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RESEARCH ARTICLE

Stenotrophomonas maltophilia from Nepal Producing Two Novel Antibiotic Inactivating Enzymes, a Class A β-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 β-lactamase, KBL-1 (Kathmandu β-lactamase), consisting of 286 amino acids with 52.98% identity to PSV-1. *Escherichia coli* transformants expressing bla_{KBL-1} were less susceptible to penicillins. The recombinant KBL-1 protein efficiently hydrolyzed penicillins. The genomic environment surrounding bla_{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 β-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

Stenotrophomonas 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_{L1} and bla_{L2}, that encode the two β-lactamases, L1 and L2, respectively. L1 is a broad-spectrum

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TABLE 1 Characteristics of the seven *S. maltophilia* strains in Nepal, including their antimicrobial resistance profiles and drug-resistant factors

	MIC (μg/mL)[a]													β-lactamase		
Strain	ABK	AMK	AZT	CAZ	CHL	CIP	CST	IPM	LVX	MEM	MIN	SXT	TIM	Metallo-β-lactamase	Serine β-lactamase[b]	Aminoglycoside modifying enzyme(s)
JUNP052	128	256	512	64	16	2	>128	512	2	128	0.5	2/38	128/2	L1	L2	APH(3′)-IIc
JUNP329	256	128	512	8	8	1	64	128	0.5	128	≤0.25	0.2/3.8	16/2	L1	L2	APH(3′)-IIc
JUNP349	>512	512	>512	32	16	2	>128	128	2	64	0.5	1/19	128/2	L1	L2	AAC(6′)-Iap, APH(3′)-IIc
JUNP350	>512	256	>512	32	8	2	>128	128	1	64	0.5	1/19	64/2	L1	L2	A AAC(6′)-Iap, APH(3′)-IIc
JUNP351	>512	512	>512	32	16	2	>128	512	4	256	≤0.25	1/19	64/2	L1	L2	AAC(6′)-Iak, APH(3′)-IIc
JUNP461	16	8	4	2	8	4	>512	256	0.25	128	≤0.25	0.3/4.7	32/2	L1	L2	APH(3′)-IIc
JUNP497	>512	128	>512	64	128	128	>512	256	32	64	2	8/152	128/2	L1	L2, KBL-1, PME-1	AAC(6′)-Iz, APH(3′)-IIc, APH(3′)-VI

[a]ABK, arbekacin; AMK, amikacin; AZT, aztreonam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; CST, colistin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; TIM, ticarcillin-clavulanic acid; MIN, minocycline.
[b]ESBL, extended-spectrum β-lactamase.

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 Slt) 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′)-Iak*, 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 ≥64 μg/mL for imipenem, meropenem, and colistin, and six each had MICs of ≥128 μg/mL for aztreonam, arbekacin, and amikacin.

Drug resistance genes. Assessment of β-lactamase encoding genes showed that all seven isolates harbored bla_{L1} and bla_{L2}, 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′)-Iap*, 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 ascidiaceicola*, 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 μg/mL) toward the penicillins, including ampicillin, amoxicillin,

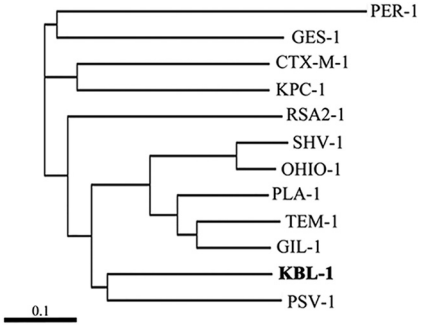

FIG 1 Dendrogram comparing class A β-lactamases with KBL-1. The dendrogram was calculated using the Clustal Omega program. Branch lengths correspond to numbers of amino acid exchanges among the proteins. The EMBL/GenBank/DDBJ accession numbers of the proteins are PER-1, WP_001100753; GES-1, WP_013250881; CTX-M-1, WP_013188473; KPC-1, AF297554; RSA2-1, NG_063889; SHV-1, NG_049989; OHIO-1, NG_049352; PLA-1, NG_049969; TEM-1, NG_050145; GIL-1, NG_049142; KBL-1, LC579778; PSV-1, NG_052626.

penicillin G, and piperacillin, with the MICs toward these penicillins being 2,048, 256, 128, and 512-fold higher, respectively, for *E. coli* expressing bla_{KBL-1} than for the vector control (Table 2). The MICs of these penicillins were significantly reduced by β-lactamase inhibitors combined with penicillins, including amoxicillin-clavulanic acid, ampicillin-sulbactam, and piperacillin-tazobactam, which had MICs of 32 to 128 μg/mL. The *E. coli* expressing bla_{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 bla_{KBL-1} and the vector control for any of these agents. The MIC of ceftazidime for *E. coli* expressing bla_{KBL-1} was low (1 μg/mL), but significantly higher than that for the vector control (0.125 μg/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 β-lactams tested, except for aztreonam (Table 3). Recombinant KBL-1 efficiently hydrolyzed the penicillins, including ampicillin, amoxicillin, penicillin G, and piperacillin with k_{cat}/k_m values of 0.422

TABLE 2 MICs of β-lactam for *S. maltophilia* JUNP497 and *E. coli* strains transformed with bla_{KBL-1}

	MIC(μg/mL)		
β-lactams	***E. coli* DH5α/ pHSG398-bla_{KBL-1}**	***E. coli* DH5α/ 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

TABLE 3 Enzymatic activities of KBL-1 recombinant protein against β-lactams[a]

β-lactams	K_m (μM)[b]	K_{cat} (s^{-1})[b]	K_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Ampicillin	37 ± 8	19 ± 1	0.532
Amoxicillin	23 ± 4	10 ± 0.4	0.455
Aztreonam	NH[c]		
Penicillin G	21 ± 5	24 ± 2	1.166
Cephradine	605 ± 45	2.23 ± 0.13	0.004
Cefoxitin	57 ± 6	0.032 ± 0.002	0.001
Ceftazidime	45 ± 12	0.013 ± 0.003	0.0003
Cefotaxime	31 ± 5	1.332 ± 0.053	0.044
Cefepime	79 ± 2	0.751 ± 0.029	0.010
Imipenem	85 ± 7	1.132 ± 0.143	0.013
Meropenem	39 ± 6	0.443 ± 0.056	0.011
Piperacillin	52 ± 7	22 ± 1	0.422
Moxalactam	49 ± 4	0.392 ± 0.009	0.008

[a]The proteins were initially modified with a His-tag, which was removed after purification.
[b]K_m and k_{cat} values represent the means ± standard deviations of the results of three independent experiments.
[c]NH: no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentration up to 700 nM.

to 1.166, whereas it slightly hydrolyzed cephalosporins and carbapenems with k_{cat}/k_m 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 μM clavulanic acid and 1.2 μM sulbactam.

A novel 6′-*N*-aminoglycoside acetyltransferase AAC(6′)-Iap. The AAC(6′)-Iap protein was found to consist of 155 amino acids. Multiple sequence alignments among AAC(6′) enzymes revealed that AAC(6′)-Iap was 85.0% identical to AAC(6′)-Iz (7), 83.0% identical to AAC(6′)-Iam (9), and 79.1% identical to AAC(6′)-Iak (10) (Fig. 2). Compared with vector control, *E. coli* expressing AAC(6′)-Iap 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′)-Iap (Fig. 3). These results indicated that *aac(6′)-Iap* 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′)-Iap*. Although gentamicin and kanamycin were acetylated by AAC(6′)-Iap, the MICs were not higher than those of *E. coli* harboring pSTV28-*aac(6′)-Iap*. 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′)-Iap.

Genomic environments surrounding *bla*$_{KBL-1}$ and *aac(6′)-Iap*. The genomic environment surrounding *bla*$_{KBL-1}$ was a unique structure, consisting of *orfA-orfB*-IS*91-msrE-istB-bla*$_{KBL-1}$-IS*91*-IS*5-orfC-orfD-orfE* (Fig. 4A). The *bla*$_{KBL-1}$ surrounding region, *msrE-istB-bla*$_{KBL-1}$, was flanked by IS*91*. BLAST analysis did not identify any similar structure in GenBank, suggesting that this structure may be unique.

The genomic environment surrounding *aac(6′)-Iap* consisted of *hemE-orfF-aroB-aroK-orfG-orfH-pdxH-aac(6′)-Iap-prpR-prpB-prpC-acnD*, which was 90.99% (nucleotides [nt] 3368404 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′)-Iap* were identical to those in *S. maltophilia* K279a harboring *aac(6′)-Iam* (9), IOMTU250 harboring *aac(6′)-Iak* (10). and ATCC13637 harboring *aac(6′)-Iz* (7) and JV3 (accession no. CP002986) (Fig. 4B). These results suggested that the genetic structures surrounding *aac(6′)-Iap* are widely conserved among *S. maltophilia* samples obtained in various countries.

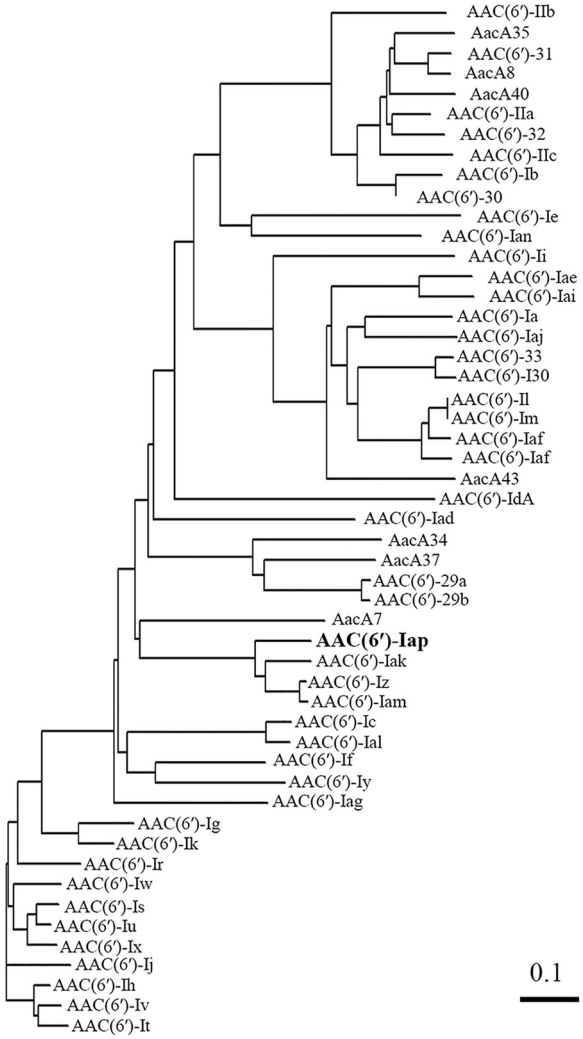

FIG 2 Dendrogram comparing 6′-N-aminoglycoside acetyltransferases [AAC(6′)s with AAC(6′)-Iap]. The dendrogram was calculated using the Clustal Omega program. Branch lengths correspond to numbers of amino acid exchanges among the proteins. The EMBL/GenBank/DDBJ accession numbers of the proteins are AAC(6′)-Ia, M18967-1; AAC(6′)-Ib, M23634; AAC(6′)-Ic, M94066; AAC(6′)-Id, X12618; AAC (6′)-Ie, M13771; AAC(6′)-If, X55353;AAC(6′)-Ig, L09246; AAC(6′)-Ih, L29044; AAC(6′)-Ii, L12710-1; AAC(6′)-Ij, L29045; AAC(6′)-Ik, L29510; AAC(6′)-Il, U13880; AAC(6′)-Im, AF337947; AAC(6′)-Iq, AF047556-1; AAC(6′)-Ir, AF031326; AAC(6′)-Is, AF031327; AAC(6′)-It, AF031328; AAC(6′)-Iu, AF031329; AAC(6′)-Iv, AF031330; AAC (6′)-Iw, AF031331; AAC(6′)-Ix, AF031332; AAC(6′)-Iy, AF144880; AAC(6′)-Iz, AF140221; AAC(6′)-Iad, AB119105; AAC(6′)-Iae, AB104852; AAC(6′)-Iaf, AB462903; AAC(6′)-Iag, AB472901; AAC(6′)-Iai, EU886977; AAC(6′)-Iaj, AB709942; AAC(6′)-Iak, AB894482; AAC(6′)-Ial, AB871481; AAC(6′)-Iam, AB971834; AAC(6′)-Ian, NG_047282; AAC(6′)-Iap, LC536747; AAC(6′)-IIa, M29695; AAC(6′)-IIb, L06163; AAC(6′)-IIc, AF162771; AAC(6′)-29a, AF263519; AAC(6′)-29b, AF263519; AAC(6′)-30, AJ584652; AAC(6′)-31, AJ640197; AAC(6′)-32, EF614235; AAC(6′)-33, GQ337064; AAC(6′)-I30, AY289608; AacA7, U13880; AacA8, AY444814; AacA34, AY553333; AacA35, AJ628983; AacA37, DQ302723; AacA40, EU912537; and AacA43, HQ247816.

BLAST analysis revealed that the genetic structure surrounding *aac(6′)-Iap*, *pdsX-aac (6′)-Iap-prpR*, was identical to the structures in environmentally arising *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 *aac(6′)-Iam/-Iak/-Iz*, *pdxH-aac(6′)-Iam/-Iak/-Iz-orfI-orfJ-orfK* were identical to those in *S. maltophilia* strains obtained from medical settings. The genomic structure surrounding *aac(6′)-Iap* seems to be new, combining structures detected in medical settings and in environmental sources.

Phylogenetic analysis of *S. maltophilia* in Nepal. Phylogenetic analysis based on whole genome sequences revealed that *S. maltophilia* can be divided into three clades,

TABLE 4 MICs of aminoglycosides for *S. maltophilia* JUNP350 and *E. coli* strains transformed with *aac(6′)-lap*[a]

Strain	MIC (μg/mL)[b]											
	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
E. coli DH5α/pSTV28-*aac(6′)-lap*	8	2	2	4	≤0.25	0.5	2	1	0.5	0.5	2	1

[a]The MICs for *S. maltophilia* and *E. coli* strains were determined with Mueller-Hinton broth preparations and individual aminoglycosides.
[b]ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin; PRM, paromomycin.

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

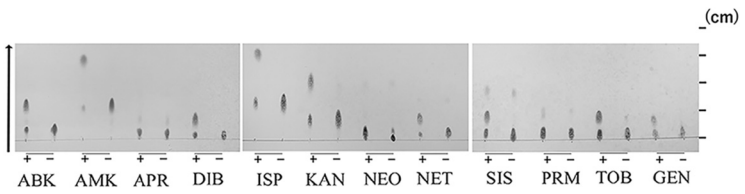

FIG 3 Analysis of acetylated aminoglycosides by thin-layer chromatography. AAC(6′)-lap and various aminoglycosides were incubated in the presence (+) or absence (-) of acetyl coenzyme A. ABK, arbekacin; AMK, amikacin; APR; apramycin; DIB, dibekacin; ISP, isopamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; PRM, paromomycin; TOB, tobramycin; GEN, gentamicin.

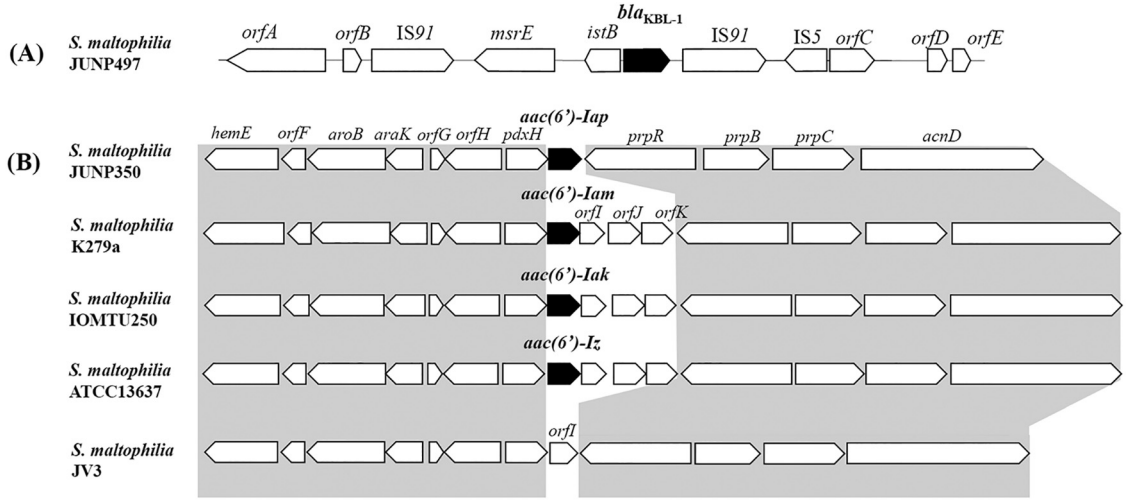

FIG 4 Genetic environments surrounding (A) bla_{KBL-1} and (B) *aac(6′)-Iap*, both of which were located on the *S. maltophilia* chromosome. (A) The bla_{KBL-1} surrounding region, *msrE-istB-*bla_{KBL-1}, was flanked by IS*91*. *orfA*, ATP-binding protein encoding gene; *orfB*, phosphoglucosamide mutase encoding gene; and *orfC*, *D and E*, hypothetical proteins encoding genes. (B) The genetic environment surrounding *aac(6′)-Iap* was similar to that of *S. maltophilia* JV3 obtained in Brazil (GenBank accession no. CP050452). *orfF*, WGR domain-containing protein encoding gene; *orfG*, dodecin family protein encoding gene; *orfH*, d-glycerate 3-kinase encoding gene; *orfI*, hypothetical protein encoding gene; *orfJ*, DoxX family protein encoding gene; *orfK*, SMI1/KNR4 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 *gyrA* and *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 *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 *S. maltophilia* K279a isolated in the United Kingdom in 1998 (GenBank accession no. AM743169), and a phylogenetic tree was constructed using kSNP3.0.

***Escherichia coli* transformants expressing bla_{KBL-1} and *aac(6′)-Iap*.** The open reading frame of bla_{KBL-1} was PCR amplified using the primers EcoRI-KBL-1-1F (5′-ATGAATTCATGCGTCTTACATTTCCCTTCG-3′) and PstI-KBL-1-R (5′- ATCTGCAGTTAGCGCCTTGCTTGGATTTCG-3′). The open reading frame of *aac(6′)-Iap* and its promoter region was PCR amplified using the primers EcoRI-AAC(6′)-Iap-F (5′-ATGAATTCAGTCGAA GACGCTTGCAACGCG -3′) and BamHI-AAC(6′)-Iap-R (5′-ATGGATCCCTACCCCCGCGTGACCGCGTCC -3′). *E. coli* transformants expressing bla_{KBL-1} and *aac(6′)-Iap* were produced as previously described (10). The PCR product of bla_{KBL-1} was digested with EcoRI and PstI and ligated into pHSG398 (TaKaRa Bio), whereas the PCR product of *aac(6′)-Iap* was digested with EcoRI and BamHI and ligated into pSTV28 (TaKaRa Bio). *E. coli* DH5α was transformed with each plasmid, and the transformants were selected on Luria-Bertani agar containing 30 μg/mL chloramphenicol. The susceptibilities of these transformants to various β-lactams and aminoglycosides were assayed.

Purification of AAC(6′)-Iap and KBL-1. The open reading frames of KBL-1 and 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′-ATGAATTCGAAAACCTGTATTTCCAAGGCGAAGCCGCCCGCGCGCTC GAGG-3′) and SalI-KBL-R (5′-ATGTCGACTTAGCGCCTTGCTTGGATTTCG-3′) for KBL-1, and BamHI-AAC(6′)-Iap-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCGCTGCATGGTCGCAGCTGCGCGTGGGCCTG-3′) and XhoI-AAC (6′)Iap-R (5′-ATCTCGAGCTACCCCCGCGTGACCGCGTCCAGCGGCAT-3′) for AAC(6′)-Iap. *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) was transformed with each of these plasmids, and the recombinant KBL-1 and 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 KBL-1 and AAC(6′)-Iap, as determined by SDS-PAGE, were each over 90%.

Catalytic activities of KBL-1 recombinant protein. The β-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 β-lactam hydrolysis were determined from Lineweaver-Burk plots of triplicate analyses. Fifty percent inhibitory concentrations (IC_{50}s) 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 (acetyl-CoA), and 50 μg/mL AAC(6′)-Iap in 20 μL

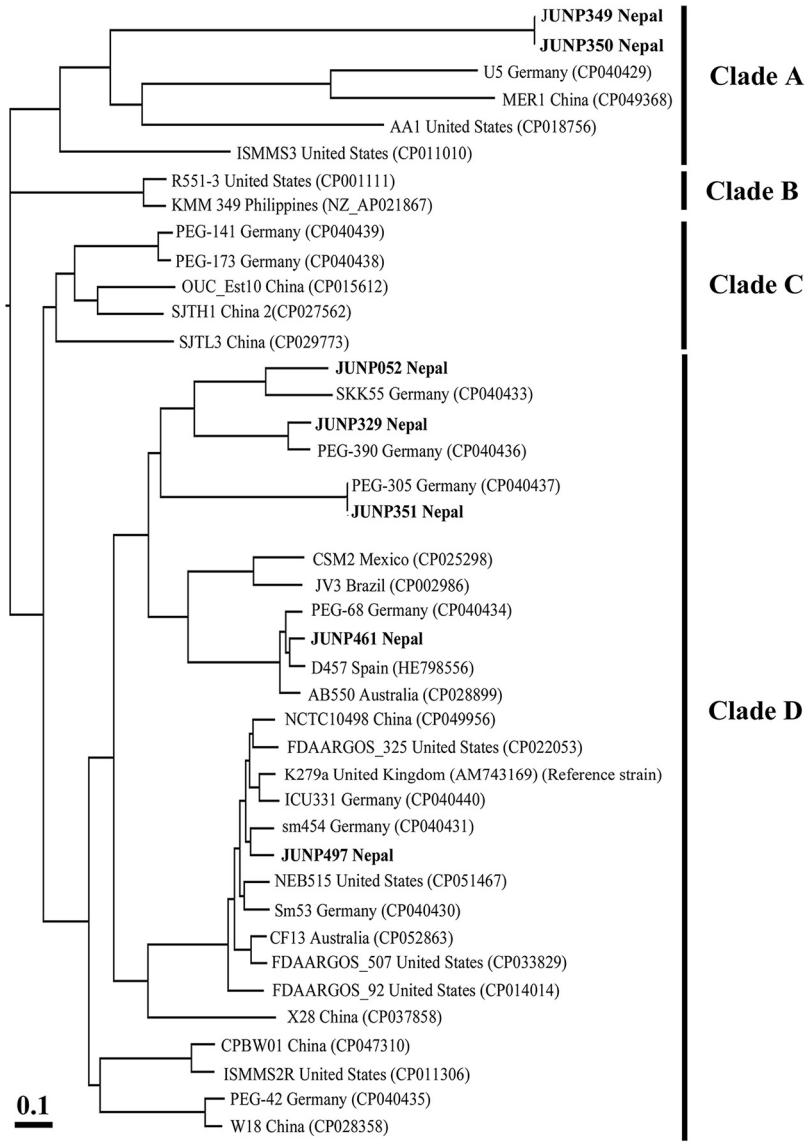

FIG 5 Molecular phylogenetic tree of *S. maltophilia* strains based on the whole genome sequences of the seven *S. maltophilia* strains obtained in Nepal (shown in bold) and 35 *S. maltophilia* strains obtained in various countries, including Australia, Brazil, China, Filipin, Germany, India, Mexico, Spain, and the United States, and registered in GenBank.

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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