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ORIGINAL PAPER

Isolation and partial purification of an antimicrobial agent from halotolerant alkaliphilic *Streptomyces aburaviensis* strain Kut-8

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Abstract A halotolerant alkaliphilic actinomycete, Kut-8, was isolated from saline desert of Kutch, Western India. It has been identified as *Streptomyces aburaviensis* based on the chemotaxonomic characteristics, including cell wall constituents. Kut-8 is Gram-positive having a spiral sporophore with dark green and fluffy spore mass. It was able to grow with 15%, w/v NaCl with optimum being in the range of 5–10%. It grew optimally at pH 9 with slow growth at neutral pH. The cell wall contained L-diaminopimelic acid and no diagnostic sugars. It produced an antibiotic that selectively inhibited the growth of Gram-positive bacteria, with *Bacillus subtilis* being the most sensitive. Kut-8 secreted the antibiotic optimally during mid-stationary phase (on day 14 of growth in liquid culture). The crude antibiotic metabolites were separated by various solvent systems with hexane–methanol–water giving the best separation. The results of bioautographs revealed the presence of single active compound in the Kut-8 antibiotic filtrate. Partial purification of antibiotic metabolite by charcoal absorption and methanol extraction resulted in enhanced antimicrobial activity by 4.16-fold. The study holds significance as only few salt-tolerant alkaliphilic actinomycetes from saline deserts have been explored and information on their antimicrobial potential is still scarce.

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Introduction

Microbial natural products are the origin of most of the antibiotics on the market today. There is an alarming scarcity of new antibiotics currently under development in the pharmaceutical industry. Still, microbial natural products remain the most promising source of novel antibiotics, although new approaches are required to improve the efficiency of the discovery process. Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances (Takahashi 2004). These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes (Lam 2006; Zhao et al. 2009; Olano et al. 2009). About 61% of all the bioactive microbial metabolites were isolated from actinomycetes especially from Streptomycetes and also from some rare actinomycetes (non-Streptomycetes).

It is believed that the desert soil may harbor a large population of halophilic and alkaliphilic actinomycetes (Badji et al. 2006), but attention has been focused on them just recently by the researchers (Chanal et al. 2006). Further, the phylogeny, diversity and biotechnological potential of these actinomycetes are still in infancy. Recent findings from culture-dependent and culture-independent methods have demonstrated that there is tremendous diversity and novelty among the halophilic and alkaliphilic actinomycetes present in saline and alkaline

environments (Maldonado et al. 2005). The desert counterpart might thus be valuable for the isolation of novel strains of actinomycetes, which could potentially yield useful new products.

The Great Rann of Kutch (India) comprises a unique geomorphic entity of the Indian sub-continent. Spanning the border of India and Pakistan on the Arabian Sea, the Rann of Kutch has been described as 'a desolate area of unrelieved, sun-baked saline clay desert'. The saline and arid region of Kutch is rich in diversified actinomycetes, which is an inexhaustible resource that has not been properly exploited. However, the full potential of this domain as the basis for biotechnology, particularly in India, remains largely unexplored. So far as microbial diversity is concerned, it is possible that it may harbor a huge fraction of unexplored genetic wealth having novel secondary metabolites of commercial interest. However, the reports on actinomycetes isolated from saline deserts are quite limited (Song et al. 2005; Li et al. 2006; Norovsuren et al. 2007), while those indicating their antimicrobial potential are rare (Badji et al. 2006).

During the last few years, interest in saline desert microbes has increased due to investigations on novel bioactive metabolites, especially antibiotics and enzymes. Many of these metabolites possess antimicrobial activities and have the potential to be developed as therapeutic agents. Desert actinomycetes are a prolific but underexplored source for the discovery of novel secondary metabolites. The present report highlights the screening and purification of an antimicrobial agent from a new halotolerant alkaliphilic *Streptomyces aburaviensis* strain Kut-8, isolated from a geographically rare habitat, the saline desert of Kutch, Western India.

Materials and methods

The organism

The halotolerant alkaliphilic actinomycete, Kut-8 was isolated from saline soil of the Great Rann of Kutch, Gujarat, India. The saline soil (10 g) was incubated at 45 °C with $CaCl_2$ (1 g) for 1 week. The soil suspension was serially diluted (1:100, 1:1,000, and 1:10,000) and plated on starch casein agar (g/L: starch, 10; casein, 10; peptone, 5, yeast extract, 5; NaCl, 100; agar, 30). The pH of the medium was adjusted to 9 by adding separately sterilized Na_2CO_3 (20%, w/v). After the incubation of 6 days at 30 °C, a typical dark green colony was picked up and re-streaked to ensure the purity of the colony. The culture was maintained at 4 °C on starch casein agar slants (5% w/v NaCl and pH 9).

Chemotaxonomic characterization of the organism

The haloalkaliphilic actinomycete, Kut-8 was characterized with respect to its salt (0–20%, w/v) and pH (6–10) tolerance in starch casein agar. The organism was identified on the basis of morphological features, pigment production, cell wall sugars and amino acids and biochemical characteristics including carbon utilization tests. Analysis of diaminopimelic acid and cell wall sugars was performed by the method of Myertons et al. (1988).

The antimicrobial potential of Kut-8

Antimicrobial activity of *Streptomyces aburaviensis* strain Kut-8 was detected using starch casein agar (5% w/v NaCl, pH 9). Kut-8 was spotted on the medium and incubated for 4 days at 28 °C till the beginning of the sporulation. Thereafter, molten nutrient agar with activated test culture, i.e. Gram-positive organisms such as *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis* and Gram negative organisms; *Escherichia coli*, *Enterobacter aerogens*, *Proteus vulgaris*, *Shigella dysentry*, *Pseudomonas aeruginosa* and *Salmonella typhosa para B*, was poured onto the already grown Kut-8. After the incubation of 24 h at 37 °C, the zone of inhibition was measured for each test organism.

Effect of NaCl and pH growth and antibiotic production

The effect of the salt on growth and antibiotic production was studied on starch casein agar at varying salt concentrations (0–20% w/v) at pH 9. The spore suspension of Kut-8 was spotted on the plates followed by incubation at 28 °C for 4 days. The actively growing culture of *Bacillus subtilis* was poured on it and the plates were incubated at 37 °C for 24 h followed by the measurement of zone of inhibition. Similarly, the effect of pH on antibiotic production was studied in the range of pH 6–11 on starch agar having 5%, w/v NaCl.

Antibiotic production by Kut-8 in liquid culture

The spore suspension (5%, v/v) of Kut-8 was inoculated into 100 mL starch casein broth (5%, w/v NaCl and pH 9). Samples were withdrawn at regular time intervals and the cells were separated by filtration. The growth was measured as dry weight per ml of the sample withdrawn. The antimicrobial activity of antibiotic filtrate was determined by agar well method in the nutrient agar plate already seeded with *Bacillus subtilis*. The well was filled with the filtrate and the plate was incubated at 37 °C for 24 h. Next day the zone of inhibition of the test organism was measured.

Separation of the antibiotic metabolites using paper chromatography

The movement of the antibiotic metabolites in specific solvent systems was used to develop the chromatograms by following the method of Odakura et al. (1984). About 50 µL of the antibiotic filtrate was applied 3 cm from the lower edge of the chromatography paper strips (Whatman No. 1) and then air dried. The chromatography paper strips were immersed to a depth of 1 cm in the solvents. The solvents tested for separation of the antibiotic metabolites included; solvent system 1 (hexane–methanol–water, 4:3:3 v/v), solvent system 2 (hexane–butanol–water, 65:25:10 v/v), solvent system 3 (methanol-*n*-hexane, 60:40 v/v) and solvent system 4 (butanol–acetic acid–water, 50:25:25 v/v). About 30 mL of each solvent was placed in chromatography chamber and ascending development was allowed without preliminary saturation of the chromatograms with the vapors of the solvents. The ascending development of the chromatograms was stopped when the solvent fronts reached a distance of 15 cm from the origin. The solvent fronts were marked, measured and the paper strips were aseptically processed for bioautography.

Bioautography of the antibiotic metabolites using paper chromatography

The solvent systems managed to move and separate the crude antibiotics into their different components. The bioautography set-up consisted of a base layer of 20 mL of sterile N-agar in plate left for a while to set and solidify. About 8 mL of sterile molten N-agar seeded with test organism *Bacillus subtilis* was evenly poured onto the basal layer. The seeded layer was allowed to set and then the developed chromatogram strips were placed on the surface of the seeded agar ensuring good contact to allow the antibiotic to diffuse from the paper. The plates were incubated at 37 °C for 24 h. The presence of inhibition, as evidenced by the clear zones around where active components were present, was determined. The distance from the point of antibiotic application to the centre of the clear zones were measured and recorded.

Partial purification of the antibiotic metabolites in culture filtrates

The partial purification of the antibiotic metabolites was carried out by modified method of Mutitu et al. (2008). Charcoal (10 g) was dried and ground coarsely. It was activated in an oven at 200 °C for 1 h and then cooled to room temperature (22 ± 2 °C). The cell free culture filtrates were mixed with 10% of the powdered charcoal (w/v) and stirred for 30 min to allow absorption of the antibiotics onto the charcoal particles. Whatman No. 1 filter paper was used to filter the mixture. The antibiotic-containing charcoal left in the funnel was eluted with 10 mL absolute methanol. Eluate which contained antibiotics dissolved in methanol was then concentrated in an oven at 70 °C to about 2 mL. This partially purified antibiotic was then bioassayed against *Bacillus subtilis* using the agar well method.

Results and discussion

It is revealed from literature that the diversity of halophiles and alkaliphiles has been studied mostly from the Soda Lakes and marine sediments (Cai et al. 2008; Qvit-Raz et al. 2008; Wu et al. 2009; Bian et al. 2009; Tian et al. 2009). The saline desert of Kutch has wide range of salinities and was selected as an ecosystem for studying the diversity of actinomycetes and their antimicrobial properties. Up to the present, only a few species have been isolated from such habitats and only limited metabolic types have been described (Okoro et al. 2009; Li et al. 2009a).

Taxonomic characterization of the organism

One of the more efficient ways of discovering novel metabolites from microorganisms is through the isolation of new microbial species. With this perspective, Kut-8, a halotolerant alkaliphilic actinomycete was isolated from the saline desert of Kutch. It was Gram-positive, having filamentous, long threadlike structure. It started sporulation on starch casein agar after 3 days of incubation with a fluffy mass of spores that was grayish green in color. Kut-8 has been identified as *Streptomyces aburaviensis* based on the morphological, physiological and biochemical characteristics, including cell wall constituents (Table 1). The original strain of *Streptomyces aburaviensis* was isolated by Nishimura et al. (1957) from soil. Till date, none of the strains of *Streptomyces aburaviensis* has been reported to grow under the extremities of salt and pH. The trends and initial results, however, suggest Kut-8 to be a novel strain.

Kut-8 utilized sucrose, inositol, rhamnose and mannitol as sources of carbon along with acid production. Tests for starch hydrolysis, casein hydrolysis, gelatin hydrolysis, nitrate reduction and H_2S production showed positive results, but utilization of phenol and methionine showed negative results. The cell wall contained L-diaminopimelic acid and diagnostic sugars were not present in the cell wall fraction (Table 1). Kut-8 was halotolerant and was able to grow up to 15% w/v NaCl, with the optimum being in the range of 5–10%. Kut-8 was also capable of growth with 0% salt indicating the halotolerant nature of the organism. This result is quite comparable with *Haloglycomyces albus* gen.

Table 1 Morphological and Biochemical characteristics of Kut-8

Properties	Kut-8
Morphological characters	
Sporophore morphology	Spiral
Color of aerial mycelium	Dark green
Color of substrate mycelium	Coffee brown
Color of spore mass	Dark green
Sporulation starts after…	4 days
Morphology of spores	
Shape	Elongated
Surface	Smooth
Number in chain	Long chain
Biochemical characteristics	
Indole production	–
Methyl red	–
Voges Proskauer	–
Citrate utilization	+
H_2S production	+
Nitrate reduction	+
Urease	–
Catalase	+
Oxidase	+
Melanin production	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Lipid hydrolysis	+
Casein hydrolysis	+
Anaerobic growth	–
Optimum NaCl for growth	5–10% w/v
Optimum pH for growth	9
Chemotaxonomic characters	
Whole cell sugar analysis	No diagnostic
Cell wall amino acid	L-DAP
Carbon source utilization	
Sucrose	+
Inositol	+
Mannitol	+
Rhamnose	+
Nitrogen source utilization	
Methionine	–
Phenylalanine	+
Arginine	+

"+" positive; "–" negative; L-DAP L-diaminopimelic acid

nov., sp. nov. (Guan et al. 2009) and *Saccharomonospora saliphila* sp. nov. (Syed et al. 2008). However, the salt requirement of our isolate was much less compared to *Prauserella salina* sp. nov., a truly halophilic actinomycete (Li et al. 2009b). Kut-8 was able to grow optimally at pH 9 with slow growth at neutral pH. Recently, *Nesterenkonia alba* sp. nov., an alkaliphilic actinobacterium was reported to grow with an optimum pH of 9–10 (Luo et al. 2009).

The antimicrobial potential of Kut-8

Halotolerant alkaliphilic actinomycetes may provide a valuable resource for novel products of industrial interest including enzymes and antimicrobial agents (Uyeda 2004; Fiedler et al. 2005; Thumar and Singh 2007; Hong et al. 2009). The antimicrobial activity of Kut-8 was screened against various Gram-positive and Gram-negative organisms. Kut-8 showed a narrow spectrum antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *B. megaterium* and *B. subtilis* (Fig. 1). However, it did not affect the growth of Gram-negative organisms. Among the Gram-positive organisms, *Bacillus subtilis* was most sensitive to the antimicrobial agent. The similar antagonistic approach has also been reported in salt-tolerant alkaliphilic *Streptomyces sannanensis* strain RJT-1 (Vasavada et al. 2006). Very recently, Dhanasekaran et al. (2009) reported a *Streptomyces* sp. secreting a broad spectrum antibiotic against some pathogenic bacteria and fungi. Earlier studies carried out by Kokare et al. (2004), Li et al. (2005), Manam et al. (2005) and Sarkar et al. (2008) also revealed that halophilic actinomycetes from saline (marine) habitats are rich in bioactive antibiotics.

Effect of NaCl and pH on growth and antibiotic production

Kut-8 grew and secreted antibiotic optimally with 5–10% w/v NaCl. However, it grew but did not secrete antibiotic at the salt concentrations above 10 (Fig. 2). Recently, Huang et al. (2009) reported the secretion of Erythronolides H and I from the new halophilic actinomycete *Actinopolyspora*

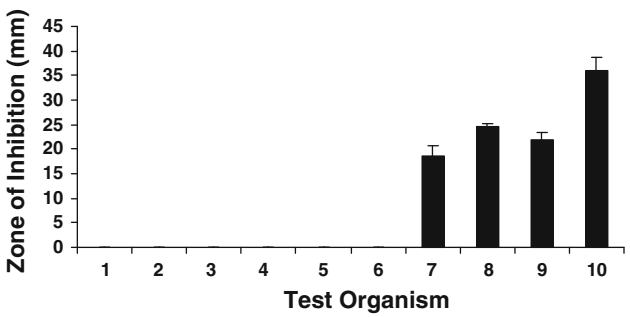

Fig. 1 Antibiotic production by *Streptomyces aburaviensis* strain Kut-8 against test organisms such as 1, *Escherichia coli*; 2, *Enterobacter aerogenes*; 3, *Proteus vulgaris*; *4*, *Shigella dysenteriae*; *5*, *Pseudomonas aeruginosa*; 6, *Salmonella typhosa para B*; *7*, *Staphylococcus aureus*, 8, *Bacillus cereus*, *9*, *Bacillus megaterium*, *10*, *Bacillus subtilis*

sp. YIM90600 at high salt concentrations. Similarly, Imada et al. (2007) described the secretion of an antibacterial compound by *Streptomyces* sp. in the presence of sea water. Kut-8 secreted the antibiotic in the wide range of pH 7–9, while poor growth was evident at pH values below the neutral (Fig. 3). A novel alkaliphilic *Streptomyces* strain has been reported to secrete pyrocoll, an antimicrobial compound, under alkaline conditions (Dietera et al. 2003). Our results are also comparable with some *Streptomyces* species that are recorded to secrete antibiotics against bacteria, fungi and yeasts at higher salinity and alkaline pH (Basilio et al. 2003).

Kut-8 secreted the antibiotic in liquid culture after 7 days of incubation under shaking conditions at 30 °C with optimum being on day 14. The production started during mid stationary phase that confirmed the compound to be a secondary metabolite (Fig. 4).

Separation and bioautography of the antibiotic metabolites using paper chromatography

Bioautographs are generally used to determine the active components on paper chromatograms. The crude antibiotic solution was separated with paper chromatography using different combinations of the solvents. The solvents tested namely methanol, butanol, *n*-hexane and acetic acid eluted the antibiotic from the culture filtrates of Kut-8. The results of the bioautography showed that R_f values were different for different solvents. The calculated R_f value was highest with solvent system 2 (0.89) followed by solvent system 3, 4 and 1 (Table 2). A single sharp zone was evident in bioautography indicating the presence of a single compound that is active against *Bacillus subtilis*. However, further analysis with HPLC should be conducted to confirm this finding, as suggested by Remya and Vijayakumar (2008). Recently, an antibiotic secreted by *Nomomuraea* sp. NM94 was extracted with dichloromethane and detected by bioautography against *Bacillus subtilis*. The results

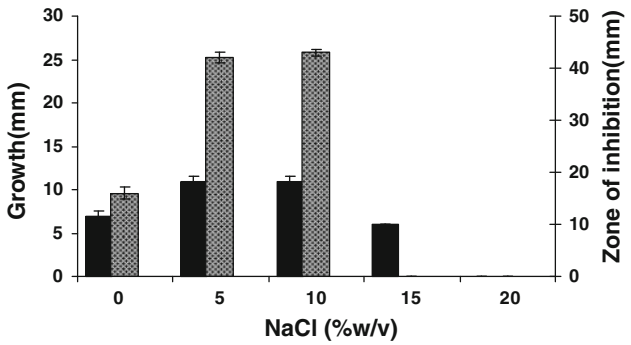

Fig. 2 Efect of salt on growth (*filled squares*) and antibiotic production (*netted squares*)

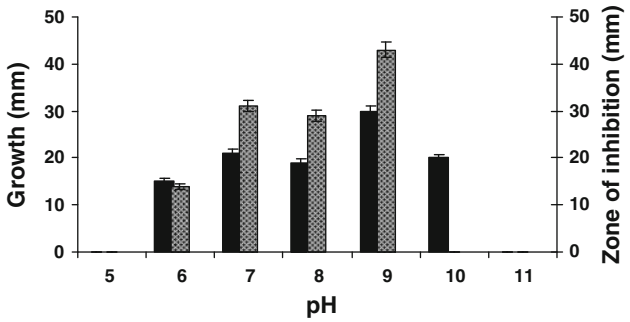

Fig. 3 Effect of pH on growth (*filled squares*) and antibiotic production (*netted squares*)

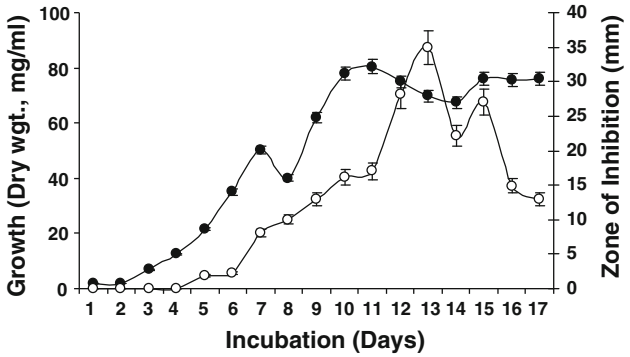

Fig. 4 Growth kinetics of *Streptomyces aburaviensis* strain Kut-8 with reference to antibiotic production against *Bacillus subtilis*. Growth (Dry wt., mg/mL; (*filled circles*)); Antibiotic production (zone of inhibition, mm; (*open circles*))

indicated the presence of five active compounds (Badji et al. 2006).

Partial purification of antibiotic metabolite

For complete characterization of an antibiotic, it should be purified as a single component. Partial purification of the antibiotic, from the culture filtrate of Kut-8 was carried out by charcoal absorption followed by extraction in methanol. The results of bioassay against *Bacillus subtilis* represented an increase of 4.16-fold as compared to the activity of crude antibiotic. The size of the inhibition zone diameter increased from 7.2 mm per 1 mL of crude filtrate to 30 mm per 1 mL of partially purified antibiotic. Remya and

Table 2 R_f values of antibiotic metabolites secreted by Kut-8 in various solvent systems

Sr. no.	Solvent system	R_f value
1	Hexane–methanol–water	0.65
2	Hexane–butanol–water	0.89
3	Methanol–hexane	0.83
4	Butanol–acetic acid–water	0.75

Vijayakumar (2008) reported that ethyl acetate extract of *Streptomyces* strain RM42 showed maximum activity against *Escherichia coli* followed by *Salmonella typhi*, *Stphylococcus aureus*, *Candida albicans* and *Bacillus subtilis*. Similarly, the extraction of antibiotics has been carried out from actinomycetes by using various solvents including ethyl acetate and methanol (Taechowisan et al. 2005; Ilic et al. 2005; Saha et al. 2005).

Conclusion

The search for novel antibiotics and other bioactive microbial metabolite, for potential agricultural, pharmaceutical and industrial applications, has been, and still is, important. Recently, the frequency of searching new antibiotics is going on worldwide because of the serious problem of antibiotic resistance among the microbes. The recent discovery of novel primary and secondary metabolites from taxonomically unique populations of extremophilic actinomycetes suggest that these organisms could add a new dimension to microbial natural product research. However, only a little information is available on the actinomycetes of the desert environment which is one of the most productive ecosystems with regard to the occurrence of novel microbial flora. Our results strongly support the idea that species of desert actinomycetes, capable of growing under selective conditions of pH and salinity, possess a significant capacity to produce compounds having unique antibacterial activity. Search for new actinomycete species seems likely to lead to discovery of potentially beneficial secondary metabolites.

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