Stenotrophomonas maltophilia from Nepal Producing Two Novel Antibiotic Inactivating Enzymes, a Class A $\beta$-Lactamase KBL-1 and an Aminoglycoside 6'-N-Acetyltransferase AAC(6’)-Iap

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ABSTRACT Seven drug-resistant strains of Stenotrophomonas maltophilia were isolated from patients at two university hospitals in Nepal. S. maltophilia JUNP497 was found to encode a novel class A $\beta$-lactamase, KBL-1 (Kathmandu $\beta$-lactamase), consisting of 286 amino acids with 52.98% identity to PSV-1. Escherichia coli transformants expressing bla\textsubscript{KBL-1} were less susceptible to penicillins. The recombinant KBL-1 protein efficiently hydrolyzed penicillins. The genomic environment surrounding bla\textsubscript{KBL-1} was a unique structure, with the upstream region derived from strains in China and the downstream region from strains in India. S. maltophilia JUNP350 was found to encode a novel 6’-N-aminoglycoside acetyltransferase, AAC(6’)-Iap, consisting of 155 amino acids with 85.0% identity to AAC(6’)-Iz. E. coli transformants expressing aac(6’)-Iap were less susceptible to arbekacin, amikacin, dibekacin, isepamicin, neomycin, netilmicin, sisomicin and tobramycin. The recombinant AAC(6’)-Iap protein acetylated all aminoglycosides tested, except for apramycin and paromomycin. The genomic environment surrounding aac(6’)-Iap was 90.99% identical to that of S. maltophilia JV3 obtained from a rhizosphere in Brazil. Phylogenetic analysis based on whole genome sequences showed that most S. maltophilia isolates in Nepal were similar to those isolates in European countries, including Germany and Spain.

IMPORTANCE The emergence of drug-resistant S. maltophilia has become a serious problem in medical settings worldwide. The present study demonstrated that drug-resistant S. maltophilia strains in Nepal harbored novel genes encoding a class A $\beta$-lactamase, KBL-1, or a 6’-N-aminoglycoside acetyltransferase, AAC(6’)-Iap. Genetic backgrounds of most S. maltophilia strains in Nepal were similar to those in European countries. Surveillance of drug-resistant S. maltophilia in medical settings in Nepal is necessary.

KEYWORDS Stenotrophomonas maltophilia, drug resistance mechanisms, KBL-1, AAC(6’)-Iap

S. maltophilia is a globally emergent, multidrug-resistant Gram-negative pathogen frequently associated with respiratory tract and bloodstream infections in immunocompromised patients (1). Between 1997 and 2016, a total of 6,467 S. maltophilia isolates were reported from 259 medical settings in 43 countries worldwide, including Asia-Pacific, Latin America, Europe, and North America. These isolates were obtained from hospitalized patients with pneumonia (55.8%), bloodstream infections (33.8%), skin infections (7.8%), urinary tract infections (1.2%), and intra-abdominal infections (1.0%) (2).

The chromosome of S. maltophilia intrinsically harbors two genes, bla\textsubscript{L1} and bla\textsubscript{L2}, that encode the two $\beta$-lactamases, L1 and L2, respectively. L1 is a broad-spectrum
metallo-β-lactamase that hydrolyzes carbapenems, whereas L2 is a serine β-lactamase that hydrolyzes cephalosporins (3). *S. maltophilia* also harbors two sets of genes associated with intrinsic multidrug resistance, including genes encoding lytic transglycosylases (MltA, MltB1, MltB2, MltD1, MltD2, and Stl) and genes encoding an efflux system (SmeD, SmeE, and SmeF); however, these factors do not affect susceptibility to aminoglycosides, such as amikacin, gentamicin, kanamycin, streptomycin, and tobramycin (4, 5). On the other hand, evaluation of aminoglycoside resistance due to modification enzymes showed that *S. maltophilia* is likely to intrinsically harbor a gene, *aph* (3′)-IIc, encoding an aminoglycoside phosphotransferase enzyme that significantly decreases bacterial susceptibility to kanamycin, neomycin, butirosin, and paromomycin (6), and that most *S. maltophilia* isolates harbor a gene, *aac(6′)-Iz*, encoding an aminoglycoside acetyltransferase that reduces susceptibility to amikacin, netilmicin, and tobramycin (7, 8). *S. maltophilia* isolates also harbor two genes, *aac(6′)-Iam* and *aac(6′)-Iap*, closely related to *aac(6′)-Iz* (9, 10).

The present study describes two clinical isolates of *S. maltophilia* obtained from hospitalized patients in Nepal, one harboring a gene encoding a novel class A β-lactamase, KBL-1, and the other harboring a gene encoding a novel 6′-N-aminoglycoside acetyltransferase, AAC(6′)-Iap.

### RESULTS AND DISCUSSION

**Drug susceptibilities of *S. maltophilia* isolates.** Of the seven *S. maltophilia* isolates, five were resistant to ceftazidime, three were resistant to ticarcillin-clavulanic acid, and one each was resistant to chloramphenicol, levofloxacin, and sulfamethoxazole-trimethoprim (Table 1). All seven isolates had MICs of $\leq 64 \, \mu g/mL$ for imipenem, meropenem, and colistin, and six each had MICs of $\geq 128 \, \mu g/mL$ for aztreonam, arbekacin, and amikacin.

**Drug resistance genes.** Assessment of β-lactamase encoding genes showed that all seven isolates harbored *bla*$_{A}$ and *bla*$_{B}$, genes intrinsic to *S. maltophilia* (3). In addition, one isolate, JUNP497, harbored two other genes encoding β-lactamases, *bla*$_{PME-1}$ and *bla*$_{KBL-1}$, a gene encoding a novel class A β-lactamase. Evaluation of genes encoding aminoglycoside modifying enzymes showed that all seven isolates harbored genes encoding APH(3′), including *aph*(3′)-IIc and *aph*(3′)-IV. In addition, four isolates harbored genes encoding AAC(6′), such as *aac(6′)-Iz*, *aac(6′)-Iak* and *aac(6′)-lap*, a novel gene encoding an aminoglycoside acetyltransferase.

**A novel class A β-lactamase KBL-1.** The novel class A β-lactamase KBL-1 consisted of 286 amino acids. A comparison of its sequence to the amino acid sequences of 10 representative class A β-lactamases showed that KBL-1 were closest to PSV-1, with 52.98% sequence identity (Fig. 1). PSV-1 had previously been identified in *Pseudovibrio ascidiaeicola*, obtained from a species of sponge, *Aplysina aerophoba*, in Spain (11). Compared with the vector control, *E. coli* expressing *bla*$_{KBL-1}$ showed much higher MIC values (256 to 4,096 $\mu g/mL$) toward the penicillins, including ampicillin, amoxicillin,
penicillin G, and piperacillin, with the MICs toward these penicillins being 2,048, 256, 128, and 512-fold higher, respectively, for \(E. coli\) expressing \(\text{bla}_{\text{KBL-1}}\) than for the vector control (Table 2). The MICs of these penicillins were significantly reduced by \(\beta\)-lactamase inhibitors combined with penicillins, including amoxicillin-clavulanic acid, ampicillin-sulbactam, and piperacillin-tazobactam, which had MICs of 32 to 128 \(\mu\)g/mL. The \(E. coli\) expressing \(\text{bla}_{\text{KBL-1}}\) showed lower MICs for the monobactam aztreonam; the cephalosporins cefepime, cefotaxime, cefoxitin, ceftazidime, cefozopran, cephradine, and moxalactam; and the carbapenems doripenem, imipenem, meropenem, and panipenem. Moreover, except for ceftazidime, there were no significant differences in the MICs of \(E. coli\) expressing \(\text{bla}_{\text{KBL-1}}\) and the vector control for any of these agents. The MIC of ceftazidime for \(E. coli\) expressing \(\text{bla}_{\text{KBL-1}}\) was low (1 \(\mu\)g/mL), but significantly higher than that for the vector control (0.125 \(\mu\)g/mL), suggesting that measurement conditions, such as salinity, temperature, and pH, may affect the hydrolysis of ceftazidime.

Recombinant KBL-1 protein had hydrolytic activities against all the \(\beta\)-lactams tested, except for aztreonam (Table 3). Recombinant KBL-1 efficiently hydrolyzed the penicillins, including ampicillin, amoxicillin, penicillin G, and piperacillin with \(k_{\text{cat}}/k_{m}\) values of 0.422

### Table 2 MICs of \(\beta\)-lactam for \(S. maltophilia\) JUNP497 and \(E. coli\) strains transformed with \(\text{bla}_{\text{KBL-1}}\)

| \(\beta\)-lactams     | MIC(\(\mu\)g/mL) | \(E. coli\) DH5\(\alpha\)/pHSG398-\(\text{bla}_{\text{KBL-1}}\) | \(E. coli\) DH5\(\alpha\)/pHSG398 | JUNP497 |
|---------------------|------------------|-------------------------------------------------|---------------------------------|--------|
| Ampicillin          | 4.096            | 2                                               | 128                             |        |
| Amoxicillin         | 1.024            | 4                                               | 256                             |        |
| Aztreonam           | 0.063            | 0.063                                           | >512                            |        |
| Penicillin G        | 2.048            | 16                                              | 128                             |        |
| Cephradine          | 8                | 8                                               | 128                             |        |
| Cefoxitin           | 4                | 4                                               | 16                              |        |
| Ceftazidime         | 1                | 0.125                                           | 64                              |        |
| Cefotaxime          | 0.031            | 0.031                                           | 128                             |        |
| Cefepime            | 0.016            | 0.016                                           | 32                              |        |
| Cefozopran          | 0.125            | 0.063                                           | 128                             |        |
| Imipenem            | 0.125            | 0.125                                           | 256                             |        |
| Meropenem           | 0.031            | 0.031                                           | 32                              |        |
| Piperacillin         | 256              | 0.5                                             | 64                              |        |
| Moxalactam          | 0.25             | 0.25                                            | 1                               |        |
| Clavulanic acid/amoxicillin | 32           | 8                                               | 128                             |        |
| Sulbactam/ampicillin| 128              | 2                                               | 128                             |        |
| Tazobactam/piperacillin | 64         | 0.5                                             | 32                              |        |
| Panipenem           | 0.125            | 0.125                                           | 256                             |        |
| Doripenem           | 0.031            | 0.031                                           | 128                             |        |
to 1.166, whereas it slightly hydrolyzed cephalosporins and carbapenems with $k_{cat}/k_{on}$ values of 0.001 to 0.044. IC$_{50}$ determinations performed with penicillin G as a substrate showed that KBL-1 activity was very well inhibited by 0.21 $\mu$M clavulanic acid and 1.2 $\mu$M sulbactam.

**A novel 6'-N-aminoglycoside acetyltransferase AAC(6')-lap.** The AAC(6')-lap protein was found to consist of 155 amino acids. Multiple sequence alignments among AAC(6') enzymes revealed that AAC(6')-lap was 85.0% identical to AAC(6')-Iz (7), 83.0% identical to AAC(6')-lam (9), and 79.1% identical to AAC(6')-lak (10) (Fig. 2). Compared with vector control, *E. coli* expressing AAC(6')-lap showed decreased susceptibilities to arbekacin, amikacin, dibekacin, isepamicin, neomycin, netilmicin, sisomicin, and tobramycin (Table 4). Thin-layer chromatography (TLC) analysis revealed that all the aminoglycosides tested, except for apramycin and paromomycin, were acetylated by AAC(6')-lap (Fig. 3). These results indicated that $aac(6')$-lap is a functional acetyltransferase that modifies the 6'-NH$_2$ position of aminoglycosides and is involved in aminoglycoside resistance. The TLC data for apramycin and paromomycin were consistent with the MICs of the aminoglycosides for *E. coli* with pSTV28-$aac(6')$-lap. Although gentamicin and kanamycin were acetylated by AAC(6')-lap, the MICs were not higher than those of *E. coli* harboring pSTV28-$aac(6')$-lap. Gentamicin includes gentamicins C1, C2, and C1a, with gentamicin C1 having no amino group at the 6'-position, suggesting that gentamicin may only have been partially acetylated by AAC(6')-lap.

**Genomic environments surrounding bla$_{KBL-1}$ and $aac(6')$-lap.** The genomic environment surrounding $bla_{KBL-1}$ was a unique structure, consisting of orfA-orfB-15S1-mstE-istB-bla$_{KBL-1}$-15S1-orfC-orfD-orfE (Fig. 4A). The $bla_{KBL-1}$-surrounding region, mstE-istB-bla$_{KBL-1}$, was flanked by 15S1. BLAST analysis did not identify any similar structure in GenBank, suggesting that this structure may be unique.

The genomic environment surrounding $aac(6')$-lap consisted of hemE-orfF-arob-aroK-orfG-orfH-pdxH+$aac(6')$-lap-prpR-prpB-prpC-acnD, which was 90.99% (nucleotides [nt] 338404 to 3374930; GenBank accession no. CP002986) identical to a strain of *S. maltophilia* JV3 obtained from the rhizosphere in Brazil and 90.17% (nt 3279480 to 3286016; GenBank accession no. CP050452) identical to a strain of *S. maltophilia* SoD9b obtained in the Collins Glacier beach area in Antarctica (12). The upstream (hemE-orfF-arob-aroK-orfG-orfH-pdxH) and downstream (prpR-prpB-prpC-acnD) regions of not $aac(6')$-lap were identical to those in *S. maltophilia* K279a harboring $aac(6')$-lam (9), IOMTU250 harboring $aac(6')$-lak (10), and ATCC13637 harboring $aac(6')$-lz (7) and JV3 (accession no. CP002986) (Fig. 4B). These results suggested that the genetic structures surrounding $aac(6')$-lap are widely conserved among *S. maltophilia* samples obtained in various countries.
BLAST analysis revealed that the genetic structure surrounding \textit{aac(6'-)Iap}, \textit{pdsX-aac(6')-Iap-prpR}, was identical to the structures in environmentally arising \textit{S. maltophilia} strains. Most of these strains had been obtained from environmental sources, including a laboratory sink, river water, soil, and wastewater (Table S1). In contrast, the genetic structures surrounding \textit{aac(6')-Iam/-Iak/-Iz}, \textit{pdxH-aac(6')-Iam/-Iak/-Iz-orfI-orfJ-orfK} were identical to those in \textit{S. maltophilia} strains obtained from medical settings. The genomic structure surrounding \textit{aac(6')-Iap} seems to be new, combining structures detected in medical settings and in environmental sources.

\textbf{Phylogenetic analysis of \textit{S. maltophilia} in Nepal.} Phylogenetic analysis based on whole genome sequences revealed that \textit{S. maltophilia} can be divided into three clades,
designated A, B, C and D (Fig. 5). Clade A consisted of the isolates obtained in Nepal in 2019 (JUNP349 and JUNP350), in China in 2019, in Germany in 2018, and in the United States in 2012 and 2013. The SNPs between JUNP349 and JUNP350 were 157. Clade B consisted of the isolates obtained in the Philippines in 1991 and the unknown strain R551-3. Clade D consisted of the isolates obtained in Nepal in 2012. Clade C consisted of the isolates obtained in China in 2013 and 2017, and in Germany in 2018. Clade D consisted of the isolates obtained in Nepal in 2012, 2018 (JUNP052), and 2019 (JUNP351, JUNP461, JUNP329, and JUNP497); in Australia in 2011 and 2016; in Brazil in 2011; in China in 2012, 2016, 2017, and 2019; in Germany in 2018; in India in 1964; in Mexico in 2016; in Spain in 2013; and in the United States in 2013 and 2015; and the unknown strain NEB515.

Most S. maltophilia clinical isolates in Nepal were derived from strains in European countries, including Germany and Spain, whereas the S. maltophilia strains JUNP349 and JUNP350 were indigenous to Nepal. Based on SNPs, the genetic backgrounds of the two clonal strains described in this study differed from those of other strains. The present study suggests that most S. maltophilia strains obtained in Nepal had similar genetic background to the wide-distributed strains belonging to Clade D. In several countries, including Australia, Brazil, Germany, Mexico, and the United States, where the wide-distributed strains belonging to Clade D were isolated, the isolation rates of levofloxacin-resistant S. maltophilia were relatively high, according to the SENTRY Antimicrobial Surveillance Program (1997–2016) (2). It is important to continue antimicrobial surveillance of S. maltophilia in Nepal and analyze the genetic backgrounds.

MATERIALS AND METHODS

Bacterial strains. Between April 2018 and November 2019, seven S. maltophilia isolates were obtained from seven patients treated at two hospitals in Kathmandu, Nepal (six isolates from hospital A and one from hospital B). The bacteria were identified using the biochemical API 20 NE test (bio-Mérieux, Marcy L’Etoile, France) and by sequencing their 16S rRNA genes. Of the seven isolates, three were from respiratory tracts, two from pus, one from blood, and one from cerebrospinal fluid. Escherichia coli DH5α (TaKaRa Bio, Shiga, Japan) and E. coli BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and protein expression, respectively. MICs were determined using a broth microdilution method, with the breakpoints of ceftazidime, chloramphenicol, levofloxacin, minocycline, ticarcillin-clavulanic acid, and trimethoprim-sulfamethoxazole for S. maltophilia determined according to the guidelines of the Clinical and Laboratory Standards Institute (13). Whole-genome sequencing. Genomic DNA was extracted from each of the seven isolates using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced using the MiSeq platform (Illumina, San Diego, CA) with the Nextera XT DNA library prep kit and MiSeq reagent kit version 3 (600 cycle; Illumina). More than 30-fold coverage was achieved for each isolate. Raw reads of each isolate were

| Strain | ABK | AMK | APR | DIB | GEN | ISP | KAN | NEO | NET | SIS | TOB | PRM |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| S. maltophilia JUNP350 | >512 | 256 | 512 | 512 | 16  | 16  | 16  | 128 | 256 | 256 | 128 | 32  |
| E. coli DH5α/pSTV28 | ≤0.25 | 1   | 2   | 0.25 | ≤0.25 | ≤0.25 | 1   | 0.5 | ≤0.25 | ≤0.25 | ≤0.25 | 1   |

*The MICs for S. maltophilia and E. coli strains were determined with Mueller-Hinton broth preparations and individual aminoglycosides.

ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin; PRM, paromomycin.

**FIG 3** Analysis of acetylated aminoglycosides by thin-layer chromatography. AAC(6’)-Iap and various aminoglycosides were incubated in the presence (+) or absence (−) of acetyl coenzyme A. ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; PRM, paromomycin; TOB, tobramycin; GEN, gentamicin.
Genetic environments surrounding (A) \textit{bla}_{KBL-1} and (B) \textit{aac(6')-Iap}, both of which were located on the \textit{S. maltophilia} chromosome. (A) The \textit{bla}_{KBL-1} surrounding region, \textit{msrE-istB-bla}_{KBL-1}, was flanked by IS91. \textit{orfA}, ATP-binding protein encoding gene; \textit{orfB}, phosphoglucomutase encoding gene; and \textit{orfC, D and E}, hypothetical proteins encoding genes. (B) The genetic environment surrounding \textit{aac(6')-Iap} was similar to that of \textit{S. maltophilia} JV3 obtained in Brazil (GenBank accession no. CP050452). \textit{orfF}, WGR domain-containing protein encoding gene; \textit{orfG}, dedcin family protein encoding gene; \textit{orfH}, d-glycerate 3-kinase encoding gene; \textit{orfI}, hypothetical protein encoding gene; \textit{orfJ}, DoxX family protein encoding gene; \textit{orfK}, SM1/KNM family protein encoding gene.

assembled using CLC Genomics Workbench version 10.0.1, and drug-resistant genes were identified using ResFinder 3.0 (https://cge.food.dtu.dk/services/ResFinder/). Fluoroquinolone resistance has been associated with mutations in the quinolone resistance-determining region, which includes the \textit{gyrA} and \textit{parC} genes that encode DNA gyrase and topoisomerase IV, respectively (14).

Phylogenetic analysis based on single nucleotide polymorphisms (SNPs). The complete genome sequences of 35 isolates of \textit{S. maltophilia} isolates obtained in various countries were collected from GenBank (https://www.ncbi.nlm.nih.gov/nuccore). These sequences were aligned against the sequence of \textit{S. maltophilia} K279a isolated in the United Kingdom in 1998 (GenBank accession no. AM743169), and a phylogenetic tree was constructed using kSNP3.0.

\textit{Escherichia coli} transformants expressing \textit{bla}_{KBL-1} and \textit{aac(6')-Iap}. The open reading frame of \textit{bla}_{KBL-1} was PCR amplified using the primers EcoRI-KBL-1-1F (5'-ATGAATTCTAGTGCGGAAGCCGCCCGCGCTC-3') and PstI-KBL-1-R (5'-ATCTGAGTTACGGCTTGGATTTCG-3'). The open reading frame of \textit{aac(6')-Iap} and its promoter region was PCR amplified using the primers EcoRI-AAC(6')-Iap-F (5'-ATGAATTCATGCGTCTTACATTTCCCTTCG-3') and BamHI-AAC(6')-Iap-R (5'-ATGATGCTCATACCCCCGGCGTACCGCGCTC-3'). \textit{E. coli} transformants expressing \textit{bla}_{KBL-1} and \textit{aac(6')-Iap} were produced as previously described (10). The PCR product of \textit{bla}_{KBL-1} was digested with EcoRI and PstI and ligated into pET28a (Novagen, Inc., Madison, WI, USA), whereas the PCR product of \textit{aac(6')-Iap} was digested with EcoRI and BamHI and ligated into pSTV28 (TaKaRa Bio). Recombinant \textit{KBL-1} and \textit{AAC(6')-Iap} proteins were purified using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. His-tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA, USA), and untagged proteins were purified by an additional passage over the Ni-NA agaroseX. The purities of the recombinant \textit{KBL-1} and \textit{AAC(6')-Iap}, as determined by SDS-PAGE, were each over 90%.

Purification of \textit{AAC(6')-Iap} and \textit{KBL-1}. The open reading frames of \textit{KBL-1} and \textit{AAC(6')-Iap} without signal peptide regions were cloned into pET28a expression vector (Novagen, Inc., Madison, WI, USA) with the primer sets EcoRI-KBL-F (5'-ATGAATTCTAGTGCGGAAGCCGCCCGCGCTCAGG-3') and PstI-KBL-R (5'-ATCTGAGTTACGGCTTGGATTTCG-3') for \textit{KBL-1}, and BamHI-AAC(6')-Iap-F (5'-ATGATGCTCATACCCCCGGCGTACCGCGCTC-3') and XhoI-AAC(6')-Iap-R (5'-ATCTCAGGCTTGGATTTCG-3') for \textit{AAC(6')-Iap}. \textit{E. coli} BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) was transformed with each of these plasmids, and the recombinant \textit{KBL-1} and \textit{AAC(6')-Iap} proteins were purified using Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. His-tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA, USA), and untagged proteins were purified by an additional passage over the Ni-NA agaroseX. The purities of the recombinant \textit{KBL-1} and \textit{AAC(6')-Iap}, as determined by SDS-PAGE, were each over 90%.

Catalytic activities of \textit{KBL-1} recombinant protein. The \beta-lactamase activities were monitored during the purification process using nitrocefin (Oxoid, Ltd., Basingstoke, United Kingdom). The initial rates of hydrolysis were determined at 37°C in 50 mM Tris-HCl (pH 7.4), 0.3 M NaCl buffer by UV-visible spectrophotometry (V-730, Jasco, Tokyo, Japan). Reactions were initiated by direct addition of substrate into the cuvettes of the spectrophotometer, allowing the UV absorption of the reaction mixture to be determined during the initial phase of the reaction. The Km, kcat, and kcat/Km ratios of \beta-lactam hydrolysis were determined from Lineweaver-Burk plots of triplicate analyses. Fifty percent inhibitory concentrations (IC50s) were determined for clavulanic acid and sulbactam. Various concentrations of these inhibitors were preincubated with the purified enzyme for 3 min at 30°C to determine the concentrations that reduced the hydrolysis rate of 100 µM penicillin G by 50%.

Thin layer chromatography (TLC) analysis of acetylated aminoglycosides. Mixtures containing 2 mM aminoglycoside, 2 mM acetyl coenzyme A (acyl-CoA), and 50 µg/mL \textit{AAC(6')-Iap} in 20 µL
phosphate buffer (pH 7.4) were incubated for 16 h at 37°C. Aliquots of 3 μL of each aminoglycoside mixture were spotted onto the surface of a Silica Gel 60 thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany), followed by development with a 5% phosphate potassium solution. The aminoglycosides and their acetylated products were detected by spraying the plates with 0.2% ninhydrin in acetone.

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