RELATIONSHIP OF CELL WALL STAINING TO GRAM DIFFERENTIATION

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Bartholomew and Mittwer (1951) presented photographic evidence that the gram-positive staining area of bacterial cells could be fitted internally to the area demonstrated by the Dyar cell wall stain. Their conclusion was that the bacterial cell wall was not included in the gram-positive area. Lamanna and Mallette (1954) explained Gram differentiation of intact cells on the basis that crystal violet stained and formed a dye-iodine lake in the cell walls of gram-positive cells, but did not stain and therefore did not form a dye-iodine lake in the cell walls of gram-negative cells. They reported also that if normally gram-positive cells were stained with crystal violet, and then were washed with tap water, the dye was removed from the cell wall and on subsequent completion of the Gram stain these cells were gram-negative. It was their conclusion that the cell wall of intact cells must be stained with the primary dye for the cells to result in gram-positive staining, and failure of the dye to be present in the cell wall was the explanation of all gram-negative staining. Although the evidence presented in their paper was primarily for yeast cells, they reported that similar observations had been made for several species of gram-positive and gram-negative bacteria. Hence, they believed that the above basis for Gram differentiation could be generalized for all cases of gram-positive and gram-negative staining.

The present paper concerns the results of additional studies on the relationship of cell wall staining to Gram differentiation for bacterial cells. Particularly studied was the effect of a wash step following the primary dye on apparent cell size, and also the effect of this wash step on the results obtained when the Gram stain procedure was completed. It was hoped that an explanation could be found for the conflicting viewpoints as to whether or not the cell wall was included in the gram-positive staining area, and that information could be obtained concerning the validity of the Lamanna and Mallette concept of the basis for Gram differentiation.

METHODS

Air dried and heat fixed bacterial smears on glass slides were prepared from 18 hr nutrient agar slant cultures incubated at 37 C. Sufficient slides were prepared at one time for all of the work reported in this paper. The steps used for the Gram procedure were as follows. Kopeloff-Beerman crystal violet was applied for 3 min, Burke’s iodine for 2 min, 95 per cent ethanol was used as a decolorizer for 1 min, and Burke’s safranin was applied for 10 sec as a counterstain. A 2 to 3 sec distilled water wash was used between each step, and all reagents including the wash water were kept in Coplin jars into which the slides were placed. When prolonged wash steps were used, the slide was progressively transferred at 30 sec intervals from one Coplin jar to another containing fresh distilled water until the total indicated wash time had elapsed. For nigrosin-negative staining, a 2 per cent solution of water soluble nigrosin was used. This was spread over the heat fixed smears by a blood smear technique in such a manner as to produce a standard film thickness as judged by visual comparison with a reference slide. Photographs were compared of the same bacterial field after being stained by different procedures.

An apochromatic Bausch and Lomb 1.4 N. A. oil immersion objective with an aplanatic 1.4 N. A. condenser was used for photomicrography. The photomicrographic camera was a Bausch and Lomb model L adapted to use 3½ by 4½ in. Kodak Panatomic X film. An Ortho-Illuminator B (American Optical Co.) was used as a light source. Wratten filters, numbers 22 and 58, were used to increase photographic contrast for slides stained with crystal violet and safranin respectively.

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RESULTS AND DISCUSSION

Lamanna and Mallette (1954) used the ability of the cell walls of organisms to be stained with crystal violet as the primary basis for their explanation of the Gram differentiation. Their evidence for staining of the cell wall was the appearance of cells in clusters. They considered that if the individuals in a group of stained cells appeared to touch each other, the cell walls had been stained. If the individuals in a group of stained cells did not appear to touch, then the cell walls had not been stained. Using this as their index of cell wall staining, they reported that crystal violet did not stain or form a dye-iodine lake in the walls of gram-negative cells, but did stain and form a dye-iodine lake in the walls of gram-positive cells. Although their observations included 2 species of yeast, 4 species of gram-positive bacteria, and 2 species of gram-negative bacteria, almost all of the evidence for bacterial cells was omitted from their paper. It was thought that the implications of these observations as the basis for explaining Gram differentiation were sufficiently important to warrant additional studies on bacterial cells. The following experiments, therefore, were performed.

Groups of Bacillus subtilis and Escherichia coli cells were photographed and compared following nigrosin-negative staining, and following each step in the Gram staining procedure. Representative results for groups of B. subtilis cells are presented in figure 1. This group of cells was chosen for presentation because, as occasionally occurs when smears of B. subtilis are gram stained, it contained both gram-positive and gram-negative cells. The nigrosin-negative stain was used to indicate the width of the unstained cells. All of the individual cells appeared to have similar widths prior to the alcohol step. The widths observed following crystal violet staining were the same as indicated by the nigrosin-negative stain. However, following the iodine step, all of the cells appeared slightly wider than indicated by either the crystal violet or the nigrosin-negative stain. Thus, it would seem that no discernible area of any of the cells was free of the dye-iodine lake. Although cells 1, 2, 3, and 5 touched and were of similar widths following the crystal violet or iodine steps, cells 1, 2, and 3 were gram-negative upon completion of the Gram procedure. It would not have been possible to observe the results after the crystal violet or after the iodine steps and to foretell which of the cells in this group would subsequently stain gram-negative.

A similar series of photographs for cell groups of E. coli is presented in figure 2. The cells touched following both the crystal violet and the iodine steps (see arrows in figure 2). Following the iodine step, these cells appeared slightly wider than indicated by the nigrosin-negative stain. It was evident that, as was observed with the B. subtilis cells, no discernible area of the E. coli cells was free of the dye-iodine lake, and yet all of these cells were gram-negative on completion of the Gram procedure.

These results failed to reveal any demonstrable staining difference between gram-positive and gram-negative cells at any point prior to the decolorization step of the Gram procedure. Both gram-positive and gram-negative bacterial cells appeared to be completely stained following the iodine step. Therefore, the results showed that there was no necessary correlation between the formation of a dye-iodine lake in any demonstrable outer portion of the bacterial cell and the results obtained on completion of the Gram procedure.

Lamanna and Mallette (1954) reported in their work that washing with distilled or tap water of normally gram-positive cells following crystal violet staining resulted in the removal of dye specifically from the cell wall. These cells would then appear gram-negative on completion of the Gram procedure. However, little is known concerning the effects of such a wash treatment on the dye distribution and visual appearance of bacterial cells. Therefore, the next series of experiments was designed to obtain this information. Photographs were taken of the same B. subtilis cells following staining with crystal violet, and after staining and exposure to a subsequent distilled water wash for different periods of time. Typical results for a single cell are shown in figure 3. A nigrosin-negative stain was included for width comparisons. When stained with crystal violet and blotted dry, the cell was similar in width to that indicated by the nigrosin stain. When stained and then washed for increasing periods of time, the apparent width of the cell progressively decreased, and after 15 min of washing, the cell had lost about one third of its apparent width. Although no visible or photographic trace was present of the unstained
Figure 1. Gram stain of *Bacillus subtilis*. The appearance of a group of cells following each step of the Gram procedure (A–D), and nigrosin-negative staining (E). This group contained both gram-positive and gram-negative cells. The dark appearing cells were gram-positive, the light appearing cells were gram-negative.

cell material, if the cell were restained with crystal violet and blotted dry, it then appeared identical to its original width and shape. This would indicate that the structure and staining ability of the cell had not been changed by the wash procedure.

The results shown in figure 3 demonstrate that the effects of a wash step following staining were
not restricted to only the cell wall. After a 15 min wash the cell was reduced in apparent width by approximately 0.3 μ. If the value of 0.02 μ is accepted as representing the thickness of the bacterial cell wall (Chapman and Hillier, 1953), it becomes evident that dye was lost from a much greater area than just the cell wall alone. This would be expected since several workers have reported that water or salt solutions are capable of completely decolorizing the stained bacterial cell (McCalla, 1940; Bartholomew et al. 1950; Finkelstein, 1957).

One can conclude that a tap water or distilled water wash following staining would affect the total dye content of the entire cell, and not just remove dye specifically from the cell wall. These results also would explain the observations of Lamanna and Mallette (1954) concerning the appearance of demonstrable unstained areas between bacterial cells in groups, following a wash step. It is apparent that the clear areas which they observed were not due to a loss of dye from the cell wall alone, but were due to dye loss from the cell as a whole.

The wash step following the crystal violet staining also had an effect on the results obtained on completion of the Gram stain procedure. When B. subtilis cells were stained with crystal violet and then washed for 5 min, the cells were normally gram-positive on completion of the Gram procedure. This gram-positive result was obtained despite the fact that the crystal violet had been removed from a demonstrable outer area of the cell representing about 25 per cent of the total cell width. If, however, the wash step following crystal violet staining was extended to...
15 min, most of the cells were gram-negative upon completion of the Gram procedure.\(^3\)

The wash times needed to produce a gram-negative result for \textit{B. subtilis} cells in these experiments were considerably longer than the 1

\(^3\) The present authors believe that the wash step between the crystal violet and iodine is very critical and that its importance in the Gram stain procedure has not been adequately appreciated. The directions given are often vague except for those procedures which completely omit the wash min tap water wash reported by Lamanna and Mallette (1954) for yeast cells. This would indicate that the distilled water wash used in the present study was a milder decolorization procedure than the tap water wash. Finkelstein (1957) has shown that tap water is a stronger step. If the slide is washed following the crystal violet, as indicated in the Hucker modification, this treatment should be rigidly controlled and limited to 2 or 3 sec. Care at this point contributes markedly to a successful Gram differentiation.
decolorizer for basic dyes than distilled water despite the lower pH of the latter. The decolorization power of tap water is due to the presence of dissolved cations which actively replace the bound basic dye in the cells.

Figures 1 and 2 show that the gram-negatively stained (i.e., safranin stained) cells appeared much smaller than when stained with crystal violet alone, or with the dye-iodine lake. This size difference has been observed previously (Churchman, 1927; Bartholomew and Mittwer, 1951; Lamanna and Mallette, 1954). The last named authors interpreted this staining difference as representing the inability of safranin to stain the bacterial cell wall. However, due to the thinness of the bacterial cell wall this interpretation would seem improbable as an explanation for the marked size differences as observed in figures 1 and 2 of the present paper. It was shown (figure 3) that washing in distilled water, following crystal violet staining, greatly reduced observable cell size. Since a wash step usually is used following safranin staining, it could be possible that this has a bearing on the apparent small size of safranin stained cells. Experiments were conducted therefore, to determine the comparative appearances of cells stained with safranin, both without and after the application of a subsequent wash step. The results are shown in figure 4. When blot dried the safranin stained cells were comparable in size to the nigrosin-negative stained cells. However, a 5 sec wash following the safranin was sufficient to produce an apparent reduction of about one third in cell width. When crystal violet was used (figure 3), a prolonged wash step of 15 min was necessary to result in a similar size reduction. It was concluded, therefore, that the reason for the small apparent size of safranin stained cells was the rapidity with which the dye was lost during the wash step which usually is used following a staining procedure. There was no indication that crystal violet and safranin differed in their ability to be present in any single area of the cell such as the cell wall. However, it appeared that there was a difference in the affinity of these dyes for the cell material.

On the basis of the results presented in figures 1-4, it is perhaps now possible to postulate an explanation for reconciling the conflicting viewpoints of Bartholomew and Mittwer (1951) and Lamanna and Mallette (1954) concerning whether or not the cell wall must be included in the gram-positive staining area of the cell. It was shown in figures 1 and 2 that no difference in the staining of cell areas could be seen between gram-positive and gram-negative cells following the crystal violet or the iodine steps, and that differentiation occurred primarily during the subsequent decolorization. The results of figures
3 and 4 showed that the extent of the wash following staining greatly influenced the observable size of the cells. The extent of the wash following crystal violet influenced also the Gram result of gram-positive cells. If such cells were only mildly washed, they appeared gram-positive; if washed extensively, they appeared gram-negative. Therefore, the possibility existed that the size of the gram-positive staining area was dependent, not specifically on the staining of the cell wall, but on the extent of the wash step between the crystal violet and iodine steps. This possibility is particularly pertinent if one accepts 0.02 µ as the approximate thickness of the bacterial cell wall. The optical microscope would not be able to differentiate between two cells differing only in whether or not the cell wall was stained, even if these cells were in contact. Therefore, this possibility was tested by Gram staining a single group of *B. subtilis* cells, and comparing the final apparent cell width when two different wash times were used. A 5 sec wash and a 5 min wash were chosen since it was known that such treatments would not cause the cells to be gram-negative on completion of the Gram procedure. The results of this experiment are shown in figure 5. Although the cells were gram-positive with either of the wash treatments, a considerable difference could be seen in the apparent size of the cells, and also in the dye distribution within the cells. The shorter wash resulted in cells appearing to be in contact (see arrows in figure 5), and therefore, the entire cell apparently containing the dye-iodine lake. The longer wash treatment resulted in cells which were not in contact. This indicated that the cells had lost considerable dye and had failed to form the dye-iodine lake in the outer cell area. The apparent size reduction from the longer wash was about one fourth of the cell width as observed following the 5 sec wash. Hence, the width reduction was much greater than could be accounted for if dye were lost only from the cell wall. These results demonstrated that cells can lose dye from the outer area of the cell and yet be gram-positive. They are in agreement with previous results where following a 5 min wash between the crystal violet and the iodine, *B. subtilis* had lost 25 per cent of its apparent width but still stained gram-positive on completion of the Gram procedure.

The different conclusions reached by Barthol-
omew and Mittwer (1951) and Lamanna and Mallette (1954), therefore, could be explained on the basis of the effects of a wash step between the crystal violet and iodine steps of the Gram procedure. That is, if a short or no wash step were used the gram-positive staining area would appear to include the entire cell. However, if a longer wash step were used, a sufficient amount of dye could be lost from the cell and thereby result in a definite reduction in the apparent width of the gram-positive staining area. Hence, the outer areas of the cell would appear not to be included in the gram-positive staining material. The conflicting results obtained by the above authors then, can be explained on the basis of differences in experimental procedure.

One could conclude from the above results that Gram differentiation is not necessarily due to the presence or absence of crystal violet in any part of the cell, such as the cell wall. Rather, it appears that gram-positivity is dependent in part upon the presence of a certain minimal amount of dye-iodine lake in the normally gram-positive cell in order for it to resist the decolorization treatment. This does not imply that Gram differentiation is due to a greater total crystal violet content in gram-positive cells than in gram-negative cells. On the contrary, it has been shown that E. coli actually could bind more crystal violet per g cell weight than B. subtilis or Saccharomyces cerevisiae cells (Bartholomew and Finkelstein, 1954). In the opinion of the present authors, Gram differentiation of intact cells is based on the differences in the rate of dye loss during the decolorization step between gram-positive and gram-negative cells. It is believed that this rate difference is primarily due to permeability factors.

**SUMMARY**

The results presented here have demonstrated that there is no necessary correlation between the staining of the cell wall area of a cell and its subsequent Gram result. Bacillus subtilis cells could be stained gram-positively whether or not the crystal violet had been washed out of the cell wall area. Conversely, Escherichia coli cells which appeared to be completely stained by the crystal violet and the dye-iodine lake, were gram-negative on completion of the Gram procedure. It was concluded that Gram differentiation was dependent upon more complex factors than simply the staining or failure to stain the cell wall area.

The conflicting statements in the literature, concerning whether or not the cell wall was included in the gram-positive staining area, were shown to be due to differences in experimental methods. If little or no wash were used between the crystal violet and iodine reagents, the gram-positive area might include the cell wall. If a longer wash step were used at this point, the cell wall area might not be included but the cell still would stain gram-positively. If excessive washing were used, normally gram-positive cells stained gram-negatively.

An explanation was presented for the often observed smaller size of safranin stained cells as compared to cells stained with crystal violet. It was found that safranin was more easily washed from the outer areas of the cell as compared to crystal violet. For safranin stained cells a 5 sec wash resulted in an apparent size reduction of about one third. It took 15 min of washing in distilled water to achieve a similar apparent size reduction for cells stained with crystal violet. Thus, the apparent difference in cell size resulting from safranin as compared to crystal violet staining, was due to the ease with which safranin was washed from the cell following the staining step.

**REFERENCES**


