Use of the Gram stain in microbiology

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Abstract

The Gram stain differentiates bacteria into two fundamental varieties of cells. Bacteria that retain the initial crystal violet stain (purple) are said to be ''Gram-positive,'' whereas those that are decolorized and stain red with carbol fuchsin (or safranin) are said to be ''Gram-negative.'' This staining response is based on the chemical and structural makeup of the cell walls of both varieties of bacteria. Gram-positives have a thick, relatively impermeable wall that resists decolorization and is composed of peptidoglycan and secondary polymers. Gram-negatives have a thin peptidoglycan layer plus an overlying lipid-protein bilayer known as the outer membrane, which can be disrupted by decolorization. Some bacteria have walls of intermediate structure and, although they are officially classified as Gram-positives because of their linage, they stain in a variable manner. One prokaryote domain, the Archaea, have such variability of wall structure that the Gram stain is not a useful differentiating tool.

Key words: carbol fuchsin, crystal violet, Gram-negative bacteria, Gram-positive bacteria, Gram stain, Gram-variable, iodine

The Gram stain is an important light microscopy stain for microbiology because it differentiates bacteria into two fundamental varieties of cells; thus it is useful for the initial classification of unknown isolates. For this reason the Gram stain is an important primary taxonomic tool. Certainly in this era of molecular biological probes, genera and even species can often be quickly ascertained using these modern techniques, but the Gram stain is still a primary tool for identification. Because the stain does not alter the shape and form of bacteria, it is also an easy method for determining the overall structure of the cells (e.g., cocci, rods, spirals, filaments, cubic packets, etc.). The Gram stain was developed in the late 1800s (Friedlander 1883, Gram 1884) and was first used as a diagnostic tool for clinical microbiology. It is remarkable how a young Danish clinician, Hans Christian Gram, developed a basic diagnostic tool for microbiology that has stood the test of time so well. The *International Journal for Systematic Bacteriology* (published by the Society for General Microbiology, Cambridge Press, UK) and *Bergey's Manual of Determinative Bacteriology* (Holt 1994), two important publications for prokaryotic taxonomy, still require authors to describe the Gram staining response of isolates, and the Gram stain to this day is used in all aspects of modern microbiology, including general, medical, environmental, and industrial fields.

Popescu and Doyle (1996) wrote a comprehensive article on the Gram stain in which the history and basic mechanism of the Gram stain was explained. For this reason, I will not dwell on these aspects, but will instead give a more detailed mechanistic view with respect to prokaryotic surface structure based on the work performed in my laboratory (Beveridge and Schultze-Lam 1997, Beveridge 1990, 1993, Beveridge et al. 1991, Beveridge and Davies, 1983, Davies et al. 1983).

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The Gram stain

Although the tried and true method found in microbiology textbooks is still a useful method for day to day routine, during the more than 100 years of its existence the Gram stain has been modified to conform to different specimens and is continually being improved (e.g., Noda and Toel 1992). For example, it is now possible to use fluorescent lectins (Sizemore et al. 1990) and Molecular Probes, Inc. has a ''live-dead'' Gram stain (Molecular Probes, Inc., Eugene, OR). Initially, Gram used gentian violet as the primary stain and Bismarck brown as a counterstain; we now use crystal violet and carbol fuchsin or safranin. The general method for staining follows:

1. A suspension of bacteria is placed on a glass microscope slide and attached by gentle heating.
2. A few drops of crystal violet (1.24 g in 100 ml water) are added to the specimen and allowed to stand for 0.5 min.
3. A few drops of Gram's iodine (a mixture of 0.33 g iodine and 0.67 g potassium iodide in 100 ml water) is added directly to the crystal violet on the slide to act as a mordant for 0.5 min.
4. The crystal violet-Gram's iodine mixture is poured from the slide, the slide is washed rapidly with tap water followed by a gentle flow of decolorizing fluid (95% v/v ethanol in water) for 20 sec.
5. The slide is again washed rapidly in tap water, and carbol fuchsin (or safranin) is added to the specimen for 1 min.
6. The slide is rinsed with tap water and patted dry with filter paper. The specimen is ready for microscopy using bright field illumination.

Using this staining regimen, Gram-positive bacteria, such as *Bacillus subtilis*, stain purple (Fig. 1) and Gram-negative bacteria, such as *Escherichia coli*, stain red. Evidence that the staining response relies on cell wall integrity comes from the treatment of Gram-positive bacteria with wall-degrading enzymes such as lysozyme (Fig. 2) which converts them to Gram-negative cells.

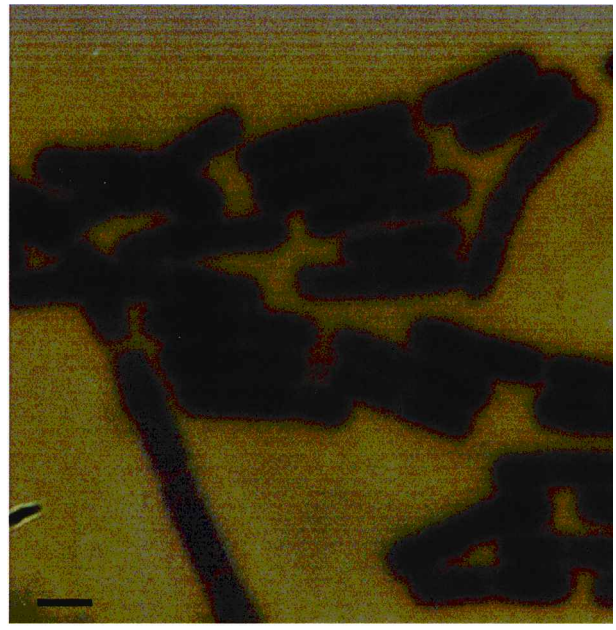

Fig. 1. Bright field light microscope image of a Gram-stained exponentially growing *Bacillus subtilis* culture. Note that these Gram-positive rods are stained purple. Scale bar = 1 μm.

Bacterial cell walls

Gram-positive walls

These cell walls can be typified by those of *B. subtilis* or *Staphylococcus aureus* (Fig. 3). The cell walls of *B. subtilis* consist of an amazingly robust network of a primary polymer (peptidoglycan) to which secondary polymers, such as teichoic or teichuronic acids, are attached. Those of *S. aureus* are more complex (Beveridge 2000). Peptidoglycan consists of linear strands of repeating N-acetyl-glucosamine–N-acetyl-muramic acid moieties to form fibers approximately 10–15 dimers long (Höltje 1998, Doyle and Koch 1987). A short peptide chain is attached to each muramyl residue and these peptides are often attached covalently to peptides on adjacent chains. The strands of peptidoglycan

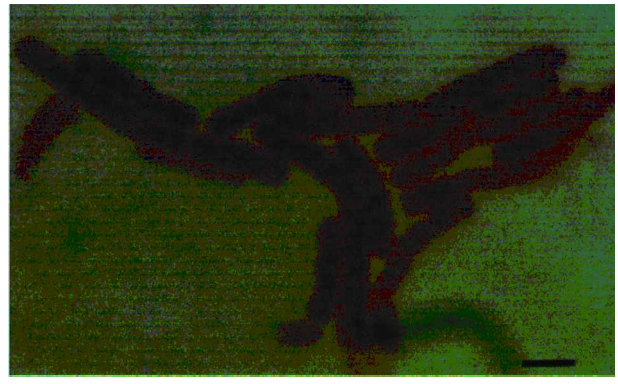

Fig. 2. Bright field light microscopy of *B. subtilis* cells treated with a cell wall-degrading enzyme (lysozyme from chicken eggs) so that the cell wall was made permeable to the crystal violet-iodide precipitate. Note that the cells now stain red because of the carbol fuchsin. Scale bar = 1 μm.

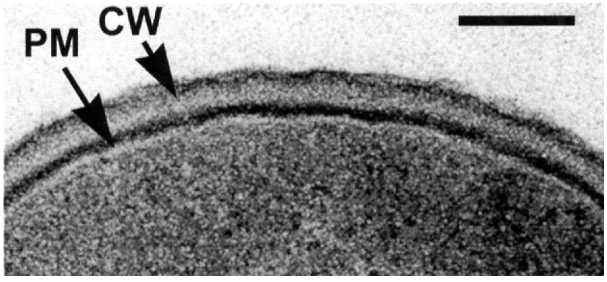

Fig. 3. Electron micrograph of a thin section of the Gram-positive cell envelope of *Staphylococcus aureus* consisting of a plasma membrane (PM) and an approximately 25 nm cell wall (CW) possessing many layers of peptidoglycan and attached secondary polymers. Scale bar = 80 nm.

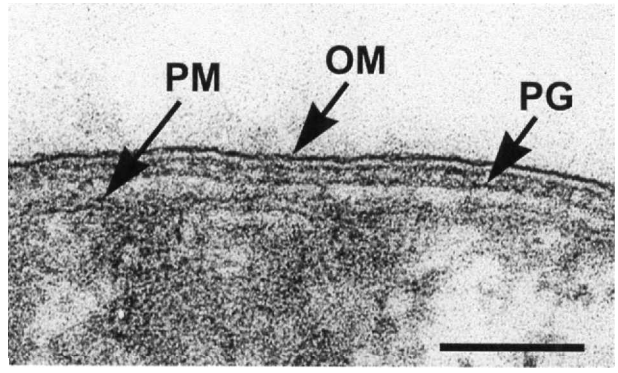

Fig. 4. Thin section of the Gram-negative *Escherichia coli* cell envelope consisting of a plasma membrane (PM), a thin peptidoglycan layer (PG) and an outer membrane (OM). Scale bar = 100 nm.

are arranged peripherally around the bacillus at right angles to the long axis of the cell somewhat like the hoops of a barrel. There are approximately 25 layers of peptidoglycan in the *B. subtilis* wall, and since they are all covalently bound together in the x-, y- and z-axes, a huge macromolecule, the peptidoglycan sacculus, is formed around the cell. This gives the cell its shape (in this case, a rod) and helps protect the cell from the outside environment. The secondary polymers are also attached to muramyl residues and are thought to flex their way throughout the interstrand spaces of the peptidoglycan network. Other molecules can also be integrated into this basic framework in bacillus walls, and other bacteria can possess subtly different peptidoglycans or secondary polymers, but for the purposes of this article, these modifications do not alter the staining response except in specific cases, which will be addressed later when discussing Gram-variable bacteria. Gram-positive walls are relatively thick, have a robust architecture, and can be quite impermeable (Beveridge 2000); these are important attributes for the Gram stain.

Gram-negative walls

These walls are more complex than the Gram-positive variety (Fig. 4) and possess a peptidoglycan sacculus that is only 1–3 layers thick (Höltje 1998, Labichinski et al. 1991, Yao et al. 1999). Gram-negative peptidoglycan is essentially similar to that discussed above except that it is much thinner and it does not have secondary polymers attached. Outside this peptidoglycan sacculus is a lipid-protein bilayer, called the outer membrane (OM), consisting of phospholipids, mainly phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol; outer membrane proteins (OMPS) such as porins, which form small aqueous channels through the bilayer; and a unique lipid called lipopolysaccharide (LPS), which is the endotoxic component of Gram-negative bacteria (see Beveridge 1999 for review). The phospholipids are mainly aligned along the inner face of the OM, whereas the LPS is on the outer face. Because OM is a relatively flexible lipid bilayer, and because the peptidoglycan is so thin, the Gram-negative wall is not as strong as the Gram-positive variety.

Mechanism of the Gram stain

The primary staining agent is crystal violet, a basic trianiline dye possessing three dimethyl-aminyl six-membered carbon rings arranged around a central carbon (hexamethyl-pararosaniline chloride). The dye resembles a three pronged boat propeller (the carbon rings being the blades of the propeller) approximately 1.6 nm in diameter and possesses an overall positive charge when dissociated in solution from its chloride salt. This cation freely enters both Gram-positive and Gram-negative bacteria. In 1983, we set out to synthesize a chemical agent that could replace the reagents in Gram's iodine solution and would have enough electron scattering power to be followed by electron microscopy (Davis et al. 1983). In this way, we hoped that each important step in the Gram reaction could be followed by high resolution transmission electron microscopy (TEM) to see exactly where the reaction deposits were located, and energy dispersive X-ray spectroscopy (EDS) to identify the elements in the reaction deposits. Nuclear magnetic resonance (^{1}H-NMR), conductance measurements (Λ^E) and infrared spectroscopy (IR) of the reaction deposit formed by the crystal violet-Gram's iodine interaction showed that the active ingredient in Gram's iodine was I^- or I^{3-},

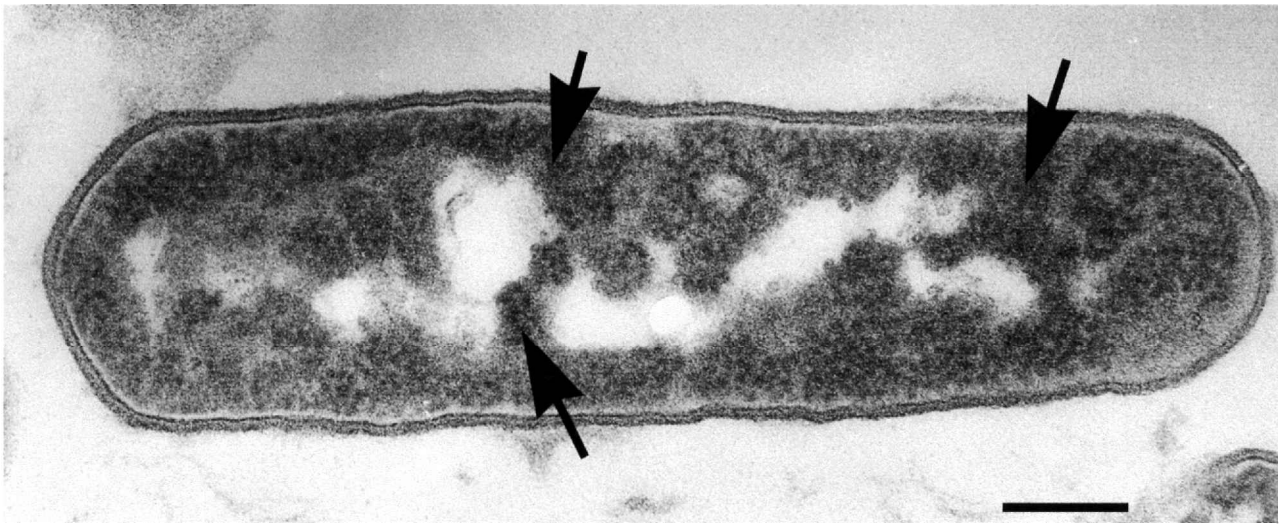

Fig. 5. Thin section of a *B. subtilis* cell after the TPt-modified Gram stain. The cell remains intact and electron dense precipitates (arrows) can be seen in the cytoplasm. Scale bar = 200 nm.

and indicated that the iodide could be replaced with an anion of similar dimensions and charge.

We settled on trichloro (η^2-ethylene) platinum II (TPt) as a replacement mordant because Pt is an effective electron scattering agent (Davies et al. 1983).

Gram-positive B. subtilis

When we stained and decolorized these cells using our modified TPt method, they stained purple by light microscopy (Fig. 1) and large electron dense precipitates could be seen in the cytoplasm by TEM (Fig. 5) (Beveridge and Davies 1983). EDS identified these as a Pt complex (TPt) and its distribution could be determined by dot-mapping when the microscope was in the scanning transmission electron microscope-EDS (STEM-EDS) mode (Fig. 6). Clearly, the crystal violet-TPt complex had randomly formed precipitates within the cytoplasm of these Gram-positive cells and was not washed out during the decolorization step. Sometimes the complex could be seen attached to the inner face of the cell wall as if it had been mobilized from the

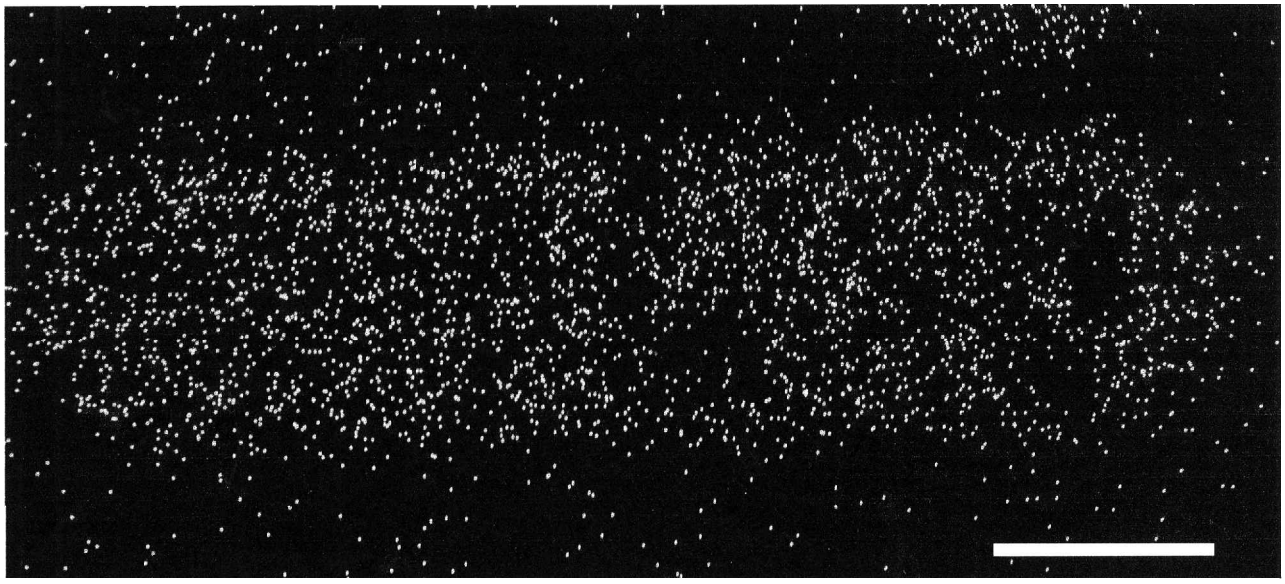

Fig. 6. Energy dispersive X-ray dot-map for Pt in the thin section of the *B. subtilis* cell shown in Fig. 5. The white dots denote where the crystal violet-TPt precipitates are located and confirm that the precipitate remains within the cell. Scale bar = 500 nm.

cytoplasm during decolorization, but the wall had prevented it from leaving the bacterium. The thickness and relative impermeability of the wall ensured that the reaction deposits remained within the cells so that they stained purple (Fig. 1; Beveridge 1993). Chemical analysis of the interaction between the dye and the mordant (either I^-/I^{3-} or TPt) suggested that the precipitate was formed via a metathetical anion exchange forming a more neutral charge transfer complex with the pi-bonds of the dye which was unstable in aqueous solution (Davis et al. 1983). Thus the exchange between the chloride of the crystal violet (the soluble salt) and the iodide (or TPt) of Gram's iodine forms an unstable crystal violet-iodide (or TPt) complex which precipitates (Davies et al. 1983).

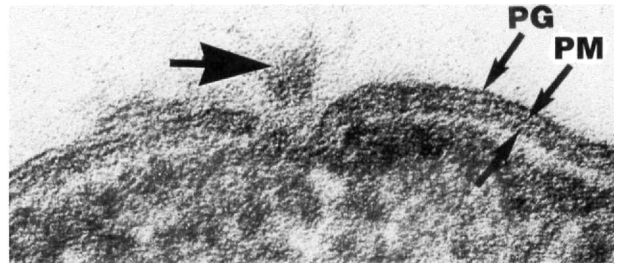

Fig. 7. Thin section of the cell envelope of *E. coli* during the decolorization step of the Gram stain showing that the envelope has been disrupted. The large arrow indicates a crystal violet-TPt precipitate that is leaving the cell. The outer membrane (cf. Fig. 4) has been dissolved and only the thin peptidoglycan layer (PG) and remnants of the plasma membrane (PM) remain. The thickness of the PM (between the two arrows) is 7.5 nm.

Gram-negative E. coli

The Gram stain is much more damaging to these cells (Beveridge 1993, Beveridge and Davies 1983). These bacteria have a thin peptidoglycan matrix and an OM lipid bilayer. The former is not strong or thick enough to retain the crystal violet-iodide (or TPt) complex, and the latter is particularly sensitive to disruption by organic solvents such as ethanol. The decolorization step is the most damaging. Many cells lyse so that most of the cytoplasmic components and the crystal violet-TPt precipitates are removed (Fig. 7). Often only the ghosts of cells remain (Fig. 8) so that carbol fuchsin, as a counterstain, can only anneal to the remaining structures thereby rendering them a red color.

Gram-variable bacteria

Sometimes bacteria that are traditionally thought to belong to a Gram-positive family, stain in a Gram-negative manner. As a result, these bacteria are especially difficult to classify taxonomically. As with the former explanations for the staining responses, this is a cell wall phenomenon (Beveridge 1990). When some Gram-positive bacteria are growing rapidly, which is often the case with exponential phase cultures in rich laboratory medium, their cell wall turnover (Doyle and Koch 1987) can scarcely keep up with their growth. Because bacteria are surrounded by a cell wall, they cannot increase their size as they grow unless more material is added to the walls to accommodate

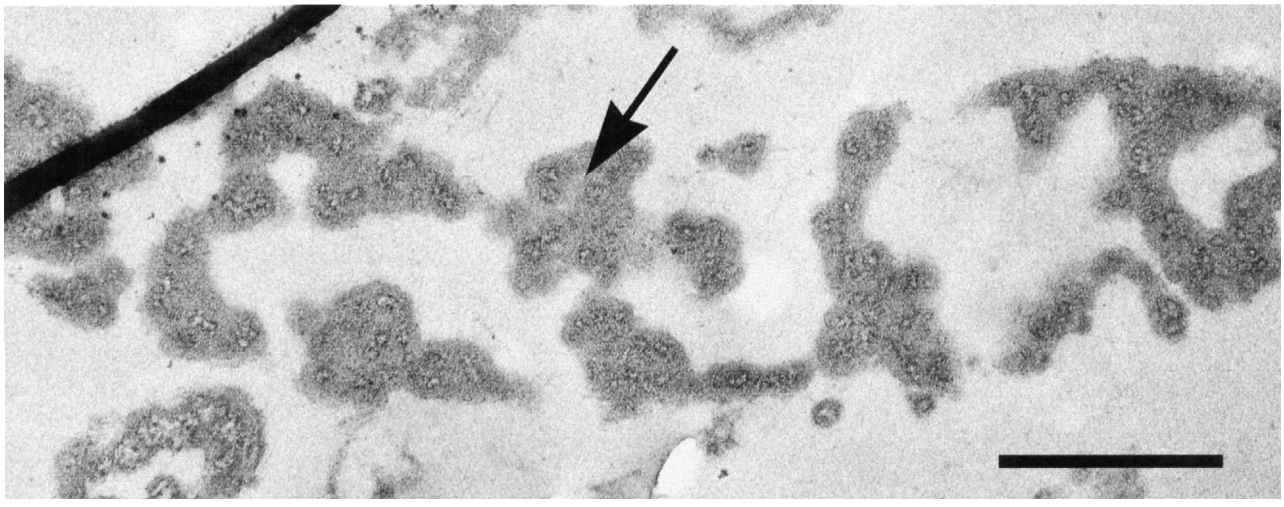

Fig. 8. Thin section of an *E. coli* cell after Gram staining. Only a cell ghost containing small remnants of cytoplasm remains (arrow). Scale bar = 500 nm.

cellular expansion. This is a delicate process because bacteria have a semipermeable membrane, the plasma membrane, internal to the cell wall that allows the cell to concentrate organic and inorganic substances inside so that substantial turgor pressure is built up. Without the wall to resist this pressure, the cells would explode or lyse. Cell wall turnover is a process whereby new peptidoglycan is added to the inner face of the wall in an unstressed, compressed manner, while old peptidoglycan is solubilized and removed from the outer face (Doyle and Koch 1987). The middle region of the peptidoglycan network is the stress bearing region and is stretched almost to its breaking point. As cells grow, there is a continuous input of new peptidoglycan at the inside face of the wall and an output at the outer face producing a dynamic inside-to-outside growth pattern for the wall. As the new, unstressed condensed fabric moves into the middle region it becomes stressed and is expanded, thereby allowing the cell to enlarge and growth to occur.

For Gram-variable bacteria, the process of cell wall turnover is disjointed and more outer wall is solubilized than is accumulated at the inner face; the wall becomes thinner during rapid growing periods (Beveridge 1990). For this reason, the bacteria are more sensitive to the trauma of the Gram stain and they lyse during staining. This type of Gram-variable reaction is frequently seen in certain *Bacillus*, *Butyrivibrio* and *Clostridium* species. Because of this, these bacteria often possess an extra surface layer (S-layer; Sleytr and Beveridge 1999), which is more permeable than peptidoglycan, to help buoy up the underlying wall.

Another group of Gram-positive bacteria are also prone to lysing and staining as Gram-negative bacteria. These include members of the *Actinomyces – Arthrobacter – Corynebacterium – Mycobacterium – Propionibacterium* group (Fig. 9; Beveridge 1990). In this case, the cell wall becomes fragile at the divison site where the septum, which partitions the two dividing daughter cells, joins the side wall of each cell (Fig. 10). Because of this fragility and the cell's turgor pressure, these cells are susceptible to ''blow-out'' during Gram staining and release cytoplasmic components as well as the crystal violet-TPt complex (Fig. 10).

Archaea

The cell walls of Archaea have the widest chemical and structural range of all prokaryotic cells (König 1988). Some possess thick cell walls that resemble those of *Bacillus,* such as those of *Methanobacterium* or *Methanosarcina.* The walls of these two genera,

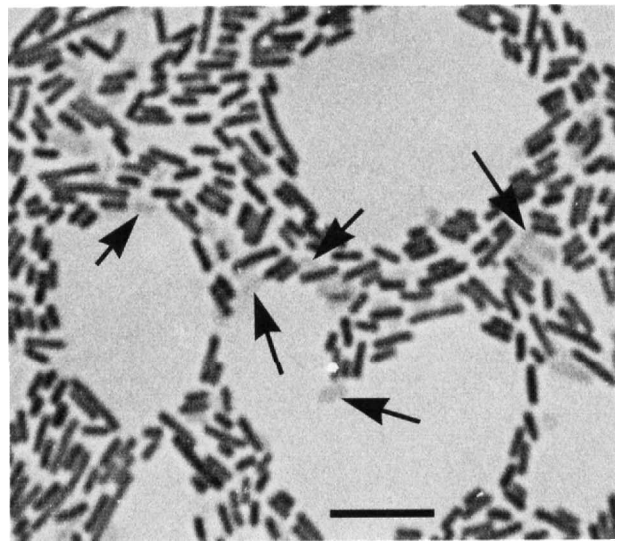

Fig. 9. Gray scale bright field light microscopy image of a *Propionibacterium acnes* exponentially growing culture showing that, although most cells stain Gram-positive, some cells are Gram-negative (the arrows point to some of these). This is due to ''blow-out'' at the septal regions (cf. Fig. 10). Scale bar = 5 μm.

Fig. 10. Thin section of the septal region of a *P. acnes* cell from the same culture as shown in Fig. 9. The arrow indicates a septum-wall junction that is suffering from ''blow-out'' so that much of the cytoplasm and crystal-violet-TPt precipitate beneath this junction is washing out. Scale bar = 50 nm.

however, are composed of entirely different substances, pseudomurein, a polymer analogous to peptidoglycan, and methanochondroitin, a polymer similar to human chondroitin, respectively. Each of these bacteria give Gram-positive staining responses (Beveridge and Schultze-Lam 1997). Other archaea, such as *Methanococcus, Sulfolobus* and *Thermoproteus,* have simple walls in that they have a single S-layer above their plasma membrane (Sleytr and Beveridge 1999, König 1998). These walls are easily disrupted by the Gram stain, the cells lyse and a Gram-negative response ensues. Some archaea give unexpected responses. For example, *Methanospirillum hungatei* has individual cells bounded by a single S-layer, but they are packaged into chains by a thick resilient sheath (also an S-layer) which is impermeable to all but the smallest molecules such as methane. The cells are separated from one another by multilamellar cell-spacers (many of the lamellae are S-layers) so that each cell is individually encased by an S-layered wall, sheath and several S-layered lamellae of the cell-spacers (Fig. 11; Beveridge et al. 1987). Typically, each chain of *Methanospirillum* is approximately nine cells long, but only the two terminal cells at each end of a filament stain Gram-positive (Fig. 11). This is because the crystal violet is too large a molecule to pass through the sheath and can only penetrate through the terminal spacers at the end of each chain of cells (Beveridge et al. 1991). Because the carbol fuchsin complexes with the surface of the sheath, the cells in the middle of the chain stain red.

Remarkably, the staining response of archaea, like bacteria, depends on the cell wall structure. The diversity of cell wall chemistry and structure in the Archaea is so broad, however, that the Gram stain is not a good taxonomic probe at this time. Perhaps as our experience with these archaeal Gram-staining factors increases, it will become a more useful tool.

Conclusions

The Gram stain retains its usefulness for most bacteria and remains an important method in microbiology. Certainly, it continues to be a primary taxonomic tool for differentiating the two fundamental types of bacterial cells, and its staining result is still a required characteristic when describing a new species. One must be careful to recognize that Gram-variable bacteria exist and that these are Gram-positive cells with problems in cell wall turnover or septation. At present, the Gram stain remains an elusive probe for accurately differentiating archaea, but in these organisms, like bacteria, the staining response depends exclusively on cell wall character.

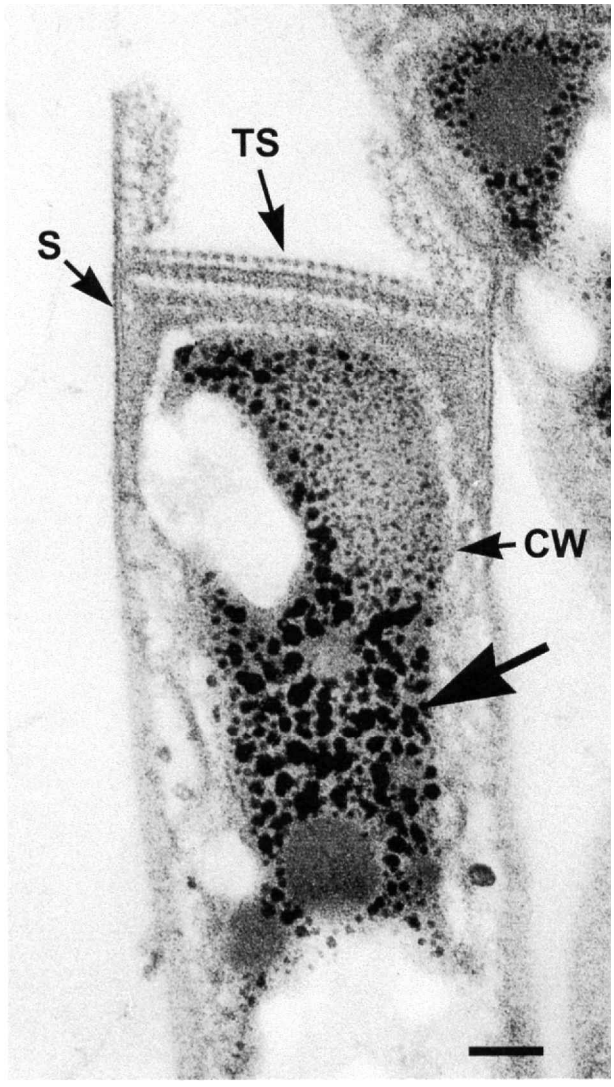

Fig. 11. Thin section of a terminal cell of *Methanospirillum hungatei* that has stained Gram-positive. The staining reagents have entered the end of the cell through the multiple lamellae of the terminal spacer (TS) so that the reagents precipitated in the cytoplasm (large arrow). The S-layered cell wall (CW) has been denatured, but the sheath (S) remains intact. The cells farther along the filament, toward the middle of the filament stain Gram-negative because the staining reagents do not penetrate that far. Scale bar = 100 nm.

Acknowledgments

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