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Antagonistic potential of marine Actinobacteria against fish and shellfish pathogens

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Abstract: A total of 94 marine actinobacterial isolates were found to be associated with 4 sponges: *Callyspongia diffusa, Mycale mytilorum, Tedania anhelans*, and *Dysidea fragilis*. Among the cultures 7 strains showed antagonism against fish and shellfish pathogens. Analysis of morphological, physiological, biochemical, and protein profile characteristics suggested that the actinobacterial strains belonged to the genus *Streptomyces*. The initial screening of the isolates by the spot inoculation method exhibited antibacterial activity against *Aeromonas hydrophila*. In vitro screening of the submerged culture extracts showed positive inhibition against the fish and shellfish pathogens *Aeromonas hydrophila*, *Serratia* sp., and *Vibrio* spp. The screening of bioactive compounds by UV spectrum confirmed the production of polyene substances, which resulted in absorbance peaks ranging from 225 to 245 nm. In TLC analysis the R_f values ranged from 0.40 to 0.78. The results indicate that the *Streptomyces* strains isolated from marine sponges produce potential bioactive compounds against fish and shellfish pathogens.

Key words: Marine sponges, Actinobacteria, *Streptomyces*, bioactive compounds, fish and shellfish pathogens

Introduction

Marine microorganisms are of considerable interest as a new and promising source of biologically active compounds (1). They produce a variety of metabolites, some of which can be used for drug development (2). Marine Actinobacteria is a very important group of bacteria that has considerable value as a prolific producer of antibiotics and other therapeutic compounds. Actinobacteria produce over half of the bioactive compounds in the Antibiotic Literature Database (3). The isolation of Actinobacteria from marine environments has been a fruitful area of research in the past decade. However, little is known about the diversity of Actinobacteria from marine samples as compared to the Actinobacteria isolated from terrestrial environments (4). Increasing numbers of both culture-based studies and culture-independent molecular studies show that many Actinobacteria exist in marine environments such as sediments, seawater, and marine invertebrates (5,6).

Many marine actinobacterial taxa in ocean sediments were found to have widespread persistent populations in ocean systems, and some taxa have no counterparts in terrestrial environments. It is becoming evident that marine habitats are an abundant source of the Actinobacteria that could form the basis of new natural products. Many promising antibiotics were discovered in marine Actinobacteria, in particular *Streptomyces* (7). Recently several antibiotics

including trioxacarcin (8), allophycocyanin (9), piperazimycin (10), chalcomycin (11), streptochlorin (12), phaeochromycins (13), marinopyrroles (14), essramycin (15), sisomicin (16), and T-muurolol (17) have been reported in marine *Streptomyces*. The antibiotics produced are entirely new and unique when compared to those derived from terrestrial sources. Of the marine inhabitants investigated, marine invertebrates, particularly sponges, are of great interest for discovering novel Actinobacteria. As sessile filter-feeding animals sponges are the largest source of marine bioactive metabolites, accounting for up to 40% of all known natural marine products (18). Few studies have investigated the diversity, distribution, and ecology of Actinobacteria from marine sponges, although several reports point out their abundance (19). There are several reports on the bioactivity of Actinobacteria, namely *Streptomyces*, from marine sediments against human and fish pathogens (20,21). There have been a few reports on antagonistic activity of marine sponges associated with *Streptomyces* against human pathogenic bacteria and fungi (22,23). The present investigation deals with the antagonistic potential of sponges associated with the Actinobacteria *Streptomyces* against fish and shellfish pathogens.

Materials and methods

Microorganism and maintenance of culture

The microorganisms used in the study were *Streptomyces* strains isolated from the marine sponges *Callyspongia diffusa*, *Mycale mytilorum*, *Tedania anhelans*, and *Dysidea fragilis*. Identification of the sponges and isolation of the *Streptomyces* strains were mentioned in an earlier report (24). The isolated strains were maintained as glycerol asparagine agar slant cultures at 28 ± 2 °C. The inocula used in all the experiments were 7-day-old cultures unless otherwise stated.

Characterisation of the *Streptomyces* isolates

The strains were characterised using the methods developed for the International *Streptomyces* Project (25). The microorganisms were characterised by acid-fast staining and Gram's staining techniques. The isolates were also studied by employing the various parameters detailed below.

Pigmentation of mycelia and spore morphology – The cultures were grown in a petri dish containing casein-starch-peptone-yeast extract (CSPY) agar medium with a cover slip inserted at an angle of 45°. The cover slip was removed after 7 days of incubation, and the cultures were air dried and observed under a scanning electron microscope (26).

Melanoid production – The cultures were streaked onto peptone-yeast extract-iron agar slants and incubated at 28 °C for 48 h.

Utilisation of carbon sources – Carbon sources (glucose, xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, and sucrose) were mixed at a concentration of 1% each to 10 mL of basal mineral salt medium and incubated at 28 °C for 7 days.

Physiological and biochemical characteristics were studied according to the procedures described by Buchanan (27).

Preparation of whole-cell proteins

A loopful of 7-day-old cultures of the strains was transferred to 1.5 mL Eppendorf tubes, centrifuged for 3 min at 12,000 rpm, and the collected cells were washed 3 times with distilled water. The washed cells were stirred after adding 25 µL SDS-samples buffer (0.06 M Tris-HCl, 2.5% Glycerol, 0.5% SDS, 1.25% β-mercaptoethanol), and the proteins were denatured by boiling the tubes for 5 min.

SDS PAGE

Solubilised proteins were subjected to SDS-PAGE in gel slabs of 1 mm thickness (5% stacking and 10% resolving gels) as described in Laemmli (28). Electrophoresis was performed with a discontinuous buffer system in a Biotech vertical slab gel apparatus (Biotech-Yercaud, India). The gel was run at 50 mA until the bromophenol blue marker reached to the bottom. A standard marker (14-80 kD) was also run along the stained proteins. The gel was stained with Coomassie Brilliant Blue R-250, and documentation was carried out to find the molecular weight of the respective proteins (Lark biosciences).

Antimicrobial assay

Spot inoculation method

Preliminary screenings for bioactivity were done using the method described in Shomurat et

al. (29). The strains were spot inoculated in glycerol asparagine medium for 7 days. After 7 days 1 mL of chloroform was added and made to stand for 40 min to arrest the growth of the inoculated colonies. Then they were overlaid with 5 mL of sloppy agar (0.6%), a layer previously seeded with 1 of the test organisms. Here the selected test organism was *Aeromonas hydrophila*. It was incubated for 24 h at 37 °C, and the diameter of the incubation zone was recorded in millimetres.

In vitro screening of isolates for antimicrobial activity (disc method)

The isolates were inoculated as submerged culture in 500 mL Erlenmeyer flasks containing 100 mL of the liquid medium (0.8 g NaCl, 1 g NH_4Cl, 0.1 g KCl, 0.1 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2.2H_2O$, 2 g glucose, and 3 g yeast extract in 1 L of distilled water, pH 7.3). These cultures were grown in a rotary shaker at 200 rpm at 28 °C for 120 h under the standard conditions of aeration and agitation. The resultant cultures were centrifuged for 15 min. The culture filtrates were solvent extracted with ethyl acetate (1:1) in the separating funnel and shaken vigorously for 20 min. The upper organic layers were collected and evaporated to dryness in a vacuum at 40 °C. Crude gummy extracts were obtained. The crude extracts were suspended in ethyl acetate at a concentration of 1 mg per mL for antimicrobial studies. Sterile filter paper discs 6 mm in diameter (HiMedia, India) were impregnated with 50 µL (50 µg crude extract) suspensions, dried, and placed on the plates previously seeded with the fish and shellfish pathogens *Aeromonas hydrophila, Serratia* sp., and *Vibrio* spp. The selective medium Thiosulphate–citrate–bile salts–sucrose (TCBS) was used for cultivation of *Vibrio* spp. (*V. alginolyticus, V. harveyi*, and *V. parahaemolyticus*). Tryptone soya agar medium was used for culturing *Aeromonas hydrophila,* and nutrient agar medium was used for *Serratia* sp. The plates were incubated for 24 h at 37 °C for *Aeromonas hydrophila* and *Serratia* sp. and 24 h at 28 °C for *Vibrio* spp., and the zone of inhibition diameters were measured (30). The in vitro screening was repeated 3 times.

Screening of polyene substances from the submerged culture extracts

Polyene compounds were recovered from the culture filtrates by solvent extraction with ethyl acetate in a ratio of 1:1 (v/v) and shaking for 1 h (31). The ethyl acetate phase was separated and evaporated to dryness in water bath at 80-90 °C, and the residue was weighed and redissolved with a little volume of ethyl acetate. The absorption spectrum was determined in UV and Visible region (200-600 nm) by using a UV/VIS spectrophotometer 2101 (Systronics).

Thin layer chromatographic analysis of antibacterial compounds

The extracts were spotted on the baseline of the silica gel plates (stationary phase) at 1 cm and then allowed to dry at room temperature (32). The plates were placed in a TLC chamber pre-saturated with the mobile phase butanol:acetic acid:water (4:1:2). The chromatogram was developed and visualised under UV light and the spots were marked. The R_f values for each spot were measured.

Results and discussion

In a previous report 94 cultures of the marine Actinobacteria *Streptomyces* were found to be associated with 4 sponges, and they produced antibacterial and antifungal activity against human pathogens (24). In the present investigation the antagonistic potential of these symbiotic strains against fish and shellfish pathogens was assessed. Among 94 cultures 7 strains exhibited positive antagonism against fish and shellfish pathogens. They were characterised by the methods recommended by the International *Streptomyces* Project. The colonies were chalky, folded, and aerobic. The colonies appeared after 7 days, and completion of the growth of the culture occurred on the 14th day. The strains were acid-fast negative and Gram-positive. The aerial mycelial colour pattern of the strains was a white, grey, and yellow series. The vegetative mycelial colour differed for all the strains. The strains do not produce melanoid pigments. The morphological characteristics of the aerial mycelia, vegetative mycelium, sporophore, and the spores of the strains could be clearly observed. In an observation using the scanning electron microscope spore morphology showed smooth spore surface and rectiflexibiles (RF) hyphae (Table 1). The strains showed the typical

Table 1. Characterisation and carbon utilisation of *Streptomyces* strains (+ positive results; - negative results; ± doubtful results; RF, rectiflexibilis; S, smooth).

Streptomyces strains	Color of aerial mycelium	Color of vegetative mycelium	Melanoid pigments	Spore surface	Spore chain	No carbon source (negative control)	D- xylose	L-arabinose	Rhamnose	D- fructose	D- galactose	Raffinose	D- mannitol	Inositol	Sucrose	D- glucose (positive control)
AQBCD03	Grey	Yellow	-	RF	S	-	+	+	+	+	-	-	+	-	-	+
AQBCD11	White	White	-	RF	S	-	+	+	-	+	-	+	-	-	-	+
AQBCD24	White	Orange	-	RF	S	-	+	-	+	+	+	-	-	-	±	+
AQBMM35	White	Pale yellow	-	RF	S	-	+	+	-	-	+	+	-	-	±	+
AQBMM49	Yellow	White	-	RF	S	-	+	+	-	-	+	-	+	-	-	+
AQBTA66	White	Red	-	RF	S	-	+	+	-	+	+	-	+	-	±	+
AQBDF81	Grey white	Yellow	-	RF	S	-	+	-	-	+	+	-	+	-	±	+

morphology of streptomycetes when the shape and spore chains were analysed under the scanning electron microscope as reported earlier (26).

The nutritional characteristics of the strains were studied using criteria carbon utilisation. The utilisation of carbon sources is displayed in Table 1. The strains grew well in media containing glucose and xylose, but the strains did not assimilate inositol. In a medium with sucrose the growth of the strains was weak or absent. The physiological and biochemical

Table 2. Physiological and biochemical characteristics of *Streptomyces* strains (+ positive results, - negative results).

Parameters	AQBCD03	AQBCD11	AQBCD24	AQBMM35	AQBMM49	AQBTA66	AQBDF81
Starch hydrolysis	-	-	-	+	+	-	-
Production of H_2S	+	+	+	-	-	+	+
Degradation of cellulose	-	-	-	+	+	-	-
Liquefaction of gelatin	+	+	+	+	-	-	+
Coagulation of milk	+	+	-	+	+	+	+
Peptonization of milk	+	+	-	+	+	+	+
Degradation of urea	-	+	-	+	-	-	-
Citrate utilisation	+	+	-	+	+	+	-
Indole production	+	+	+	-	-	+	+
Catalase	+	-	+	-	+	+	+

characteristics of the strains are displayed in Table 2. All the strains were able to grow in 22-45 °C and pH 4-10. With 2 exceptions, AQBMM49 and AQBTA66, the strains were able to liquefy gelatine. The strain AQBCD24 cannot solidify milk and peptone, while the others can. Strains AQBMM35 and AQBMM49 were able to hydrolyse starch and cellulose, but unable to produce hydrogen sulphide. Degradation of urea was effectively done by AQBCD11 and AQBMM35. Positive utilisation of citrate was confirmed in the strains AQBCD03, AQBCD11, AQBMM35, AQBMM49, and AQBTA66, but not AQBCD24 or AQBDF81. Indole production was observed in all the strains, with the exceptions of AQBMM35 and AQBMM49. Finally, catalyse activity was effectively seen in all the strains but AQBCD11 and AQBMM35. Nutritional uptake as well as physiological and biochemical characteristics clearly confirm the classification of *Streptomyces* as reported earlier (25). The identification of *Streptomyces* is a very complex process. The former *Streptomyces* classification system was mainly dependent on characteristics such as the form of spores, melanoma, and use of carbon. The method of classifying *Streptomyces* used in this study is one recognised around the world (27).

The protein profiling was done to identify differentiation among the strains that exhibited bioactivity. The protein profiles of the 7 strains showed different bands of molecular weight ranging from 9 to 80 kD (Table 3, Figure 1). Bacterial whole-cell protein profiles can be analysed by polyacrylamide gel electrophoresis to generate complex banding patterns that can be used to delimit species and subspecies groups. Proteins can be analysed either by using 1-dimensional (1-D) or 2-dimensional protein electrophoresis. Many bacterial strains can be compared rapidly and reproducibly with 1-D protein electrophoresis (33). Previous investigations used SDS-PAGE to analyse and compare the cellular protein profiles of 32 *Streptomyces* strains and 5 *Streptoverticillium* strains (34). Taxonomic correlations were found between the profiles obtained and the phenotypic groupings observed earlier (35).

Preliminary identification of antagonism against pathogen was done by the spot inoculation method, and the strains showed more than 10 mm of inhibition zone against *Aeromonas hydrophila* (Figure 2). Later, in vitro screening of the culture extracts was carried out by the disc method, which resulted in more than 10 to 30 mm diameter inhibition zones (Table 4). Inhibition zones over 30 mm were recorded against *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, and *Vibrio harveyi*. The strains AQBCD03, AQBCD24, AQBMM35, and AQBDF81 exhibited less activity

Table 3. Documentation results of SDS-PAGE (MW, molecular weight; R_f, retention factor).

MARKER		AQBCD03		AQBCD11		AQBCD24		AQBMM35		AQMM49		AQBTA66		AQBDF81	
MW	R_f	MW	R_f	MW	R_f	MW	R_f	MW	R_f	MW	R_f	MW	R_f	MW	R_f
80	0.171	79.869	0.175	47.963	0.601	78.931	0.201	80.327	0.166	80.573	0.163	78.265	0.218	79.383	0.188
66	0.381	75.034	0.283	23.741	0.84	76.916	0.248	71.877	0.326	78.064	0.223	73.895	0.3	75.259	0.279
56	0.454	71.556	0.329	12.929	0.949	75.967	0.267	69.036	0.355	77.319	0.24	62.467	0.406	69.036	0.355
50	0.54	69.426	0.351			69.93	0.346	63.68	0.398	72.984	0.312	53.811	0.475	60.025	0.424
40	0.651	58.831	0.432			66.313	0.378	56.589	0.449	61.768	0.411	50.263	0.531	49.556	0.561
29	0.689	55.037	0.463			54.161	0.471	52.755	0.487	53.054	0.483	45.973	0.621	45.973	0.621
25	0.826	50.142	0.535			50	0.54	48.868	0.585	50.818	0.518	32.321	0.677	23.814	0.737
20	0.874	46.986	0.612			48.052	0.6	39.333	0.654	48.815	0.586	23.798	0.777	23.798	0.788
17	0.925	23.798	0.749			37.191	0.661	31.253	0.681	41.243	0.646	22.29	0.852	21.28	0.86
14	0.942	23.798	0.814			23.798	0.783	23.798	0.754	25.835	0.706	18.915	0.9	9.071	0.971
		21.28	0.86			19.76	0.878	21.28	0.86	23.798	0.835	9.5	0.968		
		16.455	0.929			12.071	0.953	11.857	0.955	19.076	0.895				
										14	0.942				

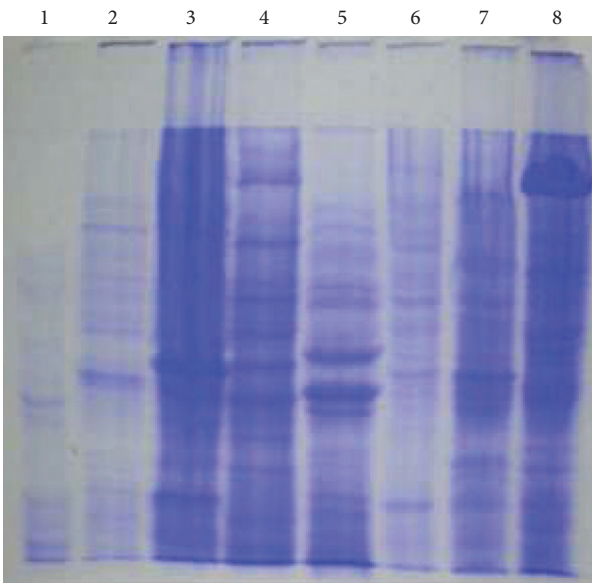


Figure 1. SDS-PAGE of *Streptomyces* strains (Lane1-marker, 2- AQBCD03, 3- AQBCD11, 4- AQBCD24, 5- AQBMM35, 6- AQBMM49, 7- AQBTA66, and 8- AQBDF81).

against *V. parahaemolyticus*, *V. alginolyticus*, and *V. harveyi* with 10 mm inhibition zones. Some strains showed inhibition against *V. harveyi* (Figure 3). The fish and shellfish pathogens (*Aeromonas hydrophila, Serratia* sp., and *Vibrio* spp.) used in this study have caused serious trouble for aqua-culturists for the past several decades. The fish pathogen *Aeromonas hydrophila* causes diseases such as motile aeromonas septicemia, hemorrhagic septicemia, and ulcer disease or red-sore disease (36). There are several strains of *Serratia* that cause diseases in fish as well as food poisoning in humans who consume the affected sea food (37). A major disease impacting shrimp aquaculture, vibriosis, is caused by the *Vibrio* species, in particular *Vibrio alginolyticus*, *V. harveyi*, and *V. parahaemolyticus* (38,39). There are some reports of actinobacterial strains from marine sediments being used against shrimp pathogens like *Vibrio* spp. (40).

The screening of bioactive compounds by UV spectrum confirmed the production of polyene substances in the submerged culture extracts, which resulted in absorbance peaks ranging from 225 to 245 nm. In TLC analysis the R_f values ranged from 0.40 to 0.78 (Table 5). This indicates that the isolates produce different bioactive compounds that inhibit the fish and shellfish pathogens. The UV spectral data and TLC analyses of the strains were clearly explained in the previous report (24) and correlate very well with the previous findings (41).

In conclusion, marine-sponge-associated *Streptomyces* strains represent a promising source of antibacterial agents against fish and shellfish pathogens. This is the first study reporting on the antagonism of marine *Streptomyces* against fish and shellfish pathogens. The screened *Streptomyces* strains were also found to have the potential to be used as probiotics in aquaculture (42). There are very limited reports on the use of marine Actinobacteria as antibacterial agents against fish and shellfish

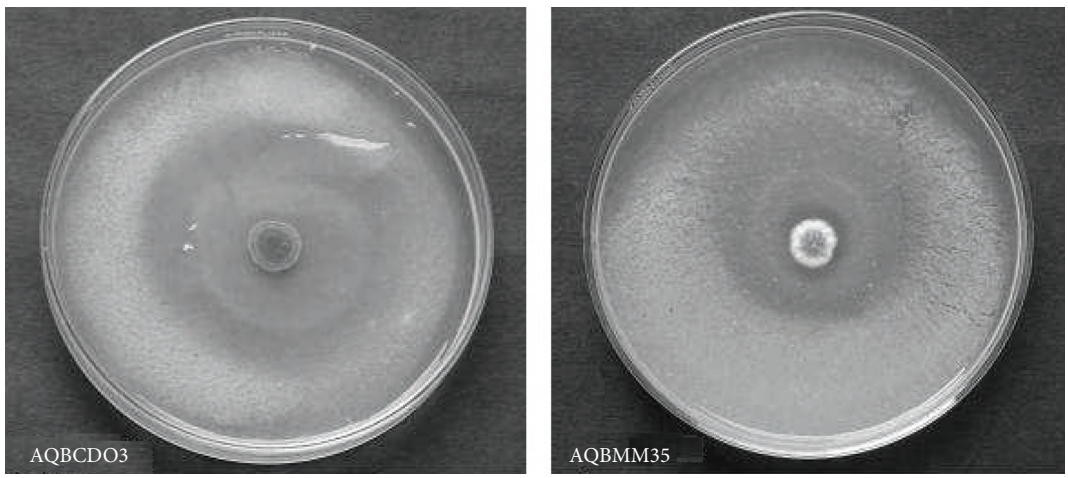


Figure 2. Inhibition zone against *Aeromonas hydrophila* by spot inoculation method.

Table 4. Antagonistic activity of *Streptomyces* strains against fish pathogens (inhibition zone: +, < 10 mm; ++, 10–20 mm; +++, 21–30 mm; ++++, > 30 mm; no inhibition zone: -).

Strains	Disc method					Spot inoculation method
	Aeromonas hydrophila	*Serratia* **sp.**	*Vibrio harveyi*	*Vibrio parahaemolyticus*	*Vibrio alginolyticus*	
AQBCD03	++++	++	++++	+	+++	++++
AQBCD11	++	++	+++	++	+++	+++
AQBCD24	++	++	+++	++++	+	++++
AQBMM35	++++	++++	++	++	+	++++
AQBMM49	+++	+++	++	+	++	+++
AQBTA66	++	+++	++	+++	++	+++
AQBDF81	+++	+	++	++	++	++

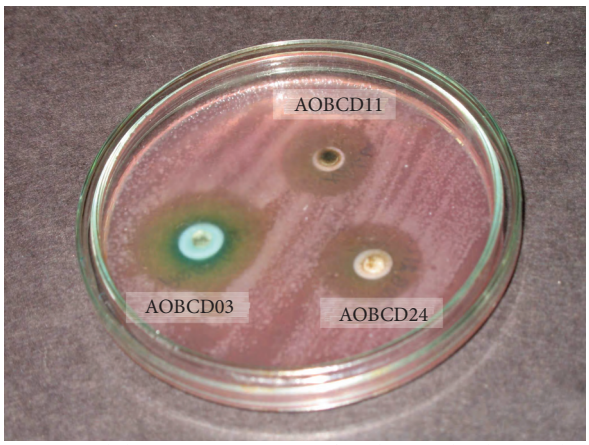


Figure 3. Inhibition zone against *Vibrio harveyi* by disc method.

pathogens. The development of new classes of antibiotics is necessary due to the increased incidence of multiple resistances to drugs that are currently in use among pathogenic microorganisms. Antibiotics developed from marine microbes are particularly attractive because they have high potency when compared with their terrestrial counterparts.

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Table 5. UV absorption and R_f values of the strains.

Strain	Maximum (nm)/ Absorbance values	Shoulder (nm)/ Absorbance values	R_f values
AQBCD03	237.8-2218	275.6-1.498 326-1.102	0.40, 0.46,0.78
AQBCD11	245-2.400	329.6-0.876	0.61, 0.77
AQBCD24	225.2-0.350	329.6-0.019	0.50, 0.54
AQBMM35	228.8-1.064	329.6-0.307	0.46, 0.61
AQBMM49	241.4-2.148	278.6-1.221 332.2-0.264	0.51, 0.63, 0.70
AQBTA66	245-2.290	331.4-0.437, 365.6-0.772,	0.51, 0.77, 0.78
AQBDF81	236-1.431	282.8-0.240	0.46, 0.51

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