

SCIENTIFIC REPORTS



OPEN

Occurrence of viable, red-pigmented haloarchaea in the plumage of captive flamingoes

Received: 16 May 2015
Accepted: 12 October 2015
Published: 10 November 2015

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Flamingoes (*Phoenicopterus* spp.) whose plumage displays elegant colors, inhabit warm regions close to the ocean throughout the world. The pink or reddish color of their plumage originates from carotenoids ingested from carotenoid-abundant food sources, since flamingoes are unable to synthesize these compounds de novo. In this study, viable red-colored archaeal strains classified as extremely halophilic archaea (i.e., haloarchaea) and belonging to the genera *Halococcus* and *Halogeometricum* were isolated from the plumage of flamingoes in captivity. Detailed analysis for haloarchaeal community structure in flamingo feathers based on metagenomic data identified several haloarchaeal genera and unclassified sequences of the class Halobacteria at the genus level. Carotenoid pigment analyses showed that a bacterioruberin precursor carotenoid in haloarchaea was identical to one of the pigments found in flamingo plumage. To the best of our knowledge, this is the first report of viable extremophilic archaea in avian plumage, thus contributing to our understanding of the ecology of haloarchaea. The potential influence of haloarchaea as an environmental factor determining avian plumage coloration should be investigated in further studies.

Flamingoes are pink wading birds that are found in warm regions close to the ocean on many continents. The six species of flamingo, which include *Phoenicopterus roseus* (Greater), *P. minor* (Lesser), *P. chilensis* (Chilean), *P. jamesi* (James's), *P. andinus* (Andean), and *P. ruber* (American), inhabit salt lakes and estuaries such as brackish shallow lagoons and mud flats. The reddish color of flamingo plumage is acquired from carotenoids in aquatic organisms such as brine shrimp or cyanobacteria upon which the birds feed^{1–4}. The birds are unable to synthesize carotenoids de novo^{3,5}.

Extremely halophilic (i.e., salt-loving) archaea known as haloarchaea are widely distributed in hypersaline environments and samples, e.g., soda and saline lakes, solar salterns and salt-fermented foods as well as the non-freshwater habitats of flamingoes. Our previous studies have characterized haloarchaeal diversity using culture-independent^{6,7} and culture-dependent^{8–18} approaches. These extremophilic microorganisms require a NaCl concentration of at least 1.5 M for proliferation, with optimal growth observed at 3.5–4.5 M^{19,20}, and produce red or pink carotenoid pigments, including bacterioruberin^{21–25}.

Not only carotenoids from the diets affect the color of the avian plumage, but also other environmental factors may take part in the development of the plumage color. In this study we isolated 13 different haloarchaeal strains present in red feathers obtained from captive flamingoes, analyzed metagenomic

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Strain	Closest taxon (accession no.)	16S rRNA gene similarity (%)	Minimum concentration (mM) of Mg ²⁺ for growth
CBA1201	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.1	10
CBA1202	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.1	5
CBA1203	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.1	5
CBA1204	<i>Halogeometricum pallidum</i> BZ256 ^T (HM185493)	99.2	0
CBA1205	<i>Halococcus salifodinae</i> Blp ^T (AB004877)	99.9	100
CBA1206	<i>Halococcus sediminicola</i> CBA1101 ^T (JX989265)	99.9	0
CBA1207	<i>Halococcus salifodinae</i> Blp ^T (AB004877)	99.4	10
CBA1208	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.3	5
CBA1209	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.1	5
CBA1210	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.1	5
CBA1211	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.4	10
CBA1212	<i>Halococcus salifodinae</i> Blp ^T (AB004877)	100.0	20
CBA1213	<i>Halococcus dombrowskii</i> H4 ^T (AJ420376)	99.4	0

Table 1. Identification of haloarchaeal strains isolated from flamingo feathers. The 16S rRNA gene sequence comparisons were carried out based on sequences in the EzTaxon-e database⁵⁴. The percent similarity between the isolated strains and known haloarchaea taxa is indicated.

sequences of the feathers for targeting feather-attached haloarchaea, and compared their pigments to those found in flamingo feathers and feed.

Results

Haloarchaeal strains isolated from flamingo feathers. A total of 13 strains of viable red-colored microorganisms were isolated from the birds' feathers with hypersaline DBCM2 or M954 medium, but not from the artificial food consumed by flamingoes in captivity, using a culture-dependent approach, and were designated CBA1201–CBA1213 (Table 1). All of the isolated strains grew well at 37 °C and required 0–100 mM Mg²⁺. According to their phylogenetic marker (16S rRNA) gene sequences, the strains belonged to the class Halobacteria in the phylum Euryarchaeota of the domain Archaea; 12 were assigned to the genus *Halococcus* (*Hcc.*) in the family Halobacteriaceae of the order Halobacteriales and one was to the genus *Halogeometricum* (*Hgm.*) in the family Haloferacaceae of the order Haloferacales²⁶. The strains formed four clusters in the phylogenetic trees constructed based on 16S rRNA sequences (Fig. 1) and were closely related to four known haloarchaea: *Hcc. salifodinae* (99.4%–100.0% similarity), *Hcc. dombrowskii* (99.1%–99.4%), *Hcc. sediminicola* (99.9%), and *Hgm. pallidum* (99.2%).

Metagenomic analysis of microbes in the flamingo feathers. For a detailed investigation of archaeal community structure, of the total number of sequence reads analyzed in the metagenome dataset, 7,549 were identified as belonging to the domain Archaea, and 2,012 (26.7%) were attributed to haloarchaea. A phylogenetic classification of haloarchaeal sequences was carried out, and the 13 haloarchaeal genera (98.9% of haloarchaeal sequences) and unclassified sequences of the class Halobacteria (1.1%) were identified at the genus level (Fig. 2). Sequences belonging to the genera *Haloquadratum* (*Hqr.*), *Haloarcula* (*Har.*), *Natronomonas* (*Nmn.*), *Halorubrum* (*Hrr.*), and *Halogeometricum* (*Hgm.*) collectively comprised 59.8% of haloarchaea (15.5%, 14.2%, 11.0%, 9.8%, and 9.3%, respectively), and were identified at the species level as *Hqr. walsbyi*, *Har. marismortui*, *Nmn. pharaonis*, *Hrr. lacusprofundi*, and *Hgm. borinquense*.

Carotenoid pigment analyses. Carotenoids from isolated haloarchaea, red flamingo feathers, and assorted artificial feed were analyzed by micro-Raman spectroscopy. Consistent Raman spectra were obtained across replicates for each type of sample, with only minor shifts within each group. The haloarchaeal strains and feathers showed similar profiles, whereas the spectrum for feed samples differed (Fig. 3). Bacterioruberin, the major carotenoid pigment responsible for the red color of haloarchaea and a biomarker for these organisms in hypersaline environments, showed two strong Raman peaks at 1505–1508 cm⁻¹ and 1148–1152 cm⁻¹ corresponding to C=C and C–C stretching as well as a minor peak at 996–1001 cm⁻¹ corresponding to C=CH bending in bacterioruberin^{21,22,27}. The major Raman bands of bacterioruberin in the haloarchaea samples (marked as A, B and C in Fig. 3) were observed in the feather samples, but not in the feed samples. Although the typical major peaks in the Raman spectra are consistent with the presence of bacterioruberin, they do not prove its presence in feather samples, since other carotenoids could exhibit similar resonances due to the low spectral resolution of Raman spectroscopy²⁸. To confirm the presence of specific haloarchaeal carotenoids such as bacterioruberin

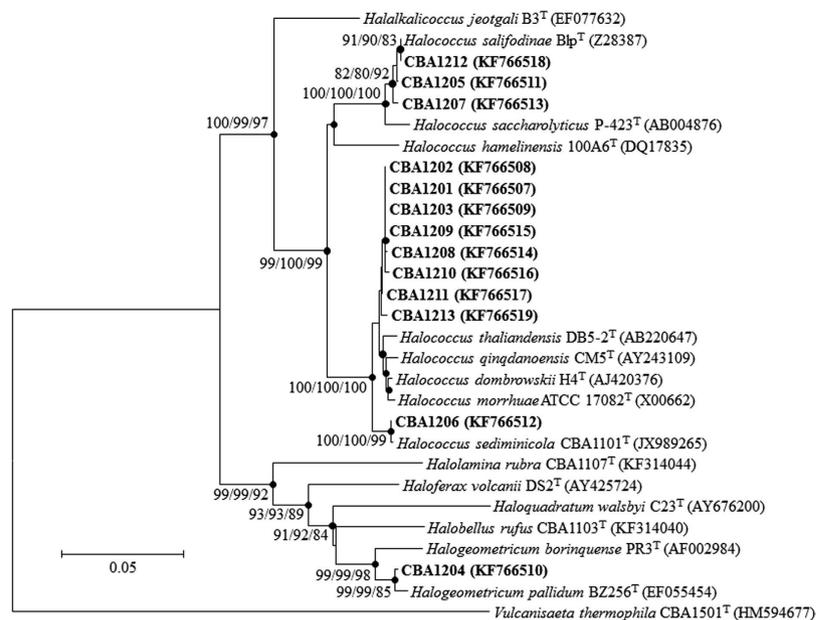


Figure 1. Phylogenetic tree derived from 16S rRNA gene sequences of 13 haloarchaeal isolates from flamingo feathers and phylogenetically related taxa. Bootstrap values (>70%) calculated from probabilities using neighbor-joining, minimum-evolution, and maximum likelihood algorithms are shown at the branch points. *Vulcanisaeta thermophila* CBA1501^T served as the outgroup. Bar, 0.05 accumulated changes per nucleotide.

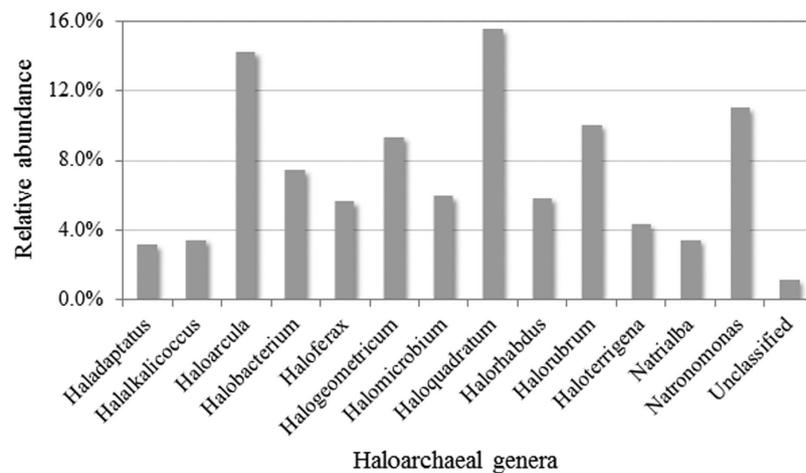


Figure 2. Relative abundance of haloarchaeal genera assigned to the class Halobacteria among metagenomic sequences isolated from flamingo feathers.

or its precursors monoanhydrobacterioruberin (MABR), bisanhydrobacterioruberin (BABR), and isopenityldehydrorhodopin (IDR)²⁹ in the feather samples, carotenoid pigments extracted from feathers, haloarchaea, and feed, together with the appropriate standards, cyanobacterial carotenoid (echinenone), and microalgal carotenoid (astaxanthin) were analyzed by Ultra Performance Liquid Chromatography (UPLC). The UPLC elution profiles corresponding to the extracted carotenoids are shown in Fig. 4. The 3 randomly selected haloarchaeal strains CBA1203, CBA1204 and CBA1209 showed the same elution profile, with 4 peaks that could be assigned to bacterioruberin and its precursors, MABR, BABR, and IDR, in order of retention time, based on a previous study that identified carotenoids from a haloarchaeon²⁹. The second peak obtained from the haloarchaeal strains, with a retention time of 7.15 ± 0.01 min, was identified as the bacterioruberin precursor MABR, consistent with the peak found only in the feather samples.

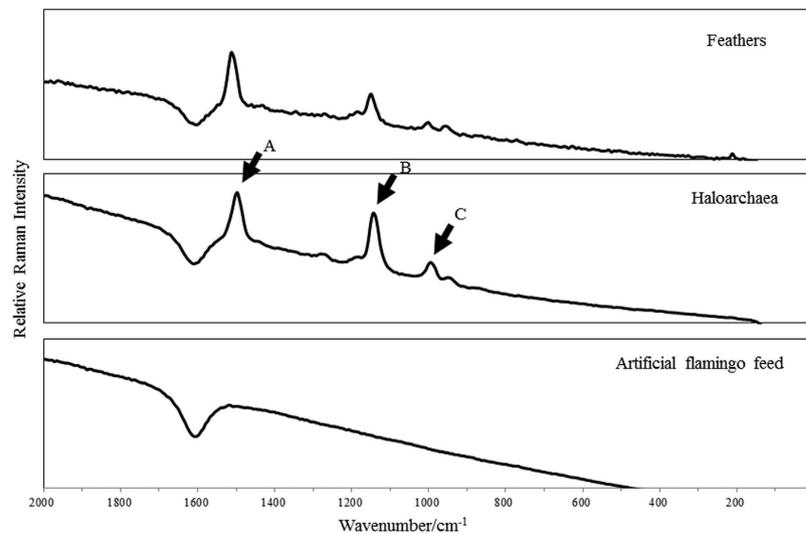


Figure 3. Raman spectra of flamingo feathers, isolated haloarchaeal strains, and artificial flamingo feed. Raman peaks were detected corresponding to (A) C=C stretching, (B) C–C stretching, and (C) C=CH bending of the pigment bacterioruberin.

Discussion

This study was based on the observation that flamingo plumage displays elegant colors similar to haloarchaeal carotenoid-based pigments. The viable red-colored haloarchaea belonging to the genera *Halococcus* and *Halogeometricum* were cultured and isolated directly from the feathers of flamingoes in captivity, using a culture-dependent approach. This is the first report of viable extremophilic archaea present in avian plumage, and provides a novel perspective on fundamental aspects of feather microbiology. Haloarchaea are currently classified into 48 genera with 177 species in the List of Prokaryotic Names with Standing in Nomenclature database³⁰. Species of the *Hcc.* and *Hgm.* genera are pink- or red-pigmented, require at least 8%–15% NaCl for growth and have optimal growth at 30°C–42°C. *Hcc.* species are not lysed in distilled water, unlike most other haloarchaeal cells³¹. Culture-independent approach was also applied to investigate detailed haloarchaeal community structures in the flamingo feathers from which the haloarchaeal strains were isolated. A culture-independent approach that circumvents PCR amplicon sequencing biases using a next-generation sequencing platform was also applied to metagenomes obtained from flamingo feathers where the haloarchaeal strains were isolated to investigate detailed haloarchaeal community structure. Metagenomic analysis of flamingo feathers showed 13 haloarchaeal genera and unclassified sequences of the class Halobacteria at the genus level. It is interesting that no isolated *Halococcus*-related phylotypes were found in the metagenome sequences; it is possible that this discrepancy between culture-dependent and culture-independent data is caused by inefficient breakage of the thick polysaccharide layer in the cell walls of the *Halococcus* spp. using our chosen DNA extraction method.

The halotolerant *Bacillus* strain 2–9–3, isolated from a 250 million-year-old salt crystal, has been reported in 2000 as the oldest viable organism³². Since then, several criteria for ensuring the authenticity of isolates or DNA from novel and unusual places have been outlined in the course of the debate on the isolation of ancient DNA^{33,34}. The presence of haloarchaea in the plumage of birds reported in this study could constitute a comparable microbiological issue since the existence of haloarchaea in bird feathers has not been previously demonstrated. The authenticity of the haloarchaeal isolates is supported by the following points regarding intra-laboratory contamination. First, the haloarchaea were isolated with extreme care and based on extensive hands-on experience with haloarchaeal cultivation. Moreover, the haloarchaea were successfully isolated only from the feather samples, but not from either pond water or soil samples in the flamingo cage. Second, none of the archaeal strains present in our laboratory are identical to the haloarchaea isolated from the feathers, as determined based on 16S rRNA gene sequences. In addition, other evidence of the presence of haloarchaea in the feathers was obtained from metagenomic sequencing of DNA extracts showing diverse haloarchaeal phylotypes.

Flamingoes at the zoo were fed mainly an assortment of artificial food from which no haloarchaeal strains were isolated. In addition, the birds were not exposed to hypersaline environments since they were imported; it was therefore possible that the flamingoes had transported the haloarchaeal cells from their native hypersaline habitats. For instance, a previous study proposed that nostril salt glands of the migratory seabird *Calonectris diomedea* was a potential habitat for haloarchaea that contributed to their dispersal through the Earth's geography³⁵. It was also suggested that *Cyanobacteria* migrate between different habitats via Lesser flamingoes³⁶. Flamingoes are social birds that live in colonies that can include thousands of individuals, have a behavior to rub the feathers with their heads, and drink water and wash

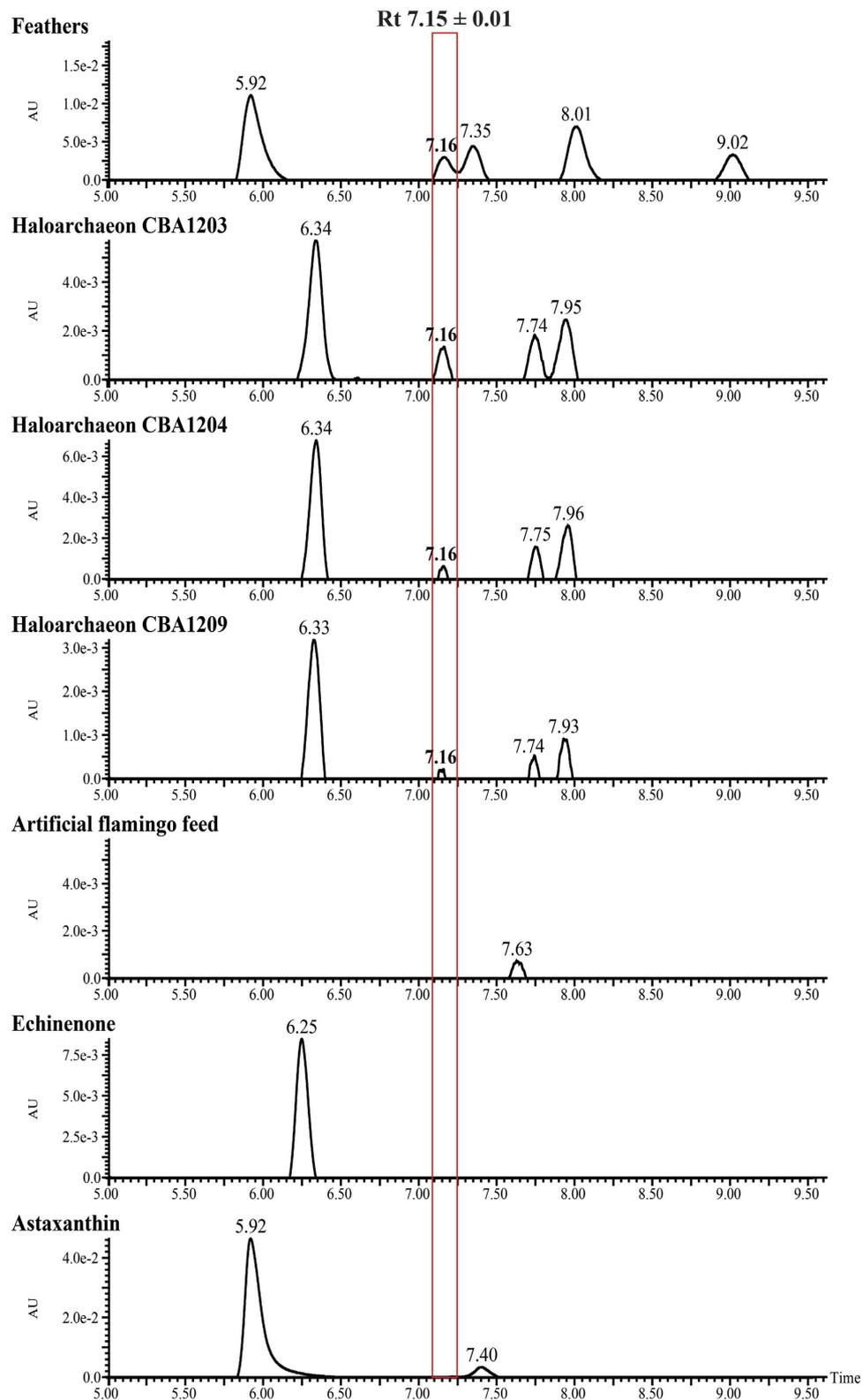


Figure 4. UPLC elution profiles of carotenoids extracted from flamingo feathers, three haloarchaeal strains (CBA1203, CBA1204, and CBA1209), and artificial flamingo feed, as well as echinenone and astaxanthin standards. Absorbance was measured at 450 nm.

their feathers in saline waters where haloarchaea thrive. Thus, haloarchaeal cells may adhere to feathers and be transferred between individuals by physical contact. There is some overlap in the global distribution of flamingoes and haloarchaea isolation sites (Fig. 5). The flamingoes mostly inhabit seashores or

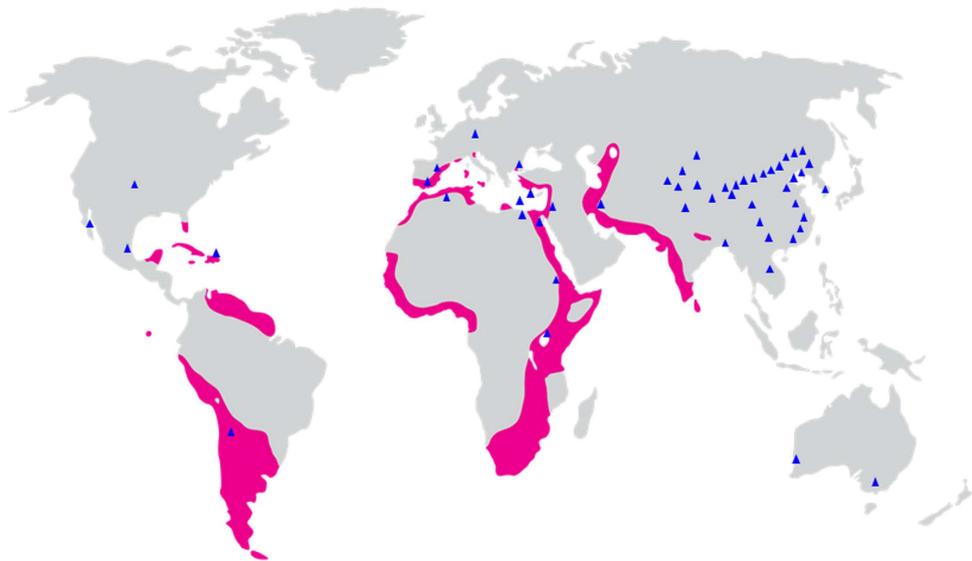


Figure 5. Map of the global distribution of flamingo habitats and haloarchaeal isolation sites. Flamingo habitats are indicated by a pink color and haloarchaeal sites are shown by blue triangles. The map was created using Adobe Illustrator software (Adobe Systems, Mountain View, CA, USA). The sources of haloarchaeal isolation sites can be found in Supplementary Information. The information of flamingo habitats was obtained from Wikimedia Commons (http://commons.wikimedia.org/wiki/File%3AFlamingo_range.png).

salt and soda lakes and haloarchaea were isolated from hypersaline environments such as salt lakes and solar salterns whose salt concentrations fit the growth conditions of haloarchaea. For example, *Hrr. vacuolatum*^{37,38} and *Natronococcus amylolyticus*³⁹ were isolated from Lake Magadi, an alkaline soda lake that is also an optimal habitat for millions of flamingoes in Kenya, Africa. *Hrr. ezzemoulense*⁴⁰ was collected from Ezzemoul, a Greater flamingo breeding site in Algeria⁴¹. In addition, *Halomicrobium katesii*⁴² and *Hrr. tebenquichense*⁴³ were isolated from Lake Tebenquiche, the northern part of the Atacama Saltern in Chile where Andean flamingoes flock.

For verification of correlation between pigments of flamingo plumage and the isolated haloarchaea, carotenoid-based pigments from the red flamingo feathers and haloarchaea were analyzed using Raman spectroscopy and UPLC. The main skeletal C=C and C–C stretching bands and C=CH bend of the carotenoid Raman spectra confirmed that bacterioruberin and its precursors constitute the main carotenoids in haloarchaea, and the bacterioruberin precursor carotenoid identified by UPLC in haloarchaea was identical to one of the pigments in the feathers, but not to pigments in the assorted artificial flamingo feed. Plumage pigments have been characterized in swallows, bluebirds, penguins, chickens^{44,45}, parrots^{46,47}, and mallards⁴⁸, and mostly consist of melanins and carotenoids^{3,49}. While the former are synthesized independently of ingested nutrients, carotenoids—which emerged in archaea as lipophilic biochemicals reinforcing cell membranes and serving as antioxidants and confer a red, orange, or yellow color to avian feathers—are only acquired by ingestion of carotenoid feeding^{1,3,5}. The pink or reddish colors of flamingo plumage originated from the carotenoids by carotenoid-abundant feed often in hypersaline lakes where is one of the haloarchaeal habitats. Carotenoid-based plumage coloration occurs through a mechanism involving carotenoid transport to blood and uptake by follicle cells in feathers^{1,50–52}. Analysis of the major carotenoid pigments of haloarchaea^{21–25,29} revealed that the isoprenoid-derived carotenoids, bacterioruberin and its precursors, are present in the haloarchaea membrane, giving rise to the characteristic bright pinkish-red color.

In this study, haloarchaeal community structures in red flamingo feathers were analyzed by culture-dependent and culture-independent approaches. The unusual existence of the extremophilic archaea in the avian plumage can provide fundamental insight to the feather microbiologist, as well as birder and ornithologist. The bacterioruberin precursor responsible for the red coloration of haloarchaea was consistent with one of the carotenoid pigments found in flamingo feathers. The carotenoid-based plumage coloration of the molting flamingoes that have been mostly fed the restricted diet without the carotenoid pigments at the zoo is not explained satisfactorily with the known coloration mechanisms. The coincidence of the carotenoid-based pigments in the haloarchaeal cells and the avian feathers suggests that haloarchaea may be considered as an environmental factor affecting the plumage coloration. The biological significances of the findings as well as the possible mechanisms for carotenoid-based plumage coloration by microorganisms need to be investigated in future studies.

Methods

Sampling of flamingo feathers and feed. Flamingo feathers and feed were obtained from the Seoul Zoo of Grand Park in the Republic of Korea on July 5, 2013. Chilean (*Phoenicopterus chilensis*), Lesser (*P. minor*), Greater (*P. roseus*), and American (*P. ruber*) flamingoes were raised together in a cage and were fed an assorted, commercial, artificial feed (45% carbohydrate, 20% protein, 5.5% lipid, 8% mineral, and 10% water) along with shrimp irregularly (for a while), not always. Most of the flamingoes were imported from Cuba in 2004 and still remain in the same cage, and there were no records of the countries of origin for the remaining individuals. Flamingo feathers were randomly collected from the birds and these along with the feed were transported directly to the laboratory. The animal breeding protocols were approved by the institutional committee of the Seoul Zoo of Grand Park and the sampling methods were carried out in accordance with the approved guidelines.

Isolation and characterization of haloarchaeal strains. DSMZ-German Collection of Microorganisms and Cell Cultures Medium no. 371 (M371), 372 (M372) and 954 (M954), Japan Collection of Microorganisms medium no. 788 (M788), and DBCM2²⁰ were used to culture haloarchaeal strains. The pH of the media was adjusted to pH 7.0 for M372, M954, and DBCM2 and pH 9.0 for M371 and M788. Flamingo feathers (0.2–0.5 g) and feed (1.0 g) were soaked in each broth medium (30 ml) in triplicate and incubated on a shaker for 5–10 min, and 100- μ l aliquots were plated onto the same medium containing 1.5% (w/v) agar. The plates were incubated at 37 °C for >2 months until pink or red colonies appeared. Colonies were transferred to fresh plates at least three times in order to obtain a pure culture. The isolated strains were cultured on various media and their growth capacity was determined. The Mg²⁺ requirement for growth was tested in M372 containing different Mg²⁺ concentrations (0, 5, 10, 20, 50, 100, 200, and 500 mM).

Phylogenetic analysis of isolated haloarchaeal strains. The 16S rRNA gene sequences of the isolates were analyzed by colony PCR using the primer set arch20F and 1492R⁵³. Purified PCR amplicons were sequenced by Sanger sequencing using arch20F, arch344F, arch520R, arch787F, 1059R, and 1492R primers. The partial 16S rRNA gene sequences were assembled using SeqMan (DNAStar, Madison, WI, USA). Nucleotide similarity values were calculated using EzTaxon-e server⁵⁴. The 16S rRNA gene sequences of related taxa were obtained from EzTaxon-e and used for phylogenetic analysis. Sequence alignments were performed using SILVA Incremental Aligner (Bremen, Germany), taking into consideration the secondary structure of the rRNA gene⁵⁵. Phylogenetic trees were constructed with the neighbor-joining⁵⁶, minimum evolution⁵⁷, and maximum likelihood⁵⁸ algorithms based on Kimura's two-parameter model⁵⁹ with 1,000 randomly selected bootstrap replicates using MEGA5⁶⁰.

Analysis of microbial community structure in flamingo plumage. Metagenomes were extracted from flamingo feathers using the G-spin Genomic DNA Extraction kit (iNtRON Biotechnology, Seongnam, Korea) and sample libraries were constructed using the Ion Plus Fragment Library kit (Thermo Fisher Scientific, Waltham, MA, USA). Crude libraries were size-selected for 400- and 450-bp fragments with the E-Gel SizeSelect 2% agarose gel system (Thermo Fisher Scientific), and the final libraries were quantified using a Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) with high-sensitivity DNA chips. Emulsion PCR was performed using an Ion Torrent OneTouch 400 Template kit (Thermo Fisher Scientific) and libraries were sequenced using the Ion Torrent PGM sequencer (400-bp library)⁶¹ with the 318D sequencing chip according to the manufacturer's instructions. Microbial community analysis was carried out using the metagenomics RAST server⁶².

Carotenoid analysis by Raman spectroscopy. For carotenoid analysis, cells from isolated haloarchaeal strains, red flamingo feathers, and commercial feed were lyophilized, and carotenoid content was analyzed in triplicate for each sample by micro-Raman spectroscopy (Nanofinder 30; Tokyo Instruments, Tokyo, Japan) according to a previously described method²¹ with the following modifications: a 488-nm laser was used for excitation at a laser power of 2.014 mW, with a spectral resolution of 7 cm⁻¹.

Carotenoid analysis by UPLC. Carotenoids were extracted under dim light from three randomly selected haloarchaeal strains and commercial feed according to a previously described method⁶³ with some modifications. Samples (0.1 g) were ground using liquid nitrogen, extracted with 3 ml of acetone, and shaken for 20 min at 60 rpm. Next, 1 ml of hexane, 3 ml water and 0.1 g NaCl were added to the acetone extract. The supernatant was transferred to a vial to remove the water soluble compounds, kept in the dark for 20 min, and then shaken for 20 min at 60 rpm. The hexane extract was evaporated with nitrogen gas, eluted with 1 ml of methanol and filtered through a 0.2 μ m filter. Carotenoid extraction from red feathers was performed under dim light as follows: the feathers were ground using liquid nitrogen and the ground sample (0.2 g) was added to 2 ml of solution (methanol:water, 100:10) and 2 ml petroleum ether in a bottle. The bottle was rotated for 20 min and the upper layer was discarded. The residue was heated in boiling water for 5 min, cooled in a water bath at 37 °C, evaporated with nitrogen gas, eluted with 1 ml of methanol and filtered. Echinenone (ChromaDex, Santa Ana, CA, USA) and astaxanthin (Sigma Chemical Co., St. Louis, MO, USA) were used as carotenoid standards. All fractions

were analyzed by Acquity UPLC (Waters, Wilmslow, UK). Chromatographic separation was performed at 40 °C on an Acquity UPLC BEH C18 column (2.1 mm ID × 100 mm, 1.7 μm particles; Waters). The mobile phase for the analysis method consisted of 10 mM ammonium acetate buffer (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Samples were analyzed using a 15 min (total) gradient: gradient profile (% A) was set as 95% mobile phase A at 0 min, 20% A over 2 min followed by a linear increase to 100% B over 13.5 min, then an isocratic elution with 95% A between 14 and 15 min. The flow rate was set at 0.4 ml/min, and the injection volume was 5 μl. Detection was performed at 450 nm using a PDA detector (Waters).

Data access. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the isolated haloarchaeal strains are KF766507–KF766519. The metagenome sequences of the flamingo feathers have been deposited in the MG-RAST server under accession numbers 4547548.3, 4556310.3, and 4556311.3.

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Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2012R1A1A2040922), by a grant from the Korea Food Research Institute (project no. E0143023840), and by a project fund (C34703) to J.S. Choi from the Center for Analytical Research of Disaster Science of Korea Basic Science Institute. The authors would like to thank Dr. Michael L. Dyall-Smith for providing constructive advices.

Author Contributions

S.W.R. and Y.-D.N. conceived the project and designed the experiments; S.W.R., K.J.Y., J.K., K.-S.O., H.-W.L., H.S.S. and E.-J.S. carried out all experiments; S.W.R., J.K., I.-T.C. and Y.-D.N. interpreted the results; J.R.R. and M.L.S. assisted with sample preparation; S.W.R., Y.-D.N., J.K., J.-K.R., J.-S.C., H.-J.C. and S.-J.L. discussed the results; S.W.R., I.-T.C. and J.-K.R. prepared the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yim, K. J. *et al.* Occurrence of viable, red-pigmented haloarchaea in the plumage of captive flamingoes. *Sci. Rep.* **5**, 16425; doi: 10.1038/srep16425 (2015).



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