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Siderophores of halophilic archaea and their chemical characterization

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Nine halophilic archaea *viz*., *Halobacterium salinarum, Halobacterium* sp.1, *Halobacterium* sp.2, *Halobaculum* sp., *Halococcus saccharolyticus*, *Halorubrum saccharovorum*, *Haloterrigena turkmenica*, *Halogeometricum* sp. and *Natrialba* sp. isolated from marine salterns around Bhavnagar coast were screened for siderophore production. Five isolates viz., *Halococcus saccharolyticus*, *Halorubrum saccharovorum*, *Haloterrigena turkmenica*, *Halogeometricum* sp. and *Natrialba* sp. produced siderophores as evidenced by positive reaction in $FeCl_3$ test, CAS assay and CAS agar plate test. Determination of chemical nature of siderophores by chemical assays and bioassays identified them as carboxylates. Quantification of siderophores indicated *Halorubrum saccharovorum* to be the maximum siderophore producer (2.62 RE mg/ml**)** and *Halococcus saccharolyticus* to be the least (1.33 RE mg/ml). The present study is the first report on siderophore production in Indian haloarchaeal strains. Mechanism of iron assimilation in four non-siderophore isolates still needs to be investigated further.

Keywords: Amphiphilic siderophores, Carboxylate, Catecholate, Halophilic archaea, Hydroxamate, Siderophores.

Siderophores are low molecular weight (<1000 Da) iron chelating compounds produced by micro-organisms (bacteria and fungi) to combat low iron stress[1, 2]. Rationale for siderophore biosynthesis besides overcoming the insolubility and immobility of ferric ions, is to regulate its uptake as iron at higher concentrations, wrecks the cellular chemistry. Although iron is the second most abundant transition metal on earth, the solubility of iron is very low at physiological *p*H as it forms insoluble stable complexes (10^{-17} *M*) of ferric oxyhydroxide (FeOOH), severely limiting the bioavailability of iron[3]. Hence, microorganisms have developed a sophisticated strategy to overcome the insolubility and immobility of ferric ions through chelation by siderophores.

Microbial siderophores are either hydroxamates, catecholate, carboxylates or mixed type. Hydroxamates are produced by bacteria and fungi, catecholates only by bacteria, carboxylates are produced by a few bacteria (*Rhizobium meliloti* and *Staphylococcus hyicus*)[4] and exclusively by fungi belonging to *Mucorales*. Mixed types are produced by fluorescent psuedomonads that contain both hydroxamate and catecholate groups. Recently, unique amphiphilic siderophores termed marinobactins and aquachelins produced by marine bacteria *Marinobacter* sp. and *Halomonas aquamarina* have been discovered[5]. The distinguishing structural feature of these siderophores is an additional fatty acid 'tail' attached to common peptidic iron chelating head group. Rest is cyclic or linear hexapeptides.

Extreme halophilic archaea are red pigmented and non-pigmented forms that form part of domain *Archaea* that require high salt concentration for their growth (4.5*M*), ten times the salinity of sea water.

Since, little is known on the assimilation of iron in archaea in general and extreme halophiles in particular, this study has been undertaken to investigate (1) the production of siderophores by halophilic archaea (2) their chemical characterization and (3) their quantification

Materials and Methods

Organisms—Nine haloarchaea isolated from marine salterns around Bhavnagar coast, out of which eight non-alkaliphilic isolates *viz. Halobacterium salinarum, Halobacterium* sp.1, *Halobacterium* sp.2, *Halobaculum* sp., *Halococcus saccharolyticus, Halorubrum saccharovorum, Haloterrigena turkmenica, Halogeometricum* sp. while one alkaliphilic *Natrialba* sp., were examined for siderophore production.

Iron decontamination—All glass wares were soaked overnight in HCl (6*M*) and rinsed with distilled water several times to remove traces of iron.

Medium for siderophore production—TYES medium[6] containing sea salt was used for siderophore production. The medium was decontaminated of iron by adding 8–hydroxyquinoline dissolved in chloroform to ensure complete removal of Fe^{3+} (Ref. 7).

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Growth—The test isolates were inoculated into TYES medium and incubated at their optimum growth temperatures determined earlier (data not shown) *viz*., 30ºC for *Halobacterium* sp.1, 37ºC for *Halobacterium salinarum, Haloterrigena turkmenica, Halogeometricum* sp., *Halococcus saccharolyticus* and rest four *viz,. Halobacterium* sp.2, *Halobaculum* sp., *Halorubrum saccharovorum, Natrialba* sp. were incubated at 45ºC. Cultures (10-12 days old) were centrifuged at 10,000 rpm for 15-20 min, and the culture supernatants were examined for extracellular siderophore production.

Screening for siderophore production

$FeCl_3$ test[8]—The formation of red or purple colour on addition of 1-5ml of 2% $FeCl_3$ indicated the presence of siderophore.

Chrome azurol sulphonate (CAS) assay[9]—It is a universal test for detection and determination of siderophores, as even 0.02 µm of siderophores can be determined. CAS assay is based on the observation that when a strong chelator (eg. a siderophore) removes iron from CAS/iron/detergent complex, the release of free dye is accompanied by change in colour from blue to orange. The dye can thus be used in solution or can be incorporated into agar plates. The change in colour from blue to orange after 2-4 min., on addition of 1ml of CAS solution to 1ml of culture supernatant, indicated siderophore production.

CAS agar plate test[9]—At 10µ*M* Fe (III) concentration, the agar develops a rich blue colour. The siderophores excreted by iron-starved microorganisms generally exceed this level. This results in a complete change to orange colour. Thus, formation of orange coloured halos around the fungal growth, indicated siderophore production.

Chemical characterization of siderophores

Isolates that gave positive reaction in $FeCl_3$ test and CAS assay indicating the siderophore production were grown in 50ml TYES medium, dispensed in 500ml conical flasks and incubated at their optimum growth temperatures as mentioned above. Cultures (10-12 days old) were centrifuged at 10,000 rpm for 15-20 min and the culture supernatants were examined for hydroxamate nature by $FeCl_3$ and tetrazolium tests, catecholates by Arnow's and $FeCl_3$ tests, while carboxylate nature was determined by spectrophotometric test.

Detection of hydroxamate siderophores

$FeCl_3$ test[10]—The formation of orange coloured ferric hydroxamates, showing maxima at 420-450 nm indicated presence of hydroxamate siderophores.

Tetrazolium test[11]—This test is based on the capacity of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate group in presence of strong alkali. Instant appearance of a deep red color on addition of tetrazolium salt and NaOH to the test sample indicated presence of a hydroxamate siderophore.

Detection of catecholate siderophores

Arnow's test[12]—Catecholate siderophores on reaction, in succession with nitrous acid, molybdate and alkali, yield a pink chromogen that absorbs maximally at 515nm. Catecholate siderophores were quantified by noting the absorbance at 515nm and using 2,3-dihydroxybenzoicacid as standard.

$FeCl_3$ test[10]—Catecholate siderophores from a wine coloured complex with λ_{max} at 495nm, on addition of ferric chloride.

Detection of carboxylate siderophores

Spectrophotometric test[13]—Carboxylate siderophores were detected by formation of copper complex which was observed for absorption maximum between 190–280 nm. There is no specific wavelength at which the copper complex gets absorbed. The entire wavelength 190–280nm was scanned to observe the peak of absorption of the siderophore.

Bioassays[14]

Bioassays of siderophores are more sensitive than the chemical assays[15]. Specific indicator strains were used for the various types of siderophores as *Arthrobacter flavescens* JG9 for hydroxamates, *Salmonella typhimurium* enb7 for catecholates and *Morganella morganii* SBK3 for carboxylates. (*A.flavescens* JG9 and *S. Typhimurium* enb7 were kindly supplied by Sally Leong, USA and *M. morganii* SBK3 was supplied by Rolf Reisbrodt, Germany).

The indicator strains, which are non-siderophore producers, were iron-starved and seeded on TYES medium. Formation of visible growth surrounding the disc loaded with culture of test organisms, indicated siderophore production.

Quantification of siderophores

Siderophores were quantified using rhizoferrin (standard for carboxylate siderophore) kindly supplied by Gunther Winklemann, Germany and noting its absorbance at 630 nm after 1h of incubation. Absorbance of rhizoferrin was extrapolated from the

standard graph and the absorbance of the samples were calculated from the standard values and denoted as rhizoferrin equivalent (RE mg/ml)[16,17].

Results and Discussion

Out of nine halophilic isolates examined for siderophore production, five viz., *Halococcus saccharolyticus, Halorubrum saccharovorum, Haloterrigena turkmenica, Halogeometricum* sp. and *Natrialba* sp. produced siderophores as evidenced by positive reaction in CAS assay and negative $FeCl_3$ test (Table 1). Negative $FeCl_3$ test was due to methodological reasons, as $FeCl_3$ complex was obstructed due to precipitation. It shows non-suitability of $FeCl_3$ test for assaying haloarchaeal siderophores. The isolates showing positive reaction in CAS assay also produced orange halos on CAS agar plate further confirming siderophore production.

Non-production of siderophores has been reported in certain bacteria *Lactobacillus* spp. and *Legionella* spp. and fungi as *Saccharomyces cerevisiae* and *Candida albicans*[3,18]. Non-production of siderophores in fungi viz., aspergilli has been reported earlier[19,20]. Attempts to detect siderophores in extreme halophiles have failed. Non-production of siderophores by *Halobacterium salinarum* has been reported earlier, but it is able to utilize exogenous hydroxamate siderophore transacetylfusarinine produced by a fungus[21]. The present study is probably the first report on siderophore production by five Indian strains of halophilic archaea.

Non-production of siderophores by four haloarchael isolates may be attributed to the fact that they may not be relying much on siderophore efficiency in hypersaline aquatic environment. Diffusion can make it an unprofitable exercise to produce siderophores. The alternative to siderophore production in certain marine and aquatic forms is replacement of iron compounds, as is known for certain marine organisms that use flavodoxin in place of ferre-doxin[22,23]. Extremely halophilic forms may either be utilizing xenosiderophores produced by other organisms or they may reduce Fe (III) to the soluble form Fe (II). But, it is not straightforward because of total abandonment of iron, as all iron-dependent respiratory chains are an imperative need of aerobic life[24] "Non–detection" could perhaps be the better way of explaining the situation than "non-production". Certain aquatic and marine bacteria as *Marinobacter* sp. and *Halomonas aquamarina* have been reported to produce novel amphiphilic lipopeptide cell–bound siderophores marinobactins and aquachelins respectively which are difficult to be detected by the chemical tests employed for the production of siderophores[25].

Table 1—Siderophore production by haloarchaea

Test Isolates	Growth temperature °C	$FeCl_3$ test	CAS assay	CAS agar plate test
Non–alkaliphilic				
Halobacterium salinarum	37	-	-	-
Halobacterium sp.1	30	-	-	-
Halobacterium sp.2	45	-	-	-
Halobaculum sp.	45	-	-	-
Halococcus saccharolyticus	37	-	+	+
Halorubrum saccharovorum	37	-	+	+
Haloterrigena turkmenica	37	-	+	+
Halogeometricum sp.	37	-	+	+
Alkaliphilic				
Natrialba sp.	45	-	+	+

(+ positive, – negative)

Detection of chemical nature of siderophores (Table 2) indicated that all the five siderophore producers synthesized carboxylate siderophores as evidenced by positive Shenker's test (λ_{max} between 190-280nm) and negative tests for hydroxamate and catecholate siderophores. Bioassays (Table 3) are in agreement with chemical tests for determination of chemical nature of siderophores (Table 2), as the isolates produced growth zones with indicator organism *Morganella morganii* SBK3 indicating its carboxylate nature. None of the isolates formed growth zones with *Arthrobacter flavescens* JG9 and *Salmonella typhimurium* enb7, indicating absence of hydroxamate and catecholate siderophores.

Our results, thus, clearly demonstrated the presence of carboxylate siderophores in extreme halophiles.The line is, thus, drawn among the three domains *Bacteria, Archaea* and *Eukarya* (fungi). Hydroxamates are produced by both bacteria and fungi (Eukarya), catecholates only by bacteria while, novel carboxylate (= complexone) siderophores by fungi belonging exclusively to *Mucorales,* a few bacteria as *Rhizobium meliloti* and *Staphylococcus hyicus* and the halophilic archaea.

Among the five isolates that produced carboxylate siderophores, maximum siderophore production was seen in *Halorubrum saccharovorum* (2.62 RE mg/ml), followed by *Haloterrigena turkmenica* (1.86 RE mg/ml), *Halogeometricum* sp. (1.58 RE mg/ml)

Table 2—Detection of chemical nature of siderophores

Test Isolates	$FeCl_3$ Test		Tetrazolium test (Hydroxamate)	Arnow's test (Catecholate)	Shenker's test (Carboxylate)
	Peak at (420-450nm) (Hydroxamate)	Peak at (495nm) (Catecholate)			
Non-alkaliphilic					
Halobacterium salinarum	-	-	-	-	-
Halobacterium sp.1	-	-	-	-	-
Halobacterium sp.2	-	-	-	-	-
Halobaculum sp.	-	-	-	-	-
Halococcus saccharolyticus	-	-	-	-	+
Halorubrum saccharovorum	-	-	-	-	+
Haloterrigena turkmenica	-	-	-	-	+
Halogeometricum sp.	-	-	-	-	+
Alkaliphilic					
Natrialba sp.	-	-	-	-	+

(+ positive, – negative)

Table 3—Bioassays of siderophore using indicators strains for hydroxamate, catecholate and carboxylate nature

Test Isolates	*Arthrobacter flavescens* JG9 (hydroxamate)	*Salmonella typhimurium* enb7 (catecholate)	*Morganella morganii* SBK3 (carboxylate)
Non- alkaliphilic			
Halococcus saccharolyticus	-	-	+
Halorubrum saccharovorum	-	-	+
Haloterrigena turkmenica	-	-	+
Halogeometricum sp.	-	-	+
Alkaliphilic			
Natrialba sp.	-	-	+

(+ positive, – negative)

and *Natrialba* sp. (1.52 RE mg/ml). However, minimum sidrophere production was observed in *Halococcus saccharolyticus* (1.33 RE mg/ml); (Table 4).

The present study is the first of its kind on siderophore studies in extremophiles and particularly extreme halophiles. This pioneering study has revealed many facets of siderophore-mediated iron acquisition including production by five haloarchaeal isolates amongst nine, their carboxylate nature and their quantification. Our discovery of carboxylate siderophores produced by haloarchaea extends the continuum of iron (III)-chelation strategy by microorganisms.

Table 4—Quantity (RE mg/ml) of carboxylate siderophores produced

Test Isolates	CAS assay (OD 630nm)	Siderophore (RE mg/ml)
Non–alkaliphilic		
Halococcus saccharolyticus	0.354	1.33
Halorubrum saccharovorum	0.699	2.62
Haloterrigena turkmenica	0.497	1.86
Halogeometricum sp	0.422	1.58
Alkaliphilic		
Natrialba sp.	0.405	1.52

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