Streptomyces sp. SCBT isolated from rhizosphere soil of medicinal plants is antagonistic to pathogenic bacteria

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Abstract

The *Streptomycetes* sp. SCBT strain Gen Bank accession number EU143270 isolated from the rhizosphere soil of medicinal plants was collected from the Kolli hills of Tamil Nadu, India. The strain *Streptomyces* is designated as School of Chemical and Biotechnology (SCBT), capable of inhibiting the growth of a wide range of Gram-negative and Gram-positive bacteria. An almost complete 16S rRNA gene sequence of the isolate was generated and compared with sequences of representative *Streptomyces* spp. The 16S rRNA data supported the classification of the strain within the genus *Streptomyces*, and showed that the SCBT strain was closely related to *Streptomyces albogriseolus* ATCC AJ494865 with a sequence similarity of 99%. Despite the high sequence similarity, SCBT was phenotypically different from *S. albogrieseolus* ATCC AJ494865. When the strain was cultured, its culture broth was extracted with ethyl acetate and the contents of the residue were subjected to Liquid Chromatography Mass Spectrum (LCMS) profiling. The chromatographic purity of the peak exhibiting the antimicrobial activity at a Retention time (RT) of 16.2 min was 48%, and the compound was purified. The mass/change (m/z) value of the peak exhibiting antimicrobial activity at the RT of 16.2 min was 391 isotopic ion peak (M+1).

Keywords: Antimicrobial activity; Carbendizim; LCMS; Rhizosphere soil; 16S rRNA sequence

INTRODUCTION

Filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important microorganisms because of their ability to produce many kinds of secondary metabolites such as antibiotics (Mellouli *et al*., 2003). Indeed the Gram-positive strepromycetes produce approximately 75% of the commercially and medically useful antibiotics (Miyadoh, 1993). In the course of screening for new antibiotics, several research studies are currently oriented towards isolation of new *Streptomyces* species from different soil samples. Streptomycetes are soil bacteria, which have distinct features such as DNA with a high G+C content (Anderson and Wellington, 2001). More than 500 *Streptomyces* species and subspecies have been described, the largest number of any bacterial genus (Lee *et al*., 2005). Several methods have been developed to identify *Streptomyces* species. These include culturing methods using the selective plating technique, construction of genetic marker systems, a combination of chemical markers, and the presence of LL-diaminopimelic acid and the absence of characteristic sugars in the cell wall (Sujatha *et al*., 2005). The 16S rRNA analysis has proved to be a very important tool in *Streptomyces* systematics, and also helpful in the assigning the newly isolated strain to the genus *Streptomyces*. The isolation of novel *Streptomyces* species are in great need as they are very potent producers of secondary metabolites (Mellouli *et al*., 2003).

Many bacteria are intimately associated with plant roots. Rhizodeposition of various exudates provide an important substrate for the soil microbial community and there is a complex interplay between this community and the quantity and type of compounds released (Kandeler *et al*., 2002; Marschner and Baumann, 2003).

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Hence, this paper describes the isolation of a *Streptomyces* sp. from the rhizosphere soil of medicinal plants, showing activities against Gram-positive and Gram-negative bacteria. The investigation also involves evaluation of the effect of the systemic fungicide, carbendizim on bacterial growth and diversity of actinomycetes recovered on media, taxonomy of the antibiotic producing strain, its fermentation, HPLC analysis of culture filtrate and partial characterization of the bioactive compounds produced by this strain.

MATERIALS AND METHODS

Plants: The *Streptomyces* sp. used in this study was isolated from the rhizosphere soils of medicinal plants namely *Aloe indica*, *Curcuma domestica*, *Gloriosa superba*, *Embelia ribes*, *Eclipta alba,* collected from Kolli hills, Tamil Nadu, India.

Isolation of *Streptomyces*: The rhizosphere soils were incubated at 60°C for 40 min and re-suspended in 5 ml of saline. The re-suspended mixture was diluted with 45 ml of saline containing 1.5% (v/v) phenol and shaken for 30 min at 28°C (Kuster and Willaims, 1964). The media were supplemented with carbendizim (400 mg/l) after sterilization. The starch casein agar plates were incubated for 14 days at 30ºC and the resulting colonies were subcultured on Yeast extract-malt extract agar.

Antifungal agents: Antifungal agents used in growth media could represent another bias. This is particularly critical when screening and isolating large numbers of actinomycetes from various soil samples. This study also aims at evaluating the effects of systemic fungicide-carbendizim on bacterial growth and diversity of bacteria recovered on media. Hence, the effect of carbendizim concentrations ranging from 0-400 mg/l on was tested was on starch casein nitrate agar medium to control the growth of fungi.

Assay for antagonistic activity: A modified version of the antibiotic assay by Anita *et al*. (2004) was followed in this procedure. The spore suspensions of individual isolates were spot inoculated (10 µl per spot) on Muller Hinton agar plates, and incubated at 28°C for 3 days. The dotted isolates were killed by inverting the uncovered Petri dishes over 4 ml of chloroform in a watch glass for 1 h. Watch glasses were removed and plates were aerated in a fume hood for 30 min to evaporate chloroform. The Plates were subsequently over laid with 15 ml of medium containing 1% (w/v) agar, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract and inoculated with 100 µl of test isolates *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhi* ATCC 6539, *Salmonella abony* NCIM 2257, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Proteus vulgaris* ATCC 9484, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC 13525, *Staphylococcus epidermidis* (NCIM 2493). The resulting cleared zones were measured after overnight incubation.

Taxonomy of the bioactive compound producing strain

Morphological and cultural characterizations: The morphology of aerial hyphae, substrate mycelium and spore chains of a 14 day culture sample of the SCBT strain were examined by light and scanning electron microscopy. Spore morphology was studied by examining gold-coated dehydrated specimens using the JEOL (JSM-5610LV), Japan make scanning electron microscope. The cultural characteristics of the strain grown at 28°C on different media for 14 days were examined. The presence of soluble pigments and the melanoid pigment was investigated on yeast extract-malt extract agar international Streptomyces project medium 2 (ISP medium 2) and peptone yeast extract ion agar (ISP medium 6).

Chemotaxonomic and physiological tests: The diagnostic isomers of diaminopimelic acid (DAP) and whole-organism sugars of the test strain were analyzed by the procedure developed by Becker *et al*. (1964). Utilization of various carbon and nitrogen sources was examined. Each source was added at a final concentration of 1% (w/v) and 0.1% (w/v) respectively. Growth of the strain SCBT in the presence of 0.1% (w/v) phenol was tested in ISP-medium 2. Degradation of starch was determined according to Gordon *et al*. (1974). Hydrogen sulfide production and coagulation of milk were determined as described by Cowan and Steel, (1974). Growth in the presence of sodium chloride was determined according to Tresner *et al*. (1968) and temperature and pH requirements were determined on ISP medium 2.

Sequencing of 16S rRNA: The 16S rRNA gene fragments were amplified by using PCR Kit (GENEI Pvt.Ltd, India). The conditions used for thermal cycling were as follows, the initial denaturing step for 5 min at 95°C, and 0.25 µl of Taq polymerase was added to the sample and kept in PCR Kit for denaturation at 95ºC for 1 min, primer annealing at 56ºC for 1 min and primer extension at 72ºC for 3 min. This was repeated for 35 cycles. Then the reaction mixture was kept at 72°C for 5 min and then cooled at 4°C. The amplified DNA was detected by using gel electrophoresis. Five 5 µl of amplified DNA was added with 1 µl of loading dye and loaded in lane 1. Marker DNA (2 µl) (HIND III digested DNA) was kept in lane 2. The tray was filled with 0.5% Tris-borate-EDTA (TBE) buffer. The gel loaded with sample DNA and marker DNA were placed submerged in buffer. And a potential difference was applied via electric field. After 45 min, the DNA bands present in the gel were visualized by staining with ethidium bromide (10 mg/ml in H_2O) and examined under the UV light. The purified DNA fragments were sequenced using sequencer model ABI 3100 sequencer according to manufacturers' instructions (ABI PRISM 3100 Genetic Analyzer User's Manual).

Phylogenetic analysis: Sequence similarity search of the 16S rRNA sequence from the SCBT strain was carried out using BLAST (NCBI). An evolutionary tree was inferred by using the neighbour-joining method (Saitou and Nei, 1987). The clustal X program (Thompson *et al*., 1997) was used for multiple alignments and phylogenetic analysis.

Fermentation: The SCBT spores were inoculated in 200 ml of a seed medium composed of starch 1% (w/v), glucose 0.5% (w/v), yeast extract 0.2% (w/v), tryptone 0.5% (w/v), K_2HPO_4 1% (w/v), $MgSO_4.7H_2O$ 0.05% (w/v). The pH was adjusted to 7.0 and incubated at 28ºC for 3 days. The inoculum was used to seed an 8 l fermenter containing 6 l of the production medium composed of starch 1% (w/v), glucose 0.5% (w/v), yeast extract 0.2% (w/v), proteose peptone 0.5% (w/v), soyabean oil 0.15% (v/v) K_2HPO_4 0.1% (w/v), $MgSO_4.7H_2O$ 0.05% (w/v), $CaCO_3$ 0.3% (w/v) Fermentation was carried out for 72 h, after which the yield of antibiotic had reached its maximum and the culture broth was subsequently harvested (as mentioned below).

Product recovery: The fermented broth was centrifuged at 8000 rpm for 10 min. The culture filtrate was extracted twice with ethyl acetate (EtOAc) and the biomass once with acetone. The extracts from the culture filtrate and mycelium were mixed and concentrated in vacuo to dryness. After dehydration with anhydrous Na_2S0_4, the EtOAc solution was further concentrated in vacuo.

Mass spectrometry: The agilent LC-MSD VL mass spectrometry (MS) (Agilent, USA) system was used for analysis of concentrated yellow antibiotic compound extracted from *Streptomyces* sp. SCBT using a symmetry column (150 × 4.6 mm, 5 mm) column and 10 mM ammonium acetate and acetonitrile as mobile phase in the ratio of 70:30 v/v was used. The chromatography was performed with mobile phase flow rate of 0.5 ml/min and injection volume of 1 ml as instrumental parameters and the peaks were monitored at a wavelength of 245 nm.

Isolation of antibiotic compound extracted from Streptomyces sp. SCBT by preparative chromatography: The concentrated yellow antibiotic compound extracted from *Streptomyces* sp. SCBT was purified and the antibiotic was isolated by preparative chromatography. The yellow antibiotic compound extracted from *Streptomyces* sp. SCBT was isolated by Agilent preparative system using reverse phase YMC-ODS-A C18 (150 × 50 mm, 25 µ) column with methanol and water as mobile phase in the ratio 70:30 v/v. The concentrated antibiotic extract was dissolved in methanol and water (50:50) and the chromatography was performed at a wavelength of 254 nm with ultra-violet detector with a flow rate of 30 ml/min. The separated compounds were collected separately, vacuum evaporated and lyophilized at -70ºC. The antibiotic activity of the lyophilized fraction was determined as mentioned above.

RESULTS

Effect of antifungal agents: Carbendizim decreases fungal counts on starch casein nitrate agar**.** The aim of this work was also, to evaluate the effect of carbendizim on fungal growth. The fungal growth was controlled by Carbendizim in the experimental conditions;

Table 1. Cultural characteristics of Strain SCBT.

Agar medium	Growth	Aerial mycelium	Substrate mycelium
Tryptone-yeast extract agar (ISP NO. 1)	Good	White	Cream
Yeast extract malt extract agar (ISP NO. 2)	Good	None	Cream-Tan
Glycerol-asparagine agar (ISP NO. 5)	Good	None	Ash-Tan
Tyrosine agar (ISP NO. 7)	Good	White	Golden yellow
Nutrient agar	Good	White	Golden yellow
Potato dextrose agar (PDA)	Good	White	White

increasing concentrations up to 400 mg/l improved the inhibition of fungal growth. The findings of the study demonstrated that Carbendizim concentrations up to 400 mg/l are required to control fungi without disturbing the colony forming units of *Streptomycetes*. A total of 20 morphologically different *Streptomyces* sp were isolated from the rhizosphere soil of the medicinal plants.

Antagonistic assay: The ability to inhibit the growth of Gram positive and Gram negative bacteria was observed. The antibacterial activity of the test isolates was varied. Out of 20 *Streptomyces* sp. subjected for antagonistic screening process, a single strain was shown to have very efficient antibacterial activity against both Gram positive and Gram negative bacteria. Hence the SCBT was selected for taxonomical investigation and bioactive compound isolation.

Taxonomy of the bioactive compound producing strain: The cultural characteristics of the test strain SCBT on various media after incubation at 28°C for 14 days are given in Table 1. The morphological and chemotaxonomic characteristics of strain SCBT indicated that it belongs to the genus *Streptomyces*. Spores were short compact spirals, arranged four to six spiny surfaced oval shaped spores per turn. (Fig. 1), whereas *S. albogriseolus* possessed hairy spore surfaces (Preobrajenskaya *et al*., 1957). The cell wall components analysis of the strain SCBT shown to possess LL-DAP and glycine in their cell walls. The presence of LL-DAP along with glycine with no characteristic sugars indicates the cell wall chemotype I. Comparison of the 16S rRNA nucleotide gene sequence of strain SCBT (1490 bp) with corresponding *Streptomyces* sequences clearly showed that the organism form a distinct phyletic line in the *Streptomyces* spp. (Fig. 2). The isolate was closely related to the type strain of *S. albogriseolus* (GenBank Accession Number AJ494865) sharing a 16S rRNA gene sequence similarity of 99%. In addition, the strain SCBT showed some differences with respect to morphological, physiological and biochemical properties (Table 2). The Phenetic and genetic results support the classification of the isolate SCBT as a new strain.

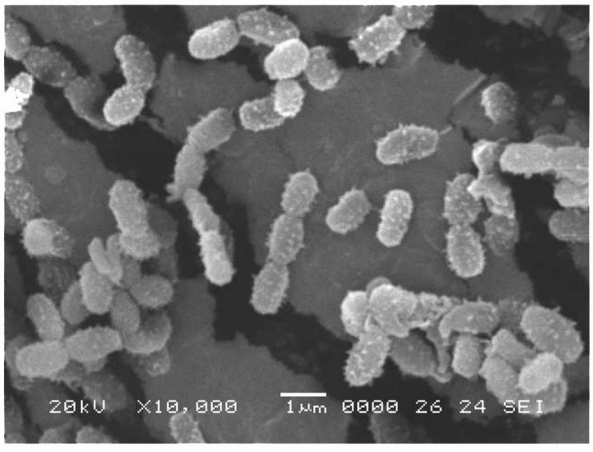

Figure 1. Scanning electron micrographs of *Streptomyces* sp. SCBT strain grown on ISP-2 agar at 27ºC for 14 days.

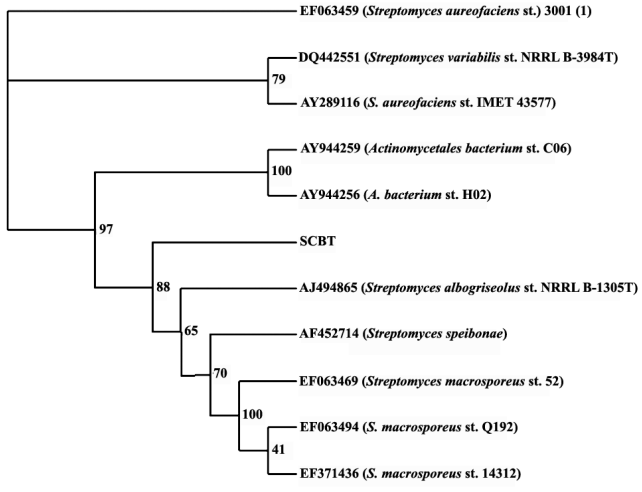

Figure 2. Neighbor-joining tree based on 16s rDNA gene sequences showing Relationship between the strain SCBT and other *Streptomyces* sp.

Table 2. Comparison of the *Streptomyces* sp. SCBT with *S. albogriseolus*.

S. No	Characters studied	*Streptomyces* sp. SCBT	*S. albogriseolus*
1	Colour of the aerial mycelium	Grey	Grey
2	Melanoid pigment	Negative	Negative
3	Reverse side pigment	Negative	Nd
4	Soluble pigment	Negative	Nd
5	Spore surface	spiny	hairy
6	Spore chain morphology	Rectiflexibles	Nd
7	Gelatin liquefaction	Positive	Positive
8	Nitrate reduction	Positive	Positive
9	Starch hydrolysis	Positive	Positive
10	Milk coagulation	Positive	Positive
11	Growth in the presence of 0.1% phenol	Negative	Nd
12	H_2S Production	Negative	Negative
13	Cellulose degradation	Positive	Nd
14	Utilisation of carbon sources		
	Arabinose	positive	positive
	Fructose	Positive	positive
	D-xylose	Positive	positive
	Inositol	Positive	positive
	Mannitol	Positive	positive
	Rhamnose	Positive	positive
	Sucrose	Positive	positive
	Raffinose	Positive	positive
15	Utilisation of nitrogen sources		
	L-aspragine	Very slight growth	Abundant growth
	L-Hydroxypyroline	Slight growth	Abundant growth
	L-Histidine	Abundant growth	Abundant growth
	L-phenyl alanine	Abundant growth	Abundant growth

Nd: Not determined

Fermentation and isolation of compounds: The whole-cultured broth of the producing strain SCBT was extracted with EtOAc and the extract was evaporated to dryness resulting in a yellow oily residue (2.36 g). When the EtOAc extracts were analyzed in Agilent LC-MSD VL mass spectrometry the mass value of the compound in the extract was identified and the purification and separation of the compounds was done by using preparative chromatography. An antibiotic activity test was done for different samples separated and (Fig. 3). The chromatographic purity of the active peak at a RT of 16.2 min was 48%, which was then purified. The m/z value of the peak exhibiting antimicrobial activity at the RT of 16.2 min was 391 (M+1) (Fig. 4).

DISCUSSION

The presence of LL- DAP and the absence of characteristic sugars in whole cell hydrolysates show that strain SCBT have wall type I which assign the strain into the genus *Streptomyces* (Lechevalier and Lechevalier, 1970). The morphological, physiological, and biochemical parameters of the strain SCBT were compared with the *Streptomyces* sp available in the Nonomura key and Bergey's manual of systematic bacteriology. Both strains have been found to have similar growth patterns on carbon compounds when grown in the synthetic medium of Pridham and Gottlieb (1948). The spores as observed by electron micrography are spiny rather than the hairy outgrowths of those from *S. albogriseolus*. Furthermore, SCBT differs from *S. albogriseolus* in the utilization of L- asparagine and L-Hydroxyproline as described by Waksman, 1961. These data, in conjunction with gene sequencing indicate that the isolate SCBT can be considered as a new strain of *Streptomyces*, designated *Streptomyces sp*. SCBT.

Due to the relative abundance of fungi and bacteria

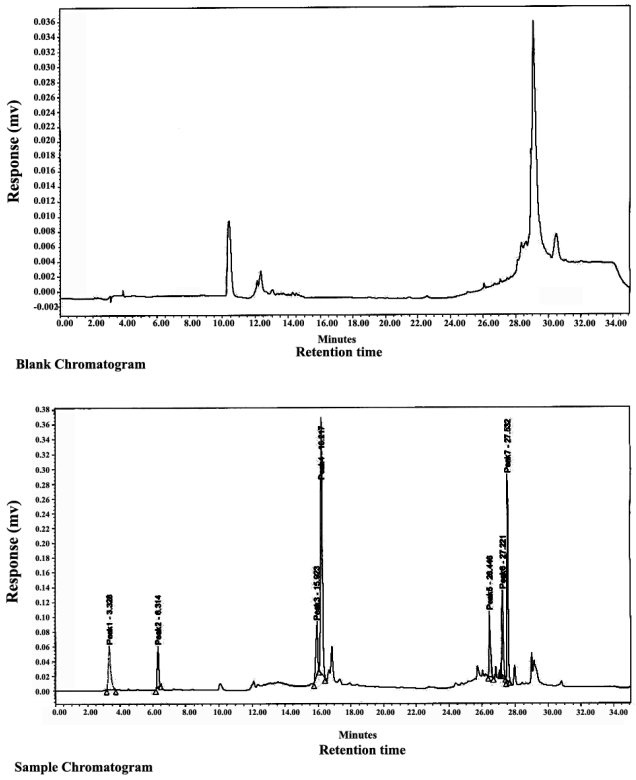

Figure 3. HPLC Chromatogram of Culture filtrate of *Streptomyces* sp. SCBT.

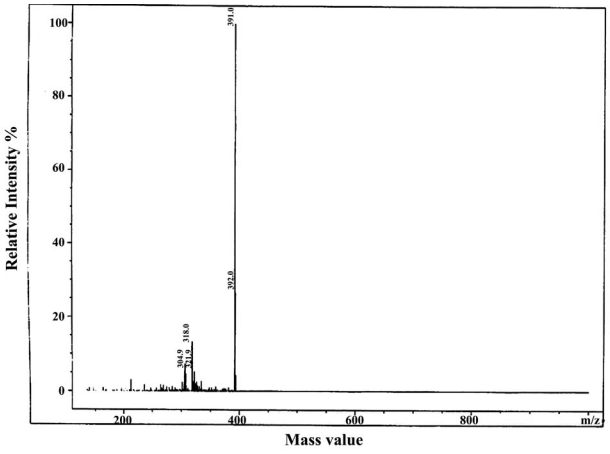

Figure 4. LCMS profiling.

in soil, it is often necessary to control the growth of some fast growing fungi on plates. This is particularly critical when particularly trying to screen and isolate large numbers of actinomycetes in various soil samples. The fungicide currently used is cycloheximide which is considered to be very toxic. Cycloheximide is an inhibitor of protein synthesis and induces apoptosis in mammalian cells (Busch *et al*., 2004; Chao *et al*., 1999). Natamycin has also been used as an alternative for isolating bacteria present in low numbers in the environment. (Ahmed Nasr Mohamed *et al*., 2005) Their results demonstrated that natamycin concentrations inhibits the growth of fungi on the media, have a small but significant inhibitory effect on the number of bacterial colony forming units and suggesting that high concentration of natamycin cannot be used for isolation of bacterial strains with the aim of studying biodiversity and for the selection of strains for practical applications. Hence, the effect of the alternative antifungal agent carbendizim has also been evaluated in this study. The effect of carbendizim on actinomycete community structures was never estimated.

In order to discover a novel compound of pharmaceutical interest, the search for novel metabolites especially from *Streptomyces* spp. requires a large number of isolates (over a thousand) (Oskay *et al*., 2004) The search will be more promising if diverse *Streptomyces* are sampled and screened. For this reason, soils were specifically collected from the rhizosphere soil of medicinal plants. This is based on the hypothesis that *Streptomyces* diversity may be influenced by the diversity of plant species. Furthermore, different plants produce different types of secondary metabolites and some of these chemical compounds are toxic to soil microorganisms including *Streptomyces*. However, adaptation has in turn allowed the *Streptomyces* to produce their own secondary metabolites. Although the collection sites have mainly been limited, i.e., the rhizosphere soil of medicinal plants, yet many *Streptomyces* have been found to have both higher and lower occurrences in rhizosphere soil than in bulk soil, depending on the plant species (Dickinson and Dooley, 1967; McCarthy and Williams, 1990). The plant exudates may play the role in the occurrence of microbial diversity and hence the rhizopshere soil of medicinal plants were chosen for this study. A number of researchers have pointed out a possible correlation between occurrence of antagonistic *Streptomyces* and nature of the soil from which they are isolated. Rouatt *et al*. (1951) have found a greater percentage of active streptomycetes in the rhizosphere than in the surrounding soil.

In summary the results of this study show that the isolated *Streptomyces* sp. SCBT strain exhibits wide antibacterial activity, which proves that the rhizosphere soil of medicinal plants can serve as an effective source of pharmaceutically important microorganisms.

The antimicrobial substance produced by *Streptomyces* sp. SCBT, when purified has been shown to have antibacterial activity against several species of human pathogens including both Gram-positive and Gram-negative bacteria. These findings indicate that antibiotics produced by the strain SCBT could act as alternative tools for controlling human diseases. There is ample scope for carrying out further research on the structural elucidation of the antibiotics produced by *Streptomyces* sp. SCBT, besides enriching scientific knowledge with regard to the mode of action of antibiotic compounds on pathogenic microbes.

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