Indian Journal of Experimental Biology
Vol. 59, December 2021, pp. 834-841

Promising antimicrobial protein from *Klebsiella*

Ashwini A. Shahadani, Rajyalakshmi M, Ashoka Hadagali & Saisha Vinjamuri*
Department of Biotechnology, BMS College of Engineering, Bengaluru, Karnataka, India

Received 31 May 2021; *revised 03 August 2021*

Increasing antibody resistance necessitates the use of new antimicrobial products. Antimicrobial peptides (AMP`s) And Proteins are a promising alternative to antibiotics currently being used due to their high degree of specificity & wide range of action Here we report a novel antimicrobial protein from iron reducing bacteria isolated from the coffee estate soils of Coorg district, Karnataka, India. Iron reducing bacteria are known to be antagonistic to others due to the presence of Pseuderophores. It makes them a good source of antimicrobial active principles. Fifteen isolates were obtained by selectively screening for iron reduction. These isolates were tested for antimicrobial activity against Gram positive [*Staphylococcus aureus* (MTCC3160), *Bacillus cereus* (MTCC6620)] & Gram negative [*Escherichia coli* (MTCC1650), Salmonella typhi (MTCC3224) and *Pseudomonas aeruginosa* (MTCC2453)] bacteria. Of the 15 isolates, one isolate, CBI-8, which showed good antimicrobial activity, was selected for further studies. The isolate was identified by MALDI-TOF & 16S rDNA sequencing as *Klebsiella variicola*/quasipneumonae (strain IS93) (genebank - MN814029). To induce and augment the production of antimicrobial molecules, CBI-8 was grown in the presence of *Salmonella typhi* and the spent medium was subjected to ammonium sulphate precipitation. The fractions which showed promising activity were further subjected to preparatory HPLC. The distinct bands obtained in the SDS-PAGE corresponding to the HPLC peak that showed antimicrobial activity from the induced sample was analyzed by Mass Spectrometry. Antimicrobial Peptide Database (APD3) calculator, Collection of Anti-Microbial Peptides (CAMPR3) software used to predict AMPs in the proteins identified from MS data.

Keywords: Antimicrobial peptides, APD3 software, Bacteriocins, CAMPR3 software

Development of resistance by the microbes to the antibiotics necessitated search for newer molecules[1-4]. A number of antimicrobial proteins[5] lectins[6] and peptides (AMPs) have been investigated as alternative to the antibiotics[5]. Antimicrobial proteins are part of the innate immune system of metazoans and for bacteria they are essential for competitive survival.

Bacteriocins, the antimicrobial proteins produced by bacteria, are capable inhibiting growth of closely related bacteria of the same species as well as unrelated species, without harming themselves[5, 7], for eg. Klebicins from *Klebsiella* sp.[8].

Bacteriocins are classified into various groups viz. (i) higher molecular weight colicins and lower molecular weight microcins from Gram negative bacteria; and (ii) class I, II and III from Gram positive bacteria. The mechanism of action of bacteriocins include enzyme inhibition, pore formation, prevention of cell wall-formation, DNA/RNA/ protein/peptido-glycan digestion, and inhibition of protein/nucleic acid synthesis[9]. Bacteriocins have been successfully used for food preservation[10]. Bacteriocins producing probiotic bacteria and purified bacteriocins have been reported to have application in combating gut, veginal and skin related infections[11, 12]. Colicin M like klebicin from *K. varricola* inhibited the growth of antibiotic resistant clinical isolates of *Klebsiella*[8]. Some bacteriocins exhibit antitumor activity[13]. It has been reported that colicin producing bacteria inhibit human colorectal carcinoma[14, 15].

The disadvantages of antimicrobial proteins are large size and susceptibility to degradation in gut. There is need for newer cost effective antimicrobials which are resistant to protease digestion and those that are active at broad pH and temperature ranges. Almost all bacterial sps produce at least one antimicrobial protein and most of them are yet to be identified[11].

Antimicrobial peptides (AMPs), with more than 100 amino acids, are also promising alternative to antibiotics due to their specificity, broad spectrum activity and ability to act on multidrug resistant

*Correspondence:
Phone: +91 9448093424 (Mob.)
E-Mail: saishav.bt@bmsce.ac.in

strains as well as on biofilms[16]. They are variously categorized into several groups based on charge, origin, and activity[6,9]. Some AMPs are immunomodulatory and anticancerous[17,18]. Of the 5000 (approx.) antimicrobial peptides reported so far, only five (gramicidin, daptomycin, nisin, polymyxins and melittin) are in clinical use due to issues related to toxicity and pharmacokinetics[19]. In view of this, there is requirement of design, development and assessment of smaller peptides with desirable physicochemical and pharmacokinetic properties[20-25].

In an effort to identify newer antimicrobials, here, we isolated iron reducing bacteria from soil and tested antimicrobial activity of the culture supernatants. We report inhibitory activity of one the isolate to *Salmonella typhi* (MTCC3224). The bacterial supernatants were further subjected to ammonium sulphate precipitation and HPLC fractionation followed by mass spectroscopic analysis. The antimicrobial peptide regions of the identified proteins were predicted using APD3 & CAMPR3 prediction tools.[26-29]and structure of the peptides predicted using PHYRE software.

Material and Methods

Selective isolation of bacteria

We isolated 15 strains of bacteria from soil collected from two different sources. The approach for isolation involved the following:

Collection of soil sample

Soil samples were collected from a depth of 15 cm from coffee estates of (Coorg and the Talakaveri districts river basin) Karnataka, India

Selective media and culture

One gram of the soil sample was serially diluted and inoculated into 'enrichment broth', i.e., modified 9KSilvermann medium: ammonium sulphate 3 g, potassium chloride 10 g, dipotassium hydrogen orthophosphate 0.50 g, magnesium sulphate 0.50 g, calcium nitrate 0.01 g, distilled water(DW) 700 mL, sulfuric acid 1.0 mL, ferrous sulphate 300 mL (14.34%w/V solution), pH 6.0 [30].

For selective isolation of the organisms, citrate broth M728 (0. 5 g each of ammonium sulphate, sodium nitrate, magnesium sulphate, dipotassium hydrogen orthophosphate , 0.2 g calcium chloride and 10 g ferric ammonium citrate in 1000 mL of DW, pH adjusted to 6.8) was inoculated with the 1.0 g of soil. Growth was allowed at 37°C for 48-72 h. Next, 0.1 mL of this culture was added to 20 mL of citrate agar medium, poured into Petri plates and incubated at 37ºC for 48-72 h under facultative anaerobic conditions. The colonies were screened for both iron reduction and rapid growth & were sub-cultured[31].

Antagonistic profile of bacterial isolates

Antagonistic profile of bacterial isolates was studied against *Escherichia coli* (MTCC1650), *Bacillus cereus* (MTCC6620), *Salmonella typhi* (MTCC3224), *Pseudomonas aeruginosa* (MTCC2453) and *Staphylococcus aureus* (MTCC3160). Well diffusion method was followed on a well developed uniform lawn of the pathogenic bacterial isolates. About 50 µL of the sample was introduced to the well and plates were incubated at 37°C for 18-20 h. Diameter of inhibition zone exclusive of the diameter of the well (5 mm) was measured. Ampicillin (100 µg/mL) was used as standard and distilled water as negative control[32-34].

Protein purification

The isolates showing antimicrobial activity were inoculated to citrate broth and were incubated at 37°C for 48-72 h under anaerobic conditions. Alternately, they were grown in presence of *Salmonella typhi* (kept separated in a dialysis bag) for 72-92 h. Culture supernatants were obtained by centrifugation at 5000 g for 10 min and proteins were precipitated using different concentrations of (30, 40, 50, 60 and 70% saturation) ammonium sulphate[35]. The precipitates were collected by centrifugation (10,000 g for 10 min), dialyzed against 0.1 M phosphate buffer (pH 7.0) and were tested for antimicrobial activity.

HPLC fractionation

The proteins that were precipitated with 70% ammonium sulphate were lyophilized at −108°C. The lyophilized powder was reconstituted in DW and subjected to HPLC (C18 Column, Schimadzu) analysis. The analytical HPLC procedure was standardized with binary gradients: Buffer A 0.1% TFA in DW, Buffer B 0.1% TFA in acetonitrile. Preparatory HPLC was done using same buffers with Gradient (10% B for 5 min, 10-80% B for 10 min, 80% B for 5 min, 80-10% B for 10 min), at 10.8 mL flow rate[34]. Peaks were collected, lyophilized and their antimicrobial activity was checked[36].

SDS-PAGE

The HPLC fractions were analyzed in SDS PAGE (15% Separating gel and 7% stacking gels). Gels were stained with silver stain[37]. The gel bands were washed

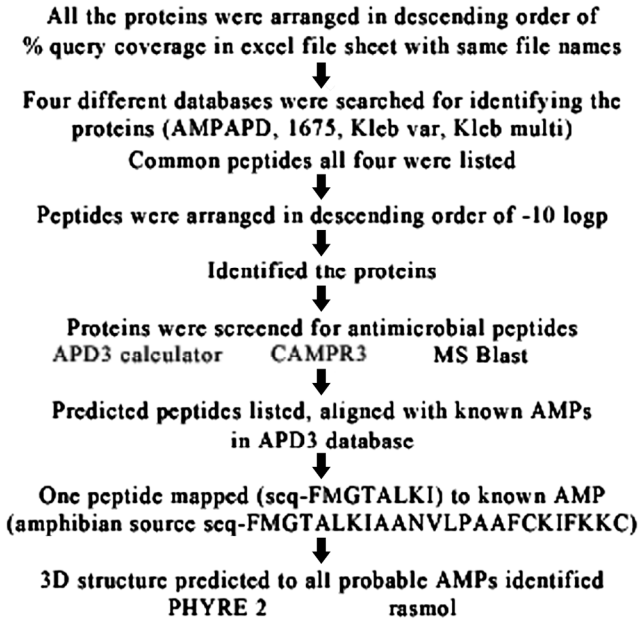
three times in distilled water and were stored in 1% acetic acid. These bands (Rf value 0.625) were subjected to the mass spectrometry (MS) analysis.

Mass Spectrometry (MS)

The MS analysis was done at NCBS-CCAMP, Bengaluru, using FT-ICR/Orbitrap system (in-gel trypsin digestion, ESI Nano-spray) & the data was analyzed by PEAKS 8 software.[26] The proteins collected from MS data were identified and were screened for antimicrobial peptides using CAMPR3 and APD3 database following the scheme1[27-29]

Results

We selectively isolated soil bacteria of which fifteen were tested for antimicrobial activity (culture supernatants) against gram positive and gram negative bacteria, as well as fungal organisms. Of the tested 15 isolates, CBI-8, identified as facultative anaerobic Gram negative rods, exhibited antimicrobial activity against *Staphylococcus aureus* (MTCC3160) and *Salmonella typhi* (MTCC3224). Ammonium sulphate (60 and 70%) precipitated and dialyzed culture supernatants of CBI-8 also showed activity against *Salmonella typhi* (MTCC3224) (Fig. 1 and hence, were taken for further fractionation in HPLC (Fig. 2). One of the preparatory HPLC peaks (peak 5) showed inhibitory activity to *S. typhi*. The purity of the samples obtained is given as supplementary data (Suppl. Table S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*)



Scheme 1 — Flow Chart showing the analysis of MS data

The protein bands that corresponded to the peak 5 of preparatory HPLC were prominently enhanced in the induced sample (Fig. 3). These protein bands were subjected to in-gel digestion with trypsin and analyzed in MS. Three proteins were identified by Peptide mapping (Table 1) using BLASTp and MS Blast tools. These proteins were scanned for

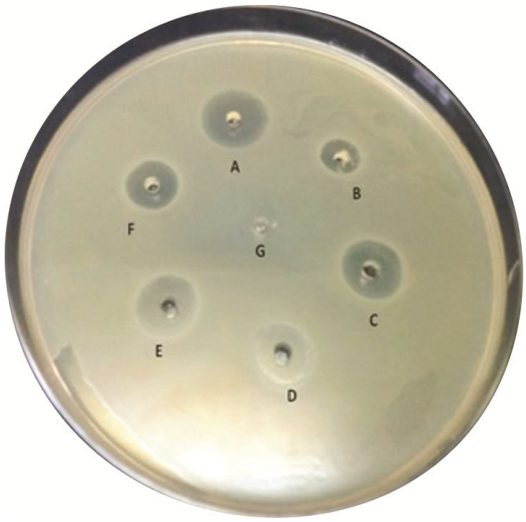


Fig. 1 — Inhibitory activity on *Salmonella typhi:* 50 µL of sample was added to the well and incubated for 18 h. (A)Ampicillin (100 µg); (B): HPLC peak 5 (14.237 min); (C-F) are ammonium sulfate precipitates dialyzed v/s phosphate buffer. [The bacterial culture either induced in presence of *Salmonella* or uninduced), C-70% induced, D-70% uninduced, E-60% induced, F=60% uninduced, G-blank (DW)]

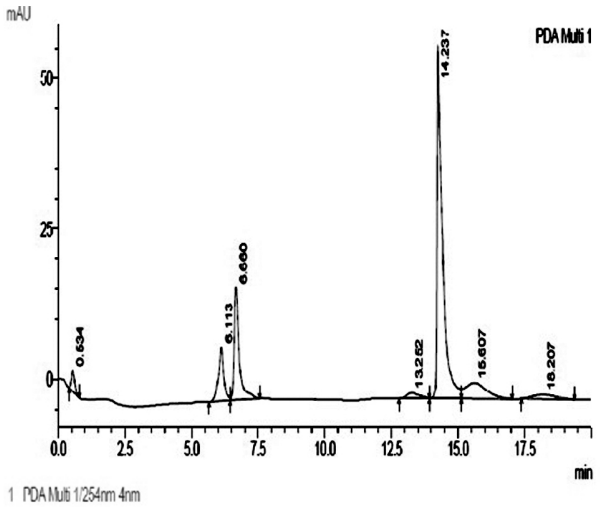


Fig. 2 — Preparatory HPLC for the proteins obtained from 70% ammonium sulphate precipitated and dialysed sample: Flow rate 10.8 mL/min, binary gradient elution: Solution A= Water: TFA (100:0.1), Solution B = Acetonitrile :TFA (100:0.1), Run conditions: in gradient (B solution ranging from 10-80% for 10 min, 80% for 5 min and 80-10% for 10 min). Peak at 14.237 minute represents peak 5

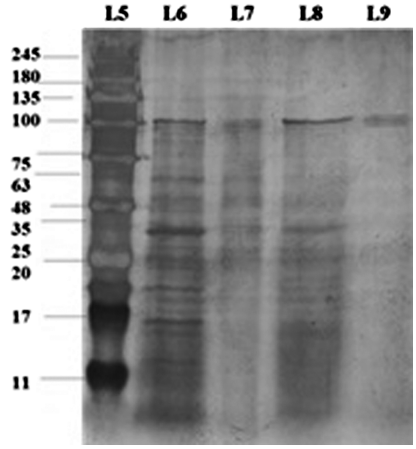


Fig. 3 — Silver stained SDS PAGE gels. [separating gel -15%, L5=Mol. Wt. marker, Lane 6 to 8 are culture supernatants precipitated with ammonium sulphate and dialyzed v/s phosphate buffer, either induced (ES) in the presence of *Salmonella* or uninduced (E): L6=70%ES, L7=70%E, L8=70%ES, L9= HPLC Peak 5 of 70%ES]

imicrobial amino acid stretches using CAMPR3 & APD3 tools (Table 2)[27-29](Suppl. Fig. S1- S6).The secondary structures of the peptides were predicted using PEP- FOLD tool (Table 3).

To identify the isolate CBI-8, DNA was isolated and 16s rDNA sequence was amplified (Primers: Forward Primer AGAGTTTGATCCTGGCTCAG & Reverse Primer AAGGAGGTGATCCAGCCGCA) (Eppendorf Master Cycler, Nexus PCR Cycler) (Fig 4). The PCR band was sequenced and the obtained sequence was queried in the data base to confirm that the organism was *Klebsiella variicola.* Additionally, the CBI-8 isolate was reported to be *Klebsiella variicola/quasipneumoniae*, strain IS93 (Sequence ID: >8756/8850 (IS93), in 16S RNA sequencing by MTCC Chandigarh (NCBI-GENBANK, sequence Id MN814029) (Fig 5)[38] and in MALIDI TOF Bacterial Identification by 'Charles river Accugenix India' ltd, Mumbai.

Table 1 — Peptide mapping of the Mass spectrometry data analysed by BLASTp for *Klebsiella* taxid (570)

MS data search file name	No of peptides mapped	% of coverage	Accession no from MS data & NCBI	Mapped peptide sequence	Protein name
27052020-AMPs-APDs_proteins	01	29	AP02279	1.FMGTALKI	AMP Brevinin
7052020_Klebisella-Variicola_proteins	12	21	tr|A0A2N4Z017 |A0A2N4Z017_KLEVA & WP_131032931.1	2.R.TANLTSNTSFNNSR.F 3.K.GYYNLNDALYAVDQEK.N 4.R.M(+15.99)FTDTQTSIVLAAYR.Y 5.R.LSWSASAQR.V 6.R.ISLNFSYPLWFGDNR.T 7.K.QYQVSYNNSFGR.L 8.R.LNISVPQIYEDQR.L 9.K.NYVSPEFWDK.G 10.R.SYAPEIR.G 11.K.EADGSVEVFSVPYASVAQLLRPGM (15.99)TR.Y 12.R.INAVEIDPK.G 13. R.TPWTTLNGSYSQGEGYR.Q	Fimbrial biogenesis outer membrane usher protein(Fragment) OS= *Klebsiella variicola*
12032020_ rid_1675_proteins	8	12	tr|A0A2N4Z017 |A0A2N4Z017_KLEVA & WP_131032931.1	14.R.TANLTSNTSFNNSR.F 15.K.GYYNLNDALYAVDQK.N 16.R.M(+15.99)FTDTQTSIVLAAYR.Y 17.R.LSWSASAQR.V 18.R.ISLNFSYPLWFGDNR.T 19.K.QYQVSYNNSFGR.L 20.R.LNISVPQIYEDQR.L 21.K.NYVSPEFWDK.G	Fimbrial biogenesis outer membrane usher protein(Fragment) OS= *Klebsiella variicola*
27052020-GCF_000828055.2 _proteins Multi	16	27	WP_008806367.1& WP_131032931.1	22.R.TANLTSSRF 23.K.GYYNLDALYAVDQEK.N 24.R.M(+15.99) FTDTQTSIVLAAYR.Y 25.R.LSWSASAQR.V 26.R.ISLNFSYPLWFGDNR.T 27.K.QYQVSYNNSFGR.L 28.R.LNISVPQIYEDQR.L 29.K.NYVSPEFWDK.G 30.R.SYAPEIR.G 31.K.EADGSVEVFSVPYASVAQLLR PGM (+5.99)TR.Y 32.R.INAVEIDPK.G 33.R.TPWTTLNGSYSQGEGYR.Q 34.G.QENPGTVYQFNDGFIVGSR.E 35. D.GWGGFYLSGR.V 36.R.TVAQVVPWEGSVVK.V 37.R.GVNLSTDDNMFPDGMR.S	Multispecies: type 3 fimbria usher protein MrkC [Klebsiella]
	3	23	WP_040968738.1 & WP_147715994.1	38 R.GYYGSVSHDVK.A 39 R.YINDQTLR.M 0 R.NFYLTGAQELR.N	Multispecies: MBL fold metallo-hydrolase [Klebsiella]

Table 2 — Probable antimicrobial peptides obtained by CAMPR3 & analyzed by APD3 tool

Protein name	Accession no.	Position of peptide	Peptide sequence	CAMPR3				APD3		
				SVM	RF	ANN	DM	% HP	Net charge	Sec. structure
AP02279	AP02279 & WP_100781525.1	1-8 & 192-204	FMGTALKI and FMGTVLAGLALKI	0.907 0.977	0.3765 0.9855	AMP AMP	0.686 0.994	66	+4	helix
Putative	SXE20359.1	7-26	GLFKLSTIFLAMLPALLSGL	0.919	0.761	AMP	0.944	65	+1	Alpha helix
Fimbrial		282-301	RGYAPIIRGIAKTNARVVIK	0.930	0.945	AMP	0.984	45	+5	Alpha helix
Outer		283-302	GYAPIIRGIAKTNARVVIKQ	0.951	0.893	AMP	0.979	45	+4	Alpha helix
Membrane		285-304	APIIRGIAKTNARVVIKQNG	0.909	0.968		0.979	45	+4	Alpha helix
Protein		287-306	IIRGIAKTNARVVIKQNGYQ	0.933	0.921	AMP	0.974	40	+4	Alpha helix
type 3	WP_131032931.1	2-21	KQRSFCPGRLSTAIAIALCC	0.895	0.6775	AMP	0.927	55	+3	Alpha helix
fimbria		3-22	QRSFCPGRLSTAIAIALCCF	0.810	0.7395	AMP	0.969	60	+2	Alpha helix
usher		4-23	RSFCPGRLSTAIAIALCCFP	0.944	0.886	AMP	0.989	60	+2	Alpha helix
protein		5-24	SFCPGRLSTAIAIALCCFPP	0.919	0.566	AMP	0.965	60	+1	Alpha helix
MrkC		6-25	FCPGRLSTAIAIALCCFPPF	0.957	0.7875	AMP	0.972	63	+1	Alpha helix
		7-26	CPGRLSTAIAIALCCFPPFS	0.923	0.7725	AMP	0.981	60	+1	Alpha helix
		8-27	PGRLSTAIAIALCCFPPFSS	0.887	0.5295	AMP	0.932	55	+1	Beta helix
		9-28	GRLSTAIAIALCCFPPFSSG	0.940	0.7375	AMP	0.975	55	+1	Beta helix
		10-29	RLSTAIAIALCCFPPFSSGQ	0.919	0.582	AMP	0.903	55	+1	Beta helix
		287-306	IRGVAQSNALVTVRQGSNII	0.935	0.731	AMP	0.945	45	+2	Alpha helix
		795-814	FISDANAKRAIVKWSGGQCS	0.824	0.926	AMP	0.910	45	+2	Alpha helix
		796-815	ISDANAKRAIVKWSGGQCSV	0.820	0.8995	AMP	0.788	45	+2	Alpha helix
MBL fold	WP_147715994.1	2-21	KLTPIVKGLALAGLLNSLAF	0.939	0.889	AMP	0.979	60	+2	Alpha helix
Metallo		3-22	LTPIVKGLALAGLLNSLAFA	0.937	0.9095	AMP	0.993	65	+1	Alpha helix
Hydrolase		5-24	PIVKGLALAGLLNSLAFAAS	0.9	0.664	AMP	0.994	65	+1	Alpha helix
		6-26	IVKGLALAGLLNSLAFAASA	0.98	0.9705	AMP	0.998	70	+1	Alpha helix
		4-23	TPIVKGLALAGLLNSLAFAA	0.74	0.6075	AMP	0.991	65	+1	Alpha helix

[$CAMP_{R3}$ –Collection of antimicrobial peptides; APD3- Antimicrobial peptide database; SVM-Support vector machine; RF-Random forest; ANN-Artificial neural network; and DA-Discriminant analysis]

Table 3 — 3D structure of the peptides from predicted by PEP-FOLD3 software

S. No	Peptide	Structure by PEP-FOLD3	Link for the structure
1	GLFKLSTIFLAMLPALLSGL		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/U21759408386946/bestModels.html?save
2	RGYAPIIRGIAKTNARVVIK		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/H25023302048922/bestModels.html
3	GYAPIIRGIAKTNARVVIKQ		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/H25023302048922/bestModels.html?save
4	APIIRGIAKTNARVVIKQNG		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/M32759525793076/bestModels.html
5	IIRGIAKTNARVVIKQNGYQ		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/H03574969645023/bestModels.html
6	KQRSFCPGRLSTAIAIALCC		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/U07255986037016/bestModels.html
7	QRSFCPGRLSTAIAIALCCF		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/H09654989496946/bestModels.html
8	RSFCPGRLSTAIAIALCCFP		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/V11795559809923/bestModels.html
9	SFCPGRLSTAIAIALCCFPP		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/H16984574719906/bestModels.html
10	FCPGRLSTAIAIALCCFPPF		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/O19216079364061/bestModels.html
11	CPGRLSTAIAIALCCFPPFS		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/S23401860394955/bestModels.html

(Contd.)

Table 3 — 3D structure of the peptides from predicted by PEP-FOLD3 software (*Contd*.)

S. No	Peptide	Structure by PEP-FOLD3	Link for the structure
12	PGRLSTAIAIALCCFPPFSS		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/V27362387969971/bestModels.html
13	GRLSTAIAIALCCFPPFSSG		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/E31222764504910/bestModels.html
14	RLSTAIAIALCCFPPFSSGQ		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/F02593387437105/bestModels.html
15	IRGVAQSNALVTVRQGSNII		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/S05942064203978/bestModels.html
16	FISDANAKRAIVKWSGGQCS		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/C09797137883902/bestModels.html
17	ISDANAKRAIVKWSGGQCSV		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/W13480524537086/bestModels.html
18	KLTPIVKGLALAGLLNSLAF		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/L16166016710043/bestModels.html
19	LTPIVKGLALAGLLNSLAFA		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/F18745395405054/bestModels.html
20	PIVKGLALAGLLNSLAFAAS		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/F23775145787954/bestModels.html
21	IVKGLALAGLLNSLAFAASA		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/J26176620567083/bestModels.html
22	TPIVKGLALAGLLNSLAFAA		https://mobyle.rpbs.univ-paris-diderot.fr/data/jobs/PEP-FOLD3/I29223918771982/bestModels.html

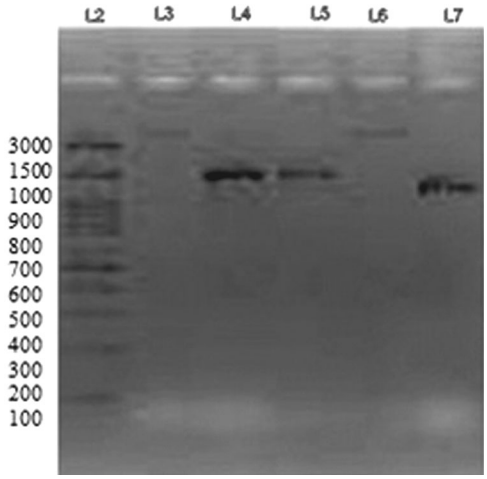


Fig. 4 — Agarose (2%) gels of PCR amplified 16s rDNA sequences (isolate CBI-8). [Lane 2- DNA ladder, Lane 3- Genomic DNA, Lane 4, 5, and 7 are triplicates of PCR amplified products displaying 1500bp (approx.)]

Discussion

In an effort to identify newer antimicrobial proteins and peptides from soil bacteria, we isolated 15 strains and tested antimicrobial activity of their culture supernatants. Of the 15 strains only one isolate (CBI-8) showed inhibitory effect on *Salmonella typhi* and *Staphylococcus aureus*. This organism was identified by MALDI-TOF as *Klebsiella variicola*. Analysis of 16s rDNA sequence resulted in the identification of the strain as *Klebsiella variicola/quasepneumonae* strain, IS93. All the three identified proteins from the MS data mapped to *K. variicola* protein sequences. *K. variicola* organisms are known to adapt to plants, animals and humans[39].

The preparatory HPLC peaks that showed antimicrobial activity to *S. typhi* were subjected to MS analysis that identified the following proteins: (i) Fimbrial outer membrane usher protein; and (ii) MBL fold metallo-hydrolase. Bacterial 'fimbrial outer membrane usher' proteins are pore forming proteins (molecular weight 86 to 100 kDa). They are part of chaperone-usher system that facilitates secretion of the fimbrial proteins. Since the protein is a membrane protein, whether its metabolic / cleavage products showed antimicrobial need to be further investigated[40]. So far, the antimicrobials reported from the *K. variicola* sps are klebicins, whose molecular weight range is 25-40 kDa[8] whereas, our proteins are 100kDa in size.

The second protein identified is MBL (metallo-beta-lactamase) fold metallo-hydrolase. This belongs to a large family of proteins known to have wide range of substrate specificity and hydrolyze beta-lactamases, RNA and DNA[41]. Hence, we suppose that the inhibitory activity observed may be due this protein. The Antimicrobial peptide regions of the

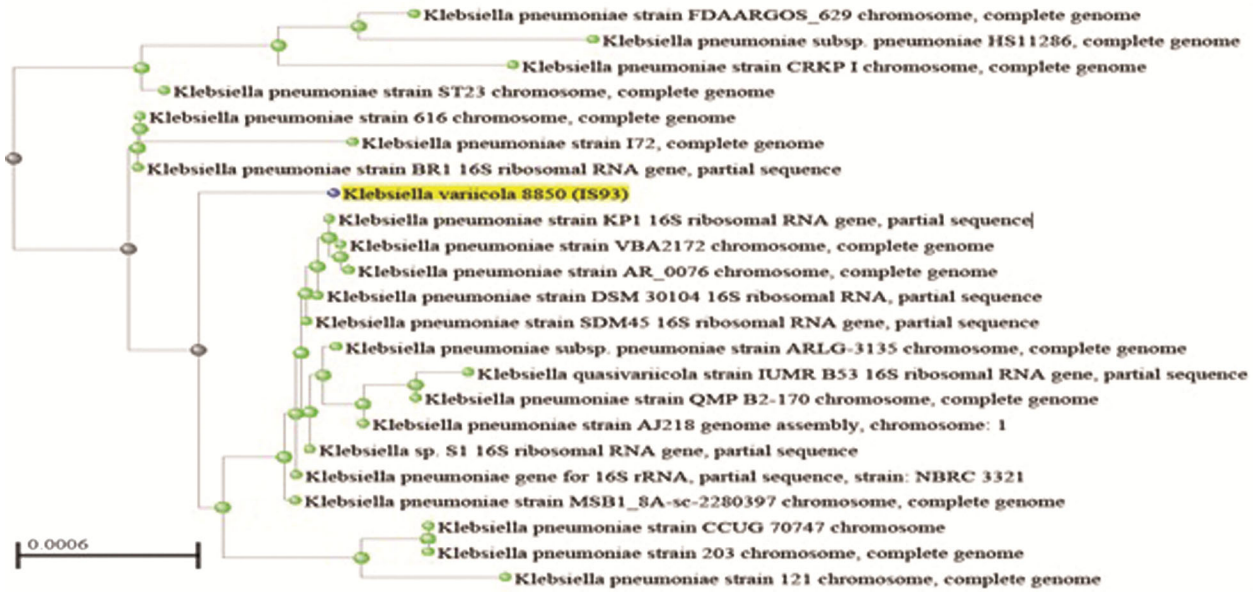


Fig. 5 — Phylogenetic tree of *Klebsiella variicola /quasipneumonae* (IS93 strain)

above two proteins were predicted using CAMPR3 and APD3.

One of the MS data identified peptide (FMGTALKI), mapped to Brevinin (APD3 database) from amphibians (FMGTALKIAANVLPAAFCKIFK KC, APD no. AP02279). BLASTp search in *Klebsiella* sp. with FMGTALKI, resulted in identification of the sequence FMGTVLAGLALKI (100% mapping with gap) (LysE translocase protein, acc.no WP_100781525.1), which is predicted to be antimicrobial in CAMPR3. Further sequence confirmation of the proteins needs to be done by PCR amplification and sequencing of the corresponding genes. Antimicrobial activity of the predicted peptides needs experimental verification

Conclusion

Bioassay guided purification and MS analysis of proteins from new isolate of *Klebsiella variicola* led to identification of two antimicrobial proteins namely Fimbrial outer membrane usher protein and MBL (metallo-beta-lactamase) fold metallo-hydrolase protein. The antimicrobial peptide regions of the above two proteins were predicted using CAMPR3 and APD3 database. One peptide FMGTALKI was identified which mapped to Brevinin from Amphibians in APD3 database and was predicted antimicrobial in CAMPR3.

Further work needs to be done for determination of the sequence function and the antimicrobial activity needs experimental validation.

Acknowledgement

The work done in this paper is a part of the Ph.D. work of Ashwini A. Shahadani. She acknowledges the TEQIP II & TEQIP III for their financial support. She acknowledges Dr Chhaya Patole from NCBS- AMP for the MS data analysis.

Conflict of Interest

Authors declare no competing interests.

References

1 Santajit S & Indrawattana N, Mechanisms of antimicrobial resistance in ESKAPE pathogens. *Biomed Res Int*, 2016 (2016). 2475067 https://doi.org/10.1155/2016/2475067

2 Evelina Tacconelli, WHO Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotic. (2017). https://doi.org/10.1016/S1473-3099(17)30753-3

3 Parish T, Steps to address anti-microbial drug resistance in today's drug discovery. *Expert Opin Drug Discov*, 14 (2019) 91. https://doi.org/10.1080/17460441.2019.1550481

4 De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, Paterson DL & Walker MJ, Antimicrobial Resistance in ESKAPE Pathogens. *Clin Microbiol Rev*, 33 (2020) 181. https://doi: 10.1128/CMR.00181-19.

5 Yang SC, Lin CH, Sung CT & Fang JY, Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Front Microbiol*, 5 (2014) 1.

6 Singh RS, Jain P & Kaur HP, Characterization and antimicrobial activity of lectins from *Penicillium* sp. *Indian J Exp Biol*, 51 (2013) 984.

7 Himandri Malik, Banani Sur, Neha Singhal & Vinod Bihari, Antimicrobial protein from *Streptomyces fulvissimus* inhibitory to methicillin resistant *Streptococcus aureus*. *Indian J Exp Biol*, 46 (2008) 254.

8 Denkovskienė E, Paškevičius Š & Misiūnas A, Broad and Efficient Control of Klebsiella Pathogens by Peptidoglycan-Degrading and Pore-Forming Bacteriocins Klebicins. *Sci Rep*, 9 (2019). https://doi.org/10.1038/s41598-019-51969-1.

9 Kumariya R, Garsa AK, Rajput YS, Sood SK, Akhtar N & Patel Seema, Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microb Pathog*, 128 (2019) 171.

10 Célia C, Silva G,, Sofia P, M Silva & Ribeiro SC, Application of Bacteriocins and Protective Cultures in Dairy Food Preservation. *Front Microbiol*, 9 (2018). https://doi.org/10.1016/S1473-3099(17)30753-3

11 Manuel Montalbán-López, Marina Sánchez-Hidalgo, Eva Valdivia, Manuel Martínez-Bueno & Mercedes Maqueda, Are Bacteriocins Underexploited? Novel Applications for old Antimicrobials. *Cur Pharma Biotech*, 12 (2011) 1205.

12 Nete Bernbom, Tine Rask Licht, Carl-Henrik Brogren, Birthe Jelle, Anette H. Johansen, Iker Badiola, Finn K. Vogensen & Birgit Nørrung, Effects of Lactococcus lactis on Composition of Intestinal Microbiota: Role of Nisin. *App Env Microbiol,* 72 (2006) 239.

13 Chumchalová J & Šmarda J, Human tumor cells are selectively inhibited by colicins. *Folia Microbiol,* 48 (2003) 111.

14 K. Hannah Farkas-Himsley & Rose Cheung, Bacterial Proteinaceous Products (Bacteriocins) as Cytotoxic Agents of Neoplasia. *Cancer Res,* 36 (1976) 3561.

15 L. Kaur S & Kaur S, Bacteriocins as Potential Anticancer Agents. *Front Pharmacol*, 6, (2015). https://doi.org/ 10.1016/S1473-3099(17)30753-3

16 Chung PY & Khanum R, Antimicrobial peptides as potential ant biofilm agents against multidrug-resistant bacteria. *J Microbiol Immun Infect*, 50 (2017) 405.

17 Vizioli J & Salzet M, Antimicrobial peptides from animals: focus on invertebrates. *Trends in Pharm Sci*, 23 (2002) 494.

18 Michel L Leitea, Nicolau Bda, Cunhaa Fabricio & F Costaabcdef, , Antimicrobial Peptides, Nanotechnology and Natural Metabolites as Novel Approaches for Cancer Treatment. *Pharmacology& Therapeutics*, 183 (2017) 160.

19 Gabrielle S, Dijksteel, Magda MW, Ulrich, Esther Middelkoop, Bouke KH & L. Boekema, Lessons Learned from Clinical Trials Using Antimicrobial Peptides (AMPs). *Front Microbiol,* 12 (2021). https://doi.org/10.1016/S1473-3099(17)30753-3

20 Travkova OG, Moehwald H & Brezesinski G, The interaction of antimicrobial peptides with membranes. *Adv Colloid Interface Sci*, 247 (2017) 521.

21 Lee EY, Fulan BM, Wong GC & Ferguson AL, Mapping membrane activity in undiscovered peptide sequence space using machine learning. *Proc Natl Acad Sci USA*, 113 (2016) 13588.

22 Ong ZY, Wiradharma N & Yang YY, Strategies employed in the design and optimization of synthetic antimicrobial peptide amphiphiles with enhanced therapeutic potentials. *Adv Drug Deliv Rev*, 78 (2014) 28.

23 Da Costa JP, Cova M, Ferreira R & Vitorino R, Antimicrobial peptides: an alternative for innovative medicines?. *Appl Microbiol Biotechnol*, 99 (2015) 2023.

24 Magana Maria Magana, Muthuirulan Pushpanathan, Ana L Santos, Leon Leanse, Michael Fernandez, Anastasios Ioannidis, Marc A Giulianotti, Yiorgos Apidianakis, Steven Bradfute, Andrew L Ferguson, Artem Cherkasov, Mohamed N Seleem, Clemencia Pinilla, Cesar de la Fuente-Nunez, Themis Lazaridis, Tianhong Dai, Richard A Houghten, Robert E W Hancock & George P Tegos, The value of antimicrobial peptides in the age of resistance. *The Lancet Infect Dis*, 20 (2020) 216.

25 Mookherjee N, Anderson MA, Haagsman HP & Davidson DJ, Antimicrobial host defense peptides: functions and clinical potential. *Nat Rev Drug Discov*, 19 (2020) 311.

26 M. Hayes, R. P. Ross, G. F. Fitzgerald, C. Hill, &C. Stanton, Casein-Derived Antimicrobial Peptides Generated by Lactobacillus acidophilus DPC6026. *Appl Env Microbiol* , (2006) 2260.

27 Wang Z & Wang G, APD: the antimicrobial peptide database. *Nucleic Acids Res*, 32 (2004) 590.

28 Wang G, Li X & Wang Z, APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res, 44* (2016) 1087.

29 Waghu, F. H., Barai, R. S., Gurung, P. & Idicula-Thomas, S. CAMPR3: A database on sequences, structures and signatures of antimicrobial peptides. *Nucleic Acids Res* 44 (2015) 1094.

30 Melvin P. Silvermann & Donald G. Lundgren, Studies on the Chemoautotrophic Iron Bacterium Ferrobacillus ferooxidans An Improved Medium and a Harvesting Procedure for Securing High Cell Yields. *J Bacte*, 77 (1959) 642.

31 M728, Citrate Agar, HI media Technical Data.

32 Agrawal, N & Prakash, A. Isolation of Lactic Acid Bacteria from Fermented Milk Products and Their Antimicrobial Activity against Staphylococcus aureus. *Int J Food Safety*, 15 (2013) 39.

33 Bauer A, Kirby W, Sherris JC & Turck M, Antibiotic Susceptibility Testing by A Standardized Single Disk Method *Am J Clin Pathol*, 45 (1966) 493.

34 Cihan Darcan, Özge Kaygusuz, Sema Leblebici, Erdem Gülümser & Zeki Acar, Antimicrobial activity of leaf extracts of Bituminaria sp. Genotypes at different growth stages. *Ind J Exp Biol*, 59 (2021) 302.

35 Krisna C Duong-Ly & Sandra B. Gabelli, Salting out of Proteins Using Ammonium Sulphate Precipitation. *Methods Enzymol*, 541 (2014) 85.

36 Ernest J. Sobkow, Noriko Shoji, Akiko Matsui, Takashi Sato, Chie okoyama, Takatomo Takai & Naohiro Kuriyama, *The efficient method development for the analysis of proteins, peptides and synthetic oligonucleotides with a novel hybrid reversed phase column*, poster presented to Eastern analytical Symposium & Exposition, New Jersey, USA, 12-15 November, 2012.

37 Thierry Rabilloud, Laurent Vuillard, Claudine Gilly & Jean Jacques Lawrence, Silver-staining of proteins in polyacrylamide gels: a general overview. *Cell Mol Biol*, 40 (1994) 57.

38 Vinjamuri S, Rajyalakshmi M & Ashwini,S. Klebsiella pneumoniae Strain IS 93 16S Ribosomal RNA Gene, Partial Sequence Genbank: MN814029.1, NCBI, Dec 2019, https://www.ncbi.nlm.nih.gov/nuccore/MN814029.1/

39 Rodríguez-Medina N, Barrios-Camacho H, Duran-Bedolla J & Garza-Ramos U, Klebsiella variicola: an emerging pathogen in humans. *Emerg Microbes Infec*, 8 (2019) 973.

40 Kato Y, Takahashi M, Seki M, Nashimoto & Shimizu-Ibuka A, RNA-hydrolyzing activity of metallo-β-lactamase IMP-1. *PLoS ONE*, 15 (2020). https://doi.org/10.1371/ journal.pone.0241557

41 Gabriel Waksman & Scott J. Hultgren, Structural biology of the chaperone–usher pathway of pilus biogenesis. *Nature Rev Microbiol*, 7 (2009) 765.