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Pseudooceanicola algae sp. nov., isolated from the marine macroalga *Fucus spiralis*, shows genomic and physiological adaptations for an algae-associated lifestyle

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ABSTRACT

The genus *Pseudooceanicola* from the alphaproteobacterial *Roseobacter* group currently includes ten validated species. We herein describe strain Lw-13e^T, the first *Pseudooceanicola* species from marine macroalgae, isolated from the brown alga *Fucus spiralis* abundant at European and North American coasts. Physiological and pangenome analyses of Lw-13e^T showed corresponding adaptive features. Adaptations to the tidal environment include a broad salinity tolerance, degradation of macroalgae-derived substrates (mannitol, mannose, proline), and resistance to several antibiotics and heavy metals. Notably, Lw-13e^T can degrade oligomeric alginate via PL15 alginate lyase encoded in a polysaccharide utilization locus (PUL), rarely described for roseobacters to date. Plasmid localization of the PUL strengthens the importance of mobile genetic elements for evolutionary adaptations within the *Roseobacter* group. PL15 homologs were primarily detected in marine plant-associated metagenomes from coastal environments but not in the open ocean, corroborating its adaptive role in algae-rich habitats. Exceptional is the tolerance of Lw-13e^T against the broad-spectrum antibiotic tropodithietic acid, produced by *Phaeobacter* spp. co-occurring in coastal habitats. Furthermore, Lw-13e^T exhibits features resembling terrestrial plant-bacteria associations, i.e. biosynthesis of siderophores, terpenes and volatiles, which may contribute to mutual bacteria-algae interactions. Closest described relative of Lw-13e^T is *Pseudopuniceibacterium sediminis* CY03^T with 98.4% 16S rRNA gene sequence similarity. However, protein sequence-based core genome phylogeny and average nucleotide identity indicate affiliation of Lw-13e^T with the genus *Pseudooceanicola*. Based on phylogenetic, physiological and (chemo)taxonomic distinctions, we propose strain Lw-13e^T (=DSM 29013^T = LMG 30557^T) as a novel species with the name *Pseudooceanicola algae*.

Introduction

The genus *Pseudooceanicola* of the alphaproteobacterial *Roseobacter* group (*Rhodobacteraceae*) currently comprises ten validated species of aerobic or facultatively anaerobic Gram-negative rods. All so-far described *Pseudooceanicola* spp. originate from seawater, deep-sea sediment or invertebrates. We herein describe the first *Pseudooceanicola* representative, designated Lw-13e^T, from the surface of a marine macroalga. Strain Lw-13e^T has been isolated from *Fucus spiralis*, a brown macroalga with broad distribution in tidal areas along the European and North American Atlantic coast. *Rhodobacteraceae* constitute almost a quarter of the epibacterial community on *Fucus* spp. [58,76] and physiological properties of *Rhodobacteraceae* obtained from *Fucus* surfaces indicate adaptations to an epiphytic lifestyle [26].

Organisms inhabiting tidal flats encounter regular desiccation and rewetting events that require distinct adaptations to swiftly changing environmental conditions such as salt, oxidative and heat stress. Bacteria associated with coastal macroalgae are poten-

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tially enriched in adaptive features like production of compatible solutes to deal with osmotic stress [91] as well as high tolerance to antibiotics and heavy metals [83] that can accumulate in algal biomass from terrestrial input [33]. Furthermore, the lifestyle of host-associated bacteria is likely shaped by biological interactions, including mechanisms of commensalism, mutualism or parasitism between bacteria and algae [28].

One relevant aspect of bacteria-algae interactions is the utilization of algal constituents by bacterial epibionts, with specialization for high- or low-molecular-weight compounds [35]. Moreover, bacteria-algae interactions can rely on chemical communication and secondary metabolites, e.g. via the bacterial production of siderophores to access insoluble Fe^{3+} [72] and vitamins for auxotrophic algae [20], known for *Rhodobacteraceae* and their microalgal hosts [1]. Stimulatory mechanisms include the promotion of algal growth by bacterial phytohormones [69], however, both bacteria and algae can also exert inhibitory mechanisms, e.g. through antifouling compounds shaping the epibiotic community [64]. Production of iron-scavenging molecules and toxins as well as antibiotic resistances were identified as relevant for both mutualistic and antagonistic relationships between bacteria and algae [13].

Here, we identified traits in the *Fucus*-derived strain *Pseudooceanicola* Lw-13e^T that adapt for life on a coastal macroalgae. We expanded preliminary insights on physiological adaptations of *Fucus* epibionts [26] by detailed investigation of genomic and physiological features of strain Lw-13e^T. Its specific adaptations to life on macroalgae in tidal areas, partially resembling terrestrial plant-bacteria interactions, distinguish strain Lw-13e^T from seawater-, sediment- and invertebrate-derived *Pseudooceanicola* spp. Together with detection of a closely related phylotype in *Fucus* epibiota [26] and alginate lyase homologs in algae-rich coastal habitats, our study reveals a range of specific adaptations of strain Lw-13e^T. Supported by (chemo)taxonomic distinctions and genomic analyses, we propose strain Lw-13e^T as a novel species within the genus *Pseudooceanicola*.

Materials and methods

Sample collection and bacterial isolation

Specimens of the brown macroalga *Fucus spiralis* were collected at the German North Sea coast in Neuharlingersiel (53°42′17.0″N 7°42′16.1″E) on June 27th 2013 during low tide. Algal specimens were transported to the lab in a container at 4 °C within two hours and washed three times with sterile seawater to remove loosely attached bacteria. Subsequently, surfaces of algal receptacles were swept over agar plates with Marine Broth Difco 2216 prepared with slight modifications (hereafter referred to as MB) to avoid precipitation of medium components [7]: 12.6 g $MgCl_2$*$6H_2O$ and 2.38 g $CaCl_2$*$2H_2O$ L^{-1} and for the trace element solution 7 mg Na-silicate*$5H_2O$ and 21.2 mg boric acid L^{-1}. Plates were incubated at 20 °C in the dark for three days and single colonies re-streaked four times on fresh plates for purification, resulting in the isolation of strain Lw-13e^T [26].

Morphological and physiological characterization

Cell morphology and motility were examined by light microscopy (Axio Lab A1; Zeiss, Germany) in exponential and stationary phase, both in MB and artificial seawater (ASW) medium [90] supplemented with 3% MB. The supplementation was found to be obligatory for growth of strain Lw-13e^T in ASW (hereafter referred to as ASW + MB). Cell motility and potential triggering by exogenous substances was investigated in ASW + MB supplemented with 10 mM sodium acetate, glucose, proline, maltose, *N*-acetylglucosamine, mannose, arabinose, fructose or dimethylsulfoniopropionate (DMSP), as well as with 0.1% (w/v) polymeric and oligomeric alginate, oligomeric β-D-mannuronate or *Fucus* powder [dried and shredded algal material], respectively. For transmission electron microscopy, 50 µL of a culture grown in MB were placed on a copper grid (200 mesh; Plano, Germany), negatively stained using uranyl acetate, and analyzed with an EM 902A electron microscope (Zeiss, Germany). Gram-staining, cytochrome oxidase and catalase activity as well as production of bacteriochlorophyll *a* were assayed as described elsewhere [43]. Analyses of respiratory quinones and cellular fatty acids were carried out by the German Collection of Cell Cultures and Microorganisms (DSMZ, Braunschweig, Germany) (see Supplementary Methods for details).

Growth experiments

Tests with liquid cultures were performed in triplicates in test tubes each containing 5 mL medium, shaken at 150 rpm. Each tube was inoculated to a starting OD_{600} of 0.001 with cells from a pre-culture grown for 48 h in MB. Unless stated otherwise, all growth experiments were carried out at 20 °C in the dark. Growth was followed daily by OD_{600} measurements, including media and substrate controls. Range for growth at different pH values was tested between pH 4 and 10.5, in increments of 0.5 and determined in ASW + MB with 5 mM proline. The pH was adjusted to 4–8 with 1 M NaOH and with glycineNaOH to 8.5–10.5, followed by sterile-filtration. Salinity tolerance was tested in NaCl-free ASW + MB with 5 mM proline, adjusted to 0, 0.5, 1–10 (in 1% increments), 12.5, 15, 17.5 and 20% NaCl using sterile 30% NaCl solution. Before inoculation, cells from the pre-culture were washed twice in NaCl-free ASW. Temperature range was analyzed in MB at 4, 7, 9, 15, 20, 24, 26, 28, 30, 34, 36 and 40 °C. Maximum growth rate (μ_{max}) and doubling time ($t_d = \ln 2/\mu_{max}$) were determined under optimal growth conditions: inoculation to a starting OD_{600} of 0.001 in 100 mL MB, incubated in 500 mL baffled Erlenmeyer flasks at 28 °C, pH 7.6 and 150 rpm in the dark. Growth rate and doubling time were determined based on OD_{600} measurements every two hours, using linear regression of a semi-logarithmic plot of mean optical density (from three replicates) versus time.

Utilization of different carbon sources (dissolved in water and sterile-filtered) was determined with final concentrations of 0.1% (w/v) for alginate substrates (polymeric and oligomeric alginate, oligomeric β-D-mannuronate and *Fucus* powder) or 10 mM for sugars (glucose, D-mannitol, D-mannose, *N*-acetylglucosamine, D-maltose, sucrose, fructose, L-arabinose) and amino acids (alanine, proline, serine, valine, lysine, methionine, arginine, aspartate) after five days of incubation. The pre-culture was grown for 48 h in MB and washed twice in ASW prior to inoculation. Cells grown in ASW + MB without further addition of carbon source served as negative control. Growth was scored as negative when equal to or less than the negative control, and as positive after two transfers and repeated growth in the same medium. Reduction of nitrate and nitrite was tested in anoxic ASW + MB containing 0.5 g/L resazurin [23] (see Supplementary Methods).

Antibiotic and heavy metal susceptibility

Antibiotic susceptibility was tested in triplicates using an antibiotic disc assay on MB agar plates [11] with penicillin G, tetracyclin, streptomycin sulfate, chloramphenicol, kanamycin sulfate, spectinomycin, gentamicin and ampicillin (final concentrations 1 mM) and the marine broad-spectrum antibiotic tropodithietic acid (0.1, 0.3, 0.5, 0.6 and 1 mM). Plates were inspected daily for inhibition zones. Controls included solvents of the antibiotics (water or 50% ethanol) as well as MB. Heavy metal tolerance was tested in mod-

ified liquid and on solid MB with 0.04, 0.075 and 0.1 mM $CuCl_2$ or 1 mM of arsenate/arsenite (Supplementary Methods). Tests were performed in triplicates, with plates or tubes without heavy metals serving as controls.

Production of secondary metabolites

Production and excretion of hemolysins was tested using a plate-based blood hemolysis test. A cell culture was grown for 48 h in MB at 20 °C and 100 rpm. Subsequently, 50 μL cell suspension was inoculated into a pierced well in Columbia blood agar plates (Merck Millipore, Germany, No. 146559) and occurrence of a yellow, clear ring around the well within two weeks was scored as β-hemolysis. Production of volatile organic compounds and acyl-homoserine lactones was analyzed by GC/MS of CLSA and XAD culture extracts (see Supplementary Methods).

Chemotactic triggering of motility

Chemotaxis was tested on soft (0.25 %) agar plates with 10% MB as carbon source. Ten μL of a bacterial culture grown for 48 h in MB were inoculated on one side of the plate and ten μL of the tested substances opposite. Swimming of bacteria towards or away from the substance was scored as chemotactic response. Substances analyzed for chemotactic response were: *Fucus* powder, 1 M of sodium-acetate, glucose, proline, 500 μM of *N*-acetyl glucosamine, maltose, mannose, arabinose, fructose, DMSP, a B-vitamin solution [4], 1% of polymeric alginate and polymeric β-D-mannuronate. Soft agar plates inoculated with bacteria but no substance served as control of motility without triggering.

Genome sequencing and functional analysis

Genomic DNA of Lw-13e^T was isolated from a culture grown in MB for 48 h using the innuPREP DNA Mini kit (Analytik Jena, Germany). Unless stated otherwise, all subsequent steps were performed according to the manufacturer's instructions. Extracted DNA was used to generate Illumina paired-end sequencing libraries (2 × 300bp) with the Nextera XT sample preparation kit (Illumina, San Diego, CA). Generated libraries were sequenced with a MiSeq instrument and sequencing kit v3 with 600 cycles (Illumina, San Diego, CA, USA). For genome closure, high-molecular-weight DNA was isolated with the MasterPure Complete DNA & RNA Purification Kit (Biozym, Hessisch Oldendorf, Germany). Quality of isolated DNA was checked by agarose gel electrophoresis and validated on an Agilent Bioanalyzer 2100 using the High Sensitivity DNA 12000 Kit (Agilent Technologies, Waldbronn, Germany). DNA concentration and purity were checked with a Nanodrop ND-1000 (PeqLab Erlangen, Germany), followed by exact quantification using the Qubit® dsDNA HS Assay Kit (Life Technologies GmbH, Darmstadt, Germany). For Nanopore sequencing 1.5 μg high-molecular-weight DNA was used to prepare three independent libraries using the Ligation Sequencing Kit 1D (SQK-LSK109) and the Native Barcode Expansion Kit (EXP-NBD103). Sequencing was performed for 72 h using a MinION device Mk1B and a SpotON Flow Cell R9.4.1 (Oxford Nanopore Technologies) using MinKNOW software v19.05.0 for sequencing and Guppy v3.0.3 [81] for demultiplexing, resulting in 19,955 reads. Unicycler v0.4.7 [88] was used with default settings to perform a hybrid assembly, resulting in a closed chromosome (3,732,046 bp) and three closed plasmids with sizes of 202,410 bp, 111,009 bp and 49,258 bp respectively. Replicons were validated using Bandage v2.1 [87] and the genome was annotated with Prokka v1.13.3 [68]. Putative biosynthetic gene clusters were predicted using AntiSMASH v4.1.0 [9], genomic islands using Islandviewer v4 [6], and carbohydrate-active enzymes using dbCAN2 [92]. Genome visualization and comparison with other *Pseudooceanicola* spp. was done using BRIG [2]. Homologs of the unique PL15 alginate lyase (Psal_36760) were searched in 77 metagenomes from different macroalgae and seagrasses from diverse coastal habitats in IMG [16] as well as 243 prokaryotic metagenomes from the TARA expedition (http://bioinfo.szn.it/tara-blast-server-help-page/), only considering hits with minimum 65% query coverage and 60% amino acid identity.

Phylogenetic analysis and unique genes

Phylogenetic analysis based on the full-length 16S rRNA gene (retrieved from the complete genome) as well as 873 core-genes was performed in context with closely related strains identified by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The genome of *Pseudooceanicola lipolyticus* (PGTB00000000) was excluded from further analyses due to high fragmentation (442 contigs with a medium size of 11 kb, including many truncated genes and assembly contigs with kmer < 5). Whole-genome phylogeny was performed on 873 single-copy orthologous gene sequences retrieved using OrthoFinder v2.3.3 [29]. 16S rRNA gene sequences and single-copy orthologs were aligned using MAFFT v7.427 [55] and concatenated. The concatenated alignment was automatically curated for blocks represented by >50% sequence information using GBlocks v0.19 [79] and the resulting 188,268 amino acid positions were back-translated to nucleotide data for better alignment resolution using the emboss package [66]. The concatenated, curated and back-translated alignment is shown in Supplementary data file 1. Maximum-likelihood phylogenies of 16S rRNA and ortholog gene alignments were calculated using RAxML v8.2.12 [73] with the GTRCAT model and 1000 or 100 bootstrap replicates, respectively, followed by tree visualization using MEGA-X v10.0.5 [45]. *Ruegeria meonggei* served as outgroup. Digital DNA-DNA hybridization (dDDH) was calculated by the genome-to-genome distance calculator GGDC 2.1 [53] and average nucleotide identities (ANI) using FastANI [40].

To identify core, accessory and unique genes, the genomes of Lw-13e^T, related *Pseudooceanicola* spp. and *Pseudopuniceibacterium sediminis* as the closest relative of Lw-13e^T (Table 1) were analyzed using OrthoFinder, with a 30% amino acid identity threshold. Accessory and unique genes were functionally annotated using eggNOG-mapper v2 [37] and manually curated. Of the 3421 unique genes in the ten strains, 32% were functionally annotated to defined KEGG categories (Table S1). Enrichment of specific KEGG categories in the genomes of Lw-13e^T and other *Pseudooceanicola* spp. was analyzed as the ratio of the specific KEGG category compared to all unique functionally annotated genes in the respective strain. Gene annotations were checked by sequence comparison against the UniProtKB database [82]. Analysis of homologies of single genes or gene clusters was done using Geneious v11.0.2 (Biomatters Ltd., Auckland, New Zealand). We use the abbreviations *Po.* for *Pseudooceanicola*, *Pp.* for *Pseudopuniceibacterium* and *Ph.* for *Phaeobacter* throughout the text.

Results and discussion

General physiological, phylogenetic and genomic features of strain Lw-13e^T

Isolation and morphological characterization

Strain Lw-13e^T was isolated from the receptacle surface of the common brown macroalga *Fucus spiralis*, collected at the German North Sea coast in summer when brown algae have their highest physiological activity and most intense interaction with epibiotic microbes [26,27]. Lw-13e^T was among the first organisms forming colonies on MB agar plates. After two days, colonies on MB

Table 1
Genome statistics of Lw-13e^T and related *Pseudooceanicola* used for comparative analysis, including source of isolation, GenBank accession numbers, G+C content and genome size (in Mbp) as well as the fraction of core, accessory and unique genes. * excluded from the comparative analysis due to a high fragmentation of the assembly; ** excluded from the comparative analysis because it is only distantly related to *Pseudooceanicola* on whole-genome level (Fig. 3B).

Organism	Locus tag	Isolation source	Genome assembly	G+C	Mbp	Genes	Core (%)	Accessory (%)	Unique (%)
Pseudooceanicola algae Lw-13e^T	**PSAL**	**Brown macroalga *Fucus spiralis*, North Sea, Germany**	CP060436 – CP060439	**64.1**	**4.09**	3776	**31.3**	**57.3**	**11.4**
Pseudooceanicola antarcticus Ar-45^T	CVM39	Seawater (50 m), Southern Ocean	GCF_002786285.1	66.1	4.30	3992	29.6	61.1	9.3
Pseudooceanicola atlanticus 22II-S11g^T	ATO9	Seawater, Atlantic Ocean	GCF_000768315.1	64.1	4.94	4633	25.5	66.0	8.5
Pseudooceanicola batsensis HTCC2597^T	OB2597	Seawater, Western Sargasso Sea	GCF_000152725.1	66.1	4.44	4138	28.5	63.1	8.4
Pseudooceanicola flagellatus DY470^{T**}	B8991	Seawater, Pacific Ocean	GCF_900176485.1	60.1	4.41	4143			
Pseudooceanicola lipolyticus 157^{T*}		Seawater, Philippine Sea	GCF_002786325.1	64.6	5.24	4826			
Pseudooceanicola marinus CECT7751^T	PSM7751	Coastal seawater (4–5 m), Taiwan	GCF_900172385.1	66.8	4.53	4244	27.8	62.5	9.7
Pseudooceanicola nanhaiensis SS011B1-20^T	Q344	Sediments (1100 m), South China Sea	GCF_000688295.1	67.9	4.66	4442	26.6	64.8	8.7
Pseudooceanicola nitratireducens JLT1210^T	BMX99	Beibu Gulf, South China Sea	GCF_900109195.1	64.2	4.06	3840	30.7	62.9	6.4
Pseudooceanicola onchidii XY-99^T	E4S58	*Onchidium* sp. (sea slug), South China Sea	GCF_004959925.1	65	3.67	3528	33.5	61.8	4.7
Pseudopunicei-bacterium sediminis CY03^T	DL237	Sediment (25 m water depth), Yellow Sea, China	GCF_003575965.1	63.7	4.45	4036	29.2	62.8	8.0
Pseudooceanicola pacificus 216_PA32_1^T	GLS40	Deep-sea sediment (5788 m), Pacific Ocean	GCF_009789075.1	66.3	3.89	3526	33.5	56.7	9.9

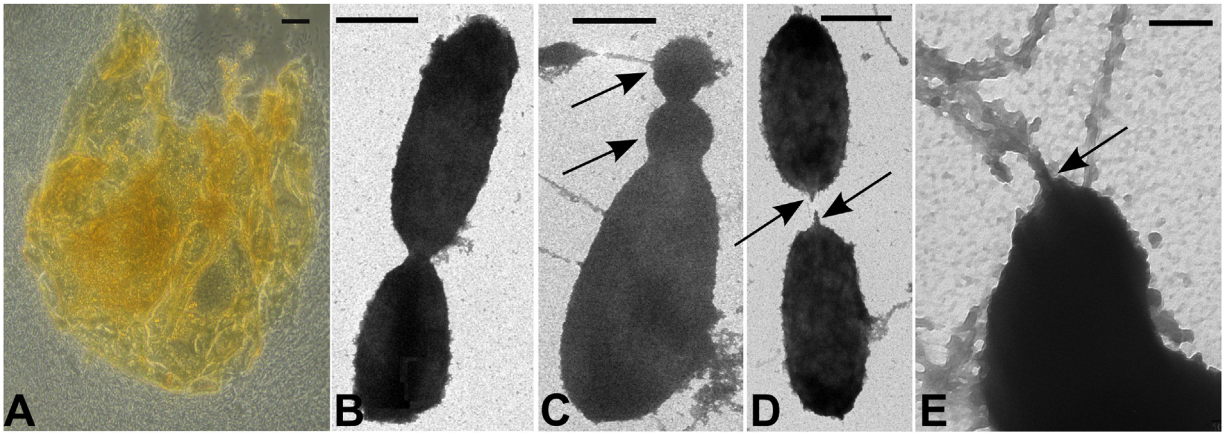

Fig. 1. Transmission electron and light microscopy of Lw-13e^T. Dense cell aggregates of Lw-13e^T are characterized by orange coloration, potentially through pigment production (A). Heterogenic cell morphology, dividing via binary fission (B) and potential budding (arrows in C) and often connected by pilus-like structures (arrows in D). Some cells display flagellum-like structures (arrow in E) and seemed repeatedly connected, but these structures were destroyed during sample preparation (also note cell debris in the samples). Bars represent 20 μm (A), 1 μm (B, C, D) and 0.2 μm (E).

appear cream-colored, circular and convex, with a shiny surface and a diameter of up to 0.5 mm. After one week, colonies turn yellowish with fuzzy edges and diameters up to 3 mm. In liquid media (MB and ASW + MB), Lw-13e^T grows creamy-yellowish, with cells clumping in sticky hard-to-disrupt aggregates, partly appearing orange when concentrated by centrifugation (Fig. 1A). Single cells are irregular elongated rods, 1.5–3 μm long and approximately 1 μm wide, displaying heterogeneous morphologies in liquid cultures. Cells propagate through both binary fission (Fig. 1B) and budding (arrows in Fig. 1C), which might relate to CtrA-based phosphorelay (see below). Most cells were connected by pilus-like structures as observed by light microscopy, which were probably disrupted during preparation for transmission electron microscopy (arrows in Fig. 1D). Detection of several flagellum-like appendages (Fig. 1E) corresponds to the presence of flagella-encoding genes in the chromosome (Fig. 2, Table S2) with homology to the *fla1* gene cluster of *Dinoroseobacter shibae* (Fig. S1). However, cells were non-motile, independent of culture media or growth phase, and motility was triggered neither by addition of algal material or algal-derived substances to the medium. The genus *Pseudooceanicola* was previously described as nonmotile, although polar or subpolar flagella were sporadically reported [38,94] and *Pseudooceanicola* genomes harbor flagella-encoding genes, with motility indicated for two strains [5]. The prevalence of flagella genes in genomes of *Pseudooceanicola* spp. might thus be a common feature, but it remains to be determined if those are expressed only under yet unknown conditions or used for purposes other than motility, e.g. surface attachment [71]. Discriminative growth characteristics of strain Lw-13e^T to related strains are summarized in Table 2. A complete overview of general growth parameters is shown in the species description (Table 3).

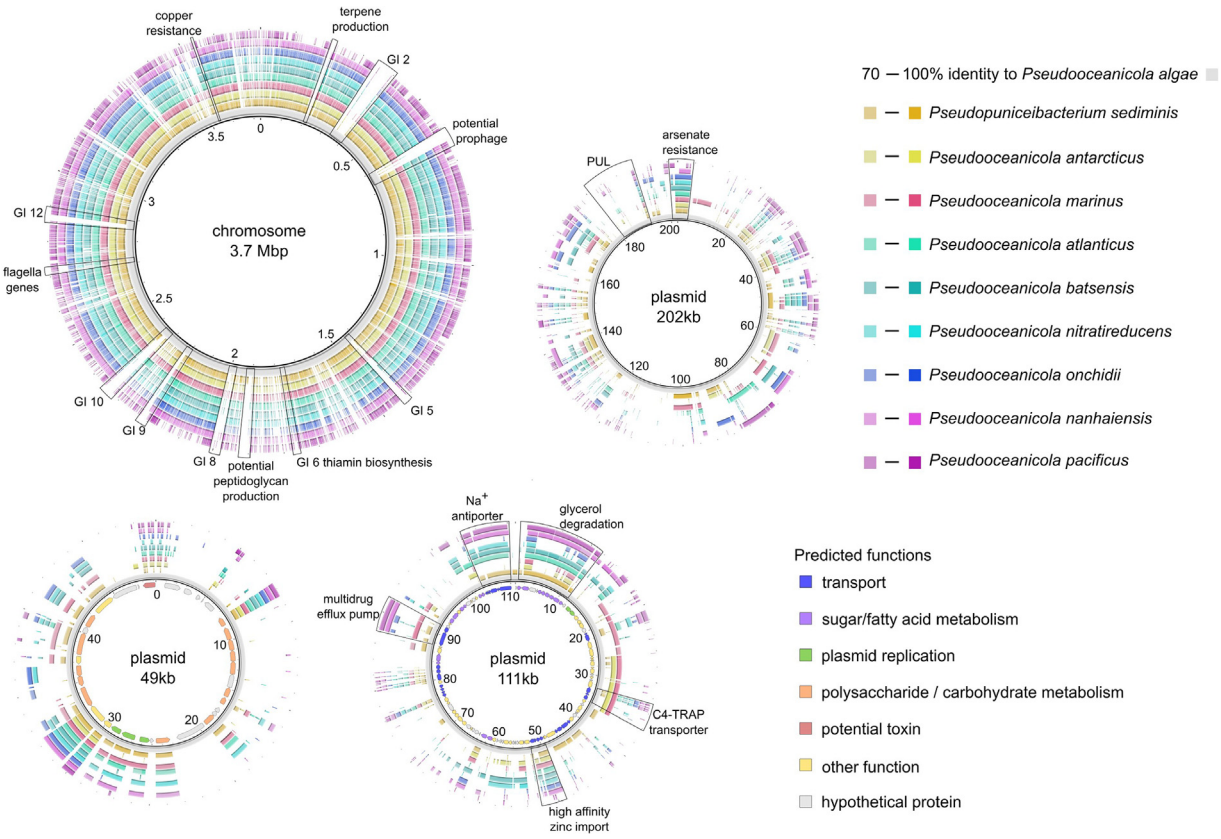

Fig. 2. Graphical representation of the complete genome of *P. algae* Lw-13e^T. Colored circles show blastn comparison to other *Pseudooceanicola* strains as described in the legend, with % identity illustrated by color gradients. Functional annotations of the genes on smaller plasmids (49 kb, 111 kb) are depicted in the inner circle by coloration as given in the legend. Genomic islands (GIs) predicted using IslandViewer v4 (Table S3) are indicated.

Table 2
Differential characteristics of strain Lw-13e^T compared to related type strains. 1: Lw-13e^T; 2: *Pp. sediminis* CY03^T; 3: *Po. antarcticus* Ar-45^T; 4: *Po. marinus* AZO-C^T; 5: *Po. atlanticus* 22II-S11g^T (type species). +, positive; –, negative; w, weak; n.d., not determined; r, resistant; s, sensitive. Concentrations of tested substances are given in the materials and methods section; * results are based on validly published Biolog data. Although this only allows an indirect comparison of substrate utilization, our complementary results provide conclusive evidence of ecological differentiation in line with species separation; **Complete fatty acid compositions are shown in Table S8. Results for the other type strains were obtained from the species descriptions [38,48,49,93]. General characteristics of strain Lw-13e^T shared with other *Pseudooceanicola* spp. are shown in Table 3.

Characteristic	1	2	3	4	5
Isolation source	surface of *Fucus spiralis*	Marine sediment (25 m)	Seawater (50 m)	Seawater (4–5 m)	surface seawater
Cell size (μm)	1 × 1.5 – 3	0.8 – 1.3 × 1.4 – 2.2	0.6 × 1	0.5 × 1	1 × 2.5
DNA G + C content (mol%)	64.1	62.8	62	70.9	64.1
Colony color	yellow cream	opaque	cream	cream white	faint yellow
Temperature range (°C)	4–34	5–40	4–40	4–42	10–41
Temperature optimum (°C)	20–28	30	35–37	28–35	25–28
Salinity range (% NaCl)	0.5–17.5	0.5–9	0.5–10	2–8	0.5–9
Salinity optimum (% NaCl)	0.5–7.5	1.5–2	0.5–3	3–5	1–7
Substrates used:					
Alanine	+	n.d.	–*	+	n.d.
D-mannose	+	+	+*	–	–*
N-acetyl-glucosamine	+	n.d.	+*	–	–*
Hydrolysis of:					
Tween 80	–	–	+*	–	–
Antibiotic susceptibility:					
Ampicillin	s	s	s	r	s
Gentamicin	w	n.d.	s	s	s
Kanamycin	r	n.d.	s	s	s
Penicillin G	s	s	s	r	s
Streptomycin	r	n.d.	s	s	s
Major fatty acids (>10%) (in order of abundance)**	$C_{18:1\ \omega6c/\omega7c}$ (84%)	$C_{19:0\ cyclo\ \omega8c}$ (35%), $C_{16:0}$ (28%), $C_{18:1\ \omega6c/\omega7c}$ (17%)	$C_{16:0}$ (34%), $C_{19:0\ cyclo\ \omega8c}$ (33%), $C_{18:1\omega6c/\omega7c}$ (21%)	$C_{18:1\omega6c/\omega7c}$ (49%), $C_{19:0\ cyclo\ \omega8c}$ (25%), $C_{16:0}$ (15%)	$C_{18:1\ \omega6c/\omega7c}$ (55%), $C_{16:0}$ (16%), 11-methyl $C_{18:1\ \omega7c}$ (11%)

Table 3
Species description of strain Lw-13e^T following the digital protologue standard.

Species name	*Pseudooceanicola algae*
Specific epithet	algae
Species status	sp. nov.
Species etymology	al'gae, L. gen. n. algae, of an alga, seaweed; referring to the isolation source from algae
Description of the new taxon and diagnostic traits	Cells are pleomorphic and non-motile, despite genomic and microscopic evidence of flagella. Single cells are irregularly elongated rods, 1.5–3 μm long and approximately 1 μm wide, displaying heterogeneous morphologies in liquid cultures. Cells propagate through binary fission and budding. Colonies are shiny cream-colored, circular and convex, with a diameter of up to 0.5 mm. After one week, colonies turn yellowish with fuzzy edges and diameters up to 3 mm. In liquid medium, Lw-13e^T grows creamy-yellowish, with cells clumping in sticky aggregates that are hard to disrupt and appear orange when concentrated by centrifugation. Growth occurs at 4–34 °C (optimum 20–28 °C), at pH 5.5–9.0 (optimum 6.5–8.0) and with 0.5–17.5 % (optimum 0.5–7.5) NaCl. Under optimal conditions, the maximal specific growth rate (μ_{max}) is 0.062 h^1, with a doubling time of 11.2 h (Fig. S2D). Tests for catalase and oxidase are positive, reduction of nitrate and nitrite are negative. The sole respiratory quinone is ubiquinone-10, widely distributed in Alphaproteobacteria. Strain Lw-13e^T does not hydrolyze gelatin, starch or Tween 80. It is susceptible to ampicillin, chloramphenicol, penicillin G and weakly to gentamicin, but is resistant to streptomycin and kanamycin as well as to the broad range antibiotic tropodithietic acid (TDA). Strain Lw-13e^T grows on glucose, D-mannitol, alanine, D-mannose, *N*-acetyl-glucosamine, proline, D-maltose, D-fructose, sucrose and weak on serine and L-arabinose. Production of iron-chelating siderophores, vitamin B_{12}, terpenes, and volatiles was detected. A peculiar feature is the predominance of $C_{18:1\ \omega7c}$ and $C_{18:1\ \omega6c}$ fatty acids (84%), contrasting to a greater diversity of fatty acids in other *Pseudooceanicola* spp.
Country of origin	Germany
Region of origin	Neuharlingersiel, North Sea coast
Date of isolation	27/06/2013
Source of isolation	Surface of the marine macroalga *Fucus spiralis*
Sampling date	27/06/2013
Latitude	53°42′17.0″N
Longitude	7°42′16.1″E
16S rRNA gene accession number	KM268063
Genome accession numbers	CP060436–CP060439
Genome status	complete
Genome size	4,094,723
GC mol%	64.1
Number of strains in study	1
Designation of the Type Strain	Lw-13e
Strain Collection numbers	DSM 29013 = LMG 30557
Miscellaneous, extraordinary features relevant for the description	production of orange pigment; heterogenic shape of cells, propagating through binary fission and budding; non-motile, despite genomic and microscopic evidence of flagella

Genomic and phylogenetic analysis

The closed genome of Lw-13e^T consists of a chromosome (3732 kb) and three plasmids (202, 111 and 49 kb) (Fig. 2), with an overall G+C content of 64.1 %. The genome contains three rRNA gene clusters, 52 tRNA genes, one tmRNA gene, 2951 genes encoding proteins with predicted functions, and 826 genes encoding hypothetical proteins. Furthermore, 18 genomic islands were predicted (Table S3), demonstrating multiple prior genetic transfer events.

Phylogeny based on 16S rRNA genes demonstrated that Lw-13e^T falls between its closest described relative *Pseudopuniceibacterium sediminis* CY03^T (98.4% 16S rRNA gene sequence similarity) and a cluster containing *Pseudooceanicola, Oceanicola* and *Phaeobacter* species (Fig. 3A). However, protein-based core genome phylogeny shows clear affiliation of Lw-13e^T with the genus *Pseudooceanicola*, forming a subcluster with *Po. antarcticus*, *Po. marinus* and *Pp. sediminis* (Fig. 3B, orange coloration) whereas other *Pseudooceanicola* spp. form two separate clusters (green and blue coloration). *Po. flagellatus* was excluded from comparative analyses, as it clusters with *Salipiger* spp. and *Puniceibacterium* spp. in whole-genome phylogeny. Deep branching from both genera suggests future reclassification of *Po. flagellatus*. Average nucleotide identities (ANI) of 79.6–79.9% with *Pp. sediminis*, *Po. marinus* and *Po. antarcticus* (Table S4) and <25% similarities using digital DNA-DNA hybridization (Table S5) illustrate that Lw-13e^T is a distinct species in accordance with accepted thresholds of ≤83% ANI [40], and suggests taxonomic reclassification of *Pp. sediminis* as another *Pseudooceanicola* species. The reclassification of *Po. flagellatus* and *Pp. sediminis* is, however, beyond the scope of this study.

Genome analysis of Lw-13e^T and related species

We performed a comprehensive genomic analysis for strain Lw-13e^T and related *Pseudooceanicola* spp. (Table 1) to identify features that may reflect adaptations to the tidal habitat and algal association of Lw-13e^T. The 1180 core genes of all *Pseudooceanicola* spp. constitute 26–34 % of protein-coding genes within all genomes, whereas 57–66 % of identified orthologous genes are accessory (Table 1). The three *Pseudooceanicola* subclusters forming by core-genome phylogeny (Fig. 3B; orange, green and blue coloration) had few cluster-specific genes, i.e. phylogenetic relatedness does not correspond to functional similarity (Fig. 3C). This observation suggests a versatile lifestyle and dynamic adaptability of each strain, driven by specific evolutionary pressure related to their particular environmental origin (Table 1). Across all strains, the majority of unique genes was affiliated with KEGG categories 'Transport' and 'Metabolism' (Fig. 3D), providing further evidence that members of the *Roseobacter* group encode a considerable diversity of transporters mediating strain-specific substrate adaptations [12].

Unique genes of strain Lw-13e^T (i.e. genes not detected in any other strain with >30% amino acid identity) constituted 11.4% of all genes, being the highest fraction of all *Pseudooceanicola*spp. analyzed (Table 1). Compared to related *Pseudooceanicola* spp., unique genes of Lw-13e^T are enriched in functions related to defense

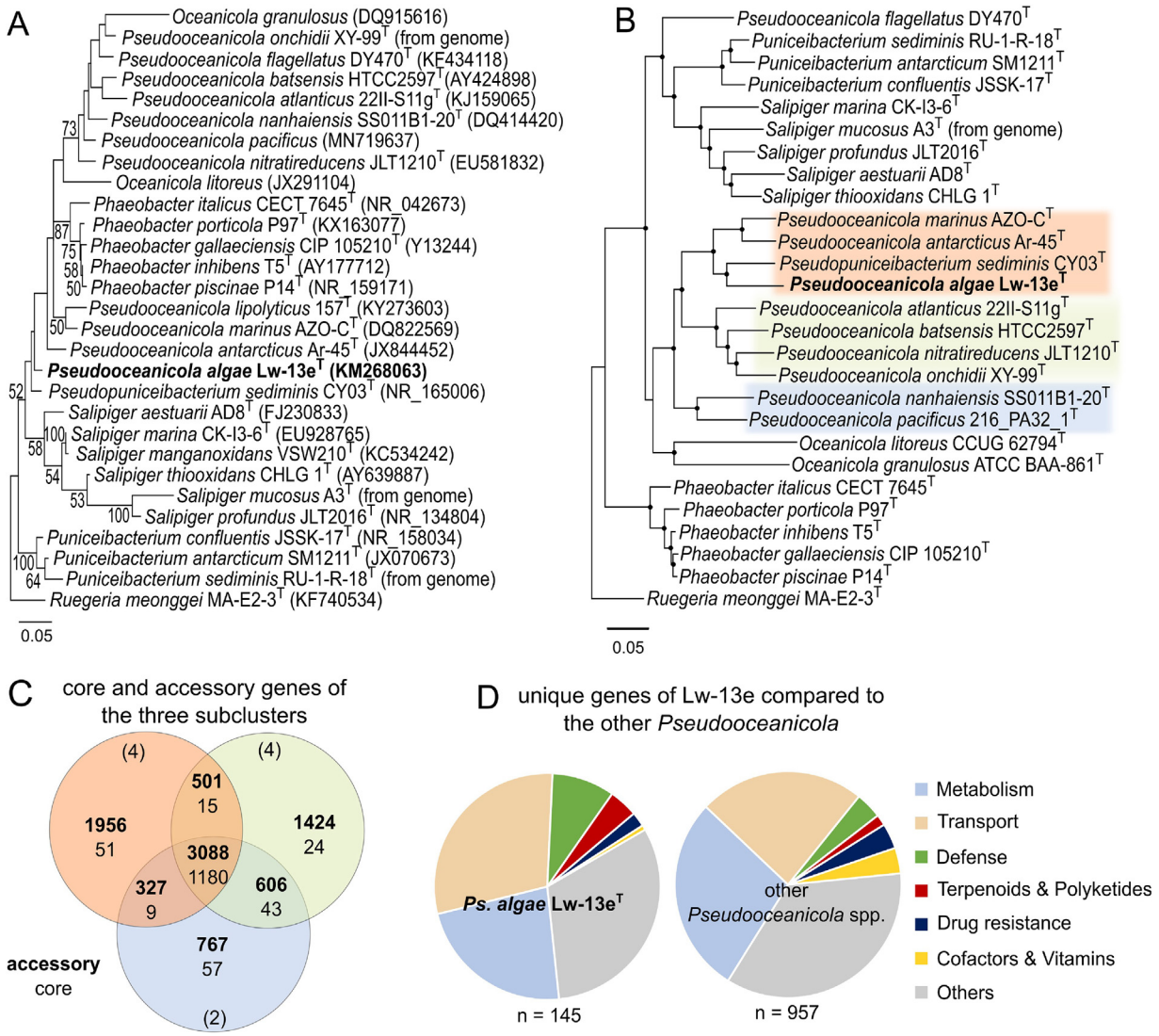

Fig. 3. Phylogenetic placement of strain Lw-13e^T and related strains with *Ruegeria meonggei* MA-E2-3^T as outgroup, and KEGG categorization of unique genes of Lw-13e^T compared to related bacteria. (A) Maximum-Likelihood phylogeny calculated on 16S rRNA gene sequences with 1312 nucleotide positions. Bootstrap values >50% are shown based on 1000 replications. Bar: 0.05 substitutions per nucleotide position. (B) Maximum-Likelihood phylogeny based on protein sequences of 873 core genes (overall 188,268 amino acid positions). Nodes with dots are supported by 100 bootstrap replicates. Colored groups reflect the three subclusters of *Pseudooceanicola* for which fractions of core and accessory genes are shown in (C). Bar: 0.05 substitutions per amino acid position. (C) Venn diagram showing the distribution of accessory (bold) and core genes of the three *Pseudooceanicola* subclusters (see Fig. 3B). Number of genomes within each subcluster are given in parentheses. (D) Fraction of unique genes associated with different KEGG categories for strain Lw-13e^T and related strains. KEGG categories are shown as fractions of unique genes with defined functional KEGG annotation (32% of all unique genes retrieved by OrthoFinder). The category 'Metabolism' includes 'Amino acid, Carbohydrate, Energy, Lipid and Nucleotide metabolism', 'Metabolism' and 'Metabolism of other amino acids'. The category 'Transport' includes 'Membrane transport' and 'Transport and Catabolism'. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

strategies (salt, heat and oxidative stress as well as restriction modification systems for phage defense; Fig. 3D, green coloration) and a unique ability for the production of terpenoids (red coloration). In contrast, unique genes of other *Pseudooceanicola* spp. are enriched in the production of specific drug resistance mechanisms (dark blue) and cofactors and vitamins (yellow). Nevertheless, strain Lw-13e^T does encode an accessory cluster for vitamin B_{12} biosynthesis. The previously shown production of this vitamin for the B_{12} auxotroph *F. spiralis* underlines the association of Lw-13e^T to this alga [26].

Habitat-related adaptations

Tolerance to challenging environmental conditions

Nearly one third of the unique genes in Lw-13e^T encode for membrane transport proteins (Fig. 3D). A set of unique ABC-type transporters predicted for transport of compatible solutes including glycine/betaine and taurine (Table S2) potentially enables strain Lw-13e^T to withstand the osmotic stress in tidal habitats, supported by growth in a salinity range of 0.5–17.5% (Table 2). This broad salinity range of epibiotic community members might likewise enable acclimation of brown algae when encountering freshwater inflow [25]. Other unique transporter genes from the group of multidrug exporters enable Lw-13e^T to tolerate a wider range of antibiotics than related *Pseudooceanicola* spp. (Table 2). Furthermore, strain Lw-13e^T shows considerable tolerance against the broad-spectrum marine antibiotic tropodithietic acid (TDA), without growth limitation until 0.6 mM of TDA (Fig. S2A). This tolerance is in the same range as in TDA-producing *Phaeobacter* spp. [11], whereas sensitive strains are normally inhibited by 0.1 mM [61]. Tolerance to TDA by non-TDA producing strains is rarely described to date [61] and possibly also relates to the abundance of multidrug exporters, as the

proposed resistance genes *tdaR1-R3* from *Phaeobacter* spp. [89] are absent in Lw-13e^T. TDA-producing *Phaeobacter* spp. are also prevalent on surfaces and in tidal habitats [11] and might hence co-occur with Lw-13e^T, stimulating the evolution of resistance. Growth with 1 mM arsenate (Fig. S2B) and up to 0.1 mM copper (Fig. S2C) corresponds to the presence of related resistance genes (Fig. 2, Table S2). The complete set of copper and arsenate resistance genes are shared with *Po. batsensis*, isolated from the Sargasso Sea (Table 1) that harbors vast amounts of seaweed [85]. These traits might thus be specific for bacteria from environments with high abundance of macroalgae, known to be enriched in heavy metals [33].

Oligo-alginate degradation

A specific adaptation of strain Lw-13e^T to macroalgal association is the presence of a unique alginate lyase gene encoded in a polysaccharide utilization locus (PUL) on the 202 kb plasmid (Figs. 2 & 4 A). Alginate is a linear polysaccharide composed of α-L-guluronate (G) and β-D-mannuronate (M) and a major component of the cell wall matrix in brown algae, constituting ~50% of the dry weight of *F. spiralis* [51]. The alginate lyase of Lw-13e^T is predicted as an exolytic oligo-alginate lyase from the family PL15 [50]. Accordingly, strain Lw-13e^T grows on oligomeric but not on polymeric alginate (Fig. 4B). Interestingly, the oligo-alginate lyase of strain Lw-13e^T shows a preference for mixed oligo-GM rather than mannuronate-rich oligomers (oligo-M), compared to the degradation of all oligomeric alginate types by a related PL15 from the terrestrial plant-associated *Agrobacterium fabrum* C58 (Fig. 4C) [56]. The PUL also encodes *kdgF* and *kdgK* genes for downstream processing of alginate monomers as well as a predicted *fabG* dehydrogenase/reductase gene, which might replace *dehR* analogous to other alginolytic bacteria [39,44,78]. Besides homology to the PL15 of *A. fabrum* C58, we detected homologous gene clusters in roseobacters isolated from phytoplankton surfaces or from environments with high concentrations of macro- or microalgae (Fig. 4C) [8,17,46,47]. In *Marinovum algicola* DG898, the PUL is located on a chromid devoted to carbohydrate transport and biosynthesis, enabling the strain to thrive in carbon-rich phycosphere environments [30]. Presence of the PUL on the linear chromosome of *A. fabrum* C58 suggests that the PUL was exchanged between the terrestrial and the marine environment, reflecting transfer events of marine plasmids across biogeographic and phylogenetic barriers [59].

As the alginate lyase lacks a signal peptide, a possible scenario is that oligomers are taken up by the PUL-encoded putative sugar ABC transporter (type I) into the periplasm for exolytic cleavage. The degradation of oligo-alginate suggests that Lw-13e^T is a secondary consumer on algal surfaces, utilizing oligomers released by other associates encoding lyases specific for complex alginate polymers. Such cross-feeding on algal cell wall constituents illustrates a partitioning of different taxa into pioneers and harvesters [3]. The presence of a PUL in Lw-13e^T is noteworthy as members of the *Roseobacter* group are rarely described as oligosaccharide degraders. Algae adaptation of Lw-13e^T is underlined by the use of various other algal substrates, including *Fucus* powder and proline (Fig. 4B), mannose (Table 2) and mannitol [26], all being enriched in brown algal biomass [42].

Production of volatile organic compounds, terpenes and siderophores

Bacterially produced volatile compounds mediate interspecies and interkingdom communication [67] as well as plant development and defense in terrestrial habitats [41]. Strain Lw-13e^T produces numerous volatiles, including dimethyl di- and trisulfides, acetoin derivatives, aromatic compounds including nitrogenous compounds such as pyrazines, as well as aliphatic ketones (Table S6 & Fig. S3). Some of these are also produced by other surface-associated roseobacters [34,80], indicating a considerable potential for chemical communication. Dimethyl di- and trisulfides are known growth promoters or defense molecules against reactive oxygen species (ROS) from terrestrial plants [14,54], suggesting that production by Lw-13e^T might mediate comparable defenses against ROS produced by macroalgae [86]. Of further interest are the aromatic compounds 2-aminoacetophenone and phenol, not commonly described for bacteria of the *Roseobacter* group. In *Pseudomonas aeruginosa*, 2-aminoacetophenone regulates antibiotic tolerance via quorum sensing [62], whereas phenol production is known from enteric and lactic acid bacteria [19]. The red algae-associated *Pseudovibrio* sp. D323 was postulated as provider of phenolic-based defense compounds common in brown algae [95] with e.g. fish deterrent effects [74]. Thus, provision of phenol by Lw-13e^T could be another adaptive factor for macroalgal association by strengthening algal defense. Detection of saturated and unsaturated do- and tridecanones as major volatiles matches the frequent detection of aliphatic ketones and alcohols originating from fatty acid biosynthesis in marine and other bacteria [24].

Detection of the volatile terpenes limonene, nerolidol and farnesol (Table S6) is consistent with enrichment of genes from KEGG category 'Terpenoid and Polyketide Synthesis' (Fig. 3D) and unprecedented for roseobacters to date. Terpene production of Lw-13e^T may have ecological implications as feeding deterrent, membrane stabilizer, anti-oxidant, signaling or antagonistic molecule [32,60]. Production of limonene could provide antimicrobial defense for the algal host [77], while farnesol inhibits quorum sensing of *Pseudomonas aeruginosa* [22] and might hence influence bacterial communication within macroalgal epibiota. Polyketide production in *Roseobacter* group bacteria was indicated before [52] but requires further investigation. Terpene metabolism in Lw-13e^T includes a unique biosynthetic gene cluster with 40% amino acid identity to a squalene-producing gene cluster in *Rhodopseudomonas palustris* ATCC BAA-98 (*Rhizobiales*) (Fig. 2 & Table S2). This homology indicates potential transfer between habitats, comparable to the alginate lyase described above. Production of squalene/lycopene as precursors for hopanoids and carotenoids [57], known to influence cell wall rigidity [10], might explain the orange pigmentation of the Lw-13e^T cell pellet (Fig. 1A). Terpene production among *Rhodobacteraceae* was so far only indicated for a *Tateyamaria* isolate from bobtail squid [18] and an uncultured metagenome-assembled genome associated with microalgae [63]. Accordingly, in 75 analyzed genomes, homologs of the terpene-related gene cluster were only detected in *Limimaricola hongkongensis* DSM 17492 from a coastal seven-day old biofilm (~50% sequence identity; e-value $<10^{-5}$).

Non-ribosomal peptide synthesis in Lw-13e^T corresponds to a siderophore-related cluster (Table S2) with >42% amino acid identity to enterobactin synthesis genes in *E. coli* K12 [21]. Enterobactin is one of the strongest bacterial siderophores [65], explaining the previously shown high iron-chelating activity of strain Lw-13e^T [26]. Siderophore production may be beneficial for the bacterium and its algal host in the typically iron-limited marine environment [72].

Importance of adaptive traits for distribution and ecology in situ

We detected at least one homolog (≥ 65% coverage and ≥ 60% amino acid identity) of the PL15 in approximately 75% of epibacterial metagenomes derived from diverse coastal macroalgae and seagrasses (Table S7). The primary detection of PL15 homologs on seagrasses, macroalgae and the surrounding seawater supports the notion that oligo-alginate degradation is a common characteristic of algae association in coastal environments. In contrast, homologs were only retrieved in three of 243 open-ocean TARA metagenomes (Table S7). The longest alignment with the highest identity (99% coverage and 65% identity) was retrieved from the epibiotic com-

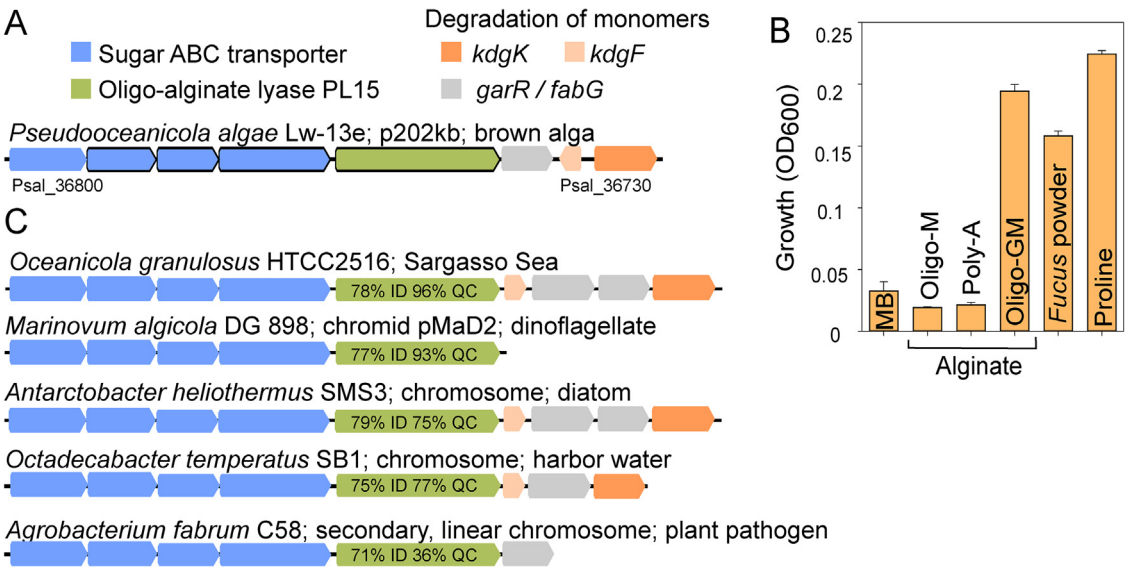

Fig. 4. Unique polysaccharide utilization locus (PUL) in strain Lw-13e^T and growth on oligomeric alginate. (A) Plasmid-encoded PUL harboring PL15 oligo-alginate lyase (green), a type-I sugar ABC transporter (blue) and genes for alginate monomer processing (orange and grey). Framed genes are unique to Lw-13e^T compared to other *Pseudooceanicola* spp. (B) Growth experiments demonstrated that Lw-13e^T degrades mixed guluronate-mannuronate oligomers (Oligo-GM) but not mannuronate-rich oligomers (Oligo-M) and polymeric alginate (Poly-A), supporting functionality of the PUL. In addition, Lw-13e^T was able to grow on *Fucus* powder. Proline was used as a positive control for macroalgal-derived substrates. (C) Homologous clusters were detected in few related roseobacters isolated from phytoplankton surfaces or habitats rich in algal biomass, as well as the terrestrial plant-associated *Agrobacterium fabrum*. ID, % identity; QC, % query coverage of the gene alignment. As the genome of *Oceanicola granulosus* HTCC2516 is a draft genome, the location of the PUL homolog either on the chromosome or a mobile genetic element cannot be specified. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

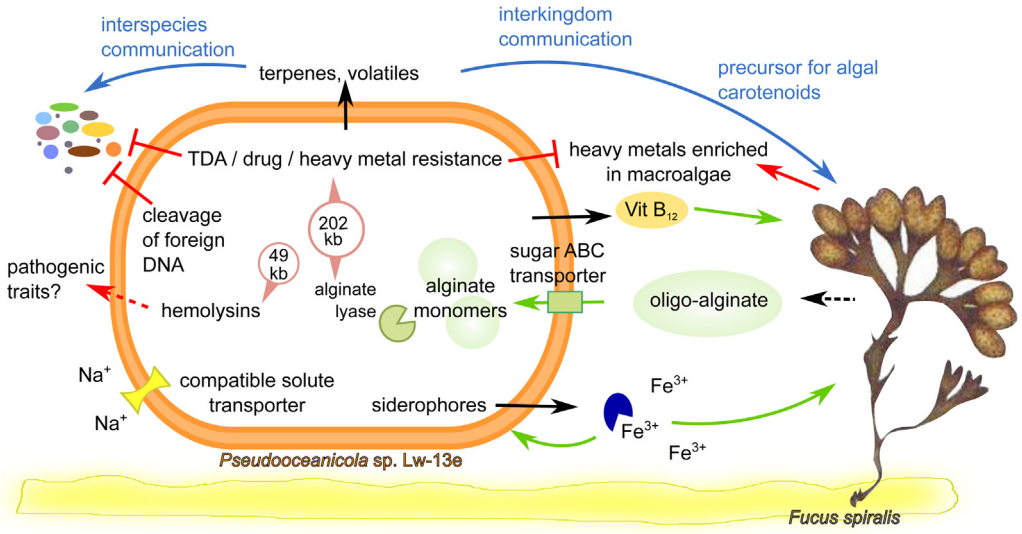

Fig. 5. Summary of adaptations of Lw-13e^T to macroalgae association and tidal habitats. From lower left counterclockwise: Broad salinity tolerance based on transporters for compatible solutes (yellow); production of iron-chelating siderophores (blue circle); degradation of oligo-alginate by PL15 alginate lyase; production and release of vitamin B_{12}; heavy metal resistance; interspecies and interkingdom communication through production of terpenes and volatiles; hemolysin production as potential mean of pathogenicity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

munity of the brown alga *Ecklonia radiata* (Botany Bay, Australia), which contains up to 30% of alginate and mannitol [75] and hence substrates accessible for Lw-13e^T. Detection of PL15 homologs in Australia indicates that adaptive features enable growth of related *Pseudooceanicola* spp. on algal surfaces worldwide. Furthermore, a 16S rRNA gene phylotype with 99% sequence similarity to Lw-13e^T was detected at 0.02% relative abundance in the epibacterial community of *F. spiralis* in the North Sea [26], from where Lw-13e^T was isolated. Overall, these environmental data illustrate that adaptive traits indeed enable niche occupation *in situ*, allowing the colonization of coastal algae-rich habitats on wide geographic scales.

Other distinct traits

Potential pathogenicity

Strain Lw-13e^T has pathogenic potential by secreting membrane-destructing hemolysins, corroborated by β-hemolysis on blood agar plates (data not shown). This observation corresponds to two unique genes coding for hemolysin production, one contained in a type-1 secretion system (Table S2). Hemolysin production can drive virulence in macroalgal pathogens under elevated temperatures [31] and a comparable mechanism was demonstrated for *Ph. inhibens*, changing from mutual to pathogenic behavior upon sensing of infochemicals [70]. This suggests that

Lw-13e^T might likewise express pathogenicity under specific conditions.

Budding morphology and regulatory mechanisms of Lw-13e^T

As described above, cells of Lw-13e^T exhibit a heterogenic morphotype, propagating through both binary fission and budding (Fig. 1B&C). In *Dinoroseobacter shibae*, this phenotype is regulated via a quorum sensing-driven phosphorelay including the *ctrA-chpT-cckA* cascade [84]. Also, Lw-13e^T harbors all genes of this response cascade (Table S2), suggesting a comparable regulation of budding. The phosphorelay of Lw-13e^T is supposedly not regulated via *N*-acyl homoserine lactone-mediated quorum sensing, as a *luxI*-synthase gene is not encoded in the genome and AHLs were undetectable following XAD extraction. However, Lw-13e^T harbors accessory and unique *luxR*-like regulators, possibly enabling response to foreign AHLs, a process known as eavesdropping [15]. Alternatively, the detection of genes relating to autoinducer-2 (Table S2) suggests this compound as regulator of cellular communication, proliferation and biofilm formation [36].

Conclusions

Our complementary analysis reveals an array of pheno- and genotypic traits in strain Lw-13e^T as specific adaptations to tidal areas and association with macroalgae (Fig. 5). Adaptations comprise the ability to counteract osmotic and chemical stress and to utilize macroalgae-derived substrates. The synthesis and excretion of secondary metabolites, including potential signaling molecules, highlight the capability for chemical communication that might strengthen the macroalgae-associated lifestyle and interactions within associated microbiota. Moreover, production of hemolysin suggests that strain Lw-13e^T can turn into a pathogen under specific conditions. These traits clearly separate Lw-13e^T from other *Pseudooceanicola* strains. Supported by phylogenetic data, we propose Lw-13e^T as the type strain of a new *Pseudooceanicola* species with the name *Pseudooceanicola algae* (al'gae. L. gen. n. algae, of an alga, seaweed; referring to the isolation source). The complete species description is shown in Table 3.

Accession numbers

The closed genome of strain Lw-13e^T is deposited at NCBI under BioProject PRJNA449592.

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CRediT authorship contribution statement

Laura A. Wolter: Conceptualization, Investigation, Writing - original draft, Visualization, Data curation. **Matthias Wietz:** Investigation, Writing - review & editing, Resources. **Lisa Ziesche:** Investigation, Writing - review & editing. **Sven Breider:** Investigation. **Janina Leinberger:** Investigation. **Anja Poehlein:** Investigation, Writing - review & editing, Data curation. **Rolf Daniel:** Supervision, Resources, Writing - review & editing. **Stefan Schulz:** Supervision, Resources, Writing - review & editing. **Thorsten Brinkhoff:** Conceptualization, Supervision, Resources, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2020.126166.

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