Cloning and Sequence Analysis of $bla_{BIL-1}$, a Plasmid-Mediated Class C $\beta$-Lactamase Gene in *Escherichia coli* BS

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The extended-spectrum, plasmid-borne $\beta$-lactamase gene $bla_{BIL-1}$, which was discovered in *Escherichia coli*, has been cloned. Unusually for a plasmid-borne $\beta$-lactamase, $bla_{BIL-1}$ encodes a novel class C enzyme and appears to have originated from the chromosomal ampC gene of *Citrobacter freundii*.

$\beta$-Lactamases are microbial enzymes which inactivate $\beta$-lactam antibiotics by hydrolyzing the $\beta$-lactam ring. Comparison of the amino acid sequences of $\beta$-lactamases creates four molecular classes, A, B (1), C (10), and D (4). This paper is primarily concerned with class C $\beta$-lactamases, which together with class A $\beta$-lactamases are thought to derive from cell-wall-synthesizing enzymes (12). The class C $\beta$-lactamases are primarily cephalosporinases, but because they are often produced at high levels, they can mediate resistance to both cephalosporins and penicillins. Most gram-negative bacteria produce class C $\beta$-lactamases, and therefore, these enzymes create one of the most serious problems for the clinical use of $\beta$-lactams and in particular broad-spectrum cephalosporins (11).

Class C $\beta$-lactamases have previously been referred to as chromosomally mediated enzymes (25). However, in 1989 clinically derived strains of *Klebsiella pneumoniae* which exhibited transferrable resistance to a broad range of $\beta$-lactam antibiotics including cephaplycins were isolated.

The $\beta$-lactamase (MIR-1) responsible for this resistance was partially sequenced and shown to be a plasmid-mediated class C $\beta$-lactamase (23).

In the same year, a multiply resistant strain of *Escherichia coli* was isolated in London, United Kingdom, from raw area swabs and a biopsy specimen from a patient with 35% burns. This strain was shown to produce a plasmid-mediated $\beta$-lactamase (BIL-1) (29) which exhibited biochemical characteristics similar to those of MIR-1 (24). Here we present the cloning and primary sequence of $bla_{BIL-1}$ and by homology studies suggest the likely origin of this gene.

*E. coli* K-12 J62.2(5446.89) (24), a transconjugant of *E. coli* BS (29), the original clinical isolate producing BIL-1, was shown to produce BIL-1 by isoelectric focussing (19). A plasmid in excess of 70 kbp was isolated from *E. coli* J62.2(5446.89) by the alkaline lysis method of Timmis et al. (26) and partially digested with Sau3A I, generating fragments with an average size of 2 to 4 kb. These fragments were ligated into the BamHI site of the plasmid pACYC184 (3).

Recombinant plasmids were introduced into the $\beta$-lactamase-deficient strain *E. coli* 44 (21) by electroporation (BioRad Gene Pulser), and transformants producing BIL-1 were selected by resistance to ampicillin (50 $\mu$g/ml). A number of transformants were further characterized by resistance to cefazidime (3 $\mu$g/ml) and by identification of BIL-1 production by isoelectric focussing. Preliminary restriction endonuclease analysis of recombinant plasmids revealed plasmid instability with plasmids increasing in size after subculture of transformants. Primary recombinant plasmids containing $bla_{BIL-1}$ were therefore transferred to the *E. coli* recA1 host

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**FIG. 1.** Cloning and localization of the $bla_{BIL-1}$ gene.
DH1 (9, 17, 20). Further restriction enzyme analysis of several plasmids containing blaBIL$_1$ identified a common region of DNA flanked by an EcoRV site. Deletion of the EcoRV fragment in pBROC442 produced the plasmid pBROC443 (Fig. 1), which no longer conferred ceftazidime resistance on E. coli DH1. The EcoRV site was therefore inferred to lie within the blaBIL$_1$ gene.

An initial sequencing primer derived from pACYC184 sequences adjacent to the EcoRV site was used to sequence (Sequenase kit, version 2 [U.S. Biochemical Corp.]) one end of the cloned DNA in pBROC443.

Comparison of this preliminary nucleotide sequence with the sequence data base confirmed that the EcoRV site did indeed lie within a beta-lactamase gene. Subsequently, sequencing primers were derived from the known blaBIL$_1$ sequence, and the sequence of the entire blaBIL$_1$ gene was determined on both strands in pBROC442. The complete sequence of blaBIL$_1$ together with the upstream flanking sequence is shown in Fig. 2. The DNA sequence was analyzed with the Genetics Computer Group programs of the University of Wisconsin (5), and the blaBIL$_1$ open reading frame (ORF), comprising 1,146 nucleotides and encoding a protein of 381 amino acids, was identified. After cleavage of the predicted signal sequence, the mature protein of 361 residues has a molecular mass of 39,936 Da and a predicted pI of 10.04. This is in agreement with the high value of the experimentally determined pI (24).

Two of the characteristic amino acid fingerprints of penicillin-binding proteins and beta-lactamases are present in the blaBIL$_1$ ORF: the active-site serine (serine 70 in the Ambler classification scheme [1]) in the motif SXXK at residues 64 to 67 of the mature protein and the motif KTG at residues 315 to 317 (12). This second motif plays an essential role in the formation of the tertiary structure of the active site. A third motif, SXR, which is present approximately 80 residues carboxy-terminal to the active-site serine in class A beta-lactamases and penicillin-binding proteins, is not present in this ORF. However, the tyrosine residue at position 150, which forms part of the motif YAN, has been postulated to perform the same function as the serine in the SXR motif (22).

Comparison of both the nucleotide sequence of blaBIL$_1$ and the predicted amino acid sequence of BIL-1 with the appropriate data bases revealed extensive homology to class C beta-lactamases. BIL$_1$ showed greatest homology to the chromosomally encoded class C beta-lactamases of Citrobacter freundii with 93% DNA identity to the ampC genes of C. freundii OS60 (14) and C. freundii GN346 (27) and 94% identity at the amino acid level.

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**FIG. 2.** Nucleotide sequence of the blaBIL$_1$ gene. The deduced amino acid sequence of the BIL-1 protein is also presented. SD, ribosome binding site; ▼, limit of sequence homology to ampC genes; ▲, signal sequence cleavage site.
acid level, suggesting that bla\textsubscript{BIL-1} originated from this species. Therefore, it appears that BIL-1 could have originally been a \textit{C. freundii} class C enzyme which migrated from the chromosome to a plasmid. This gene migration could have been mediated by transposable elements, which are normal constituents of most bacterial genomes and of many extrachromosomal plasmids and bacteriophages (13). Transposons promote DNA rearrangements and are known to play a role in the dissemination of antibiotic resistance genes (8, 18). However, preliminary studies were unable to demonstrate transposition of an element containing bla\textsubscript{BIL-1}, indicating a transposition frequency of less than $10^{-9}$ for the putative bla\textsubscript{BIL-1}-containing element (6).

The \textit{ampC} gene of \textit{C. freundii} encodes an inducible \beta-lactamase. Induction is regulated by AmpR, a trans-acting protein encoded by the \textit{ampR} gene which lies immediately upstream of, and is transcribed in the direction opposite to that of, the \textit{ampC} gene (15). The \textit{ampR} gene has been sequenced, and the divergent \textit{ampC} and \textit{ampR} promoters have been shown to overlap (16). AmpR is a transcription activator, binding to a DNA region that is immediately upstream of the \textit{ampC} promoter and which overlaps the \textit{ampR} promoter (16). Sequences upstream of bla\textsubscript{BIL-1} homologous to sequences upstream of \textit{C. freundii} OS60 ampC extend for 117 bp and include both \textit{ampC} and \textit{ampR} promoter sequences. However, only 31 bp of the 38-bp region protected by the AmpR protein are present upstream of bla\textsubscript{BIL-1}, and there is no evidence for an ORF homologous to the \textit{C. freundii} ampR ORF. The absence of an ORF with homology to \textit{ampR} implies that bla\textsubscript{BIL-1} is not regulated in the same way as \textit{C. freundii ampC}. Indeed, biochemical evidence points towards the constitutive production of BIL-1 \beta-lactamase (24).

Only 150 nucleotides of bla\textsubscript{MIR-1} have been published (23); however, comparison of this limited sequence data reveals that BIL-1 and MIR-1 are related but distinct enzymes: bla\textsubscript{BIL-1} and bla\textsubscript{MIR-1} are 75% identical, but whereas bla\textsubscript{BIL-1} shows greatest homology to the \textit{ampC} genes of \textit{C. freundii}, bla\textsubscript{MIR-1} shows 90 to 91% identity to the \textit{ampC} genes of \textit{Enterobacter cloacae} (7). Until recently, these were the only proven examples of plasmid-mediated class C enzymes worldwide. However, two more cases have recently been reported (2, 28), both isolated from \textit{K. pneumoniae}. It is probable that more plasmid-mediated class C \beta-lactamases exist, albeit currently undetected. Whilst not a current clinical problem, the existence of these plasmid-mediated class C \beta-lactamases must be a prime consideration for the design of new \beta-lactam antibiotics and \beta-lactamase inhibitors.

The nucleotide sequence of the bla\textsubscript{BIL-1} gene has been deposited in the EMBL data library under accession number X74512.

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