Journal of Global Antimicrobial Resistance 39 (2024) 6–11

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

KSA-1, a naturally occurring Ambler class A extended spectrum β-lactamase from the enterobacterial species *Kosakonia sacchari*

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ARTICLE INFO

Article history:
Received 6 February 2024
Revised 5 July 2024
Accepted 16 July 2024
Available online 13 August 2024

Editor: Stefania Stefani

Keywords:
ESBL
KSA-1
Kosakonia sacchari

ABSTRACT

Background: Several bacterial species belonging to the *Gammaproteobacteria* possess intrinsic class A β-lactamase genes that may represent a source of further dissemination and acquisition to other Gram-negative species. Here we characterised KSA-1 class A β-lactamase, the gene of which was identified within the chromosome of an environmental Enterobacterales species, namely *Kosakonia sacchari*, which was also recently identified as the progenitor of an MCR-like colistin-resistance determinant.
Methods: In silico analysis using the GenBank database identified a class A β-lactamase gene within the chromosome of *K. sacchari* SP1 (GenBank accession no. WP_017456759). The corresponding protein KSA-1 shared 63% amino acid identity with the intrinsic CKO-1 from *Citrobacter koseri* and 53% with TEM-1. Using the *K. sacchari* DSM 100203 reference strain as a template, bla_{KSA-1} was amplified, cloned into the plasmid pUCp24 and expressed in *Escherchia coli* TOP10. Minimal inhibitory concentrations and kinetic parameters were obtained from the purified enzyme.
Results: *K. sacchari* strain SP1 conferred resistance to amino-, carboxy- and ureido-penicillins only. Once produced within *E. coli*, KSA-1 showed a typical clavulanic acid-inhibited extended spectrum β-lactamase associated with a peculiar temocillin resistance profile. Kinetic assays were performed using a purified extract of KSA-1 and demonstrated a high hydrolysis rate for benzylpenicillin and piperacillin, as well as weakly extended spectrum cephalosporins. Determination of inhibitory constants showed 50% inhibitory concentration values of 2.2, 3 and 1.8 nM for clavulanic acid, tazobactam and avibactam, respectively. Analysis of sequences surrounding the bla_{KSA-1} gene did not reveal any mobile element that could have been involved in the acquisition of this β-lactamase gene in that species.
Conclusion: KSA-1 is a class A extended spectrum β-lactamase distantly related to known extended spectrum or broad-spectrum Ambler class A β-lactamases, which is highly resistant to temocillin. The bla_{KSA-1} gene could be considered as intrinsic within the species.

1. Introduction

A majority of the extended spectrum or broad-spectrum Ambler class A β-lactamases (ESBLs) are either derivatives of narrow-spectrum TEM and SHV types of β-lactamases or they belong to the CTX-M group of β-lactamases [1,2]. They are characterised by their hydrolytic activity against β-lactams including second- and third-generation cephalosporins, as well as aztreonam. Such enzymes, when acquired, are often encoded by genes associated with mobile genetic elements as they are plasmid-mediated and disseminated among clinical isolates of *Enterobacterales* and less commonly in other Gram-negative species. In addition, some other ESBLs are defined as "minor" due to their more limited spread, among which PER, VEB and GES are the most frequently identified [3]. Ambler class A β-lactamases comprise two main biochemical branches—A1 and A2—based on their conserved amino acid residues [4].

Gram-negative *Proteobacteria*, a major group of bacteria producing various chromosomal and plasmid-encoded β-lactamases, is represented principally the *Gammaproteobacteria*, including *En-*

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https://doi.org/10.1016/j.jgar.2024.07.008

terobacterales, Pseudomonales and *Vibrionales.* Several phyla of *Gammaproteobacteria* possess genes encoding type A1 ESBLs in their chromosome such as BPS-1 (*Burkholderia pseudomallei*), ERP-1 (*Erwinia persicina*), FONA-1 (*Serratia fonticola*), HugA (*Proteus penneri*), KLUA-1 (*Kluyvera ascorbata*), KLUC-1 (*Kluyvera cryocrescens*), KLUG-1 (*Kluyvera georgiana*), RAHN-1 (*Rahnella aquatilis*), SED-1 (*Citrobacter sedlakii*), SFC-1 (*Serratia fonticola*) and SMO-1 (*Ewingella* sp.), among others [5-16].

Enterobacter sacchari was the first identified isolate recovered in 1994 from the inner tissues of a *Saccharum officinarum* L. (sugar cane) stem grown in Nanning, China [17]. This plant-associated bacterial species belonging to the family *Enterobacteriaceae* and in the genus *Enterobacter* was later reclassified as the novel genus *Kosakonia* as *Kosakonia sacchari* comb. nov. [18,19]. *Kosakonia* species may be recovered from clinical samples but mainly derive from environmental sources including soil and plants. Several species of this genus are known to promote plant growth by fixing nitrogen.

In this study we have characterised a novel class A1 β-lactamases from the chromosome of *K. sacchari*, which has recently been identified as a progenitor of an MCR-type (phosphoethanolamine transferase) colistin-resistance determinant [20].

2. Materials and methods

2.1. In silico analysis

DNA sequence analysis of the genome of *Kosakonia sacchari* SP1 (GenBank accession no. WP_017456759) revealed an open reading frame (ORF) of 867 bp encoding a putative class A β-lactamase, hence named KSA-1. KSA-1 was compared with known β-lactamases using the NCBI BLAST alignment tool.

Phylogenetic analysis was performed using alignment software SeaView (Prabi, La Doua, France).

2.2. Bacterial strains and plasmids

K. sacchari strain DSM100203 was obtained from the Leibniz Institute DSMZ (Brunswick, Germany) and cultured on tryptic soy agar at 37 °C for 18 h [21]. The plasmid pUCp24, encoding *LacZ* as a selectable colour marker and a gentamicin acetyltransferase-3–1 gene encoding resistance to gentamicin, was used for cloning and expression in *E. coli* TOP10.

2.3. Molecular characterisation and cloning experiments

The isoelectric point and molecular weight of KSA-1 were determined via Expasy (www.web.expasy.org/compute_pi). Identification of the KSA-1 subclass was performed by analysing conserved amino acid residues according to Phillipon et al. and Bush-Jacoby-Medeiros classifications schemes [4,22,23].

In order to analyse the hydrolysis proprieties of the novel β-lactamase KSA-1, the entire sequence and adjacent DNA sequences of the bla_{KSA-1} gene were amplified by polymerase chain reaction using outward-specific designed primers KSA-SacI-Fw (5′- GAT GAG CTC TGT TGC TCT GTT TGA ACA AG -3′) and KSA-*Bam*HI-Rv (5′- GAT GGA TCC AGT GGC AGA GAT TTT TCT CTC -3′) using the *K. sacchari* DSM 100203 reference strain as a template. The bla_{KSA-1} gene template, previously digested with a restriction enzyme (SacI and *Bam*HI), was cloned using DNA T4 ligase into the cloning vector pUCp24 and expressed in *E. coli* TOP10, giving rise to a recombinant strain of *E. coli* (pKSA-1).

Selection was made on Mueller-Hinton agar plates supplemented with 100 µg/ml ampicillin and 10 µg/ml gentamicin.

2.4. Susceptibility testing and β-lactamase content

Antibiotic susceptibility testing was performed using disc diffusion and including amoxicillin, amoxicillin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, temocillin, cephalothin, cefotaxime, ceftazidime, cefepime, aztreonam, cefoxitin, meropenem, imipenem and gentamicin (Bio-Rad, Cressier, Switzerland). The recombinant strain was tested for ESBL activity using Rapid ESBL NP [24]. The minimal inhibitory concentrations (MICs) of amoxicillin, amoxicillin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, temocillin, cephalothin, cefotaxime, ceftazidime, cefepime, aztreonam, cefoxitin, meropenem, imipenem and ertapenem were determined by Etest (bioMérieux, La Balme-les-Grottes, France) on Mueller-Hinton agar plates incubated at 37 °C for 18 h and interpreted according to EUCAST v.14 [25]. Combinations of ceftazidime with fixed respective concentrations of clavulanic acid (2 µg/ml) or avibactam (4 µg/ml) were also tested by broth microdilution, following EUCAST recommendations.

2.5. β-Lactamase purification

E. coli TOP10 (pKSA-1) was grown overnight in 1 L of Luria-Bertani broth supplemented with 100 µg/ml ampicillin and 10 µg/ml gentamicin. The culture was centrifuged for 10 min at 4200 rpm at 4 °C. After resuspension in 20 ml of 50 mM phosphate buffer (pH 6.9), the bacterial cells were disrupted by sonication using a Vibra-Cell sonicator (Sonics and Materials Inc., Newton, MA, USA) with 30 s off, 30 s on and an amplitude of 50%. Protein extract was obtained via centrifugation, followed by 0.4-µm filtration of the supernatant using a nitrocellulose filter and concentration using a Vivaspin 50 kDA MWCO concentrator (Cytiva Life Sciences, Marlborough, MA, USA). Protein extract was dialysed against the purification buffer as follows. Enzyme purification was carried out by ion-exchange chromatography (AKTA Prime, Cytiva Life Sciences) in two steps, first with 20-mM piperazine buffer (pH 9.1) (buffer A-Q), second with 50-mM phosphate buffer (pH 6.9; buffer A-S). Protein extract was loaded first onto a pre-equilibrated anion Q-sepharose column (Cytiva Life Sciences). β-Lactamase was eluted with a linear NaCl gradient (from 0 to 1 M; buffer B-Q) and fractions with the highest β-lactamase activity, assessed via nitrocefin test, were pooled and dialysed overnight in buffer A-S. The second step implies performing the same procedure onto a cation exchanger (S-Sepharose). The final protein extract was concentrated 10-fold with a Vivaspin concentrator.

2.6. Determination of molecular relative mass and concentration of β-lactamase

The protein purification rate as well as the relative molecular mass of the purified KSA-1 were estimated by SDS-12% PAGE gel electrophoresis analysis, which involves boiling purified extract and marker proteins in 1% SDS-3% β-mercaptoethanol solution prior to electrophoresis.

Protein concentration was calculated using a Bradford protein assay (Sigma-Aldrich, St. Louis, MO, USA). The estimated purification coefficient was calculated by dividing the specific activities of the purified protein over the crude extract with 200 µM benzylpenicillin as a substrate.

2.7. Kinetic measurements

Hydrolysis assays were performed in 100 mM phosphate buffer (pH 7.0) supplemented with 50 µM Zn^{2+} using a Genesys 10S UV–visible spectrophotometer (Thermo Fisher Scientific, MA, USA), as previously described [26,27]. Catalytic constant (k_{cat}) and Michaelis

constant (K_m) values were determined by analysing β-lactam hydrolysis under initial-rate conditions by using Eadie-Hoffstee linearisation of the Michaelis-Menten equation. The following wavelengths and absorption coefficients $\wedge\varepsilon$ were used: benzylpenicillin, 232 nm and −1100 M^{-1} cm^{-1}; amoxicillin, 240 nm and −1100 M^{-1} cm^{-1}; ticarcillin, 235 nm and −1050 M^{-1} cm^{-1}; piperacillin, 235 nm and −1070 M^{-1} cm^{-1}; cephalothin, 262 nm and −7960 M^{-1} cm^{-1}; cefoxitin, 265 nm and −7380 M^{-1} cm^{-1}; cefotaxime, 265 nm and −6260 M^{-1} cm^{-1}; cefepime, 264 nm and −8240 M^{-1} cm^{-1}; aztreonam, 318 nm and −640 M^{-1} cm^{-1}; imipenem, 297 nm and −9210 M^{-1} cm^{-1}; and ertapenem 297 nm and −9210 M^{-1} cm^{-1}.

The inhibition constant K_i values were determined by direct competition assays using 200 µM benzylpenicillin ([S]). Inverse initial steady-state velocities ($1/V_o$) were plotted against the inhibitor concentration ([I]) to obtain a straight line. K_i was determined by dividing the value of the *y* intercept by the slope of the line. K_i was corrected by considering benzylpenicillin affinity using the following equation: K_i (corrected) = K_i (observed)/ (1 + [S]/K_m). The K_i values of ceftazidime and temocillin were measured by competitive inhibition using 200 µM benzylpenicillin.

2.8. Sensitivity to β-lactamase inhibitors

The 50% inhibitory concentration values for clavulanic acid, tazobactam and avibactam were determined by determining the concentration of inhibitors reducing 50% of the hydrolysis rate of KSA-1 using 200 µM of benzylpenicillin as a substrate, using an inhibition time of 3 min.

3. Results

3.1. K. sacchari genome analysis

K. sacchari is an environmental species closely related to the genus *Enterobacter* and recovered from plants. Analysis of the genome of *K. sacchari* SP1 available under Genbank accession number no. NZ_CP007215 revealed a putative chromosomal class A β-lactamase gene (GenBank accession number no. WP_017456759) of 867 bp, termed bla_{KSA-1}. The G + C content of this ORF was 56.7%. Close analysis of the genetic environment of bla_{KSA-1} did not reveal any mobile elements, suggesting an intrinsic origin.

The putative enzyme KSA-1 of 289 amino acids shared 63% amino acid identity with the intrinsic CKO-1 from *Citrobacter koseri*, 54% with TEM-1, 53% from OKP-A from *Klebsiella quasipneumoniae* subsp. q*uasipneumoniae* and 51% with HER-1 from *Escherichia hermannii*, as well as with the intrinsic SHV-like β-lactamases from *Klebsiella pneumoniae* (Fig. 1). KSA-1 was encoded in all sequenced genomes of *K. sacchari* with more than 99% of amino acid identity and in related genomes such as *Kosakonia pseudosacchari, Kosakonia oryzendophytica* and *Kosakonia cowanii* with an amino acid identity of 94%, 81% and 73%, respectively.

3.2. Resistance profile

The *K. sacchari* DSM100203 strain was resistant to amino-, carboxy- and ureido-penicillins but not to extended spectrum cephalosporins nor carbapenems.

However, the rcombinant *E. coli* TOP10 strain (pKSA-1) displayed a typical ESBL phenotype with a synergistic effect between clavulanic acid and broad-spectrum cephalosporins, and was found positive using the Rapid ESBL NP test. It was resistant to all tested penicillin and showed increased MICs for ceftazidime, cefotaxime, aztreonam and cefepime (Table 1). It also remained susceptible to β-lactam/β-lactam inhibitor combinations including amoxicillin-clavulanic acid, piperacillin-tazobactam and ceftazidime-avibactam, but showed resistance to ticarcillin-clavulanic acid. Notably, KSA-1 conferred high resistance to temocillin (256 µg/ml) once its gene was cloned and expressed in *E. coli*, which is an uncommon feature for ESBLs (Table 1) and a feature not seen in any other enzyme of its type.

3.3. Molecular characterisation

According to its functional properties, KSA-1 could be included in β-lactamase subclass A1 according to Phillipon et al. and within the 2e group of the Bush-Jacoby-Medeiros classification scheme [4,22,23]. The following motifs, or their analogues, are highly conserved among class A β-lactamases: S70XXK, S130DN, E166 and K34TG. Moreover, the following conserved residues of subclass A1

Table 1
Minimum inhibitory concentrations of β-lactams for *Kosakonia sacchari* and *Escherichia coli* strains.[a]

β-lactam(s)[b]	*Kosakonia sacchari* DSM 100203	MIC[c] (µg/ml)	
		Escherichia coli TOP10 (pKSA-1)	*Escherichia coli* TOP10
Amoxicillin	>512	>512	8
Amoxicillin + CLA	1	8	8
Ticarcillin	>512	>512	4
Ticarcillin + CLA	0.5	32	1
Piperacillin	32	>512	1
Piperacillin + TZB	0.25	8	0.5
Temocillin	2	256	8
Temocillin + CLA	2	16	8
Cefoxitin	2	8	4
Ceftazidime	0.06	2	0.25
Ceftazidime + AVI	0.03	0.5	0.125
Ceftazidime + CLA	0.03	0.5	0.125
Cefotaxime	0.015	0.5	0.125
Cefepime	0.015	1.5	0.06
Aztreonam	0.015	2	0.06
Imipenem	0.125	0.125	0.125
Meropenem	0.03	0.06	0.06
Ertapenem	0.03	0.03	0.03

[a] *Kosakonia sacchari* DSM 100203 isolate harbouring bla_{KSA-1}, *Escherichia coli* TOP10 recombinant strain producing KSA-1, and *E. coli* TOP10 recipient strain.
[b] Clavulanic acid (CLA) was added at 2 µg/ml, tazobactam (TZB) was added at 4 µg/ml, avibactam (AVI) was added at 4 µg/ml.
[c] Data of minimal inhibitory concentrations by microdilution/E-test.

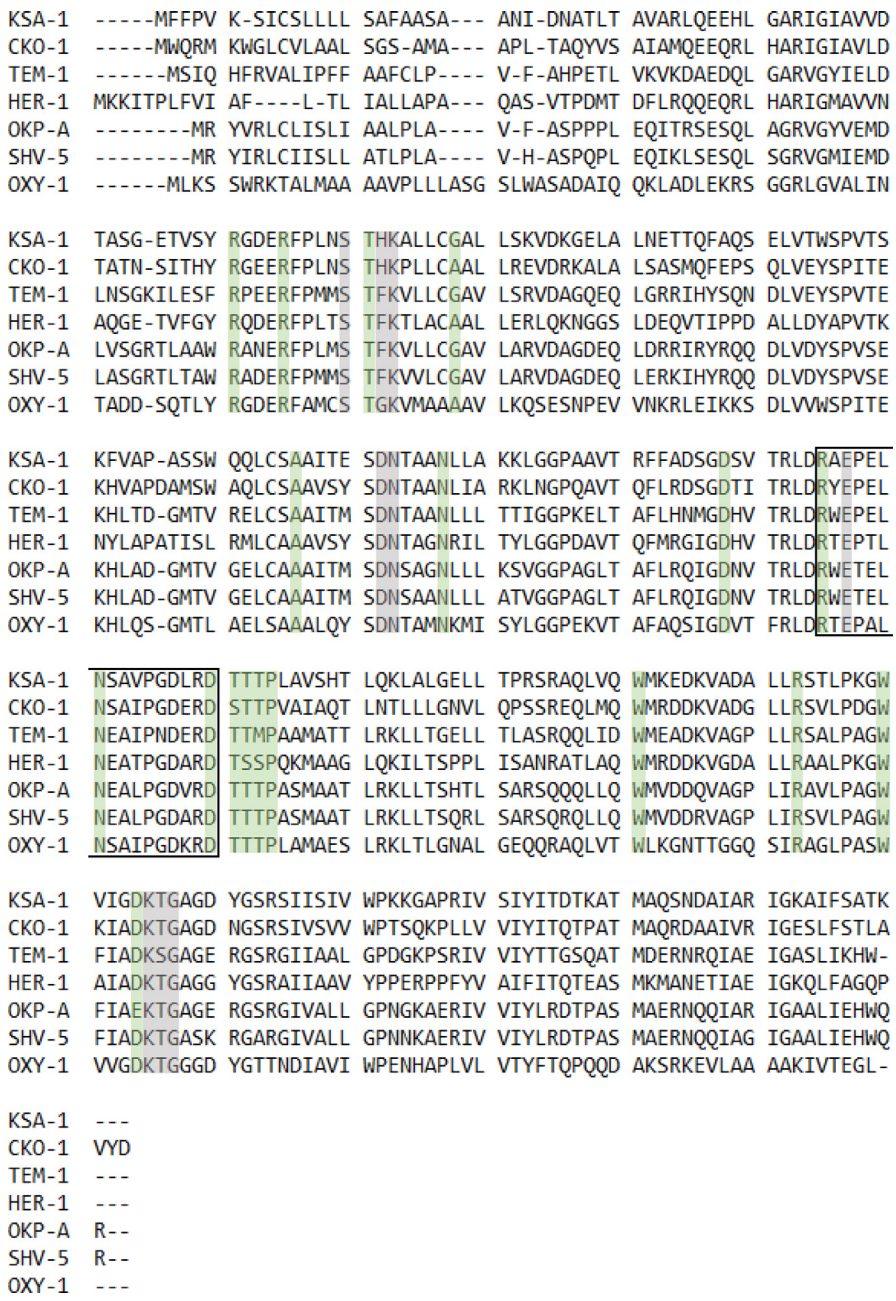

Fig. 1. Amino acid alignment of KSA-1 with the closest β-lactamases: CKO-1 (63%) from *Citrobacter koseri*, TEM-1 (54%) from unknown origin, HER-1 (51%) from *Escherichia hermannii*, OKP-A (53%) from *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* and SHV-5, a variant of SHV-1, from *Klebsiella pneumoniae*. The motifs, S70XXK, S130DN, E166 and K34TG, indicated by grey shading, are conserved motifs of Ambler class A β-lactamases. The residues highlighted in green are specific to the subclass A1 (R61, R65, T71, G78, A125, N136, D157, R164, N170, D179, T180, T181, T182, P183, W210, R222, W229 and D233). The following residues are considered equivalent: A and G; S and T; D and E; I, L, M and V; F, Y and W; K, R and H; N and Q [21,22]. Ω-loop is bracketed, whereas asterisks indicate a stop codon.

β-lactamases have also been identified, namely R61, R65, T71, G78, A125, N136, D157, R164, N170, D179, T180, T181, T182, 183, W210, R222, W229 and D233 [22,23].

The pI of KSA-1 was calculated as 8.93 and the molecular weight of the mature protein as 30.6 kDa. The concentration of the purified protein extract was estimated to be 1.9 µg/ml. The estimated purification coefficient was calculated by dividing the specific activities of the purified protein (99.78 µmol.min^{-1}.mg of protein^{-1}) over the crude extract (3349.3 µmol min^{-1}.mg.protein^{-1}) with 200 µM benzylpenicillin as the substrate (data not shown), with the KSA-1 purification factor determined to be approximately 30-times.

3.4. Kinetic study

Measurements of kinetic parameters were performed using a purified KSA-1 enzyme (Table 2). This enzyme showed the highest level of activity against benzylpenicillin and piperacillin (k_{cat} = 2940 and 1108s^{-1}, respectively) and the lowest against cefotaxime, cephalothin and cefepime (k_{cat} = 0.0003, 0.4 and 0.2 s^{-1}, respectively). Hydrolysis was not detected for temocillin, ceftazidime, aztreonam and carbapenems. However, both temocillin and ceftazidime have a binding affinity for KSA-1, as shown by the inhibitory constant K_i and the increased MIC values (>256 and 2 µg/ml, respectively) observed for the recombinant strain.

Table 2
Kinetic parameters of purified β-lactamase KSA-1.

β-lactam (s)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_i (μM)
Benzylpenicillin	2940	133	22	–
Amoxicillin	282	60	4,7	–
Ticarcillin	843	74	11,4	–
Piperacillin	1108	60	19	–
Cephalothin	4,5	11	0,4	–
Cefepime	11	56	0,2	–
Cefotaxime	0,4	1200	0,0003	–
Ceftazidime	–	–	–	0.13
Temocillin	–	–	–	0.017
Imipenem	ND	ND	ND	–
Ertapenem	ND	ND	ND	–
Aztreonam	ND	ND	ND	–

Standard deviations were below 15%. ND, not determined due to a low initial rate of hydrolysis. k_{cat}, catalytic efficiency; K_m, Michaelis constant; k_{cat}/K_m, specificity constant; K_i, relative k_{off}/k_{on} ratio.

Table 3
50% inhibitory concentrations of various β-lactamase KSA-1 inhibitors.

β-lactamase	IC_{50} (nM)[a]		
	Clavulanic acid	Tazobactam	Avibactam
KSA-1	2.2	3	1.8

[a] IC_{50} represents the required concentration of a drug for 50% inhibition of the enzymatic activity. IC_{50},50% inhibitory concentration.

3.5. Sensitivity to inhibitors

The 50% inhibitory concentration values for clavulanic acid, tazobactam and avibactam were 2.2, 3 and 1.8 nM, respectively (Table 3).

4. Conclusion

In silico analysis of the *K. sacchari* genome revealed a chromosomally encoded β-lactamase, namely KSA-1, characterised as an Ambler class A β-lactamase distantly related to the closest β-lactamases CKO-1 and TEM-1. Further analysis did not reveal any mobile element in the genetic environment of bla_{KSA-1}, suggesting its natural occurrence within the core genome of that species. As is the case for many intrinsic class A ESBLs, their expression does not confer any clinically significant degree of resistance to extended spectrum cephalosporins.

Kinetic parameter determination confirmed the ESBL properties of this enzyme. The β-lactamase KSA-1, like other ESBLs, is capable of hydrolysing activity against first-, second- and third-generation cephalosporins, spearing monobactams and carbapenems, and being sensitive to β-lactamase inhibitors such as clavulanic acid, tazobactam and avibactam.

The low MIC values of β-lactams observed in the original *K. sacchari* strain suggests the occurrence of the bla_{KSA-1} gene as a single copy, hence a natural reservoir. This report expands the knowledge on Gram-negative species that reservoirs of ESBL genes, which further may be identified in more significant Gram-negative clinical species such as, for example *E. coli* and *K. pneumoniae* or non-fermenters.

Funding

This work was funded by the University of Fribourg and the Swiss National Science Foundation [project FNS- 407240_177381].

Ethical approval

Not required.

Declaration of Competing Interest

None declared.

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