

Chromosomal cephalosporinase in *Enterobacter hormaechei* as an ancestor of ACT-1 plasmid-mediated AmpC β -lactamase

Kyoung Ho Roh,¹ Wonkeun Song,² Hae-Sun Chung,³ Yang Soon Lee,³ Jong Hwa Yum,⁴ Ha Na Yi,⁵ Jong Sik Chun,⁵ Dongeun Yong,³ Kyungwon Lee³ and Yunsop Chong³

Correspondence
Dongeun Yong
deyong@yuhs.ac

¹Department of Laboratory Medicine, Korea University College of Medicine, Seoul, Republic of Korea

²Department of Laboratory Medicine, Hallym University College of Medicine, Seoul, Republic of Korea

³Department of Laboratory Medicine, Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul, Republic of Korea

⁴Department of Clinical Laboratory Science, Dong-eui University, Busan, Republic of Korea

⁵School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul, Republic of Korea

In this study of the diversity of AmpC β -lactamase in clinical isolates of *Enterobacter* spp., a strain was found carrying the plasmid-mediated AmpC β -lactamase ACT-1 gene on its chromosome. The strain was identified as *Enterobacter hormaechei* using phylogenetic analysis of 16S rRNA and *hsp60* genes. In addition, the species was confirmed by DNA–DNA hybridization. The genetic environment of the *bla*_{ACT-1} gene was characterized, including the *ampR* and *ampG* genes, using a two-step PCR. The amino acid sequences of AmpR at serine 35, arginine 86, glycine 102, aspartic acid 135 and tyrosine 264 were conserved. Measurement of the transcription level of the *bla*_{ACT-1} gene using real-time quantitative PCR showed that it increased 1.98-fold following cefoxitin induction. These results suggest that the plasmid-mediated *bla*_{ACT-1} gene originated from the chromosome of *E. hormaechei*.

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INTRODUCTION

Cephamycin resistance in organisms such as *Enterobacter cloacae*, *Citrobacter freundii* and *Serratia marcescens* can be caused by the induction or hyperproduction of AmpC β -lactamase (Livermore *et al.*, 2001). Induction of this enzyme requires three additional gene products, AmpG, AmpR and AmpD (Reisbig *et al.*, 2003). AmpG is a permease that transports mucopeptides into the cytoplasm during cell-wall recycling. These peptides cause a conformational change in AmpR, resulting in induction of *ampC* transcription. Mutations in AmpD lead to derepression of the chromosomal *ampC* gene (Reisbig *et al.*, 2003).

Initially, AmpC β -lactamase was chromosomally encoded; it expanded its host range by incorporating the genes into plasmids. Whilst most plasmid-mediated AmpC β -lactamases

(PABLs) are expressed constitutively, the *bla*_{ACT-1}, *bla*_{DHA-1}, *bla*_{DHA-2} and *bla*_{CMY-13} genes are reported to be inducible by the *ampR* genes upstream of *ampC* (Jacoby, 2009). Among these PABLs, ACT-1 was found initially in imipenem-resistant *Klebsiella pneumoniae* with the loss of an outer-membrane protein (Bradford *et al.*, 1997). Although an ancestral association of ACT-1 with *Enterobacter asburiae* was proposed, the nucleotide and amino acid sequence identities were only 96.5 and 98 %, respectively (Rottman *et al.*, 2002).

Currently, the taxonomy of the *E. cloacae* complex is based on whole-genome DNA–DNA hybridization and phenotypic characteristics (Brenner *et al.*, 1986). *E. asburiae*, *E. cloacae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii* and *Enterobacter nimipressuralis* have been assigned to this complex. Accurate species identification is desirable because these organisms are increasingly isolated as nosocomial pathogens and are often not identified further to species level, perhaps because of the difficulty of achieving this (Paauw *et al.*, 2008). The heat-shock protein 60 (*hsp60*) gene has been proposed as a promising marker for identification of *Enterobacter* spp. (Paauw *et al.*, 2008).

Abbreviations: RT-qPCR, real-time quantitative PCR; PABL, plasmid-mediated AmpC β -lactamase.

The GenBank/EMBL/DDBJ accession numbers for the sequences of 16S rRNA, *ampG*, *ampR*, *ampC*, *bhc* and *sugE* genes reported in this study are HQ215203, HQ215206 and HQ235645.

While determining the diversity of AmpC β -lactamases in *Enterobacter* strains isolated from clinical specimens, we found a strain carrying the *bla*_{ACT-1} gene on its chromosome. The strain was identified as *E. hormaechei* using nucleotide sequencing of the 16S rRNA and *hsp60* genes. In addition, DNA–DNA hybridization was performed to confirm the species. We characterized the genetic environment of the *bla*_{ACT-1} gene because its induction was not observed in disk approximation tests. We also measured the transcription level of the *bla*_{ACT-1} gene following cefoxitin induction using a cephalothin hydrolysis assay and real-time quantitative PCR (RT-qPCR). We propose that the plasmid-mediated *bla*_{ACT-1} gene originated from the chromosome of *E. hormaechei*.

METHODS

Bacterial strain and antimicrobial susceptibility testing. An *Enterobacter* strain (YMC/KN/03/21) was isolated from urine collected from an intensive care unit patient in a Korean teaching hospital. Species identification and antimicrobial susceptibility tests were performed using the VITEK2 GN system (bioMérieux). Clinical and Laboratory Standards Institute guidelines (CLSI, 2010) were used to interpret the results.

Phenotypic detection and spectrophotometric assay for ACT-1 induction. Inducibility was screened using cefoxitin disk approximation tests with cefotaxime, ceftazidime and aztreonam disks (Song *et al.*, 2006) because cefoxitin is a strong inducer and is stable against hydrolysis. Cephalothin hydrolysis was assayed using crude enzyme extract obtained from a sonicated suspension following a 2 h induction assay with 10 μ g cefoxitin ml⁻¹ (Sanders *et al.*, 1986). The change in absorbance resulting from the opening of the β -lactam ring was measured using a UV-1601PC spectrophotometer (Shimadzu).

16S rRNA and *hsp60* gene sequencing and DNA–DNA relatedness. 16S rRNA gene sequencing was performed using primers 8F

and 1541R (Table 1) (Zhou *et al.*, 1995) and the results were analysed using the EzTaxon server (Chun *et al.*, 2007) and MEGA software (<http://www.megasoftware.net>). The primers for PCR and sequencing of the *hsp60* gene were Hsp60 For and Hsp60 Rev (Table 1; Hoffmann & Roggenkamp, 2003). The DNA–DNA relatedness between strain YMC/KN/03/21 and the type strains of *E. hormaechei* CCUG 27126^T and *E. asburiae* CCUG 25714^T were evaluated using DNA–DNA hybridization. The DNA–DNA similarity value was calculated using the equation given by De Ley *et al.* (1970). A threshold value of 70% DNA–DNA similarity was used to define bacterial species.

Measurement of mRNA levels of the *bla*_{ACT-1} gene by RT-qPCR.

Five colonies of clinical isolates of YMC/KN/03/21 were cultured in 10 ml Luria–Bertani broth overnight at 35 °C. A 2 h induction assay with cefoxitin (Sanders *et al.*, 1986) was performed with 5 ml Luria–Bertani culture. Total RNA was extracted using an RNeasy Protect Bacteria Mini kit and an RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. An Omniscript RT kit (Qiagen) with random primers (Promega) and RNase inhibitor (GenDEPOT) were used to produce cDNA. Primers Act1 F and Act1 R were then used for amplification, together with the Act1 probe (Table 1). The 5' and 3' ends of the probe were labelled with 6-carboxyfluorescein (FAM) and a fluorescence quencher dye (BHQ1), respectively. A capillary real-time thermal cycler (LightCycler; Roche Diagnostics) was used. The reaction conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 50 °C for 30 s and 72 °C for 1 min, with cooling for 30 min at 40 °C. Expression levels of the *bla*_{ACT-1} gene with or without cefoxitin induction were normalized against expression of the 16S rRNA gene (using primers 16S rRNA F and 16S rRNA R and the 16S rRNA probe; Table 1) and compared.

Sequencing of the AmpC β -lactamase, *ampR* and *ampG* genes.

AmpC β -lactamase genes were investigated using multiplex PCR (Pérez-Pérez & Hanson, 2002). The nucleotide sequences and deduced amino acid sequences were compared with sequences in the EMBL-EBI database (<http://www.ebi.ac.uk/Tools/sss/fastaf/>). Two-step PCR was performed to determine the flanking nucleotide sequences in the upstream and downstream regions of the *ampC* gene (Sørensen *et al.*, 1993), because induction of the ACT-1 enzyme was not observed in

Table 1. Primers used in this study

Primer	Sequence (5'→3')	Purpose	References
8F	AGAGTTTGATCMTGGCTCAG	Species identification	Zhou <i>et al.</i> (1995)
1541R	AAGGAGGTGWTCCARCC	Species identification	Zhou <i>et al.</i> (1995)
Hsp60 For	GGTAGAAGAAGGCGTGGTTGC	Species identification	Hoffmann & Roggenkamp (2003)
Hsp60 Rev	ATGCATTTCGGTGGTATCATCAG	Species identification	Hoffmann & Roggenkamp (2003)
Act1 F	CTGGGAAATGCTTAAC	RT-qPCR sense	This study
Act1 R	GGTGGATTCACCTTCTCTC	RT-qPCR antisense	This study
Act1 probe	AACCTTATTGTCGCTACCTTCAACC	RT-qPCR probe	This study
16S rRNA F	AGCTAGAGTATGGGAGAGGATGG	RT-qPCR sense	This study
16S rRNA R	TTCGTACCTCAGCGTCAGTATTAG	RT-qPCR antisense	This study
16S rRNA	TGCCTTCGCCATCGGTATCTCCTCAGA	RT-qPCR probe	This study
R0	TTCCAACAGGTCCTTAAG	Two-step PCR	Sørensen <i>et al.</i> (1993)
R1	CAGTTCAAGCTTGCCAGGAATTCNN- NNNNNGGCCT	Two-step PCR	Sørensen <i>et al.</i> (1993)
ACT flank L1	CCFCCACCGCCATACCCGGA	Two-step PCR	This study
ACT flank L2	CACCTCAGCCAGCTGTTTTT	Two-step PCR	This study
ACT flank R1	CCTCCGCGCCTCCGGTCAA	Two-step PCR	This study
ACT flank R2	AAGCTATCCAACCCAGCAC	Two-step PCR	This study
Enterob ampG F1	GTGGTGATCGCCTTCTGTTC	<i>ampG</i> sequencing	Schmidt <i>et al.</i> (1995)
Enterob ampG R1	GTATATTCAGCGTCTGGCG	<i>ampG</i> sequencing	Schmidt <i>et al.</i> (1995)

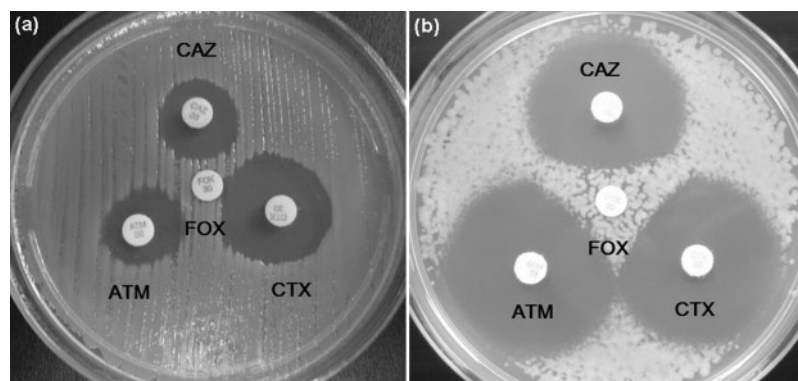


Fig. 1. A disk approximation test with ceftazidime (CAZ), cefotaxime (CTX) and aztreonam (ATM). (a) Strain YMC/KN/03/21; (b) *E. cloacae* ATCC 23355.

ceftazidime disk approximation tests. The primers used were R0, R1, ACT flank L1, ACT flank L2, ACT flank R1 and ACT flank R2 (Table 1). The PCR conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 45 °C for 30 s and 72 °C for 1 min, and for the second-round PCR were 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 50 °C for 30 s and 72 °C for 1 min.

To investigate the *ampG* gene, which encodes a transporter of muropeptides required for AmpC induction and is essential for high-level expression of AmpC β -lactamase in *E. cloacae*, primers Enterob ampG F1 and Enterob ampG R1 were used for PCR and nucleotide sequencing (Schmidt *et al.*, 1995).

Genetic localization of the *bla*_{ACT-1} gene using PFGE and Southern blot hybridization. To confirm the genetic location of the *bla*_{ACT-1} gene, transconjugation experiments were performed using *Escherichia coli* J53 (azide resistant). Genomic DNA was prepared in low-melting-point agarose blocks and digested with the endonucleases S1 or I-CeuI (Sigma-Aldrich). DNA fragments were separated using a CHEF-DR II apparatus (Bio-Rad). Southern blot hybridization with an *ampC* probe was performed using a digoxigenin DNA labelling and detection kit (Roche Diagnostics).

RESULTS

Bacterial strain and antimicrobial susceptibility testing

The strain was initially identified as *Enterobacter cloacae* with 96% probability using the VITEK2 GN system

(bioMérieux). The strain was resistant to ampicillin ($\geq 32 \mu\text{g ml}^{-1}$), amoxicillin/clavulanic acid ($\geq 32 \mu\text{g ml}^{-1}$), cephalothin ($\geq 64 \mu\text{g ml}^{-1}$), ceftazidime ($\geq 64 \mu\text{g ml}^{-1}$), cefotaxime ($4 \mu\text{g ml}^{-1}$), ceftazidime ($16 \mu\text{g ml}^{-1}$) and aztreonam ($32 \mu\text{g ml}^{-1}$), but had intermediate resistance or was susceptible to cefepime ($\leq 1 \mu\text{g ml}^{-1}$), imipenem ($\leq 1 \mu\text{g ml}^{-1}$) and meropenem ($\leq 0.25 \mu\text{g ml}^{-1}$).

Phenotypic detection and spectrophotometric assay for ACT-1 induction

The disk approximation test showed no antagonism between the ceftazidime disk and the ceftazidime, cefotaxime and aztreonam disks compared with *E. cloacae* ATCC 23355 (Fig. 1). To measure the change in ACT-1 enzyme activity following ceftazidime induction, spectrophotometric hydrolysis assays were repeatedly performed using cephalothin as a substrate. The hydrolysis activity increased from 98.8 to 189.0 mU (mg protein)⁻¹ with ceftazidime induction, thus showing a 1.9-fold increase.

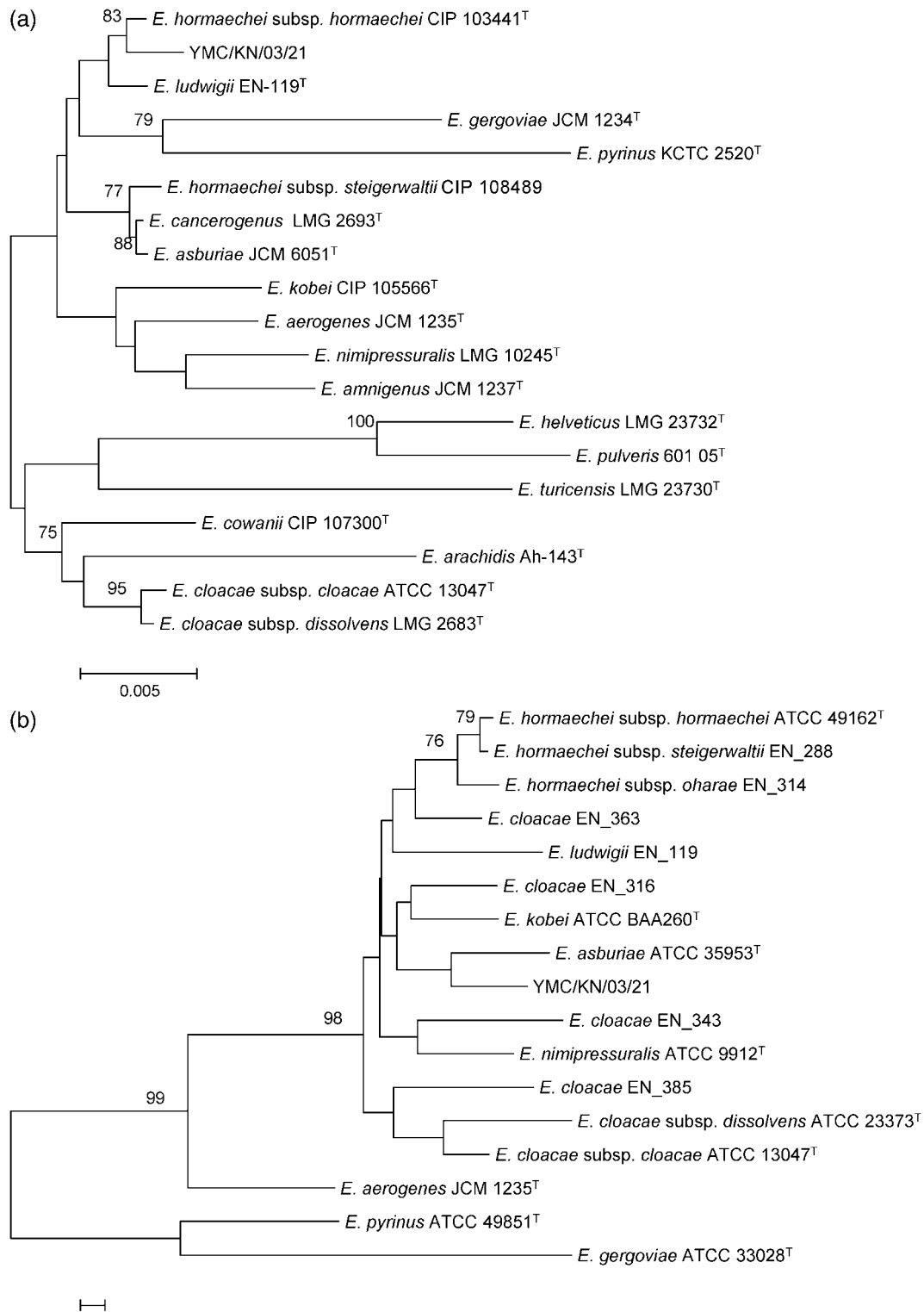
16S rRNA and *hsp60* gene sequencing and DNA-DNA relatedness

The best-matching 16S rRNA gene sequence for strain YMC/KN/03/21 was that of *E. hormaechei* ATCC 49162^T (CCUG 27126^T), showing 99.61% (1287/1292) identity. A

Fig. 2. (a) Phylogenetic tree of 16S rRNA gene sequences in the *E. cloacae* complex. The GenBank accession numbers are: *E. hormaechei* subsp. *hormaechei* CIP 103441^T, AJ508302; *E. ludwigii* CIP 108491^T, AJ853891; *Enterobacter gergoviae* JCM 1234^T, AB004748; *Enterobacter pyrinus* KCTC 2520^T, AJ010486; *Enterobacter cancerogenus* LMG 2693^T, Z96078; *E. asburiae* JCM 6051^T, AB004744; *E. hormaechei* subsp. *steigerwaltii* CIP 108489, AJ853890; *E. aerogenes* JCM 1235^T, AB004750; *E. kobei* CIP 105566^T, AJ508301; *Enterobacter amnigenus* JCM 1237^T, AB004749; *E. nimipressuralis* LMG 10245^T, Z96077; *Enterobacter helveticus* LMG 23732^T, DQ273688; *Enterobacter pulveris* 601/05^T, DQ273684; *Enterobacter turicensis* LMG 23730^T, DQ273681; *E. cloacae* subsp. *cloacae* ATCC 13047^T, AJ417484; *E. cloacae* subsp. *dissolvens* LMG 2683^T, Z96079; *Enterobacter cowanii* CIP 107300^T, AJ508303; *Enterobacter arachidis* Ah-143^T, EU672801. (b) Phylogenetic tree of *hsp60* genes in the *E. cloacae* complex. The GenBank accession numbers are: *E. hormaechei* subsp. *hormaechei* ATCC 49162^T, AJ417108; *E. hormaechei* subsp. *steigerwaltii* EN_288, AJ543769; *E. hormaechei* subsp. *oharae* EN_314, AJ543782; *E. cloacae* EN_363, AJ543819; *E. cloacae* EN_316, AJ543784; *E. kobei* ATCC BAA260^T, AJ567899; *E. ludwigii* EN_119, AJ417114; *E. asburiae* ATCC 35953^T, AJ417141; *E. cloacae* EN_343, AJ543804; *E. nimipressuralis* ATCC 9912^T, AJ567900; *E. cloacae* subsp. *dissolvens* ATCC 23373^T, AJ417143; *E. cloacae* subsp. *cloacae* ATCC 13047^T, EU643113; *E. aerogenes* JCM 1235^T, AB004750; *E. gergoviae* ATCC 33028^T, AJ567897; *E. pyrinus* ATCC 49851^T, AJ567901. Bars indicate nucleotide substitutions per site.

neighbour-joining phylogenetic tree of the 16S rRNA gene sequences showed that the sequence of YMC/KN/03/21 did not cluster with those of the 18 *Enterobacter* species except for *Enterobacter hormaechei* subsp. *hormaechei* ATCC

49162^T (CIP 103441^T; Fig. 2a). The phylogenetic tree of *hsp60* sequences showed that YMC/KN/03/21 belonged to a cluster most closely related to *E. asburiae* ATCC 35953^T (CCUG 25714^T) (Fig. 2b).



The DNA–DNA similarity of YMC/KN/03/21 with *E. hormaechei* CCUG 27126^T and *E. asburiae* CCUG 25714^T was 84 and 46 %, respectively.

RT-qPCR to measure mRNA levels of the *bla*_{ACT-1} gene

To confirm whether expression of the *bla*_{ACT-1} gene was induced by cefoxitin, RT-qPCR was performed. After cefoxitin exposure, the expression levels of the *bla*_{ACT-1} gene increased 1.98-fold, supporting the result of the cephalothin hydrolysis assay.

Sequencing of the AmpC β-lactamase, *ampR* and *ampG* genes

The nucleotide sequence of the AmpC β-lactamase gene showed 100 % identity with *bla*_{ACT-1} of *K. pneumoniae* (GenBank accession no. U58495). Intact *ampR* genes were found in the upstream region of the *bla*_{ACT-1} gene (Fig. 3a). The amino acid sequences of AmpR at positions serine 35, arginine 86, glycine 102, aspartic acid 135 and tyrosine 264 were conserved in the index strain (Fig. 3b). A comparison of the internal transcribed spacer promoter regions with those of inducible ACT-1 PABL reported in *K. pneumoniae*

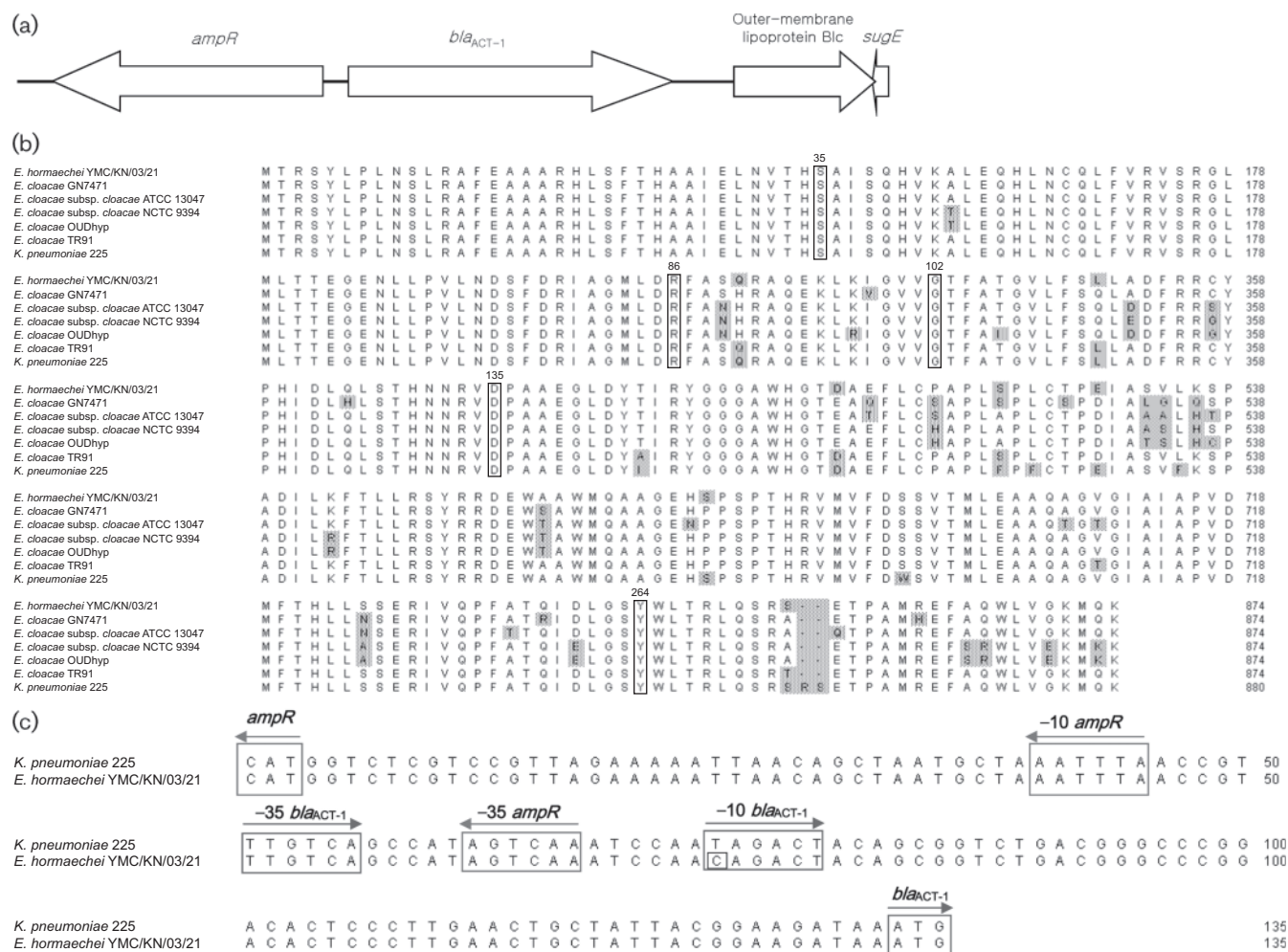


Fig. 3. (a) Schematic map of ACT-1 and its flanking regions in YMC/KN/03/21 (3029 bp, GenBank accession no. HQ235645). Open arrows indicate ORFs. (b) Alignment of the amino acid sequences of AmpR of YMC/KN/03/21 and other related strains that produce various AmpC enzymes. Conserved residues are indicated in boxes. Non-identical residues are shaded. (c) The intergenic region of the ACT-1 and *ampR* genes. The promoter elements and start codons are indicated in boxes. The GenBank accession numbers are: *E. cloacae* GN7471, AB016612; *E. cloacae* subsp. *cloacae* ATCC 13047^T, CP001918; *E. cloacae* subsp. *cloacae* NCTC 9394, FP929040; *E. cloacae* OUDhyp, AJ278995; *E. cloacae* TR91, DQ478697; *K. pneumoniae* 225, AF362955.

strain 225 (GenBank accession no. AF362955) (Reisbig & Hanson, 2002) is shown in Fig. 3(c).

The amino acid sequence of AmpG of the index strain YMC/KN/03/21 exhibited 98.9 and 98.5 % identity to those of *E. cloacae* subsp. *cloacae* ATCC 13047^T (GenBank accession no. CP001918) and *E. cloacae* strain 55 (GenBank accession no. AB194784), respectively. The sequence downstream of the *bla*_{ACT-1} gene (817 bp) showed the highest identity of 87.1 % with *E. cloacae* subsp. *cloacae* ATCC 13047^T.

Genetic localization of the *bla*_{ACT-1} gene using PFGE and Southern blot hybridization

In several conjugation experiments, the *bla*_{ACT-1} gene was not transferred to *Escherichia coli* J53 (azide resistant). When the S1 or I-CeuI nuclease-treated PFGE gels were blotted with the *ampC* probe, the results clearly showed that the *bla*_{ACT-1} gene was located on the chromosome of *E. hormaechei* strain YMC/KN/03/21 (data not shown).

DISCUSSION

Strain YMC/KN/03/21 was initially misidentified as *E. cloacae* using an automated biochemical identification system because the list of organisms that can be identified by the system included *E. cloacae*, *Enterobacter aerogenes*, *E. amnigenus*, *E. asburiae*, *E. cancerogenus*, *E. gergoviae* and *Enterobacter sakazakii*, but not *E. hormaechei*.

We determined the DNA–DNA relatedness, a reference method for taxonomic classification, between strain YMC/KN/03/21 and *E. hormaechei* CCUG 27126^T and *E. asburiae* CCUG 25714^T, because they were the most closely related organisms in the phylogenetic analysis using 16S rRNA and *hsp60* gene sequences. *E. asburiae* has previously been proposed as an ancestor of ACT-1 PABL (Rottman *et al.*, 2002). Strain YMC/KN/03/21 was confirmed as *E. hormaechei*.

When bacteraemic patients infected with *Enterobacter* strains are treated with oxyimino-cephalosporins for which they have tested susceptible, there is a 20 % probability that the drugs may select for a resistant mutant due to derepression of the β -lactamase (Chow *et al.*, 1991).

The standard method to measure the induction of AmpC enzymes is to assay the β -lactamase activity with or without β -lactams as an inducer. Simple and efficient methods such as a disk approximation test can be an alternative. However, induction of AmpC β -lactamase was not observed in disk approximation tests, which could be explained by too low a cefoxitin concentration for maximal induction. In contrast, a slight enhancement was noted between the disks, probably due to the additional inhibitory effect (Fig. 1a).

A twofold increase in the expression level of *bla*_{ACT-1} using RT-qPCR was not detectable in the disk approximation test, indicating that this assay was not sensitive enough to measure low-level ACT-1 induction. This might be explained by suboptimal cefoxitin concentrations in the

disk diffusion assay, and is perhaps also related to low permeability or high efflux in this strain.

Three other gene products, AmpR, AmpG and AmpD, are important for AmpC β -lactamase induction (Reisbig *et al.*, 2003). AmpR is a DNA-binding protein that positively regulates the expression of the *ampC* gene. AmpG allows muropeptides to enter the cytoplasm for cell-wall recycling, causing a conformational change in AmpR and resulting in AmpC induction. In contrast, inactivation of AmpD causes the derepressed hyperproduction of the AmpC enzyme. Therefore, in this study, the *ampR* and *ampG* genes were sequenced.

The amino acid sequences of AmpR at positions serine 35, arginine 86, glycine 102, aspartic acid 135 and tyrosine 264, which are important for AmpC induction, were conserved in the index strain (Hanson & Sanders, 1999). Comparison of the internal transcribed spacer promoter regions with those of *K. pneumoniae* strain 225 showed that the promoter sequences were also conserved (Reisbig & Hanson, 2002). The nucleotide sequences at positions 452, 803 and 1118 of *ampG*, which are essential for induction of chromosomal AmpC β -lactamase (Hanson & Sanders, 1999), were conserved in the index strain, reflecting the complexity of ACT-1 induction in *E. hormaechei*.

In summary, we have identified an *E. hormaechei* strain carrying the ancestral AmpC β -lactamase of plasmid-mediated ACT-1 by using PCR, nucleotide sequencing, phylogenetic analysis of 16S rRNA and *hsp60* genes and DNA–DNA relatedness studies. It is also interesting that the production of ACT-1 was weakly induced by cefoxitin, even though it had the *ampR* gene in the upstream region. The –10 and –35 promoters in the intergenic regions were intact, reflecting the complexity of ACT-1 induction in *E. hormaechei*.

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