APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1995, p. 130–137 Vol. 61, No. 1
0099-2240/95/$04.00+0

Unusually Stable Spinae from a Freshwater *Chlorobium* sp.†

J. S. BROOKE,[1] S. F. KOVAL,[1]* AND T. J. BEVERIDGE[2]

Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1,[1] and Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1,[2] Canada

Received 22 August 1994/Accepted 25 October 1994

A green *Chlorobium* sp. with spinae, strain JSB1, was isolated from an enrichment culture previously obtained from Fayetteville Green Lake, N.Y. (J. S. Brooke, J. B. Thompson, T. J. Beveridge, and S. F. Koval, Arch. Microbiol. 157:319–322, 1992). Cells were gram-negative, nonmotile rods which contained bacteriochlorophyll *c* and chlorosomes. Spinae were best seen by transmission electron microscopy in thin sections of cells fixed in the presence of tannic acid. High-resolution scanning electron microscopy showed the spinae randomly distributed at the cell surface and at the junctions between cells. Spinae were physically sheared from cells and isolated from the culture supernatant by ultrafiltration. As observed by electron microscopy, spinae demonstrated unusual structural stability when exposed for 1 h at 37°C to chemical treatments such as hydrogen bond-breaking agents, detergents, metal-chelating agents, proteases, and organic solvents. They were stable for 1 h at 37°C over the pH range 2.3 to 9.9 and in 1 M HCl and 1 M NaOH. The structural integrity of the spinae was also maintained when spinae were subjected to harsher treatments of autoclaving in 2% (wt/vol) sodium dodecyl sulfate and exposure to dithiothreitol at pH 9 for 1 h at 100°C. Partially dissociated spinae were obtained after 5 h at 100°C in 1 M HCl and 1 M NaOH. In acid, the tubular spinae became amorphous structures, with no helical striations visible. In alkali, the spinae had dissociated into irregular aggregates of disks. Since both high temperature and extremes of pH were required to achieve partial dissociation of the spinae, the strength of the structure presumably comes from covalent bonding.

Spinae are nonprosthecate, rigid, columnar projections that extend from the bacterial cell surface outward into the external environment (7). Spinae have been observed on a taxonomically wide range of bacteria, including heterotrophs and phototrophs in freshwater and marine environments. Therefore, these appendages probably reflect an adaptation to a particular ecological niche (7). Spinae are randomly dispersed on the cell surface and vary in number per cell. The exact physical nature of their attachment to the cell is unknown. The size and morphology of spinae vary among different bacterial genera; the three main morphological types of spinae are cylinders, cylinders with a conical base, and cones (7). To date, no function for spinae of any organism has been experimentally proven. However, since spinae can almost cover the cell surface, their synthesis and assembly must be a major metabolic commitment and indicate an important function.

We have previously reported the occurrence and ultrastructure of spinae on a freshwater *Chlorobium* sp. isolated in enrichment culture from Fayetteville Green Lake, N.Y. (5). These spinae are cylindric without a conical base. They are striated with a shallow, right-handed helix and a hollow center. Their width is 39.4 ± 2.6 nm. They are attached to but do not penetrate the outer membrane. The aim of this paper is to describe the isolation, purification, and morphology of *Chlorobium* strain JSB1 and to assess the structural stability of the spinae.

MATERIALS AND METHODS

Bacterial strains. *Chlorobium* strain JSB1 was isolated from Fayetteville Green Lake, N.Y., by culture enrichment techniques (5) and obtained in pure culture by repeated application of the agar shake dilution technique (20). *Chlorobium limicola* f. sp. *thiosulfatophilum* 6230 (DSM 249, Tassajara) was isolated at the Hopkins Marine Station, Pacific Grove, Calif. (6), and *C. phaeobacteroides* 9230 (DSM 268) is a previous isolate from Fayetteville Green Lake (22). N. Pfennig, University of Konstanz, kindly provided both cultures.

Growth of *Chlorobium* strain JSB1. Biebl and Pfennig medium (4) was modified by the substitution of 10 ml of 7.7 M HCl for Na_2 EDTA and 2.1 g of $FeSO_4 \cdot 7H_2O$ for $FeCl_2 \cdot 4H_2O$ in the trace element solution (21). Freshly inoculated cultures were placed in the dark for 18 h at room temperature and then incubated at 10 to 20 lux (incandescent lamp) at room temperature. To avoid a long lag phase of growth, cultures were fed sulfide 48 h after inoculation.

Bacteriochlorophyll analysis. Two methods were used to determine the bacteriochlorophyll content of strains. In the first, sucrose absorption spectra, the method of Pfennig and Trüper (23) was used without modification on intact cells. In the second, methanol-acetone extraction of pigments, 1 ml of a *Chlorobium* strain JSB1 culture was placed in a foil-covered 30-ml Corex centrifuge tube, and 5 ml of an ice-cold mixture of 7:2 (vol/vol) acetone-methanol was added. Extraction of the pigments was allowed to occur at −20°C in the dark for 4 to 6 h, the sample was centrifuged at 20,190 × *g* for 10 min, and the relative absorbance of the supernatant was measured from 900 to 300 nm against a blank of 2 ml of the acetone-methanol mixture. Absorption spectra were measured with an LKB model 4050 Ultrospec II spectrophotometer.

Transmission electron microscopy (TEM). For negative staining, samples were negatively stained with 0.5% (wt/vol) uranyl acetate (pH 4.4; Eastman Kodak, Rochester, N.Y.) containing 50 μg of bacitracin per ml as a wetting agent. For production of thin sections, cells were fixed in 4% (wt/vol) tannic acid–2.5% (vol/vol) glutaraldehyde and washed in 0.1 M sodium phosphate buffer (pH 7.2) containing 7% (wt/vol) sucrose. After being enrobed in agar, the cells were postfixed with 1% (vol/vol) osmium tetroxide in the phosphate-sucrose buffer. All cells were dehydrated through an ethanol series and embedded in Spurr embedding medium. Sections were cut and poststained with uranyl acetate and lead citrate. For freeze-substitution, cells were freeze-plunged and freeze-substituted by the osmium tetroxide-uranyl acetate in acetone method of Graham and Beveridge (12) as described by Beveridge et al. (2). After freeze-substitution, samples were brought to room temperature, embedded in Epon 812 (Can EM, Guelph, Canada), cured for 48 h at 60°C, and sectioned. The sections were stained with uranyl acetate and lead citrate (12). Samples were examined with a Philips EM300 transmission electron microscope operating at 60 kV under standard conditions.

Scanning electron microscopy (SEM). *Chlorobium* strain JSB1 cells were allowed to settle onto polylysine-coated silicon chips and then fixed in 1.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). Cells were postfixed in 1% (wt/vol) osmium tetroxide in buffer, dehydrated in ethanol and then acetone, and then critical point dried. Samples were gold coated and examined with an ISI DS-130 scanning electron microscope, using the upper stage.

Isolation of spinae. Spinae were recovered from the culture supernatant and cell surface by first centrifuging 2 liters of cells at 7,000 × *g* for 30 min. The

* Corresponding author. Phone: (519) 661-3439. Fax: (519) 661-3499. Electronic mail address: skoval@julian.uwo.ca.

† This paper is dedicated to Professor R. G. E. Murray on the occasion of his 75th birthday.

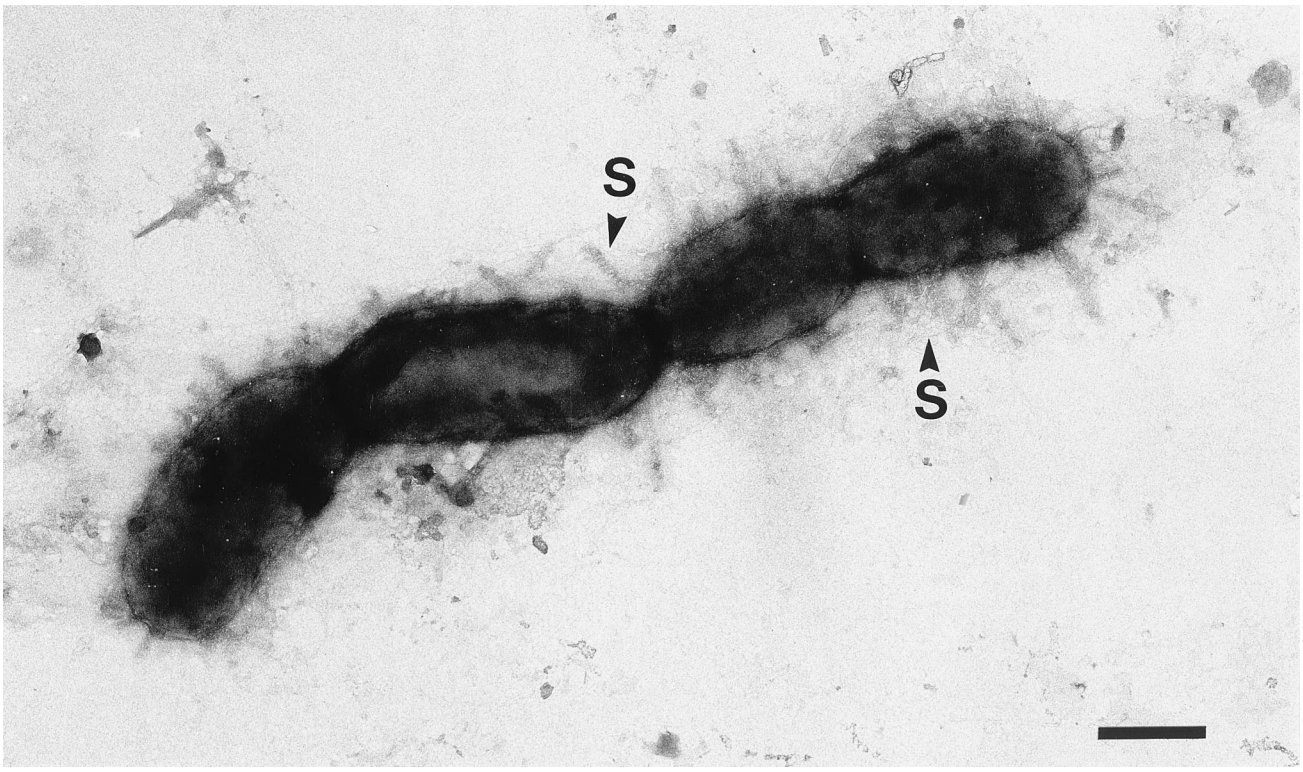

FIG. 1. Negative stain of *Chlorobium* strain JSB1. S, spinae. Bar, 500 nm.

culture supernatant was recentrifuged to pellet any remaining whole cells and stored at 4°C with sodium azide. The cell pellets were resuspended in a total volume of 50 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.4). The cell suspension was subjected to shear forces twice for 90 s each in a Servall Omni-mixer (Sorvall, Inc., Norwalk, Conn.) at a rheostat setting of 60. The sheared cells were pelleted by centrifugation at 4,000 × *g* for 30 min, and this supernatant was combined with the cell-free culture supernatant obtained as described above. The spinae in these supernatants were concentrated to approximately 20 ml by ultrafiltration with stirred ultrafiltration cells (Amicon Corp., Lexington, Mass.) and XM100 membranes. The surface of the membrane was gently scraped with a rubber policeman to remove any spinae which had settled during concentration. All fractionation steps were monitored by TEM.

Protein assay. Cells from 7.5 ml of culture were harvested (7,000 × *g* for 20 min), washed twice with double-distilled water, resuspended in 2.5 ml of 5% (vol/vol) trichloroacetic acid (Fisher Scientific), and boiled at 100°C for 10 min. The sample was then put on ice for 1 h to precipitate the cell protein. The sample was centrifuged in an Eppendorf microcentrifuge for 2 min at maximum speed, and each pellet was resuspended in 500 μl of 1 N NaOH. After incubation at 37°C for 25 min, the samples were combined and brought to a final volume of 1 ml with double-distilled water. Protein was determined by the modified protein assay of Lowry et al. (19) with NaOH excluded from the assay mixture because

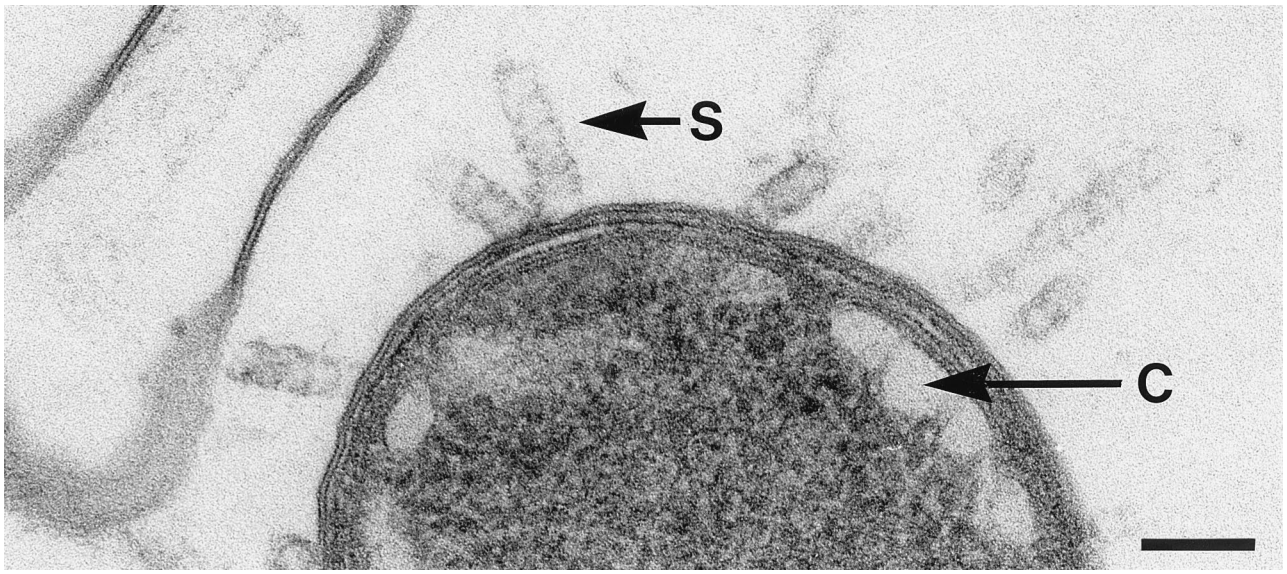

FIG. 2. Thin section of *Chlorobium* strain JSB1 prepared by freeze-substitution. S, spinae; C, chlorosome. Bar, 100 nm.

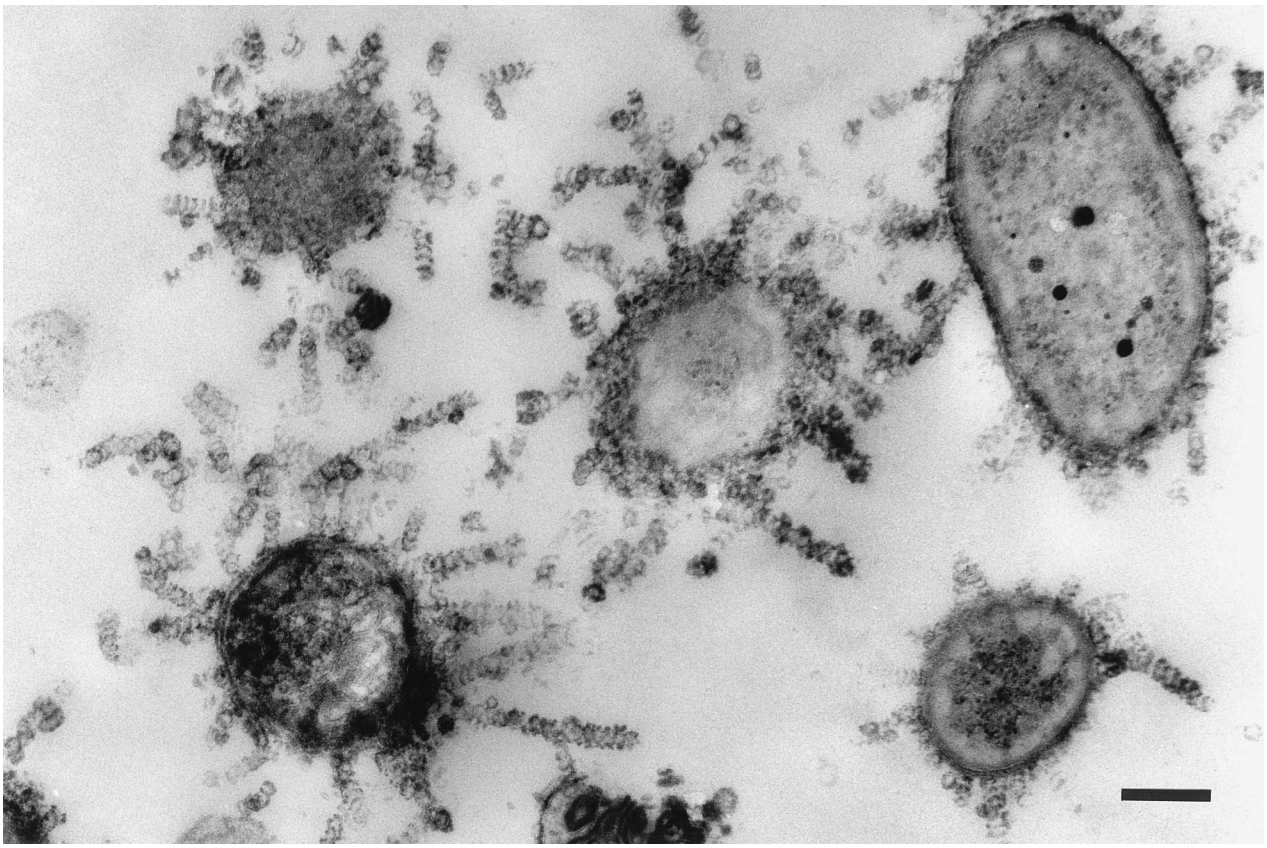

FIG. 3. Thin section of *Chlorobium* strain JSB1 cells fixed in the presence of tannic acid. The spinae appear as electron-dense coils projecting from the outer membrane or unattached to the cell surface. Bar, 250 nm.

NaOH had already been included during the preparation of the sample. The protein content of the spina-enriched preparations was determined in the presence of NaOH in the assay mixture.

Chemical treatments of spinae. Preliminary studies indicated that spinae were very stable surface structures. Therefore, whole cells or spina-enriched fractions were subjected to numerous chemical treatments to assess this structural stability. Reaction mixtures contained 100 μl of sample (80 to 100 μg of protein) mixed with 100 μl of the two-times-concentrated test solution. (i) The hydrogen-bond-breaking agents guanidine hydrochloride (Caledon Laboratories, Georgetown, Canada) and urea (Fisher Scientific, Fair Lawn, N.J.) were prepared as 6 M aqueous solutions. For these reagents, spinae were first pelleted and then resuspended in 6 M solutions. (ii) The following detergents were prepared as 2% (wt/vol) aqueous solutions: sodium dodecyl sulfate (SDS; 95% practical grade; J. T. Baker, Phillipsburg, N.J.); Sarkosyl (sodium lauryl sarcosinate), Brij 58 (polyoxyethylene-20-cetyl ether), and Tween 20 (polyoxyethylenesorbitain monolaurate) (all from Sigma Chemical Co., St. Louis, Mo.); zwitterionic detergent 3-14 (Calbiochem-Behring, La Jolla, Calif.); Triton X-100 (Mallinckrodt, Pointe-Claire, Canada); and Triton X-100 containing 50 mM Na_2EDTA (Fisher Scientific). (iii) The chelating agents EDTA, ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Sigma), and ethylene diamine-*N*,*N*′-diacetic acid (EDDA; a gift from C. Trick, University of Western Ontario) were prepared as 50 mM aqueous solutions at pH 4.8, 7.0, and 4.5 respectively. (iv) Protease XIV (pronase E from *Streptomyces griseus*; activity, 4 U/mg) and thermolysin (from *Bacillus thermoproteolyticus* rokko; activity, 50 to 100 U/mg), both obtained from Sigma, were prepared as solutions of 20 μg/100 μl in 0.05 M Tris-HCl buffer (pH 7.4) containing 2 mM $CaCl_2$. Trypsin (Fisher Scientific) and chymotrypsin (type II from bovine pancreas, crystallized three times from four-times-crystallized chymotrypsinogen; activity, 40 to 60 U/mg) (Sigma) were also prepared as solutions of 20 μg/100 μl in 0.05 M Tris-HCl buffer (pH 7.4) containing 2 mM $CaCl_2$. Papain (from papaya latex, two-times crystallized; activity, 16 to 40 U/mg) (Sigma) was prepared as a solution of 20 μg/100 μl in 0.05 M sodium phosphate buffer (pH 5.4). Proteinase K (from *Tritirachium album*; activity, 10 to 20 U/mg) (Sigma) was prepared as a solution of 20 μg/100 μl in 0.05 M Tris-HCl (pH 7.4) containing 2 mM $CaCl_2$. Reaction mixtures were incubated at 37, 25, 25, 60, and 60°C for protease XIV, trypsin, chymotrypsin, papain, and proteinase K, respectively. (v) The organic solvents alcohol, acetone, chloroform (95% [vol/vol] aqueous solution), and phenol (90% [vol/vol] aqueous solution) were used to treat cells. The mixtures were incubated at room temperature for 24 h except for the mixture containing phenol, which was incubated at 60°C for 1 h. (vi) The enzymes lipase (from wheat germ; Nutritional Biochemicals, Cleveland, Ohio), phospholipase C (type I from *Clostridium perfringens*; activity, 10 to 20 U/mg) (Sigma), and β-glucosidase (type II from almonds) were prepared as solutions of 20 μg/100 μl in 0.05 M Tris-HCl buffer (pH 7.4) containing 2 mM $CaCl_2$. (vii) The reducing agent 10 mM dithiothreitol in 40 mM bicine buffer (pH 9) was used.

Effect of pH on spinae. To test the stability of the spinae over a range of pH values, the following buffers were prepared: 0.2 M glycine-hydrochloride buffer (pH 2.3), 0.2 M sodium acetate buffer (pH 4.0), 0.2 M citrate-phosphate buffer (pH 6.4), 0.2 M sodium phosphate buffer (pH 7.8), and 0.2 M glycine-sodium hydroxide buffer (pH 9.9).

RESULTS

Enrichment and isolation. The selective enrichment culture technique described previously (5) resulted in cultures which contained approximately 90% *Chlorobium* cells and produced dark-green cell suspensions. In agar shake tubes, colonies of strain JSB1 were disk shaped and dark green with defined edges. Yellow-sectored colonies were sometimes observed. Selection of dark-green colonies into small volumes of medium resulted in the purification of strain JSB1.

Morphology and photosynthetic pigments. Strain JSB1 was commonly observed as small aggregates or chains of two to four short, rod-shaped cells (Fig. 1). In thin sections prepared by freeze-substitution (Fig. 2), the resolution of the envelope layers was sharply defined but the structure of chlorosomes was not preserved better than in conventional thin sections. A periplasmic gel could not be distinguished between the inner and outer membranes.

We have previously detected spinae on strain JSB1 by neg-

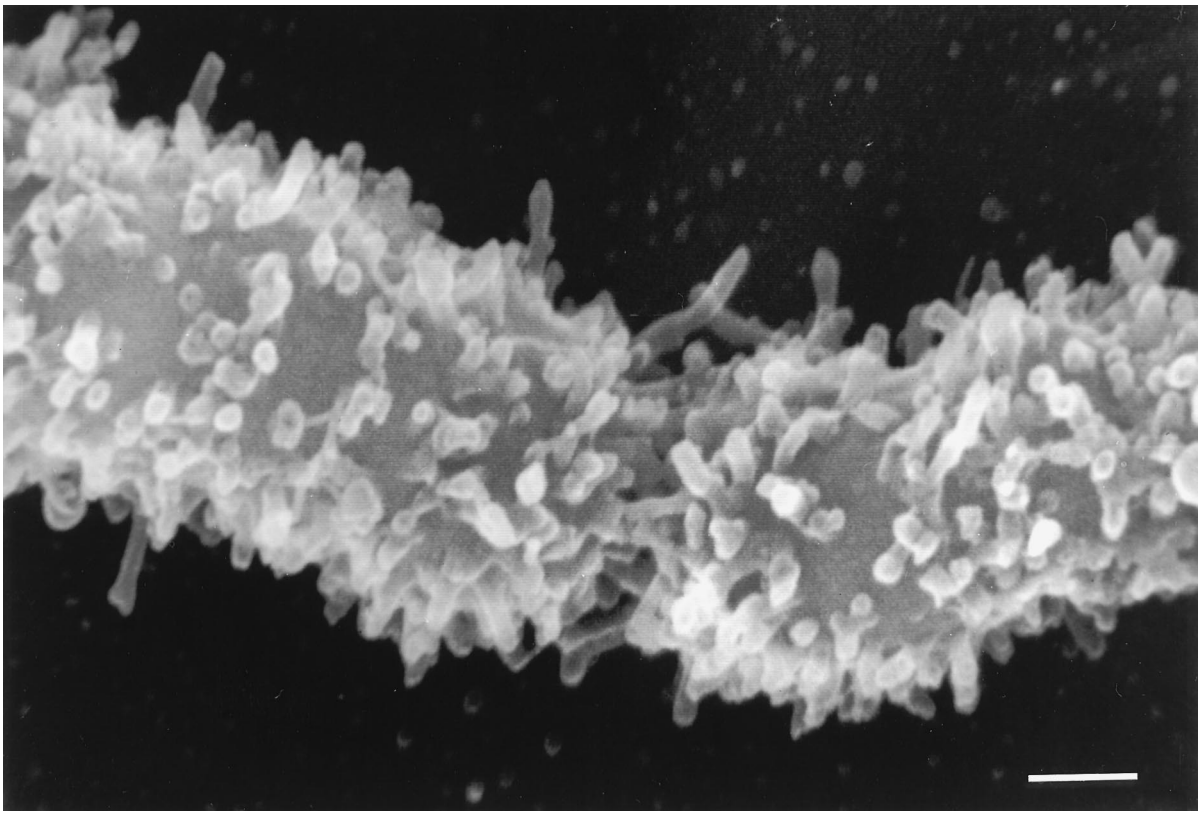

FIG. 4. Scanning electron micrograph of *Chlorobium* strain JSB1. The spinae are randomly dispersed at the cell surface and at the junction between the cells. Bar, 250 nm.

ative staining, metal-shadowing, and freeze-fracturing techniques (5). Spinae can be identified in thin sections of cells fixed by conventional techniques, but they are not very electron dense. Use of tannic acid during the fixation procedure increased the electron density of both the cell wall layers and the striations in the spinae which are a result of the shallow helical arrangement of subunits (Fig. 3). The visualization of spinae was thus greatly enhanced. The general use of tannic acid in embedding procedures for TEM of samples from nature would aid in the identification of spinae on other *Chlorobium* isolates.

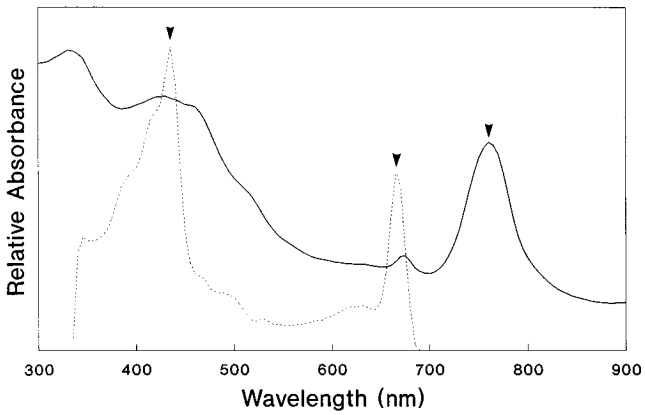

FIG. 5. Absorption spectra of intact cells (solid line) and methanol-acetone extract (dotted line) of cells of *Chlorobium* strain JSB1. Arrowheads indicate absorption maxima for bacteriochlorophyll *c*.

Spinae were also clearly identified over the cell surface by SEM (Fig. 4). Here, the spinae were randomly arranged as protrusions from the surface and were also found at the junction between cells.

Absorption spectra of living cells and extracted photosynthetic pigments were used to determine if the strain JSB1 isolate was a member of the green or brown *Chlorobium* spp. The absorption spectrum of intact cells showed an absorption maximum for bacteriochlorophyll *c* at 760 nm (Fig. 5). The absorption spectrum for extracted photosynthetic pigments was downshifted by ~60 to 100 nm from the whole-cell spectrum. It showed absorption maxima for bacteriochlorophyll *c* at 665 and 436 nm. Comparison of strain JSB1 with the green (strain 6230) and brown (strain 9230) *Chlorobium* spp. (grown under the same light conditions) showed that its two absorption curves closely resemble those obtained for *C. limicola* f. sp. *thiosulfatophilum* 6230 (data not shown). Thus, strain JSB1 is a member of the green *Chlorobium* spp.

Isolation of spinae. Although initial studies on the stability of spinae were performed with whole cells, a method was developed to isolate a spina-enriched fraction for subsequent studies. Initially, cells were disrupted in a French pressure cell, and a fraction which was enriched in spinae, with smaller amounts of plasma membrane vesicles, chlorosomes, and cell wall fragments, was obtained after differential centrifugation (data not shown). However, the spinae, when pelleted, formed aggregates that were very difficult to resuspend. This preparation was not suitable for subsequent density gradient centrifugations. Interpretation of dissociation studies was also difficult with aggregates of spinae, especially since penetration of the dissociation agents was suspect. Therefore, spinae were con-

TABLE 1. Chemical agents with no effect on the spinae of *Chlorobium* strain JSB1

Agent[a]
6 M guanidine hydrochloride
6 M urea
1% SDS
1% Sarkosyl
1% Brij 58
1% zwitterionic detergent 3-14
1% Tween 20
1% Triton X-100
1% Triton X-100 plus 5 mM EDTA
25 mM EDTA
25 mM EGTA
25 mM EDDA
Protease XIV (10 μg/100 μl)
Thermolysin (10 μg/100 μl)
Trypsin (10 μg/100 μl)
Chymotrypsin (10 μg/100 μl)
Proteinase K (10 μg/100 μl)
Papain (10 μg/100 μl)
Ethanol (47%, vol/vol)
Acetone (50%, vol/vol)
Chloroform (50%, vol/vol)
Phenol (45%, vol/vol)
β-Glucosidase (10 μg/100 μl)
Lipase (10 μg/100 μl)
Phospholipase C (10 μg/100 μl)
5 mM dithiothreitol (pH 9)
pH 2.3–9.9
1 M NaOH
1 M HCl

[a] These agents had no effect on spinae after 1 h at 37°C.

centrated from the culture supernatant and from sheared cells by ultrafiltration. The spinae in this preparation were longer than those in the French press lysate and were dispersed. This light-green preparation contained a small number of chlorosomes and plasma membrane vesicles. The final protein concentration of this preparation was usually 0.8 to 1.5 mg/ml.

Stability of spinae. To gain some insight into the chemical composition of the spinae, a wide variety of chemical agents were used in attempts to dissociate the spinae (Table 1). The effect of these treatments on the structural stability of the spinae was monitored by TEM with negative stains. Untreated spinae are shown in Fig. 6. The spinae proved to be stable to all the agents tested at 37°C for 1 h. They were also resistant to heat and were not dissociated after autoclaving at 121°C for 15 min even in the presence of 2% (wt/vol) SDS. They were not solubilized at 100°C in dissociation buffer used for SDS-polyacrylamide gel electrophoresis, and thus this technique was not used to monitor their disruption. Partial dissociation of spinae was obtained with 1 M HCl and 1 M NaOH at elevated temperatures. After 1 h at 60 or 100°C in 1 M HCl, the helical striations of spinae appeared "swollen" or expanded (Fig. 7A). Some amorphous areas were also seen. After 18 h at 60°C or 5 h at 100°C, all the tubular spinae were amorphous, with no helical striations visible (Fig. 7B). The spinae were not dissociated into smaller, amorphous aggregates or fibers. After 1 h at 100°C (but not 60°C) in 1 M NaOH, the structure of some spinae was disrupted into aggregates of "disks" (Fig. 8A). Other spinae still retained their tubular, helical structure. After 18 h at 60°C or 5 h at 100°C, all the spinae had dissociated into irregular aggregates of disks (Fig. 8B).

DISCUSSION

The identification of strain JSB1 as a member of the green *Chlorobium* spp. was unexpected, since the brown *C. phaeobacteroides* 9230 had also been previously isolated from the chemocline of Fayetteville Green Lake (22). Trüper and Pfennig (25) list examples of meromictic lakes where brown members

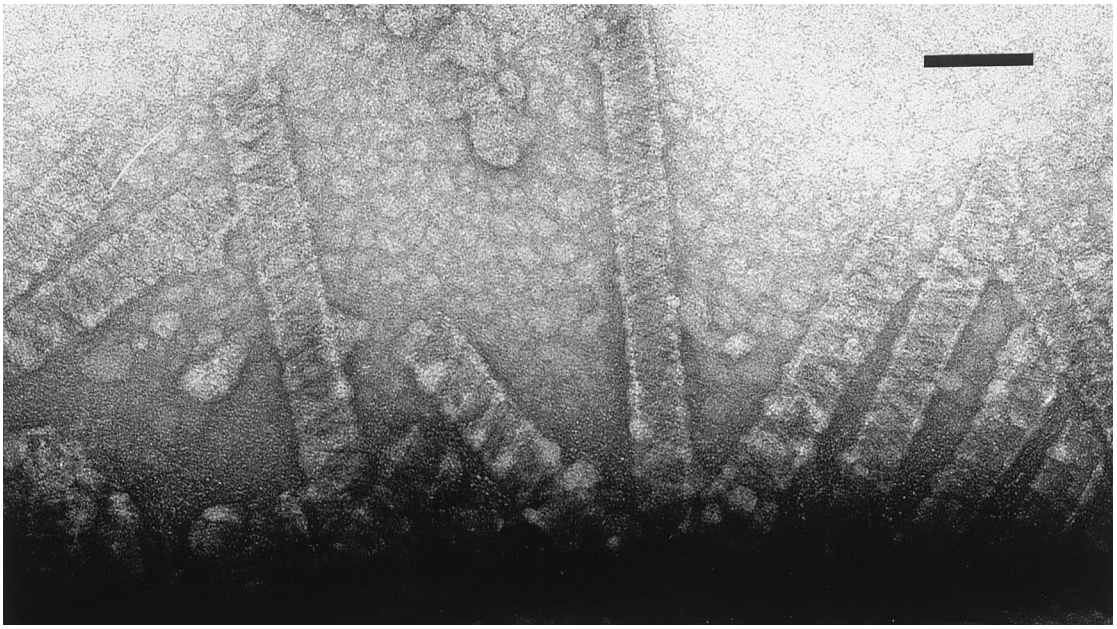

FIG. 6. Negative stain of spinae of *Chlorobium* strain JSB1. Bar, 100 nm.

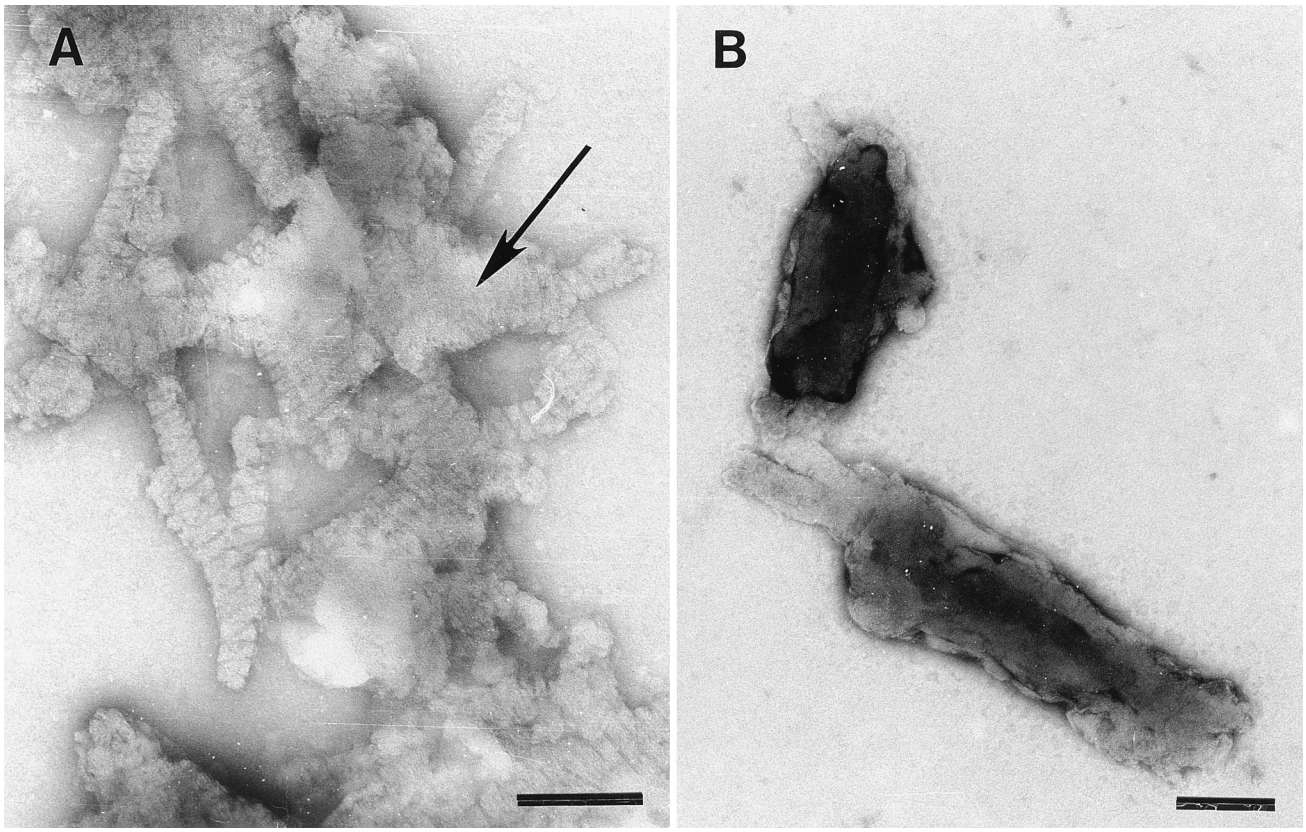

FIG. 7. Negative stains of spinae treated with 1 M HCl at 100°C for 1 h (A) and 5 h (B). The arrow indicates amorphous material. Bar, 200 nm.

of the family *Chlorobiaceae*, in particular *C. phaeobacteroides* and *C. phaeovibrioides*, are the dominant species.

We have observed spinae on strains of both green and brown *Chlorobium* species (5), including the *C. phaeobacteroides* 9230 used in this study. However, the *C. limicola* f. sp. *thiosulfatophilum* 6230 that we used did not possess spinae. Strain 6230 is the neotype strain of *C. limicola* f. sp. *thiosulfatophilum* and is the Tassajara strain described by Cohen-Bazire et al. (6). In this study on the fine structure of green bacteria (6), spinae are present in thin sections of the Tassajara strain. During subculture of strain 6230, a variant which has lost the ability to produce spinae must have been selected. It is important to note that Cohen-Bazire et al. (6) incorrectly identified the spinae as pili. The same micrograph was used by Kondratieva et al. (14) in the second edition of *The Prokaryotes*.

The isolation of spinae from strain JSB1 was difficult because of their propensity to aggregate after centrifugation. Attempts to dissociate these aggregates sufficiently for either sucrose or CsCl density gradient centrifugation were unsuccessful. The spina-enriched fraction obtained after ultrafiltration provided a good source of dispersed spinae for stability studies and for the purification of spinae. Pure spinae could not be obtained on a sucrose gradient because the spinae were too dense and formed a pellet even at the highest concentration of sucrose (55% [wt/vol]) used. Work is in progress to find conditions under which the spinae will form a band on a CsCl gradient. Once obtained, the band of purified spinae will be used for further biochemical studies.

The results of chemical treatments (Table 1) suggested that the spinae of strain JSB1 are unusually stable bacterial surface structures. The spinae of a marine pseudomonad, strain D71, were not so difficult to dissociate and, accordingly, are the only other spinae to have been characterized in detail (8). There are ultrastructural differences between the spinae of these two strains. Strain JSB1 spinae have a right-handed helix, a width of approximately 39 nm, and no physically expanded base at the junction with the outer membrane. Those of strain D71 are wider (65 nm) and have a conical base. The purified spinae from strain D71 often form ribbon-like structures, derived by the unwinding of chains of spinin subunits along their helical axis when exposed to shear and surface tension forces acting on them during specimen preparation (8). Such structures have never been observed in negatively stained preparations of the spinae from strain JSB1. Both the spinae of strain JSB1 (see above) and those of strain D71 (8) were resistant to proteases, EDTA, organic solvents, guanidine hydrochloride, 1 M HCl, and 1 M NaOH at room temperature, suggesting that they had some structural similarities. The resistance of strain JSB1 spinae to the reducing agent dithiothreitol suggests a lack of disulfide bonds, which corresponds to the absence of cystine in the spinae of strain D71. Easterbrook and Coombs (8) observed solubilization of strain D71 spinae that were treated with 0.1% (wt/vol) SDS at 80°C for 5 min. In contrast, the spinae of our strain (JSB1) resisted this treatment and a more harsh treatment of autoclaving in 2% (wt/vol) SDS. This difference in response to SDS must reflect differences in molecular composition and, presumably, intermolecular bonding. Dissociation of spinae with 1 M NaOH at 100°C for 5 h (Fig. 8B) may prove to be a suitable condition for studying the eventual reassembly of the JSB1 structures. Spinae treated with 1 M HCl under the same conditions (Fig. 7B) were irre-

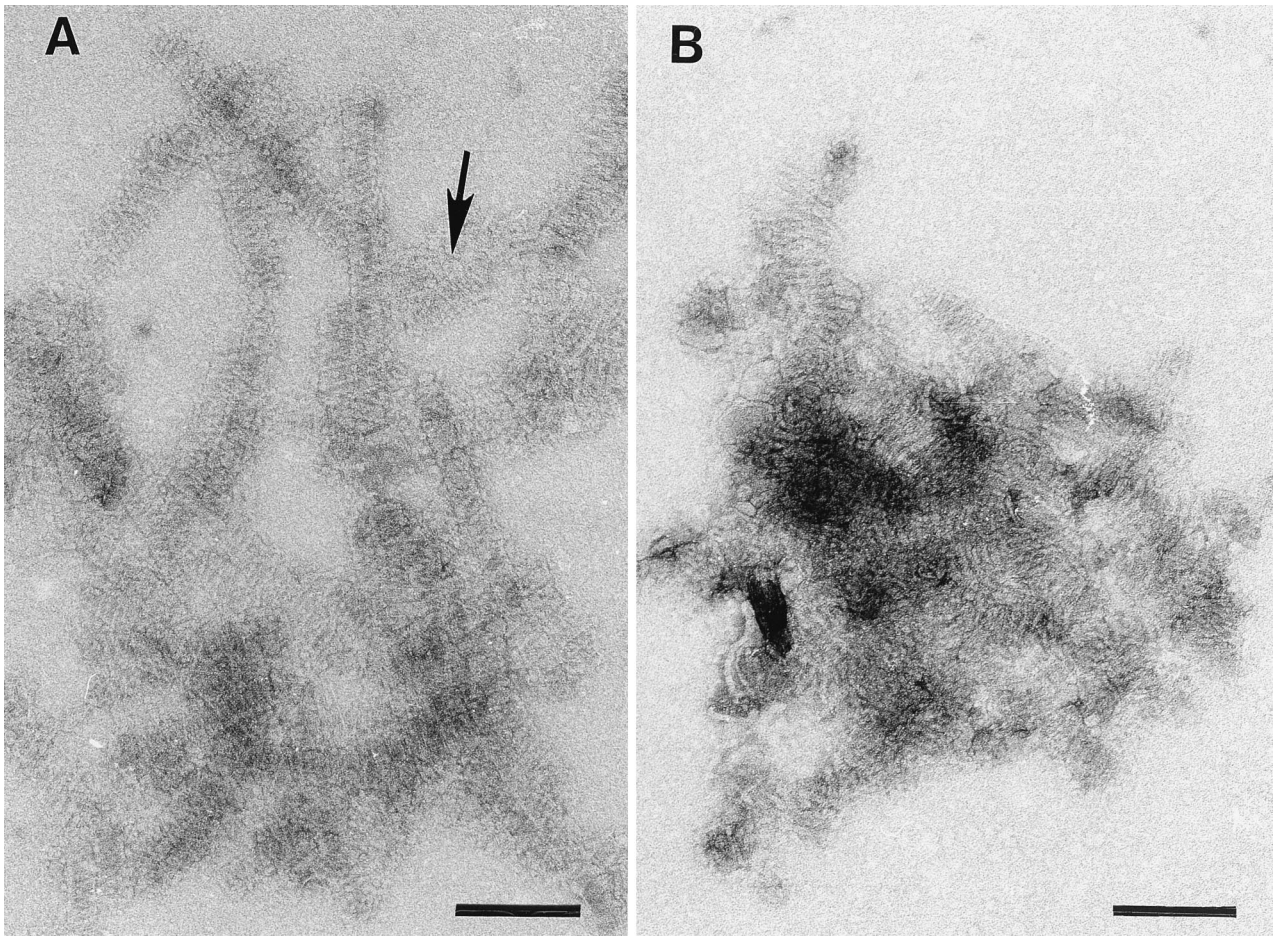

FIG. 8. Negative stains of spinae treated with 1 M NaOH at 100°C for 1 h (A) and 5 h (B). The arrow indicates dissociated spinae. Bar, 200 nm.

versibly dissociated to a large amorphous mass with little evidence of substructure.

On the basis of the observations presented in this study, the resistance of the spinae of strain JSB1 bears some resemblance to that of other resilient bacterial structures such as type I pili of *Escherichia coli* (16); the sheaths of *Methanothrix concilii* GP6 (17), *Methanospirillum hungatei* (3, 17, 24), and *Leptothrix discophora* (11); and the spore coat of *Bacillus subtilis* (13). The dissociation of these resistant structures required a combination of techniques, e.g., low or high pH, elevated temperature, detergents, organic solvents, and reducing agents. Such an approach is being used in combination with SDS-polyacrylamide gel electrophoresis with the spinae of strain JSB1 to identify the component macromolecule(s).

It is possible that the chemical resistance of the spinae of strain JSB1 reflects their function. Many suggestions have been proposed for the function of bacterial spinae. Spinae may serve a general function of providing an increased cell surface area useful for absorption of nutrients (18). It was also suggested (18) that spinae may act as buoyancy structures to maintain the cells at an optimum water depth in situ. Easterbrook and Rao (9), however, found that nonspinate marine pseudomonad D71 cells differed only slightly in buoyant density under natural simulations and did not occupy a preferential growth region in tube cultures. This possible buoyancy function should be reevaluated under conditions that better mimic in vivo environmental factors. Spinae could provide bacterial cells with the potential for cellular association. Spinate *Chlorobium* cells are often found in aggregates. Recently, Bayer and Easterbrook (1) examined cultures of strain D71 by SEM and TEM. Some cells were connected by spinae over a distance of up to 7 μm, producing cell clusters. This three-dimensional array of interconnected bacteria may permit exchange of cell-to-cell signals (1). Spinae have also been considered antipredation devices (10). As part of a study on predation of eubacteria by *Bdellovibrio bacteriovorus* and protozoa (15), we have assayed the ingestion of strain JSB1 by *Tetrahymena thermophila*. Some spinate cells were ingested into food vacuoles, but the rate of ingestion was lower than that with unspined bacteria (15a). These results are in agreement with other observations (10) that unspined bacteria were preferred to spined bacteria by various protozoa. In our grazing studies, we used a single bacterial species as the food source. In nature, a mixed population of bacteria, at lower cell densities, is the available food source, and spinae may have a greater impact as antipredation structures under these natural conditions. The clusters and chains of *Chlorobium* cells may also hinder ingestion, since monodisperse cells are ingested more efficiently.

ACKNOWLEDGMENTS

We are grateful to N. Pfennig for providing *Chlorobium* cultures and to R. G. E. Murray for helpful discussions. The technical assistance of Dianne Moyles and Steve Smith with electron microscopy, Lori Gra-

ham with freeze-substitution, and Anuradha Saxena with the biochemical studies is greatly appreciated.

This work was supported by a grant from the Medical Research Council of Canada to S.F.K. and from the Natural Sciences and Engineering Research Council of Canada (NSERC) to T.J.B. Some TEM and the freeze-substitution was performed at the NSERC Guelph Regional STEM Facility at the University of Guelph, which is partially supported by a NSERC infrastructure grant to T.J.B. The SEM was performed at the Surface Science Western Facility at the University of Western Ontario. J.S.B. was a recipient of a University of Western Ontario graduate scholarship.

REFERENCES

1. **Bayer, M. E., and K. B. Easterbrook.** 1991. Tubular spinae are long-distance connectors between bacteria. J. Gen. Microbiol. **137:**1081–1086.
2. **Beveridge, T. J., T. J. Popkin, and R. M. Cole.** 1994. Electron microscopy, p. 53–54. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
3. **Beveridge, T. J., M. Stewart, R. J. Doyle, and G. D. Sprott.** 1985. Unusual stability of the *Methanospirillum hungatei* sheath. J. Bacteriol. **162:**728–737.
4. **Biebl, H., and N. Pfennig.** 1978. Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate-reducing bacteria. Arch. Microbiol. **117:**9–16.
5. **Brooke, J. S., J. B. Thompson, T. J. Beveridge, and S. F. Koval.** 1992. Frequency and structure of spinae on *Chlorobium* spp. Arch. Microbiol. **157:**319–322.
6. **Cohen-Bazire, G., N. Pfennig, and R. Kunisawa.** 1964. The fine structure of green bacteria. J. Cell Biol. **22:**207–225.
7. **Easterbrook, K. B.** 1989. Spinate bacteria, p. 1991–1993. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.
8. **Easterbrook, K. B., and R. W. Coombs.** 1976. Spinin: the subunit protein of bacterial spinae. Can. J. Microbiol. **22:**438–440.
9. **Easterbrook, K. B., and D. V. S. Rao.** 1984. Conical spinae associated with a picoplanktonic procaryote. Can. J. Microbiol. **30:**716–718.
10. **Easterbrook, K. B., and S. Sperker.** 1982. Physiological controls of bacterial spinae production in complex medium and their value as indicators of spina function. Can. J. Microbiol. **28:**130–136.
11. **Emerson, D., and W. C. Ghiorse.** 1993. Role of disulfide bonds in maintaining the structural integrity of the sheath of *Leptothrix discophora* SP-6. J. Bacteriol. **175:**7819–7827.
12. **Graham, L. L., and T. J. Beveridge.** 1990. Effect of chemical fixatives on accurate preservation of *Escherichia coli* and *Bacillus subtilis* structure in cells prepared by freeze-substitution. J. Bacteriol. **172:**2150–2159.
13. **Hiragi, Y.** 1972. Physical, chemical and morphological studies of the spore coat of *Bacillus subtilis*. J. Gen. Microbiol. **72:**87–99.
14. **Kondratieva, E. N., N. Pfennig, and H. G. Trüper.** 1992. The phototrophic prokaryotes, p. 312–330. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.
15. **Koval, S. F.** 1993. Predation on bacteria possessing S-layers, p. 85–92. *In* T. J. Beveridge and S. F. Koval (ed.), Advances in bacterial paracrystalline surface layers. Plenum Publishing Corp., New York.

15a. **Koval, S. F.** Unpublished data.

16. **McMichael, J. C., and J. T. Ou.** 1979. Structure of common pili from *Escherichia coli*. J. Bacteriol. **138:**969–975.
17. **Patel, G. B., G. D. Sprott, R. W. Humphrey, and T. J. Beveridge.** 1986. Comparative analyses of the sheath structures of *Methanothrix concilii* GP6 and *Methanospirillum hungatei* GP1 and JF1. Can. J. Microbiol. **32:**623–631.
18. **Perkins, F. O., L. W. Haas, D. E. Phillips, and K. L. Webb.** 1981. Ultrastructure of a marine *Synechococcus* possessing spinae. Can. J. Microbiol. **27:**318–329.
19. **Peterson, G. L.** 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal. Biochem. **100:**201–220.
20. **Pfennig, N.** 1989. Green bacteria, p. 1682–1687. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.
21. **Pfennig, N.** 1989. Personal communication.
22. **Pfennig, N.** 1992. Personal communication.
23. **Pfennig, N., and H. Trüper.** 1992. The family Chromatiaceae, p. 3200–3221. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.
24. **Sprott, G. D., T. J. Beveridge, G. B. Patel, and G. Ferrante.** 1986. Sheath disassembly in *Methanospirillum hungatei* GP1. Can. J. Microbiol. **32:**847–854.
25. **Trüper, H. G., and N. Pfennig.** 1992. The family Chlorobiaceae, p. 3583–3592. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.