Composition of Cell Walls of Ageing *Pseudomonas aeruginosa* and *Salmonella bethesda*

By F. M. COLLINS

Department of Microbiology, University of Adelaide, Adelaide, South Australia

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SUMMARY

Anaerobic cultures of *Pseudomonas aeruginosa* died rapidly in the absence of nitrate and death was normally followed by extensive autolysis. A mutant which did not undergo extensive autolysis was isolated. Anaerobic cultures of *Salmonella bethesda* did not die or lyse even after prolonged incubation. The protein and lipid content of the parent *P. aeruginosa* cell walls altered during ageing in contrast to *S. bethesda* walls which did not alter greatly as the organisms aged. The amino acid and amino sugar content of the three strains was determined. The diaminopimelic acid, glycine, alanine, glutamic acid, glucosamine, muramic acid and glucose content of the parent cell walls decreased by 50 % as the organisms aged. The mutant strain of *P. aeruginosa* and *S. bethesda* walls showed no such change in ageing. Chemical changes similar to ageing could be produced in the cell walls of *P. aeruginosa* by incubation with an 'autolysin' obtained from old cultures of the parent strain.

INTRODUCTION

Pseudomonas aeruginosa is a strict aerobe which can grow under anaerobic conditions when nitrate is added to the medium to act as an alternative to oxygen as hydrogen acceptor. Under these conditions rapid growth occurs until the exhaustion of the nitrate supply after which all growth ceases and the culture rapidly passes into the decline phase. Such cultures afford an opportunity to examine some of the changes which occur when non-proliferating organisms are held in an otherwise non-toxic environment.

Rapid death and extensive autolytic changes have previously been observed in anaerobically incubated cultures of *Bacillus subtilis* (Nomura & Hosoda, 1956). Kaufmann & Bauer (1958) noted rapid lysis of anaerobically incubated cultures of *B. subtilis* and isolated an autolytic enzyme from the medium. They suggested that this 'autolysin' was released in an active form by the organisms when the normal cellular respiration was inhibited. Lysis of several species of sporing bacilli has been examined in some detail (Strange, 1959) and a bacterial lysozyme has been shown to be involved in some instances (Richmond, 1959). The cell walls of Gram-negative bacilli are more complex in nature than those of the Gram-positive bacteria (Salton, 1958, 1960) and in general, lysozyme treatment of Gram-negative bacteria does not result in cell lysis except under special conditions (Warren, Gray & Bartell, 1955; Repaske, 1958). A number of Gram-negative bacteria undergo autolysis on ageing, but for the most part little information is available on the mode of action of the enzymes involved. The similarities between

the rapid death and autolysis observed in the anaerobic cultures of *Pseudomonas aeruginosa* and that reported for the corresponding anaerobically incubated *Bacillus* cultures suggested that a similar mechanism may be responsible for both phenomena. An 'autolysin' has been found in the culture medium of old *P. aeruginosa* cultures and comparison of the composition of cell walls isolated from ageing organisms with that of young cell walls treated with the enzyme showed similar changes to occur in both.

METHODS

Organisms. *Pseudomonas aeruginosa* NCTC 6750 and *Salmonella betesda* strain Md. 2 (kindly supplied by Dr N. Atkinson, University of Adelaide) were grown on nutrient agar slopes at 37° for 24 hr and stored at 4°. Subcultures were prepared from these slopes from time to time. A mutant strain of *P. aeruginosa*, which was not subject to extensive autolysis on ageing, was isolated from an old broth culture of NCTC 6750.

Medium. The growth medium contained the following nutrients: acid hydrolysate of casein (Oxoid Ltd., England), 5.0 g.; sodium nitrate, 5.0 g.; ammonium sulphate, 1.0 g.; magnesium sulphate, 0.1 g.; trace elements solution, 1.0 ml. (Meiklejohn, 1950); 0.067 M-2-amino-2-hydroxymethyl propane-1,3-diol (Tris) buffer (pH 7.0), 1 l. The medium was autoclaved at 115° for 15 min.

Cultural conditions. The medium was dispensed in 1 l. quantities in screw-capped serum bottles. Each bottle was inoculated with approximately 10⁷ viable bacteria. The air was removed with a vacuum pump after the insertion of a sterile hypodermic needle through the rubber wad. A positive gas pressure was introduced by returning sterile pure nitrogen into the bottle and removing the needle. The positive pressure ensured that air did not leak into the cultures during the prolonged incubation period. All cultures were incubated at 37°.

Counting methods. Viable bacteria were counted by the method of Miles & Misra (1938). Total counts were made by the Williams (1952) method.

Quantitative analyses. Total-nitrogen, protein (Folin-Ciocalteu method and biuret method), total reducing sugar, total hexosamine and total phosphorus determinations were made by methods described by Kabat & Mayer (1961). Nitrate was estimated as previously described (Collins, 1956).

Individual sugars were identified chromatographically after hydrolysis of the walls with 2 N-sulphuric acid at 100° for 2 hr. The descending chromatograms were run in ethyl acetate + acetic acid + water (3 + 1 + 3 by vol.) for 18 hr at 20°. The papers were sprayed with aniline phthalate reagent (Cramer, 1954) and the sugar content of each spot estimated by the method of Baar (1954). Muramic acid was estimated by the method described by Strange & Kent (1959).

Amino acids were identified, after hydrolysis of cell walls with 6 N-HCl at 105° for 18 hr, by two-dimensional chromatography as described by Salton (1953). The intensities of the colours obtained with ninhydrin were compared with those for standard spots of known amino acids developed under identical conditions.

Lipids were estimated by the method described by Salton (1953).

Preparation of cell walls. Organisms were washed twice in saline and once in distilled water and suspended in cold distilled water at a concentration equivalent to 20 mg. dry wt./ml. The cells were then exposed for 15 min. to sonic vibration in

a Raytheon disintegrator with an output of 50 W. at 9 kc./sec., the suspension being kept at 0° throughout the experiment. The cell walls were washed by the method of Munoz, Ribi & Larsen (1959) and their purity checked by the examination of palladium shadowed preparations with a Philips model EM 100 electron microscope. The cell walls were suspended in distilled water and stored at -20° until required.

Concentration of autolysin from old cultures of Pseudomonas aeruginosa. Eight-day anaerobic cultures of *P. aeruginosa* were freed from whole cells by centrifugation at 8000 rev./min. for 30 min. The supernatant fluid was adjusted to pH 7.0 with N-acetic acid. Solid ammonium sulphate was slowly added in the cold to a final concentration of 3M. The precipitate which formed was spun down, washed with 3M-ammonium sulphate and re-dissolved in 0.067 M-Tris buffer (pH 7.0). The preparation was dialysed against 0.067 M-Tris buffer (pH 7.0). The precipitate in the dialysis sac was spun off and discarded. Nucleic acids were removed with protamine sulphate (Korkes, del Campillo & Ochoa, 1950). The autolysin was re-precipitated with 3 M-ammonium sulphate and dialysed against 0.067 M-Tris buffer (pH 7.0). After centrifugation the clear supernatant fluid was assayed for protein and diluted with buffer until it contained 5 mg. protein/ml. Three ml. of the enzyme preparation was added to the equivalent of 200 mg. dry wt. 24 hr *P. aeruginosa* NCTC 6750 cell walls suspended in 5 ml. of 0.01 M-Tris buffer (pH 8.0) and the volume made up to 10 ml. with Tris buffer (pH 8.0). The preparation was incubated at 45° for 20 hr and the cell walls were spun down at 20,000 g for 30 min. The walls were washed twice in cold 0.067 M-Tris buffer (pH 7.0) and stored in distilled water at -20° until required for assay.

RESULTS

The growth of Pseudomonas aeruginosa and Salmonella bethesda in anaerobic cultures

Both the parent and the mutant strain of *Pseudomonas aeruginosa* grew vigorously in the anaerobic cultures. A maximum viable population of $3-5 \times 10^9$ organisms/ml. was maintained until exhaustion of the nitrate supply (shown by an arrow in Fig. 1) whereupon a rapid and extensive decline in viable count was regularly observed (Fig. 1). Following the decrease in viable count the *P. aeruginosa* NCTC 6750 culture showed 40-50% lysis on ageing, whereas the mutant showed very little lysis (5-10%; see Fig. 1). The maximum viable population of *Salmonella bethesda* remained constant at $4-5 \times 10^8$ organisms/ml. throughout the experiment and the culture did not undergo any observable autolytic changes during ageing. The extensive lysis observed in the older cultures of *P. aeruginosa* NCTC 6750 suggested the presence in those cultures of an autolytic enzyme and so lysis experiments with young washed suspensions in the presence of cell-free culture filtrates were attempted. Preliminary experiments showed that some lytic activity was associated with the fluid of old cultures; to obtain significant results it was necessary to first concentrate the 'autolysin' by ammonium sulphate precipitation.

Incubation of young resting cell suspensions of *Pseudomonas aeruginosa* NCTC 6750 with the crude autolysin concentrate resulted in the lysis of 40-50% of the organisms in 10-15 min. Total counts confirmed that after 60 min. only 20% of the original organisms were still intact. Control suspensions which were incubated with heated autolysin (100° for 10 min.) showed little or no lysis during this time (Fig. 2). The addition of ethylenediaminetetraacetic acid (EDTA) to the test mixture greatly

increased the rate of lysis of the parent *Pseudomonas* and the mutant so that 60–70 % lysis was observed within 2–4 min. Control suspensions (containing EDTA only) decreased by 10 % over the 10 min. period. Ammonium sulphate precipitation of cell-free culture filtrates of aged mutant broth cultures yielded preparations with little or no lytic activity for parent or mutant *Pseudomonas* either in the presence or absence of EDTA. Lysis presumably resulted from the hydrolysis of the cell wall by the autolysin and attempts were, therefore, made to determine the possible substrates of the enzyme by making chemical analyses of the cell walls of the ageing bacteria.

Fig. 1. Growth curves of *Pseudomonas aeruginosa* NCTC 6750, *P. aeruginosa* (mutant) and *Salmonella bethesda* in casein hydrolysate medium incubated anaerobically at 37°. The arrow indicates the time the nitrate supply became exhausted. ×—× = viable count; ●—● = total count. Nitrate was still present in the *S. bethesda* culture at 192 hr.

Composition of ageing bacterial cell walls

The details of the balance sheets for washed cell walls of the three organisms are set out in Tables 1 and 4.

Total nitrogen and protein. The total-N content of the walls of the three organisms accounted for 8–11 % of the dry weight and showed very little change with age.

The cell walls of young *Pseudomonas aeruginosa* NCTC 6750 cultures contained 50–60 mg. protein/100 mg. dry wt. wall and this rose to almost 70 mg. in old organisms. By contrast, the cell walls of the *Pseudomonas* mutant together with

Fig. 2. Lysis of resting suspensions of *Pseudomonas aeruginosa* NCTC 6750 and *P. aeruginosa* (mutant) by crude autolysin in the presence of 0.01 M-Tris (pH 8.0) and 100 µg. ethylenediaminetetraacetic acid (EDTA)/ml. ●—●, *P. aeruginosa* NCTC 6750 + autolysin + EDTA; ○—○, *P. aeruginosa* NCTC 6750 + autolysin; +—+, *P. aeruginosa* mutant + autolysin + EDTA; ×—×, *P. aeruginosa* mutant + autolysin; △—△, *P. aeruginosa* + EDTA; ▽—▽, *P. aeruginosa* control.

Table 1. Composition of cell walls of ageing bacteria

Age (hr)			Total-N	Protein*	Total reducing sugar (mg./100 mg. dry wt. cell walls)	Total-P	Lipid	
							Free	Total
24	} <i>Pseudomonas aeruginosa</i> NCTC 6750	{	8.4	54	8.0	1.7	5.2	15
48			8.4	50	—	1.1	—	15
96			8.8	61	—	1.4	—	12
192			8.7	64	6.1	1.6	5.4	4
24	Enzyme treated†		—	63	6.0	—	7.6	10
24	} <i>P. aeruginosa</i> (mutant)	{	10.1	70	12.1	1.6	6.3	16
96			10.5	72	—	1.4	—	14
120			10.0	69	—	1.0	—	11
192			10.9	70	13.5	1.0	5.8	13
48	} <i>Salmonella bethesda</i>	{	8.0	77	18.5	1.7	8.7	12
96			9.0	63	—	1.7	6.0	11
192			9.0	63	15.4	1.6	7.2	10

* Average of Folin-Ciocalteu and biuret determinations.

† 24 hr *P. aeruginosa* NCTC 6750 cell walls incubated at 45° for 20 hr in 0.01 M-Tris buffer (pH 8.0) with the enzyme preparation.

those of *Salmonella bethesda*, contained 65–70 mg. protein/100 mg. dry wt. wall irrespective of the age of the cells (Table 1).

Amino acids. The amino acid composition of the cell walls altered significantly as the cells aged (Table 2). Although the over-all recovery of amino acids increased with age, considerable decreases were observed in the amounts of diaminopimelic acid (DAP), alanine, glutamic acid and glycine present in the older cell walls of the parent strain. The amino acid composition of the cell walls of young mutant *Pseudomonads* closely resembled that of the parent but there was no corresponding decrease in the DAP, alanine, glutamic acid or glycine content as the organisms aged (Table 3). The amino acid composition of the *Salmonella bethesda* cell walls differed quantitatively but not qualitatively from that of the *Pseudomonas aeruginosa* preparations.

Table 2. *Amino acid composition of Pseudomonas aeruginosa* NCTC 6750 cell walls

Amino acid	Age of cell walls (hr)				Enzyme treated* 24
	24	40	88	192	
	(mg./100 mg. dry wt. cell walls)				
Phenylalanine	6.0	7.8	9.0	9.5	6.3
Leucine and isoleucine	2.2	2.9	3.5	3.5	2.4
Valine	3.0	2.5	2.6	2.0	1.7
Tyrosine	3.3	2.6	3.4	2.8	2.4
Arginine	0.9	0.6	0.6	0.9	0.9
Lysine	1.1	0.8	0.9	1.1	0.8
Alanine	7.2	7.5	4.2	3.3	3.1
Glycine	11.0	11.4	6.3	6.6	5.6
Serine	5.0	—	4.0	3.4	4.1
Glutamic acid	6.6	5.7	4.7	3.2	4.6
Aspartic acid	14.4	11.6	9.6	11.4	10.9
Diaminopimelic acid	2.8	3.0	1.7	1.2	0.9

* As in Table 1.

Table 3. *Amino acid composition of Pseudomonas aeruginosa* (mutant strain) and *Salmonella bethesda* cell walls

Amino acid	<i>P. aeruginosa</i> (mutant)			<i>S. bethesda</i>		
	Age of cell walls (hr)					
	24	96	192	48	88	192
	(mg./100 mg. dry wt. cell walls)					
Phenylalanine	7.3	9.3	10.8	4.1	6.6	7.2
Leucine and isoleucine	2.8	3.2	4.0	1.6	2.5	2.7
Valine	3.9	6.8	4.7	2.0	2.3	2.6
Tyrosine	4.2	6.4	5.4	2.5	3.0	4.2
Arginine	1.3	1.9	1.7	0.4	0.3	0.7
Lysine	1.7	2.5	2.3	0.5	0.5	0.6
Alanine	5.1	6.2	7.3	4.3	3.5	3.5
Glycine	7.1	8.4	8.7	5.5	8.0	14.0
Serine	5.4	8.4	8.9	2.6	2.0	3.0
Glutamic acid	5.8	8.6	6.0	4.9	4.9	7.8
Aspartic acid	9.3	8.9	7.0	14.5	15.0	22.5
Diaminopimelic acid	3.2	5.5	5.1	2.1	1.9	2.9

Carbohydrate. The details of the total reducing sugar content of the three cell wall preparations are given in Table 4. *Pseudomonas aeruginosa* NCTC 6750 showed a decrease in total reducing sugar content with ageing whilst the cell walls of the mutant did not show any ageing effect. However, the cell walls of the mutant contained more reducing sugar residues than did the walls of the parent strain. The *Salmonella bethesda* cell walls contained 18.5 mg. reducing sugar/100 mg. wall and this decreased slightly with ageing (Table 4). The walls of all three organisms contained 1.5–2.0 mg. of amino sugar/100 mg. wall, of which about half was muramic acid (Table 4). The amino sugar content of *P. aeruginosa* NCTC 6750 cell walls decreased by 50 % as the organisms aged. Little change in the amino sugar content of the other two organisms was noted. Chromatographic identification of the individual cell wall sugars and amino sugars disclosed the presence of glucose, mannose, rhamnose, glucosamine and muramic acid (Table 4). The cell walls of *P. aeruginosa* NCTC 6750 contained approximately equimolar amounts of glucose and rhamnose. The glucose content of the walls decreased by 60 % as the organisms aged, whereas the rhamnose and the mannose content changed very little (Table 4). The glucose and mannose content of the cell walls of the mutant was almost double that of the parent and this offers an explanation for the higher reducing sugar content observed with the cell walls of the mutant. The sugar content of the *S. bethesda* cell wall was quantitatively quite distinct from both *Pseudomonads* and there was no detectable alteration in sugar content on ageing.

Table 4. *Carbohydrate and amino sugar composition of cell walls of ageing bacteria*

Age (hr)	Strain	(mg./100 mg. dry wt. cell walls)				
		Glucos- amine	Muramic acid	Glucose	Mannose	Rhamnose
24	<i>Pseudomonas aeruginosa</i> NCTC 6750	0.7	0.8	2.9	0.3	2.2
64		—	—	2.7	0.3	2.0
88		—	—	1.8	0.3	2.0
192		0.2	0.6	1.1	0.3	1.8
24	Enzyme treated*	tr.	0.4	0.6	0.3	1.9
24	<i>P. aeruginosa</i> (mutant)	0.7	0.8	5.0	0.6	3.0
72		—	—	4.4	0.7	3.0
192		0.5	0.9	3.4	0.5	2.8
48	<i>Salmonella bethesda</i>	1.1	1.0	3.9	3.7	9.6
88		—	—	4.8	3.3	8.2
192		1.6†	—	4.1	3.0	8.1

* As in Table 1.

† Estimated only as total hexosamine.
tr. = trace.

Lipid and total phosphorus. The phosphorus content of the cell walls of the three strains varied from 1 to 2 mg./100 mg. wall and there was no change on ageing. The free lipid content varied between 5.2 and 8.7 mg./100 mg. wall and did not alter with ageing. However, the total lipid content of the parent pseudomonad walls decreased by almost 25 % (Table 1). The lipid content of the walls of the mutant and of *Salmonella bethesda* showed very little change on ageing. The total recovery

of wall material varied from 80 to 100 %. The high recoveries for the mutant walls were mainly due to the unexplained 15–20 mg./100 mg. wall increase in the protein content of these walls. The incomplete recoveries for *Pseudomonas aeruginosa* NCTC 6750 and *S. bethesda* suggest that there may be other undetected minor components in these walls.

Autolysis of washed Pseudomonas aeruginosa cell walls

Incubation of young *Pseudomonas aeruginosa* cell walls with the crude autolysin obtained from old cultures resulted in a change in opacity of 15–20 % in 18 hr. Chemical assays carried out on the hydrolysed cell walls disclosed changes in composition similar to those recorded above for aged cell-wall preparations. The protein content increased somewhat but the total reducing sugar was not apparently affected (Table 1). However, chromatographic examination of the cell-wall hydrolysates disclosed that the amino acids DAP, alanine, glycine and glutamic acid decreased by 50 % (Table 2). Similarly, the glucosamine and muramic acid content of the walls decreased sharply (Table 4). The total lipid content of the walls also decreased considerably (Table 1). The composition of a control cell wall suspension treated with boiled autolysin resembled, within experimental error, the initial 24 hr old preparation recorded in Table 1.

DISCUSSION

The observed lysis of the anaerobically incubated *Pseudomonas aeruginosa* NCTC 6750 was due to the presence of an extracellular autolysin. Cultures of the mutant strain and *Salmonella bethesda* did not produce detectable amounts of autolysin in ageing cultures, although the mutant was still susceptible to lysis. Thus the mutation appeared to involve the loss of ability to produce an extracellular autolysin, rather than the formation of a lysis-resistant cell wall. The mutant cells were still strictly aerobic and were subject to a rapid and extensive degree of killing when incubated under anaerobic conditions. The absence of subsequent lysis of these cells suggests that the lethal and lytic processes observed in cultures of *P. aeruginosa* NCTC 6750 are distinct and not directly inter-related.

The gross chemical composition of the cell walls of the three strains of bacteria underwent a number of changes as the cultures passed through their growth phases. The most interesting alterations occurred in the amino acid and amino sugar content of the ageing cell walls of the parent strain. The over-all amino acid content of the walls increased slightly as the cells aged. This was to be expected from the observed increases in total protein. The coincidence of the sharp decline in the DAP, alanine, glycine and glutamic acid content of the walls of the parent strain with the onset of autolysis suggested a causal relationship between the two phenomena. Significantly, the cell walls of neither the mutant strain nor *Salmonella bethesda* showed any comparable decrease in these amino acids. In view of the known importance of these amino acids in the cell wall mucopeptides of other Gram-negative bacteria (Salton, 1958, 1960; Brown, 1958) it seems reasonable to presume that the autolysin present in the cultures of the parent strain removed part of the rigid mucopeptide layer from the *Pseudomonas aeruginosa* cell walls so that cell lysis resulted. The removal of at least 50 % of the muramic acid and glucosamine from the aged

P. aeruginosa NCTC 6750 cell walls was taken as confirmation that the entire mucopeptide moiety was affected. However, the *Pseudomonas* autolysin was not a simple bacterial lysozyme since the crude autolysin preparation was unable to lyse *Micrococcus lysodeikticus* suspensions.

The removal of complex lipid from the cell walls of the parent strain during ageing suggested the presence of an esterase in the autolysin. Sierra (1957) demonstrated the presence of several esterases in *Pseudomonas aeruginosa* cells and noted a correlation between their presence in the culture medium and the subsequent induction of cellular lysis. In the present study, both the whole organisms and the autolysin concentrate were found to have lecithinase activity when tested against serum lecithin. The loss of lipid from the ageing cell walls of the parent but not the mutant organism suggested that lysis of the cell was initiated only after some lipid complex in the wall had been removed. This was confirmed by the increased autolysis of *P. aeruginosa* following treatment of the cells with acetone or Teepol. Increased autolysis following treatment of cells with lipid solvents has previously been observed by Warren *et al.* (1955) and others. Presumably the removal of a lipid moiety exposed the mucopeptide layer which could then be attacked by other enzymes present in the autolysin. Thus it seems likely that the *P. aeruginosa* autolysin consists of a mixture of at least two enzymes and is distinct from the autolysins produced by the Gram-positive bacteria. Further purification of the autolysins of this and other Gram-negative bacteria would, therefore, be useful and should reveal whether or not the autolysin of *P. aeruginosa* is typical of the enzymes produced by other Gram-negative micro-organisms.

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