JOURNAL OF BACTERIOLOGY, Nov. 1995, p. 6176–6183
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 21

# A Periplasm in *Bacillus subtilis*

RAFAEL MERCHANTE, HAROLD M. POOLEY, AND DIMITRI KARAMATA*

*Institut de Génétique et de Biologie Microbiennes, 1005 Lausanne, Switzerland*

Received 12 June 1995/Accepted 24 August 1995

**The possibility of there being a periplasm in *Bacillus subtilis*, in the distinct cell compartment bounded by the cytoplasmic membrane and the thick cell wall, has been investigated quantitatively and qualitatively. Cytoplasmic, membrane, and protoplast supernatant fractions were obtained from protoplasts which were prepared isotonically from cells grown under phosphate limitation. The contents of the protoplast supernatant fraction represent an operational definition of the periplasm. In addition, this cell fraction includes cell wall-bound proteins, exoproteins in transit, and contaminating cytoplasmic proteins arising through leakage from, or lysis of a fraction of, protoplasts. The latter, measured by assay of enzyme markers and by radiolabeled RNA and protein, was found to represent 7.6% of total cell protein, yielding a mean of 9.8% ± 4.8% for *B. subtilis* 168 protein considered periplasmic. Qualitatively, after subjection of all cell fractions to polyacrylamide gel electrophoresis, RNase and DNase, zymographs revealed that (i) each cell fraction had a unique profile of nucleases and (ii) multiple species and a major fraction of both nucleases were concentrated in the periplasm. We conclude that the operationally defined periplasmic fraction corresponds closely, both quantitatively and qualitatively, to the contents of the periplasm of *Escherichia coli*. We discuss evidence that the maintenance of the components of this surface compartment in *B. subtilis* is compatible with the thick negatively charged cell wall acting as an external permeability barrier.**

The existence in the envelopes of gram-negative organisms of a distinct cell compartment, the periplasm, is evidenced by electron micrographs revealing a clear separation between the inner and the outer membranes and by the release, through selective damage to the outer membrane barrier, of a distinct population of proteins and enzymes. Many of the latter are of a scavenging kind, including multiple phosphatases and nucleases (3). Estimates for the protein content of the periplasm of *Escherichia coli* range from 4% (28) to 16% (1) of total cell protein. A condition for the existence of this outer cell compartment is the presence of two permeability barriers, which, in the case of the envelopes of gram-negative organisms, are a distinctive outer membrane and the ubiquitous cytoplasmic membrane. The selective outward as well as inward traffic through the outer barrier is achieved through specialized structures such as porins and various protein export factors (20) identified in a wide range of bacterial species, including *Erwinia*, *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, and *Neisseria* species.

For gram-positive organisms, whose surfaces differ both morphologically and chemically from those of gram-negative bacteria, the question of the existence of a periplasm has generally been avoided (4). It has been widely accepted that the cytoplasmic membrane represents the main, if not the only, barrier to traffic in and out of a gram-positive cell (6, 27, 38) and that, having translocated this membrane, proteins of up to about 60 kDa (47) can freely diffuse into the surrounding medium. Support for a relatively high porosity of the cell walls of gram-positive organisms has been seen in the capacity of many of them to export large amounts of certain proteins (32, 38). An S-layer, which is a regular array of proteins on the external surface of the cell wall present in many gram-positive organisms, can act as a molecular sieve (35, 36). There is no evidence for such a protein layer being present in the generally used laboratory strains of *Bacillus subtilis*.

Nevertheless, despite the absence of an observable periplasmic space and an outer membrane, certain authors proposed the existence of a functional periplasm in gram-positive bacteria (4, 15). Previous studies with *B. subtilis* as well as related bacilli provided evidence (26) in favor of the latter point of view. In *B. subtilis*, an endoribonuclease (5), a β-glucanase (45), and a nucleotide diphosphate sugar hydrolase (26) were released into the soluble fraction during isotonic formation of protoplasts. An alkaline phosphatase of *Bacillus licheniformis* has a similar location (10, 13). Interestingly, in *E. coli*, several of these activities are specifically located in the periplasm.

In this communication, we describe an operational definition of a protein fraction that would potentially comprise the periplasm of the gram-positive organism *B. subtilis*. We provide quantitative estimates of this protein fraction, as well as the identification of some of its specific enzyme components. Finally, we discuss the question of the permeability of the thick cell wall in gram-positive organisms.

## MATERIALS AND METHODS

**Strains.** *B. subtilis* 168 *trpC2* and W23 were used.

**Media and growth conditions.** The defined liquid medium tris-low-phosphate (TLP), modified from that of Mauck and Glaser (26), contained the following: ammonium sulfate (9 mM), sodium citrate (0.75 mM), Tris-HCl (100 mM), and sodium glutamate (54.4 mM). The final pH was 7.6. To 100 ml of medium 0.1 ml of a sterile salts solution was added. The latter, slightly modified from that described by Pollock (31), contained magnesium sulfate (2 M), ferrous sulfate (3.6 mM), zinc sulfate (3.5 mM), manganese sulfate (0.5 mM), cupric sulfate (40 μM), and potassium dichromate (6.8 μM). Histidine, leucine, arginine, isoleucine, phenylalanine, tyrosine, proline, alanine, methionine, tryptophan, threonine, lysine, valine, serine, glycine, and cysteine were each added at a final concentration of 20 μg $ml^{-1}$ together with glucose (27.7 mM) and dipotassium phosphate at concentrations indicated below.

Strains maintained as spore preparations were streaked out on Luria agar medium.

Overnight (14- to 16-h) cultures, grown at 26°C, were obtained by inoculating TLP containing 1 mM $K_2HPO_4$ with isolated colonies from fresh LA plates. For growth under phosphate-limited conditions, a 10- to 50-fold dilution of the overnight cultures, with a nephelometric density (ND) generally in the range of 100 to 500, was made to inoculate TLP containing 0.22 or 0.44 mM phosphate.

* Corresponding author. Mailing address: Institut de Génétique et de Biologie Microbiennes, Rue César-Roux 19, CH-1005 Lausanne, Switzerland. Phone: (41.21)3206075. Fax: (41.21)3206078. Electronic mail address: dkaramat@igbm.unil.ch.

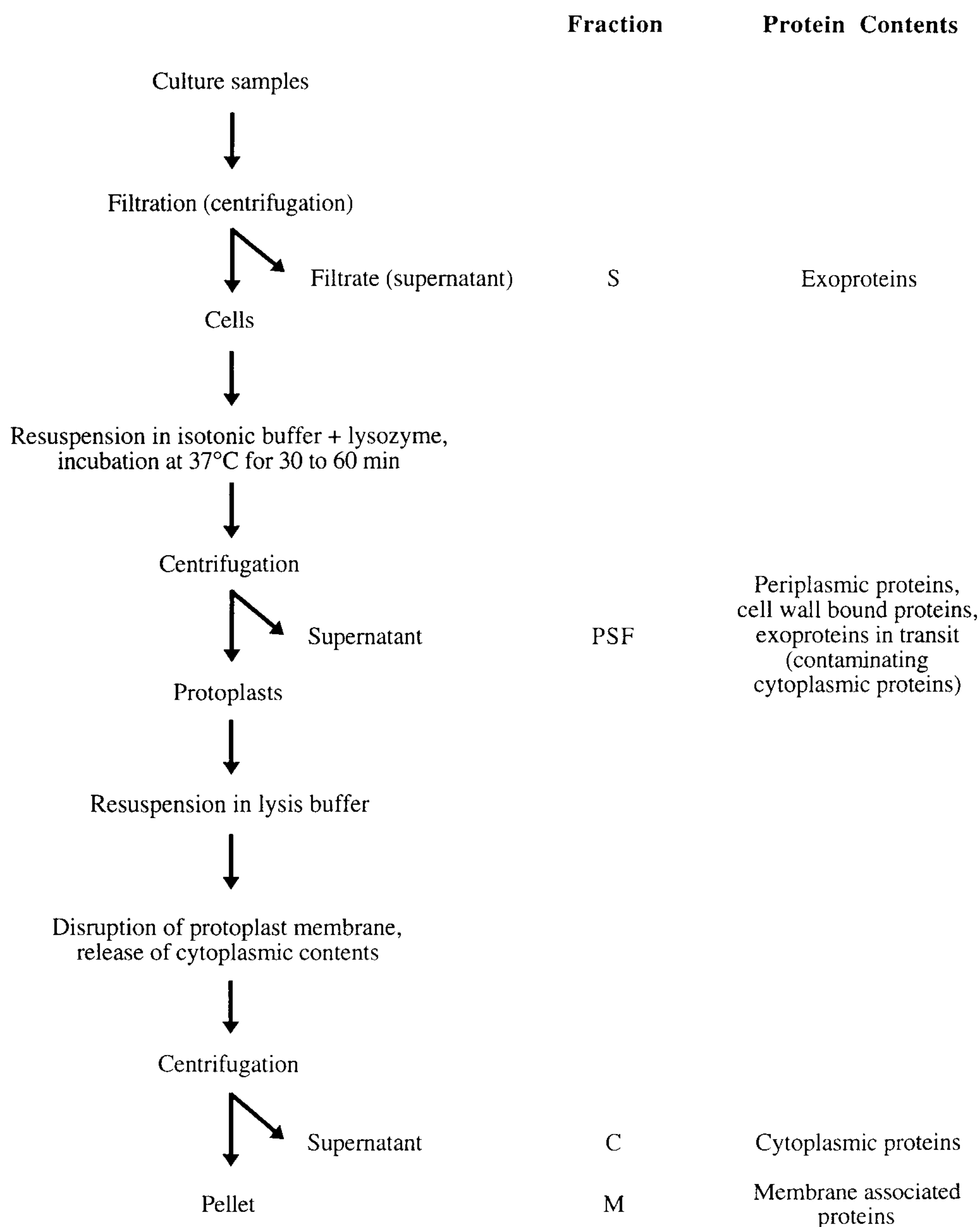


FIG. 1. Schematic representation of the cell fractionation procedure. S, C, and M represent culture supernatant, cytoplasm, and membrane.

Cultures (15 to 25 ml) in glass tubes (internal diameter, 17 mm), aerated by bubbling, were grown at 37°C, and the cell mass was monitored by ND measured on a Unigalvo (Corning EEL) nephelometer. For an exponentially growing culture, an ND reading of 100 corresponds to 0.11 mg of bacteria (dry weight) $ml^{-1}$.

**Cell fractionation.** The preparation of various cell fractions is shown schematically in Fig. 1. From early exponential to late stationary phase, 25-ml culture aliquots were withdrawn at given times. Cells were harvested by filtration on a manifold, washed four times with 2 ml of TLP, and immediately resuspended in, usually, 0.5 ml of a freshly prepared isotonic buffer solution (TMS) containing Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (16 mM), and sucrose (66% or, occasionally, 33% [wt/vol]). Lysozyme (100- to 200-μg $ml^{-1}$ final concentration) and phenylmethylsulfonyl fluoride (0.1 mM final concentration) were added, and the suspension was incubated at 37°C. Usually after 30 to 60 min, more than 99% of the cells had been converted into protoplasts, as judged by phase-contrast microscopy. Protoplasts were pelleted (15 min, 21,000 × *g*), and the protoplast supernatant fraction (PSF) was carefully collected. Rarely, if signs of lysis were evident, such as increased viscosity or difficulty in obtaining a compact pellet, the sample was discarded. Protoplasts were lysed by resuspension in lysis buffer containing Tris-HCl (50 mM, pH 8.0) and $MgSO_4$ (5 mM), followed by repeated passage through a 1-ml micropipette tip. Occasionally, sonication was used to reduce the viscosity due to release of high-molecular-weight DNA. A subsequent centrifugation (1 h, 21,000 × *g*) allowed separation of cytoplasmic and membrane fractions. In some initial experiments, cells were harvested by centrifugation and, without a washing step, the pellet was immediately resuspended in TMS.

When cells were harvested by centrifugation, the S fraction, representing the culture medium, was filtered to remove any remaining cells. Proteins were precipitated with trichloroacetic acid (final concentration, 6% [wt/vol]) following addition of yeast RNA (50-μg $ml^{-1}$ final concentration) as a carrier. After 1 h at 0°C, precipitated proteins were recovered by centrifugation in a swing-out rotor (20 min, 13,000 × *g*). Alternatively, for in situ screening for nucleases after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below), trichloroacetic acid was not added and protein in fraction S was concentrated by ultrafiltration (Centricon 10 ultrafiltrator; Amicon). When the S fraction was screened for the presence of RNA, either lysozyme or bovine serum albumin (50-μg $ml^{-1}$ final concentration) was added as a carrier.

**Isocitrate dehydrogenase assay.** Formation of reduced NADPH was coupled

with dehydrogenase activity and monitored spectrophotometrically at $A_{340}$ as described previously (17). The reaction mixture contained 10 mM $MgCl_2$, 1 mM isocitrate, 0.4 mM NADP, potassium phosphate buffer (50 mM, pH 7.0), and 0.8 ml of the cell fraction (equivalent to 0.5 to 1 ml of culture). The reaction was initiated by the addition of the substrate, and the $A_{340}$ was monitored for 15 min at room temperature. One milliunit of isocitrate dehydrogenase (EC 1.1.1.42) activity is defined as that causing an increase in the $A_{340}$ of 0.001 per min. The background dehydrogenase activity, measured on incubation mixtures in the absence of added substrate, was subtracted for each cell fraction.

**Glucose 6-phosphate dehydrogenase assay.** The glucose 6-phosphate dehydrogenase (EC 1.1.1.43) assay (23) is based, as for isocitrate dehydrogenase, on the reduction of NADP to NADPH. The reaction mixture contained 10 mM $MgCl_2$, 1 mM glucose 6-phosphate, 0.4 mM NADP, Tris-HCl buffer (33.3 mM, pH 7.5), and an appropriate volume of each cell fraction (equivalent to 0.5 to 1 ml of culture). The reaction, carried out at room temperature (about 23°C) and initiated by the addition of the substrate, was checked by monitoring the $A_{340}$ for 15 min. One unit of activity is measured as increase of the $A_{340}$ of 0.001 per min. The background dehydrogenase activity, measured on incubation mixtures in the absence of added substrate, was subtracted for each cell fraction.

**RNase activity assay.** The procedure followed was essentially that described by Lopes et al. (24). The reaction mixture (0.3 ml) contained potassium phosphate buffer (30 mM, pH 7.0), 2 mM EDTA, and, as a substrate, [$^3$H]poly(A) (50 nCi; 17.5 to 63 Ci mmol$^{-1}$; Amersham) together with 32.5 μg of unlabeled poly(A) (Sigma). A cell fraction equivalent to 0.25 or 0.5 ml of culture was added. The reaction mixture was incubated at 37°C for 30 min. Unsolubilized [$^3$H]poly(A), precipitated during 15 min at 0°C following addition of 30 μl of 100% (wt/vol) perchloric acid and 3 μl of yeast RNA (50 mg ml$^{-1}$) as a carrier, was sedimented (10 min, 21,000 × *g*), and supernatants (200 μl) were transferred to a scintillation minivial containing 5 ml of Optifluor (Packard) scintillation cocktail for measurement of soluble radioactivity.

**Chemical determination of RNA content.** The RNA content of each fraction was measured by the orcinol-based method (9). RNA in cell extracts, precipitated with perchloric acid (0.25 N) for 30 min at 0°C, was centrifuged. The pellet, after being washed two or three times with 0.25 N perchloric acid and twice by resuspension in 0.5 N perchloric acid to remove any contaminating sugar, which interferes with the assay, was incubated for 15 min at 70°C to extract RNA. To 0.5 ml of the RNA-containing extract equal volumes of 6% (wt/vol) orcinol (dissolved in ethanol) and 0.1% (wt/vol) $FeCl_3$ dissolved in 37% (wt/vol) HCl were added, and samples were heated for 30 min at 90°C. Formation of the chromogen was monitored colorimetrically at $A_{665}$. Yeast RNA or ATP was used as an RNA standard.

**Protein assay based on incorporation of [$^3$H]leucine.** Cultures (25 ml) were grown in TLP containing limiting phosphate (0.44 mM), unlabeled leucine (30 μg ml$^{-1}$), and 0.1 μCi of [$^3$H]leucine (37 mCi mmol$^{-1}$; Amersham) ml$^{-1}$. At various times during growth, triplicate 1-ml aliquots were withdrawn. Cells were harvested by filtration on a manifold (Millipore), washed with 4 volumes (2 ml) of TLP containing a 10-fold-higher leucine concentration (200 μg ml$^{-1}$), and resuspended in 0.5 ml of TMS (60% sucrose). Apart from the fact that TLP was inoculated with filtered exponentially growing cells to avoid any possible carryover of RNase in the culture medium, PSF and the cytoplasmic and membrane fractions were obtained as described above and as shown in Fig. 1. Proteins present in the culture filtrate (S fraction) were precipitated with 6% trichloroacetic acid and centrifuged in a swing-out rotor as described above. To eliminate unincorporated soluble [$^3$H]leucine, the pellet was washed with 5% trichloroacetic acid (containing unlabeled leucine). To optimize counting efficiency for membrane samples, NaOH was added to a final concentration of 0.2 M and the mixture was incubated at 60°C for 1 h; incubation was followed by addition of the scintillation cocktail.

**Double labeling with [$^3$H]uracil and [$^{14}$C]leucine.** Double labeling of a given culture permitted direct comparison of protein and RNA contents in the same sample of each fraction. Cultures were supplemented with [$^3$H]uracil (5 μCi ml$^{-1}$), unlabeled uracil (400 μg ml$^{-1}$), [$^{14}$C]leucine (0.1 μCi ml$^{-1}$), and unlabeled leucine (30 μg ml$^{-1}$). Triplicate 1-ml samples were treated as described above for [$^3$H]leucine-labeled cultures.

**Detection of RNase and DNase activities in SDS-PAGE gels.** The method described by Spanos and Hübscher (42) was used. SDS-PAGE gels were prepared (22) with acrylamide concentrations of 8, 12, or, occasionally, 15%, depending on the molecular weights of the proteins of interest. 16S and 23S rRNA from *E. coli* (Boehringer) or herring sperm DNA (Sigma), at a final concentration of 10 μg ml$^{-1}$, was incorporated into the separation gel solution. For preparation of adequately concentrated extracts for SDS-PAGE, 25-ml aliquots of cultures, harvested at an appropriate cell density, were centrifuged (5 min, 10,000 × *g*) and cells were immediately resuspended in 0.5 ml of TMS (60% sucrose). Cell fractions were prepared as described above. Proteins in the S fraction were concentrated as described previously. After samples were boiled for 5 min, wells were loaded with samples of each cell fraction, containing the protein equivalent of 1 ml of culture, dissolved in a sample buffer lacking β-mercaptoethanol. After passage through the stacking gel, separation gels were run at 12 V cm$^{-1}$ for 6 h or at 3.5 V cm$^{-1}$ for 15 h. After an overnight washing in distilled water with agitation at 4°C, the gels were incubated in a solution containing Tris-HCl (50 mM, pH 7.5) and 1 mM EDTA solution (7 mM $MgCl_2$ replaced EDTA in DNase gels) at 37 or 47°C for periods ranging from 1 to 24 h. The gels were stained with ethidium bromide, and bands were visualized in a 254-nm UV transilluminator. Dark bands appearing against a bright background indicated areas of nucleic acid dissolution by separated nucleases.

**Detection and staining of heme proteins.** A standard method was followed (8, 19).

## RESULTS

**Operational definition of the periplasmic fraction.** One established method for the release of the periplasmic contents of *E. coli* involves lysozyme digestion of the peptidoglycan layer under osmotic buffering conditions (25). To obtain a fraction enriched for the contents of a hypothetical periplasm, this procedure was adapted for *B. subtilis* (Fig. 1).

To limit possible anaerobiosis and any consequent onset of autolysis prior to osmotic protection, cells were harvested rapidly by filtration and washed on the filter with complete medium. Occasionally, they were centrifuged and resuspended in the same buffer without a prior washing step. The cell supernatant (S fraction), containing the proteins secreted into the medium, was kept. The cells were resuspended in an isotonic buffer solution containing lysozyme. Following solubilization of the cell wall, protoplasts were spun down, and the PSF, which should contain any soluble periplasmic proteins, was recovered. Finally, protoplasts were lysed by resuspension in the lysis buffer and the lysate was partitioned by centrifugation (60 min, 21,000 × *g*) into the membrane pellet and the cytoplasm-containing supernatant.

In addition to putative periplasmic proteins, the PSF inevitably contains (i) the cell wall-bound proteins (7, 44); (ii) the proteins secreted across, and thus transiently present in, the cell wall; and (iii) contaminating proteins of cytoplasmic or membrane origin arising during cell fractionation (see below).

In the presence of 20 mM $Mg^{2+}$ and 50 mM Tris in the protoplasting medium, the bulk of lipoteichoic acid was expected to remain attached to the protoplast membrane (2, 18) and thus to cosediment with the protoplasts. Cytoplasmic membrane-derived so-called mesosomal vesicles, likely to be formed during the plasmolysis that accompanies osmotic upshift (12) produced by resuspension in hypertonic medium, are also expected to cosediment with the protoplasts (33, 37).

**Measurement of the extent of protoplast lysis during cell fractionation.** Inspection of protoplast preparations (see above [Fig. 1]) by phase-contrast microscopy revealed, among the majority of phase-dark protoplasts, a very minor fraction of phase-light ghosts, as well as possible membrane debris. Therefore, limited lysis and contamination of the PSF by cytoplasmic and, possibly, membrane material must have occurred.

To quantify this unwanted, but unavoidable, lysis inherent to the protoplast formation step, we assayed all cell fractions of exponentially growing cultures of strain W23 for several cytoplasmic markers. More than 90% of G6PDH was located in the cytoplasmic fraction, while, on the basis of three independent experiments, a mean of 8.1% ± 0.9% of total cell activity was found in the PSF. As expected, the culture medium S, obtained after the cells were filtered, was devoid of this activity, indicating an absence of whole-cell lysis. Assay of a second cytoplasmic marker enzyme, isocitrate dehydrogenase, employed as a marker for detecting cell lysis (17), gave an identical result, with a mean of 8.4% ± 2.9% in the PSF. In two independent experiments, assay for RNA, another cytoplasmic marker that has been used to detect cell lysis (11, 21), yielded a mean value of 6.4% ± 3.5% for the PSF, which is comparable to that found for the enzyme markers. The amounts of three separate cytoplasmic markers in the PSF—with a mean value of 7.6%—consistently point to the lysis, during protoplasting, of a small but significant fraction of the cell population.

TABLE 1. RNA and protein distributions among different cell fractions in *B. subtilis* 168[a]

| Substance | ND | % of total cell fraction[b] | | | | PΔ (%)[c] |
|---|---|---|---|---|---|---|
| | | S | PSF | C | M | |
| RNA | 142 | 0.5 | 7.8 | 67.7 | 24.0 | |
| | 270 | 0.9 | 4.8 | 59.1 | 35.1 | |
| | 451 | 1.2 | 8.1 | 49.1 | 41.8 | |
| Protein | 142 | 0.2 | 18.8 | 58.9 | 22.0 | 11.0 |
| | 270 | 0.3 | 18.7 | 55.8 | 24.9 | 13.9 |
| | 451 | 0.4 | 12.5 | 55.5 | 31.6 | 4.4 |

[a] Cultures were grown in TLP supplemented with [$^{14}$C]leucine (30 μg ml$^{-1}$; 0.1 μCi ml$^{-1}$) and [$^{3}$H]uracil (400 μg ml$^{-1}$; 5 μCi ml$^{-1}$). Samples taken at each ND were trichloroacetic acid precipitated and counted for $^{14}$C and $^{3}$H.

[b] S, PSF, C, and M refer to different cell fractions, as defined in Fig. 1. Values are means for triplicate 1-ml samples. Standard deviations of the means shown did not exceed 8% for cytoplasmic and membrane fractions or 17% for the PSF and ranged from 20 to 60% for the S fraction.

[c] Net protein attributable to periplasm. The part of cytoplasmic protein originating from lysed protoplasts and contaminating the PSF proteins proper was subtracted by assuming that the ratio of RNA to protein in the cytoplasmic and membrane fractions is equal to the ratio of RNA to contaminating protein in the PSF.

Therefore, to determine the actual proportion of putative periplasmic proteins in the PSF, an accurate correction for contaminating proteins of cytoplasmic origin was necessary.

Initial experiments (data not shown) revealed a certain sample-to-sample variability of the PSF protein contents measured in cell fractions obtained from cultures grown in the presence of [4.5-$^{3}$H]leucine. To eliminate possible sampling errors, we have resorted to a double labeling method. Cell fractions were obtained from a culture of *B. subtilis* 168 grown under phosphate-limiting conditions while continuously labeled with [$^{14}$C]leucine and [$^{3}$H]uracil specifically incorporated into protein and RNA (Table 1). Samples were taken from cultures in the exponential phase and about 1 and 3 h after phosphate depletion. Table 1 shows the contents of the PSFs of these samples. The contamination of the PSF by other cytoplasmic components, including protein, is assumed to be comparable to that by RNA. After correction for contaminating cytoplasmic protein, significant amounts, i.e., a mean figure of 9.8 ± 4.8%, of protein could be considered periplasmic.

In an independent experiment, a culture of *B. subtilis* 168 was split into two parts; [4.5-$^{3}$H]leucine was added to one and [$^{3}$H]uracil was added to the other. Samples, withdrawn at regular intervals from the parallel cultures, were harvested, and the cells were fractionated. For samples taken at the end of the exponential growth phase and about 3 h after phosphate depletion from the medium, the PSF contained 14 and 17%, respectively, cell protein, the corresponding figures for RNA being 8.9 and 2.5%. It was calculated that protein originating from the periplasmic fraction represented 5.1 and 14.5%, respectively.

From this and the previous experiment (Table 1), the protein contents of the periplasm of *B. subtilis* 168, corrected for cytoplasmic contamination, were at a mean of 9.8% ± 4.8% of the total cell protein. These amounts were generally higher than the RNA content of the PSF and, therefore, we assume, than that of the contaminating cytoplasmic proteins also.

After correction for a cytoplasmic contamination of 7.6% (see above), a lower proportion of protein, between 2.5 and 3% (data not presented), was determined to be present in the PSF of *B. subtilis* W23. Incidentally, comparison of the overall distributions of protein among different cell fractions for the two strains revealed that strain W23 contained a significantly higher proportion of protein associated with membrane preparations, i.e., a mean of 28.7% ± 4.9% versus 20.3% ± 2.5% for strain 168 (data not presented for three independent experiments).

Cytochromes have been shown to be membrane proteins in *B. subtilis* (46). By screening all cell fractions for the presence of heme containing cytochromes after SDS-PAGE, their presence was detected exclusively in the cytoplasmic membrane fraction of *B. subtilis* (data not presented). They were not detected in the PSF, whereas one cytochrome species was reported for the osmotic shockate of *Neisseria* (19).

**Ribonuclease activities in different cell fractions.** The presence, in the PSF, of a significant proportion of cell protein, which is compatible with the existence in *B. subtilis* of a periplasm, prompted us to screen this fraction for two enzyme markers commonly found (29) in the periplasms of gram-negative organisms.

Our first choice was RNase, a characteristic periplasmic activity. Initially, global RNase activity was measured in all cell fractions obtained from a batch culture of *B. subtilis* W23 grown under phosphate-limiting conditions (Table 2). Prior to phosphate depletion from the medium, while cultures were still growing exponentially in the presence of a low phosphate concentration, a large portion (more than 45%) of the cellular ribonuclease activity was found in the PSF. In samples taken 2 h after phosphate depletion, while total RNase activity had increased nearly sixfold, the increase did not take place uniformly in all cell fractions. There was a very limited absolute increase in the cytoplasmic fraction and a more pronounced one in the PSF. However, there was a nearly 10-fold increase in the culture supernatant fraction, which eventually held 75% of the overall RNase activity.

Thus, in response to phosphate starvation, the induction of nucleases in *B. subtilis* and their accumulation at the cell surface and release to the culture medium paralleled the behavior reported to occur in *B. licheniformis* for other phosphate-scavenging enzymes, notably the alkaline phosphatases (13). One noteworthy difference between *B. licheniformis* and *B. subtilis* W23, however, was the virtual absence of detectable RNase activity associated with membrane preparations of the latter (Table 2). In the absence of membrane washing steps (Fig. 1), the small amount of activity detected in isolated membranes was most likely due to contamination by the RNases from other fractions.

**RNase detection in polyacrylamide gels.** SDS-PAGE on renaturing, RNA containing gels revealed the presence of mul-

TABLE 2. Relative amounts of RNase activity in cell fractions of strain W23

| ND | Total activity ($10^4$ dpm) | Distribution among fractions[b] (%) | | | | Relative RNase activity[c] |
|---|---|---|---|---|---|---|
| | | S | PSF | C | M | |
| 46 | 6 | 1.8 (30) | 2.7 (45) | 1.3 (22) | 0.2 (3) | 1 |
| 93 | 10.2 | 1.9 (19) | 4.7 (46) | 3.4 (33) | 0.2 (2) | 1.7 |
| 180 | 11.2 | 1.6 (14) | 5.0 (45) | 4.0 (36) | 0.6 (5) | 1.9 |
| 410 | 26.1 | 14.4 (55) | 6.2 (24) | 5.0 (19) | 0.5 (2) | 4.4 |
| 600 | 32.7 | 24.4 (75) | 5.3 (16) | 3.0 (9) | 0 (0) | 5.5 |

[a] Cultures were grown in TLP with a phosphate concentration of 0.44 mM. The latter becomes exhausted from the medium at an ND of around 160. At indicated NDs, 1-ml aliquots were collected and fractionated. RNase activity was measured as the amount of soluble degradation products of [$^{3}$H]poly(A) as described in Materials and Methods.

[b] S, PSF, C, and M refer to different cell fractions, as defined in Fig. 1. Values are means for triplicate samples. For more than two-thirds of the samples, the standard deviations were within ±16%.

[c] Total RNase relative to that present at an ND of 46.

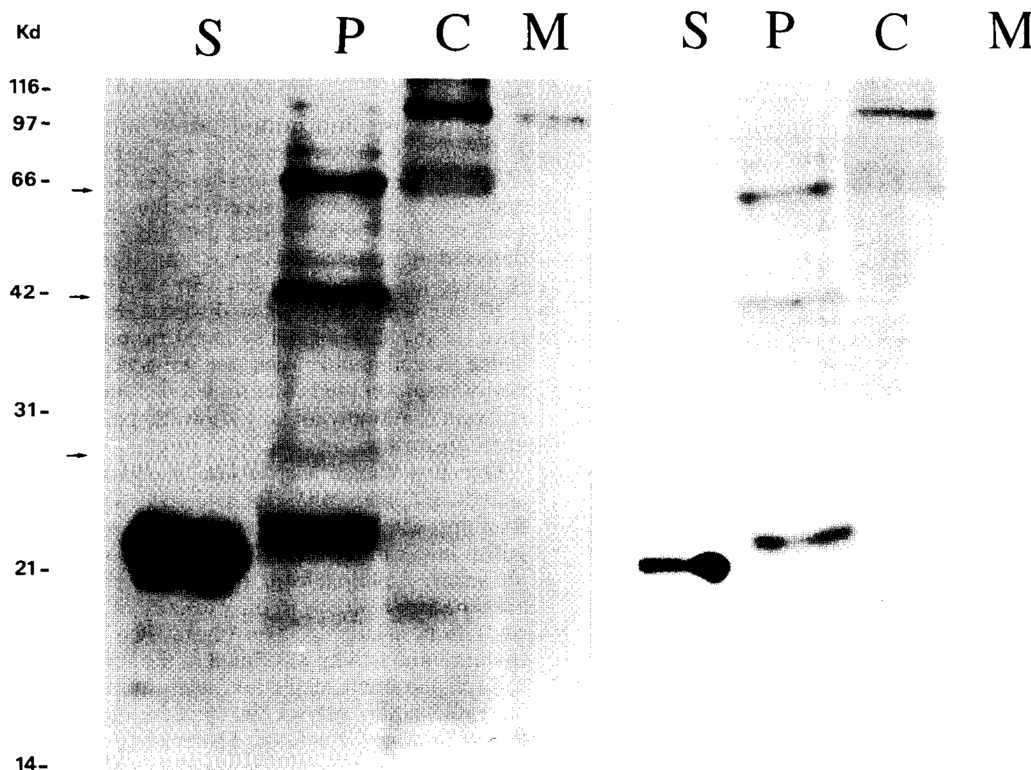


FIG. 2. Ribonucleases in different cell fractions of *B. subtilis* W23. Protein bands with RNase activity are detected after separation by renaturing SDS-PAGE with RNA as a copolymer, followed by a washing and renaturing step to remove detergent. S, P, C, and M (Fig. 1) are, respectively, the culture supernatant containing secreted proteins, the periplasm or the PSF, the cytoplasm, and the membrane fraction. The culture was harvested about 2 h after phosphate depletion, at an ND of about 700. Each well was loaded with the equivalent of 1 ml of culture. After an overnight washing in water with gentle agitation to remove SDS, the gel was photographed after 1 and 5 h of inculation (right and left panels, respectively) at 47°C at pH 7.5. In the presence of ethidium bromide, RNase activity was revealed as an unstained zone. Molecular mass standards are shown, in kilodaltons. RNase bands specific to, or most abundant in, the periplasmic fraction are indicated by arrows.

tiple bands of RNase activities in cell extracts obtained from broken cells of *B. subtilis* (34, 42). To determine their distribution among various cell fractions obtained by the fractionation procedure used here (Fig. 1), we have applied the method of Rosenthal and Lacks (34). Phosphate-starved cultures of *B. subtilis* W23 were fractionated and screened for RNases. Inspection of zymographs revealed multiple RNase bands most evidently in the periplasmic fraction (Fig. 2). The profile of RNase of the PSF, which was very distinct from that of both the culture medium, and, particularly, the cytoplasmic fraction, provided strong evidence for the PSF being a unique cell fraction. Such a marked concentration of nucleases in a fraction which represents 10% or less of cell protein reinforces the parallels with the periplasm of *E. coli* and strongly argues in favor of the PSF containing the periplasm of the gram-positive organism *B. subtilis.*

The limited contamination of the PSF by cytoplasmic proteins, due to protoplast lysis, is compatible with the presence in the PSF of a very faint RNase band of about 105 kDa which, possibly, corresponds to the major cytoplasmic RNase of the same size. This observation is in good agreement with the measurements of PSF contamination by cytoplasmic enzyme and RNA markers (see above).

The periplasmic and the culture medium fractions were compared; particularly noteworthy was the absence of release to the medium of several periplasmic RNases, of which at least four have molecular sizes in the range of 50 to less than 20 kDa. One RNase band only, of approximately 23 kDa, appears in significant quantities in the culture medium, as well as in the periplasm-containing fraction. The presence of this band in the medium, clearly prominent 1 h after phosphate depletion, was paralleled in the PSF by the appearance of a major band with a slightly higher molecular mass, ca. 25 kDa, which was barely detectable at earlier times (data not shown); this is consistent with the latter being a proenzyme precursor of the released activity. Proenzyme precursors of a small (11.4-kDa) extracellular RNase (barnase) of *Bacillus amyloliquefaciens* have been detected in a cell-bound form (30).

**DNase activities in different cell fractions of *B. subtilis.*** The specific profile of RNases that distinguishes the PSF from all other cell fractions prompted a screening of cell fractions for a second marker nuclease activity, DNase. Cell fractions from phosphate-starved cultures were screened for DNase activity after SDS-PAGE on DNA-containing gels (Fig. 3). Inspection of the gels revealed that the periplasmic fraction contains an important proportion, possibly the major part, of the DNase activity of the cell. The periplasmic nucleases exhibit a clearly distinct profile, although certain bands, found mainly in the periplasm, seem also to be present in both the membrane and the cytoplasmic fractions. Two rather faint bands at about 33 and 41 kDa are unique to the PSF, while a very prominent series of bands between 50 and 90 kDa are essentially absent from the cytoplasm and the membrane.

In marked contrast to the absence of RNases (Fig. 2), the cytoplasmic membrane was well endowed with DNases. Interestingly, there were parallels with the nucleases of competent cells (40, 41); DNases of the membrane fraction of cells obtained under growth conditions very different from those used

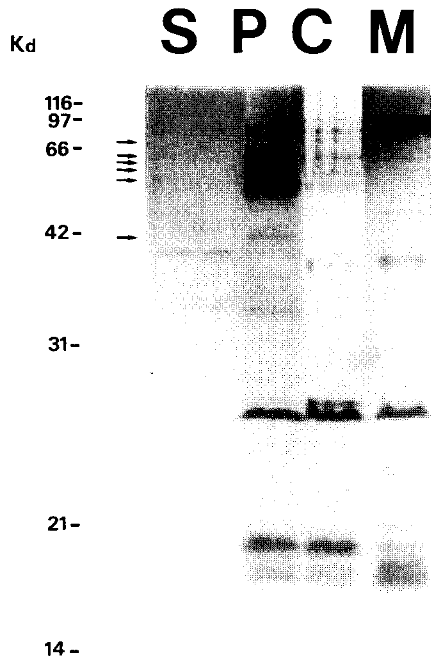


FIG. 3. Deoxyribonuclease activity in different cell fractions of *B. subtilis* 168 detected after protein separation in renaturing, DNA-containing SDS-PAGE gels. Cells were harvested 1 h after phosphate depletion, at an ND of about 300. Each well was loaded with the equivalent of 1 ml of culture. S, P, C, and M refer to culture supernatant, PSF, and cytoplasmic and membrane fractions. After electrophoretic separation of proteins, gels were washed to remove detergent and incubated for 3 to 6 h at 37°C in a $Ca^{2+}$- and $Mg^{2+}$-containing buffer at pH 7.6. Arrows indicate the positions of bands specific to the periplasmic fraction. Molecular mass markers are specified, in kilodaltons. DNase bands specific to, or most abundant in, the periplasmic fraction are indicated by arrows.

here were apparently associated with bands of identical molecular sizes, ranging from below 20 to about 80 kDa. DNases were much less abundant in the cytoplasmic fraction, which contained mainly two bands at around 20 and 25 kDa. In view of the data provided in Table 1, the presence, in very comparable amounts, of apparently identical bands in the cytoplasm and the PSF cannot be due to contamination since the latter contains less than 10% of the cytoplasmic material. With the exception of these two bands, the DNase profiles of the periplasmic and the cytoplasmic fractions are quite distinct. Finally, released into the medium were two DNase species associated with very faint bands with molecular masses of about 34 and 40 kDa. Incidentally, no differences between strains 168 and W23 were observed, since, under the same growth conditions, cell fractions obtained from the latter strain yielded a pattern of DNase activities that was virtually superimposable on that of the former strain (data not presented).

Comparison of nuclease activities in the periplasmic fraction (Fig. 2 and 3) clearly revealed that RNases and DNases correspond to different sets of bands. Thus, it appears that, with a possible exception of one or two bands with higher molecular masses, distinct proteins are involved rather than ones associated with a relatively unspecific nuclease activity.

In conclusion, our observations imply that in *B. subtilis*, under the conditions used here, it is likely that the products of an important number of nuclease genes are specifically directed to a cell fraction that largely corresponds, by its operational definition and its enzymatic composition, to a periplasm.

## DISCUSSION

Electron microscopic observations of sections of gram-positive bacteria, obtained either by classical methods or with cryofixed and dehydrated cells, leave little room for a periplasmic space (4, 15). *Staphylococcus aureus*, for which structures present in an interlayer between the cytoplasmic membrane and the cell wall were reported (12), appears to be an exception. Evidence in favor of a distinct cell compartment in *B. subtilis* lying outside the cytoplasmic membrane, presented above, rests on a physiological, operational definition. Indeed, protein assay and enzymatic analyses of the soluble fraction obtained by isotonic dissolution of the cell wall and removal of the resulting protoplasts reveal a PSF consisting of a substantial fraction of cell protein, i.e., a mean of 17.0% (range, 8 to 19%) for *B. subtilis* 168. Assay of the PSF for the presence of several markers revealed a cytoplasmic contamination, with a mean value corresponding to 7.6% of total cell protein. The latter was attributed to the lysis of a small fraction of, or leakage from, protoplasts. In an earlier study, Mauck and Glaser (26) found that the PSF of *B. subtilis* W23 contained 6, 9, and 14% of three cytoplasmic enzyme markers, with an overall mean of 9.7%, a figure for cytoplasmic contamination in excellent agreement with that obtained in the present study.

After correction for cytoplasmic contamination, we found that up to 9 and 3% of total cell protein were associated with the periplasmic fraction of *B. subtilis* 168 and W23, respectively. Quantitatively, these figures fall within or close to the range reported for the *E. coli* periplasmic protein, i.e., 4 (28) and 16% (1). According to its operational definition (Fig. 1), the PSF of *B. subtilis* also comprises the cell wall-bound proteins, which, however, amount to less than 0.5% of total cell protein (43).

Our observations cast light neither on the possible existence of a supramolecular organization nor on the nature of the protein-protein interactions within what is likely to be a rather densely packed (4) periplasm. The presence in the PSF of proteins that, in the intact cell, are closely associated with the outer face of the cytoplasmic membrane is not unlikely; nevertheless, the PSF and membrane fraction, as prepared here, exhibit very distinct profiles of nucleases.

Artifacts inherent to procedures involved in cell fractionation cannot be excluded. In particular, the osmotic shock and the resulting plasmolysis that accompany cell resuspension in hypertonic medium are known to generate the so-called mesosomal vesicles, structures that derive from the cytoplasmic membrane. Whether the majority of such vesicles remain associated with the membrane or are released to the PSF depends upon the ionic environment of the suspension buffer (33, 37). In either case, these vesicles are likely to contain proteins, possibly including those of periplasmic origin, engulfed during their formation. Part of the protein found in the pellet, enriched for these vesicles by sedimentation of the PSF at 100,000 × *g* for 1 h (data not presented), is likely to be an experimental artifact, since the periplasmic RNases (see Results) detected in this pellet are absent from cytoplasmic-membrane preparations (Fig. 1).

The potential existence of a distinct cell compartment external to the cytoplasmic membrane has implications for cell fractionation procedures commonly employed for gram-positive organisms. Indeed, the so-called cytoplasmic fraction obtained by disruption of whole cells means that components of the cytosol will be harvested together with those of the periplasm and that unambiguous assignment of any given component to the cytosol is not possible.

If the foregoing reveals a reasonable quantitative correspon-

dence between protein in the PSF of *B. subtilis* and the periplasm of *E. coli*, how comparable are these fractions qualitatively, in terms of specific enzyme components? The clearly differentiated profiles for RNases and for DNases shown by the different cell fractions in the present study and the presence, in the PSFs of both *B. subtilis* strains, of multiple nuclease activities (which in several cases appear to be specific to this fraction, which contains 10% or less of cell protein) argue strongly in favor of a close convergence with the *E. coli* periplasm for several major scavenging enzymes. Furthermore, the specificity of enzyme profiles of different cell fractions confirms the limited degree of contamination of the PSF by the cytoplasmic membrane or the cytoplasm. This led us to conclude that the PSF of *B. subtilis* includes the contents of a distinct compartment that, within the limits of what is known, has close parallels with the periplasmic space of *E. coli*, different cell envelope structures notwithstanding. Previously, the absence of crypticity in whole cells for specific enzymes, considered evidence for their having a periplasmic location in *E. coli* (3), has been shown to be true for a nucleoside diphosphate sugar hydrolase in *B. subtilis* W23 (26). In addition, for bacilli, there have been several reports of the presence of phosphatases, notorious as periplasmic scavenging enzymes in *E. coli* (13, 16), while DNases were shown to be associated with the cell surface of *B. subtilis* and specifically released into the supernatant during isotonic formation of protoplasts (5).

While in the cell envelopes of both gram-negative and gram-positive organisms the cytoplasmic membrane forms the inner boundary of the periplasmic space, in the absence of the outer membrane in a gram-positive bacterium we consider it possible that the external barrier function could be accomplished by the much thicker, generally teichoic acid-endowed cell wall layer, as has been suggested previously (14). What evidence is there that the cell wall of *B. subtilis* could act as a permeability barrier? The absence from the medium of a large number of nucleases belonging to the periplasm (see above), with molecular masses ranging from over 100 down to 20 kDa and below, is consistent with the wall acting as a permeability barrier to proteins of this size range. However, one other possibility, at least, could provide an alternative explanation for lack of secretion of periplasmic components. It is not impossible that protein complexes, formed through the action of Van der Waals forces, could preclude access of individual proteins to the secretion pathways through the cell wall matrix. Nonetheless, previous results obtained with a related organism, *Bacillus megaterium*, are consistent with the proposed barrier function. Indeed, direct measurements of the bulk porosity of the isolated cell walls to a series of carbohydrate probes with defined molecular masses yielded an upper exclusion limit of 1.2 kDa (39). This figure, which is practically identical to the exclusion limit for molecules able to pass, via porins, the outer membrane of the envelopes of gram-negative organisms, is consistent with the hypothesis that the *Bacillus* cell wall layer can act as a barrier for molecules of greater size. That the bulk of the cell wall could exclude molecules of such low molecular masses is, however, not incompatible with the fact that large molecules such as proteins are able to cross this barrier to be released into the medium. For proteins to penetrate and transit the thick cell wall of bacilli from the bulk of which they are excluded, specialized structures or singularities would be necessary (43). The nature of such structures or any possible parallels with mechanisms for the transit of the gram-negative outer membrane are at present only speculative. Nevertheless, an equivalent situation is observed for gram-negative organisms, including *Klebsiella*, *Erwinia*, *Proteus*, and *Pseudomonas* species, which have a variety of specialized export systems that allow specific subsets of proteins to transit the outer membrane permeability barrier (20).

## ACKNOWLEDGMENTS

We acknowledge the support of the Fonds National Suisse de la Recherche Scientifique (Subside 31.991.92).

We also thank Eduard Kellenberger for constructive discussion.

## REFERENCES

1. **Ames, G. F.-L., C. Prody, and S. Kustu.** 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. **160:**1181–1183.
2. **Archibald, A. R.** 1974. The structure, biosynthesis and function of teichoic acids. Adv. Microb. Physiol. **11:**53–95.
3. **Beacham, I. R.** 1979. Periplasmic enzymes in Gram-negative bacteria. Int. J. Biochem. **10:**877–883.
4. **Beveridge, T. J.** 1995. The periplasm space and the periplasm in gram-positive and gram-negative bacteria. ASM News **61:**125–130.
5. **Birnboim, H. C.** 1966. Cellular site in *Bacillus subtilis* of a nuclease which preferentially degrades single-stranded nucleic acids. J. Bacteriol. **91:**1004–1011.
6. **Chang, S.** 1988. Engineering for protein secretion in Gram-positive bacteria. Methods Enzymol. **153:**507–516.
7. **Foster, S. J.** 1993. Molecular analysis of three major wall-associated proteins of *Bacillus subtilis* 168: evidence for processing of the product of a gene encoding a 258 kDa precursor two-domain ligand-binding protein. Mol. Microbiol. **8:**299–310.
8. **Francis, R. T., and R. R. Becker.** 1984. Specific indication of hemoproteins in polyacrylamide gels using a double staining process. Anal. Biochem. **136:** 509–514.
9. **Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips.** 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
10. **Ghosh, A., S. Vallespir, and B. K. Ghosh.** 1983. Specificity of subcellular distribution of alkaline phosphatase in *Bacillus licheniformis* 749/C. Can. J. Microbiol. **30:**113–125.
11. **Giesbrecht, P., M. Franz, D. Krüger, M. Labichinski, and J. Wecke.** 1993. Bacteriolysis of staphylococci is only a side-effect of penicillin-induced death, p. 353–363. *In* H. Kleinkauf and H. von Döhren (ed.), 50 years of penicillin application—history and trends. Video Press, Prague.
12. **Giesbrecht, P., J. Wecke, and B. Reinicke.** 1977. The demonstration of the existence of an interlayer between the cytoplasmic membrane and the cell wall proper of staphylococci. Arch. Microbiol. **115:**25–35.
13. **Glynn, J. A., S. D. Schaffel, J. M. McNicholas, and F. M. Hulett.** 1977. Biochemical localization of the alkaline phosphatase of *Bacillus licheniformis* as a function of culture age. J. Bacteriol. **129:**1010–1019.
14. **Gould, A. R., B. K. May, and W. H. Elliot.** 1975. Release of extracellular enzymes from *Bacillus amyloliquefaciens*. J. Bacteriol. **122:**34–40.
15. **Graham, L. L., T. J. Beveridge, and N. Nanninga.** 1991. Periplasmic space and the concept of periplasm. Trends Biochem. Sci. **16:**328–329.
16. **Hansa, J. G., M. Laporta, M. A. Kuna, R. Reimschuessel, and F. M. Hulett.** 1981. A soluble alkaline phosphatase from *Bacillus licheniformis* MC14. Biochim. Biophys. Acta **657:**390–401.
17. **Harwood, C. R., R. D. Coxon, and I. C. Hancock.** 1990. The *Bacillus* cell envelope and secretion, p. 327–390. *In* C. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. Wiley, Chichester, United Kingdom.
18. **Hughes, A. H., I. C. Hancock, and J. Baddiley.** 1973. The function of teichoic acids in cation control in bacterial membranes. Biochem. J. **132:**83–93.
19. **Judd, R. C., and S. F. Porcella.** 1993. Isolation of the periplasm of *Neisseria gonorrhoeae*. Mol. Microbiol. **10:**567–574.
20. **Koronakis, V., and C. Hughes.** 1994. Secretion of hemolysin and other proteins out of the Gram-negative bacterial cell, p. 425–446. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier, Amsterdam.
21. **Labischinski, H. H., H. Maidhof, M. Franz, D. Krüger, T. Sidow, and P. Giesbrecht.** 1988. Biochemical and biophysical investigations into the cause of penicillin-induced lytic death of staphylococci: checking predictions of the murosome model, p. 242–257. *In* P. Actor, L. Daneo-Moore, M. L. Higgins, M. R. J. Salton, and G. D. Shockman (ed.), Antibiotic inhibition of bacterial cell surface assembly and function. American Society for Microbiology, Washington, D.C.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Langdon, R. G.** 1966. Glucose 6-phosphate dehydrogenase from erythrocytes. Methods Enzymol. **9:**126–131.
24. **Lopes, J., S. Gottfried, and L. Rothfield.** 1972. Leakage of periplasmic enzymes by mutants of *Escherichia coli* and *Salmonella typhimurium*: isolation of "periplasmic leaky" mutants. J. Bacteriol. **109:**520–525.
25. **Malamy, M. H., and B. L. Horecker.** 1964. Release of alkaline phosphatase from cells of *Escherichia coli* upon lysozyme spheroplast formation. Biochemistry **3:**1889–1893.

26. **Mauck, J., and L. Glaser.** 1970. Periplasmic nucleoside diphosphate sugar hydrolase from *Bacillus subtilis*. Biochemistry **9:**1140–1147.
27. **Nagarajan, V.** 1990. System for secretion of heterologous proteins in *Bacillus subtilis*. Methods Enzymol. **185:**214–222.
28. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241:**3055–3062.
29. **Oliver, D.** 1987. Periplasm and protein secretion, p. 56–69. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
30. **Paddon, C. J., N. Vasantha, and R. W. Hartley.** 1989. Translation and processing of *Bacillus amyloliquefaciens* extracellular RNase. J. Bacteriol. **171:**1185–1187.
31. **Pollock, M. R.** 1965. Purification and properties of penicillinases from two strains of *Bacillus licheniformis*: a chemical, physico-chemical and physiological comparison. Biochem. J. **94:**666–675.
32. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41:**711–753.
33. **Reaveley, R. A., and H. J. Rogers.** 1969. Some enzymic activities and chemical properties of the mesosomes and cytoplasmic membranes of *Bacillus licheniformis* 6346. Biochem. J. **113:**67–79.
34. **Rosenthal, A. L., and S. A. Lacks.** 1977. Nuclease detection in SDS-polyacrylamide gel electrophoresis. Anal. Biochem. **80:**76–90.
35. **Sára, M., D. Pum, and U. B. Sleytr.** 1992. Permeability and charge-dependent adsorption properties of the S-layer lattice from *Bacillus coagulans* E38-66. J. Bacteriol. **174:**3487–3493.
36. **Sára, M., and U. B. Sleytr.** 1987. Molecular sieving through S layers of *Bacillus stearothermophilus* strains. J. Bacteriol. **169:**4092–4098.
37. **Sargent, M. G.** 1973. Membrane synthesis in synchronous cultures of *Bacillus subtilis* 168. J. Bacteriol. **116:**397–409.
38. **Sarvas, M.** 1986. Protein secretion in bacilli. Curr. Top. Microbiol. Immunol. **125:**103–125.
39. **Scherrer, R., and P. Gerhardt.** 1971. Molecular sieving by the *Bacillus megaterium* cell wall and protoplasts. J. Bacteriol. **107:**718–735.
40. **Smith, H., W. de Vos, and S. Bron.** 1983. Transformation in *Bacillus subtilis*: properties of DNA-binding-deficient mutants. J. Bacteriol. **153:**12–20.
41. **Smith, H., K. Wiersma, G. Venema, and S. Bron.** 1984. Transformation in *Bacillus subtilis*: a 75,000-dalton protein complex is involved in binding and entry of donor DNA. J. Bacteriol. **157:**733–738.
42. **Spanos, A., and U. Hübscher.** 1983. Recovery of functional proteins in sodium dodecyl sulfate gels. Methods Enzymol. **91:**263–277.
43. **Studer, R. E.** 1988. Caractérisation de la paroi native de *Bacillus subtilis* et étude des protéines qui lui sont associées. Ph.D. thesis. University of Lausanne, Lausanne, Switzerland.
44. **Studer, R. E., and D. Karamata.** 1988. Cell wall proteins in *Bacillus subtilis*, p. 146–150. *In* P. Actor, L. Daneo-Moore, M. L. Higgins, M. R. J. Salton, and G. D. Shockman (ed.), Antibiotic inhibition of bacterial cell surface assembly and function. American Society for Microbiology, Washington, D.C.
45. **Thirunavukkarasu, M., and F. G. Priest.** 1984. Purification and characterization of an extracellular and a cellular $\alpha$-glucosidase from *Bacillus licheniformis*. J. Gen. Microbiol. **130:**3135–3141.
46. **von Wachenfeldt, C., and L. Hederstedt.** 1990. *Bacillus subtilis* 13-kilodalton cytochrome *c*-550 encoded by *cccA* consists of a membrane-anchor and a heme domain. J. Biol. Chem. **265:**13939–13948.
47. **Williamson, R., and J. B. Ward.** 1981. Deficiency of autolytic activity in *Bacillus subtilis* and *Streptococcus pneumoniae* is associated with a decreased permeability of the wall. J. Gen. Microbiol. **125:**325–334.