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Original Article

پتانسیل شکارگری *دلوویبریو باکتریووروس* علیه جدایه های پاتوژن بالینی دارای مقاومت دارویی بسیار گسترده

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چکیده

سابقه و هدف: باکتری های مشابه *دلوویبریو* (BALOs) گروهی از باکتری های شکارچی هستند که منظور تکثیرشان، سایر باکتری های گرم منفی را به عنوان طعمه مورد تهاجم قرار می دهند. ماهیت باکتری کشی *دلوویبریوها* (*Bdellovibrios*) آنها را به یکی از جایگزین های امیدوارکننده برای آنتی بیوتیک های رایج تبدیل کرده است. در این مطالعه، جداسازی و شناسایی مولکولی *دلوویبریو باکتریووروس* سویه SOIR-1 توصیف شد. الگوی مقاومت آنتی بیوتیکی برخی از جدایه های بالینی تعیین و پتانسیل شکارگری SOIR-1 علیه آنها ارزیابی گردید.

مواد و روش ها: برای جداسازی، ارزیابی ریخت شناسی، و شناسایی مولکولی SOIR-1 به ترتیب از روش های آگار دولایه، میکروسکوپ الکترونی عبوری و واکنش زنجیره ای پلیمراز علیه جایگاه ژنتیکی اختصاصی *دلوویبریوها* (*hit*) استفاده شد. به دنبال تعیین الگوی مقاومت آنتی بیوتیکی جدایه های بالینی، فعالیت باکتری کشی SOIR-1 علیه آنها از طریق ارزیابی تشکیل پلاک و سنجش لیز در کشت های هم زمان مایع ارزیابی گردید.

نتایج: SOIR-1 توسط بررسی میکروسکوپ الکترونی عبوری و واکنش زنجیره ای پلی مراز اختصاصی به عنوان یک سویه *دلوویبریو باکتریووروس* شناسایی شد. تمام جدایه های بالینی دارای ویژگی مقاومت بسیار گسترده در برابر دارو (XDR) بودند و پلاک های شاخص *دلوویبریو* بر روی گستره سلولی همه آنها تشکیل شد. در میان جدایه های بالینی، SOIR-1 بیشترین و کمترین راندمان شکارگری را به ترتیب علیه *آسینتوباکتر باومانی* (٨٤/٣٣٪) و *سودوموناس آئروجینوسا*-٣٦٩ (٥٥/١٦٪) دارا بود.

نتیجه گیری: این مطالعه پتانسیل بالای SOIR-1 را برای لیز جدایه های بالینی XDR صرف نظراز وضعیت مقاومت دارویی آنها آشکار ساخت. بنابراین، *دلوویبریو باکتریووروس* در موارد عفونت های ناشی از باکتری های مقاوم در برابر چندین دارو می تواند به عنوان یک آنتی بیوتیک زنده محسوب گردد.

واژگان کلیدی: مقاومت ضدمیکروبی، فعالیت باکتری کشی، *دلوویبریو*، شناسایی، شکارگری.

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The predatory potential of *Bdellovibrio bacteriovorus* against clinically isolated pathogens with extensively drug-resistance (XDR)

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Abstract

Background & Objectives: *Bdellovibrio*-and-like organisms (BALOs) are a group of predatory bacteria which invade other Gram-negative bacterial cells for growth. The bacteriolytic nature of *Bdellovibrios* makes them one of the promising alternatives for conventional antibiotics. In this study, the isolation and molecular identification of *Bdellovibrio bacteriovorus* strain SOIR-1 was described. The antibiotic resistance pattern of some clinically isolated Gram-negative pathogens was determined, and the predatory potency of SOIR-1 toward them was evaluated. *Material & Methods:* Double-layer agar technique, transmission electron microscopy, and PCR targeting the *Bdellovibrios*-specific *hit* locus were used for the isolation, morphological investigation, and molecular identification of SOIR-1, respectively. Following the antibiotic resistance profile determination of clinical isolates, the bacteriolytic activity of SOIR-1 against them was evaluated through the plaque formation assay and lysis analysis in the broth co-cultures. *Results:* SOIR-1 was identified as a strain of *Bdellovibrios bacteriovorus* through the transmission electron microscopy examination and specific PCR detection. All clinical isolates showed the properties of extensively drug-resistant (XDR) and typical *Bdellovibrios* plaques were developed on their lawns of cells. The SOIR-1 had the highest and lowest predation efficiency among the clinical isolates toward *Acinetobacter baumannii* (84.33%) and *Pseudomonas aeruginosa*-369 (55.16%), respectively.

Conclusion: This study highlights the great potential of SOIR-1 to prey and lyse XDR pathogens, regardless of their antimicrobial resistance state. So, *B. bacteriovorus* can be considered as a living antibiotic in the cases of infections caused by multidrug-resistant bacteria.

Keywords: Antimicrobial resistance, Bacteriolytic activity, *Bdellovibrio*, Identification, Predation.

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Introduction

Simultaneously with the discovery of antibiotics in the mid-20th century, the phenomenon of resistance also emerged. Microorganisms have acquired many diverse mechanisms for tolerance or resistance to antimicrobial agents. Genetic determinants associated with antibiotic resistance can be spread among bacterial communities, both intra -species and inter-genus, leading to the expansion of antibiotic resistance. Nowadays, drug-resistant infections due to the extensive use and abuse of antimicrobial agents in the medical, animal, agricultural, and aquaculture fields are global challenges. Besides, the incomplete administration of antimicrobial medication regimens by physicians or failure to complete the course of treatment by patients is another effective factor in the development of drug resistance phenomena. Recently, multidrug-resistant (MDR) pathogenic bacteria are the main causal of human infections with high mortality, especially in nosocomial infections, even in developed countries (1-4). The most clinical interest drug-resistance Gram-negative bacteria are species of *Enterobacteriaceae* family (*Escherichia*, *Klebsiella*, *Shigella*, *Salmonella*, *Proteus*, *Enterobacter*, *Serratia*, and *Citrobacter*), *Campylobacter* spp., *Acineto bacter baumannii*, *Burkolderia cepacia*, *Stenotrophomonas maltophil-ia*, *Pseudomonas aeruginosa*, *Morganella morganii*, *Yersinia pestis*, and *Haemophilus influenzae* (3, 5-7). Several alternative therapeutic strategies for countering the drug resistance have been proposed; antibiotic resistance breakers (2), bacteriocins, essential oils, quorum-sensing inhibitors, antibodies, nanotherapeutics, (7, 8), bacterial cell wall hydrolases, antimicrobial peptides, enzybiotics, bacteriophage (8-11), and predatory prokaryotes bacteria (12-14). Predatory bacteria acquire their required biosynthetic materials and energy from other live bacterial prey cells (13). The wolf-pack, epibiotic, endobiotic, and periplasmic invasions are the main predatory mechanisms employed by predatory prokaryotes based on the nature of the interactions between the prey cell and predator. Almost all wild-type *Bdellovibrio*-and -like organisms (BALOs) are obligate predators and utilize the periplasmic invasion fashion in which the predator cell invades and grows solely within the periplasmic space of Gram-negative prey bacterium (13, 15). Three taxonomic families are proposed for BALOs; *Bacteriovoraceae* (marine halophilic *Bacteriovorax*), *Peridibacterace-ae* (*Peredibacter*), and *Bdellovibrionaceae* (terrestrial *Bdellovibrios*) (16, 17). Small size (about 0.2–0.5 μm wide and 0.5–2.5 μm long), Gram-negative vibrio-shaped cells, and a single sheathed polar waveform flagellum are the main features of *Bdellovibrios* (18, 19). Assessment of the genome and proteome composition of the *Bdellovibrio bacteriovorus* (the most studied BALOs) revealed its incredible potential for encoding molecular arsenals and hydrolysis enzymes required for an obligate predatory life-style. However, the actual functions and regulatory pathways of these components are not yet fully known (18, 20). *Bdellovibrio bacteriovorus* has a biphasic life cycle (Supplementary Figure S1), a free-swimming attack phase in the search

for prey, and an intraperiplasmic growth phase within the prey cell. Attachment of *Bdellovibrio* to the outer membrane of susceptible prey is followed by penetrating the prey periplasmic space using various degradative enzymes. The prey cell loses its viability and turns into a round-up structure called "bdelloplast" where the *Bdellovibrio* cell inserts the hydrolytic enzymes into the prey cytoplasm to digest prey macromolecules as a source of nutrients and energy. The intraperiplasmic growth phase proceeds with the synthesis of *Bdellovibrio* progenies in a filamentous and non-septated structure. The bdelloplast is lysed as soon as depleting the prey cell's cytoplasm, and individual flagellated motile progeny cells are released to resume new life cycles (17, 18, 21-23). The unique predatory nature of *Bdellovibrios* makes them attractive candidates as a potential living antibiotic and biocontrol agents towards human, animal, plant, and aquatic Gram-negative pathogens (12, 14, 18, 24-36). In this study, the isolation and identification of one native *Bdellovibrio bacteriovorus* were described. The predatory potency of isolated *Bdellovibrio* against some antibiotic-resistant clinical strains was also determined.

Materials and Methods

A) *Bacterial strains and antimicrobial susceptibility testing*

Six characterized and clinically isolated pathogenic bacteria, resistant to the conventional antibiotic therapy, were obtained from the Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, and cultured in the Blood agar plates. Antibiotic susceptibility pattern of the isolates was preliminary determined during the hospitalization of patients. it was completed by us using Kirby-Bauer disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2020) (37, 38) for different antibiotic categories (Table 1). Briefly, some fresh colonies of each isolate were mixed and suspended in the sterile physiological serum to achieve turbidity equivalent to 0.5 McFarland standard. The adjusted bacterial suspensions were streaked on all over the surface of Mueller-Hinton Agar (Condalab, Madrid, Spain) plates using a sterile cotton swab dipped into the suspensions. Selected antibiotic discs were placed aseptically on the surface of the inoculated media after 5 minutes using a sterile pair of forceps. The diameters of inhibition zones were determined and interpreted according to the CLSI (2020) guidelines after incubation of plates at 37 °C for 24 h. Resistance frequency (%) of each prey was calculated using the following formula:

$$\text{Resistance frequency (\%)} = \frac{A}{B} \times 100$$

Where A is the number of resistance and intermediate features among all evaluated antibiotics (B), approved by the US Food and Drug Administration (FDA).

B) Preparation of preys

The TSBY broth medium [Tryptic Soy Broth (Merck, Darmstadt, Germany) supplemented by 0.2% Yeast Extract (HiMedia, Mumbai, India)] was used for culturing of bacterial

Table 1. The antibiotic discs used in this study for antibiogram profiling [(a)].

Antimicrobial class / subclass	Antibiotic discs	Abbreviation and concentration (µg)	Antimicrobial class / subclass	Antibiotic discs	Abbreviation and concentration (µg)
Aminoglycosides	Amikacin	AN (30)	**Cephems / Cephamycins**	Cefoxitin	FOX (30)
	Gentamicin	GM (10)			
	Tobramycin	TOB (10)			
Penicillins / Aminopenicillins	Amoxicillin	AMX (25)	**Cephems /3rd and 4th generation cephalosporins**	Cefepime	FEP (30)
	Ampicillin	AM (10)		Cefixime	CFM (5)
				Cefotaxime	CTX (30)
				Ceftazidime	CAZ (30)
Aminopenicillins + *β*-lactamase inhibitors	Amoxicillin-Clavulanic acid	AMC (20/10)		Ceftriaxone	CRO (30)
	Ampicillin-Sulbactam	SAM (10/10)		Ceftizoxime	CT (30)
Penicillins / Ureidopenicillins	Piperacillin	PIP (100)	**Cephems / 3rd and 4th generation cephalosporins + β-lactamase inhibitors**	Cefotaxime-Clavulanic acid	CTC (30/10)
Penicillins / Carboxypenicillins + *β*-lactamase inhibitors	Ticarcillin-clavulanic acid	TCC (75/10)		Ceftazidime-Clavulanic acid	CZA (30/10)
Ureidopenicillins + *β*-lactamase inhibitors	Piperacillin-Tazobactam	PTZ (100/10)	**Cephems / 1st and 2nd generation cephalosporins**	Cefuroxime	XM (30)
				Cephalexin	CN (30)
Penems / Carbapenems	Imipenem	IPM (10)		Cefazolin	CZ (30)
	Meropenem	MEN (10)			
Monobactams	Aztreonam	AZT (30)	**Quinolones / Fluoroquinolones**	Ciprofloxacin	CP (5)
				Levofloxacin	LEV (5)
Phenicols	Chloramphenicol	C (30)	**Folate pathway antagonists**	Trimethoprim-sulphamethoxazole	SXT (1.25/23.75)
Lipopeptides / Polymyxins	Colistin (Polymyxin E)	CS [(b)]	**Tetracyclines / Glycylcyclines**	Tigecycline	TGC (15)
	Polymyxin B	PB [(b)]			
Tetracyclines	Tetracycline	TE (30)	**Lincosamide**	Clindamycin	CC (2)
	Doxycycline	D (30)	**Macrolides**	Azithromycin	AZM (15)

[(a)] All antibiotic discs were obtained from PADTAN TEB CO., Tehran, Iran.
[(b)] Determined by broth microdilution method.

strains (Table 2). All cultures were incubated at 37 °C with shaking at 160 rpm until the end of exponential to early stationary phase. Cells were harvested by centrifugation at 7,000*g* for 20 min at 4 °C, and the resultant pellets were then washed twice and re-suspended in the sterile 25 mM HEPES (HM) buffer (4-[2-hydroxyethyl]-1-piperazineethanesulfonic

acid) (Fisher Scientific, Fair Lawn, New Jersey, USA) supplemented by 0.22-µm pore size filter sterilized (JET-BIOFIL, Guangzhou, China) 3 mM CaCl2×2H2O and 2 mM MgCl2×6H2O, final pH 7.4. The prey cell suspensions were then adjusted to an optical density of 2.0 at 600nm (OD600) (~109 colony forming units per ml [CFU/ml]). *Escherichia coli*-PTCC 1270 served as prey strain for primary isolation and propagation of the *Bdellovibrio bacteriovorus*, as well as a positive control in the predation assessments. In this regard, the negative control strain was *Bacillus subtilis* subsp. *subtilis* PTCC 1720; a Gram-positive bacterium which is not used as prey by *Bdellovibrios*.

C) Isolation and identification of Bdellovibrio bacteriovorus strain SOIR-1

Bdellovibrio bacteriovorus strain SOIR-1 was isolated in our laboratory from rhizosphere soil using *E. coli*-PTCC 1270 as prey. The double-layer agar plating technique (see below) was used for preliminary isolation of SOIR-1 according to the previously described procedure (39) with some modifications (unpublished data). The isolation process eventually resulted in the formation of lysis plaques. One of the best plaques that had the most characteristics of *Bdellovibrios* plaques was selected and purified by three successive double-layer agar plating techniques. Transmission electron microscopy (TEM) and *Bdellovibrio*-specific PCR-based assessments were used for identification of isolated *Bdellovibrio*.

Transmission electron microscopy

A Formvar carbon-coated 300-mesh copper microscope grid was loaded with one drop of freshly prepared attack-phase *Bdellovibrio* cell suspension (see below) for 5 min at 25 °C. Staining was done using 1% (w/v) solution of uranyl acetate (pH 4.0) for 10 min. The prepared sample was then examined with a Philips CM10 transmission electron microscope at an accelerating voltage of 100 KV (Laboratory of transmission electron microscopy, School of Veterinary Medicine, Shiraz University, Shiraz, Iran).

Bdellovibrio-specific PCR-based detection

The CinnaPure-DNA Kit for Gram-negative bacteria (SinaClon, Tehran, Iran) was used for extraction of genomic DNA from the attack-phase *Bdellovibrio*

Table 2. The bacterial strains used in this study and their sensitivity to the predation by *Bdellovibrio bacteriovorus* SOIR-1.

Species	Strain information (isolate number)	Source [a]	Lysis [b] by SOIR-1
Escherichia coli	Enteropathogenic *E. coli* O111: K58	PTCC 1270	Yes
Bacillus subtilis subsp. *subtilis*	Type strain, Marburg strain	PTCC 1720 (ATCC 6051)	No
Acinetobacter baumannii	578	Clinically isolated [c]	Yes
Escherichia coli	586	Clinically isolated [c]	Yes
Klebsiella pneumoniae	604	Clinically isolated [c]	Yes
Pseudomonas aeruginosa	566	Clinically isolated [c]	Yes
Pseudomonas aeruginosa	369	Clinically isolated [c]	Yes
Pseudomonas aeruginosa	2946	Clinically isolated [c]	Yes
Bdellovibrio bacteriovorus strain SOIR-1	(GenBank accession number: MG230309.1) [d]	Our laboratory [d]	---

[a] PTCC (Persian Type Culture Collection, Tehran, Iran), ATCC (American Type Culture Collection, Manassas, Virginia, USA).
[b] Indicated by plaque formation through double-layer agar plating technique.
[c] Obtained from the Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[d] Isolated and characterized previously in our laboratory from rhizosphere soil (unpublished data).

cell suspension (~109 cell/ml). The amplification of *Bdellovibrio* specific *hit* (host interaction) locus was carried out using the Hit-FW (5/-GACAGATGGGATTACTGTCTTCC-3/) and Hit-RW(5/-GTGTGATGACGACTGTGA ACGG-3/) primers (40) in a PCR reaction with the final volume of 20 µl containing: 10 µl Taq DNA polymerase master mix red 2X (Ampliqon, Odense, Denmark), 0.5 µl of each primer (20 µM), 1.5 µl template DNA (20 ng/µl), and 7.5 µl sterile deionized distilled water. The following thermal conditions were provided by a DNA thermo-cycler (BIO-RAD, Hercules, CA, USA): initial template denaturation at 95 °C for 5 min, followed by 20 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min, and the final extension step at 72 °C for 10 min. The PCR products were electrophoresed on 1.8% Tris-Borate-EDTA (TBE) agarose gel and visualized using a U: Genius3 GelDoc system (SYNGENE, Cambridge, United Kingdom).

D) Bdellovibrio bacteriovorus SOIR-1 *in the attack-phase*

One pure and fresh plaque of *Bdellovibrio bacteriovorus* SOIR-1 and surrounding prey region maintained on the HM-based double-layer agar plates was lifted and inoculated into 40 ml of *E. coli* PTCC 1270 prey cell suspension in HM buffer (~109 CFU/ ml). The co-culture was incubated at 30 °C with shaking at 200 rpm, and monitored for prey lysis caused by SOIR-1 growth after 72-96 h (clearing of prey suspension along with the reduction of initial OD600). Remaining prey cells and derbies were removed sequentially by centrifugation of lysate (4,000*g* for 10 min at 4 °C) and passing the resultant supernatant three times through a 0.45-µm pore size syringe filter (Orange scientific, Braine-l'Alleud, Belgium) to generate a filtrate lysate containing attack-phase SOIR-1. Centrifugation at 27,000*g* for 25 min at 4 °C was used to concentrate the attack phase SOIR-1 cells. The SOIR-1 concentration was adjusted to ~109 plaque-forming units per ml (PFU/ml) using re-suspending of resultant pellet in appropriate HM buffer (109 PFU/ml gave OD600 nm ca. 0.15).

E) Predatory and lytic activity of Bdellovibrio bacteriovorus SOIR-1

The predatory potency and lytic activity of SOIR-1 toward potential prey (Table 2) were evaluated via two separate assessments; the plaque formation on a lawn of bacterial cells and lysis analysis in broth co-cultures.

Plaque assay

Double-layer agar plating technique was employed. Fresh filtrate lysate of attack-phase SOIR-1 (~109 PFU/ml) was serially diluted 10-fold, and 300 µl of each dilution was mixed with 900 µl of each bacterial cell suspension (~109 CFU/ml) in six ml of molten HM top agar (25 mM HEPES buffer amended with 0.7% agar). The mixtures were immediately spread over the surface of HM bottom agar (25 mM HEPES buffer amended with 1.5% agar), and the plates were incubated for ten days at 37 °C. The appearance of typical clear lytic plaques on the double-layer agar plates, with a

progressive increase in the size, indicated the susceptibility of bacterial prey to the predation by SOIR-1.

Lysis in broth co-cultures

Twenty-five ml of each fresh bacterial cell suspension in HM buffer (~109 CFU/ml) was inoculated with 400 µl of fresh filtrate lysate of attack-phase SOIR-1 (final cell density of ~104 PFU/ml). The co-cultures were incubated at 37 °C with shaking at 200 rpm. The CFU/ml (using standard spread plate count method), PFU/ml (through double-layer agar plating technique as described above), and OD600 parameters were monitored at 24-h intervals. The bacteria were considered as susceptible if significant changes occurred in the initial OD600, CFU/ml, and PFU/ml of co-cultures. Bacterial cell suspensions without inoculated SOIR-1 and SOIR-1 cell suspensions without any prey served as additional negative controls. Killing rate (%) of each prey at each interval was calculated using the following formula:

$$\text{Killing rate } (\%) = \left[\frac{N0 - Ni}{N0}\right] \times 100$$

where N0 and Ni are the number of bacterial cells at the day 0 (starting day) and day i (evaluation day), respectively.

F) Statistical analysis

All experiments were carried out in triplicate, and the results were presented as the mean ± standard deviation (SD) error. The analysis of variance (ANOVA) and posthoc Tukey test were used for comparing the means, determining the statistically significant differences, and multiple comparisons between groups (GraphPad Prism v6.07 for Windows, GraphPad Software, La Jolla, California, USA). Differences were considered significant at the P-value≤0.05 level. The drawing of graphs and diagrams was done by the GraphPad Prism v6.07 software and Microsoft Office for Windows© tools, 2013 (Microsoft, Redmond, Washington, USA). Other mathematical and statistical analyses were performed using Microsoft Excel© for Windows©.

Results

A) *Antimicrobial susceptibility profile*

Table 3 demonstrates the antibiotic susceptibility profile of clinically isolates. *E. coli* 586 and *K. pneumoniae*-604 showed the extended-spectrum β-lactamase (ESBL) properties. Some additional antibiotics with intrinsic resistance for prey were also incorporated and evaluated in this study. These can be helpful in at least three ways. Firstly, they provide a way to assess the accuracy of testing methods. Secondly, they aid in recognition of common phenotypes. Thirdly, they can assist with the verification of cumulative antimicrobial susceptibility test data (38). Regardless of these intrinsic resistance antibiotics, the frequency of antibiotic resistance for preys were 69.69% (23/33, *E. coli* 586), 81.81% (27/33, *K. pneumonia* 604), 92.30% (24/26, *A. baumannii* 578), 85% (17/20, *P. aeruginosa* 566), 60% (12/20, *P. aeruginosa* 2946), and 70% (14/20, *P. aeruginosa* 369).

B) Identification of Bdellovibrio bacteriovorus strain SOIR-1

One of the plaques developed during

Table 3. Antibiotic susceptibility of MDR isolates.

Species	Susceptibility patterns			
	Susceptible	**Intermediate**	**Resistant**	**Intrinsic resistant**
***E. coli* 586**	AN FOX CP IPM MEN LEV TOB PB C TGC	PTZ	XM AM AMC SAM CAZ CT AZT CTC CZA CZ CFM CTX AMX D CRO PIP GM CN TE TCC FEP SXT	CC AZM
***K. pneumonia* 604**	LEV TOB C GM AN IPM	FOX CP PTZ TGC MEN AZT	CN CZ AMX AMC CZA CT SAM PB CTC XM FEP CFM CRO TCC SXT TE D CS CTX PIP CAZ	CC AM AZM
***Ac. baumannii* 578**	PB CS	PTZ	CFM AN SAM FEP CT CZA CTX CTC CN D LEV GM TE MEN PIP IPM TGC SXT TCC TOB CP CRO CAZ	AMX AMC AM AZM AZT CZ CC XM C FOX
***P. aeruginosa* 566**	PTZ PB CS	AN AZT	CAZ D FEP CFM MEN CP CN CT TCC LEV IPM GM CZA PIP TOB	CC AMX AMC AM TE SAM AZM CZ TGC CTX CTC FOX SXT CRO XM C
***P. aeruginosa* 2946**	GM IPM AZT LEV CP PB CS TOB	AN PTZ	CFM CAZ CZA CT AMX AZM D MEN PIP TCC AMC CTX AM FEP CN CTC CRO XM C CC TE TGC SXT	
***P. aeruginosa* 369**	CP PIP CS PTZ GM PB	TOB	AZT FEP CFM CAZ AMX AMC AM SAM CT CN D IPM AZM CZ CTX CTC MEN TCC LEV CZA FOX CRO XM C AN CC TE TGC SXT	

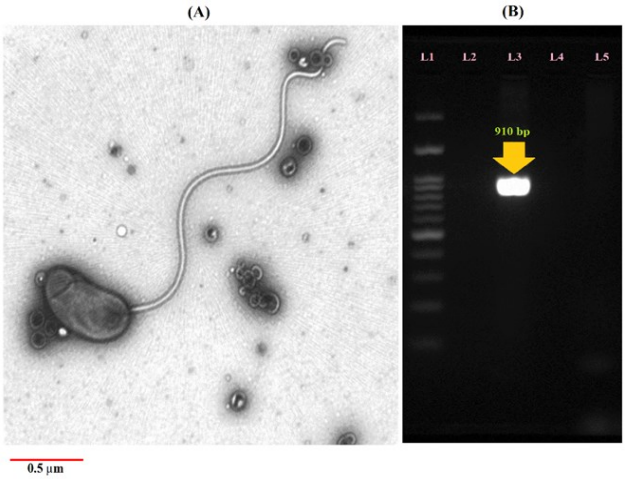

Figure 1. **(A)** Transmission electron microscope image of *Bdellovibrio bacteriovorus* strain SOIR-1 in the attack phase. **(B)** Agarose gel electrophoresis of PCR products using *Bdellovibrio*-specific Hit-FW and Hit-RW primers. L1; DNA ladder marker (100 bp), L2; Negative control (distilled water), L3; SOIR-1 genomic DNA, L4; Empty well, L5; *E. coli* genomic DNA..

the isolation process with the most *Bdellovibrios* plaque characteristics (see below) was selected for purification and identification. Transmission electron microscope evaluation showed that the isolated predator had the distinctive features of *Bdellovibrios*. It was small vibrioid-shaped cell (~ 0.8 µm in length and 0.25 µm in width) with a terminal single polar sheathed flagellum (~ 2.3 µm) (Figure 1A). *Bdellovibrio*-specific PCR detection targeting the *hit* locus (Figure 1B) was also performed. An expected PCR product (910 bp) was generated and confirmed that the isolated SOIR-1 was a strain of *Bdellovibrio bacteriovorus* since the *hit* locus has been proposed to be restricted to *B. bacteriovorus*. This genetic locus contains

genes encoding the required proteins for attachment and invasion of *Bdellovibrio* to the prey cell (17, 23, 41). Furthermore, 16S rRNA gene sequence was also analyzed and deposited in the GenBank database of National Center for Biotechnology Information (NCBI) under the accession number of MG230309.1 (unpublished data).

C) Plaque formation by Bdellovibrio bacteriovorus SOIR-1

The double-layer agar plating technique was used for the development of *Bdellovibrios* lytic plaques. All Gram-negative strains, including MDR clinically isolates, were susceptible to the attack and lyse by SOIR-1 (Table 2). The SOIR-1 developed typical plaques of *Bdellovibrios*-type; regular with sharp boundaries, circular, clear, and without any colony in their centers. The tiny plaques (1mm in diameter) appeared after 3-4 days, progressively increased in size (5-20 mm in diameter) upon a more extended incubation period, and eventually covered almost the entire lawn of prey cells in plates through the attachment of plaques to each other (Figure 2). The growing plaque is the unique feature of *Bdellovibrios*, caused by the high motility of the *Bdellovibrio* cell within the soft (top) agar (19). No plaque was developed in the case of *B. subtilis* subsp. *subtilis* since the Gram-positive bacteria are not in the prey range of *Bdellovibrios*.

D) Lytic activity of Bdellovibrio bacteriovorus SOIR-1 *in broth co-cultures*

No significant changes were observed in the OD600 and CFU/ml of all non-inoculated preys with SOIR-1 during 7-days incubation ($p>0.05$) (Supplementary Figure S2 and S3). Whereas in the co-cultures inoculated with SOIR-1, the OD600 and CFU/ml decreased significantly ($p<0.05$), except for *B. subtilis* subsp. *subtilis* as the negative control ($p>0.05$) (Figure 3 and 4, Supplementary Figure S2 and S3). In the case of susceptible preys inoculated with SOIR-1, the cell density of SOIR-1 (PFU/ml) increased significantly ($p\leq0.05$) at the same time as the number of prey cells (CFU/ml) decreased remarkably ($p\leq0.05$) (Supplementary Figure S3). The most intense descending changes in the OD600 occurred mainly within the first 24 hours ($p\leq0.05$), and then the slope of these changes became milder ($p>0.05$) (Figure 5). Regardless of the first 24 hours, the OD600 differences between consecutive days were insignificant ($p>0.05$). Exceptions are *E. coli*-PTCC 1270 and *E. coli*-586 in which the OD600 was decreased significantly for two successive days ($p\leq0.05$). The OD600 changes of *P. aeruginosa*-369 as another exception was significant between the second and third days ($p\leq0.05$) (Supplementary Figure S2). Similarly,

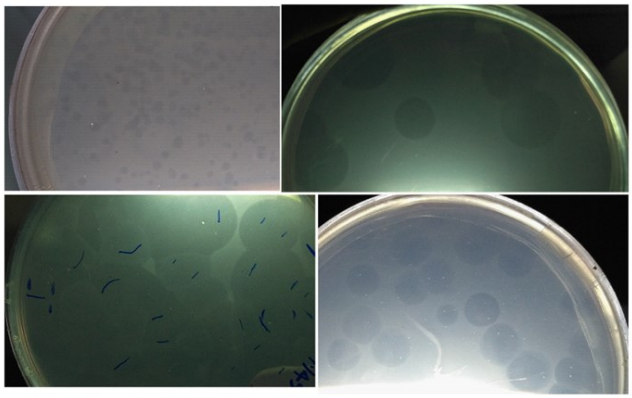

Figure 2. Some typical, tiny, growing, and attached lytic plaques developed by *Bdellovibrio bacteriovorus* SOIR-1 on the lawns of prey cells.

the sharpest decline in the CFU/ml and the most SOIR-1 particle production (PFU/ml) was during the first day after incubation with SOIR-1 (Figure 5, Supplementary Figure S3). A comparative view of the ΔOD600 of preys and killing rate (%) of SOIR-1 is presented in Figure 6. Regarding the first day after the inoculation of preys with SOIR-1, *A. baumannii*-578 showed the highest OD600 reduction rate (1.33 ± 0.04), and

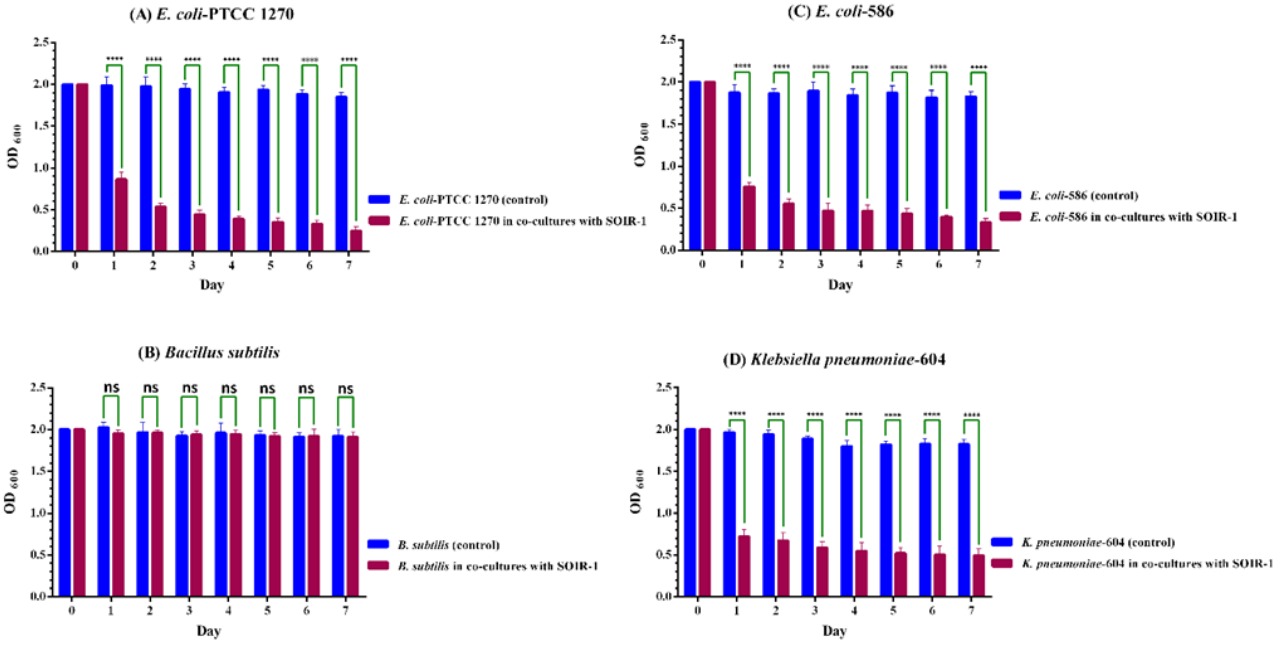

Figure 3. The changes in the OD_{600} of *E. coli*-PTCC 1270 (positive control), *B. subtilis* (negative control), *E. coli*-586, and *K. pneumoniae*-604 in the co-cultures with SOIR-1.

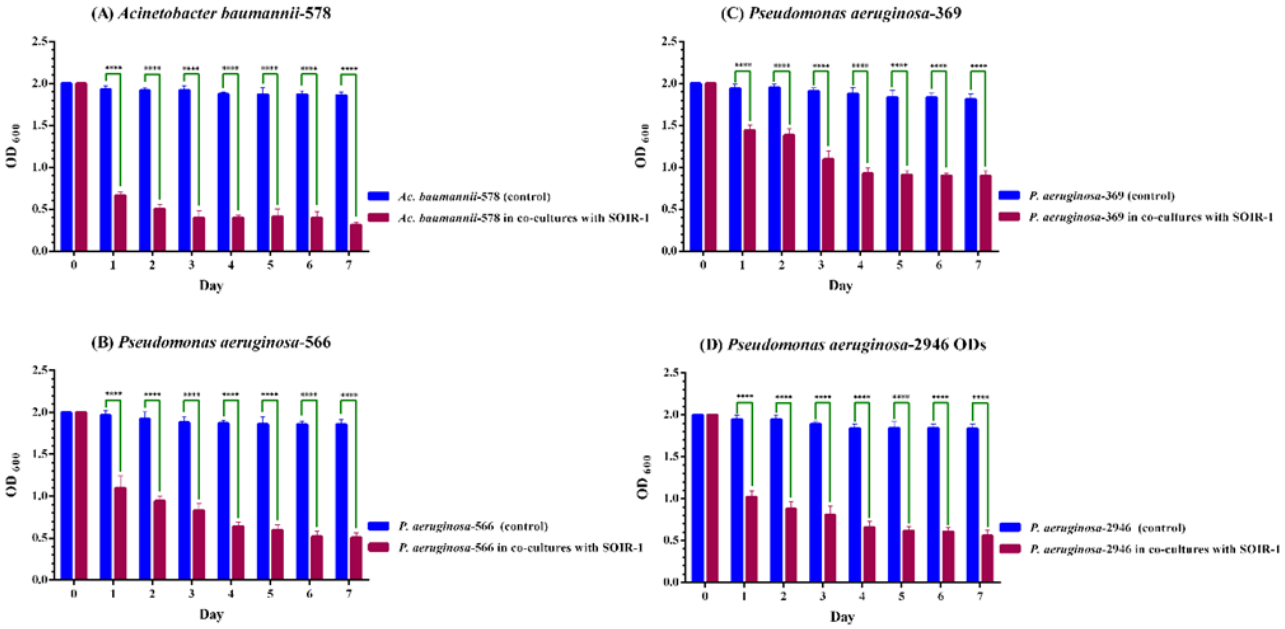

Figure 4. The changes in the OD_{600} of *A. baumannii*-578, *P. aeruginosa*-566, *P. aeruginosa*-369, and *P. aeruginosa*-2946 in the co-cultures with SOIR-1.

P. aeruginosa-369 had the lowest ones (0.55 ± 0.06). However, the highest OD600 reduction rate at the endpoint of the evaluation period (day 7) was recorded for *E. coli*-PTCC 1270 (1.75 ± 0.04), and this was still lower than other preys in the case of *P. aeruginosa*-369. Mutually, the higher and lower killing rate (%) of SOIR-1 toward susceptible prey at the first evaluation point (day 1) was for *A. baumannii*-578 (66.83 ± 2.02) and *P. aeruginosa*-369 (27.83 ± 3.01), respectively. The final killing rate of SOIR-1 was as follows (from the highest to lowest); *E. coli*-PTCC 1270 (87.5 ± 2.17), *A. baumannii*-578 (84.33 ± 1.6), *E. coli*-586 (83.33 ± 2.25), *K. pneumoniae*-604 (75.16 ± 3.81), *P. aeruginosa*-566 (74.66 ± 2.75), *P. aeruginosa*-2946 (72 ± 3.27), and *P. aeruginosa*-369 (55.16 ± 3.01).

Discussion

The present study described the predatory potential of isolated and identified *Bdellovibrio bacteriovorus* strain SOIR-1 toward some MDR clinically isolated Gram-negative pathogens. The results indicated the great potential of SOIR-1 to prey and lyse MDR pathogens, regardless of their antimicrobial resistance state. We concluded that *B. bacteriovorus* could be considered a living antibiotic in the cases of infections caused by multidrug-resistant bacteria. There are several definitions for describing the intensity of drug resistance in bacteria. According to one of the most approved standards, MDR is referred to as a bacterium with resistance against at least one antimicrobial agent in more than 3 antimicrobial categories. A bacterium with resistance against at least one critical antimicrobial agent in most antimicrobial categories is defined as extensively drug-resistant (XDR). Resistance to all commercially available antimicrobial classes for empirical treatment is called pan-drug resistant (PDR). It is estimated that the worldwide annual mortality rate due to the infections caused by these "superbugs" will exceed 10 million in 2050 (5, 6). Given this definition and the resistance status of bacteria, we can conclude that the clinically isolated strains in this study are XDR, and there is a risk for them to become more resistant (the PDR state). Expanding multidrug-resistant pathogens makes it increasingly necessary to re-search and re-evaluates alternatives to conventional antimicrobials. If we do not care about the

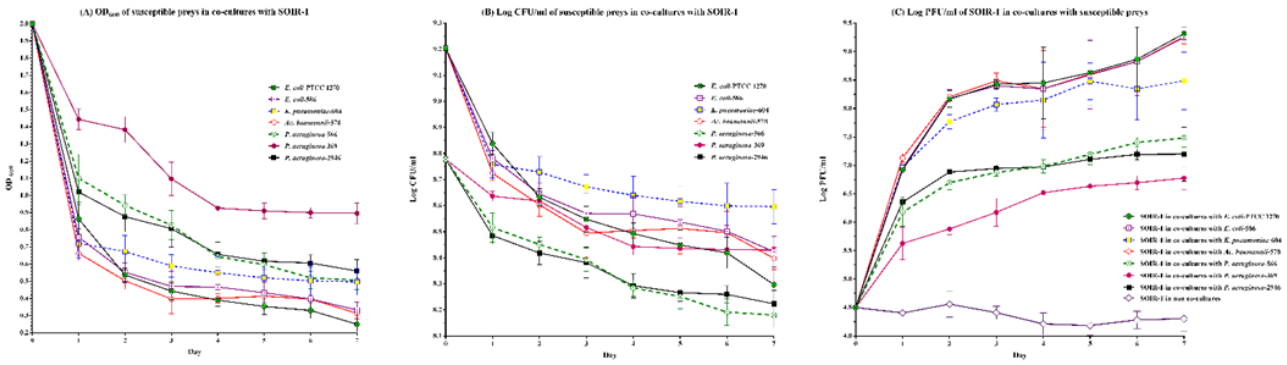

Figure 5. The gradual changes in the OD_{600} **(A)**, CFU/ml **(B)**, and PFU/ml **(C)** parameters in the co-cultures of susceptible preys with SOIR-1.

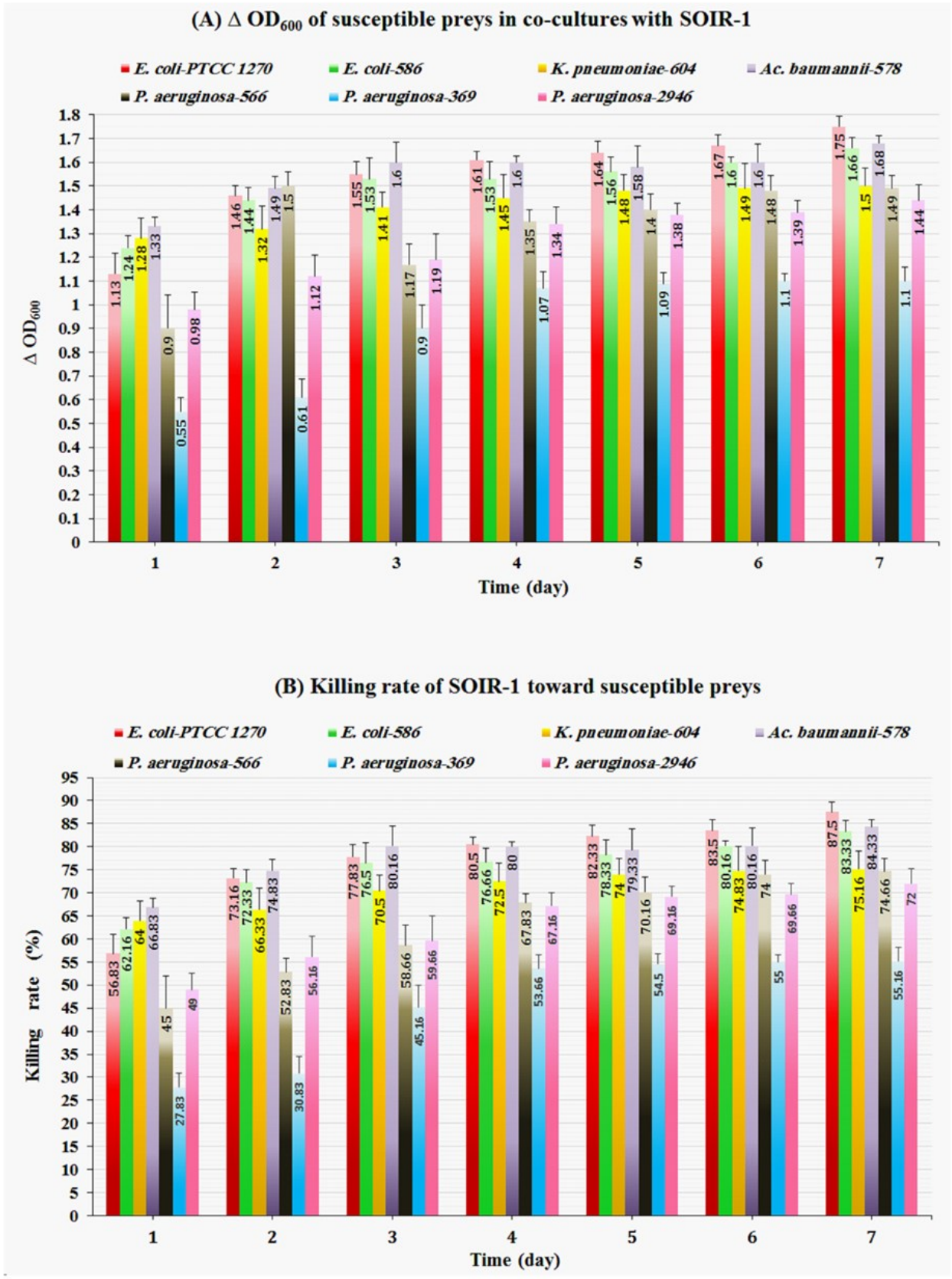

Figure 6. The ΔOD_{600} and killing rate parameters in the co-cultures of SOIR-1 with preys.

issue of MDR, we will return to the dark ages, the pre-antibiotic era. Predatory bacteria, especially *Bdellovibrios*, are one of the promising alternatives for conventional antibiotics. The potential of native *B. bacteriovorus* strain SOIR-1 to act as a living antibiotic against the most challenging clinically isolated MDR Gram-negative pathogens was investigated. Although with different predation efficiency, SOIR-1 was able to kill and lyse all MDR preys. The different predation efficiency is the result of preferential predation; an undeniable fact of *Bdellovibrios*, which has been mentioned in previous studies. Rogosky et al. (2006) documented that the *B. bacteriovorus* 109J kills some preys more readily than others, and linked the predation efficiency to the attachment efficiency (42). Li et al. (2011) stated that the preys used for primary isolation of particular BALO strain are the most susceptible preys (43), a hypothesis that ruled out in our previous study (unpublished data). The interaction between *Bdellovibrios* and their prey is affected by some complex and unknown mechanisms. One of the most critical factors involved in the attachment and preferential predation by *Bdellovibrios* is the type and accessibility of potential specific receptors on the prey cell surface. These receptors are not yet fully characterized and are presumably located in the core portion of lipopolysaccharide (LPS) of the prey cell wall (44). Two hypotheses are conceivable for the wide prey range of most *Bdellovibrios* toward Gram-negative bacteria. Firstly, *Bdellovibrios* can recognize diverse receptors on the surface of various prey cells. Secondly, *Bdellovibrios* recognize common motifs on the surface of different prey cells as the receptor, which are essential for the viability of prey cells (22, 45). The later hypothesis expresses a kind of survival strategy for *Bdellovibrios*, which means that it is not rational for *Bdellovibrios* to use just a simple receptor, which can easily lead to the prey cell resistance by mutations, unless that it be essential for the viability of prey cell. The differential predation observed in this study can be interpreted as the role of prey receptors; since *E. coli*, *K. pneumonia*, and *A. baumannii* are closer to each other phylogenetically compared with *P. aeruginosa* isolates, the structure of their probable surface receptors is more similar to each other than the *P. aeruginosa* isolates. So, the SOIR-1 attacks them with almost a same efficiency. Alternatively, *Bdellovibrios* may use diverse receptors with various affinities. Considering this hypothesis, the lower killing rate of SOIR-1 toward *P. aeruginosa* isolates can be due to either the lower distribution of main receptors in the cell surface of *P. aeruginosa* isolates or the use of alternative receptors with lower affinity by SOIR-1. Theoretically, antibiotic resistance and its severity can affect the predation efficiency of *Bdellovibrios*, especially where the bacterial cell surface structures change due to antibiotic resistance. Although this hypothesis needs further evaluations, in our study, antibiotic resistance had a minor effect on the predation efficiency of SOIR-1. For example, although the *P. aeruginosa*-566 is resistant against more antibiotics than *P. aeruginosa*-2946 (Table 3), the killing rate of SOIR-1 toward them is not

significantly different ($p>0.05$) (Figure 6). In terms of receptor accessibility, it has been reported that S-layers, but not capsules, protect prey cells from predation by *Bdellovibrios*. S-layers may block the access of *Bdellovibrios* to the potential receptors located in the prey cell wall (46, 47). Predation efficiency of *Bdellovibrios* is also depended on the number of successful collisions between prey and *Bdellovibrios* provided by the swimming speed of *Bdellovibrios* cells in the free-living attack phase. Therefore, disrupting the structure and function of the flagellum or slowing its velocity by any factor can affect the predation efficiency of *Bdellovibrios* (45, 48, 49). The killing rate of SOIR-1 toward *P. aeruginosa*-369 was significantly lower than other *Pseudomonas* isolates ($p\leq0.05$) (Figure 6). As mentioned above, this is not related to the different antibiotic resistance status of *Pseudomonas* isolates. *Pseudomonas aeruginosa*-369 produced high amounts of exo-biopolymers as it grew and released it into the extracellular environment. We concluded that these exo-biopolymers might attenuate the *Bdellovibrios* predation efficiency through two probable mechanisms; covering the possible prey cell surface receptors or reducing the *Bdellovibrios* speed. Our previous unpublished study approved the attenuation effect of viscose exo-biopolymers produced by some certain phytopathogenic bacteria on the *Bdellovibrios* predation efficiency. There are several reports regarding the effects of *Bdellovibrio*s on the MDR Gram-negative pathogenic bacteria and the ineffectiveness of prey's drug resistance on the *Bdellovibrio*s predation comparable to the results of this study. Kadouri et al. (2013) approved the predation ability of *B. bacteriovorus* and *Micavibrio aeruginosavorus* against 14 MDR Gram-negative clinical strains, including *A. baumannii*, *E. coli*, *K. pneumoniae*, and *Pseudomonas* spp., possessing extended-spectrum ß-lactamase, KPC-type carbapenemase, AmpC-type ß-lactamase, and Metallo-ß-lactamase (12). Fluoroquinolone-resistant clinical isolates of *P. aeruginosa* and *Serratia marcescens*, associated with ocular infections, were successfully controlled by *Micavibrio aeruginosavorus* and *B. bacteriovorus* (36). Sun et al. (2017) evaluated the predation efficacy of *B. bacteriovorus* on clinical MDR or XDR Gram-negative pathogens, including *A. baumannii*, *Escherichia coli*, *K. pneumoniae*, and *P. aeruginosa*. All prey were susceptible to the predation by *B. bacteriovorus* both in planktonic and biofilm states (50). Dharani et al. (2018) demonstrated the susceptibility of clinically relevant colistin-resistance *A.baumannii*, *E.coli*, *K. pneumoniae*, and *P. aeruginosa* to predatory bacteria, *Micavibrio aeruginosavorus* and *B.bacteriovorus* (30). Some studies have shown that *Bdellovibrios* are safe. They have been isolated from the guts of healthy mammals, where they are likely to have a probiotic role (40, 41). *Bdellovibrios* do not invade mammalian cell lines (18, 21, 36, 51). Furthermore, *Bdellovibrio*s have a unique and less immunogenic lipid A structure in their LPS with lower binding affinity to the LPS receptors presented in the surface of human cells (52). Ingestion, injection, and topical usage of

B. bacteriovorus in animal models do not show any harmful side effects (15, 53-55).

Conclusion

The purpose of this study was to evaluate the bacteriolytic activity of native *B. bacteriovorus* SOIR-1 against MDR Gram-negative pathogenic bacteria. The successful lysis of target bacteria nominates SOIR-1 as a promising bio-agent for the control and treatment of infections caused by these pathogens. Although SOIR-1 showed differential predation, the antibiotic resistance did not affect the predation ability of SOIR-1. However, the viscose exo-biopolymer produced by *P. aeruginosa*-369 attenuated the predation efficiency of SOIR-1. *Bdellovibrios* are considered as "amphibiotic", which represents their dual probiotic and antibiotic nature (15). It can be concluded from these findings that predatory bacteria, especially *Bdellovibrios*, might be part of future therapeutic strategies as biocontrol agents, probiotic, or living antibiotics. However, there are still some challenges in this way that need to be further addressed. Firstly, *Bdellovibrios* are not able to completely eradicate the prey population, i.e., there are still some non-attacked prey cells. Such transient resistance is due to a plastic phenotype rather than permanently genetically encoded one, and the resistance to the *Bdellovibrios* is lost quickly (56). Nevertheless, we have this hypothesis that at least a significant part of the prey population cells are eradicated by the *Bdellovibrios* attack. This can lead to the disruption of quorum sensing networks, which play a considerable role in the expression of virulence factors and pathogenesis. Secondly, *Bdellovibrios* have a broad and non-specific prey range toward Gram-negative bacteria compared to bacteriophages. This can be a useful property, mainly when the infections are caused by mixed bacterial species or by an XDR pathogen. Collectively, there is still a long way for the actual use of predatory bacteria in the treatment cycle, and further *in-vivo* evaluations of their safety, performance, and prey resistance should be undertaken. Alternatively, predatory bacteria are a source of antimicrobial substances such as lytic enzymes that can be produced through recombinant technology in the biotechnological processes.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix: Supplementary Data

Supplementary **Figure S1**.

Supplementary **Figure S2**.

Supplementary **Figure S3**.

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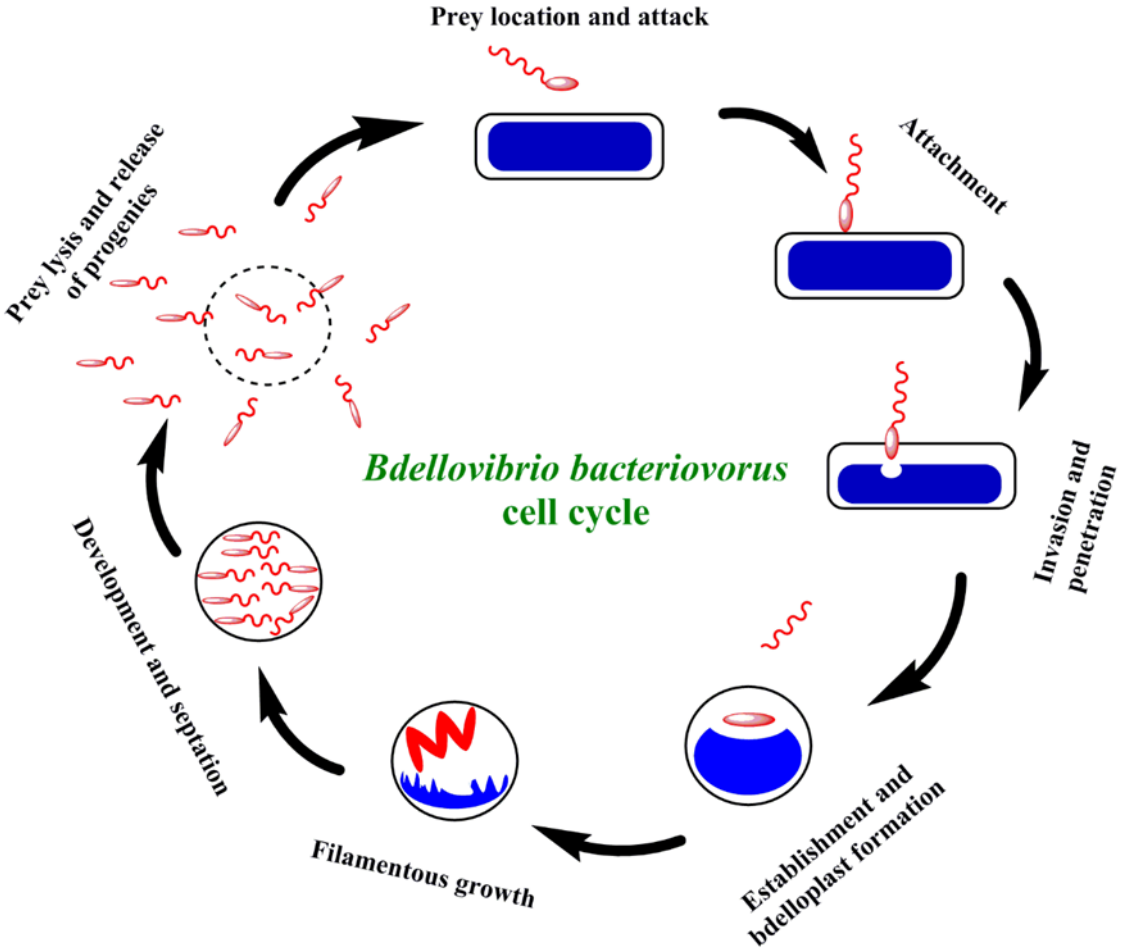
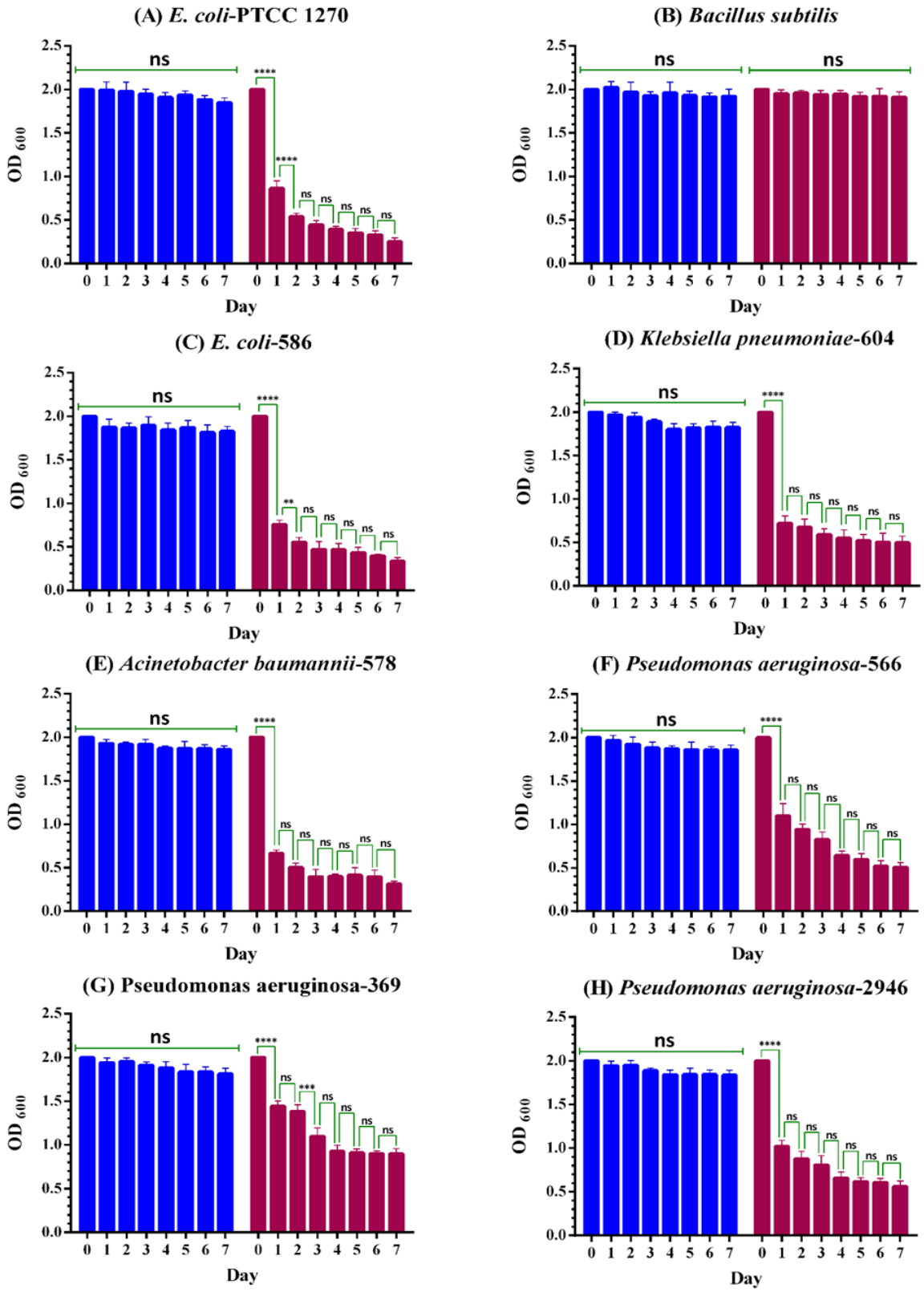
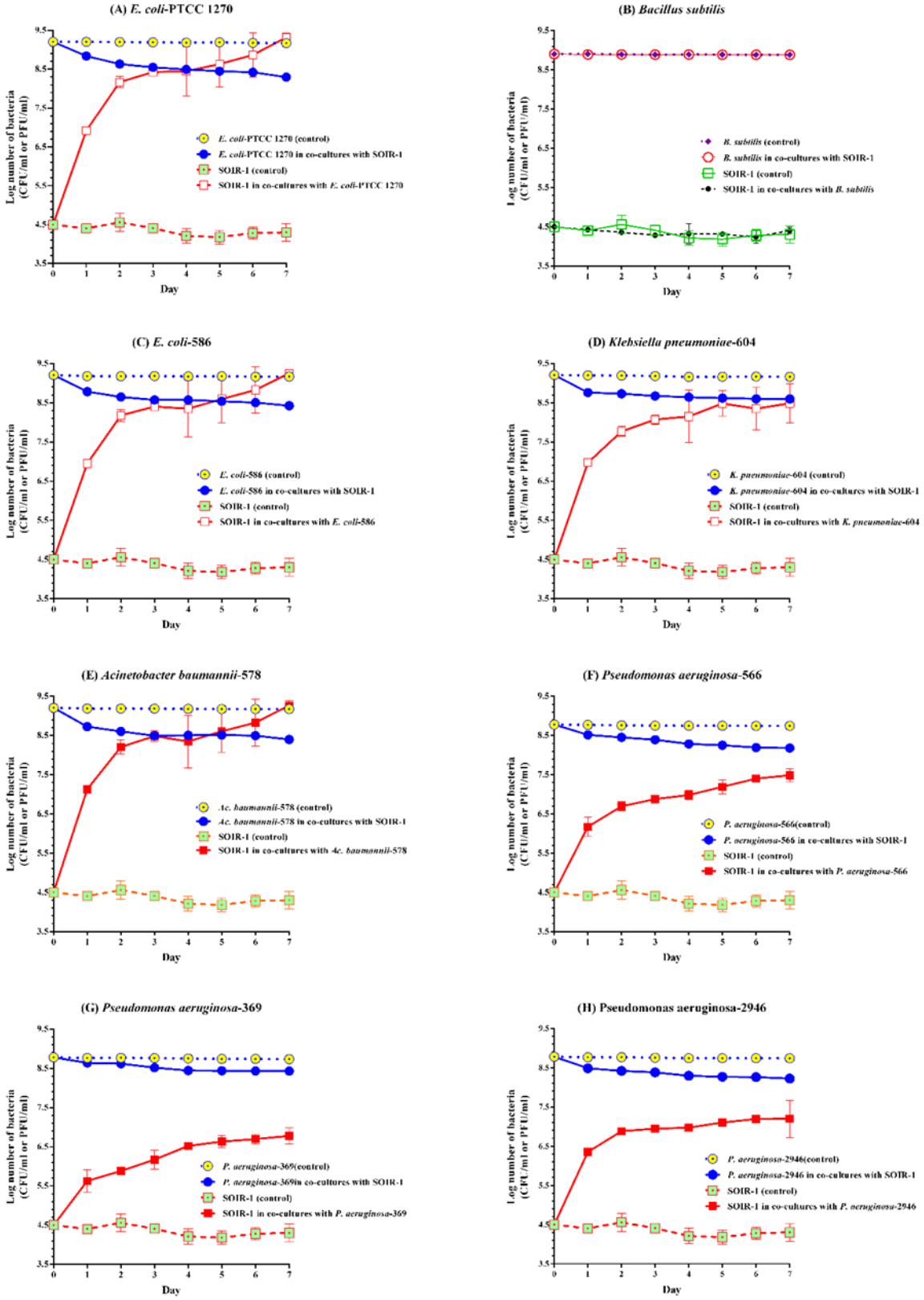


Supplementary Figure S1. The predatory life cycle of *Bdellovibrio bacteriovorus*.



Supplementary Figure S2. The changes in the OD_{600} of preys in the broth co-cultures. Blue column: non-inoculated preys; Red column: inoculated preys with SOIR-1.
ns: Non-significant ($p>0.05$).
*: Significant ($p<0.05$).



Supplementary Figure S3. The synchronized changes in the number of preys (CFU/ml) and SOIR-1 predator (PFU/ml) cells upon predation in the broth co-cultures.