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Antimicrobial Activity of Heterotrophic Bacterial Strains of Marine Origin

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A B S T R A C T

Background: Bacterium-bacterium antagonistic interactions could be important in the ecology of marine bacteria. Antimicrobial properties of microorganisms are exploited in various fields of human activities.
Objectives: Antagonism of heterotrophic bacteria from different marine environments of tropical and temperate zones was examined.
Materials and Methods: Bacteria were isolated from biofilm samples, tissues of hydrobionts and sea water. Isolates were characterized by phenotypic and 16S rRNA phylogenetic analyses. Agar diffusion assay was applied to investigate inhibitory interactions. 5 type strains and 21 strains of marine origin were used as test cultures.
Results: 68.97% of isolates from temperate zone and 56.76% of tropical zone showed antimicrobial activity. The most active strains belonged to genera *Pseudomonas* and *Pseudoalteromonas.*
Conclusions: Bacterial interspecies growth inhibition is widely distributed in marine environments. Marine bacteria, especially *Vibrio* spp., may be good probiotics which are active against pathogenic bacteria.

Keywords: Aquatic Organisms; Antimicrobial Activity; Probiotics

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Implication for health policy/practice/research/medical education:
Our results demonstrated selective activity of isolates from different climatic zones against human pathogens. It may help in search strategy for new probiotics of marine bacterial origin.

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1. Background

Bacterium-bacterium interactions, diverse in form and content are widely seen in water column with different microparticles. Over one half of marine bacteria examined so far have displayed antagonistic activity towards other pelagic bacteria. Antimicrobial interactions influence, first, the structure of the microbial community and second, the functioning of microbial cenoses (1). Microbial antagonism results from the effects of antibiotic substances inhibiting the growth of microorganisms or killing them. Bacterial production of secondary metabolites, in particular growth inhibitors, is one of the adaptation mechanisms, which gives advantage in competition for available nutrients and living space.

Searching for previously unknown microbial strains is an effective approach to obtain new biologically active substances. Marine bacteria are producers of unique substances which have never been found in terrestrial organisms (2, 3). Secondary metabolites of microbial origin are widely used in various fields of human activities, such as medicine, agriculture, pharmaceuticals, food processing, chemical industries and many others. In addition to production of antibiotics and lytic enzymes (4), the antimicrobial properties of microorganisms are used to work out biofilms with anticorrosion and antifouling properties (5, 6) and to enhance crop protection against phytopathogenic bacteria (7) and fungi (8).

In aquaculture, the usage of live cultures of antagonistic bacteria as probiotics is developing to prevent outbreaks of diseases in aquatic organisms (9, 10). The interest to biological control in the past two decades has increased dramatically due to expanding opportunities for synthesis of biological products and highly competitive chemical preparations which could often inflict enormous damage to the environment (11).

2. Objectives

The aim of our study was to investigate the peculiarities of bacterium-bacterium antagonistic interactions among heterotrophic bacteria of marine origin in tropical and temperate zones. This would be helpful to clarify some problems concerning the role of biosynthesis of antimicrobial substances in natural bacterial communities and could also be helpful to develop a strategy of search for new physiologically active substances of bacterial origin.

3. Materials and Methods

3.1. Sampling

Heterotrophic bacteria were isolated from different marine objects in Nha Trang Bay, South China Sea in June–July 2008 and January 2009; in the pelagic part of the East China Sea in April 2010; and in Peter the Great Bay, Sea of Japan, in August 2008, 2009 and 2010 in Nha Trang Bay. Invertebrates and algae were collected by scuba divers from depths of three to 23 meters. Microflora samples of the fouling of copper-containing and aluminum plates were selected on a test bench of Marine Corrosion Station, Primorsky Branch of Russian-Vietnamese Tropical Center, Nha Trang (Vietnam). Water samples were taken directly at exposure points of metal plates. The samples of algae included the green alga *Caulerpa lentillifera* and brown algae *Padina* spp., *Turbullaria* spp. and *Sargassum* spp. The samples of animals included the ascidian *Didemnum molle*, bivalves *Pinctada margaritifera* and *Crassostrea gigas* and unidentified species of sponges (three samples).

3.1.1. Open Part of the East China Sea

Water samples were collected using a Niskin bottle at the points with coordinates 29 ° 40, 647 'N, 124 ° 27, 909 'E and 26 ° 46, 326 'N, 121° 41, 916 'E.

3.1.2. Peter the Great Bay

The samples of algae, animals and water were collected by scuba divers in Avangard Bight of Peter the Great Bay. The examined animals included bivalves *Crenomytilus grayanus, Modiolus dificillus* and *C. gigas*; echinoderms, sea urchins *Strongylocentrotus nudus* and *S. intermedius*; starfishes *Patiria pectinifera* and *Asterias amurensis*, and sea cucumber *Apostichopus japonicus* and the ascidian *Halocynthia aurantium*. The seaweed *Laminaria japonica* was examined from the plants. As test cultures the following type strains were applied: *Escherichia coli ATCC 15034, Bacillus subtilis BKM B501, Candida albicans KMM 455, Pseudomonas aeruginosa KMM 433* and *Staphylococcus aureus ATCC 21027*.

3.2. Isolation of Bacteria in Pure Cultures and Phenotypic Characterization of the Isolates

Isolation of bacteria from hydrobionts and seawater and preservation of bacterial strains were conducted as described elsewhere (12). Bacteria were isolated from internal tissues of ascidians and sponges, coelomic fluid and digestive tract of sea urchins, bivalves and starfishes and digestive tract of holothurians. Biofilm samples (8 cm2 in area) were scrapped off from the surface of each metal plate using a stencil and a sterile tool and then carefully taken with a sterile absorbent cotton stick. The stick with the microbial mass was placed into a test tube containing 2 ml of sterile seawater.

Serial dilutions of homogenates, biofilm suspensions and water samples (0.1 ml) were plated on solid Youschimizu–Kimura medium (13) with the following composition: peptone (5.0 g), yeast extract (2.0 g), glucose (1.0 g), K2HPO4 (0.2 g), MgSO4•7H2O (0.1 g), agar (12.0 g), distilled water (500 ml), and seawater (500 ml); the pH of the medium equaled 7.8 to 8.0. Cetrimide agar (Serva) supplemented with glycerol in proportion of 10 g/l was used to isolate bacteria of the genus *Pseudomonas*. The plates

were incubated for three to ten days at 28 °C for tropical isolates and at 23 °C for isolates from temperate zone.*E. coli, C. albicans, S. aureus, P. aeruginosa* and *B. subtilis* were cultured on tryptic soya agar (TSA, Difco).

Motility and cell morphology were observed by the hanging-drop method. Gram-reaction, oxidase and catalase activities, presence of nitrate reductase, sodium ion requirements and tolerance to different NaCl concentrations (0–12% NaCl), growth at different temperatures (4–42 °C), acid production from sugars, production of lysine and ornithine decarboxylases and arginine dehydrolase and gelatinase, DNA base composition and resistance to antibiotics were tested as described elsewhere (14). The following antibiotics were used for tests: ampicillin (Amp), erythromycin (Ery), gentamicin (Gen), lincomycin (Lin), rifampicin (Rif), oleandomycin (Ol), polymyxin B (Pol) and vibriostatic agent O-129 (2,4-diamino-6,7-di-isopropylpteridine) with Oxoid disks. Additional biochemical tests with API-20NE and API-20E test kits (bioMérieux) were performed as described by the manufacturers, except that strains were suspended in 3% NaCl.

3.3. 16S rRNA Gene Sequence Analysis of Bacterial Isolates

Total DNA was isolated using the standard technique (15). A fragment of 16S rRNA gene sequence was amplified in 25 μl of reaction mixture comprising 2.5 μl of 10 × PCR buffer, 2 μl of 10 mM dNTP mixture (2.5 mM each), 2.5 μl of each primer (2.5 μM), 10 ng of DNA and 1 unit of Taq DNA polymerase (Fermentas). Primers amplification were performed according to Lane (16). The PCR amplification (GeneAmp PCR System 9700, Applied Biosystems) was performed using the following scheme: an initialization hold at 95 °C for three minutes, 35 cycles each comprising 30 seconds at 94 °C, one minute at 56 °C and 1.5 minutes at 72 °C and the final hold at 72 °C for five minutes.

The purity and size of products were estimated in 1% agarose gel. The purified amplification products were applied as a matrix for sequencing, which was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The purified products of sequencing were subjected to electrophoresis with an ABI Prism 3130 genetic analyzer, on a 50 cm capillary cartridge. The obtained direct and inverse sequences for each tested species were aligned using SeqScape v2.6 (Applied Biosystems) software. The obtained fragments of 16S rRNA gene sequences were deposited in NCBI/GenBank (GenBank accession numbers. GU579451, GU579452, GU726840–GU726880, JN679843–JN679865). Phylogenetic trees were developed with the Neighbor-Joining Method (NJ) (17), using Kimura's two-parametric model of nucleotide substitutions (K2P) (18) and MEGA 5 software (19). Cluster stability was estimated using bootstrap analysis (1000 iterations) (20).

3.4. Screening of Isolates for Inhibitory Interactions

Antimicrobial activity was tested in strains isolated in Vietnam from the surface of metal plates fouling and seawater and in Russian isolates associated either with different hydrobionts or with free-living in seawater. As test cultures strains were used isolates in tropical and temperate zones from seawater and different hydrobionts and five type strains belonging to Gram-positive bacteria, Gram-negative bacteria and yeasts. Antimicrobial activity of the isolates was assayed with a slightly modified method of Long and Azam (1). A lawn of a target isolate was prepared by mixing 25 ml of molten (44 °C), 0.6% Marine agar with 0.5 ml of isolate suspension. The suspension was prepared by dilution of an MA-grown daily culture in physiological solution down to concentration of 109 cells/ml, according to the McFarland Turbidity Standard. From 12 to 16 strains of potential growth inhibitors were spotted on the lawn. The plates were incubated face up for six days at 28 °C for tropical isolates and at 23 °C for temperate zone and examined daily for zones of inhibition. Potential producers were considered positive, if the diameter of the inhibition zone was at least 4 mm greater than that of the colony formed by the potential producer.

4. Results

4.1. Identification of Bacteria

Among 66 strains analyzed for antimicrobial activity, 61 strains were identified using results of 16S rRNA gene sequencing (*Figure*).

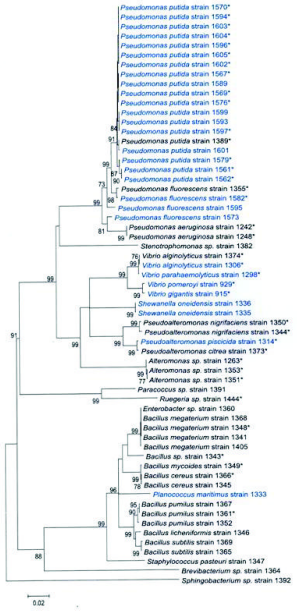

Figure. Strains Isolated from Peter the Great Bay, Sea of Japan, Russia and Nha Trang Bay, South China Sea, Vietnam Are Shown in Blue and Black, Respectively.

The strains used as test cultures and five antagonistic strains were identified by phenotypic characteristics. The obtained data was compared with available literature to corroborate the identification of some bacterial strains. To specify the taxonomic position of some strains, antibiotics sensitivity test was applied. For example, the strain 1541 *Pseudoalteromonas sp*, tested for antimicrobial activity, showed oxidative metabolism pattern, possessed cytochrome oxidase, demonstrated low G/C DNA molar percentage ratio (39.5%) and had dark brown pigment which diffused into the medium; it hydrolyzed agar and did not possess nitrate reductase. It was sensitive to rifampicin, ampicillin, erythromycin, gentamicin and polymyxin, but resistant to lincomycin and oleandomycin.

Proceeding from phylogenetic and phenotypic analysis the strains examined for antimicrobial activity were divided into five different phylogenetic groups as follow: γ-*Proteobacteria* (66.7%), *Firmicutes* (27.3%), α-*Proteobacteria* (3%), *Actinobacteria* (1.5%), and *Bacteroidetes* (1.5%). Most of γ-*Proteobacteria* belonged to genera Pseudomonas and *Pseudoalteromonas*; bacteria of genera *Vibrio, Shewanella, Alteromonas, Enterobacter, and Stenotrophomonas* were registered as minor groups (*Table 1*). *Firmicutes* mostly constituted of species of the genus Bacillus and also included significantly fewer members of genera *Staphylococcus* and *Planococcus*.

The group of α-Proteobacteria included genera Ruegeria and Paracoccus; while phyla Bacteroidetes and Actinobacteria included one registered member each, namely the genera Sphingobacterium and Brevibacterium, respectively. Most Russian isolates obtained from different hydrobionts were categorized as γ- *Proteobacteria*, whereas more than a half of tropical isolates from the surface of copper-containing plates belonged to *Firmicutes*. Most of 21 strains identified by phenotypic characteristics belonged to genera *Bacillus* and *Vibrio* (*Table 2*). Genera *Xanthomonas, Enterobacter, Planococcus* and *Serratia* included one registered species each. The taxonomic position of strains 1335 and 1336 (*S. oneidensis*) and the strain 1355 (*P. fluorescens*), which were examined for capability to produce antimicrobial compounds and also were used as test cultures was determined using the results of 16S rRNA gene sequencing.

Table 1. Sources and Taxonomic Position of Bacterial Strains Tested for Antimicrobial Activity.

Strain No./ Gene Bank Ref. No.	Source of Isolation	Close Phylogenetic Relatives	Identity, %
Vietnam, Nha Trang Bay, South China Sea			
1242/GU726840[a]	brass	*P*	100
1248/GU726841	brass	*P. aeruginosa*	100
1263/GU726843	bronze	*Alteromonas sp.*	100
1341/GU726850	brass	*B*	100
1343/GU726852	brass	*Bacillus sp.*	99,6
1344/GU726853	brass	*P*	96,6
1345/GU726854	brass	*B*	100
1346/GU726855	brass	*B*	100
1347/GU726856	brass	*S*	100
1348/GU726857	brass	*B*	100
1349/GU726858	brass	*B*	99,8
1350/GU726859	bronze	*P*	97,3
1351/GU726860	bronze	*Alteromonas sp.*	99.9
1352/GU726861	bronze	*B*	100
1353/GU726862	bronze	*Alteromonas sp.*	99,9
1355/GU726863	copper	*P*	100
1360/GU726864	copper	*Enterobacter sp.*	99,5
1361/GU726865	copper	*B*	100
1364/GU726866	copper	*Brevibacterium sp.*	99,9
1365/GU726867	copper	*B*	100
1366/GU726868	copper	*B*	99,9
1367/GU726869	brass	*B*	100
1368/GU726870	brass	*B*	100
1369/GU726871	brass	*B*	100
1373/GU726872	brass	*P*	99,9

1374/GU726873	seawater	*V*	99,6
1382/GU726874	aluminum	*Stenotrophomonas sp*	100
1389/	aluminum	*P*	100
1391/GU726876	copper	*Paracoccus sp.*	99,1
1392/GU726877	brass	*Sphingobacterium sp.*	99,3
1405/GU726879	sponge	*B*	99,9
1441	seawater	*Staphylococcus sp.*	n/d[b]
1442	seawater	*Staphylococcus sp.*	n/d
1443	seawater	*Vibrio sp.*	n/d
Vietnam, Nha Trang Bay, South China Sea; The East China Sea			
1444	seawater	*Ruegeria sp.*	*99,9*
1541	seawater	*P*	*n/d*
1542	seawater	*Vibrio sp.*	*n/d*
Peter the Great Bay, Japan Sea, Russia			
915/EU579451	trepang	*V*	*99.8*
929	trepang	*V*	*99.1*
1298/GU726844	sea urchin	*V*	*99,9*
1306/GU726845	laminaria	*V*	*99,9*
1314/GU726846	soil	*P*	*100*
1333	ouster	*P*	*99,6*
1335/GU726848	ouster	*S*	*99,8*
1336/GU726849	ouster	*S*	*99,8*
1561	seawater	*P*	99,5
1562	seawater	*P*	99,5
1567	starfish	*P*	99.9
1569/JN679848	starfish	*P*	99.9
1570	sea urchin	*P*	99.9
1573	starfish	*P*	97.3
1576	starfish	*P*	100
1579	bivalve	*P*	99.2
1582	starfish	*P*	99.6
1589	starfish	*P*	99.9
1593	starfish	*P*	99.9
1594	sea urchin	*P*	99.9
1595	trepang	*P*	97.1
1596	bivalve	*P*	99.9
1597	holothurian	*P*	99.9
1599	bivalve	*P*	99,9
1601	bivalve	*P*	99.8
1602	sea urchin	*P*	99.9
1603	sea urchin	*P*	99.9
1604	sea urchin	*P*	99.9
1605	sea urchin	*P*	99.9

[a] The numbers of 16S rRNA gene sequences deposited in NCBI/GenBank
[b] no data

Table 2. Sources and Taxonomic Position of Bacterial Test-Culture Strains .

Strain No.	Source of Strain	Close Phylogenetic Relative	Identity, %
1420	Vietnam, holothurian	*Bacillus*	n/d
1421	Vietnam, holothurian	*Bacillus*	n/d
1422	Vietnam, holothurian	*Bacillus*	n/d
1423	Vietnam, holothurian	*Vibrio*	n/d
1424	Vietnam, holothurian	*Vibrio*	n/d
1425	Vietnam, holothurian	*Vibrio*	n/d
1427	Vietnam, ouster	*Vibrio*	n/d
1430	Vietnam, algae	*Bacillus*	n/d
1437	Vietnam, algae	*Bacillus*	n/d
1438	Vietnam, algae	*Bacillus*	n/d
1478	Vietnam, seawater	*Bacillus*	n/d
1537	Vietnam, seawater	*Vibrio*	n/d
1543	Vietnam, seawater	*Xanthomonas*	n/d
1306 / GU726845	Russia, laminaria	*V*	99.9
1331	Russia, trepang	*Enterobacter sp.*	n/d
1333 / GU726847	Russia, ouster	*P*	99.6
1335	Russia, ouster	*S*	99.8
1336	Russia, ouster	*S*	99.8
1355	Vietnam, copper	*P*	100
1410	Vietnam, aluminum	*S*	*99.9*
1530	Vietnam, bivalve	*Vibrio sp.*	n/d
ATCC 15034	KMM, PIBOC [a]	*E*	n/d
KMM 455	KMM, PIBOC	*C*	n/d
ATCC 21027	KMM, PIBOC	*S*	n/d
B	KMM, PIBOC	*B*	n/d
KMM 4	KMM, PIBOC	*P*	n/d

[a] The strains are deposited in the Collection of Marine Microorganisms (KMM) of the Pacific Institute of Bioorganic Chemistry, Far Eastern Division, Russian Academy of Sciences, Vladivostok, Russia

4.2. Antimicrobial Activity

68.97% of isolates from temperate zone and 56.76% of Vietnamese strains showed antimicrobial activity. The strains that showed the greatest activity were of tropical origin (*Table 3*). Regarding taxonomic position of the most active strains, in both temperate and tropical zones, the undisputed leaders belonged to families *Pseudomonadaceae* and *Pseudoalteromonadaceae* (*Table 3* and *Table 4*). Among Vietnamese isolates the activity against most test cultures, besides *P. aeruginosa and P. nigrifaciens*, was also demonstrated by *Ruegeria sp.* and *Bacillus* spp.

All tropical strains of *Alteromonas spp.*, *Pseudomonas spp.*, *Pseudoalteromonas spp.* and *Vibrio spp.* tested for antimicrobial activity appeared active against two or more test cultures. 35.7% of the genus *Bacillus* members showed antimicrobial activity. Two strains of Russian origin, *P. putida* no. 1567 and no. 1602, suppressed growth in 10 and 11 test cultures respectively (Table 4). Among *pseudomonads* 71.4% of strains demonstrated antimicrobial activity. A strain of *P. piscicida* suppressed growth in 10 test cultures. All *Vibrio* strains showed antimicrobial activity in respect to 1–4 test cultures. No antimicrobial activity was detected in the examined strains of *S. oneidensis and P. maritimus* and certain strains of *Pseudomonas* spp.

Vietnamese isolates were the most active against bacteria of genera *Bacillus* and *Vibrio*; Russian strains showed the greatest activity against *Bacillus spp., S. marcescens, S. oneidensis and Vibrio spp*. Tropical isolates suppressed more actively the growth of *E. coli* and *S. aureus*, whereas strains from temperate area were more active against *P. aeruginosa*. None of Russian isolates showed activity against *P. fluorescens and C. albicans*, whereas tropical strains of *P. citrea and Ruegeria sp.* suppressed the growth of *P. fluorescens*, while *P. nigrifaciens* and *Alteromonas* sp. were active against *C. albicans*.

Table 3. Antagonistic Activity of Bacterial Isolates From Nha Trang Bay, South China Sea, Vietnam

	1420	1421	1422	1423	1424	1425	1427	1430	1437	1438	1478	1537	1543
1242	+ [a]	+	+	+	-	+	+	+	+	-	+	+	+
1248	+	+	+	+	+	+	+	+	-	-	+	+	+
1263	- [b]	-	-	-	-	-	+	-	-	-	-	-	-
1343	-	-	-	-	-	-	-	-	-	-	-	-	-
1344	+	+	+	+	-	-	-	+	-	-	+	-	-
1348	+	-	-	-	-	-	+	-	-	-	-	-	-
1349	-	+	-	-	-	-	+	-	-	-	-	-	-
1350	+	-	-	+	-	+	-	+	+	-	+	-	-
1351	-	-	-	-	-	-	+	-	-	-	-	+	-
1353	+	-	-	+	-	-	-	+	-	-	-	-	-
1355	-	-	-	-	-	-	-	+	+	-	-	-	-
1361	+	-	+	-	-	+	-	-	-	-	-	-	+
1366	-	-	-	-	-	-	+	-	-	-	+	+	-
1373	-	-	-	-	+	-	-	+	-	-	-	-	+
1374	-	-	-	-	-	-	+	-	-	-	+	-	-
1389	+	-	-	+	-	-	-	-	-	-	+	-	-
1442	-	-	-	-	-	-	-	-	-	+	-	-	-
1443	+	+	-	-	+	-	-	-	-	-	-	-	-
1444	+	+	+	+	-	-	+	-	+	+	+	-	-
1541	-	+	-	+	-	-	-	+	+	-	-	-	-
1542	-	-	-	-	-	-	-	+	-	+	-	-	-
	E. coli	*C. albicans*	*S. aureus*	*P. aeruginosa*	*B.subtilis*	*1306*	*1331*	*1333*	*1335*	*1336*	*1355*	*1410*	*1530*
1242	-	-	+	-	+	+	+	-	+	+	-	+	+
1248	-	-	+	-	+	+	+	-	+	-	-	+	+
1263	-	-	-	-	-	+	-	-	-	-	-	-	+
1343	+	-	-	-	-	-	+	-	-	-	-	-	-
1344	-	+	-	+	-	-	-	-	-	-	-	-	-
1348	+	-	-	-	+	-	-	-	-	-	-	-	-
1349	-	-	+	-	-	-	+	-	-	-	-	+	-
1350	-	-	+	-	-	-	-	+	-	-	-	-	-
1351	-	-	-	-	-	+	-	-	-	-	-	-	-
1353	-	+	-	-	-	-	-	-	-	-	-	-	-
1355	+	-	-	-	+	-	+	-	-	-	n/d [c]	+	+
1361	+	-	+	-	-	-	-	-	-	-	-	-	-
1366	+	-	-	-	-	+	-	-	+	-	-	-	+
1373	-	-	-	-	-	-	-	-	-	+	+	-	+
1374	-	-	-	-	-	-	-	-	-	-	-	-	+
1389	+	-	-	-	-	-	-	-	-	-	-	+	-
1442	-	-	-	-	-	-	-	-	-	-	-	-	-
1443	-	-	-	-	-	-	-	-	-	-	-	-	-
1444	+	-	+	-	-	-	-	+	+	+	+	-	-
1541	-	-	-	-	-	-	-	-	+	+	-	-	-
1542	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Grown inhibition zone was more than 5 mm in diameter; [b] Grown inhibition zone was absent; [c] n/d – no data

Table 4. Antagonistic Activity of Bacterial Isolates From Peter the Great Bay, Sea of Japan, Russia

	1420	1421	1422	1423	1424	1425	1427	1430	1437	1438	1478	1537	1543
915	-	-	-	-	-	-	+	-	-	-	-	-	-
929	-	-	-	+	-	+	-	-	-	-	-	+	-
1298	-	-	-	-	-	-	+	-	-	-	-	+	-
1306	-	-	-	-	-	-	-	-	-	-	-	+	-
1314	-	-	-	+	-	-	+	-	-	-	+	+	-
1561	-	-	-	-	-	+	-	-	-	-	-	+	-
1562	-	-	-	-	-	-	-	-	-	-	-	-	-
1567	+	+	+	+	-	-	-	+	-	+	+	-	-
1569	-	+	+	-	-	+	-	+	+	+	-	-	+
1570	-	-	-	-	-	-	-	+	+	-	-	+	-
1576	-	+	-	-	-	-	-	-	-	-	-	-	+
1579	-	+	-	-	-	-	-	-	-	-	+	-	-
1582	+	+	-	-	-	-	-	-	-	-	-	-	-
1594	+	+	+	-	-	-	-	+	+	+	-	-	-
1596	-	+	-	-	+	-	-	+	-	-	-	-	-
1597	-	-	-	-	-	-	-	-	-	-	-	-	+
1602	+	+	+	-	+	-	-	+	+	+	-	-	-
1603	-	+	+	-	+	-	-	+	-	-	-	-	-
1604	-	+	+	-	+	-	-	+	-	+	-	-	-
1605	+	-	+	-	+	-	-	+	-	-	-	-	-
915	-	-	-	-	-	-	-	-	-	-	-	-	-
929	-	-	-	-	-	+	-	-	-	-	-	-	-
1298	-	-	-	-	-	+	-	-	-	-	-	-	+
1306	-	-	-	-	-	n/d	-	-	-	-	-	-	-
1314	+	-	+	-	-	-	+	-	+	-	-	+	+
1561	-	-	-	-	+	+	+	-	+	-	-	+	+
1562	-	-	+	-	+	+	+	+	-	-	-	+	-
1567	-	-	-	-	+	+	-	+	-	-	-	-	-
1569	-	-	-	-	-	+	+	+	-	-	-	-	-
1570	-	-	-	-	-	+	-	-	+	+	-	+	+
1576	-	-	-	-	-	-	-	-	-	-	-	-	-
1579	-	-	-	-	-	-	-	-	-	-	-	-	-
1582	-	-	-	-	-	-	-	-	-	-	-	-	-
1594	-	-	-	-	-	-	-	-	+	-	-	-	-
1596	-	-	-	-	-	-	-	-	+	-	-	+	-
1597	-	-	-	-	-	-	-	-	-	-	-	-	-
1602	-	-	-	+	-	-	-	-	+	+	-	+	-
1603	-	-	-	+	-	-	-	-	+	+	-	-	-
1604	-	-	-	+	-	-	-	-	+	-	-	-	-
1605	-	-	-	+	-	-	-	-	-	+	-	-	-

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Authors' Contribution

None declared.

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