PS/20 and a 65.6-fold increase with respect to AG100 (Table 4, Fig. S1); therefore, it is possible that mppA might be upregulated by sdiA. SdiA indeed accelerates cell division in E. coli (Wei et al., 2001ab), which results in an increased usage of peptides derived from murein turnover or recycling (Park et al., 1998) including those secreted by bacteria into the growth medium that are poor AmpG substrates (Cheng & Park, 2002; Maqbool et al., 2011). Therefore, one could hypothesize that mppA being part of sdiA regulon might be overexpressed when sdiA accelerates cell division to improve the uptake of such murein-derived peptides that cannot be efficiently recovered by AmpG. Likewise, it was previously proposed that MppA would serve some purpose other than recycling, perhaps acting also as a periplasmic binding protein that could mediate signal transduction in the quorum-sensing response (Park et al., 1998). The results in the present study seem to indicate that there was no relationship or dependence between either ampG and mppA expression or ampG and sdiA expression. In this regard, despite PS/20, Caz/20-1 and Caz/20-2 strains showing the same amino acid sequences in AmpG, including three mutations Ala420Glu, Val436Ile and Thr491Met, differences in the transcript levels of mppA and sdiA were observed between PS/20 and Caz/20-2 mutant (Table 4, Fig. S1).

In the present work the frequent appearance of multiple mutations during in vitro-selection with ceftazidime was striking and it could be due to the inhibition of PBP3 by ceftazidime and its previously described relationship with the production of mutator phenotypes (Pérez-Capilla et al., 2005; Blázquez et al., 2006). It has been previously demonstrated that the PBP3 inhibition results in the arrest of cell-wall synthesis, which, in turn, induces the transcription of SOS genes and error-prone DNA-polymerase, which results in mutator phenotypes that are overexpressed (Pérez-Capilla et al., 2005; Blázquez et al., 2006).

The acquisition of possible mutations in QRDR of gyrA, gyrB, parC and parE genes responsible for the increase in quinolone MICs in Caz/20-2 with respect to those in PS/20 was also analysed. No mutations other than silent nucleotide substitutions were found in the amplified fragments of the gyrA, gyrB and parC genes in PS/20, Caz/20-1 and Caz/20-2. Nevertheless, two mutations at

![Fig. 1. Outer-membrane protein extracts separated in a 6 M urea 11% polyacrylamide gel and stained with Imperial Protein Stain: size markers, lane 1; PS/20, lane 2; Caz/20-1, lane 3; Caz/20-2, lane 4; Caz/20-2 grown with NaCl 1.35 %, lane 5; KL16, lane 6; JF703, lane 7; size markers, lane 8. Size markers from top to bottom (the thickest bands) correspond to: albumin, bovine serum (66 kDa); ovalbumin, chicken egg (45 kDa); carbonic anhydrase, bovine erythrocytes (29 kDa). The position (height) of OmpC, OmpF and OmpA in the gel are indicated as C, F and A.](image-url)
codons 443 and 446 were identified in the amplified fragment of the ParE subunit (covering codons 365 to 525) from PS/20 and both Caz mutants. They consisted of an A→C transversion in the codon AAC together with a C→A transversion in the codon AAG, which resulted in Lys-443→Thr and Asn-446→Lys substitutions, respectively (Table 3) located outside but adjacent to QRDR of ParE Asp420 to Lys441, which could result in increases in nalidixic acid and norfloxacin MICs in Caz/20-2 (Tables 2 and 3) when their effect was multiplied by OmpF loss and increased active efflux. Along this line, an eightfold increase in norfloxacin MIC, such as that seen in Ca/20-2 mutant, was in the range of a previous description where an increase in norfloxacin MIC, such as that seen in Ca/20-2 mutant, was the contribution of active efflux to quinolone MICs (Yang et al., 2003). Furthermore, other mutations different to the above ones have been also described outside the ParE QRDR associated with norfloxacin MICs between 0.047 and 1.5 μg ml⁻¹ (Ruiz et al., 1997; Komp Lindgren et al., 2005; Morgan-Linnell et al., 2009).

In conclusion, a single mutation in acrR and related acrB overexpression resulted in only a fourfold increase in ceftazidime MIC in Caz/20-1. Nevertheless, mutations found in ftsl (PBPs) in both the catalytic penicillin-binding and N-terminal non-catalytic modules together with OmpF loss and increased expression (6.1- to 17.8-fold) of one mono-component and two-component efflux pumps were in a coordinated way responsible for a 128-fold increase in ceftazidime MIC in Caz/20-2 mutant in absence of ESBLs and a high-level production of AmpC beta-lactamase.

ACKNOWLEDGEMENTS

We employed the services of Genetics and Molecular Diagnosis Service at the University of Las Palmas de Gran Canaria for DNA sequencing. This work was supported by the Canary Foundation for Research and Health (40/2009-FUNCIS). The work of V.D.A. was supported by a grant awarded by the Carolina Foundation (Spanish Agency for International Cooperation).

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