Induction of Spheroplasts in *Capnocytophaga ochracea*

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A gentle technique for preparing spheroplasts of *Capnocytophaga ochracea* strain 25 is described. Cells in the exponential phase were washed with 1.0 M-NaCl, agitated in 1.0 M-NaCl for 2 h at 30 °C and exposed to lysozyme in a Tris/salts buffer, pH 7.0. This procedure resulted in 98% spheroplast formation with complete removal of the peptidoglycan layer as detected by both phase-contrast and electron microscopy in combination with chemical analysis.

INTRODUCTION

Capnocytophaga ochracea is a member of the newly classified genus of non-fruiting, gliding, capnoid, Gram-negative, rod-shaped bacteria (Leadbetter *et al.*, 1979). The members of this genus are found in the gingival sulci and supragingival plaque of healthy human mouths (Leadbetter *et al.*, 1979; Socransky *et al.*, 1979; Holt, 1982). They are the predominant cultivatable Gram-negative micro-organisms involved in juvenile periodontitis (Bick *et al.*, 1981; Celesk *et al.*, 1979; Newman *et al.*, 1976; Newman & Socransky, 1977; Slots, 1976; Newman, 1979; Hammond & Stevens, 1982). In addition *Capnocytophaga* strains have been isolated from periodontal abscesses (Newman & Sims, 1979) and from the gingival pockets of patients with advancing periodontitis (Slots, 1977; Van Palenstein Helderman, 1981). There is also evidence that these micro-organisms cause neutrophil abnormalities (Shurin *et al.*, 1979), promote diminished neutrophil chemotaxis (Lindhe & Socransky, 1979) and inhibit human fibroblast cell proliferation (Stevens *et al.*, 1980). There have been only a few studies investigating the cellular structure of *Capnocytophaga* (Holt *et al.*, 1979; Poirier *et al.*, 1979; Poirier & Holt, 1983). These studies examined whole cells, spheroplasts and cell wall fragments by electron microscopy, alone or in combination with sonication and osmotic shock, and have shown the cells to have a typical Gram-negative morphology.

This investigation was undertaken to develop a gentle procedure for preparing spheroplasts of *C. ochracea* strain 25 and utilizing phase-contrast and electron microscopy in combination with chemical analysis to determine the results of each step.

METHODS

Bacteria, growth medium and cultural conditions. *C. ochracea* strain 25, a gift from S. S. Socransky, Forsythe Dental Center, Boston, Mass., USA, was initially cultivated anaerobically (5% CO₂, 95% N₂) on Trypticase soy agar (BBL) supplemented with 5% (v/v) defibrinated sheep blood at 37 °C. Then it was cultivated anaerobically at 37 °C in Trypticase soy broth (BBL) supplemented with 1% (w/v) yeast extract (Difco) and 0.1% NaHCO₃ for 17 h and harvested. Bacteria were stored as freeze-dried stocks at -50 °C. Culture purity was judged on the basis of Gram-staining, morphology, and aerobic incubation without 10% CO₂ of duplicate cultures plated on Trypticase soy agar supplemented with 5% defibrinated sheep blood to rule out facultative anaerobic bacterial contamination.

Abbreviation: KDO, 2-keto-3-deoxyoctanoic acid.

Preparation of spheroplasts. Cells were harvested in the exponential phase by centrifugation at 8000 *g* at 4 °C, washed three times by suspension in 1 M-NaCl in volumes equal to that of the original culture and centrifuged at 8000 *g* at 4 °C. The cells were then resuspended in 1 M-NaCl in volumes equal to one-half that of the original culture, and incubated with agitation for 2 h at 30 °C. Following incubation the cells were centrifuged at 8000 *g* at 4 °C, resuspended in a medium consisting of 0.3 M-NaCl, 0.05 M-MgSO₄, 0.01 M-KCl, 0.01 M-Tris and 200 µg lysozyme (egg albumin; Sigma) at a volume equal to one-half that of the culture volume and incubated with agitation for 20 h at 30 °C. Changes in gross morphology were followed by phase-contrast microscopy. At the end of each treatment, samples of the centrifuged cells were removed for fixation and embedding.

Preparation of extracted cellular material. Supernates from each of the three sequential treatments involved in the preparation of spheroplasts were centrifuged at 16000 *g* for 10 min at 5 °C to remove any remaining whole cells. Each supernate was then concentrated, exhaustively dialysed and centrifuged at 135000 *g* for 2 h at 5 °C. Each of the resulting pellets was removed for fixation and embedding and freeze-dried for chemical analysis.

Chemical analysis. Extracted cellular materials, spheroplasts and whole cells were analysed for 2-keto-3-deoxyoctanoic acid (KDO) by the method of Karkhanis & Zeltner (1978), for muramic acid by the method of Hadzija (1974) and for protein by the Markwell *et al.* (1978) modification of the Lowry method. Prior to analysis, whole cells were disrupted by sonication at 10 to 15 MHz (Biosonik 111, Bronwill Scientific, Division of Will Scientific, Inc., Rochester, NY, USA). The cell suspension was held at 5 °C and sonicated for periods of 10 s with a 3 min cooling period between each treatment. Sonication was stopped when >90% of cells were ruptured as observed by phase-contrast microscopy. The sonicated cells were then freeze-dried.

Preparation for electron microscopy. Bacterial preparations were sedimented by centrifugation and fixed by covering the pellet with 2% (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.3, at room temperature. The pellets were gently broken into pieces of approximately 1 mm diameter using a wooden spatula. The pieces were allowed to settle, the fixative replaced by fresh fixative, and the specimens allowed to stand at room temperature 1 h before being stored at 5 °C. Specimens were then washed three times in 0.1 M-phosphate buffer (pH 7.3) and postfixed for 1 h in 2% (w/v) OsO₄ in phosphate buffer at room temperature. After washing several times in distilled water, specimens were dehydrated in a graded ethanol series, and embedded in Epon. Thin sections were cut on an LKB Ultratome III, and poststained with uranyl acetate and Sato's triple lead stain (Sato, 1968). Sections were examined on a Hitachi HU-11E transmission electron microscope at 75 kV.

RESULTS

The normal cellular morphology of *C. ochracea* strain 25 in the exponential phase of growth prior to any treatment is shown in Fig. 1. The rod-shaped cells showed a double-tracked outer membrane, a thick electron-transparent peptidoglycan layer and a double-tracked cytoplasmic membrane. The outer membrane was closely adjacent to the other components of the cell envelope. The peptidoglycan was intact, maintaining the rod shape. The cytoplasm was dense, maintaining its integrity.

After being washed with NaCl, the cells, still maintained their normal structure although the surfaces showed some wrinkling, indicating that the outer membrane was becoming less organized and taut (Fig. 2*a*). Cell envelope materials were lost as indicated by the detection of muramic acid, KDO and protein in the washings (Table 1).

After 2 h exposure to NaCl with agitation, the cells showed varying losses of outer membrane fragments. In some cells, the outer double track was not only separated from the other layers of the cell envelope, but ballooned out (Fig. 2*b*). Chemical analysis of extractable cellular materials in the suspending medium showed the presence of muramic acid and KDO (Table 1). Electron microscopy of the pellet from the suspending medium showed double-tracked fragments of the outer membrane (Fig. 3).

Phase contrast microscopy of cells following exposure to NaCl showed no spheroplast formation (Fig. 4*a*). It was necessary to expose the cells to Tris/salt/lysozyme solution for 20 h in order to obtain 98% spheroplast formation (Fig. 4*b*). Electron microscopy of the spheroplasts showed the outer double track to be separated from the remaining cell envelope components and ballooned out (Fig. 5). Also some inner and outer double-tracked fragments appeared to be rounding up on themselves. Although the inner double track remained intact, the cytoplasm appeared to have become less dense. Chemical analysis of the extractable cellular material in the Tris/salts/lysozyme solution revealed only the presence of muramic acid (Table 1). The amount of muramic acid in addition to that released earlier in the procedure approximated that found in

Fig. 1. Electron micrograph of exponential phase cells of *C. ochracea* grown in Trypticase soy broth supplemented with 1% yeast extract and 0.1% NaHCO_3 . Bar, 0.5 μm .

Table 1. *Chemical analysis of fractions from Capnocytophaga ochracea obtained during conversion to spheroplasts*

The results are averages of three independent experiments, and are shown as means $\pm$ SEM.
Amount (μg) of:

Fraction	Muramic acid	KDO	Protein
Supernates following NaCl washes	4.0 ± 0.54	22 ± 0.95	20 ± 1.77
Supernate following 2 h NaCl agitation	3.0 ± 0.53	22 ± 0.95	trace
Supernate following Tris/salts/lysozyme	8.5 ± 0.72	0	trace
Spheroplasts	0	24 ± 1.05	124 ± 2.24
Untreated whole cells	16.5 ± 0.68	65 ± 1.35	144 ± 2.85

whole cells. The equivalence between the amount released and the amount found in whole cells was supported by the absence of muramic acid in spheroplasts (Table 1). The total amounts of muramic acid, KDO and protein found for the whole cells taken through the spheroplast preparation technique were very similar to the values found for a comparable amount of untreated whole cells (Table 1). Also there was minimal loss of cells during the various treatments since the number of spheroplasts approximated to the initial cell numbers (not shown).

Two alternative procedures for preparing spheroplasts were also tested. In one, washing the cells three times with 0.5 M-sucrose followed by a 2 h exposure to 0.5 M-sucrose solution containing 0.01 M-EDTA and a subsequent 20 h exposure to the Tris/salts/lysozyme solution produced no spheroplasts. The other procedure, though, in which the cells were washed three times with 1.0 M-NaCl followed by a 2 h exposure to the sucrose/EDTA solution and subsequent exposure to the Tris/salts/lysozyme solution produced 30–40% spheroplasts.

Fig. 2. Electron micrographs of *C. ochracea*. (a) Following washings in 1.0 M-NaCl. (b) After exposure to 1.0 M-NaCl for 2 h with agitation, showing a ballooning out of the outer double-track membrane. Bars, 0.5 μ m.

Fig. 3. Electron micrograph of the fragments of the cell wall material present in the supernatant after 2 h exposure of *C. ochracea* to 1.0 M-NaCl with agitation. Bar, 0.5 μ m.

Fig. 4. Phase-contrast micrographs of *C. ochracea* prior to (a) and after (b) exposure to Tris/salts/lysozyme solution for 20 h. Nomarski interference contrast was employed for (b). Bar, 5 μ m (magnification the same in a and b).

Fig. 5. Electron micrograph of *C. ochracea* after exposure to Tris/salts/lysozyme solution for 20 h. Bar, 0.5 μ m.

DISCUSSION

The purpose of the present work was to devise a gentle procedure for preparing spheroplasts of *C. ochracea* which would lead to the separation of the various layers of the cell envelope.

Our results show that cells washed three times with 1.0 M-NaCl and then agitated for 2 h in 1.0 M-NaCl maintained their rod shape, but that these treatments had a marked effect on the integrity of the cell envelope. The NaCl washes (particularly the first) caused the release of protein, possibly from the periplasmic space and/or from an outer periodic macromolecular monolayer or S-layer. A possible explanation for the marked effect of the NaCl washes and suspension on the cell envelope is an antagonistic effect between Na⁺ and Mg²⁺ ions, since the latter are known to stabilize Gram-negative bacteria.

In addition to the initial release of protein, the NaCl treatments caused substantial changes in the still rod-shaped cell structures, which closely resembled the mureinoplasts formed with the Gram-negative marine bacterium *Alteromonas haloplanktis* (Forsberg *et al.*, 1970*a*). Considerable outer membrane material was damaged and peeled off, being subsequently found in the supernate. Even though there was some release of muramic acid during the NaCl treatments, the cells retained their rod shape and the total concentration of cells was equal to that at the beginning of the experiment.

After the Tris/salts/lysozyme treatment, at least 98% of the rod-shaped structures were converted to spheroplasts, and the remaining muramic acid was removed at this stage as expected. Again, there was little or no reduction in cell number during this conversion.

MacLeod and co-workers found that by manipulating the environment it was possible to gently remove the various cell envelope layers of *A. haloplanktis* (Forsberg *et al.*, 1970*a, b*; Costerton *et al.*, 1967; summarized by Laddaga & MacLeod, 1983). However, in order to obtain complete conversion of the rod-shaped mureinoplasts to spheres, the cells had to be suspended in sucrose rather than NaCl before the lysozyme step. When they used a procedure similar to ours (replacing the sucrose suspension with an NaCl suspension), all their cells retained the outer membrane and no spheres were formed.

When Poirier & Holt (1983) used an analogous procedure to MacLeod and co-workers, involving suspension of four strains of *Capnocytophaga* in Tris, sucrose, EDTA and lysozyme, the conversion rate of the rod-shaped cells to spheroplasts ranged from 73.8% to 100%. The spheroplast preparation procedure reported here provides an alternative method by which the *Capnocytophaga* cell envelope can be gently separated into the component parts. Since *Capnocytophaga* is a principal causative agent of periodontal disease, the ultimate extension of this work will be the further characterization and purification of the various cell envelope fractions and determination of the immunological importance of these fractions in the overall disease process.

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REFERENCES

- BICK, P. H., CARPENTER, A. B., HOLDEMAN, L. V., MILLER, G. A., RANNEY, R. R., POLCANIS, K. G. & TEW, J. G. (1981). Polyclonal B-cell activation induced by extracts of Gram-negative bacteria isolated from periodontally diseased sites. *Infection and Immunity* **34**, 43-49.
- CELESK, R. A., MCCABE, R. M. & LONDON, J. (1979). Colonization of the cementum surface of teeth by oral Gram-negative bacteria. *Infection and Immunity* **26**, 15-18.
- COSTERTON, J. W., FORSBERG, C., MITULA, T. I., BUCKMIRE, F. L. A. & MACLEOD, R. A. (1967). Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, spheroplasts and related forms from a Gram-negative marine bacterium. *Journal of Bacteriology* **94**, 1764-1777.
- FORSBERG, C. W., COSTERTON, J. W. & MACLEOD, R. A. (1970*a*). Separation and localization of cell wall layers of a Gram-negative bacterium. *Journal of Bacteriology* **104**, 1338-1353.
- FORSBERG, C. W., COSTERTON, J. W. & MACLEOD, R. A. (1970*b*). Quantitation, chemical characteristics, and ultrastructure of the three outer cell wall layers of a Gram-negative bacterium. *Journal of Bacteriology* **104**, 1354-1368.
- HADZIJIA, O. (1974). A simple method for the quantita-

- tive determination of muramic acid. *Analytical Biochemistry* **60**, 512–517.
- HAMMOND, B. F. & STEVENS, R. H. (1982). *Capnocytophaga* and *Actinobacillus actinomycetemcomitans*: occurrence and pathogenic potential in juvenile periodontitis. In *Host-Parasite Interactions in Periodontal Diseases*, pp. 46–61. Edited by R. J. Genco & S. E. Mergenhagen. Washington, DC: American Society for Microbiology.
- HOLT, S. C. (1982). Bacterial surface structures and their role in periodontal disease. In *Host-Parasite Interactions in Periodontal Diseases*, pp. 139–150. Edited by R. J. Genco & S. E. Mergenhagen. Washington, DC: American Society for Microbiology.
- HOLT, S. C., LEADBETTER, E. R. & SOCRANSKY, S. S. (1979). *Capnocytophaga*: new genus of Gram-negative gliding bacteria. II. Morphology and ultrastructure. *Archives of Microbiology* **122**, 17–27.
- KARKHANIS, Y. D. & ZELTNER, J. Y. (1978). A new and improved microassay to determine KDO in LPS of Gram-negative bacteria. *Analytical Biochemistry* **85**, 595–601.
- LADDAGA, R. A. & MACLEOD, R. A. (1983). Factors affecting the release of outer membrane from a Gram-negative marine bacterium. *Canadian Journal of Microbiology* **29**, 659–663.
- LEADBETTER, E. R., HOLT, S. C. & SOCRANSKY, S. S. (1979). *Capnocytophaga*: a new genus of Gram-negative gliding bacteria. I. General characteristics, taxonomic considerations and significance. *Archives of Microbiology* **122**, 9–16.
- LINDHE, J. & SOCRANSKY, S. S. (1979). Chemotaxis and vascular permeability produced by human periodontopathic bacteria. *Journal of Periodontal Research* **14**, 138–146.
- MARKWELL, M. A., HAAS, S. M., BIEBER, L. L. & TOBERT, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* **87**, 207–210.
- NEWMAN, M. G. (1979). The role of microorganisms in the etiology of gingival and periodontal disease. In *Glickman's Clinical Periodontology*, 5th edn, pp. 374–405. Edited by F. A. Carranza. Philadelphia: W. B. Saunders Co.
- NEWMAN, M. G. & SIMS, T. N. (1979). The predominant cultivable microbiota of the periodontal abscess. *Journal of Periodontology* **50**, 350–354.
- NEWMAN, M. G. & SOCRANSKY, S. S. (1977). Predominant cultivable microbiota in periodontosis. *Journal of Periodontal Research* **12**, 120–128.
- NEWMAN, M. G., SOCRANSKY, S. S., SAVITT, E. D., PROPOS, D. & CRAWFORD, A. (1976). Studies on the microbiology of periodontosis. *Journal of Periodontal Research* **12**, 120–128.
- POIRIER, T. P. & HOLT, S. C. (1983). Acid and alkaline phosphatases of *Capnocytophaga* species. III. The relationship of the enzymes to the cell wall. *Canadian Journal of Microbiology* **29**, 1369–1381.
- POIRIER, T. P., TONELLI, S. J. & HOLT, S. C. (1979). Ultrastructure of gliding bacteria: scanning electron microscopy of *Capnocytophaga sputigena*, *Capnocytophaga gingivalis* and *Capnocytophaga ochracea*. *Infection and Immunity* **26**, 1146–1158.
- SATO, T. (1968). A modified method for lead staining of thin sections. *Journal of Electron Microscopy* **17**, 158.
- SHURIN, S. B., SOCRANSKY, S. S., SWEENEY, E. & STOSSEL, T. P. (1979). A neutrophil disorder induced by *Capnocytophaga*, a dental microorganism. *New England Journal of Medicine* **301**, 849–854.
- SLOTS, J. (1976). The predominant cultivable organisms in juvenile periodontitis. *Scandinavian Journal of Dental Research* **84**, 1–10.
- SLOTS, J. (1977). The predominant cultivable microflora of advanced periodontitis. *Scandinavian Journal of Dental Research* **85**, 14–21.
- SOCRANSKY, S. S., HOLT, S. C., LEADBETTER, E. R., TANNER, A. C. R. & HAMMOND, B. F. (1979). *Capnocytophaga*: new genus of Gram-negative gliding bacteria. III. Physiological characterization. *Archives of Microbiology* **122**, 29–33.
- STEVENS, R. H., SELA, M. N., SHAPIRA, J. & HAMMOND, B. F. (1980). Detection of fibroblast proliferation inhibitory factor from *Capnocytophaga sputigena*. *Infection and Immunity* **27**, 271–275.
- VAN PALENSTEIN HELDERMAN, W. H. (1981). Microbial etiology of periodontal disease. *Journal of Clinical Periodontology* **8**, 261–280.