Goblet-shaped Sub-units from the Wall of a Marine Gliding Microbe

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SUMMARY

Goblet-shaped sub-units from the wall of a gliding marine bacterium have been isolated. They are apparently largely proteinaceous, having a buoyant density in CsCl solution of 1.31 g/ml at 25 °C. Their ultrastructure revealed by electron microscopy allows the hypothesis that they function *in vivo* as secretory organelles.

INTRODUCTION

The subject of bacterial wall topography has been reviewed extensively (Glauert & Thornley, 1969). Ordered arrays of globular macromolecules, commonly arranged tetragonally or hexagonally, have been observed on the surface of both Gram-positive and Gram-negative bacteria. Non-globular 'wineglass-shaped' sub-units have been reported on the outer surface of the photosynthetic purple sulphur bacteria *Chromatium buderii* (Remsen, Watson & Trüper, 1970) and *Amoebobacter bacillosus* (Cohen-Bazire, Kunisawa & Pfennig, 1969) and of the methane-utilizing bacterium *Methylobacterium albus* (Wilkinson, 1971). At least in *C. buderii*, the sub-units are arranged hexagonally. We report similar 'wineglass-shaped' structures (goblets) discovered among stable particulate products released during spontaneous autolysis of a marine *Flexibacter* sp.

METHODS

Organism and maintenance. An obligately marine *Flexibacter* sp. (Flexibacteraceae) was isolated from a decaying littoral ascidian, *Didemnum* sp., collected in September 1971 in Baja California, Mexico. The organism was filamentous, glided rapidly (at rates up to 8.0 µm/s), and synthesized a light-inducible orange carotenoid pigment. Organisms were routinely grown under fluorescent lights at approximately 1000 lux. Since the organisms autolysed readily, frequent subculture was necessary. Subsequent to its isolation in pure culture, it was subcultured every 2 to 3 days in the following growth medium: 5.0 g monosodium glutamate (Matheson Co., Los Angeles, California, U.S.A.); 1.0 g vitamin-free Casamino acids (Difco); 1.0 g Bacto-tryptone (Difco); 0.1 g sodium glycerophosphate (Matheson Co.); 1.0 µg cobalamin; 1.0 ml trace mineral solution (Reichle & Lewin, 1967); 1000 ml filtered sea water (FPB medium). Solid medium (FPA) was prepared from FPB by the addition of 1.0 % (w/v) agar (Difco).

Topography of autolysed organisms. A 1.0 ml portion of exponential-phase *Flexibacters* was inoculated into 120 ml of sterile FPB medium in a 250 ml Erlenmeyer flask and incubated at 23 °C on a reciprocating shaker (120 oscillations/min) until autolysis was complete, about 5 days. Clumped autolysed organisms ('ghosts') were pelleted at 4000g/15 min/4 °C

in a Sorvall RC-2B centrifuge and the supernatant suspension centrifuged at 20000g/15 min/4 °C to sediment non-aggregated ghosts. Non-aggregated goblets were resuspended in aqueous potassium phosphate buffer (0.05 M, pH 7.0), water, or sea water. A volume of this preparation, diluted to an appropriate ghost concentration, was mixed with an equal volume of aqueous 3% (w/v) potassium phosphotungstate (PTA) at pH 7.0 (adjusted using N-KOH). After 30 s, one drop of this suspension was transferred to a collodion-coated, carbon-stabilized 400-mesh copper grid, and excess liquid removed after 30 s. Grids were air-dried and examined immediately in a Philips EM-300 at an accelerating voltage of 60 kV. Purified goblets were visualized by the same staining method. Two per cent (w/v) aqueous uranyl acetate (UA), pH 4.2, was used successfully as an alternative to PTA.

Electron microscopy of non-autolysed organisms. For isolation of single colonies, Flexibacters were streaked on FPA medium and incubated at 23 °C. After 48 h drops of sterile filtered sea water were placed on well-isolated colonies. Such colonies were easily dislodged from the agar surface, transferred intact to 1% (w/v) OsO₄ in 75% sea water and left overnight at room temperature (about 23 °C). Fixed colonies, washed once in a large volume of sea water, were transferred to 2% aqueous UA (at pH 4.2) and left for 50 min. Rapid dehydration was accomplished in a graded acetone series. Material was infiltrated and embedded in Araldite, which was polymerized for 3 days at 65 °C. Thin sections were cut on a Sorvall MT-2B ultramicrotome and stained with 2% (w/v) aqueous UA and Reynolds lead citrate (Reynolds, 1963). Grids were examined in a Philips EM-300 at an accelerating voltage of 60 kV.

Scanning electron microscopy. A Petri plate containing FPA medium, overlaid with a sterile 90 mm Nucleopore membrane filter (5.0 µm pore size), was inoculated by streaking with a suspension of Flexibacters and incubated at 23 °C. After 48 h, 15 to 20 ml of 2% (v/v) glutaraldehyde (Ladd, Burlington, Vermont, U.S.A.) in 70% sea water was gently layered over the surface. Following overnight fixation at room temperature, 1 cm squares of the filter, bearing adhering colonies, were cut out, washed three times in water, dehydrated in a graded ethanol series, and critical-point dried (by the method of Cohen, Marlow & Garner, 1968) using 'Freon' 113 as an intermediate transitional fluid. Dried squares were mounted on aluminium stubs and shadowed (while being continuously rotated) with gold-palladium (60:40). Specimens were viewed in a Cambridge S4 scanning electron microscope and photographed on Polaroid PN55 film.

Partial purification of goblets. A 120 ml portion of fully autolysed Flexibacters, grown in FPB medium, was homogenized mechanically using a Potter-Elvehjem homogenizer to break up chains and clumps. Remaining clumps were removed by centrifugation in a Sorvall RC-2B centrifuge at 4000 g/15 min/4 °C, the residue discarded or recycled as above, and the supernatants pooled. The supernatant mixture was spun at 27000g/20 min/4 °C and the residue discarded. The 27000g supernatant fluid was spun at 80000g/90 min/4 °C in a Spinco Model L ultracentrifuge (no. 40 fixed-angle rotor) and the pellet resuspended in 20 ml of potassium phosphate buffer (0.05 M, pH 7.0). Following gentle homogenization, MgSO₄ (5×10^{-3} M), DNase I (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A., 100 µg/ml), and bovine pancreatic RNase (Calbiochem, La Jolla, California, U.S.A., 100 µg/ml) were added and the mixture incubated at 30 °C. After 90 min, the solution was centrifuged again at 80000g/90 min/4 °C and the opalescent orange residue re-suspended in 1.0 to 1.5 ml cold phosphate buffer. Nineteen drops (about 0.5 ml) from a Pasteur pipette were carefully layered on an approximately linear continuous CsCl (American Potash and Chemical Corp., Los Angeles, California, U.S.A.) density gradient (performed by the method of May, 1966) made up in the phosphate buffer. Gradients were spun at 120000g/6 h/4 °C

Fig. 1

Fig. 2

Fig. 1. Partial purification of *Flexibacter* goblets by CsCl equilibrium density-gradient centrifugation. The goblet-containing fraction (as assayed by electron microscopy) corresponded to the protein maximum. Buoyant density of the goblet fraction was 1.31 g/ml at 25 °C.

Fig. 2. U.v.-absorption spectrum of the purified *Flexibacter* goblet fraction. Dialysed goblet fraction (0.06 ml) was diluted to 1.0 ml with phosphate buffer (0.05 M, pH 7.0). 280/260 nm = 0.874.

in a SW-50L rotor using a Beckman Model L3-50 ultracentrifuge. Fifty to 65 fractions were obtained from each 5 ml bucket by collecting drops. CsCl was removed from the fractions by dialysis for 20 h at 4 °C against 1200 ml water or the phosphate buffer, both of which contained 1×10^{-3} M-MgSO₄ (added because Mg²⁺ ions seemed to stabilize goblet ultra-structure). U.v.-absorption of each fraction diluted in the phosphate buffer was measured in a 1.0 cm quartz cuvette at 25 °C in a Hitachi Perkin-Elmer Model 139 spectrophotometer. Protein determinations were made by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin over the range 0 to 50 µg was used to generate a nearly linear standard plot of absorption at 750 nm. An optical density of 0.3 (Bausch & Lomb Spectronic 20) at 750 nm corresponded to 41.8 µg BSA.

RESULTS

Goblet-like structures were isolated from supernatant fluids of spontaneously autolysed cultures and of growing organisms lysed by resuspension in cold water or dilute buffer. A regimen of differential centrifugation and CsCl equilibrium density-gradient centrifugation was employed for partial purification of more or less aggregated goblets (Fig. 1). Their buoyant density (approximately 1.31 g/ml), u.v.-absorption (280/260 nm = 0.874, Fig. 2), and strong Lowry reaction suggest a high proportion of protein.

Electron microscopy of banded material (Fig. 3, 4), negatively contrasted with neutral potassium phosphotungstate (PTA), reveals ultrastructural details of the goblets and their mode of aggregation, as diagrammatically represented in Fig. 5. Purified goblets fre-

(a)

(b)

Fig. 3. Electron micrograph of CoCl-banded *Flexibacter* goblets (PTA negative stain). (a) Goblets banded at a density of 1.31 showing aggregation in either close-packed spheres (arrow) or in planar loops (wedge). Several goblets are seen in plan view, filled with PTA. A single goblet is indicated by the narrow wedge. (Scale = 200 nm.) (b) Goblets bound to membrane-like fragments sedimented at a buoyant density of 1.25. Note fine axial filaments which apparently originate from goblet bases (wedges). (Scale = 100 nm.)

Fig. 4. Goblet ultrastructure. (a) Two opposed goblets associated by their bases. Each base is about 12 nm in diameter; their combined diameter is 25 nm. A filament stems from only one member of the pair. Immediately adjacent to the pair are four close-packed goblets in plan view, each showing a heavily stained centre region 5.5 nm in diameter. (Scale = 50.0 nm.) (b) Single goblets (indicated) and small aggregates. Note base units. (Scale = 100 nm.)

Fig. 5. Diagrammatic illustration of goblet aggregation. Dimensions are averages ($\pm 5\%$). Aggregation is by base units and sides of cups. Each goblet may be surrounded by a maximum of 6 equally spaced and oriented neighbours in close-packed spherical arrangements.

quently aggregated in planar closed loops with neighbouring bases and cups (see below) in apposition or in close-packed spheres which tended to collapse during the negative staining procedure (Fig. 3).

Individual goblets consisted of a cup-shaped portion, a stem, and an ellipsoidal base, best observed in goblets which exist singly or in small aggregates (Fig. 4a, b). A single narrow filament, which apparently originated from the goblet base and passed axially up

Fig. 6. Topography of autolysed *Flexibacters* (PTA negative stain). (a) Electron micrograph showing goblet-shaped sub-units comprising the surface layer. Areas of goblet detachment are indicated. Note single and aggregated filaments derived from the goblet layer. (Scale = $0.5\ \mu\text{m}$.) (b) Electron micrograph showing overall appearance of sub-units. Regions of apparent hexagonal close-packing are indicated. Repeat distance is 25 nm. (Scale = $0.5\ \mu\text{m}$.)

Fig. 7. Electron micrograph of osmium-fixed non-autolysed *Flexibacter*. Section cut tangentially through the outer wall layer showing hexagonally arranged 12.5 nm sub-units, possibly corresponding to goblet bases. Centre-to-centre spacing of the sub-units is 25 nm. (Scale = 0.25 μ m.)

Fig. 8. Scanning electron micrographs of non-autolysed organisms. (a) *Flexibacter*s grown adhering to the surface of a Nucleopore membrane filter. Filamentous mucoid material (aggregates of goblet filaments) may anchor organisms to their substratum. (Scale = 1.0 μ m.) (b) Surface of a colony showing cross-wall indentations and filaments contacting neighbouring organisms. (Scale = 1.0 μ m.)

the stem, projected outward through the open 'mouth' of the cup-shaped portion. More than one such filament per goblet has never been observed in any of our micrographs. Goblets were approximately 35 nm long from the tip of the base unit to the rim of the 'cup', and 25 nm in diameter across the mouth of the cup. Base units were 12 nm in diameter and 6 nm thick. They may be primarily responsible for the aggregative properties of goblets, since paired goblets were connected only by their base units (Fig. 4*a*). Filaments were 1.0 to 1.5 nm in diameter and up to 0.92 μm long, but of variable length, presumably due to random shear-force cleavage during purification steps. Because of their linearity, diameter and susceptibility to shear stress, these filaments were initially suspected of being nucleic acid. However, the filaments present in purified preparations of goblets were completely refractory to degradation (detectable by electron microscopy) even after prolonged treatment (90 min at 30 °C) with relatively high concentrations of DNase I and bovine pancreatic RNase (both at 100 $\mu\text{g/ml}$). Since the u.v.-absorption spectra of purified goblet fractions (Fig. 2) exhibited a 280/260 nm ratio of 0.874, the purified goblets contained less than 4.52 % nucleic acid (on the basis of the equation of Warburg & Christian, 1941). Carbohydrate may be present in the filaments since purified nuclease-treated goblets gave a positive anthrone reaction.

Preparations of autolysed Flexibacters ('ghosts'), negatively stained with PTA and examined in the electron microscope, revealed a topography which suggests that goblets may comprise a specific wall layer (possibly the outermost, see below) (Fig. 6*a, b*). It appears that in certain peripheral areas chains or sheets of aggregated (or possibly membrane-bound) goblets became detached from the lysed organism surface and subsequently circularized, generating closed loops or spherical close-packed arrangements of goblets. Although these bodies do not conform to any obvious ordered array, specific regions of the ghost surface exhibited hexagonal close-packing of goblets with a repeat distance of 25 nm (Fig. 6*b*, arrows). Tangential thin sections of osmium-fixed non-autolysed organisms provided evidence that the sub-units constituted a loose hexagonal surface array (Fig. 7). Centre-to-centre spacing of the sub-units visible in the sectioned material was about 25 nm, the sub-units themselves being 12.5 nm in diameter. These sub-units may correspond to goblet bases, which had similar dimensions and arrangement. The fixation method employed failed to demonstrate the cup-shaped portions of the goblets, which supposedly opened outwards away from the cell surface. About 30000 goblets would be required to cover a cylindrical Flexibacter 1.0 μm in diameter and 5.0 μm in length.

Much negatively stained fibrillar material was associated with the surface of ghosts (Fig. 6*a, b*). Such fibres appeared to be aggregates composed of numerous fine goblet filaments arranged parallel with one another. Scanning electron micrographs of young non-autolysed Flexibacter colonies grown attached to a Nucleopore membrane filter indicated that aggregation of such goblet filaments may aid in anchoring organisms to their substratum or to other organisms (Fig. 8).

DISCUSSION

We propose that the goblets are subcellular membrane-bound organelles responsible for filament synthesis and/or secretion. Continuous secretion of extramembranous macromolecular filaments could provide a mechanism for surface attachment and for gliding movement. This hypothesis is consistent with proposed models for interface adhesion (Corpe, 1970; Fletcher & Floodgate, 1973) and gliding motility (Weibull, 1960; Jarosch, 1962; Halfen & Castenholz, 1971).

We suspect that the 'wineglass-shaped' sub-units on the surfaces of *Chromatium buderii*, *Methylomonas albus* and *Amoebobacter bacillosus* (see Introduction), too, may specifically secrete extracellular slime (probably polysaccharide). Slime production, by mediating surface adhesion, could provide such organisms with an advantage during scavenging of interface-concentrated organic growth factors (Zobell, 1943).

Extracellular slimes and capsular mucopolysaccharides secreted by bacteria consistently possess a fibrillar ultrastructure (Pate & Ordal, 1967; Verma & Martin, 1967; Marshall, Stout & Mitchell, 1971; Fletcher & Floodgate, 1973; Springer & Roth, 1973), suggesting that they are initially secreted as individual polymeric macromolecular filaments originating from specific loci on the cell surface. Where goblet-like sub-units have not been detected, analogous wall-bound structures may be present to perform such a secretory function.

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