Systematic and Applied Microbiology 36 (2013) 244–251

Contents lists available at SciVerse ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Isolation and distribution of iridescent *Cellulophaga* and other iridescent marine bacteria from the Charente-Maritime coast, French Atlantic

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ARTICLE INFO

Article history:
Received 11 September 2012
Received in revised form 19 February 2013
Accepted 19 February 2013

Keywords:
Iridescence
Iridescent bacteria
Marine bacteria
Bacteroidetes
Flavobacteriaceae
Cellulophaga
Tenacibaculum
Aquimarina
Epibiont
Rocky shore

ABSTRACT

An intense colored marine bacterium, identified as *Cellulophaga lytica*, was isolated previously from a sea anemone surface on the Charente-Maritime rocky shore (Atlantic Coast, France), and iridescence of its colonies under direct light was recently described. In addition, iridescence intensities were found to differ strongly between *C. lytica* strains from different culture collections. However, importantly, the occurrence and distribution of iridescent bacteria in the marine environment were still unknown. Therefore, in this study, a search was undertaken for marine iridescent bacterial strains in different biotopes of the Charente-Maritime coast. Various marine samples (water, sediment, macroalgae, other macroorganisms and detritus) were collected from seven biotopes using a direct plate inoculation method. As a result, 34 iridescent strains related to the genus *Cellulophaga*, as well as 63 iridescent strains affiliated to the genera *Tenacibaculum* and *Aquimarina*, were isolated. Iridescent colors were different according to the genera but iridescent marine bacteria were widely distributed. However, a majority of strains were isolated from rocky shores and, in particular, red seaweed surfaces and mollusks. The data from the study suggested that isolates with iridescent properties were well conserved in stressful environments such as the coastal shoreline. This origin may provide an insight into the ecological and biological functions of iridescence.

Introduction

Different color processes are present in the natural environment. Pigmentation, a biochemical coloration, can be generated by molecular absorption of specific wavelengths. The exhibited color is uniform. A very different coloration process corresponding to structural colorations is referred as iridescence. An intense coloration with angle-dependent color changes is produced due to micron- and submicron-sized periodic structures interfering with light [8,33]. This appearance has been well studied in higher organisms, particularly in insects [10,29] and birds [28], but iridescence is also encountered in marine organisms such as fishes [21], ctenophores [35], macroalgae [5,9] and diatoms [24].

Bacterial iridescence to date has been a vague and poorly documented concept. However, a marine bacterium, *Cellulophaga lytica* CECT 8139, exhibiting a unique intense iridescence in reflected light on solid media has been described recently [17]. Colonies had colored centers of varying brightness distributed across the biofilm that appeared with a glitter-like character. Iridescent green was described as the dominant color, but red and blue-violet were also observed at the peripheral edges of the colonies. Iridescence of the colonies was proved using physical methods and the phenomenon was named glitter-like iridescence. Since this type of iridescence occurred under direct natural light exposure it was described as a more natural coloration effect compared to other bacterial iridescences.

We recently demonstrated that the iridescent "*Cellulophaga lytica*" CECT 8139 belonged to the species *Cellulophaga lytica* by DNA-DNA hybridization against *C. lytica* LIM-21^T (88.9% similarity, unpublished data obtained from the DSMZ collection). The *C. lytica* species is adapted to a wide range of marine biotopes in free-living, biofilm and associated forms [2,19]. Diverse intensities of iridescence have been found within the *C. lytica* strains [17]. Non-iridescent or less iridescent *C. lytica* strains Cy I2 (DSM 2039), LIM-21^T (CIP 103822 and DSM 7489) were all isolated from muddy sediment of Costa Rica beaches [13,19]. In contrast, iridescent strains *C. lytica* WFB 21 (DSM 2040) and ACEM 21 were isolated from seawater in an aquarium outflow and an estuary, respectively [19,30]. The iridescent *C. lytica* CECT 8139 was isolated from the surface of the red anemone *Actinia equine* along the rocky shore of Oléron Island on the Charente-Maritime coast, France [17]. Therefore, different ecotypes exist and, unlike the benthic origin, the epibiontic and planktonic origins seemed favorable for the

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http://dx.doi.org/10.1016/j.syapm.2013.02.004

iridescent trait. However, an extended study concerning the origins and biotopes favoring the iridescence trait has not yet been performed. In addition, even if bacterial iridescence has not yet been directly observed in the marine environment, investigating the specific distribution of iridescent strains could be of major importance for determining the possible roles of marine bacterial iridescence.

In this work, the occurrence and distribution of iridescent bacterial strains were studied on the Charente-Maritime coast, France, with special attention being paid to the *C. lytica* species. A specific observation device was employed in order to detect iridescent colonies, and isolated strains were affiliated to a genus by sequence analysis of the 16S rRNA gene.

Materials and methods

Sample collection and bacterial isolation

The sampling was performed between October and December 2011. The seven sites chosen were located on the Charente-Maritime coast in France (Fig. 1). Diverse biotopes, such as rocky shore, mudflats and rocky-sandy shore, were included (Table 1). A total of 139 samples were collected at low tide for bacterial isolation from seawater (11), sediments (20), red macroalgae (Rhodophyta) (16), green macroalgae (Chlorophyta) (11), brown macroalgae (Phaeophyta) (20), decomposed macroalgae (7), marine plants (4), sponges (5), cnidarians (8), worms (3), crustaceans (7), mollusks (19), echinoderms (6) and detritus (2).

A "fresh" inoculation protocol was defined in order to isolate environmental strains and to avoid population changes during transport. For seawater samples, a side of the agar plate was immersed directly in the water and the Petri dish was rapidly closed. For solid samples, a fragment was rinsed in artificial sterile seawater (ASW) Instant Ocean© (30 g L^{-1} in pure water) in order to remove loosely attached epibionts and it was then scraped onto the agar plate [17]. For sediment samples, approximately 1 mL was added directly onto the agar. Three days incubation at 20 °C were needed in order to observe iridescent colonies. Picked colonies were then subcultured, purified and stored at −80 °C in Cryovials© (Dutscher).

Culture conditions of iridescent marine bacteria

The solid medium Cytophaga (CYT) was used to isolate iridescent marine bacteria [13]. CYT medium contained 1 g tryptone, 0.5 g yeast extract, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, and 15 g agar in 1 L of artificial seawater (ASW) Instant Ocean© (30 g L^{-1} in pure water). The incubation temperature was fixed at 20 °C.

Iridescence observation

Plates from the cultured samples were observed using a direct illumination set up (see below) designed to elicit iridescent colonies. Additionally, iridescence of the pure strains was observed using a streaking procedure. One colony from a 24 h-old plate was subcultured onto triplicate plates by drawing thin 5 cm-linear streaks. Cultures were photographed after 24 h and 72 h growth in a dark room using an experimental arrangement of oblique epi-illumination at a fixed illumination angle of 60° [17]. The light source was an 18 watt, 5400 Kelvin lamp (Kaiser RB 218N HF copy lighting unit), and the operating voltage corresponded to AC 220–240 V at 50 Hz with an operating frequency of 40 kHz. The camera was a Nikon D1500 18–55VR using the Av program with f22, a macro lens, and large resolution (12.1 megapixels) in superfine mode.

Molecular characterization of the strains

Identification of each iridescent isolate was determined by 16S rRNA gene sequencing. The DNA suspension for the PCR consisted of colonies picked from agar plates and resuspended in 500 μL sterile water. Before carrying out PCR, three heat (incubation at 60 °C for 2 min) and cold (incubation in liquid nitrogen for 2 min) shocks were applied to the bacterial suspension in order to lyse the cells and liberate the DNA. The primers used for 16S RNA full length gene sequencing were 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′), and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) [18,22]. Reaction mixtures of 50 μL contained 20 μL of PCR master mix (DyNAzyme II, Finnzymes), 100 pmol of each primer, 2 μL of washed cells and MilliQ water to the 50 μL final volume. PCR reactions were carried out in a Labcycler SensoQuest. The thermal PCR profile was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min. The final elongation step was 9 min at 72 °C. The 16S rDNA products were analyzed by electrophoresis in 1% agarose gels. The 16S rDNA products were then sequenced with an automatic DNA analysis system (Genoscreen, Lille, France and GATC, Konstanz, Germany). Sequences were compared with those available in the GenBank database by using the BLAST (Basic Local Alignment Search Tool) service to determine their approximate phylogenetic affiliations [1]. Sequences of

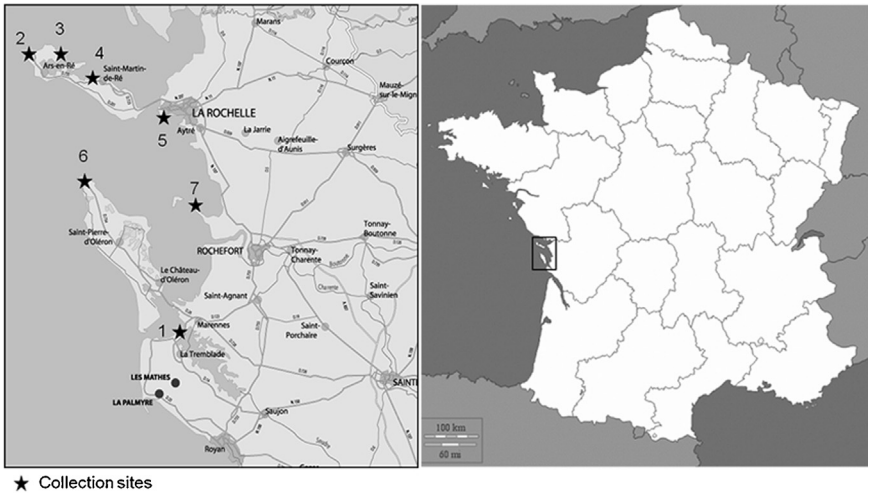

Fig. 1. Maps of the Charente-Maritime coast in France and the locations of the different sampling sites (see details in Table 1).