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Complex Regulation Pathways of AmpC-Mediated β-Lactam Resistance in *Enterobacter cloacae* Complex

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***Enterobacter cloacae* complex (ECC), an opportunistic pathogen causing numerous infections in hospitalized patients worldwide, is able to resist β-lactams mainly by producing the AmpC β-lactamase enzyme. AmpC expression is highly inducible in the presence of some β-lactams, but the underlying genetic regulation, which is intricately linked to peptidoglycan recycling, is still poorly understood. In this study, we constructed different mutant strains that were affected in genes encoding enzymes suspected to be involved in this pathway. As expected, the inactivation of *ampC*, *ampR* (which encodes the regulator protein of *ampC*), and *ampG* (encoding a permease) abolished β-lactam resistance. Reverse transcription-quantitative PCR (qRT-PCR) experiments combined with phenotypic studies showed that cefotaxime (at high concentrations) and cefoxitin induced the expression of *ampC* in different ways: one involving NagZ (a *N*-acetyl-β-D-glucosaminidase) and another independent of NagZ. Unlike the model established for *Pseudomonas aeruginosa*, inactivation of DacB (also known as PBP4) was not responsible for a constitutive *ampC* overexpression in ECC, whereas it caused AmpC-mediated high-level β-lactam resistance, suggesting a post-transcriptional regulation mechanism. Global transcriptomic analysis by transcriptome sequencing (RNA-seq) of a *dacB* deletion mutant confirmed these results. Lastly, analysis of 37 ECC clinical isolates showed that amino acid changes in the AmpD sequence were likely the most crucial event involved in the development of high-level β-lactam resistance *in vivo* as opposed to *P. aeruginosa* where *dacB* mutations have been commonly found. These findings bring new elements for a better understanding of β-lactam resistance in ECC, which is essential for the identification of novel potential drug targets.**

Species of the *Enterobacter cloacae* complex (ECC) are widely distributed in nature and are part of the commensal microbiota of the human gastrointestinal tract. For 2 decades, they have emerged as major human pathogens (1, 2). Indeed, they have become one of the leading causes of hospital-acquired infections worldwide, accounting for around 5% to 10% of intensive care unit (ICU) infections (1, 2). The ECC shows a genomic heterogeneity with 13 clusters and currently comprises six different species: *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, and *Enterobacter nimipressuralis* (3). ECC species are highly adapted to the hospital environment and are able to contaminate various medical devices. Because of its huge ability to rapidly develop multiple antimicrobial resistances, therapeutic failures are commonly observed (1, 2).

It is well known that the ECC is intrinsically resistant to ampicillin, amoxicillin-clavulanate, and first- and second-generation cephalosporins due to a low-level but inducible expression of a chromosomal *ampC* gene encoding a β-lactamase (1, 4). The production of this cephalosporinase is highly inducible in the presence of strong β-lactam inducers such as imipenem, cefoxitin, and clavulanate (4). The chromosomal β-lactamase induction mechanism is complex and involves three major gene products intimately linked to the peptidoglycan (PG) recycling pathway: AmpR (a LysR-type transcriptional regulator), AmpD (a cytosolic amidase), and AmpG (an inner membrane permease) (5–12).

In the current model of Gram-negative bacteria, during normal growth, muropeptides from PG degradation are removed from the cell wall and transported via the AmpG permease into the cytoplasm where they are cleaved by AmpD to generate free peptides. To be recycled back into the cell wall synthesis, they are converted into UDP-pentapeptides. These interact with AmpR creating a conformation that represses the transcription of *ampC* (4, 11, 12). Jacobs et al. suggested that the pentapeptide may be the AmpR ligand, since the murein precursor decreases AmpR-mediated transcriptional activation *in vitro* (13). Under inducing conditions, there is an accumulation of muropeptides in the cytoplasm, and AmpD is unable to efficiently process the high levels of cell wall fragments. Therefore, the muropeptides (inducing peptides) are thought to displace the UDP-pentapeptides (repressing peptides) from AmpR, converting it into a transcriptional activator of *ampC* expression (11, 12). In *Pseudomonas aeruginosa*, several works showed that AmpR is a global transcriptional regulator. It is a potential membrane-associated dimer that regulates genes involved in virulence, quorum sensing, and stress response (14–18). In addition, three AmpD enzymes have been found in *P. aeruginosa*. The cytoplasmic AmpD protease appear to be involved in cell wall recycling events and the antibiotic resistance pathway, whereas the periplasmic AmpDh2 and AmpDh3 enzymes exhibited marginal activities (19). Mechanisms of AmpC regulation have been extensively studied in *P. aeruginosa*, and several studies demonstrated that other genes intimately linked to the cell wall recycling system are involved in the regulation of *ampC* expression. Thus, *ampE* (coding for an inner membrane-bound

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype/relevant characteristic(s)[a]	Reference or source
Strain		
E. cloacae subsp. *cloacae* ATCC 13047	Reference strain completely sequenced and annotated	41
ECLΔ*ampC*	13047 derivative with deletion of *ampC* (ECL_00553)	This study
ECLΔ*ampR*	13047 derivative with deletion of *ampR* (ECL_00554)	This study
ECLΔ*ampD*	13047 derivative with deletion of *ampD* (ECL_00906)	This study
ECLΔ*ampE*	13047 derivative with deletion of *ampE* (ECL_00907)	This study
ECLΔ*ampG*	13047 derivative with deletion of *ampG* (ECL_01191)	This study
ECLΔ*ampH*	13047 derivative with deletion of *ampH* (ECL_01132)	This study
ECLΔ*dacB*	13047 derivative with deletion of *dacB* (ECL_04561)	This study
ECLΔ*dacB*Δ*ampC*	13047 derivative with deletion of *dacB* and *ampC*	This study
ECLΔ*dacB*Δ*ampR*	13047 derivative with deletion of *dacB* and *ampR*	This study
ECLΔ*nagZ*	13047 derivative with deletion of *nagZ* (ECL_02529)	This study
ECLΔ03254	13047 derivative with deletion of ECL_03254	This study
ECLΔ03253	13047 derivative with deletion of ECL_03253	This study
Plasmid		
pKOBEG	Recombination vector, phage λ recγβα operon under the control of the pBAD promoter, Cm^r	42
pKD4	Plasmid containing an FRT-flanked kanamycin cassette, Kan^r	43
pCP20_Gm	FLP-mediated recombination vector, Gen^r	44

[a] Cm^r, chloramphenicol resistant; Gen^r, gentamicin resistant; Kan^r, kanamycin resistant.

sensory transducer), *dacB* (coding for a D-alanyl-D-alanine carboxypeptidase also known as PBP4), and *nagZ* (coding for an *N*-acetyl-β-D-glucosaminidase) have been shown to play an important role in the regulatory network of *ampC* in *P. aeruginosa* (20–26). In contrast, the role of these different proteins on AmpC-mediated β-lactam resistance in ECC is not known, and regulatory pathways still remain to be fully elucidated.

Among ECC clinical isolates, high-level resistance to β-lactams (especially third-generation cephalosporins) is due to *ampC* constitutive overexpression (a phenomenon called derepression), mainly resulting from *ampD* mutations and much less commonly from *ampR* mutations (27–32). Alterations responsible for AmpD inactivation or decreased *ampD* expression lead to a permanent increase in concentrations of inducing muropeptides into the cytoplasm, which convert AmpR into a transcriptional activator conformation (33, 34). This development of resistance in *Enterobacter* spp. is a major concern since it appears among ca. 10% to 20% of patients treated with broad-spectrum cephalosporins (35–37). Also, once selected, AmpC overproduction is stable, and approximately 30% to 40% of ECC isolates are currently resistant to third-generation cephalosporins worldwide (38, 39).

The aim of this study was then to investigate in detail the regulation mechanisms of AmpC-mediated β-lactam resistance in ECC. First, in the genome of *E. cloacae*, we identified all of the gene products putatively involved in peptidoglycan recycling based on a *P. aeruginosa* model. Second, we constructed corresponding deletion mutants and tested their β-lactam resistance profiles and their impact on *ampC* expression. Third, we screened a collection of ECC clinical isolates for mutations putatively involved in acquisition of β-lactam resistance *in vivo*. Our results revealed that the model described for *P. aeruginosa* was not completely relevant for ECC and gave a better overview of regulatory mechanisms underlying β-lactam resistance in *Enterobacteriaceae*.

(A preliminary report of this work was presented at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 9 to 12 September 2014 [40].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The reference strain used for the construction of knockout mutant strains was *E. cloacae* subsp. *cloacae* ATCC 13047 (ECL13047), of which the genome is completely sequenced and annotated (GenBank accession numbers NC_014121, NC_014107, and NC_014108) (41). The 37 independent clinical isolates were recovered from diverse infection sites between 2013 and 2014 (CHU, Caen, France). *E. cloacae* strains were cultured by shaking at 37°C in Luria-Bertani (LB) medium.

Antimicrobial susceptibility testing. The MIC values of different β-lactams (amoxicillin, AMX; piperacillin-tazobactam, PTZ; cefoxitin, FOX; cefotaxime, CTX; ceftazidime, CAZ; cefepime, FEP; and imipenem, IPM) were determined on Mueller-Hinton agar using Etest strips (bioMérieux, Marcy-l'Etoile, France) in three independent experiments according to the manufacturer's instructions.

AmpC β-lactamase activity assay. AmpC activity was assessed using the nitrocefin hydrolysis assay as previously described (26). *E. cloacae* cultures were grown in LB medium until reaching an optical density at 600 nm (OD_{600}) of 0.5. At this time point, 50 μg/ml FOX or 25 μg/ml CTX was added for 2 h. During these treatments, no bactericidal effect was observed. One sample without antibiotic was used as a control. Nitrocefin hydrolysis was measured every minute for 15 min at room temperature by absorbance at 486 nm. AmpC activity was calculated using a nitrocefin extinction coefficient of 20,500 M^{-1} cm^{-1}. Each assay was independently performed at least three times. Statistical significance was assessed using a two-tailed Student *t* test, and a *P* value of ≤0.05 was considered statistically significant.

Construction of knockout deletion mutants. Disruption of the selected genes putatively implicated in the regulation of *ampC* was performed using the method described by Datsenko and Wanner with some modifications, using the plasmid pKOBEG (containing a gene for chloramphenicol resistance selection and a gene encoding a recombinase) (42, 43). Briefly, the plasmid pKOBEG was introduced into the ECL13047 strain by electroporation, and transformants were selected on Luria-Bertani (LB) agar with chloramphenicol (25 μg/ml) after incubation for 24 h at 30°C. A selectable kanamycin resistance gene was amplified by PCR using the pKD4 plasmid as a DNA template (42). The primers used included 5′ extensions with homology for the candidate genes and are listed in Table S1 in the supplemental material. The PCR product was intro-

TABLE 2 MICs and basal and induced *ampC* expression in wild-type *E. cloacae* ATCC 13047 and its derivative mutants

Strain	MIC (μg/ml)							Relative mRNA level of *ampC*[a]		
	AMX	PTZ	FOX	CTX	CAZ	FEP	IPM	Basal	FOX induced[b]	CTX induced[b]
Wild-type 13047	≥256	4	≥256	0.5	1	0.03	0.5	1	**21.6 ± 4.2**	**79.2 ± 4.5**
ECLΔ*ampC*	2	4	4	0.12	0.25	0.03	0.25	≤0.01	≤0.01	≤0.01
ECLΔ*ampR*	4	4	4	0.12	0.25	0.03	0.25	1.5 ± 1.0	0.67 ± 0.1	2.9 ± 0.3
ECLΔ03254	≥256	4	≥256	0.5	1	0.03	0.5	1.1 ± 0.3	**16.6 ± 3.6**	**60.4 ± 5.0**
ECLΔ03253	≥256	4	≥256	0.5	1	0.03	0.5	1.1 ± 0.2	**11.2 ± 1.6**	**46.3 ± 10.9**
ECLΔ*ampD*	≥256	32	≥256	32	32	1	0.5	**369 ± 45**	**707 ± 17**	**600 ± 106**
ECLΔ*ampE*	≥256	4	≥256	0.5	1	0.03	0.5	1.2 ± 0.1	**11.2 ± 1.4**	**145 ± 22**
ECLΔ*ampG*	4	2	4	0.12	0.25	0.03	0.25	1.0 ± 0.2	1.9 ± 0.9	0.5 ± 0.2
ECLΔ*dacB*	≥256	16	≥256	16	16	0.25	0.25	1.6 ± 0.8	**7.0 ± 2.2**	**17.4 ± 8.5**
ECLΔ*dacB*Δ*ampC*	2	4	4	0.12	0.25	0.03	0.25	≤0.01	≤0.01	≤0.01
ECLΔ*ampH*	≥256	4	≥256	0.5	1	0.03	0.5	1.1 ± 0.7	**6.0 ± 2.9**	**77.0 ± 18.2**
ECLΔ*nagZ*	16	2	256	0.25	0.5	0.03	0.25	1.1 ± 0.2	**37.8 ± 14.1**	2.1 ± 0.3

[a] Relative amount of *ampC* mRNA compared to the wild-type 13047 basal level ± standard deviation; significant changes are indicated in bold.
[b] Induction experiments carried out with 50 μg/ml of FOX or 25 μg/ml of CTX.

duced into the ECL13047/pKOBEG cells by electroporation, and after homologous recombination the disruption of the candidate gene was obtained. Selected clones were cured for the pKOBEG plasmid following a heat shock. In order to obtain deletion mutants after double crossover, the strains were transformed with the pCP20_Gm plasmid (44), which is able to express the FLP nuclease that recognizes the flippase recognition target (FRT) sequences present on either side of the kanamycin resistance gene.

PCR, sequencing, and quantification of gene expression. Genomic DNA from the ECL13047 strain and clinical isolates were extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France). Different genes (*hsp60* for species-level distinction [3], *ampR*, *ampC*, and *dacB*) were amplified by PCR with specific primers (see Table S1 in the supplemental material), and the purified PCR products were sequenced with the same sets of primers in both directions (GATC Biotech, Constance, Germany).

The levels of expression of *ampC*, *ampR*, and ECL_03254 were determined by real-time reverse transcription-PCR (RT-PCR) using specific primers (see Table S1 in the supplemental material). Bacterial cells were harvested 2 h after reaching an OD_{600} of 0.5, during which CTX (25 μg/ml) or FOX (50 μg/ml) was added for the induction experiments. Total RNAs were extracted from ECL13047 and its derivative mutants using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA). Residual chromosomal DNA was removed by treating samples with the Turbo DNA-*free* kit (Life Technologies, Saint-Aubin, France). Samples were quantified using the BioSpec-nano spectrophotometer (Shimadzu, Noisiel, France), and the integrity was assessed using the Agilent 2100 bioanalyzer according to the manufacturer's instructions. cDNA was synthesized from total RNA (~25 ng) using the QuantiFast SYBR green RT-PCR kit (Qiagen), and transcript levels were determined by the ΔΔ threshold cycle ($\Delta\Delta C_t$) method using the *rpoB* gene as a housekeeping control gene (see Table S1). Each experiment was performed in triplicate.

Transcriptomic analysis by RNA-seq. Total RNAs were extracted from ECL13047 and ECLΔ*dacB* (cultured to the late-exponential growth phase) in duplicate as mentioned above. Before library preparation, DNase-treated samples were depleted from 23S, 16S, and 5S rRNAs using the Ribo-Zero rRNA removal kit (Gram-negative bacteria) (Epicentre, Madison, WI) according to the manufacturer's instructions. To evaluate the degree of rRNA depletion, the samples were analyzed using the Agilent 2100 bioanalyzer. cDNA libraries were prepared with the strand-specific Nextflex rapid directional transcriptome sequencing (RNA-Seq) kit (dUTP-based) v2, and sequencing was performed using an Illumina HiSeq 2500 instrument with the paired-end (2 × 100 bp) multiplexing protocol (ProfileXpert-LCMT, Lyon, France).

For bioinformatic analysis, reads were mapped against the genomic sequence of ECL13047 (GenBank accession number NC_014121) using the CLC Genomics Workbench software v5.1 (Qiagen). Calculation of fold change (FC) values and statistical analysis were performed using the DESeq R package (45). Genes with an expression $\log_2$ FC superior to 2 or inferior to −2 were considered induced or repressed, respectively, and statistical significance was retained in the case of a *P* value of ≤0.05. For visualization, each gene was plotted according to its mean expression value and the differential expression (MA plot).

RESULTS AND DISCUSSION

Role and regulation of the two *ampC*-annotated genes in ECL13047. As expected, the wild-type ECL13047 strain was highly resistant to AMX and FOX (MICs, ≥256 μg/ml) but remained susceptible to the other β-lactams tested (Table 2). The AmpC enzyme affinity is indeed much higher to aminopenicillins and FOX than to third-generation cephalosporins and carbapenems (4). In parallel, the expression of the *ampC* gene in ECL13047 was induced 22- and 79-fold in the presence of FOX (50 μg/ml) and of high-concentration CTX (25 μg/ml), respectively. The β-lactamase activity of AmpC was also statistically significantly increased in the presence of FOX or CTX (Fig. 1). FOX and CTX at high concentrations should be considered β-lactam inducers of *E. cloacae ampC* expression. Because the induction by CTX was performed during a short period (2 h), this result was very unlikely due to a selection of derepressed mutants. Moreover, significant decreases in the MICs of AMX (≥128-fold), FOX (≥64-fold), and CTX and CAZ (4-fold) seen in the *ampC* deletion mutant correlated with the absence of β-lactamase activity (Table 2 and Fig. 1), confirming the crucial role of *ampC* in β-lactam resistance in *E. cloacae* (4). Detailed analysis of the *E. cloacae* genome sequence revealed the existence of another gene (ECL_03254) annotated as encoding a class C β-lactamase based on the presence of the conserved motif (CubicO group peptidase, beta-lactamase class C family, COG1680). Interestingly, the level of transcription of ECL_03254 was not modified in the presence of FOX or CTX (data not shown), while the mutant lacking ECL_03254 had the same profile of β-lactam resistance as the wild-type strain. In addition, the absence of ECL_03254 did not have any effect on basal or induced *ampC* expression and AmpC β-lactamase activity (Table 2 and Fig. 1). Altogether, these results demonstrate the minimal impact of such a protein in β-lactam resistance in ECC and its negligible role in regulation and induction pathways of *ampC*.

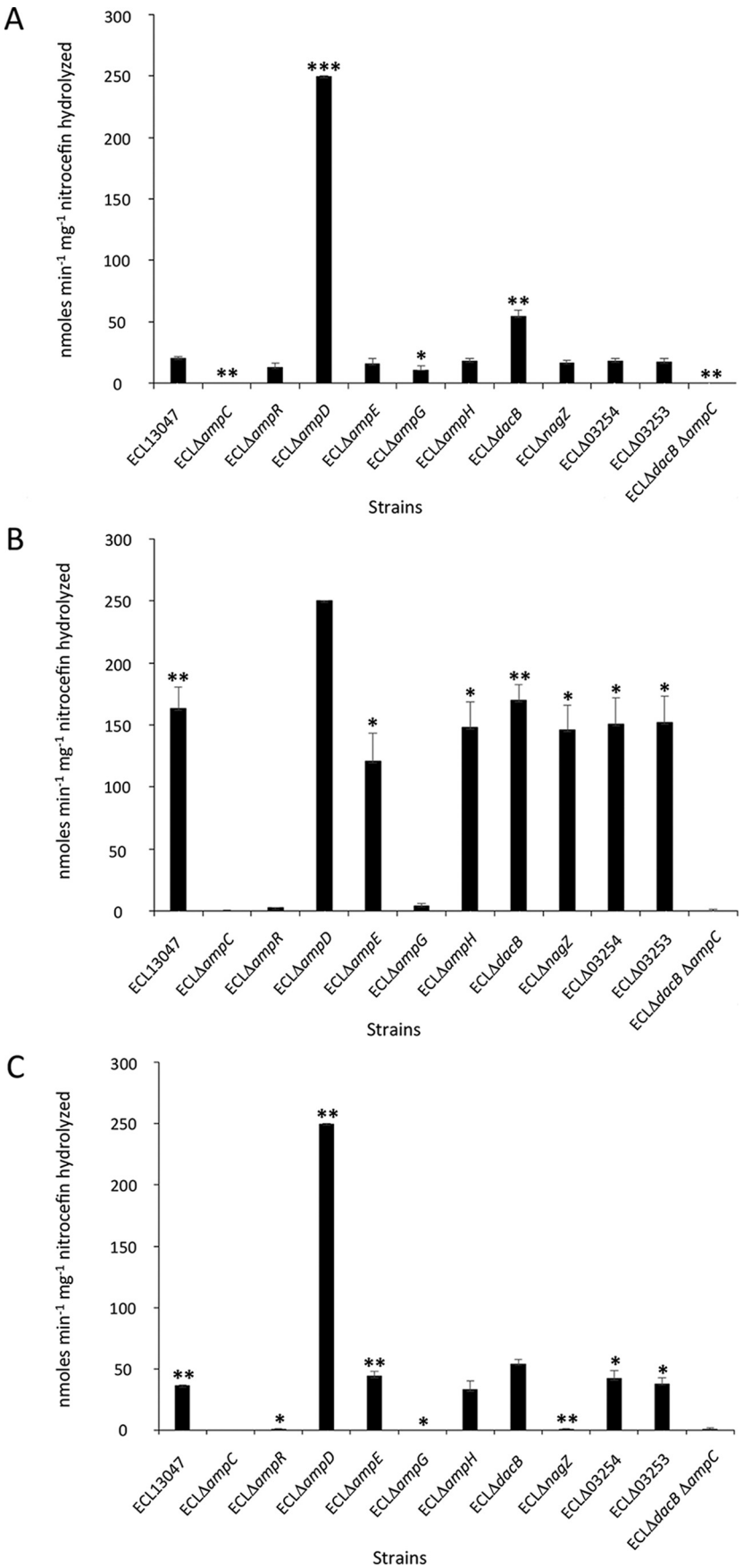

FIG 1 AmpC activity (measured in nanomoles per minute per milligram nitrocefin hydrolyzed) of *E. cloacae* ATCC 13047 grown under basal conditions (A) and induced conditions, with the medium supplemented with 50 μg/ml of cefoxitin (B) or 25 μg/ml of CTX (C) for 2 h. Bars represent the means ± standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Expression of *ampC* is regulated by AmpR, a transcriptional regulator, coded by the *ampR* gene located next to *ampC* and divergently transcribed (5–7). The ECLΔ*ampR* mutant was more susceptible to β-lactams than the wild-type strain and lost the ability to induce the transcription of *ampC* (Table 2). Transcriptomic analysis also showed that *ampR* was constitutively transcribed in the wild-type strain and in all of the isogenic mutants (data not shown), confirming that the activity of AmpR depends on the interaction with coregulators rather than on an overexpression or underexpression of *ampR*. This is in accordance with the current model where, in the absence of β-lactams, AmpR represses *ampC* transcription, and when exposed to antibiotics, cells accumulate peptidoglycan catabolites (i.e., 1,6-anhydroMurNAc-peptides), changing AmpR to an activator of *ampC* transcription (33, 46, 47). In *P. aeruginosa*, AmpR plays a role in physiological processes and influences the expression of over 500 genes, including virulence-encoding genes and other transcriptional regulators (15, 16, 48). Then, AmpR appears as a global regulator, and its possible role in the virulence of ECC should be further investigated. Note that this regulation by AmpR is not a general mechanism in *Enterobacteriaceae* since some *Escherichia coli* strains do not possess an *ampR* gene (5–7). Interestingly, ECL_03254 was preceded by a gene (ECL_03253) that coded for a transcriptional regulator that also belonged to the LysR family. Because the transcription of ECL_03254 was significantly induced (8.4-fold) in ECLΔ*3253*, ECL_03253 likely acts as a repressor under standard growth conditions (data not shown). It is tempting to speculate that the protein encoded by ECL_03254 may be functional and that the role and substrate of this enzyme and the precise regulation of the corresponding gene remain to be elucidated.

***ampD* and *ampG* mutations also affect the *ampC* expression.** In order to verify that enzymes involved in PG recycling play a role in *ampC* regulation, mutant strains affected in *ampD*, *ampE*, and *ampG* were constructed. AmpD is a cytoplasmic *N*-acetylmuramyl-L-alanine amidase leading to the production of UDP-MurNAc-pentapeptides that change AmpR to a repressor of *ampC* transcription (13, 34, 49). This explains why, in the ECLΔ*ampD* mutant, the level of *ampC* expression was extremely high (between 369- and 707-fold more than in the wild type) even in the absence of β-lactams (Table 2). This was correlated with its high-level resistance to PTZ, CTX, and CAZ (MICs, 32 μg/ml) (Table 2). Based on what is already known about *E. cloacae*, this mutant likely accumulates peptidoglycan catabolites triggering the activation form of AmpR and the overexpression of *ampC* (7, 27, 28, 32, 50). Consequently, the high level of AmpC activity correlates with the high MICs observed (Fig. 1 and Table 2).

In *E. cloacae*, the *ampE* gene is part of the *ampDE* operon and encodes a cytoplasmic membrane protein thought to act as a β-lactam-reactive sensory transducer (7). The unique deletion of *ampE* did not reveal a significant difference in terms of *ampC* regulation and β-lactam resistance compared to the wild-type strain (Table 2). Nevertheless, transcomplementation experiments performed in *P. aeruginosa* suggest that AmpE may play an indirect role in resistance and that there are other unknown genes (likely located close to the *ampDE* operon) involved in AmpC overproduction (51).

AmpG is an inner membrane permease that transports PG catabolites involved in cell wall recycling (9, 10). We confirm that, in the absence of AmpG, the levels of *ampC* mRNA were not significantly modified by the addition of CTX or FOX, while ECLΔ*ampG* was more susceptible to all of the β-lactams tested, which is similar to the ECLΔ*ampC* and ECLΔ*ampR* mutants (Table 2). In accordance with the current model of regulation, this is explained by the fact that peptides are unable to enter the cytosol that leads to the absence of molecules interacting with AmpR and preventing the modulation of the *ampC* expression (9, 10).

These results, which combine resistance phenotypes, the level of *ampC* transcription, and AmpC activity, were in agreement with the current model linking PG recycling and β-lactam resistance in Gram-negative rods.

DacB and AmpH are two PBPs involved in the regulation of *ampC* expression. DacB is a nonessential low-molecular mass penicillin-binding protein (PBP) (called PBP4) with D,D-carboxypeptidase and D,D-endopeptidase activities. In addition to its role in PG recycling, it is also involved in *E. coli* cell separation during division or bacterial morphology (52, 53). In the ECLΔ*dacB* mutant strain, we showed that the level of *ampC* transcription did not significantly change in the absence of β-lactams. However, in the presence of FOX or CTX, the *ampC* transcription remained slightly induced, but this was 3- to 4-fold lesser than that observed in the wild-type strain (Table 2). Surprisingly, whereas no substantial increase of *ampC* expression was observed, a high-level resistance to PTZ, CTX, and CAZ (MICs, 16 μg/ml) was observed in the ECLΔ*dacB* mutant (Table 2). It may be suggested that DacB regulates AmpC at a post-transcriptional level, since *dacB* deletion triggered a significant increase of AmpC β-lactamase activity without a strong upregulation of the *ampC* gene (Fig. 1 and Table 2). It has been shown that the production of AmpC in *Serratia marcescens* was controlled by the presence of a 5′-untranslated region (5′-UTR) stem-loop structure of the mRNA that may be related to RNA stability or translational efficiency (54). The 5′ end of the *ampC* transcript of *E. cloacae* did not show such specific organization, but it is conceivable that translational regulation may occur, either by interaction with a small *trans*-acting noncoding RNA or by site-specific ribosome stalling, which is a mechanism used for the control of translation of antibiotic resistance genes such as *cat* (chloramphenicol acetyltransferase) and *erm* (erythromycin ribosomal methylase) (55–57). This mechanism of regulation radically diverges from that described in the *P. aeruginosa* model, where the inactivation of PBP4 leads to a constitutive *ampC* overexpression (23). In this last species, the role of PBP4 in the *ampC* induction process is linked to the activation of the CreBC two-component system that, in turn, plays a crucial role in β-lactam resistance (58). Such a regulatory system has not been found in the genome of ECL13047, and this may explain why regulatory pathways of *ampC* expression are, in part, different from those of *P. aeruginosa*. Note that the protein coded by the *dacB* gene of *E. cloacae* shows 90% amino acid identity with that of *E. coli* K-12 but only shows 29% amino acid identity with that of *P. aeruginosa*; this may explain such differences. Mutations in *dacB* have been identified in high-level β-lactam-resistant clinical isolates of *P. aeruginosa*, and PBP4 was shown to be the main driver of the resistance (23). These data reveal the presence of alternative process in PG recycling and the existence of multiple pathways leading to the control of *ampC* expression. To assess the role of AmpC in the β-lactam resistance observed in ECLΔ*dacB*, *ampC* was disrupted in the Δ*dacB* mutant background, showing that β-lactam susceptibility was restored to the levels observed for the single ECLΔ*ampC* mutant (Table 2). This confirms that high-level β-lactam resistance in the absence of *dacB* is mediated by AmpC.

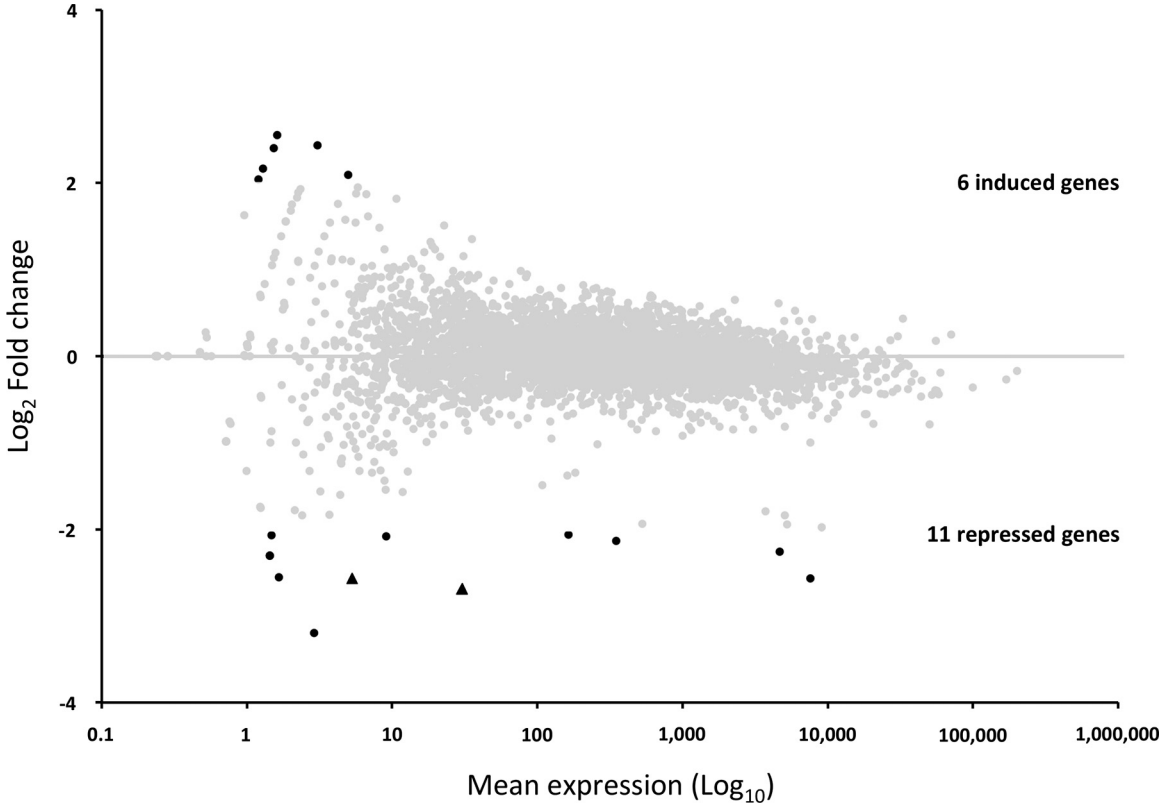

FIG 2 MA plot representing the global gene expression analysis obtained by RNA-seq. Each dot represents one gene. The *x* axis reflects the mean expression level of a gene (baseMean in Table S3 in the supplemental material). The *y* axis reflects the differential expression of a gene (as $\log_2$ fold change) between the ECLΔ*dacB* mutant and the wild-type ECL13047 strain (see Table S3). Plots corresponding to genes with no significant altered expression (i.e., $\log_2$ FC between −2 and 2) are faded. The two statistically differentially expressed genes (ECL_01582 and ECL_01584) are represented as triangles.

In order to verify whether DacB in *E. cloacae* was linked to a regulatory pathway able to modify the expression of some genes, we performed a global transcriptomic analysis by RNA-seq comparing the Δ*dacB* mutant with the wild-type strain (see Table S2 in the supplemental material). This comparison allowed us to identify only six genes of which expression was induced (but not statistically significantly) and 11 repressed genes (Fig. 2; see also Table S3 in the supplemental material). Of these 11 genes, only two exhibited statistically significantly decreased expression changes: ECL_01582 (FC, −6.4; $P = 10^{-6}$) and ECL_01584 (FC, −5.9; $P = 0.04$) (see Table S3). The two genes coded for hypothetical proteins (135 and 52 amino acids long, respectively), and no homology was found with any other bacterial protein. Noteworthy, the levels of expression of *ampC* and *ampR* were not statistically significantly different (FC, 1.1) (see Table S3), which was also confirmed by reverse transcription-quantitative PCR (qRT-PCR) experiments (data not shown).

We also tested whether another PBP, AmpH, involved in the maturation and remodeling of PG may be part of the control of AmpC production in ECC. In *E. coli*, this low-molecular-mass PBP displays bifunctional D,D-endopeptidase and D,D-carboxypeptidase activity (59). The ECLΔ*ampH* mutant exhibited similar MICs of β-lactams compared to those of the wild-type strain (Table 2). However, we observed that the induction of *ampC* due to the addition of FOX was not as high as that measured for the ECL13047 wild-type strain (6-fold versus 22-fold, respectively) (Table 2). It may be proposed that AmpH has more affinity for cefoxitin and, despite a role in PG recycling, has a minor influence in *ampC* regulation.

FOX and CTX induce the transcription of *ampC* in two different ways. Based on the *P. aeruginosa* and *E. coli* models, it is proposed that PG recycling also involves NagZ, which is a β-*N*-acetylglucosaminidase. This enzyme processes peptidoglycan degradation products in the cytoplasm, producing 1,6-anhydro-MurNAc-peptides that may activate *ampC* transcription through its interaction with AmpR (60, 61). It is thus expected that the inactivation of *nagZ* should abolish the induction of *ampC* when β-lactams are present. In ECC, this appeared true with CTX since no transcriptional induction of *ampC* was observed in the ECLΔ*nagZ* strain associated with a drastic reduction in the MIC of AMX (from ≥256 to 4 μg/ml) and, to a lesser extent, of PTZ, CTX, and CAZ (2-fold decrease) (Table 2). In *P. aeruginosa*, loss of *nagZ* reduces the capability to acquire resistance to β-lactams (62). However, the lack of *ampC* induction by the presence of CTX in the ECLΔ*nagZ* mutant that we observed is very unlikely due to the selection of mutant strains because the antibiotic treatment only lasted 2 h. In contrast, when FOX was added to the culture of this mutant, the level of *ampC* mRNA increased 38-fold compared to the basal transcription without an antibiotic (Table 2). Note that the ECLΔ*nagZ* mutant remained resistant to FOX (MIC, ≥256 μg/ml) (Table 2). Similar results were observed in *P. aeruginosa*, where *nagZ* inactivation had little effect on the induction of AmpC in the presence of FOX (62). Moreover, the nitrocefin hydrolysis activity data correlated well with the transcriptomic results

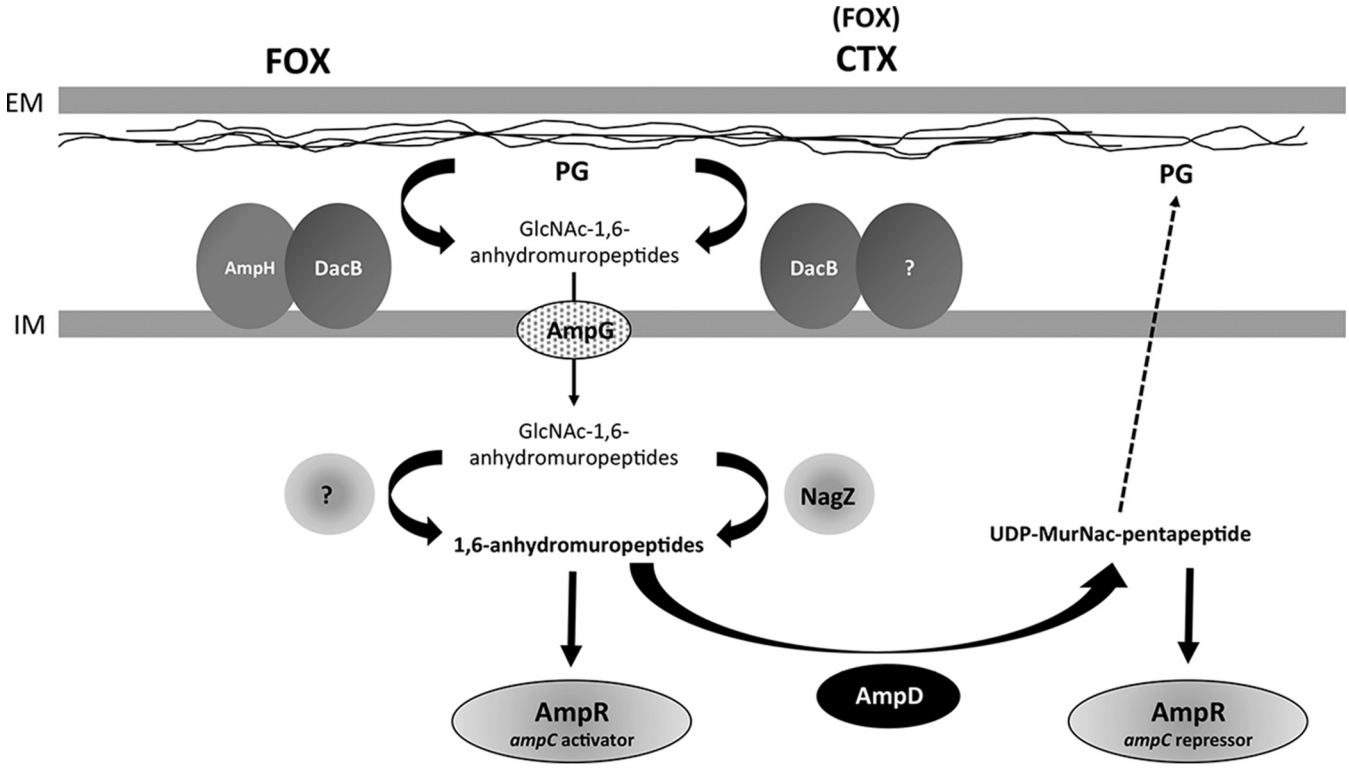

FIG 3 Model of the transcriptional induction of *ampC* following exposure to cefoxitin (FOX) (left) or cefotaxime (CTX) (right) in *E. cloacae*. EM, external membrane; GlcNAC, *N*-acetylglucosamine; IM, inner membrane; MurNac, *N*-acetylmuramic acid.

(Fig. 1). This demonstrates that at least two different pathways exist for the overexpression of *ampC*: one stimulated by CTX and involving NagZ and another triggered by FOX and independent of NagZ. *Shewanella oneidensis* mutants lacking NagZ or AmpG are still able to induce the β-lactamase BlaA, which suggests a parallel signal transduction pathway independent of NagZ (61). Note that the *blaA* gene in *S. oneidensis* is inducible by ampicillin but this species does not have a neighbor gene coding for a transcriptional regulator such as *ampR* and that the *nagZ* mutation results in increased β-lactam resistance (61).

Prevalence of *dacB* mutations in β-lactam-resistant ECC clinical isolates. Mutations in *ampD* and *ampR* genes have been shown to be responsible for AmpC overexpression and high-level β-lactam resistance in *Enterobacter* spp. for a long time (5–7, 63). However, the involvement of mutations in other genes (such as *dacB*) has never been investigated. To do that, screening of mutations in *ampD*, *ampR*, *ampC*, and *dacB* genes was done on a panel of 37 unrelated ECC clinical isolates (see Table S4 in the supplemental material). Determination of MICs showed that 31 (84%) exhibited constitutive AmpC overexpression (CAOP), while six (16%) presented a β-lactam susceptibility profile. Out of the 31 CAOP strains, 28 (90%) had at least one amino acid change in AmpD, seven of which resulted in premature termination of the protein by creation of a stop codon or by introducing a frameshift mutation (see Table S4). Some of these mutations have already been described in *E. cloacae* (50). Despite the existence of other amino acid substitutions in AmpR, AmpC, or DacB (without any sequence interruption), these data strongly argue for the crucial role of AmpD alterations in the development of high-level β-lactam resistance in *E. cloacae*. For the three CAOP strains devoid of AmpD mutation (strains 6, 20, and 30), it is tempting to speculate that the modifications observed in AmpR, AmpC, and/or DacB may play an important role in resistant phenotypes (see Table S4). In this context, the unique mutation in the DacB sequence was retrieved in strain 30. However, this strain showed MIC values significantly lower than the those of the other CAOP strains. These results were not in accordance with those reported for *P. aeruginosa*, where *dacB* mutations seem to be much more common (23).

Taken altogether, our data, which were obtained with the different mutant strains affected in genes involved in PG recycling, allowed us to propose a model in which the presence of a β-lactam leads to the expression of the AmpC β-lactamase (Fig. 3). CTX (at high concentration) and FOX were able to significantly enhance the *ampC* expression. Growth of *E. cloacae* in the presence of β-lactams increases the production of PG degradation products (1,6-anhydroMurNAc-peptides), which enter the cytoplasm via the AmpG permease and which ultimately lead to the accumulation of MurNAc-tripeptide. NagZ, which catalyzes the production of the MurNAc-tripeptide, appeared important when CTX was present, whereas another mechanism, as yet unknown, is involved in the *ampC* induction in the presence of FOX. MurNAc-tripeptide may interact with the AmpR regulator, changing its activity from a negative to a transcriptional activator of *ampC*. In the absence of β-lactams, the MurNAc-tripeptide can be reintegrated into the peptidoglycan synthesis thanks to AmpD activity. Note that mutations in AmpD, which conduces to an increase of AmpC production and consequently β-lactam resistance, seems the most frequent mechanism of resistance in ECC clinical isolates. In addition, we have shown that DacB played an important role for β-lactam resistance in *E. cloacae* but also that AmpH intervened in

ampC induction by FOX. This is the first work that describes the different steps of the regulation of AmpC expression in *E. cloacae* and that clearly diverges from those of *E. coli* (sometimes devoid of AmpR) or *P. aeruginosa* (where *dacB* inactivation leads to *ampC* overexpression). A better understanding of the molecular mechanism of resistance to β-lactams is crucial to better fight and prevent infections by this important opportunistic pathogen.

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