

Cross-Species Induction and Enhancement of Antimicrobial Properties in Response to Gamma Irradiation in *Exiguobacterium* sp. HKG 126

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Abstract A gram positive, extreme haloalkaliphilic, radioresistant bacterium was isolated from mangrove region of Kerala (India) which was characterized as *Exiguobacterium* sp. HKG-126 using morphological, physiological, biochemical and molecular characterization. Present investigation was undertaken to examine *Exiguobacterium* sp. as a potential source of broad-spectrum antimicrobial activity and enhancement in this activity was observed due to cross-species/cross-genera induction and also in response to high dose of gamma (γ) irradiation. Individual studies on the antimicrobial activity of all the co-cultivated bacterial strains before and after mixed culture fermentation, showed excellent enhancement in antimicrobial activity of *Exiguobacterium* sp. against a variety of clinical pathogens. To the best of our knowledge, this is the first report showing existence of an extremely high radioresistant strain of (up to 15 kGy) *Exiguobacterium* sp.

Keywords *Exiguobacterium* sp. · Gamma irradiation · Cross-species induction · Extraction of secondary metabolites · Antimicrobial activity

Introduction

The production of antimicrobial compounds by microbial system is usually assayed under straight forward growth conditions that are very different from their natural environments. The primary role of any antimicrobial compound is to antagonize competitors [1–5]. Microbial interaction during mixed culture involves the production of some communicating chemicals known as quorum sensing signal molecules. When these molecules from one species or genus interact with those of another, they may stimulate or inhibit a number of physiological and biochemical activities including production of secondary metabolites like antimicrobial compounds. Such type of interaction is referred to as cross-species or cross-genera induction or inhibition [6, 7]. Thus, new cultivation strategies, particularly strategies mimicking the natural habitats of these microorganisms, can be used to enhance the production of secondary metabolites. We can greatly influence the productivity of such compounds by maximizing the types of samples collected, diversifying the isolation strategies, optimizing nutrients, temperature, pH, aeration, incubation time, exploiting talented strains by co-cultivation, and genetically modifying the strains by radiation exposure [5, 8–10]. Since these bacteria are able to flourish in extreme conditions, the bioactive secondary metabolites obtained from such microorganisms, have great structural and functional diversity, including antimicrobial compounds against clinical pathogens [11]. In the present study, we showed the enhancement of antimicrobial properties in response to high dose of gamma (γ) irradiation and cross-species induction of antimicrobial activity in presence of some pathogenic micro-organisms during mixed culture fermentation.

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Materials and Methods

Sample Collection, Culture Conditions and Bacterial Identification

The bacterial strain was isolated from the Mangrove region of Kannamali, Kerala, India. Morphological, physiological and biochemical characterization of the isolated strains were performed as per Bergey's manual of determinative bacteriology [12] and finally bacterial identification was done by using 16S rRNA sequence analysis as described earlier [13]. The temperature range, pH range and effect of salinity at 37 °C was determined at a temperature range of 10–50 °C, pH range between pH 5.0 and 12 and NaCl ranging from 0 to 14 %, respectively.

Gamma Irradiation/Extraction of Secondary Metabolites from *Exiguobacterium* sp

The radioresistant property of the *Exiguobacterium* sp. HKG-126 was evaluated by exposing them to gamma (γ) irradiation various dose levels of 5–15 kGy at 22 °C as described in previous studies [13, 14]. After molecular and biochemical characterization of the mutant population as compared to non-irradiated, shake flask fermentation of both, control strain (before irradiation) as well as gamma irradiated bacterial strains was done to retrieve bioactive molecule for evaluation of antimicrobial properties. The extraction of secondary metabolites was done by using Diaion HP-20 poly aromatic adsorbent resin from culture supernatant as described previously [15] followed by antimicrobial activity test.

Determination of Antimicrobial Activity and Minimum Inhibitory Concentration (MIC)

Antimicrobial activities of all strains were performed according to the method described previously [15, 16] in which gentamicin disc (10 μ g/disc) and methanol were used as positive and negative controls, respectively. Minimum inhibitory concentration (MIC) of both extracts (0 and 15 kGy) on all sensitive test microorganisms was done by the twofold serial dilution method in 96-well microtiter plate and incubated at 37 °C overnight. Test organism without methanolic crude extract was used as negative control. As an indicator of bacterial growth, 50 μ l of 0.2 mg/ml iodinitrotetrazolium chloride (INT) (Sigma–Aldrich) was added to the wells and incubated at 37 °C for 30 min. The lowest concentrations, which did not show any growth of tested microorganism was determined as MIC. All the assays were carried out in triplicates.

HPLC Analysis

The final analysis of bioactive compounds from 0 to 15 kGy gamma irradiated *Exiguobacterium* sp. HKG-126, was carried out using high performance liquid chromatography (HPLC) column (Waters 600 controller, USA) equipped with a waters 2996 photodiode array (PDA) and waters 2475 multi-wavelength fluorescence detector. The column used was SunFire C18 reverse phase column (4.6 mm interior diameter \times 150 mm long) with a particle size of 5 μ m. HPLC of 0.22 μ filtered sample (20 μ l) was performed at room temperature with isocratic CH₃OH:H₂O (40:60) containing 0.1 % (v/v) tri-fluoro acetic acid as a mobile phase over a 20 min period.

Mixed Culture Fermentation of Microbial Strains

Four bacterial isolates: *Arthrobacter* sp. HKG 115 [15], *Bacillus sonorensis* HKG 102 [11], *Micrococcus luteus* and *Staphylococcus aureus* were randomly selected from diverse environmental sources, such as high altitude region of Leh, Himalaya region, sediment sample from Laccadive Sea, India and clinically isolated strains from patient samples, for mixed culture fermentation. Above selected isolates along with *Exiguobacterium* sp. HKG-126 were grouped in two separate batches and fermented at 37 °C for 5 days in Hi-Media nutrient broth. Individually grown bacterial cultures in nutrient broth were taken as control. After 5 days of fermentation, all retrieved bacterial strains are preliminary identified by morphological and biochemical tests and confirmation was done by using 16S rRNA sequencing. All the strains before and after mixed culture fermentation were processed for the extraction of secondary metabolites followed by antimicrobial activity test, as described earlier.

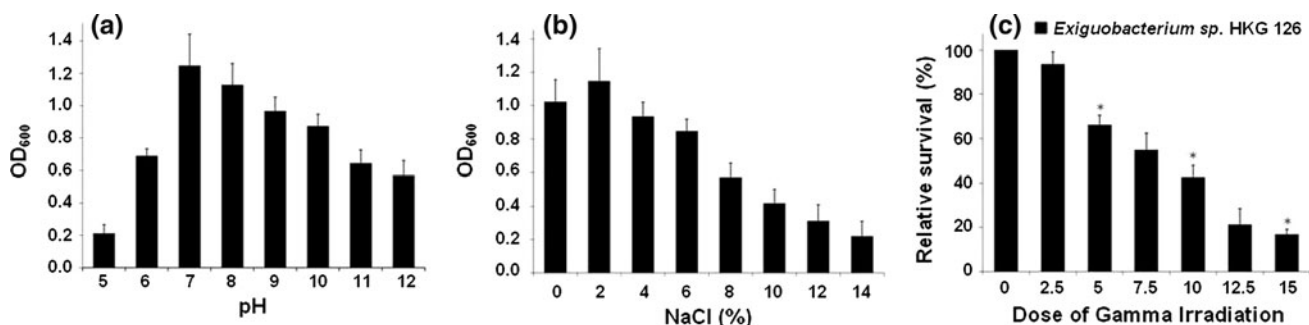
Result and Discussion

Isolation, Identification and Characterization of Bacterial Strains

We have screened a unique radiation resistant bacterial strain, isolated from mangrove region of Kannamali, Kerala (India) which was identified as *Exiguobacterium* sp. through morphological, biochemical (Table 1) and molecular characterization. The 16S rDNA sequence of strain was submitted as *Exiguobacterium* sp. HKG-126 to NCBI GenBank under accession no. HM012684. Significant growth of this bacterium was observed at temperature 10–40 °C, pH level 6–12 and in media containing 0–14 % NaCl (Fig. 1a, b), and it was found to withstand extremely high dose of gamma radiation up to 15 kGy (Fig. 1c).

Table 1 The morphological, physiological and biochemical characteristics of *Exiguobacterium* sp. HKG-126

Test	Result	Test	Result
Shape	Rod shaped	Casein hydrolysis	Positive
Gram reaction	Gram positive	Starch hydrolysis	Positive
Colonies	Circular (1–5 mm in diameter)	β -galactosidase	Positive
Colour	Orange coloured	Acid phosphatase activities	Positive
NaCl tolerance	Up to 14 %	Lipid hydrolysis	Negative
Growth temperature	10–40 °C (optimum 37 °C)	Indole test	Negative
Catalase test	Positive	Voges Proskauer test	Negative
Methyl red test	Positive	H ₂ S production	Negative
ONPG test	Positive	Reduction of NO ₃ ⁻ to NO ₂ ⁻	Negative

**Fig. 1** Characterization of *Exiguobacterium* sp. HKG 126; **a, b** Effect of pH and NaCl (%) on the growth rate of *Exiguobacterium* sp. HKG 126, respectively; **c** Relative survival (%) of *Exiguobacterium* sp. HKG 126 (unirradiated control and irradiated strains) at different

doses of gamma radiation. All growth experiments were performed in triplicates and the values are mean \pm standard deviation of three independent experiments

Enhancement of Antimicrobial Activity After Exposing them to Gamma Irradiation

As demonstrated by measuring zone of inhibition, methanol extracts showed excellent antibacterial activity against *Dietzia* sp., *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus* and *Pseudomonas aeruginosa* (Table 2). MIC was calculated only for two conditions (0 kGy: control and 15 kGy: irradiated), which were supposed to be more significant in irradiation experiment. It was observed that the lowest MIC was demonstrated against *Micrococcus flavus*, and *Dietzia* sp. (Table 2). The MIC for the antibacterial, extracted from the 15 kGy supernatant extract was less than that of 0 kGy supernatant extract of *Exiguobacterium* sp. which indicates that the antimicrobial agents from 15 kGy supernatant extract was more active than from 0 kGy extract. Earlier, it was believed that enhancement in antimicrobial activity occurs at only lower dose (up to 3 kGy) and highly decreased at higher dose level (>5 kGy), but interestingly, in this report, it was observed at both, lower and higher dose levels (up to 15 kGy). Thus, alteration in antimicrobial properties in response to γ -irradiation was found to be dose-dependent, i.e., the higher the dose used, higher the alteration in properties was observed (Table 2).

HPLC Chromatogram Based Analysis

Enhancement in one chromatographically distinct peak (peak 2) out of two chromatographically distinct peaks (peak 1 and peak 2) was observed in crude methanol extracts of *Exiguobacterium* sp. HKG-126, after exposing to 15 kGy γ -radiations (Fig. 2). It might be possible that the enhanced antimicrobial activity in γ -irradiated strain was due to the compound showing enhanced peak and not any new compound, because of similar retention time of induced peak. Although the HPLC profile is one of the basic evidences to identify antimicrobial compounds, similarities in the HPLC chromatogram might explain that compounds produced by strains may have similar structure. By comparing HPLC chromatogram of known antibiotic compounds to that of active compounds in supernatant extract might explain the ability of the *Exiguobacterium* sp. to produce those compounds.

Enhancement of Antimicrobial Activity During Mixed Culture Fermentation

In order to find more novel structures and to enhance antibiotic production from microbial systems, new ways of screening for these compounds must be applied. Knowledge

Table 2 Inhibition zones (mm) and minimum inhibitory concentration (MIC) against several pathogenic microbial test strains, caused by methanolic extracts of *Exiguobacterium* sp. (EC)

Microbial test strains	Zone of inhibition (mm) ^a					MIC (mg/ml)	
	EC ^b	EC-5 ^c	EC-10 ^c	EC-15 ^c	Gent ^d	EC ^e	EC-15 ^e
<i>Dietzia</i> sp. K44 (MTCC 7402)	8.66	10.33	12.33	16.0	23.66	3.67	1.83
<i>Micrococcus flavus</i> (NCIM 2378)	9.66	9.66	11.0	14.33	24.33	3.33	2.33
<i>Bacillus subtilis</i> (NCIM-2063)	–	–	7.33	9.0	29.0	–	3.67
<i>Escherichia coli</i> (NCIM 2739)	7.0	7.33	8.66	11.66	29.66	4.17	2.83
<i>Pseudomonas aeruginosa</i> (NCIM 2053)	9.0	8.33	9.66	12.0	29.0	3.67	2.83
<i>Staphylococcus aureus</i>	9.33	9.0	9.33	11.33	22.0	3.67	3.17
<i>Bacillus cereus</i>	8.33	8.66	10.66	12.33	21.33	3.83	2.83
<i>Salmonella typhi</i>	–	6.33	7.33	11.33	21.33	–	3.17
<i>Micrococcus luteus</i>	6.33	6.33	7.66	11.66	16.33	–	2.83

All the values are mean of three independent experiments and the values of standard deviation were in the range of 5–10 %

^a Diameter of zone of inhibition (mm), including disc diameter of 6 mm

^b Methanol extract (100 µg/disc) from *Exiguobacterium* sp. before treatment with gamma irradiation

^c Methanol extract (100 µg/disc) from *Exiguobacterium* sp. after exposing them to 5 kGy, 10 kGy and 15 kGy gamma irradiation, respectively

^d Gentamicin (10 µg/disc) as a positive control

^e MIC value (mg/ml) of methanol extract from *Exiguobacterium* sp. before and after 15 kGy gamma irradiation exposure

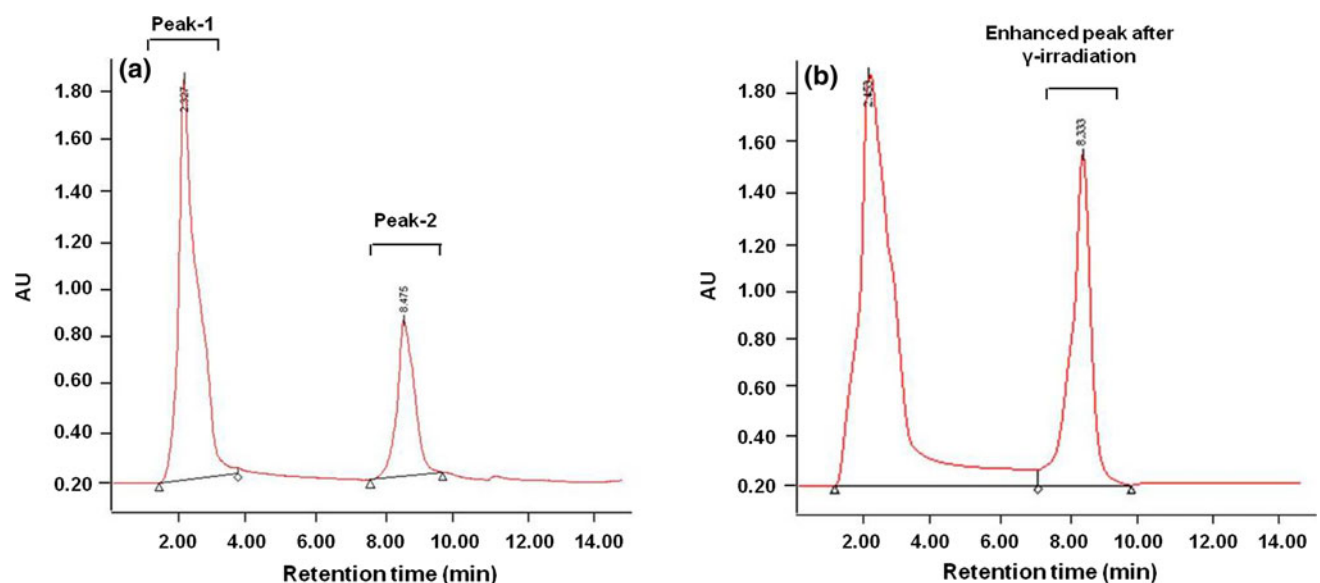


Fig. 2 HPLC chromatogram of crude methanol extracts from *Exiguobacterium* sp. HKG 126 showing enhanced peak after gamma irradiation; **a** unirradiated control; **b** 15 kGy gamma irradiated strains

concerning mixed culture fermentation, especially with regards to antimicrobial production, could be an attractive research challenge from ecological relationship and industrial points of view. To test, the effect of mixed culture fermentation on such type of enhancement in antimicrobial activity as observed during γ -irradiation exposure, we adopted co-cultivation strategy for the production and enhancement of new antibiotics. Interestingly, we observed an enhancement

in antimicrobial activity of *Exiguobacterium* sp. during and after mixed culture fermentation (Table 3a, b). It is possible that *Exiguobacterium* sp. might be able to sense the presence of any foreign species, possibly via some communication molecules secreted into the medium by the inducer strain. The antimicrobial properties of lyophilized extracts was tested before and after frozen or heated (-80 to $+60$ °C) conditions and was found stable under different pH (5–9) and

temperature conditions and data were reproducible after incubation in conditions provided.

In case of ^{f1}ML, it was not characterized and identified after mixed culture fermentation. The reason behind them supposed to be killing of ^{f1}ML during mixed culture due to antimicrobial agents produced from other co-cultivated strains like EC and AB. Keeping these in mind, it is tempting to speculate that induction and enhancement in

antimicrobial activity does not occur in all microbial strains and it depends on the pathogen used. A reason for this could be that induction by communication molecules affects only certain types of microbial population by means of quorum sensing (QS) and this response may be a defence mechanism which has evolved to deter or kill a potential competitor. There are several reports which have shown the production of such type of communication molecules from bacterial

Table 3 Determination of the antibacterial activity of crude methanolic extracts from different bacterial strains isolated before and after mixed culture fermentation and their combinations by measuring zone

of inhibition (mm) and minimum inhibitory concentration (MIC), against several pathogenic microbial test strains

Microbial test strains	Zone of inhibition (mm) ^a								MIC (mg/ml)	
	Before mix culture fermentation ^b					During mixed ^m culture fermentation		Positive Standard	Before fermentation	
	EC ^b	AB ^{b1}	ML ^{b1}	BS ^{b2}	SA ^{b2}	Comb-1 ^{m1}	Comb-2 ^{m2}	Gent ^g	EC ^b	
(a)										
<i>Dietzia</i> sp. K44 (MTCC 7402)	8.66	10.33	8.66	–	7.66	12.33	9.33	23.66	3.67	
<i>Micrococcus flavus</i> (NCIM 2378)	9.66	16.66	–	–	–	17.0	10.66	24.33	3.33	
<i>Bacillus subtilis</i> (NCIM-2063)	–	19.66	–	–	–	21.66	7.66	29.0	–	
<i>Escherichia coli</i> (NCIM 2739)	7.0	11.0	–	–	–	15.33	7.66	29.66	4.17	
<i>Pseudomonas aeruginosa</i> (NCIM 2053)	9.0	8.66	–	8.33	–	8.33	9.0	29.0	3.67	
<i>Staphylococcus aureus</i>	9.33	8.33	–	–	–	13.66	11.33	22.0	3.67	
<i>Bacillus cereus</i>	8.33	9.33	–	–	–	12.0	10.33	21.33	3.83	
<i>Salmonella typhi</i>	–	12.0	6.33	–	7.33	13.66	7.66	21.33	–	
<i>Micrococcus luteus</i>	6.33	7.66	–	7.66	8.0	8.33	10.0	16.33	–	
Microbial test strains	Zone of inhibition (mm) ^a								MIC (mg/ml)	
	After mix culture fermentation ^f							Positive standard	After fermentation	
	EC ^{f1}	EC ^{f2}	AB ^{f1}	ML ^{f1}	BS ^{f2}	SA ^{f2}	Gent ^g	EC ^{f1}	EC ^{f2}	
(b)										
<i>Dietzia</i> sp. K44 (MTCC 7402)	8.33	10.66	11.66	–	6.33	8.0	23.66	3.83	3.17	
<i>Micrococcus flavus</i> (NCIM 2378)	13.66	10.66	16.66	–	–	–	24.33	2.33	3.17	
<i>Bacillus subtilis</i> (NCIM-2063)	8.66	7.33	19.33	–	–	–	29.0	3.67	4.17	
<i>Escherichia coli</i> (NCIM 2739)	8.66	7.66	13.33	–	6.66	6.33	29.66	3.67	3.83	
<i>Pseudomonas aeruginosa</i> (NCIM 2053)	8.66	10.66	8.66	–	8.66	–	29.0	3.67	3.17	
<i>Staphylococcus aureus</i>	12.0	10.33	9.66	–	–	–	22.0	2.83	3.17	
<i>Bacillus cereus</i>	8.33	9.66	8.66	–	–	–	21.33	3.83	3.33	
<i>Salmonella typhi</i>	7.33	–	12.0	–	–	7.66	21.33	4.17	–	
<i>Micrococcus luteus</i>	7.33	8.66	7.66	–	8.33	8.33	16.33	4.17	3.67	

All the values are mean of three independent experiments and the values of standard deviation were in the range of 5–10 %

^a Diameter of zone of inhibition (mm) including disc diameter of 6 mm showing antibacterial activity of methanol extracts (100 µg/disc) from bacterial supernatant of, EC, *Exiguobacterium* sp.; AB, *Arthrobacter* sp.; ML, *Micrococcus luteus*; BS, *Bacillus sonorensis*; SA, *Staphylococcus aureus*

^{m1} Comb-1 corresponds to mixed bacterial cultures of ^bEC, ^{b1}AB and ^{b1}ML

^{m2} Comb-2 corresponds to mixed bacterial cultures of ^bEC, ^{b2}BS and ^{b2}SA

^{f1} Purified bacterial culture after mixed culture fermentation of combination-1

^{f2} Purified bacterial culture after mixed culture fermentation of combination-2

^g Gentamicin (10 µg/disc) as a positive control

organisms such as *Edwardsiella ictaluri* [17]. Besides to these QS molecules, production of some other molecules which can degrade these QS signals by inhibiting the production of QS signal molecules or degradation of the AHLs, also has been reported from bacterial organisms, called quorum quenching (QQ) [18–21].

Natural genetic transformation is also believed to be the essential mechanism for exploiting innate microbial capacity in bacterial populations, during mixed culture fermentation via horizontal gene transfer. In an early report, microbial sequencing studies and result of genome analyses indicates that the biosynthetic potential of microbial strains is much greater than that observed by traditional natural product isolation [22–24]. Therefore, with countless possible microbial combinations and increasingly sophisticated chemical isolation and structure determination methods, the potential for mixed fermentation in natural product drug discovery seems quite promising. Some of these independent studies involving particular approaches like co-cultivation and radiation exposure strategies to enhance antimicrobial activity support the findings reported here and these findings have important implications for the discovery of novel antimicrobial compounds from *Exiguobacterium* sp. HKG-126 and may allow the development of new methods for screening novel compounds active against multi-drug-resistant bacteria. In addition, the effect of mixed cultures of bacterial population on antimicrobial activity could be investigated as this would give a more in-depth picture of ecological relationships. Encouraged by these findings, further attempts are being directed towards the characterization of the mechanism involved; it is difficult to predict whether this approach will serve as a general model for producing other unusual antibiotics. In future, we are focusing on the purification of these compounds from crude extracts using HPLC and determining the exact composition and structure elucidation of compound responsible for antimicrobial property using analytical study.

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