Determination of the Gram Type Using the Reaction Between Polymyxin B and Lipopolysaccharides of the Outer Cell Wall of Whole Bacteria

By J. WIEGEL*† AND L. QUANDT

Institut für Mikrobiologie der Universität Göttingen, Grisebachstr. 8, 3400 Göttingen, Federal Republic of Germany

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The polymyxin B-dependent formation of protrusions (blebs) of lipopolysaccharides on the cell wall of whole bacteria is suggested as a specific reaction for the determination of the Gram type of newly isolated bacteria. A total of 35 different bacterial species from 27 genera were studied as well as the influence of various test parameters. The reaction was specific: all 13 species of the positive Gram type gave a negative reaction, but all bacteria of the negative Gram type gave a distinct positive reaction.

INTRODUCTION

Originally, the Gram staining method with triphenylmethane and iodine was used to visualize bacteria in tissues (Gram, 1884). It was soon established that this method divided bacteria into two groups: those which retained the stain (Gram-positive) and those which could be decolorized with alcohol (Gram-negative). Several variations have been published which should lead to more reliable staining procedures (e.g. Magee *et al.*, 1975; Bartholomew, 1962; Diena *et al.*, 1962). The mechanism of the staining reaction is still not fully understood. However, it is known that the differential staining response correlates not only with differences in cell wall structure but also, in general, with many other properties (Lamanna *et al.*, 1973), including aminopeptidase activity (Cerny, 1978) and the size and regulation of the citrate synthase (Weitzman & Jones, 1975). The differentiation by Gram staining has grown in importance as a taxonomical property and a systematic term. There are a number of organisms for which the results of the Gram staining reaction are variable or, and this is more important, contradict their taxonomic classification. Therefore, a differentiation between the term describing the results of the staining reaction (Gram reaction positive/negative/variable), and the systematic term based on a classification according to biochemical properties (Gram type positive/negative), has been suggested (Wiegel, 1981). This led to the need for methods other than the Gram staining procedure. One property which completely separates organisms of the positive and negative Gram type is the presence of lipopolysaccharides (LPS) in the outer cell wall of the negative type.

In recent years, a number of publications have dealt with the determination of LPS (also termed endotoxin or pyrogen) by the Limulus-lysate test (Levin & Bang, 1968) and its variations (e.g. Maeda & Taga, 1979; Flowers, 1979; Coates, 1977; Janda & Work, 1971). This extremely sensitive test is used mainly to detect bacteria in medical fluids. Its use for taxonomic purposes may give questionable results: a slight LPS contamination of any solutions used, even in the absence of viable bacteria, can give a positive result. A specific reaction of LPS with the basic cyclic polypeptide antibiotic polymyxin B (Newton, 1956), has been documented, e.g. for *Escherichia coli* (Bader & Teuber, 1973), *Serratia marcescens* (Brown & Tsang, 1977) and *Pseudomonas aeruginosa* (Newton, 1953; Koike *et al.*, 1969). Koike & Iida (1971) demonstrated that polymyxin B forms a complex with the LPS, but does not react with lipoproteins of *E. coli* and

† Present address: Department of Microbiology, University of Georgia, Athens, GA 30602, U.S.A.

Salmonella typhimurium. The formation of a complex between LPS and polymyxin B results in the formation of blebs (protrusions) on the cell surface, which are easily seen using the electron microscope (Schindler & Teuber, 1975; Lounatmaa *et al.*, 1976; Koike *et al.*, 1969; Wiegel & Mayer, 1978). Bleb formation is not an artefact produced during fixation of detergent-weakened membranes: the blebs are evaginations of the cell surface monolayer due to the formation of the LPS-polymyxin B complex (Schindler & Teuber, 1975).

In this paper we report a standardized application of the LPS-polymyxin B reaction to various bacteria. Since the method has proved to be reliable, specific and very sensitive, we suggest that it may be used for the differentiation of Gram type positive and negative bacteria, especially for the classification of new bacteria and in cases where other tests give questionable results.

The application of this method has led to the reclassification of *Corynebacterium autotrophicum*, now *Xanthobacter autotrophicus* (Wiegel & Mayer, 1978; Wiegel *et al.*, 1978).

METHODS

Bacteria and culture conditions. The bacteria investigated are listed in Table 1. Most were obtained from the Deutsche Sammlung von Mikroorganismen (DSM). The growth conditions were as indicated in the DSM catalogue (Deutsche Sammlung von Mikroorganismen, 1977); however, if possible, liquid or agar-containing medium with peptone, yeast extract and glucose was employed.

Incubation with polymyxin B. Bacteria were suspended in 1 ml 10 mM-Tris/HCl buffer, pH 7.2, which, in the case of halophilic bacteria, was supplemented with up to 25% (w/v) NaCl to prevent lysis. Samples (0.25 ml) of this suspension were incubated at 35 °C (thermophiles at 50 °C) in the presence of 0 (control), 160 and 1000 units polymyxin B (Sigma, no. 210-A, 8000 units mg⁻¹), respectively. After 10 and 30 min the suspensions were diluted with cold (0 °C) Tris/HCl buffer (10 mM, pH 7.2) and the cells centrifuged at 3000 g for 5 min. Cells were washed once to remove free or loosely-bound polymyxin, and then resuspended in Tris/HCl buffer to give a slightly turbid suspension. This suspension was used immediately or within 8 h for electron microscopy.

The influence of various test parameters was investigated with selected bacteria. These were *Xanthobacter autotrophicus* (DSM 432, 2267), a species which has an extremely low LPS content and shows an indistinct Gram staining reaction, a '*Salmonella minnesota*' mutant with a rudimentary LPS, an *Alcaligenes eutrophus* strain (three species of the negative Gram type), and *Bacillus megaterium* as a representative of the positive Gram type. The parameters tested and the results were:

(1) Incubation time. No basic differences in bleb formation were observed between 3 and 60 min incubation. Incubation can therefore be reduced to 3–5 min if desired (see also Schindler & Teuber, 1975).

(2) Incubation temperature. No significant difference was observed when temperature was varied between 20 and 45 °C when testing mesophiles (37 to 60 °C for the thermophilic *Thermoanaerobacter ethanolicus*).

(3) Growth stage of the cells. Early- and late-exponential and early- and late-stationary growth phase liquid cultures were tested. Only the cells of the late-stationary phase tended to lyse during negative staining, otherwise the age of the cells was of little significance in bleb formation. However, the use of cells from colonies (not too old) on agar medium is recommended; all growth phases are represented and cells from the agar surface show less debris after negative staining than those from liquid cultures.

(4) Time of storage at 0–4 °C between polymyxin B treatment and negative staining. No difference was observed up to 8 h of storage, but some bacteria tended to lyse when stored for over 20 h after treatment with the high polymyxin B concentration.

(5) Test volume. Using single colonies, the test volume could be reduced to as little as 50 µl in Eppendorf reaction vials without difficulty.

(6) Polymyxin concentration. It was important to vary the concentration of polymyxin B from 0 to 2000 units ml⁻¹, as bleb formation was dependent on polymyxin B concentration (Fig. 1). For example, with *Xanthobacter autotrophicus* 80–240 units of polymyxin gave ambiguous results, but good bleb formation was obtained with 800 units. In some cases, high concentrations of polymyxin caused cell lysis (e.g. *Thermoanaerobacter ethanolicus* of the positive Gram type), or the blebs separated from the cells (e.g. *Citrobacter*). Therefore, two concentrations of polymyxin (in addition to the zero-control) are recommended: 160 and 1000 units (ml cell suspension)⁻¹.

Electron microscopy. Negative staining was performed as described by Beuscher *et al.* (1974) and Valentine *et al.* (1968) using 3% (w/v) uranyl acetate (pH 5) dissolved in water. Electron micrographs were taken with a Philips EM 301 electron microscope. Sizes were calibrated with a cross-lined grating replica. Acceleration voltage was 80 kV. Electron microscopic examination of the samples was done in a random order to minimize the possibility of biased interpretation of the bacteria examined.

Gram staining reaction. The Difco kit was used for Gram staining, but with propanol decolorization (Bartholomew, 1962).

Table 1. *Bacterial strains investigated*

(a) Bacteria of negative Gram type		
	Part in <i>Bergey's Manual</i> *	
<i>Rhodospirillum rubrum</i> HA (DSM 107)	1	
<i>Rhodopseudomonas capsulata</i> Kb1 (DSM 155)	1	
<i>Rhodopseudomonas gelatinosa</i> (DSA 149)	1	
<i>Chlorobium limicola</i> (DSA 249)	1	
<i>Xanthobacter autotrophicus</i> 7c (DSM 432); JW21; JW33 (DSM 1618); JW50	7	
<i>Azotobacter vinelandii</i> (DSM 85)	7	
<i>Beijerinckia mobilis</i> (DSM 1720)	7	
<i>Derxia gummosa</i> (DSM 723)	7	
<i>Rhizobium meliloti</i> (DSM 30135)	7	
<i>Pseudomonas pseudoflava</i> (DSM 1034)	7	
<i>Alcaligenes eutrophus</i> H16; N9A (DSM 428; DSM 518)	7	
<i>Citrobacter freundii</i> (DSM 30039)	8	
' <i>Salmonella minnesota</i> ' R595 (E. Rietschel, Freiburg)	8	
<i>Escherichia coli</i> K12 (DSM 498)	8	
<i>Chromobacterium violaceum</i> (DSM 30191)	8	
<i>Proteus mirabilis</i> (DSM 30118)	8	
<i>Bacteroides amylophilus</i> (DSM 1361)	9	
<i>Neisseria meningitidis</i> (P.R.G. Schindler, München)	10	
<i>Acinetobacter calcoaceticus</i> (DSM 586)	10	
(b) Bacteria of positive Gram type		
	Part in <i>Bergey's Manual</i>	
<i>Bacillus megaterium</i> (DSM 32)	15	
<i>Bacillus brevis</i> (DSM 1794)	15	
<i>Clostridium thermoautotrophicum</i> (DSM 1974)	15	
<i>Clostridium thermohydrosulfuricum</i> JW102	15	
<i>Sporosarcina ureae</i> (DSM 317)	15	
<i>Sporosarcina</i> sp. (courtesy D. Claus, Göttingen)	15	
' <i>Thermoanaerobium brockii</i> ' (DSM 1457)	—	
' <i>Nocardia opaca</i> ' 1B (DSM 427)	17	
(c) Bacteria of questionable Gram type		
	Gram staining reaction	Gram type
<i>Xanthobacter autotrophicus</i> (DSM 432, 1393, 1618, 2267) (← ' <i>Corynebacterium autotrophicum</i> ')	± / +	negative
<i>Xanthobacter flavus</i> (DSM 338) (← ' <i>Mycobacterium flavum</i> ' 301)	+	negative
' <i>Corynebacterium nephridii</i> ' (DSM 21150) (→ ?)	±	negative
<i>Thermoanaerobacter ethanolicus</i> (ATCC 31550)	— / ±	positive
<i>Bacillus lentimorbus</i> (DSM 1797)	—	positive
<i>Desulfotomaculum nigrificans</i> (DSM 574)	—	positive
<i>Clostridium thermosaccharolyticum</i> (ATCC 7956)	—	positive
<i>Clostridium cellobioparum</i> (DSM 1351)	—	positive
<i>Halobacterium halobium</i> (DSM 670)	—	zero

* Buchanan & Gibbons (1974).

Aminopeptidase test. The test of Cerny (1976) was employed; the indicator strips were a gift from Merck.*Limulus amoebocyte lysate test.* The determination of LPS with the Limulus-lysate test was done according to Sigma Bulletin 210 using the E-toxate R test kit.

RESULTS

Examination of bacteria

Table 1(a) lists all the organisms investigated which are known for their definite negative Gram type and Gram-negative staining reaction. These bacteria all gave definite bleb formation with polymyxin B, whereas the organisms in Table 1(b), representatives of the positive Gram type, gave no reaction with polymyxin B.

Fig. 1. Bacteria of the negative Gram type treated with increasing amounts of polymyxin B. (a, b, c) '*Salmonella minnesota*' R595 treated with 0 (control), 160 or 1000 units, respectively. (d, e, f) *Citrobacter freundii* treated with 0, 160 or 1000 units, respectively. The bar markers represent 0.5 μm.

Table 1(c) gives examples of bacteria for which a Gram staining reaction opposite to their Gram type (systematic classification) is found. Here the polymyxin B-LPS reaction was in accordance with the systematic Gram type. Table 1(c) also includes three organisms of the so-called archaebacteria: a halobacterium and two methane-producing bacteria. This group has totally different cell wall types, i.e. a pseudomurein polymer or a peptide polymer, but no murein sacculus, and the cells do not contain LPS (Kandler, 1979; König & Kandler, 1979). Consequently we could not find bleb formation. Thus a differentiation between a positive and a zero Gram type according to Wiegel (1981) was not possible; this differentiation can only be achieved by cell wall analysis. However, the typical resistance to the common antibiotics that interfere with the synthesis of the murein sacculus may give the first indication of an archaebacterium (Hilpert *et al.*, 1981).

Morphology of the protrusions and interfering structures

Different organisms gave different blebs, presumably due to the quantity and structure of LPS in the cell wall (Figs 2, 3). The size of the blebs varied from 10 to over 100 nm in length, depending on the organism and the polymyxin concentration.

Although the morphology of the blebs varied, there was no problem in differentiating between polymyxin-dependent bleb formation and other structures of the outer cell wall layers when a control with 0 µg polymyxin was included. For example, *Neisseria meningitidis* always shows bleb-like structures of the outer cell wall (Morse *et al.*, 1979), but even the so-called polymyxin-insensitive strains (courtesy of Dr P. R. G. Schindler, München, F.R.G.) gave definite positive responses with 160 and 1000 units polymyxin B. Also, the 'polymyxin-resistant' *Proteus mirabilis* gave a positive reaction.

The possibility of interference by slime structures was tested. The slime-forming strains JW33, 7c and the slime-less mutant 7c/SF of *Xanthobacter autotrophicus* (representative of the Gram type negative bacteria) and *Bacillus megaterium* (an example of a slime-producing Gram type positive bacterium) were selected. Slime could be clearly differentiated from blebs in the negatively stained preparation examined at higher magnification (Fig. 3). High concentrations of polymyxin B may damage the cytoplasmic membrane, resulting in a release of cytoplasm. However, as already shown with *Pseudomonas aeruginosa* (Koike *et al.*, 1969), the released cytoplasm could be easily differentiated from blebs produced by the reaction of LPS with polymyxin B.

'*Salmonella minnesota*' R595 (chemotype Re), a mutant which has one of the most rudimentary LPS-structures so far known, gave a definite reaction with polymyxin B (Fig. 1 a-c). However, the number and size of the blebs were reduced, in accordance with the results of Schlecht & Fromme (1980) and Schindler & Teuber (1975) using a mutant of the chemotype Rc of *S. typhimurium*. HsuChen & Feingold (1973) reported that polymyxin B reacted with phosphatidylethanolamine in artificial membranes. Species of *Bacillus* and *Sporosarcina* contain high amounts of this compound (Ikawa, 1967; Lombardi & Fulco, 1979), but we found no interfering reactions (i.e. no bleb formation) with *Sporosarcina* sp., *S. ureae*, *Bacillus megaterium*, *B. lentimorbus* and *B. brevis*. This agrees with the results of Imai *et al.* (1975) with the lipids of *Streptococcus sanguis*.

Prefixation of the organisms with glutaraldehyde or formaldehyde did not result in a good staining reaction and/or bleb formation, and is not recommended for the standard test.

Comparison with the Limulus-lysate test

The bacteria listed in Table 1(c) were subjected to the Limulus-lysate test (Sigma, E-toxate test). The results agreed with those obtained by the polymyxin-LPS test. However, it proved difficult to perform the gel-forming Limulus-lysate test with bacteria producing copious amounts of slime, such as *Xanthobacter* strains, *Rhizobium* (grown under N₂-fixation conditions) and *Bacillus megaterium*. These bacteria had to be washed in saline of pH 10.5-11.0 to remove the slime. Gel formation was poor for *Xanthobacter* and *Rhizobium*. It is difficult to distinguish between a very low LPS content as found in these organisms and minor contaminations of LPS

Fig. 2. Bacteria treated with 1000 units or 0 units (control, as inset) polymyxin B. (a-c) Gram type negative with bleb formation: (a) *Bacteroides amylophilus*, (b) *Rhizobium meliloti*, (c) *Chlorobium limicola*. (d-f) Gram type positive with no bleb formation: (d) *Bacillus brevis*, (e) *Clostridium cellobioparum*, (f) '*Nocardia opaca*' (white areas due to cell shrinkage following evaporation of negative stain). The bar markers represent 0.5 μm ; inset magnification is identical with that of the respective main figure.

Fig. 3. Comparison of blebs and slime. (a) Blebs, *Xanthobacter autotrophicus* JW33 (DSM 1618); (b) blebs, *Citrobacter freundii*; (c) slime, *Bacillus megaterium*. The bar markers represent 0.2 µm.

or LPS-containing bacteria; some batches of yeast extract gave a positive reaction with the Limulus-lysate test.

Peptidase test versus the polymyxin-LPS test

Cerny (1976) suggested the determination of the peptidase reaction as a possible replacement for the Gram staining reaction. This method gave questionable or contradictory results with respect to the Gram type of seven species: *Xanthobacter autotrophicus*, *Alcaligenes eutrophus*, *Derxia gummosa*, *Beijerinckia mobilis*, *Chlorobium limicola*, *Bacillus brevis* and *Sporosarcina ureae*. These results are presumably due to factors leading to questionable judgements of the reactions e.g. pigment formation (Otte & Tolle, 1980), slime production and/or low peptidase activity. Since the test is based on the development of a yellow colour, bacteria with yellow pigmentation and a low peptidase activity would give an indistinct reaction.

KOH test versus the polymyxin-LPS test

According to Gregersen (1978) only Gram type negative bacteria are lysed when cells are mixed with a 3% (w/v) solution of KOH. Due to the liberation of DNA and RNA the cell suspension becomes viscous. If the reaction is positive, the viscous mixture follows an inoculation loop 0.5 to 2 cm from the surface. Many of the bacteria listed in Table 1 (a, b) gave a reaction according to their Gram type when subjected to the KOH test. However some (e.g. *Xanthobacter autotrophicus*, Gram type negative, or *Bacillus megaterium*, Gram type positive) gave questionable or wrong results. With several strains of *X. autotrophicus* the controls without KOH treatment were more viscous than the treated cells. *Xanthobacter* might be more stable to KOH than other negative Gram type organisms, perhaps due to an additional capsule-like polymer (H. König, O. Kandler & J. Wiegel, unpublished). This test also gave an indistinct reaction with some extreme thermophilic strains: *Thermoanaerobacter* and '*Thermoanaerobium*', although of the positive Gram type, tended to lyse, thus giving at least a weak positive reaction. Therefore, the test of Gregersen (1978) does not seem to be suitable for the systematic characterization of uncertain isolates from soil or extreme environments or of some slime-producing bacteria, although it might be a rapid and convenient method for routine analysis.

Internal controls

Bacteria with a conspicuous morphology can be recommended as an internal control for bleb formation by mixing these with the suspension of bacteria to be treated with polymyxin B. We found no cross-reactions if such mixed cultures were analysed. *Bacillus megaterium*, growing as large cells containing no LPS, and *Xanthobacter autotrophicus* JW21, which grows typically as large branched cell-forms on succinate-supplemented agar, and contains very low amounts of LPS, are suitable as internal control organisms. They are easily grown on nutrient broth agar plates supplemented with 0.1% succinate and can be mixed with the test organism of another morphology in appropriate ratios for the incubation with polymyxin B.

DISCUSSION

Although Newton (1956) has shown that polymyxin B is a surface-active compound which can alter the cell permeability of various organisms, there is no evidence that it produces blebs or bleb-like structures on whole cells with components other than LPS (which is only formed by bacteria of the negative Gram type). More than 30 bacterial species, from various genera described in different Parts of *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons, 1974), have been investigated. All bacteria of the negative Gram type tested gave a positive polymyxin B reaction (i.e. bleb formation); all those of the positive Gram type, irrespective of the results of the Gram staining reaction, gave no bleb formation. This demonstrates that the reaction is apparently specific. Therefore electron microscopic examination of the formation of a polymyxin B-LPS complex, which causes a typical evagination of the outer cell wall leaflet, is a valid method in systematic and taxonomic studies for distinguishing between positive and negative Gram type bacteria.

It should be stressed that adapted strains of *Pseudomonas aeruginosa* grown in the presence of 6000 units polymyxin gave no significant bleb formation; however, if, after such adaptation, these cells were grown in the absence of polymyxin, polymyxin sensitivity was recovered (Gilleland & Murray, 1976). Nicas & Hancock (1980) reported that the same organism showed increased polymyxin resistance when grown under Mg^{2+} limitation. Greenwood (1975) isolated an *Escherichia coli* strain which was adapted to 24 μg (about 160 units) polymyxin. From these results and from the existence of the *Salmonella typhimurium* mutants obtained by Vaara (1981) one can expect that bacteria might exist with an altered lipid A component or outer cell wall which barely reacts with polymyxin B.

Furthermore, we have not proved by analysing the composition of the blebs whether their formation was really due to the interactions of polymyxin B with LPS or with other components. However, although it has been reported that polymyxin reacts with phospholipids and cytoplasmic membranes (Teuber, 1973; Imai *et al.*, 1975), none of the bacteria of the positive Gram type investigated showed bleb formations similar to those described for the bacteria of the negative Gram type. Therefore, further investigations are required of bacteria of the positive Gram type, and a detailed biochemical analysis of the blebs induced by polymyxin on the outer cell wall of bacteria of the negative Gram type.

The polymyxin B-LPS test offers several advantages over methods published for the differentiation of bacteria of the negative Gram type from those of the positive Gram type as follows. (1) The Gram type classification is based on the presence or absence of an essential cell wall component of the negative Gram type bacteria, as opposed to the activity or properties of enzymes such as peptidase or citrate synthase. (2) Direct examination of single cells excludes a false reaction by minor contaminants, as is possible with other tests for LPS. (3) The assay is very sensitive, as only a single colony is required and an extremely low content of LPS or the presence of a rudimentary LPS still gives definite results. (4) The test is insensitive to interfering substances such as slime, excreted cytoplasm and apparently to lipoproteins. Modifications in temperature, pH, incubation time and variation of salt concentrations (up to 25%, w/v) did not affect the test. (5) The analysis is independent of the growth phase of the cells, although a sample from an agar plate culture is recommended. (6) The assay of several strains can be completed within an hour, at the same time as the electron microscopic examination of the flagellation

type. (7) No expensive, unstable compounds such as those for the Limulus-lysate test are required, and a time lapse of several hours between the incubation with polymyxin B and electron microscopic examination has no significant influence, since the blebs formed are quite stable. The negatively stained samples can be stored for later re-examination. (8) No LPS has to be isolated prior to the test, as required for the test of Ulitzur *et al.* (1979).

A light microscopic examination of the bacteria tested using fluorescent derivatives of polymyxin (Newton, 1955; Schindler & Teuber, 1975) is not recommended, since the direct examination of bleb formation is eliminated. Thus a differentiation between adsorption to other components such as lipoproteins or slime and an actual complex formation between polymyxin B and small amounts of LPS in whole cells could not be guaranteed. Also, the use of polymyxin B-containing agar media, on which LPS-containing bacteria cause a precipitation zone around the colony, lacks the specificity and the ease for direct microscopic examination. However, growing the bacteria on polymyxin B-containing agar might serve as a good preliminary test.

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