Spinae are tubular surface appendages broadly found in Gram-negative bacteria. Little is known about their architecture, function or origin. Here, we report structural characterization of the spinae from marine bacterium *Roseobacter* sp. strain YSCB. Electron cryo-tomography revealed that a single filament winds into a hollow flared base with progressive change to a cylinder. Proteinase K unwound the spinae into proteolysis-resistant filaments. Thermal treatment ripped the spinae into ribbons that were melted with prolonged heating. Circular dichroism spectroscopy revealed a dominant beta-structure of the spinae. Infrared spectroscopy identified the amide I spectrum maximum at a position similar to that of amyloid fibrils. Therefore, the spinae distinguish from other bacterial appendages, e.g. flagella and stalks, in both the structure and mechanism of assembly.

*The Roseobacter* clade is one of the major groups of marine *Proteobacteria*. They are broadly distributed across diverse marine environments from coastal to open oceans and from sea ice to sea floor. The members of this clade share >89% identity of the 16S rRNA genes. The *Roseobacter* clade are physiologically very versatile and play an important role in global marine biogeochemical cycles. They are often associated with organic or inorganic surfaces and their association with larvae or alga may serve as probiotics to protect these organisms against pathogenic bacteria. Various surface appendages including flagella, pili and holdfast have been observed for *Roseobacter* and play an important role in the surface adhesion and colonization of this clade of microorganisms. However, spinae have not been observed for *Roseobacter* clade despite their wide distribution in both marine and freshwater bacteria.

Spinae are hollow, tubular appendages with transverse striation and are distinct from flagella, pili and stalks. Spinae were first observed for *Agrobacterium* sp. by Moll and Ahrens in 1970, then have been found in wide range of bacteria including strain marine pseudomonad D71, *Chlorobium* spp., *Desulforhopalus singaporenensis*, *Methylcystis echinoides* and *Cyanobium* sp. with up to hundreds spinae per cell. There is species specificity in the morphology of the spinae. They vary in length and in width, and some have a conical base whereas others taper to the distal tip. The function of spinae has not been experimentally demonstrated although various roles have been hypothesized, such as buoyancy to maintain the cells in aquatic habitat, promotion of cellular aggregation and signal exchange between cells. The most extensively characterized spinae are those from the strain marine pseudomonad D71 that was renamed as *Spinomonas maritima*. They are proposed to consist of a 42 kDa spinin-specific protein with an estimated content of about 2.5 × 10^9
spinae on Roseobacter sp. YSCB cells. Despite the fact that flagella are observed for most Roseobacter spp.\(^2\), the spine-like extracellular appendage has not previously been reported for the members of this clade.

We sought to optimize growth conditions for the production of the spinae. Temperatures, shaking speed as well as the pH of the media increase neither the number of spinae on a cell nor the proportion of population with spinae. Spinae were almost absent from the cells grown on plates. Finally, we found that dilution of the Marine Broth 2216 liquid media to half strength drastically increased the production of the spinae but had no effect on flagellar production. Under these conditions, almost all cells have spinae (Fig. 1, A). The number of spinae was maximal in the stationary phase. Each cell might have a dozen of spinae up to 3 \(\mu\)m in length and resemble a long-spined sea urchin (Fig. 1, A). Further dilution of Marine Broth media slowed down bacterial growth without increasing the spine production. In contrast, increasing NaCl concentration in the diluted media progressively decreased spine production. Under all growth conditions, the spinae appeared after 2-day incubation and reached maximal number at the stationary growth phase after 4–5 day incubation. Therefore, the kinetics of spinae synthesis does not seem to be affected by the growth media.

The spinae were distributed randomly on the cellular surface when analyzed by transmission electron microscopy (TEM) and cryo-electron microscopy (cryo-EM). Scars appeared at the places where spinae were mechanically detached from a cell (Fig. 1, B). The scars seemed to be closed without the leak of the cytosol. Moreover, no internal sub-structure like the flagellar basal body was observed on the cryo-EM images (Fig. 1, C). This finding suggests that the spinae are unlikely to be a rotary apparatus and probably assembled through a mechanism different from that of the flagella. The overall morphology of the spinae resembles the Eiffel Tower, consisting of a flared base connected to a columnar or tubular structure with transverse ribs. Therefore, we also call them Eiffel Towers.

A literature search revealed that the spinae are similar to those of the strain marine pseudomonad D71\(^2\). Although the taxonomic name Spinomonas maritima has been proposed to the strain marine pseudomonad D71\(^3\), we do not know how closely related are Roseobacter sp. YSCB and marine pseudomonad D71 because of the absence of molecular genetic data, i.e. 16S rRNA gene sequence of the D71 strain. Notably, Roseobacter sp. YSCB have almost no spinae when incubated in the growth media optimized for the spinae production for the strain marine pseudomonad D71\(^4\) (data not shown). Either these two strains belong to different species or one carries a regulatory mutation affecting the spine synthesis.

**Results**

**Production of spinae by Roseobacter sp. isolates.** The Roseobacter YSCB isolates are heterotrophic, marine bacteria that have been isolated from the Yellow Sea Cold Water Mass at 70 m depth\(^2\). They showed different colony morphology, smooth for YSCB-1 and YSCB-3 and rough for YSCB-2 and YSCB-4, when they were initially isolated on Marine Broth 2216 plates. Because their 16S rRNA gene sequences are virtually identical, and they show the same features for all genetic and physiological analyses and they can all change colony phenotypes depending on the growth phase and conditions, we consider the four isolates as the same species and thus refer to them as Roseobacter sp. strains. The results described here are common to all four isolates and will not be reported for individual strains. Transmission electron microscopy (TEM) observation rarely reveals a single flagellum at one pole of some cells (Supplementary information, Figure S1, A). Consistently, we observed that less than one percent of cells in a population could swim smoothly, but most of them showed a non-directional movement, probably as a result of both inefficient flagellar propulsion and Brownian motion.

The cells were routinely grown in Marine Broth 2216 media. Occasionally, surface appendages, referred to as spinae, were observed on a few cells (Supplementary information, Figure S1, B).
Microscopy characteristics of the spinae of the *Roseobacter* sp. strains. To characterize the physico-chemical property of the spinae we have purified the spinae. After analyses with various buffers, we found that the spinae are extremely stable in water. The final protocol used for purification of the spinae consists of washing and resuspending the harvested cells with deionized water, then mechanically shearing to detach the spinae and collecting them by centrifugation (see Methods for details). Inspection by transmission electron microscopy showed highly purified, intact spinae (Fig. 2, A1). Flagella with a diameter of about 18 nm were sometimes found in the samples (arrowhead in Fig. 3, B). Amplified view of the tubular part showed obvious helical structure (Fig. 2, A2). We further analyzed the fine structure of the spinae with various microscopy techniques. Atomic force microscopy (AFM) confirmed the feature of cylinder with flared base and the shallow pitch (Fig. 2, B1 and B2). Cryo-EM preserved structures in their native state and without staining provided more and finer details (Fig. 2, C1 to C4, on line movie). The spinae are composed of a flared base with the enlarged side attached on the surface of the cells and a tubular part connected on the narrow side of the base. Clearly, the spinae are not extension of the cell wall. Based on about 100 measurements, we found that the size of the flared bases is $139.2 \pm 10.2$ nm (mean $\pm$ standard deviation) for the enlarged side, $46.5 \pm 3.9$ nm for the narrow side and $137.4 \pm 3.8$ nm for the height. The cylindrical part has a width similar to the narrow end of the base and a variable length probably exacerbated by breakage during sample preparation. The helical pitch is $11.6 \pm 1.0$ nm per turn and a striation has an inclination of $6.6 \pm 0.8^\circ$ to the short axis. Reconstitution of the spinae structure from the sections of cryo-tomographs showed clearly that a single filament winds to form the left-handed helical flared base that progressively transforms into the cylinder (Online movie). The spinae appeared to be hollow from the base to the tip (Fig. 2, C3 and C4).

Biochemical analysis of the spinae. To determine the chemical nature of the spinae, we have treated the purified spinae with trypsin (at final concentration of 1 mg/ml), proteinase K (1 mg/ml), DNase (1 mg/ml), RNase (1 mg/ml), lysozyme (1 mg/ml),
**Biophysical characterization of the spinae.** The elemental composition of the spinae was analyzed by X-ray energy dispersive spectroscopy (XEDS). As shown in Fig. 4, A1, the analyzed area.
contains virtually only spinae. The salient feature of the XEDS spectrum is that the nitrogen and oxygen were roughly estimated at a ratio less than 1:5 that is much lower than the estimated N:O ratio in spinin. Easterbrook and Coombs have reported elemental composition and amino acids distribution of the spinin of marine pseudomonad D715. Based on these data, the calculated N:O ratios are 0.51 and 0.59, respectively. In addition, we calculated, based on the data from the same report, the N:O ratios for flagellin (0.64) and

Figure 4 | Biophysical characterization of *Roseobacter* sp. YSCB spinae. Panel A1 (TEM) shows the sample from where the XEDS spectra (A2) were obtained. The peaks correspond to carbon (1), nitrogen (2), oxygen (3), sodium (4), magnesium (5), silicium (6), sulfur (7), chore (8) and calcium (9). Panel B1 is a representative DSC spectrum of the extracted spinae samples. The scan rate is 1°C per min. The thermograms of the subsequent first, second and sixth scan are presented in black (line), red (dash-line) and blue (dot-line), respectively. Panel B2 shows the CD spectroscopy results of the sample before (black line) or after (red dash-line) the first DSC scan. Panel C is IR spectra of amide I bands of the spinae extraction without treatment (black line), or treated at 100°C for 20 min (green dash-line) or with 2 mg/ml proteinase K for overnight at room temperature (blue dot-line). The spectrum of the recombined proteinase K (100 mg/ml) was measured and presented as a reference (red dash-dot-line). The curves are normalized to fit into the same scale.
pilin (0.66). As the XEDS technique used here is only qualitative, further quantitative analysis is required to verify if the main components of the spinae is different from those of the spinin, flagellin and pilins of marine pseudomonad D71. We have used Periodic acid-Schiff staining and lectin binding to assess biochemical nature of the spinae. The results obtained from these assays did not show the presence of carbohydrates (Fig. S3).

The disintegration analyses of the spinae show that proteins maintain the proteolysis-resistant filament winding into the tubular structure. In addition, thermal treatment at 100 °C ripped the tubular spinae into ribbons that were melted under prolonged heating. In order to get more information about the chemical and structural nature of the spinae we performed differential scanning calorimetry (DSC) analysis. This technique measures heat flows in or out from a sample that undergoes a physical transformation such as protein denaturing and aggregation or polymer phase transition. While it is generally dedicated to purified macromolecules and at a defined concentration, DSC analyses could provide important qualitative information about the thermal behavior of the components in the extracted spinae fractions. Changes of particle sizes and the secondary structures of the spinae sample before and after the first DSC scan were also monitored with dynamic light scattering (DLS) and circular dichroism (CD). Before the DSC experiment, the samples were composed of two major groups of particles with hydrodynamic diameters of 231.2 nm and 5323 nm. Heating scans were then performed between 30 °C and 125 °C at 1 °C per minute scan rate. No exothermic process was observed in all analysis, showing the absence of strong aggregation of the samples upon the heating22. This was further confirmed by the fact that the samples did not present any precipitation or turbidity after each scan, which indicated a thermal stability within this temperature range. Thus the reversibility of the events could be evaluated, and a sample was subjected to up to six subsequent scans at the identical scan rate. As shown in Figure 4, B1, the typical DSC thermogram of the first scan (black curve) displays a very complex melting profile with three major endothermic transitions corresponding to the three major melting peaks P1, P2 and P3. The first one (P1) shows a large irregular and complex shape with many broad transitions, and displays a maximum around 57 °C. This feature could be attributed to multi-domain protein or macromolecule unfolding-denaturation, or multiple protein denaturation as generally observed21–23. The absorption of heat at 98 °C (P2) could be due to the unfolding of a more thermally resistant macromolecule as well as the requirement of heat to rip the tubular spinae as observed with TEM. Interestingly, thermal melting of amyloid fibrils occurs at this temperature region24. The small absorption peak at 123 °C (P3) was absent from the control-scan with only water as a sample, but was repeatedly observed when the spinae extraction samples were analyzed. After the first DSC scan, DLS results demonstrated a poly-dispersion of the population. The major group of particles was split into two groups of 134.8 nm and 800.9 nm (data not shown). The minor group displayed only a small size shift to 4920 nm with a tiny increase of intensity, which falls into the normal logical ranges 11. However, prolonged sulfide starvation stimulated the small absorption peak at 1652 nm (Fig. 4, C, red spectrum). This wave number range corresponds to the amide I band and has been reported to be significantly different between native beta-sheet proteins and those in inclusion bodies, thermally induced aggregates or amyloid fibrils27. In fact, the amide I maxima in the cross-beta structure of amyloid fibrils generally shift 20–30 nm toward the short wavelength27. Therefore, the beta-structure in the spine extractions corresponds better to those of amyloid fibrils than to the beta-sheet structures found in native beta-sheet proteins. Together these biophysical data showed features distinct from those of most native proteins.

### Discussion

Bacteria produce various extracellular appendages on their outer surfaces. In addition to the well known flagella, pili, and needles, there are two kinds of appendages from Gram-negative bacteria; prosthecate and nonprosthecate (also called echinuliform) appendages. The prosthecate appendages are extensions of the cell wall containing cytoplasm30, which include the stalks of Caulobacter and the hyphae of Hyphomicrobiurn and Rhodomicrobiurn. Spinae are the representatives of the nonprosthecate appendages. Studies have been carried out with respect to their microscopic structures31,32 and the growth conditions favorable for spinae production31,33. Transmission electron microscopy clearly shows that the spinae extend without apparent connection to the cytoplasm, and are unlikely to be extensions from the cell wall. Our data, obtained in this study, are consistent with the previous reports. In addition, proteolysis analysis revealed that spinae are attached to the cell surface by proteins (Fig. S1, D), and cryo-EM images did not reveal any special structure underneath the spinae (Fig. 2). Growth conditions such as pH, salt concentration and temperature affect the synthesis of the spinae by marine pseudomonad D7134. In contrast, the production of the spinae in C. limicola strain UdG6038 was not influenced by changes in temperature, pH, salt concentration, or illumination over physiological ranges31. However, prolonged sulfide starvation stimulated spinae production in this strain. Similar to the C. limicola strain, synthesis of the spinae in Roseobacter sp. YSCB strains was unlikely.
to be controlled by temperature, oxygen content or pH, but was enhanced by the dilution of the growth media. This appears to be due to changes in salt concentration since addition of NaCl in the diluted media reduced spination. It is unknown how salt concentration influences the production of spinae. These results show that spinae production is regulated by different environmental and physiology parameters depending on the species analyzed.

Spinae are found in Gram-negative bacteria with various physiological ranging from heterotrophic to phototrophic, both aerobic and anaerobic. Spinae might be omnipresent in aquatic bacteria but only occasionally observed and their synthesis is tightly controlled by environmental parameters and nutrient availability. The Arctic seawater bacterium R7967 and Roseobacter sp. DSS-1 that are closely related to Roseobacter sp. YSCB isolates, might synthesize the spinae when they are incubated under appropriate conditions. The possible functions of spinae include improving cellular buoyancy, stabilizing capsule structure to retain sulfur colloids, allowing cell-cell contact and signal exchange, aggregation of cells, protection against predation and adhesion to nutrient particles\(^1\)\(^2\). To date, none of these hypothetical functions has been experimentally proven. We compared the sedimentation of Roseobacter sp. YSCB cells by a method similar to that used for measuring sedimentation rate of red blood cells, and did not find difference between the cells without spinae (grown in normal Marine Broth media) with those with spinae (grown in diluted media). Therefore, the spinae of the Roseobacter sp. YSCB strain are unlikely to serve as buoyancy structures to help the cells maintain a position within the stratified water column. Cryo-EM revealed the absence of the basal body structure underneath the spinae of Roseobacter sp. YSCB strain, which excludes a rotary propeller function as the flagella. Consistently, spinae were observed for non-motile bacteria such as C. limicola\(^20\)\(^\text{11}\)\(^\text{12}\). We found that dilution of growth media triggered the spination of Roseobacter sp. YSCB strain. Therefore, the spinae might increase cellular surface to improve nutrient binding and uptake. Spinae might have multiple functions depending on ecological niches where the spinae covered cells live.

Bacterial surface appendages are generated by different mechanisms. Flagella, pili, type IV pili and injectisomes are polymerized from proteinaceous subunits via a dedicated protein secretion and assembly apparatus\(^3\)\(^4\)\(^5\)\(^6\). Stalk appendages are extensions of the cell wall and disrupted with lysozyme treatment\(^7\). Compared to these appendages, the spinae exhibit some distinct features. First, cryo-EM analysis and lysozyme treatment clearly showed that the spinae are not extensions of the cell wall, hence belonging to the family of non-prosthecate appendages. Second, TEM, AFM and cryo-EM inspections showed an unambiguous helical structure of the spinae. Consistently, proteolysis treatment unwound a spine into a proteolysis-resistant filament. These findings allow us proposing the hypothetic spinae assembly mechanism, according to which a filament rotates about an axis, which is accompanied by translation along the axis. Proteins that connect neighboring turns in the helix maintain the helical structure. The distance between the neighboring turns is different according to the analysis method used. The negatively stained TEM images showed that the helix is highly compact whereas relatively large rib spacing was observed for the spinae of the cryo-EM images (Fig. 2, panels Bs versus Cs). Several hypotheses might account for this discrepancy. The uranyl acetate used in negative staining might fill up the gaps between the neighboring turns. Alternatively, the electron density generated from the proteins connecting the neighboring turns is too weak in the cryo-EM analysis. At present, we do not know how hermetic the spinae are. Finally, the thermal treatment ripped the spinae of Roseobacter sp. YSCB strain into ribbons. We interpret this observation by suggesting a cooperative rip to change curvature of the helix. The ribbons tend to curl because they consist of ribs with about 6.6\(^\text{1}\) inclination. High-resolution microscopy analysis of the filament ultra-structure might assess this hypothesis.

We have never observed columnar structure directly connected to the cell surfaces. In contrast, the large sides of flares are always linked to the cells and the narrow, distal part is connected to the columnar structure with different length. This observation would suggest that the spine production starts with the flares and then the columnar part. The synthesis of flares should be relatively fast once started or the intermediate product is highly unstable because we have never observed different stage of flare production despite of almost ten-year effort. It is partially because that we can examine the structure only under electron microscopes and the immature structure might be destroyed during the sample preparation.

We attempted to determine the nature of the spinae of Roseobacter sp. YSCB strain by various biochemical and biophysical approaches. MALDI-TOF analysis of spinae failed to give any significant peaks in the size range examined (50,000–500 Da). This is not surprising, as it seems unlikely that subunits of such a robust large presumably polymeric structure will be easily extracted and put into the gas phase in the presence of multiple interactions with neighboring subunits. Enzymatic treatment digested the polypeptides present in the spine extracts. However the relationship of these polypeptides to the spine structure is unclear as they are completely digested under conditions where the spinae remain intact. Analysis of the structural protein of the spinae by MS/MS was unfeasible as we are currently unable to obtain soluble peptide fragments for analysis. We found that the spinae are extremely resistant to proteolysis and are relatively thermal stable. The unexpectedly high transition temperature of 98°C observed by DSC has been previously reported for the unfolding transition of large amyloid aggregates\(^\text{21}\). Furthermore the rather low frequency vibration of the amide 1 band at 1600 cm\(^{-1}\) is also characteristic of amyloid like beta sheet structures\(^\text{22}\). Such a structure would also explain the proteinase resistance of the spinae. It thus seems possible that the repetitive helical structure is based on an amyloid like peptide polymerization, though the N\(\text{O}\)\(\text{O}\)\(\text{O}\) ratio indicates that the structure is not based uniquely on this. The formation of spinae might thus rely on a polymerization of beta-sheet structures similar to amyloid plaque formation. Taken together, the spinae of Roseobacter sp. YSCB strain are distinguished from other bacterial surface appendages, such as flagella, stalks and pili, by both their structure and assembly mechanisms.

**Methods**

**Purification and treatment of the spinae.** The Roseobacter sp. YSCB isolates were incubated in marine broth M2216 (Difco) at normal or diluted concentration at 28°C with 200 rpm rotary shaking. Cells were harvested by centrifugation at 6000 g for 10 min, washed once with deionized water and resuspended in deionized water. Appendages were sheared by 20 passages of the cellular suspension through 0.8 × 40 nm needles and harvested in the supernatant fraction after centrifugation at 14 000 g for 10 min. The spinae suspension was either treated directly at 100°C for indicated times, or mixed with Tris-HCl buffer at pH 8 to the final concentration of 20 mM and then treated with various enzymes and DTT. Proteinate K (recombinant PCR grade, Roche Applied Sciences) was used according to manufacturer’s instruction. Denaturing, SDS-polyacrylamide gel electrophoresis was performed as previously described\(^\text{38}\).

TEM, XEDS and AFM microscopy analyses. Transmission electron microscopic analysis was described previously\(^\text{23}\). The bacteria or purified spinae, absorbed on Formvar-carbon coated grids, were negatively stained with 1% uranyl acetate for 1 min. Examination was performed with a Zeiss EM9 microscope at 80 kV.

Alternatively, samples were examined without fixing nor staining using a transmission electron microscope (JEOL J2100F) equipped with energy dispersive X-ray spectrometry (XEDS, Joel) and scanning TEM device.

Atomic Force Microscopy was performed using a Topometrix TMX-2000 Explorer (dry scanner, tapping mode) on specimens adsorbed on freshly cleaved Mucovisc E-1 treated with 0.1% uranyl acetate and air-dried.

**Cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET).** Five microliters of purified spinae were deposited onto a glow discharge, C-flatTM holey carbon-coated grid. The excess of the solution was manually blotted, 1 second, with a filter paper. Samples were then quickly frozen into liquid ethane using Leica EM CPC equipment (Leica Microsystems). The grids were placed onto a Gatan 626
cryo-holder, and transferred into the microscope. Sample observations were performed on a Tecnai G2 LaB6 microscope (FEI Company) operating at 200 kV, at a temperature of about ~177°C, and under low dose conditions. Images were recorded on a 2k × 2k FEI Eagle CCD (FEI Company).

Cryo-ET of the spines were collected at a magnification of 14,500, with the FEI Automated Tomography, Xplore 3D (FEI Company), tilted from −65° to +65° with a step of 1°, at a defocus of about ~10 μm. Final tomogram is constituted of 131 images with an electron dose per tomogram evaluated at 120 e-/Å².

Alignment of the tilt series and tomographic reconstructions (calculated by Simultaneous Iterative Reconstruction Technique) were computed with Inspect 3D (FEI Company). Colloidal Gold particles (10 nm) were tracked as fiducial markers to compute Simultaneous Iterative Reconstruction Technique. The IR spectra of various regions were recorded and analyzed. The measurement of macromolecule sizes was analyzed by dynamic light scattering using a NanoZS (Malvern Instruments Inc., UK). For the experiments, 5 μl of each sample were diluted in 70 μl of pure water. Three records of at least three scans were recorded.

Circular dichroism analysis. CD Spectra were recorded on a J-810 spectropolarimeter (Jasco, Tokyo, Japan). Measurements were recorded between 190 and 300 nm in 10 nm steps at 20°C. Each spectrum is the sum of at least three scans, after baseline subtraction (pure water).

Dynamic light scattering analysis. The measurement of macromolecule sizes was analyzed by dynamic light scattering using a NanoZS (Malvern Instruments Inc., UK). For the experiments, 5 μl of each sample were diluted in 70 μl of pure water. Three records of at least three scans were recorded.

Microcalorimetric measurements were carried out with a high-sensitivity differential scanning calorimeter DSC 1000 (Mettler Toledo, Greifensee, Switzerland). Samples were heated at a rate of 1°C/min from 17°C to 200°C in the absence of water vapor.

Acknowledgements

We thank S. Robert and S. Canaan for advice regarding to lipid analysis, H. Celia for initial cryo-electron microscopy observation, and R. Lebrun for assistance in proteomic analysis. This work was supported by grants from CNRS and CAS to LIA-BioMNSL.

Author contributions

L.F.W. and A.B. conceived the experiments. A.B., P.B. and C.N. performed TEM, AFM and CET analyses; C.V. carried out DSC, CD and DLS analyses; N.M. for XEDS analysis and J.S. for IR spectroscopy, L.F.W. and C.L.S. performed biochemistry analysis, L.F.W. and T.X. studied spin production and the growth of strain; L.F.W., C.V. and J.S. wrote the manuscript. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports