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Cloning and Expression of a Cloxacillin-Hydrolyzing Enzyme and a Cephalosporinase from *Aeromonas sobria* AER 14M in *Escherichia coli*: Requirement for an *E. coli* Chromosomal Mutation for Efficient Expression of the Class D Enzyme

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Two β-lactamase genes, *asbA1* and *asbB1*, encoding AsbA1 and AsbB1, respectively, have been cloned from *Aeromonas sobria* AER 14M into *Escherichia coli*. AsbA1 was expressed at low but detectable levels in all *E. coli* laboratory cloning strains tested. AsbB1 was expressed well in the *E. coli* cloning strain DH5α. However, no enzyme activity could be detected from the same clone when placed in *E. coli* MC1061. Ampicillin-resistant mutants of *E. coli* MC1061 were obtained that expressed high levels of enzymatically active AsbB1. Four independent mutants were examined. All four mutations mapped to one locus, designated *blpA* (β-lactamase permissive). The *blpA* locus was distinct from other known loci that play a role in β-lactamase expression, i.e., the two loci that affect expression of the *Bacteroides fragilis* metallo-β-lactamase and the *ampC* regulatory genes, *ampD*, *ampE*, and *ampG*. Sequence analysis of *asbA1* and *asbB1* revealed that AsbA1 was a class C β-lactamase most closely related to the *Pseudomonas aeruginosa* chromosomal cephalosporinase and probably represents the common *A. sobria* cephalosporinase. AsbB1 was a class D enzyme most closely related to the oxacillin-hydrolyzing enzyme OXA-1, with 34% amino acid sequence identity. Purified AsbA1 was a typical cephalosporinase with a substrate profile that reflected high rates of hydrolysis of cephaloridine compared with benzylpenicillin. Purified AsbB1 showed strong penicillinase activity, with hydrolysis rates for carbenicillin and cloxacillin 2 to 2.5 times that for benzylpenicillin. Hydrolysis of imipenem was ≤1% of that for benzylpenicillin. Both clavulanic acid and tazobactam strongly inhibited AsbB1, while sulbactam inhibited the AsbB1 enzyme less effectively. None of the inhibitors worked well against the AsbA1 enzyme. The chelators EDTA and 1,10-*o*-phenanthroline did not affect the activity of either enzyme. *A. sobria* AER 14M was found to produce both a group 1 cephalosporinase and a novel group 2d cloxacillin-hydrolyzing β-lactamase that has been designated herein OXA-12.

The identification of *Aeromonas* species as causative agents in human disease processes has increased in recent years. In conjunction with this is an increased need to improve our understanding of antibiotic resistance among various *Aeromonas* species. β-Lactamase activity is always a serious concern when β-lactams are used as chemotherapeutic agents. In addition to a chromosomal β-lactamase found among many *Aeromonas* species, there are two reports of *Aeromonas* species that express a metallo-β-lactamase conferring imipenem resistance (2, 12, 23). *Aeromonas sobria* AER 14, a clinical isolate, was reported to express two distinct inducible β-lactamases (12). The *A. sobria* AER 14 A1 enzyme was reported to have a pI and hydrolytic properties similar to those of the A1 cephalosporinase of *Aeromonas hydrophila* AER 19. Similarly, the *A. sobria* AER 14 As2 enzyme was thought to be similar to the *A. hydrophila* AER 19 Ah2 metallo-β-lactamase and was thought to be responsible for the decreased imipenem sensitivity of *A. sobria* AER 14 (12). We sought to further characterize the *A. sobria* As2 enzyme and to determine if this enzyme was responsible for the decreased susceptibility to imipenem of *A. sobria* AER 14. Two β-lactamase genes were cloned from *A. sobria* AER 14M, a β-lactamase constitutive mutant of *A. sobria* AER 14 that encoded β-lactamases with pI values corresponding to those of the A1 and As2 enzymes. Genetic and biochemical characterization of the two β-lactamase genes and their gene products and expression of the β-lactamases in *Escherichia coli* are reported.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids are listed in Table 1. All of the cloning and β-lactamase expression experiments were performed with Luria broth or Luria broth agar (24), supplemented, when required, with kanamycin (25 μg/ml) and ampicillin (25 to 100 μg/ml).

Antibiotics and susceptibility testing. Antibiotics were supplied from their manufacturers and were freshly prepared each day. MICs were measured by serial twofold dilutions in Mueller-Hinton agar according to criteria established by the National Committee for Clinical Laboratory Standards (25).

Cloning of *asbA1* and *asbB1*. Restriction endonucleases, calf intestinal phosphatase, and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals or Bethesda Research Laboratories and were used according to the manufacturer's instructions. Basic molecular biology techniques were performed as described by Maniatis et al. (22).

Total DNA was prepared from *A. sobria* AER 14M (12) as previously described (29). The DNA was restricted with either *Bgl*II or *Eco*RI, ligated into appropriately restricted pCLL2300 (28), a cloning vector encoding kanamycin resistance, and used to transform *E. coli* DH5α. The transformed cells were plated

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic or genotype	Source or reference
Strains		
A. sobria		
AER 14	Clinical isolate producing β-lactamases with pIs of 6.7 and 8.6	Iaconis and Sanders (12)
AER 14M	*A. sobria* AER 14 constitutive for the expression of both β-lactamases	Iaconis and Sanders (12)
E. coli K-12		
DH5α	F⁻ *endA1 hsdR17*(r_k^- m_k^+) *supE44 thi-1* mutant *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169 Φ80d*lacZ*M15	Bethesda Research Laboratories
MC1061	F⁻ *araD139* Δ(*ara, leu*)*7697* Δ*lacX74 galU* mutant *galK* mutant *hsr* mutant *hsm*⁺ *strA*	Casadaban and Cohen (6)
Hfr mapping strains	Tet^r selection	Singer et al. (30)
Tn*10* fine mapping strains	Tet^r selection	Singer et al. (30)
BAR9113	MC1061 *iarA1* (class I IAR mutation) linked to *fadAB*::Tn*10*	Rasmussen et al. (29)
BAR9114	MC1061 *iarB1* (class II IAR mutation) linked to *fadR*::Tn*10*	Rasmussen et al. (29)
BAR9301	MC1061 *blpA1* pCLL2222	This study
BAR9302	MC1061 *blpA2* pCLL2222	This study
BAR9303	MC1061 *blpA3* pCLL2222	This study
BAR9304	MC1061 *blpA4* pCLL2222	This study
Plasmids		
pCLL2300	Cloning vector, Kan^r	Rasmussen et al. (28)
pCLL2221	pCLL2300 *asbA1* (15-kb *Eco*RI insert) encoding the β-lactamase AsbA1	This study
pCLL2222	pCLL2300 *asbB1* (3.6-kb *Bgl*II insert) encoding the β-lactamase AsbB1	This study

on kanamycin (25 μg/ml), and the resulting colonies were pooled. Dilutions of the pooled transformants were plated onto Luria broth agar containing ampicillin (50 or 100 μg/ml), and ampicillin-resistant isolates were identified. β-Lactamase-positive clones were identified by ampicillin resistance and/or a positive response with penicillinase assay filters (29).

Mapping of the *blp* mutations in *E. coli*. The seven tetracycline-resistant Hfr mapping strains of Singer et al. (30) were used in the initial localization of the *blpA* locus. The Hfr strains were crossed against MC1061(pCLL2222), selecting for the kanamycin resistance marker on pCLL2222 and the tetracycline resistance marker of the donor Hfr strain. Kanamycin- and tetracycline-resistant transconjugants were screened for acquisition of β-lactamase activity with penicillinase assay filters (29). The *blpA* locus was further localized within the chromosome by its linkage (cotransducibility) with 13 individual Tn*10* markers (30) spaced approximately 1 min apart on the chromosome and covering min 95 to 8. Bacteriophage P1*vir* lysates of each Tn*10* insertion strain were made and used to transduce the β-lactamase-expressing isolate BAR9301 (pCLL2222). Tetracycline-resistant transductants were screened for loss of β-lactamase activity with penicillinase assay filters.

DNA sequence analysis of *asbA1* and *asbB1*. The *asbA1* and *asbB1* genes were localized within their cloned restriction fragments by DNA restriction analysis and subcloning. The region of the clone corresponding to the β-lactamase gene was subcloned into pUC118 and/or pUC119 (31). Double-stranded DNA sequence analysis was performed with the Sequenase kit (U.S. Biochemical). The M13 −40 universal primer and other synthetic oligonucleotides complementary to determined DNA sequences were employed as primers. The complete DNA sequences of both strands were determined.

DNA and amino acid sequence analysis. Sequence analysis and homology searches were performed with the computer program DNA Star. The amino acid sequences of AsbA1 and AsbB1 were compared with those of other proteins in the Swiss Protein data bank (release 78) by using the Lipman and Pearson algorithm (20).

β-Lactamase purification. *E. coli* DH5α cells harboring either pCLL2221 or pCLL2222, expressing either AsbA1 or AsbB1, respectively, were grown in 2 liters of Trypticase soy broth. The cells were harvested and the enzymes extracted by four cycles of freeze-thaw lysis followed by centrifugation. The β-lactamases were purified from each of the resulting supernatants by anion-exchange column chromatography. AsbA1 was eluted from DEAE-Sephadex A-50 equilibrated with 10 mM phosphate buffer (pH 7.0). AsbB1 was eluted from QAE-Sephadex A-50 equilibrated with 25 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 8.0). After elution with the equilibrating buffer, fractions containing enzyme from each preparation were identified with nitrocefin and isoelectric focusing with an LKB Multiphor with PAGplates (pH range 3.5 to 9.5 [LKB]). The bicinchoninic acid assay (Pierce Biochemical) was used to determine the protein concentrations of the enzyme preparations.

Enzyme kinetic studies. Hydrolysis of β-lactams by the purified β-lactamase preparations was examined with a Gilford 250 spectrophotometer (34). The following wavelengths were used: benzylpenicillin, 240 nm; carbenicillin, 232 nm; oxacillin, 250 nm; cloxacillin, 260 nm; cephaloridine, 295 nm for AsbA1 and 260 nm for AsbB1; cephalothin, 260 nm; cefotaxime, 267 nm; and imipenem, 295 nm (34). Antibiotic solutions were freshly prepared in 50 mM phosphate buffer (pH 7.0). Kinetic parameters were derived from the initial velocity obtained with 6 to 8 substrate concentrations. K_m and V_{max} values were determined with the computer program ENZPACK (Biosoft), with results calculated according to five different plots: direct linear, Hanes-Woolf, Lineweaver-Burk, Eadie-Hofstee, and the method of Wilkinson. Enzyme inhibition was evaluated with nitrocefin as the substrate (50 μg/ml) (5). Inhibitor, at various concentrations, was preincubated with enzyme in a volume of 100 μl for 10 min at 25°C before the addition of 900 μl of substrate. The inhibitor concentration required to inhibit enzyme activity by 50% was derived from semilogarithmic plots of the percentage of control activity against the inhibitor concentration.

Nucleotide sequence accession number. The nucleotide se-

TABLE 2. Microbiological activity of various β-lactams and β-lactam-β-lactamase inhibitor combinations against *A. sobria* AER 14, *A. sobria* AER 14M, and *E. coli* DH5α harboring either vector plasmid (pCLL2300), the *asbA1* plasmid (pCLL2201), or the *asbB1* plasmid (pCLL2222)

Strain	MIC (μg/ml) of[a]:								
	PEN	AMP	PIP	PIP-TZB	TIM	CFZ	CAZ	IMI	OXA
A. sobria									
AER 14	>4	>32	>128	>128	64	>32	≤0.5	2	>4
AER 14M	>4	>32	>128	>128	>128	>32	>64	4	>4
E. coli									
DH5α(pCLL2300)	>4	2	1	0.5	≤1	≤2	≤0.5	≤0.25	>4
DH5α(pCLL2221) (*asbA1*)	>4	>32	16	8	128	>32	64	≤0.25	>4
DH5α(pCLL2222) (*asbB1*)	>4	>32	>128	>128	>128	4	≤0.5	≤0.25	>4

[a] Abbreviations: PEN, benzylpenicillin; AMP, ampicillin; PIP, piperacillin; PIP-TZB, piperacillin-tazobactam; TIM, timentin; CFZ, cefazolin; CAZ, ceftazidime; IMI, imipenem; OXA, oxacillin.

quence data reported here will appear in the GenBank nucleotide sequence database under accession number U10250 for the *asbA1* sequence and accession number U10251 for the *asbB1* sequence.

RESULTS

Cloning of the two β-lactamases from *A. sobria* AER 14M. Total DNA from *A. sobria* AER 14M was restricted with either *Eco*RI or *Bgl*II and ligated into pCLL2300 restricted with the corresponding enzyme. The ligation mixes were transformed into DH5α, and kanamycin-resistant transformants were selected. The kanamycin-resistant colonies were scraped from the plates, pooled, and stored frozen. For the *Eco*RI-restricted DNA, 1.5×10^4 to 2.0×10^4 transformed colonies were obtained. Screening of the plasmids harbored by the transformants indicated that >90% of the plasmids contained inserts with insert sizes of up to 20 kb. For the *Bgl*II-restricted DNA, 2×10^4 to 4×10^4 transformed colonies were obtained. More than 80% of the plasmids harbored by these transformants contained inserts with sizes of greater than or equal to 8 kb. Ampicillin-resistant isolates were selected by plating dilutions of the pooled transformants on ampicillin (50 and 100 μg/ml) with or without kanamycin (25 μg/ml).

For the *Eco*RI clone bank, detectable β-lactamase-positive colonies were identified on the 100-μg/ml plates and occurred at a frequency of less than 1 in 10^6. Eight ampicillin-resistant colonies were selected, and their plasmids were analyzed. Plasmids from all eight clones harbored a single 15-kb *Eco*RI fragment. One plasmid, pCLL2221, was selected for further analysis. For the *Bgl*II clone bank, numerous colonies were identified on plates containing 50 and 100 μg of ampicillin per ml. Plasmids from eight ampicillin-resistant clones from each ampicillin concentration were characterized. All 16 plasmids contained a *Bgl*II DNA fragment of approximately 3.6 kb, the same size as the vector. At least eight of the plasmids contained one or more additional *Bgl*II inserts. Five of the plasmids were selected for further characterization. Restriction analysis confirmed that all five plasmids contained a *Bgl*II insert of 3.6 kb. One of these plasmids, pCLL2222, containing only the 3.6-kb fragment, was selected for all further studies. Restriction analysis of pCLL2221 and pCLL2222 indicated that the DNA fragments harbored by the two plasmids were distinct and nonoverlapping. Isoelectric focusing of cell extracts from DH5α isolates harboring either pCLL2221 or pCLL2222 indicated that pCLL2221 encoded a β-lactamase with a pI of 6.7 and that pCLL2222 encoded a β-lactamase with a pI of 8.6. These pI values corresponded to those of the two β-lactamases expressed by *A. sobria* AER 14 and AER 14M. The β-lactamase gene harbored by pCLL2221 was designated *asbA1*, and its encoded β-lactamase was designated AsbA1, and that harbored by pCLL2222 was designated *asbB1*, and its encoded β-lactamase was designated AsbB1. The pI and other characteristics of the AsbA1 enzyme (described below) closely resembled those of the *A. hydrophila* and *A. sobria* 14 A1 β-lactamases (12), and so it has been designated AsbA1 to reflect its A1 heritage. However, although some of the characteristics of AsbB1 were similar to those of As2 (12), the two enzymes could not be precisely identified as being the same enzyme. As a result, the name AsbB1 was selected to avoid a possible confusion with enzyme As2.

Antibiotic susceptibility. Determination of the MICs (Table 2) for *A. sobria* AER 14 and AER 14M indicated that both isolates were resistant to all of the β-lactam antibiotics tested, with the exception of imipenem for both isolates and ceftazidime for AER 14. In general, AER 14M showed decreased susceptibility versus AER 14. Introduction of either *asbA1* or *asbB1* cloned β-lactamases into *E. coli* DH5α resulted in increased MICs of the majority of β-lactams tested for the *E. coli* isolate (Table 2). However, neither β-lactamase clone was able to confer resistance to imipenem, and for the *E. coli* isolate harboring *asbB1*, there was only modest or no increased resistance to ceftazidime and cefazolin.

Expression of AsbA1 and AsbB1 in *E. coli*. The β-lactamase encoded by *asbB1* was expressed well in DH5α cells; however, that encoded by *asbA1* was expressed poorly. Isolates exhibiting increased ampicillin resistance were easily obtained by plating an overnight culture on an increased ampicillin concentration of 100 μg/ml. Studies of the metallo-β-lactamase CcrA from *Bacteroides fragilis* have identified two loci, *iarA* and *iarB*, whose gene products affect the level of metallo-β-lactamase activity (29). To determine if the gene products of one or both of these loci also affected the expression of AsbA1 or AsbB1, pCLL2221 and pCLL2222 were transformed into MC1061, BAR9113, and BAR9114. The β-lactamase activity of pCLL2221-harboring cells remained low and poorly detectable in all three isolates. Surprisingly, AsbB1 activity could not be detected in any of the three strains harboring pCLL2222. However, if cultures of MC1061(pCLL2222) were grown overnight in the absence of ampicillin and an aliquot was then plated on ampicillin (100 μg/ml), β-lactamase-positive colonies appeared. Four independent MC1061(pCLL2222) ampicillin-resistant isolates (BAR9301 to BAR9304) were selected for further studies. Plasmids from each of the four ampicillin-resistant isolates were purified and transformed back into MC1061. The transformed cells were kanamycin resistant but were unable to grow on plates containing ampicillin (25

μg/ml), indicating that the ampicillin resistance was not linked to the plasmid but was due to mutation of the *E. coli* chromosome. The locus allowing for expression of enzymatically active AsbB1 has been designated *blp* for β-lactamase permissive.

Localization of the *blp* mutations on the *E. coli* chromosome. The location of the chromosomal mutations in *blp* was determined by Hfr mating followed by P1 cotransduction. Seven Hfr strains were used in the initial localization of *blp*. All seven Hfr isolates were determined to be permissive for expression of AsbB1. Therefore, to map the location of *blp*, it was assumed that there was only one permissive locus and that all seven Hfr isolates were mutated at this locus. The Hfr strains were crossed with the β-lactamase-negative MC1061(pCLL2222), and the frequency of β-lactamase-positive transconjugants was determined. From these matings, *blp* was localized to between 96 and 6 min on the *E. coli* chromosome (data not shown). Further localization was determined with a set of 13 Tn*10* insertions spaced approximately 1 min apart and spanning 95 to 8 min on the chromosome. All 13 of the Tn*10* mapping strains were not permissive for expression of AsbB1. Therefore, the P1 lysates made from these strains were used to transduce the AsbB1-expressing isolate BAR9301(pCLL2222), and the frequency of loss of β-lactamase activity was determined. The BAR9301 *blp* mutation *blpA1* was 75 to 90% cotransducible with the Tn*10* located at 0 min, from strain CAG18442, approximately 20% cotransducible with a Tn*10* located at 0.75 min, from strain CAG12093, and approximately 30% cotransducible with the Tn*10* located at 99.5 min, from strain CAG18403; this placed the *blpA* locus near 0 min. None of the loci that have been previously identified as β-lactamase expression regulatory genes mapped to this region of the *E. coli* chromosome (1, 15, 19, 29). The *blp* loci of all four independent AsbB1-expressing isolates (BAR9301 to BAR9304) showed similar cotransduction frequencies with the Tn*10*s described above. Therefore, all four β-lactamase-positive isolates appear to harbor a mutation in the same locus, which has been designated *blpA*.

DNA sequence determination and analysis of *asbA1*. The β-lactamase activity harbored on pCLL2221 was localized within the 15-kb *Eco*RI fragment. Restriction digest analysis indicated that a *Stu*I, a *Sac*I, and a *Bam*HI restriction site were each present within *asbA1*. Taking advantage of this, *asbA1* was subcloned into pUC119 (31) by using the *Bam*HI or *Sac*I restriction site to fix one end of the subclone and *Eco*RI to fix the other outlying end. The DNA sequence of *asbA1* was determined by analyzing the sequence from within the vector toward the *Bam*HI or *Sac*I restriction site directly into *asbA1* with the M13 −40 universal primer. After initial primary sequence data were acquired, synthetic primers complementary to the determined DNA sequences were used to complete the DNA sequence determination. Sequences of both strands of *asbA1* and the flanking DNA were determined (Fig. 1).

Analysis of the DNA sequence revealed an open reading frame of 1,143 nucleotides encoding a protein of 381 amino acids. Potential −10 and −35 sequences and a ribosomal binding site, determined on the basis of *E. coli* consensus sequences (9, 10), were identified upstream of the ATG initiation codon. In addition, a 30-nucleotide imperfect inverted repeat that overlapped the −10 and −35 sequences was identified. Within one arm of this inverted repeat was a nine-nucleotide direct repeat. Twenty-seven base pairs distal to the TGA termination codon was a 12-base perfect inverted repeat that could function in transcription termination (33). The full-length protein encoded by the open reading frame has a predicted molecular weight of 40,746. The amino-terminal

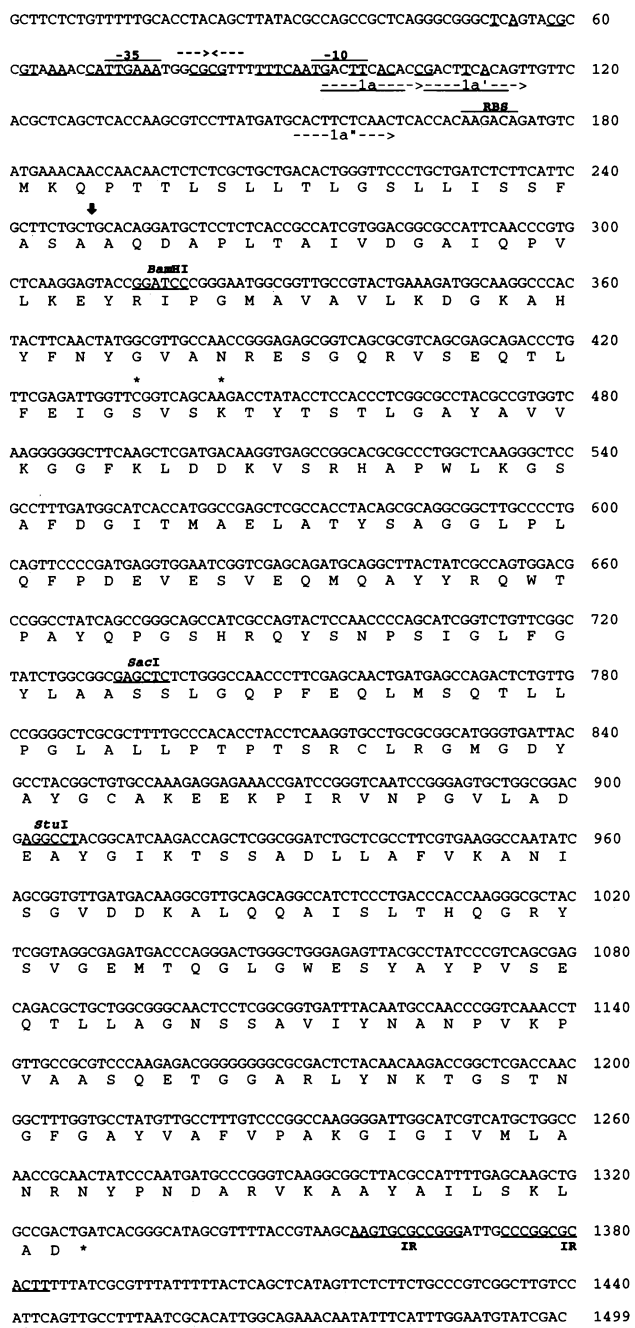

FIG. 1. DNA sequence and the deduced amino acid sequence of the *A. sobria asbA1* β-lactamase gene. Potential −35 and −10 sequences and a ribosomal binding site (RBS) (on the basis of *E. coli* consensus sequences [9, 10]) are indicated. The bold arrow indicates the potential signal sequence cleavage site. Also indicated are the matching bases within the 30-base imperfect inverted repeat (underlined) and a 9-base perfect direct repeat within the promoter region of the gene (solid line below the DNA sequence). A 12-base perfect inverted repeat (IR) 27 bases distal to the termination codon is underlined. The conserved Ser and Lys of the Ser-X-X-Lys consensus sequence are indicated with asterisks. The *Bam*HI, *Sac*I, and *Stu*I restriction enzyme cleavage sites are underlined. DNA consensus sequences identified within both the *asbA1* promoter (1a, 1a′, and 1a″) and the *asbB1* promoter are indicated with dashed arrows below the DNA sequence.

amino acid sequence has the characteristics of a signal sequence. On the basis of the consensus for signal sequence cleavage sites (32), the signal sequence was predicted to be 23 amino acids in length and was cleaved following the sequence Ala-Ser-Ala. Amino acids 85 to 88 (Ser-Val-Ser-Lys) correspond to the Ser-X-X-Lys motif characteristic of the active site serine sequence common to the majority of β-lactamases (14).

For many chromosomally encoded cephalosporinases, regulation is at the level of transcription and involves a dually active repressor/activator protein, AmpR (16–18). AmpR can bind to the β-lactamase promoter to either enhance or inhibit transcription (16–18). The *ampR* gene and β-lactamase gene usually form a divergent operon, with the two genes sharing a common promoter region but encoded on opposite strands of the DNA (16, 18, 21). In the case of *asbA1*, no *ampR* gene was identified upstream within 400 nucleotides of the initiation codon. No extended open reading frames were identified within this 400-nucleotide sequence (data not shown). In addition, a search of the amino acid sequences of all three upstream reading frames divergent from the *asbA1* coding sequence revealed no amino acid sequences that shared identity with any AmpR protein sequences present in the Swiss Protein database (release 78). Thus, it has yet to be established if a regulatory AmpR-like protein gene lies upstream of *asbA1* or if an AmpR-like protein exists for *asbA1* but is not adjacent to the *asbA1* gene.

Amino acid identity between AsbA1 and other β-lactamases. The predicted amino acid sequence of AsbA1 was analyzed for homology to other known proteins. AsbA1 was found to be most closely related to the chromosomal AmpC cephalosporinase of *Pseudomonas aeruginosa* (21). The two proteins shared 53.6% identity (Fig. 2). On the basis of this homology, AsbA1 appeared to be a class C β-lactamase (13).

DNA sequence determination and analysis of *asbB1*. An approach similar to that used to determine the sequence of *asbA1* was used to determine the sequence of *asbB1*. The *asbB1* gene was localized within the 3.6-kb *Bgl*II fragment. A *Pst*I site was identified as being within or very near *asbB1*. DNA from each side of the *Pst*I site was cloned into pUC118, and the DNA sequence was determined by analyzing the sequence in from the *Pst*I ends with the M13 −40 universal primer and synthetic primers complementary to the determined DNA sequence. The sequences of both strands of *asbB1* and the flanking DNA were determined (Fig. 3). The sequence of the *Pst*I junction in the original 3.6-kb clone was also determined to confirm the presence of only one *Pst*I site in that region of the gene. Within the DNA sequence was a large open reading frame encoding a protein of 264 amino acids with a calculated molecular weight of 28,600. Potential −10 and −35 sequences were identified upstream of the ATG start codon. However, the nucleotide spacing between these sequences was not optimal (20 nucleotides not 17) according to *E. coli* consensus sequences (10). A potential ribosomal binding site, determined on the basis of *E. coli* consensus sequences (9), was also identified. Sixty-three nucleotides distal to the TAG termination codon was a nine-base inverted repeat that could function in transcription termination (33). Analysis of the predicted amino acid sequence for the opening reading frame showed an amino-terminal sequence characteristic of a signal sequence. On the basis of the consensus for signal sequence cleavage sites (32), the signal sequence was predicted to be 22 amino acids in length and cleaved following the sequence Ala-Ser-Ala. Amino acids 51 to 54 (Ser-Thr-Phe-Lys) correspond to the active site Ser-X-X-Lys motif common to many serine β-lactamases (14).

No strong identity was found between the promoter regions

```
                                  ↓
AsbA1    MKQPTTLSLLTLGSLLISSFASAAQDAP   LTAIVDGAIQPVLKEYR
          .:  . L  :: ::LL::: :: A :AP   L.A:VD:A:QPV:K.
AmpC  MRDTRFPCLCGIAASTLLFATTPAIAGEAPADRLKALVDAAVQPVMKAND

                                                 *  *
AsbA1  IPGMAVAVLKDGKAHYFNYGVANRESGQRVSEQTLFEIGSVSKTYTSTLG
       IPG:AVA:  .G.:HYF:YG:A::E:G:RV: :TLFEIGSVSKT:T:TL:
AmpC   IPGLAVAISLKGEPHYFSYGLASKENGRRVTPETLFEIGSVSKTFTATLA

AsbA1  AYAVVKGGFKLDDKVSRHAPWLKGSAFDGITMAELATYSAGGLPLQFPDE
       :YA:.:: .:LDD:.S:H P L:GS FDGI:: :LATY:AGGLPLQFPD.
AmpC   GYALTQDKMRLDDRASQHWPALQGSRFDGISLLDLATYTAGGLPLQFPDS

AsbA1  VESVE QMQAYYRQWTPAYQPGSHRQYSNPSIGLFGYLAASSLGQPFEQL
       V:. : Q::.YYRQW P:Y.PGS:R YSNPSIGLFGYLAA.SLGQPFE:L
AmpC   VQKDQAQIRDYYRQWQPTYAPGSQRLYSNPSIGLFGYLAARSLGQPFERL

AsbA1  MSQTLLPGLALLPTPTSRCLRGMGDYAYGCAKEEKPIRVNPGVLADEAYG
       M.Q ::P:L:L .T. .    ::::YA G.:K:::P:RV.PG L..E YG
AmpC   MEQQVFPALGLEQTHLDVPEAALAQYAQGYGKDDRPLRVGPGPLDAEGYG

AsbA1  IKTSSADLLAFVKANISGV DDKALQQAISLTHQGRYSVGEMTQGLGWES
       :KTS:ADLL FV.AN:     D:: .QA:. TH:G Y.VG:MTQGLGWE:
AmpC   VKTSAADLLRFVDANLHPERCDRPWAQALDATHRGYYKVGDMTQGLGWEA

AsbA1  YAYPVSEQTLLAGNSSAVIYNANPVKPVAASQETGGARLYNKTGSTNGFG
       Y..P:S : L AGNS:::  ::::.: .::A:Q. .G.RL NKTGSTNGFG
AmpC   YDWPISLKRLQAGNSTPMALQPHRIARLPAPQALEGQRLLNKTGSTNGFG

AsbA1  AYVAFVPAKGIGIVMLANRNYPNDARVKAAYAILSKLAD
       AYVAFVP: ::G:V:LANRNYPN..RVK AYAILS L.:
AmpC   AYVAFVPGRDLGLVLLANRNYPNAERVKIAYAILSGLEQQGKVPLKA
```

FIG. 2. Comparison of the amino acid sequences of AsbA1 and the *P. aeruginosa* AmpC chromosomal cephalosporinase (21). The conserved Ser and Lys of the Ser-X-X-Lys consensus sequence are indicated with asterisks. The proposed signal sequence cleavage site for AsbA1 is indicated with a bold arrow. Amino acids that are positively related (conservative amino acid substitutions) are shown with a colon on the line between the amino acids, those with a zero value relationship (semiconservative amino acid substitution) are shown with a period, and those that are negatively related (nonconservative amino acid substitution) are blank.

of *asbA1* and *asbB1*. However, several short regions of similarity were identified. The consensus sequence C/G ACTTC/A A/T CAN C/T appeared three times within the *asbA1* promoter; the first and second repeats (1a and 1a′) were adjacent. Thirty-seven base pairs separated the third repeat (1a″) from the second (1a′). Within the promoter of *asbB1*, the consensus sequence was repeated twice (2a and 2a′). Interestingly, these two sequences were separated by 36 bp. The significance of these sequence similarities between the two promoter regions is unknown.

Amino acid identity between AsbB1 and other β-lactamases. A comparison of amino acid sequences between AsbB1 and other proteins indicated that AsbB1 shared the greatest percentage of identity with OXA-1 (27), an oxacillin-hydrolyzing β-lactamase (Fig. 4). These two enzymes were 34% identical in amino acid sequence. One divergence in sequence between AsbB1 and OXA-1 was in the sequence preceding the active serine, Asp in OXA-1 and Ala in AsbB1, the same as in all other class D enzymes (7). Thus, from amino acid sequence information, AsbB1 would be classified as a class D enzyme (11).

Enzymatic studies of AsbA1 and AsbB1. AsbA1 was an unstable β-lactamase after purification, with a low specific activity (V_{max} of 0.10 μmol of cephaloridine hydrolyzed per min/mg of protein). AsbA1 showed a hydrolytic profile similar to that expected for a class C molecular cephalosporinase (Table 3). The enzyme hydrolyzed cephalothin and cephaloridine with a V_{max} two- to threefold faster than that of benzylpenicillin. This enzyme also showed slow, but detectable, hydrolytic activity against cefotaxime and imipenem. The K_m value for cephalothin was much lower than those for cephalo-

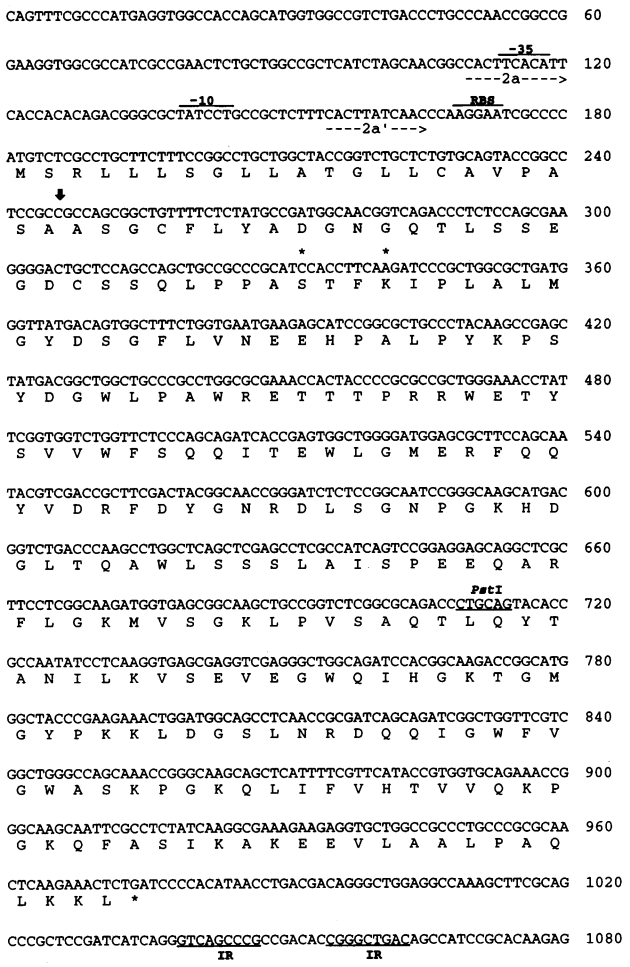

FIG. 3. DNA sequence and the deduced amino acid sequence of the *A. sobria asbB1* β-lactamase gene. Potential −35 and −10 sequences and a ribosomal binding site (RBS) (on the basis of *E. coli* consensus sequences [9, 10]) are indicated. The bold arrow indicates the potential signal sequence cleavage site. Also indicated is a 9-base perfect inverted repeat (IR) 63 bases distal to the termination codon. The conserved Ser and Lys of the Ser-X-X-Lys consensus sequence are indicated with asterisks. The *Pst*I restriction enzyme cleavage site is underlined. DNA consensus sequences identified within both the *asbA1* promoter and *asbB1* promoter (2a and 2a', respectively) are indicated with dashed arrows below the DNA sequence.

ridine and benzylpenicillin, leading to the higher catalytic efficiency, or V_{max}/K_m. It should be noted that the K_m for benzylpenicillin was much higher than that seen for other AmpC cephalosporinases (8, 12). These hydrolysis parameters are consistent with the functional classification of AsbA1 as a group 1 cephalosporinase (3).

AsbB1, with a molecular sequence similar to that of a class D β-lactamase, showed strong penicillin-hydrolyzing activity (V_{max} of 7.0 μmol of benzylpenicillin per min/mg of protein), with very slow hydrolysis rates seen for cephaloridine and ceftazidime. AsbB1 hydrolyzed carbenicillin and cloxacillin much faster than benzylpenicillin, although with lower affinity (higher K_m values). Oxacillin was hydrolyzed the most efficiently (high V_{max}, low K_m) of all the substrates tested. No detectable hydrolysis rate was observed for imipenem. On the basis of the hydrolysis profile, AsbB1 is classified as a group 2d cloxacillin-hydrolyzing β-lactamase (4).

TABLE 3. Kinetic parameters of the AsbA1 and AsbB1 β-lactamases from *A. sobria* AER 14M

Enzyme	Substrate	Relative V_{max}	K_m (μM)	Relative V_{max}/K_m
AsbA1	Benzylpenicillin	100	120	100
	Cephaloridine	310	340	110
	Cephalothin	260	25	1,220
	Cefotaxime	≤1	ND[a]	ND
	Imipenem	≤3	ND	ND
AsbB1	Benzylpenicillin	100	13	100
	Carbenicillin	160	43	48
	Oxacillin	210	6.8	400
	Cloxacillin	190	480	5.1
	Cephaloridine	14	ND	ND
	Ceftazidime	≤2	ND	ND
	Imipenem	≤1	ND	ND

[a] ND, not determined, because rates were too slow to obtain an accurate K_m value.

Inhibition of β-lactamase activity. Fifty percent inhibitory concentration values for clavulanic acid, sulbactam, and tazobactam for inhibition of AsbA1 and AsbB1 are given in Table 4. From the 50% inhibitory concentration values, it is evident that sulbactam had stronger activity against AsbA1 than tazobactam and clavulanic acid, although none of the inhibitors was highly effective at inhibiting this enzyme. This pattern resembles that of the class C S2 enzyme. Both clavulanic acid and tazobactam strongly inhibited AsbB1. Sulbactam also inhibited AsbB1, but to a lesser extent. This inhibition profile was more closely related to that of the extended-spectrum class A β-lactamases than to that of the class D oxacillin-hydrolyzing enzymes. As expected, by amino acid sequence similarities, AsbA1 and AsbB1 are not class B metallo-β-lactamases and thus were not inhibited by the chelators EDTA and 1,10-*o*-phenanthroline when tested at a concentration of 50 μM.

TABLE 4. Inhibition of AsbA1 and AsbB1 by β-lactamase inhibitors compared with that of selected β-lactamases

Enzyme	Bush group[a]	IC_{50}[b] (nM) of:		
		Clavulanate	Sulbactam	Tazobactam
Class C				
P99[c]	1	>100,000	5,600	8.5
S2[c]	1	51,000	5,200	6,000
AsbA1	1	42,000	1,600	15,000
Class A				
TEM-1[d]	2b	30	80	27
TEM-3[d]	2b'	11	21	5.0
TEM-9[d]	2b'	9.0	270	77
Class D				
OXA-1[e]	2d	1,760	4,720	1,430
PSE-2[e]	2d	810	37,000	940
AsbB1	2d	9.0	240	30

[a] Reference 3.
[b] IC_{50}, 50% inhibitory concentration.
[c] Reference 5.
[d] Reference 26.
[e] Reference 4.

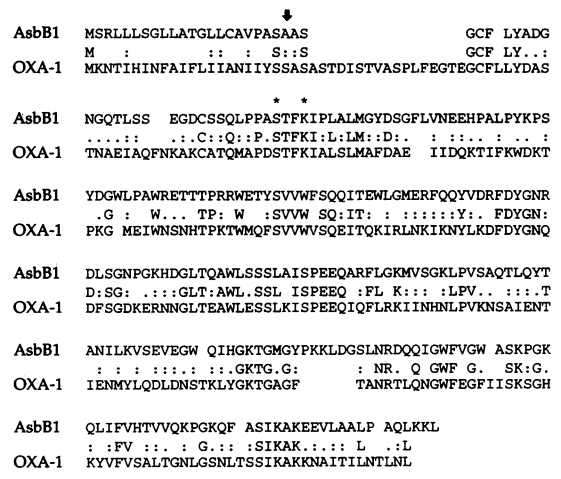

FIG. 4. Comparison of the amino acid sequences of AsbB1 and the oxacillin-hydrolyzing enzyme OXA-1 (27). The conserved Ser and Lys of the Ser-X-X-Lys consensus sequence are indicated with asterisks. The proposed signal sequence cleavage site for AsbB1 is indicated with a bold arrow. Amino acids that are positively related (conservative amino acid substitutions) are shown with a colon on the line between the amino acids, those with a zero value relationship (semiconservative amino acid substitution) are shown with a period, and those that are negatively related (nonconservative amino acid substitution) are blank.

DISCUSSION

Two β-lactamases have been cloned from *A. sobria* AER 14M. Both molecular evidence and biochemical evidence confirm that the first β-lactamase, AsbA1 (encoded by *asbA1*), is a typical group 1 cephalosporinase. The second β-lactamase, AsbB1 (encoded by *asbB1*), is not a metallo-β-lactamase but is a group 2d cloxacillin-hydrolyzing enzyme. It is proposed that AsbB1 be designated OXA-12 on the basis of both its biochemical characteristics and its genetic characteristics. The original *A. sobria* AER 14 and *A. sobria* AER 14M isolates were believed to harbor a metallo-β-lactamase with a pI value similar to that of the AsbB1 enzyme (12). When strains *A. sobria* AER 14 and *A. sobria* AER 14M were initially tested with imipenem, MICs of 2 and 16 μg/ml, respectively, were reported (12). However, the *A. sobria* isolates used in this study differed only twofold in their microbiological responses to imipenem and yielded no metallo-β-lactamase clones. It is quite possible that a metallo-β-lactamase gene was lost upon storage of the isolate. It is also possible that the initial characterization, which relied heavily on the physical similarity of the As2 enzyme to the *A. hydrophila* Ah2 metallo-β-lactamase, was not carried out in sufficient detail.

Previous studies of the production of AsbA1 and AsbB1 in *A. sobria* AER 14 indicate that both β-lactamases are inducible (12). In addition, isolates of *A. sobria* AER 14 constitutive for the production of one of the two β-lactamases are also constitutive for the expression of the second β-lactamase (12). Thus, it appears that the mechanisms of induction for the two β-lactamases share a common component. If the coordinate constitutive characteristic of the two β-lactamases is mediated through an AmpR-like regulatory protein, then one would expect to find common nucleotide sequences within the promoter regions of the two β-lactamase genes. Analysis of the two *asb* gene promoters revealed some homology between them; an 11-bp semiconserved sequence was repeated within both promoters. The repeats were separated by 37 and 36 bp in the two promoters. Whether these DNA sequences participate in the regulation of either *asbA1* or *asbB1* has yet to be determined.

Expression of *asbA1* and *asbB1* in *E. coli* was strikingly different. Expression of *asbA1* was poor but detectable in all laboratory *E. coli* strains tested. In contrast, *asbB1* was either well expressed or not detectably expressed, depending on the individual laboratory strain. High-level expression of *asbB1* was associated with mutation of one specific locus, designated *blpA*, which mapped near 0 min on the *E. coli* chromosome. None of the *E. coli* chromosomal genes known to affect β-lactamase expression in *E. coli*, *ampD*, *ampE*, *ampG*, or the class I and class II IAR mutations (15–19, 29) mapped to this region of the chromosome. The function of the *blpA* locus in *asbB1* expression and its effect on the expression of other β-lactamase genes have not yet been elucidated. Since *asbA1* was expressed equivalently in all of our *E. coli* strains, the *blpA* gene product does not appear to affect its expression in *E. coli*. The observation that *asbA1* and *asbB1* had different patterns of expression in *E. coli* demonstrates that their mechanisms of regulation may not be identical. The coordinate constitutive expression seen in *A. sobria* indicates that they may share a common component in their regulation. However, the *E. coli* findings suggest that there may be additional layers of regulation or some components in the regulatory cascade of the two genes that are not shared.

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