An ortholog of the *Leptospira interrogans* lipoprotein LipL32 aids in the colonization of *Pseudoalteromonas tunicata* to host surfaces

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The bacterium *Pseudoalteromonas tunicata* is a common surface colonizer of marine eukaryotes, including the macroalga *Ulva australis*. Genomic analysis of *P. tunicata* identified genes potentially involved in surface colonization, including genes with homology to bacterial virulence factors that mediate attachment. Of particular interest is the presence of a gene, designated *ptlL32*, encoding an ortholog to the *Leptospira* lipoprotein LipL32, which has been shown to facilitate the interaction of *Leptospira* sp. with host extracellular matrix (ECM) structures and is thought to be an important virulence trait for pathogenic *Leptospira*. To investigate the role of *ptlL32* in the colonization by *P. tunicata* we constructed and characterized a Δ*ptlL32* mutant strain. Whilst *P. tunicata* Δ*ptlL32* bound to an abiotic surface with the same capacity as the wild type strain, it had a marked effect on the ability of *P. tunicata* to bind to ECM, suggesting a specific role in attachment to biological surfaces. Loss of *ptlL32* also significantly reduced the capacity for *P. tunicata* to colonize the host algal surface demonstrating a clear role for this protein as a host-colonization factor. *ptlL32* appears to have a patchy distribution across specific groups of environmental bacteria and phylogenetic analysis of *ptlL32* orthologous proteins from non-*Leptospira* species suggests it may have been acquired via horizontal gene transfer between distantly related lineages. This study provides the first evidence for an attachment function for a LipL32-like protein outside the *Leptospira* and thereby contributes to the understanding of host colonization in ecologically distinct bacterial species.

**Keywords:** LipL32, seaweed/s, marine bacteria, *Pseudoalteromonas*, host-microbe interaction, bacterial attachment, algae

**INTRODUCTION**

Macroalgae, or seaweeds, are important ecosystem engineers in temperate marine environments and are a rich source of biologically active compounds (Egan et al., 2008, 2013b). The surface-associated microbial community (SAMC) that rapidly colonize the alga have important roles for normal morphological development, nutrient supply, and defense against unwanted colonizers (Goecke et al., 2010; Wahl et al., 2012; Hollants et al., 2013). Furthermore, the SAMC can influence the health of the host alga and members of this community have the potential to function as opportunistic pathogens (Gachon et al., 2010; Egan et al., 2013a). The composition of the SAMC for many macroalgal species has been well studied and includes both generalist epibionts and host-specific taxa that are distinct from the surrounding seawater (Longford et al., 2007; Tujula et al., 2010; Burke et al., 2011; Lachnit et al., 2011). In contrast, there remains a paucity of knowledge regarding the specific mechanisms that facilitate interaction of the SAMC with the host alga (Egan et al., 2013a).

Attachment is considered a key stage in host colonization, and facilitates the progression of both commensal and pathogenic bacterial-host interactions (Kline et al., 2009; Petrova and Sauer, 2012; Bogino et al., 2013). The abundance of specialized adhesins and pili encoded in the genomes of seaweed-associated bacteria indicates this is similarly an important aspect of macroalgal epibiosis (Thomas et al., 2008; Fernandes et al., 2011; Thole et al., 2012). The marine bacterium *Pseudoalteromonas tunicata* was originally isolated from the tunicate *Ciona intestinalis* and has since been well studied as a surface colonizer of the alga *Ulva australis* (Holmström et al., 1998; Rao et al., 2005, 2006). Attachment of *P. tunicata* to biotic and abiotic surfaces occurs within 2h of contact, and cells proceed into biofilm formation within 24 h through the production of differentiated mushroom-shaped microcolonies (Mai-Prochnow et al., 2004; Dalisay et al., 2006). With the exception of a MSHA-like pili that has been demonstrated to play a role in the attachment of the bacterium to both biotic and host surfaces (Dalisay et al., 2006), there is a lack of experimental data on the specific factors that mediate host colonization in this bacterium. Genome analysis of *P. tunicata* has identified genes with homology to putative colonization factors, lipoproteins, pili and outer membrane proteins.
A number of these genes have homology to factors that mediate specific interactions with host cells in other bacteria. One example, designated ptlL32 (locus tag PTD2_05920) encodes for a protein with 47% identity to the *Leptospira* MSCRAMM (microbial surface components recognizing adhesive matrix molecules) lipoprotein, LipL32.

*Leptospira* species are the causative agent of the endemic zoonotic infection, leptospirosis (reviewed in Levett, 2001; Adler and de la Pena Mocetzuma, 2010). LipL32 is highly conserved in pathogenic *Leptospira* species (with an average 98% amino acid identity) where it is the most abundantly expressed lipoprotein (Haake et al., 2004; Dey et al., 2007; Adler et al., 2011). The absence of LipL32 in saprophytic *Leptospira* strains and its ability to bind to extracellular matrix (ECM) structures suggest that LipL32 plays a major role in host-cell attachment during mammalian infections (Hauk et al., 2008; Hoke et al., 2008; reviewed in Murray, 2013). However the precise involvement of LipL32 in *Leptospira* pathogenesis remains unclear as recent studies using a *L. interrogans* lisp32 transposon mutant failed to demonstrate a direct role for this protein in infection models (Murray et al., 2009).

*P. tunicata* rapidly attaches to ECM structures (Hoke et al., 2011) and the tunicate, *C. intestinalis*, a natural host of *P. tunicata*, possesses the genes necessary for ECM synthesis, including those encoding for collagen type IV, fibronectin, laminin, and nidogen (Huxley-Jones et al., 2007). In addition, *Ulua itza*, a close relative of *U. australis*, possesses the genes encoding ECM-like proteins, including collagen (Stanley et al., 2005). Interestingly, recombinantly produced PttL32 bind ECM structures in a manner analogous to LipL32 from *Leptospira* sp. (Hoke et al., 2008). Moreover heterologously expressed PttL32 is immunologically cross reactive with *Leptospira* LipL32 antibodies (Hoke et al., 2008) and the amino acid sequence across the characteristic calcium-binding and putative polypeptide binding regions of LipL32 is conserved in the two proteins (Hauk et al., 2009). This biochemical information suggests that PttL32 is an ECM-binding protein, however the biological function and importance for *P. tunicata* is not established. Moreover prior to the identification of this LipL32 ortholog in *P. tunicata*, it was widely believe that LipL32 proteins were unique to pathogenic *Leptospira* spp. (Murray, 2013), raising the question as to the origin of PttL32 and its’ prevalence in other environmental bacteria. Here we use a combination of allelic exchange mutagenesis, attachment assays, colonization experiments, and phylogenetic analysis to demonstrate a role for this conserved lipoprotein in facilitating bacterial interaction with host surfaces and provide evidence that suggests LipL32-proteins may have been acquired via horizontal gene transfer from an environmental origin. This study provides the first experimental evidence for a function of LipL32-like proteins outside of the *Leptospira* genus and builds upon our current understanding of the traits that drive host colonization in marine bacteria.

**MATERIALS AND METHODS**

**ALLELIC EXCHANGE AND COMPLEMENTATION OF *P. TUNICATA* ptlL32**

A *P. tunicata* pplL32 allelic replacement mutant strain was generated using the Gene Splicing by Overlap Extension (SOE) PCR strategy (Horton, 1995) coupled with bi-parental conjugation and homologous recombination as described previously for the mutagenesis of *P. tunicata* (Egan et al., 2002; Mai-Prochnow et al., 2004). Briefly, the first section of the pplL32 gene was amplified from wild type (WT) genomic DNA with primers, first-forward, 5’ ATG AAA ATA AAC TCT GTG GTG G 3’; and first-reverse, 5’ CTG GTT TCG GTA AAT CAC CCA C 3’. In a separate PCR reaction, the second section was amplified with primers, second-forward, 5’ CAG ACA AAT TAA AAG CCG ATA AAG; and second-reverse, 5’ TTA TTT ATT GAC TGC TTT TTA TAA C 3’. A kanamycin resistance (kanR) cassette was amplified from the plasmid pACYC177 (*Table 1*) using the following primers: kanR forward, 5’ GAT TTA TAC AAG AAA GCC ACG 3’; kanR reverse, 5’ ATT TTA TAC ACA AAG CCG CCG CC 3’. A recombinant pplL32 knockout fragment (pplL32::kanR) was constructed using SOE-PCR (Horton, 1995) by “PCR splicing” the overhangs on the first and second PCR products onto the 5’ and 3’ ends of the kanR cassette, respectively. Following PCR amplification of pplL32::kanR using the first-forward and second-reverse primers, the fragment was introduced into the EcoRI site of the suicide vector pGP704 (*Table 1*) (Miller and Mekalanos, 1988) to generate the vector pLP704. Standard electroporation techniques (Ausubel et al., 1994) were used to transfer pLP704 into *E. coli* SM10. The *P. tunicata* ΔptlL32 strain was constructed using allelic exchange by conjugation of the recombinant *E. coli* SM10 pLP704 strain with *P. tunicata* WT (strR) (*Table 1*) (according to the method described in Egan et al., 2002). Exconjugants with the pplL32::kanR fragment inserted into the chromosome by homologous recombination were selected using VNSS (Marden et al., 1985) agar plates supplemented with streptomycin (200 μg ml⁻¹) and kanamycin (85 μg ml⁻¹). PCR confirmation of the recombination event was performed with a forward primer that targets a region upstream of pplL32 in the WT chromosome, 5’ AAG CAT CCA GTG TGC AGT CG 3’ and the kanR reverse primer.

To complement the ΔptlL32 strain, the pplL32 gene and surrounding potential promoter and terminator regions as was amplifed from WT genomic DNA with primers: WT forward, 5’ GCA ATA GCT TTC TTT GTT CCT C 3’; WT reverse, 5’ GAC ACA TCA GCA TCA TCA AC 3’. The pplL32 PCR product was ligated into the Smal site of the broad host range plasmid, pBBR1 MCS (Kovach et al., 1995) to generate the complementation plasmid, pBBR1. Standard electroporation was used to transfer pBBR1 into an *E. coli* SM10 donor strain (Ausubel et al., 1994), before bi-parental conjugation of the donor strain with *P. tunicata* ΔptlL32 as described previously (Egan et al., 2002). *P. tunicata* ΔptlL32 ex-conjugants with pplL32 complemented in trans were selected for on VNSS agar plates supplemented with gentamycin (50 μg ml⁻¹), kanamycin and streptomycin, and verified by PCR using the WT forward primer, and the gen resistance cassette reverse primer 5’ GCCGCGTTGTGACAATT 3’. The confirmed complemented ΔptlL32 strain was named ΔptlL32.

**BACTERIAL ATTACHMENT TO POLYSTYRENE AND MATRIGEL™ BASEMENT MEMBRANE MATRIX**

The attachment of *P. tunicata* ΔptlL32 to polystyrene was compared to the WT using a cell adhesion assay. The bacterial strains were grown at 28°C for 24 h shaking in VNSS supplemented with the appropriate antibiotics (*Table 1*). The cell suspension was
Table 1 | Plasmids and bacterial strains used in this study.

| Strain or plasmid | Relevant genotype | References |
|-------------------|-------------------|------------|
| **E. coli**        |                   |            |
| SM10 λ, pir        | RP4-2-Tc::Mu, α replicase (pir); strR kanR | Simon et al., 1983 |
| SM10 pLP704        | pGP704::ptlL32::kanR amp | This study |
| SM10 pBBS1L        | pBBS1 MCS5::ptlL32 gen | This study |
| SM10 pCJS10        | pCJS10 cat | Dalsay et al., 2006 |
| **P. tunicata D2** |                   |            |
| WT strR            | Spontaneously resistant to streptomycin | Egan et al., 2002 |
| GFP-labeled WT strR| pCJS10 cat strR | Dalsay et al., 2006 |
| ΔptlL32            | pCJS10 cat strR | This study |
| CΔptlL32           | ΔptlL32 knockout mutant; strR, pCJS10 cat | This study |
| GFP-labeled ΔptlL32| ΔptlL32 knockout mutant; strR, pCJS10 cat | This study |
| **PLASMIDS**       |                   |            |
| pBBS1L             | Broad host range mobilizable vector, gen | Kovach et al., 1995 |
| pBBS1L MCS5::ptlL32| pBBS1L MCS5::ptlL32 | This study |
| pCJS10-GFP         | RSF1010 broad host range backbone, gfpmut3, cat | Rao et al., 2005 |
| pGP704             | Suicide vector, R6K ori, mob, amp | Miller and Mekalanos, 1988 |
| pLP704             | pGP704 with pCJS10 cat | This study |
| pACYC177           | Cloning vector, amp kanR | Chang and Cohen, 1978 |

*strR, streptomycin sensitive; kanR, kanamycin sensitive; strR, streptomycin resistance; kanR, kanamycin resistance; tet, tetracycline resistance; amp, ampicillin resistance; cat chloramphenicol resistance; gen, gentamycin resistance.

centrifuged (6000 × g, 5 min), washed twice and resuspended in 1 ml of sterile NSS (Marden et al., 1985) at Abs660 nm = 1 (~10^9 CFU ml^−1). Fifty microliters of cell suspension for each strain was added to triplicate wells of a polystyrene Costar® 96 well plate (Corning™). The plate was incubated for 6 h at 28°C with gentle shaking before non-adherent cells were removed by rinsing the wells six times with sterile PBS. Twenty-five microliters of a 200 μg ml^−1 solution of trypsin were added to each well and the plate incubated at 37°C for 5 min to allow for cell detachment. Detached cells were then counted by dark field microscopy in a Helber™ bacterial counting chamber (Hawskley, Sussex, UK). Attached bacteria from a total 80 small squares (chosen based on the results of a random number generator) on the counting chamber were enumerated for each of the replicates. The experiment was performed in triplicate for each strain on three independent days. Statistical analysis was performed using SYSTAT 13 (SYSTAT Software Inc., USA) and significance was assessed using an unpaired Students two-tailed t-test.

To assess the attachment of ΔptlL32 to ECM, the assay outlined above was modified according to the protocol described in Hoke et al. (2008). The ECM preparation used in this study, BD Matrigel™ Basement Membrane Matrix (BD Biosciences, USA), is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, and is composed mainly of laminin, entactin, collagen, and mammalian growth factors (Vukicevic et al., 1992; Hughes et al., 2010). The wells of a 96 well plate were coated with 50 μL of BD Matrigel™ and incubated at 4°C overnight. The wells were then washed three times with PBS to remove excess ECM. The P. tunicata bacterial strains (Table 1) were grown, washed, and resuspended as described for the polystyrene adhesion experiment above. Fifty microliters of cell suspension for each strain was added to triplicate wells of a plate coated with BD Matrigel™, and incubated for 2 and 6 h at 28°C with gentle shaking. Non-adherent cells were then removed and detached cells enumerated as described for the polystyrene adhesion experiment. Significance was assessed using a One Way analysis of variance (ANOVA).

**BACTERIAL ATTACHMENT TO U. AUSTRALIS**

To assess the adhesion of P. tunicata ΔptlL32 to a living host surface an attachment assay to U. australis was performed as described previously (Dalsay et al., 2006). The U. australis samples were collected from Clovelly Bay, Sydney Australia and processed immediately. The algal samples were rinsed four times with 50 ml of autoclaved seawater and thallus sections of approximately 6 mm were excised from the mid thallus using a sterile scalpel. The algal surface was then cleaned to remove the majority of epiphytic bacteria. Briefly individual samples were swabbed with sterile cotton tips, containing 0.012% NaOCl for 5 min, and incubated for 24 h in an antibiotic mixture that consisted of ampicillin (300 μg ml^−1), polymyxin (30 μg ml^−1), and gentamycin (60 μg ml^−1). The algal thalli were then incubated for 1 h in 50 ml of 0.2 μm filtered-seawater to remove residue chemicals.

P. tunicata ΔptlL32 was labeled with green florescent protein (GFP) by introducing the broad host range plasmid pCJS10 as described previously for the WT strain (Dalsay et al., 2006) using bi-parental conjugation with an E. coli SM10 donor strain (Egan et al., 2002) (Table 1). GFP-labeled ΔptlL32 ex-conjugants were grown on VNSS agar plates supplemented with chloramphenicol (18 μg ml^−1) and confirmed by visual inspection under...
the GFP filter cube of an epifluorescence microscope (DM-LB Leica). For the U. australis attachment assays, GFP tagged WT (Dalisay et al., 2006) and ΔptlL32 (Table 1) were grown in VNNS supplemented with chloramphenicol for 24 h at 28°C with shaking. One milliliter of culture was harvested by centrifugation (6000 × g, 2 min), washed three times with NSS, and resuspended at an Abs600nm of 0.3. A single thallus section was placed in a well of a 6 well plate (Corning™) containing 1 ml PBS and 1 ml of bacterial culture, and incubated for 6 h with gentle shaking.

After 6 h incubation the algal samples were rinsed three times in 2 ml PBS to remove loosely attached cells and visualized immediately using a Confocal Laser Scanning Microscope (Olympus Fluoview FV1000) under 40× oil magnification and 488 nm excitation. Seven images were taken randomly across each sample and the images were analyzed using ImageJ (Schneider et al., 2012). The number of GFP-fluorescing cells attached to the surface of the algae per mm² was quantified using the “analyze particles” function in ImageJ and the results were plotted using GraphPad Prism 6. In addition the number of microcolonies was manually counted (where >10 cells clustered together was classified as a microcolony) and the results plotted using GraphPad Prism 6. The assay was replicated four times in triplicate for each bacterial strain and data analyzed as described for the polystyrene adhesion experiment.

**Table 1** | Characteristics of non-**Leptospira** species that possess orthologs of PtlL32.

| NCBI accession | Bacterial strain              | Taxonomic affiliation                      | Isolation source                        |
|----------------|-------------------------------|-------------------------------------------|-----------------------------------------|
| EAR27184       | *Pseudoalteromonas tunicata*  | C: Gammaproteobacteria                     | Surface of the macroalga *Ulva* spp. (Australia) and the invertebrate *Ciona intestinalis* (Sweden) |
| ERG54672       | *Pseudoalteromonas spongiae*  | As above                                  | Surface of the sponge *Mycale adhaerens* in Hong Kong |
| ERG42655       | *Pseudoalteromonas rubra*     | As above                                  | Mediterranean seawater of the coast of France |
| ESP92640       | *Pseudoalteromonas luteoviolacea* | As above                          | Surface of the coral *Montastrea annularis* in reef water off the coast of Florida, USA |
| ADZ89577       | *Marinomonas mediterranea*    | C: Gammaproteobacteria                     | Mediterranean seawater from the southeastern coast of Spain |
| EDM65737       | *Moritella sp. PE36*          | C: Gammaproteobacteria                     | Deep ocean waters of the Pacific Ocean San Diego, USA |
| ADV49486       | *Cellulophaga algicola*       | P: Bacteriodetes                          | Surface of a sea ice-chain forming pennate diatom, *Melosira* sp. in the Eastern Antarctic coastal zone |
| ADY29818       | *Cellulophaga lytica*         | As above                                  | Beach mud in Limon, Costa Rica         |
| CDF79332       | *Formosa agariphila*          | As above                                  | Surface of the green alga *Acrosiphonia sonder* isolated from the Sea of Japan |
| EDM44616       | *Ulvibacter sp. SCB49*        | P: Bacteriodetes                          | Surface waters of the Pacific Ocean Southern California Bight, USA |
| AEE17958       | *Treponema brennaborrense*    | P: Spirochaetes                           | Ulcerative skin lesion on a bovine foot infected with digital dermatitis, Germany |

**SEQUENCE RETRIEVAL AND PROTEIN ALIGNMENT OF PtlL32 ORTHOLOGS**

Bacterial genomes were searched for orthologs to the *P. tunicata* gene PtlL32 using a blastp search tool of both the UniProt database (http://www.uniprot.org/) and the non-redundant database of the National Centre for Biotechnology Information (NCBI) in April 2014 (Altschul et al., 1990). All non-**Leptospira** orthologs with greater than 40% amino acid sequence identity to PtlL32 from *P. tunicata* (Table 2) and a further five sequences from representative **Leptospira** species were selected (L. interrogans, ADJ95774; L. kirschneri, AAF60198; L. borgpetersenii, ABJ79303; L. santarosai, AAS21795). All sequences were aligned with ClustalX using the default parameters (Larkin et al., 2007) and the resulting alignment curated with Gblocks to remove gap positions (Talavera and Castresana, 2007). The resulting alignment of 102 amino acid positions was then subject to maximum-likelihood analysis using PhyML 3.0 with the default LG substitution model and 100 bootstraps (Criscuolo, 2011). Trees were visualized using Dendroscope (Huson and Scornavacca, 2012). Information on the phylogeny and isolation of bacterial strains was obtained from published data and using the genome browser in the Integrated Microbial Genome (IMG) browser (https://img.jgi.doe.gov/cgi-bin/er/main.cgi) in April 2014 (Markowitz et al., 2009).
RESULTS AND DISCUSSION

PtLL32 IS NOT REQUIRED FOR ATTACHMENT TO ABIOTIC SURFACES, HOWEVER CONTRIBUTES TO THE ATTACHMENT OF P. TUNICATA TO HOST SURFACES

To investigate the role of ptlL32 in attachment to abiotic surfaces a ptlL32 knock-out mutant (ΔptlL32) was constructed and was compared to the WT P. tunicata strains for its ability to adhere to a polystyrene surface. After 6 h incubation there was no significant difference (p > 0.8) between the number of cells attached for the ΔptlL32 strain compared to WT (Figure 1). These data demonstrate that a mutation in ptlL32 has no immediate impact on the ability of P. tunicata to attach to abiotic surfaces.

Attachment of the P. tunicata ΔptlL32 strain to ECM structures was also compared to WT and CΔptlL32, at times that have been characterized as early (2 h) and late (6 h) stages of irreversible attachment in other bacterial species (Hinsa et al., 2003; Palmer et al., 2007; Li et al., 2012). Figures 2A,B show the average numbers of cells attached per mm³ for the three strains after 2 and 6 h, respectively. The mutant strain ΔptlL32 exhibited 10-fold reduction (p < 0.001) in attachment compared to WT after both 2 and 6 h. There was also a clear increase in attached cells over time for the WT, but not for the mutant strain (ΔptlL32) (Figure 2). Complementation of ptlL32 in trans (strain CΔptlL32) restored the WT phenotype, excluding polar effects of the knock-out mutant. Together with the observations that mutations in ptlL32 had no effect on attachment of P. tunicata to abiotic surfaces (Figure 1), these data show that PtL32 contributes specifically to the ability of P. tunicata to adhere to complex biological surfaces.

Bacterial colonization of various marine eukaryotes has been demonstrated in other non-algal systems to be mediated by surface-specific adhesins (Mueller et al., 2007; Bulgheresi et al., 2011; Stauder et al., 2012); and we hypothesize that PtL32 may facilitate adhesion to ECM-like surfaces in the marine environment, including, but not limited to, its natural host U. australis. To further explore this role we assessed the ability of ΔptlL32 and the WT P. tunicata strains to attach to the surface of U. australis. P. tunicata ΔptlL32 demonstrated a significant reduction in the number of cells attached to the surface of the alga (Figures 3, 4A) compared to WT cells (p < 0.001). A reduction in the number of cell aggregates on the surface of U. australis in the mutant strain (Figure 4B; p < 0.001) also indicates that biofilm maturation may be impaired. The reduced attachment of ΔptlL32 cells to the surface of the alga compared to WT suggests that PtL32 may bind to specific host cell wall components. The major cell wall matrix components for Ulva species are cellulose and ulvan (Lahaye and Robic, 2007), however the structural details are unknown (Lahaye and Kaeffer, 1997; Robic et al., 2009). Genetic analysis of U. linza identified genes involved in the synthesis of hydroxyproline-rich glycoproteins, including collagen (Stanley et al., 2005), which are homologous to components in the ECM preparation used above (Hughes et al., 2010). Leptospira LipL32 has also been shown to bind a number of proteins, including different types of collagen (Hauk et al., 2008; Hoke et al., 2008; Chaemchuen et al., 2011). Therefore, the reduced adhesion of ΔptlL32 to both ECM and U. australis may be the result of interaction of PtL32 with host proteins, including different forms of collagen.

The observation that PtL32 mediates colonization only on biotic surfaces stands in contrast to what has been observed for the MSHA-like pili, which mediates adhesion to both biotic and...
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**FIGURE 3** | Representative confocal laser scanning microscopy images of GFP-labeled WT (A) and GFP-labeled ΔptL32 (B) attached to the surface of *U. australis*. Green fluorescent cells were enumerated using ImageJ software. Images were captured using a FV1000 confocal laser-scanning microscope. Scale bar represents 20 μm.

Abiotic surfaces (Dalisay et al., 2006). Changes in the membrane proteome of *P. tunicata* between cells grown on either BSA-coated or ECM-coated surfaces have recently been observed (Hoke et al., 2011) and therefore it is likely that *P. tunicata* utilizes a distinct subset of proteins to adhere to different surfaces. Thus the available data suggests a conserved function for LipL32-like proteins in facilitating interaction with ECM structures (Hauk et al., 2008; Hoke et al., 2008), a novel finding given the absence of an overlapping niche between *P. tunicata* and *Leptospira* species.

**PtiL32 ORTHOLOGS HAVE A PATCHY DISTRIBUTION ACROSS SPECIFIC GROUPS OF ENVIRONMENTAL BACTERIA**

Given that PtiL32 is important for mediating host colonization in *P. tunicata* we sought to determine the distribution and relationship of orthologous proteins across other non-*Leptospira* organisms. *In silico* analysis of publically available bacterial genomes revealed sequences similar to PtiL32 in eleven other species, of which five were isolated from eukaryotic hosts, ten are marine bacteria and only the spirochete *Treponema brennaborense* has been associated with disease (Table 2). This distribution is interesting considering that until relatively recently LipL32-like proteins were thought to be unique to pathogenic *Leptospira* (Hoke et al., 2008; Murray, 2013). Given the untapped bacterial diversity in environmental ecosystems these data further suggest that the distribution of LipL32 orthologs may be greater still.

Multiple alignment of the protein sequences revealed a total of 29 amino acids positions that are fully conserved across all sequences; with increased conservation of amino acids at positions 85–107 (Supplementary Material). The regions of sequence conservation also include components of the protein that are postulated to mediate binding of host structures in *Leptospira*, including the polypeptide binding function located in the C-terminal (Supplementary Material, amino acids 188–272) (Hauk et al., 2008; Hoke et al., 2008) and an acidic loop at amino acids 164–178 (AKPVQKLDDDDDGDD) (Hauk et al., 2009; Vivian et al., 2009). These regions of amino acid conservation were previously highlighted in the crystal structures of three non-*Leptospira* LipL32 proteins for their potential role in conferring similar binding properties to the orthologous proteins (Hauk et al., 2009; Vivian et al., 2009).

Analysis of the phylogenetic relationship between the PtiL32 orthologs provides new insight into its evolutionary origins. Most striking is the observation that the *P. tunicata* sequence clusters with the *Leptospira* and not with those from other...
Pseudoalteromonas species or Gammaproteobacteria (Figure 5). Likewise, a PtIL32 ortholog of T. brennaborense (phylum Spirochaetes) does not cluster with the other members of the Spirochaetes (i.e., Leptospira spp.) (Figure 5, Table 2). This clustering of LipL32 sequences outside of their taxonomic relatives is in support of an acquisition via horizontal gene transfer (HGT) and/ or maintenance only in the pathogenic Leptospira and a selected group of environmental organism. In support of this theory, an examination of the GC content of the lipL32 genes revealed a deviation from the genomic GC content in examples of Leptospira lipL32 genes, but not in the non-Leptospira orthologs. Given the diversity of the ecosystems in which many of the LipL32 containing strains are found (Table 2) it possible that the Leptospira LipL32 has evolved from environmental strains, where it facilitates commensal interactions, to a role in pathogenic interaction with animals. This proposed “dual function” for LipL32 is in line with observations of other environmentally acquired pathogens, where certain colonization traits mediate pathogenesis in a host-associated context, whilst facilitating microbial survival and persistence in the environment (Casadevall et al., 2003; Vezzulli et al., 2008). Indeed an environmental origin of pathogenic Leptospira has also been recently suggested and is supported by observations that the closest orthologs to many hypothetical Leptospira genes are from environmental rather than pathogenic bacteria (Murray, 2013).

CONCLUSION

Understanding of the mechanisms that facilitate bacterial interactions with marine surfaces is poorly understood compared to that of medically relevant systems. Here we have begun to fill this knowledge gap by investigating the mechanisms that facilitate interactions between the marine bacterium P. tunicata with its macroalgal host. We found that a P. tunicata ΔptIL32 strain attached with a greatly reduced capacity to the biotic surfaces (Figures 2–4), a finding that is in line with previous reports indicating that the colonization of U. australis by P. tunicata involves multiple adhesins (Dalilay et al., 2006; Thomas et al., 2008). Having multiple mechanisms for adhesion may represent a means by which the bacterium can mediate host specificity and/or modify its interaction with the host in response to environmental variability. A degree of functional overlap has also been reported for adhesive structures in a diverse range of bacteria where the apparent redundancy may reflect the ability of these organisms to interact with multiple eukaryotic hosts or tissues (Ramey et al., 2004; Clarke and Foster, 2006; Rodriguez-Navarro et al., 2007; Puzzo et al., 2008). For example, collagen is also a component of the ECM of the tunicate C. intestinalis (Vizzini et al., 2002), since P. tunicata has also been isolated from C. intestinalis it is possible that PtIL32 also has a function in mediating colonization to both macroalgae and marine invertebrates.

To the best of our knowledge this is the first study to investigate the function for a LipL32 ortholog in an organism outside the Leptospira species and the results speak to a conserved function for this protein in mediating association with host cell matrix components. Phylogenetic analysis suggests the Leptospira LipL32 was acquired from environmental bacteria and that LipL32 proteins act as “dual function” traits (Casadevall et al., 2003; Casadevall, 2006; Hoke et al., 2008; Murray, 2013) facilitating both pathogenic interactions by Leptospira spp. and host colonization by the environmental bacterium P. tunicata. This study therefore adds weight to the hypothesis presented by Egan et al. (2013a), that such dual function traits may facilitate interactions between bacteria and macroalgae; a relationship that has important implications for macroalgal health, as well as nutrient cycling, and homeostasis in the temperate ocean environment (Wahl et al., 2012; Egan et al., 2013b).

*FIGURE 5 | Maximum-likelihood tree of PtIL32 and orthologous protein sequences in non-Leptospira species. Five representative Leptospira LipL32 sequences were included for comparison with the non-Leptospira sequence. NCBI GenBank accession numbers for each protein sequence is provided in brackets. Bootstrap values for 100 replicates are shown for each node. The scale bar represents 10% sequence divergence.*
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00323/abstract

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