Composition of Cell Walls of Some Gram-Negative Cocci

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SUMMARY

Cell walls isolated from six species of *Neisseria* contained 17 amino acids, 6.3-8.3% mucopeptide, and 11.4-30.0% lipid. *Veillonella parvula* cell walls were similar, differing principally in the presence of 24.5% mucopeptide. The mucopeptide of all seven Gram-negative cocci contained glucosamine, muramic acid, glutamic acid, alanine, glycine and DL-diaminopimelic acid as principal components; the mucopeptide from *V. parvula* cell walls also contained an unidentified ninhydrin-reacting component. The marked similarity of the cell walls of the Gram-negative cocci and those of Gram-negative bacilli indicates a closer relationship between these two groups than between the Gram-negative and Gram-positive cocci.

INTRODUCTION

On the basis of progressive changes in several properties, Bisset (1959) arranged the following genera of Gram-positive and Gram-negative cocci in a supposed phylogenetic series which started with *Sarcina*, passed through *Micrococcus* and *Staphylococcus* to *Neisseria*, and terminated with *Veillonella*. In an attempt to avoid relating two such distinct groups as the Gram-positive and Gram-negative bacteria, he claimed that *Neisseria* and *Veillonella* were only superficially Gram-negative (Bisset, 1959, 1962). In view of the marked chemical and structural differences between the cell walls of Gram-positive and Gram-negative bacteria (Salton, 1960; see also Murray, 1962; Perkins, 1963) Bisset's claim implies that the cell walls of *Neisseria* and *Veillonella* would be of the Gram-positive type. However, apart from data on the presence of lipid and amino sugar in the cell walls of *Neisseria catarrhalis* (Salton, 1963), little information is available on the chemical composition of the cell walls of Gram-negative cocci. We therefore examined some of the more important components of cell walls isolated from *Veillonella parvula* and six species of *Neisseria*.

METHODS

Organisms and growth conditions. *Veillonella parvula* no. 10,790 and *Sarcina lutea* no. 9341 were obtained from the American Type Culture Collection. *Neisseria animalis* no. 10,212, *N. catarrhalis* no. 3622, *N. caviae* no. 10,293, *N. flavescens* no. 8263, *N. pharyngis* no. 4591, *N. pharyngis-flavus* no. 4590, *Staphylococcus saprophyticus* no. 7291 and *Micrococcus lysodeikticus* no. 2665 were obtained from the National Collection of Type Cultures (Colindale, London). *Escherichia coli* CL 30 was obtained from Dr B. A. D. Stocker and *E. coli* 173-25, auxotrophic for LL- or DL-diaminopimelic acid (Davis, 1952; Hoare & Work, 1955), from Dr J. Leder-

berg. The bacteria were stored as lyophilized cultures (Annear, 1962) and maintained on meat infusion agar (Mackie and McCartney's Handbook, 1960) supplemented, where necessary, with 10% (v/v) defibrinated horse blood or diaminopimelic acid 10 µg./ml.

Veillonella parvula was grown anaerobically (McIntosh and Fildes jar) at 37° for 18–24 hr in 100 ml. batches of a modification of the medium of Rogosa (1956) which contained per litre: Trypticase (Baltimore Biological Laboratory Inc.), 10 g.; yeast extract (Baltimore Biological Laboratory Inc.), 5 g.; sodium lactate (85%), 10 g. All other bacteria were grown at 37° for 18–24 hr in 400 ml. batches of meat infusion broth (supplemented with 10% (v/v) horse serum for *Neisseria animalis*, *N. pharyngis* and *N. pharyngis-flavus*) in 1-l. Erlenmeyer flasks on a reciprocal shaker (Kantorowicz, 1951).

Isolation of cell walls. Bacteria were harvested in a refrigerated centrifuge and washed three times with cold distilled water. Portions (5–10 g.) of packed wet bacteria and 10–25 g. glass beads (Ballotini no. 12) were placed in the capsule of a cell disintegrator (Nossal, 1953) and shaken for three 1 min. periods, the capsule being cooled in ice-water between runs. Phase-contrast microscopy showed that almost all the bacteria were broken by this treatment. The capsule was washed out with 50 ml. cold distilled water and the supernatant fluid decanted after the glass beads had settled. The beads were washed three times with distilled water and the combined washings centrifuged at 2000g for 20 min. to remove unbroken bacteria, steel particles and other debris. The supernatant fluid was centrifuged at 20,000g for 15 min. and the deposited cell walls washed three times with distilled water, frozen and dried *in vacuo* over P₂O₅.

Preparation of mucopeptide fraction. The mucopeptide fraction of the cell wall was isolated by a modification of the method of Schocher, Bayley & Watson (1962). Lyophilized cell walls were suspended in 20% (w/v) sodium dodecyl sulphate and incubated at 37° for 18 hr, with gentle shaking. The suspension was centrifuged at 20,000g for 30 min., and the deposited mucopeptide fraction freed from sodium dodecyl sulphate by washing with distilled water at least five times; the fraction was then frozen and dried *in vacuo* over P₂O₅.

Analytical methods

Sodium dodecyl sulphate was estimated spectrophotometrically by the method of Graham & Whitney (1959).

Paper chromatography was used to determine the amino acids and amino sugars present in acid-hydrolysed cell walls and mucopeptide fractions. Hydrolysis was done in sealed tubes with 6 N-HCl at 105° for 18 hr; subsequently the HCl was removed *in vacuo* over solid NaOH and P₂O₅ at 37°, and the dried residue dissolved in distilled water. Single-dimensional descending chromatography on Whatman no. 4 paper was done with the following solvent systems: (A) *n*-butanol + acetic acid + water (12 + 3 + 5, by vol.); (B) *n*-butanol + pyridine + water + acetic acid (60 + 40 + 30 + 3, by vol.; Primosigh, Pelzer, Maass & Weidel, 1961); (C) methanol + water + 10 N-HCl + pyridine (80 + 17.5 + 2.5 + 10, by vol.; Rhuland, Work, Denman & Hoare, 1955). Two-dimensional ascending chromatography was done on Whatman no. 1 paper with methanol + water + pyridine (80 + 20 + 4, by vol.) as the first solvent and *tert*.-butanol + methylethylketone + water + diethylamine (40 + 40 +

20 + 4, by vol.) as the second solvent (Redfield, 1953). Amino acids and amino sugars were detected by spraying with ninhydrin (0.2%, w/v, in acetone) and heating at 100° for 3 min. Amino sugars were confirmed with a modified Elson-Morgan reagent (Smith, 1958) and alkaline silver nitrate (Smith, 1958).

Isomers of diaminopimelic acid were identified by chromatography in solvent C (Rhuland *et al.* 1955), and by the growth response of *Escherichia coli* 173-25 to the diaminopimelic acid contained in discs cut from unstained strips of chromatograms developed with solvent A.

Total lipid was determined by ether extraction of cell-wall preparations after hydrolysis in 6 N-HCl for 4 hr at 100° (Salton, 1953).

RESULTS

In addition to the cell walls of the seven species of Gram-negative cocci, we also examined, for reference purposes, the cell walls of three species of Gram-positive cocci and a typical Gram-negative bacterium.

The mucopeptide content for each of the eleven species of bacteria is shown in Table 1. The walls of the six species of *Neisseria* gave an average mucopeptide yield of 6.9%; a value very close to that obtained for the *Escherichia coli* CL 30. In comparison, *Veillonella parvula* cell walls contained 24.5% mucopeptide, that is about 3.5 times more than the other Gram-negative bacteria; nevertheless, this corresponded to only about one-third of the amount found in the three Gram-positive cocci. (The estimate of 24.5% mucopeptide in cell walls of *V. parvula* was obtained by pooling the results of two independent experiments which gave 23.1% and 26.4% mucopeptide, respectively; a total of 143.4 mg. dry weight cell wall were analysed.) However, there are two possible sources of error in the determination of the proportion of mucopeptide in the cell wall: (i) adsorption of sodium dodecyl sulphate on to the mucopeptide (Putnam, 1948); (ii) loss of mucopeptide during the repeated washings following sodium dodecyl sulphate treatment of cell walls. Examination of cell walls of *Micrococcus lysodeikticus* following sodium dodecyl sulphate treatment and subsequent washings revealed no detectable (less than 0.5%) bound sodium dodecyl sulphate; on the other hand, an incubation and washing régime equivalent to that used for the isolation of mucopeptide but, with the sodium dodecyl sulphate omitted, resulted in losses of up to 15% of cell-wall preparations of *Neisseria animalis*, *N. caviae* and *Staphylococcus saprophyticus*. Thus, because of losses during washing, our estimates for the proportion of mucopeptide in cell walls are probably less than the true values.

Chromatography of acid hydrolysates of cell walls of *Veillonella parvula*, the six *Neisseria* species and of *Escherichia coli* showed the following amino acids and amino sugars: diaminopimelic acid, arginine, lysine, aspartic acid, glutamic acid, histidine (trace), glycine, serine, alanine, proline, tyrosine, valine, methionine (trace), isoleucine, leucine, phenylalanine, threonine, muramic acid, glucosamine (see Table 1). Cell walls of *V. parvula* also contained an unidentified ninhydrin-reacting constituent; this material, like diaminopimelic acid, was not always obvious in chromatograms of cell-wall hydrolysates because of its relatively low concentration. The mucopeptide isolated from cell walls of the six *Neisseria* species and of *Escherichia coli* contained diaminopimelic acid, alanine, glutamic

Table 1. *Composition of cell walls and mucopeptide fraction of Gram-negative and Gram-positive bacteria*

Bacteria	No. of amino acids in cell walls	Mucopeptide (% of dry wt. of cell wall)	Principal amino acids present in mucopeptide					Total lipid (% of dry wt. of cell wall)
			Glutamic acid	Alanine	Lysine	Diamino-pimelic acid	Glycine	
Gram-negative								
<i>Escherichia coli</i>	17	7.2 (103.1)†	+	+	—	+	—	15.6 (86.2)†
<i>Neisseria animalis</i>	17	6.4 (58.2)	+	+	—	+	—	16.8 (163.6)
<i>N. caviae</i>	17	6.4 (273.0)	+	+	—	+	—	30.0 (67.0)
<i>N. catarrhalis</i>	17	7.8 (127.6)	+	+	—	+	—	.
<i>N. flavescens</i>	17	6.3 (37.8)	+	+	—	+	—	.
<i>N. pharyngis</i>	17	6.6 (56.4)	+	+	—	+	—	.
<i>N. pharyngis-flavus</i>	17	8.3 (87.4)	+	+	—	+	—	11.4 (115.7)
<i>Veillonella parvula</i>	17*	24.5 (143.4)	+	+	—	+	—	27.0 (59.0)
Gram-positive								
<i>Staphylococcus saprophyticus</i>	4	79.5 (37.4)	+	+	+	—	+	0 (58.0)
<i>Micrococcus lysodeikticus</i>	4	96.3 (93.5)	+	+	+	—	+	.
<i>Sarcina lutea</i>	3	68.0 (29.4)	+	+	+	—	—	.

* An unidentified ninhydrin-reacting component was also present in the cell wall and mucopeptide of *Veillonella parvula*.

† The figures in parentheses show the mg. dry wt. cell wall analysed; . = not done.

acid, muramic acid and glucosamine as principal components (see Table 1); all other amino acids were either absent or present only in trace amounts. In addition to the amino acids and amino sugars found in the mucopeptide of *E. coli* and the neisserias, *V. parvula* also contained the previously mentioned unidentified ninhydrin-reacting component now present in relatively high concentration. The unknown component formed a single spot in single-dimensional chromatograms, but in the two-dimensional system, although it remained as a single spot at the origin after development with the first solvent, two spots (R_F values 0.05 and 0.13, respectively) were observed with the second solvent.

As expected, treatment with sodium dodecyl sulphate produced no obvious qualitative changes in the composition of the cell walls of the three Gram-positive cocci. The principal components detected in the cell walls of these three cocci were lysine, glutamic acid, alanine, muramic acid and glucosamine; the walls of *Staphylococcus saprophyticus* and *Micrococcus lysodeikticus* also contained glycine (see Table 1), and there were trace amounts of serine and phenylalanine in *S. saprophyticus*.

The diaminopimelic acid from *Veillonella parvula* and all six *Neisseria* species showed an R_F value corresponding to the DL or DD isomers in chromatograms developed with solvent C and permitted the growth of *Escherichia coli* 173-25. This indicated the presence of the DL isomer in the walls of all seven Gram-negative cocci examined.

All four species of Gram-negative cocci examined contained considerable amounts of lipid (Table 1): *Neisseria caviae* and *Veillonella parvula* cell walls contained about twice the amount present in cell walls of *Escherichia coli* and the other two neisserias. No lipid was detected in the cell walls of *Staphylococcus saprophyticus*.

DISCUSSION

In general, the cell walls of Gram-positive bacteria are characterized by a high proportion of mucopeptide, the presence of a few amino acids, and a low lipid content; in addition, the majority of such members of the family Micrococcaceae as have been examined contain lysine (and no diaminopimelic acid) and glycine as principal components of the cell-wall mucopeptide. On the other hand, the cell walls of Gram-negative bacteria contain a small amount of mucopeptide, a wide range of amino acids, and a substantial amount of lipid (see Salton, 1960; Perkins, 1963). In respect of the foregoing criteria, our results for *Escherichia coli* and the three species of Gram-positive cocci examined agree with previously published data, except that we were unable to detect glycine in the mucopeptide of the *Sarcina lutea* (Weidel, Frank & Martin, 1960; Perkins, 1963). Further, the cell walls of the six *Neisseria* species in so far as we have examined them, show no affinity with the cell walls of the Gram-positive cocci, but closely resemble those of the Gram-negative bacteria as typified by *E. coli* (see Table 1). *Veillonella parvula* differed only in having a somewhat higher mucopeptide content, but still well below that commonly found in Gram-positive cocci.

The cell walls of Gram-positive and Gram-negative bacteria are just as distinctive structurally as they are chemically. In thin section, the former exhibit a homogeneous outer layer adhering to the plasma membrane (see Glauert, 1962; Murray,

1962); on the other hand Gram-negative bacteria, including species of *Neisseria* and *Veillonella*, show a corrugated triple-layered membrane overlying what seems to be the rigid layer of the wall (Murray, 1963; Murray, Reyn & Birch-Andersen, 1963; Bladen & Mergenhagen, 1964). As a result of the differences in structural organization of the cell walls, the surface of Gram-positive bacteria appears smooth and that of Gram-negative bacteria markedly rugose (Zwillenberg, 1964); the *Veillonella* species examined by Bladen & Mergenhagen (1964) showed a rugose surface.

A prominent feature of the sections of the *Veillonella* examined by Bladen & Mergenhagen (1964) was the unusually thick (70Å) 'solid membrane' lying between the outer triple-layered membrane and the plasma membrane. If this middle membrane is the rigid mucopeptide layer, as suggested by Bladen & Mergenhagen, then our finding of at least 25% mucopeptide in *Veillonella* cell walls is not surprising. In view of the comparatively lower mucopeptide content of walls of all six *Neisseria* species a corresponding decrease in the thickness of the rigid layer would be expected. In fact, the rigid layer in *Neisseria* appears to be 20–30Å in thickness (Dr R. G. E. Murray, personal communication).

Thus the previously demonstrated structural relationship between the genera *Veillonella*, and *Neisseria* and other Gram-negative bacteria is complemented by the chemical data obtained in the present work. This relationship is strengthened by the close affinity of the genus *Neisseria* to the rod-shaped Gram-negative bacteria shown in the numerical taxonomic survey of Sneath & Cowan (1958). Collectively, these observations would appear to refute Bisset's proposal for the derivation of the Gram-negative cocci from their Gram-positive counterparts (Bisset, 1959, 1962). Indeed, *Veillonella*, with a relatively thick rigid layer and the ability under certain conditions to divest itself of its outer triple-layered membrane and so structurally mimic the Gram-positive cocci (Bladen & Mergenhagen, 1964), could represent a transitional stage in the development of the typically Gram-positive cocci from the typically Gram-negative cocci. Alternatively, the gross morphological resemblance between the Gram-negative and Gram-positive cocci could be the result of convergent evolution rather than direct derivation.

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