Biochimica et Biophysica Acta, 406 (1975) 60–67

BBA 77058

ALTERATIONS IN THE COMPOSITION AND BACTERIOPHAGE-BINDING PROPERTIES OF WALLS OF *STAPHYLOCOCCUS AUREUS* H GROWN IN CONTINUOUS CULTURE IN SIMPLIFIED DEFINED MEDIA

A. R. ARCHIBALD and J. E. HECKELS*

Microbiological Chemistry Research Laboratory, The University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU (U.K.)

(Received March 28th, 1975)

SUMMARY

A nutritional mutant of *Staphylococcus aureus* H has been isolated and grown in media in which the only amino acids are arginine, cysteine, glutamic acid and proline. Walls of the bacteria grown in such media in continuous culture under potassium limitation differ in composition from walls of the bacteria grown in batch culture in rich nutrient broth in that they contain less glycine, the peptidoglycan component is less highly cross-linked and the teichoic acid component contains a reduced proportion of *N*-acetylglucosaminyl substituents. Walls of the potassium-limited bacteria retain the ability to bind bacteriophage 52a but are more susceptible to the action of lytic peptidases than are wall samples in which the peptidoglycan is more highly cross-linked. Teichoic acid was present in walls of the bacteria grown under phosphate limitation in the defined medium and these walls were also able to absorb bacteriophage 52a.

INTRODUCTION

The structures of the peptidoglycan and teichoic acid components of cell walls of *Staphylococcus aureus* have been studied extensively. Characteristically the peptidoglycan is a "tight" structure [1] in which the peptide chains are extensively cross-linked through pentaglycine bridges [2, 3]. The teichoic acid consists of chains of poly(ribitol phosphate) in which *N*-acetylglucosaminyl substituents are attached to each ribitol unit and a proportion of these units also contain D-alanyl ester substituents [4, 5]. However, the composition of bacterial cell walls is subject to considerable phenotypic variation [6] and the composition of staphylococcal peptidoglycan has been shown [3, 7–9] to be influenced by the composition of the medium in which the bacteria are grown. The proportion of D-alanyl ester residues on the wall teichoic acid of *S. aureus* strain H grown in continuous culture under conditions of magnesium

* Present address: Department of Microbiology, Faculty of Medicine, The University of Southampton, Southampton, OS9 3TU, U.K.

limitation depends on the pH at which the bacteria are grown [10]; under conditions of phosphate limitation the wall teichoic acid is replaced by teichuronic acid [11]. The media used in these continuous culture studies contained tryptone though staphylococci will grow in fully defined media containing several amino acids and the continuous culture of *S. aureus* in media containing 18 amino acids has recently been described [12]. In an early study Gladstone [13] found that various staphylococci which originally required the presence of several amino acids could be "trained" to grow in media containing progressively fewer amino acids. Similar observations have been made more recently [14, 15] and we now describe the continuous culture of such a "trained" derivative of *S. aureus* H in fully defined media containing only four amino acids. Walls of the bacteria grown under such conditions differ in composition from those of the bacteria grown in batch culture in nutrient broth. The possibility that these differences might sufficiently alter the organisation and structure of the wall so as to affect the presence of bacteriophage receptor sites has been investigated.

MATERIALS AND METHODS

Amino acids and vitamins were purchased from Sigma Chemical Co., other chemicals were obtained from B.D.H. Ltd. *Myxobacter* peptidase was prepared as described by Wingard et al. [16] and the *Flavobacter* L-11 peptidase [17, 18] was a gift from Dr S. Kotani.

Bacteria were disrupted and walls were isolated as previously described [10]. Phosphate, hexosamines and amino acids were determined as previously described [19]. N-Terminal amino acids were determined after hydrolysis of dinitrophenylated cell walls [20]. Methods used for hydrolysis and chromatographic examination of teichoic acids were those described previously [21]. Bacteriophage 52a was propagated and assayed as described previously [22]. The phage adsorbing efficiency of the walls was determined in a manner similar to that described by Lovett and Shockman [23] and the phage inactivation capacity of the walls was determined as described by Chatterjee [24].

Media. The basic medium contains $NaH_2PO_4 \cdot 2H_2O$, 10.0 mM; K_2SO_4, 3.0 mM; $MgCl_2 \cdot 6H_2O$, 1.25 mM; $CaCl_2$, 100 μM; $FeCl_3 \cdot 6H_2O$, 100 μM; $MnCl_2 \cdot 4H_2O$, 25 μM; $ZnCl_2$, 25 μM; $CuCl_2 \cdot 2H_2O$, 5 μM; citric acid, 1.0 mM; L-cysteine, 0.5 g/l; L-proline, 0.5 g/l; L-arginine, 0.5 g/l; thiamine, 10 mg/l; nicotinic acid, 10 mg/l, L-glutamic acid, 7.4 g/l, D-glucose, 20 g/l. As described below various other amino acids were added to this medium in certain cases and the concentrations of certain components were altered to achieve the limitations described.

Bacteria were also grown in batch culture in the following medium: 25 g Nutrient Broth (Difco), 5 g Yeast Extract (Difco), 5 g K_2HPO_4, 10 g glucose, 1 l water.

RESULTS

Isolation of bacteria having simplified nutritional requirements

Preliminary studies showed that *Staphylococcus aureus* H could grow in batch culture in the above fully defined medium adjusted to pH 7.2 by the addition of 2 M NaOH and supplemented by the addition of L-aspartic acid, glycine, L-histidine,

L-leucine, L-phenylalanine and L-valine, all at concentrations of 0.5 mg/ml. The procedure for deletion of amino acids was similar to that described by Gladstone [13] and by Mah et al. [14] and involved inoculation of a series of flasks each containing medium lacking one of the amino acids. Bacteria from the flask which showed most growth were then inoculated into a second flask containing medium of the same composition and after incubation for 48 h at 37 °C the resultant culture was plated on medium of the same composition but solidified by the inclusion of 1.5 % (w/v) agar. This culture was then used to inoculate a series of flasks containing media lacking one further amino acid and the process was repeated as above. Amino acids were deleted in the sequence histidine, phenylalanine, aspartic acid, leucine, glycine and valine. However, growth could not be established in media which lacked arginine, cysteine or proline.

In batch culture, the medium containing glutamic acid, arginine, cysteine and proline supported growth to a density of approx. 0.15 mg per ml of culture. During growth the pH value of the medium fell to 4.8 and this low pH is probably responsible for the cessation of growth since higher cell densities were obtained in continuous culture when the pH was maintained at 7.0 by the addition of ammonia. The nutritional mutant was the same phage type (52a/79) as the original strain H (we are indebted to Mr G. Fenwick, Staphylococcal Reference Laboratory, Royal Victoria Infirmary, Newcastle upon Tyne, for phage typing these strains) and wall of both had the same composition when grown in rich nutrient media. However, the original strain would not grow in the simple media in batch culture nor was growth obtained in this medium in a 500-ml chemostat inoculated with a 25-ml culture of the original strain H which had been grown overnight in rich medium.

Growth of bacteria under continuous culture

The nutritional mutant of *S. aureus* H was grown overnight at 37 °C in Tryptic Soy Broth (Difco). This culture (50 ml) was then added to a stirred 0.5-l Porton-type chemostat [25] containing defined medium which was then pumped in so as to give a dilution rate of 0.2 h^{-1}. The temperature was set at 37 °C and the pH was maintained at 7.0 by the addition of sterile 4 M NH_4OH. Foaming was suppressed by continuous addition of sterile polyethylene glycol and aeration was maintained at 2.0 l/min. After equilibration of growth of 2–3 days bacteria were collected in a receiver kept at 4 °C and were harvested periodically over collection periods of 6 h. The four amino acid medium described above supported growth at a density of 3.8 mg (dry weight)/ml.

Walls were isolated from bacteria which had been grown in two different K^+-limiting media. Medium 1 contained 0.5 mM K_2SO_4 and medium 2 contained this same diminished concentration of K_2SO_4 but was supplemented by the addition of 0.5 mg/ml of each of glycine, DL-alanine and DL-lysine. Both media gave approx. 1.4 mg/ml (dry weight) of bacteria. Walls were also isolated from bacteria which had been grown in two different phosphate-limiting media. In medium 3 the concentration of $NaH_2PO_4 \cdot 2H_2O$ was reduced to 1.5 mM; medium 4 contained 0.6 mM NaH_2PO_4 and a reduced concentration of glutamic acid (4.0 g/l); bacterial yields of approx. 1.9 and 0.9 mg/ml (dry weight) were obtained with these two media, respectively. Walls were also isolated from the nutritional mutant and from the original strain H which had been grown in batch culture in nutrient broth-yeast extract medium.

TABLE I

COMPOSITION OF WALLS AND PEPTIDOGLYCAN FRACTIONS OF BACTERIA GROWN UNDER DIFFERENT CONDITIONS

Samples were analysed after hydrolysis in 6 M HCl at 100 °C for 16 h. Results are quoted in μmol/g of sample and are not corrected for any destruction occuring during hydrolysis. n.d., not determined.

Sample	Alanine	Glutamic acid	Glycine	Lysine	Glucosamine	Muramic acid	Phosphate
Walls of K^+-limited (medium 1) bacteria	973	557	807	462	601	207	760
Walls of K^+-limited (medium 2) bacteria	1122	541	1848	558	810	294	710
Walls of bacteria grown in nutrient broth	1201	465	2180	548	898	326	775
Walls of parent strain H grown in nutrient broth	1176	552	2507	558	958	374	760
Peptidoglycan of K^+-limited (medium 1) bacteria	1221	566	1520	610	548	359	n.d.
Peptidoglycan of bacteria grown in nutrient broth	1290	570	2820	760	545	540	n.d.
Peptidoglycan of parent strain H grown in nutrient broth	1129	612	2971	701	534	415	n.d.

Composition of walls of bacteria grown under K^+ limitation and in batch culture

Walls were analysed for phosphate and for amino compounds after hydrolysis for 16 h in 6 M HCl at 100 °C. Results are given in Table I where the composition of walls of the original strain H grown in batch culture is also shown. Examination of dinitrophenylated walls showed that 36 % of the lysine in walls of K^+-limited (medium 1) bacteria had a free ε-NH_2 group, trace quantities of free NH_2-alanine and free NH_2-glycine were also present. In walls of bacteria grown in K^+-limited medium supplemented with additional amino acids (medium 2) and in batch culture in nutrient broth, 20 and 11 %, respectively, of the lysine residues had free ε-NH_2 groups; these walls also contained trace quantities of free NH_2-alanine but free NH_2-glycine was not detected. The peptidoglycan fraction of the K^+-limited (medium 1) bacteria, and those of the mutant and original strain H grown in batch culture, obtained by removal of most of the teichoic acid and protein by extraction with 0.5 M NaOH at 22 °C for 30 min [26] was also hydrolysed and analysed for amino compounds (Table 1). Samples of the extracted teichoic acids were hydrolysed in NaOH, treated with phosphomonoesterase, N-acetylated and examined chromatographically as previously described [4]. Teichoic acid from staphylococci grown in nutrient broth gave *N*-acetylglucosaminyl ribitol only whereas teichoic acid from the K^+-limited bacteria gave, in addition, a substantial proportion of unsubstituted ribitol. Analysis showed that the former teichoic acids contained phosphate and glucosamine in the molar ratio 1 : 0.93 whereas the teichoic acid from the potassium-limited bacteria contained these components in the molar ratio 1 : 0.55. A solution of this latter teichoic acid (2 μmol P) in 0.9 % aqueous NaCl (10 ml) was mixed with antiserum prepared from rabbits which had been immunized by injection of cell walls of *S. aureus* H grown in rich nutrient medium. The mixture was incubated at 20 °C for 1 h and then at 4 °C for 70 h. The precipitate which formed was removed by centrifugation, washed with 0.9 % aqueous NaCl and suspended in 10 % (w/v) aqueous trichloroacetic acid (2 ml). The suspension was shaken at 20 °C for 30 min and then centrifuged. After removal of trichloroacetic acid by extraction with ether (six times) the supernatant solution was evaporated to dryness. A sample of the extract was hydrolysed in 4 M HCl and analysed for phosphate and glucosamine; these components were present in the molar ratio 1 : 1.06. The remaining extract was hydrolysed in alkali, incubated with phosphomonoesterase and N-acetylated as before. Chromatographic examination showed the presence of *N*-acetylglucosaminyl ribitol but no free ribitol.

Walls of phosphate-limited bacteria

Walls from bacteria grown in media 3 and 4 contained 2.9 and 1.9 % phosphate, respectively. Acid hydrolysis of the material which was extracted with 5 % (w/v) trichloroacetic acid gave products characteristic of *S. aureus* teichoic acid, the presence of which was also shown by the ability of the walls to absorb bacteriophage 52a (Table II). No products characteristic of teichuronic acid were detected.

Phage-binding properties

Walls (0.25–4 μg) were incubated with phage 52a ($8 \cdot 10^4$ p.f.u. plaque-forming units) in 0.4 ml Tryptic Soy Broth (Difco) at 37 °C for 1 h. Unadsorbed phages were assayed after appropriate dilution and the concentration of walls giving adsorption of 50 % of the phage particles [23] was determined graphically. Results are given in

TABLE II

PHAGE-BINDING PROPERTIES OF WALL SAMPLES

Sample	Phage absorbing efficiency (concentration^{-1} (μg/ml) of walls absorbing 50 % added phage 52a under standard conditions [27])	Phage absorption capacity (total number phage 52a bound/μg wall at equilibrium [28])
Walls of K^+-limited (medium 1) bacteria	0.20	$0.88 \cdot 10^9$
Walls of K^+-limited (medium 2) bacteria	0.30	$1.7 \cdot 10^9$
Walls of phosphate-limited (medium 3) bacteria	0.88	$2.6 \cdot 10^9$
Walls of phosphate-limited (media 4) bacteria	0.40	$1.6 \cdot 10^9$
Walls of bacteria grown in nutrient broth	0.32	$1.8 \cdot 10^9$

Table II. Walls (25 μg/ml) and phage particles ($1.2 \cdot 10^{11}$/ml) were also incubated at 37 °C and appropriately diluted samples were assayed after 1 and 2 h. Adsorption was complete in 1 h under these conditions and the inactivation capacities [24] are given in Table II.

Digestion of walls with Myxobacter AL-1 enzyme

A solution (20 μl, 400 units) of *Myxobacter* AL-1 peptidase was added to suspensions of walls (4 mg) in 0.06 M Tris · HCl buffer, pH 9.0 (4 ml) at 37 °C and the absorbance at 550 nm was measured at intervals. Results are shown in Fig. 1.

Digestion of bacteria with Flavobacter peptidase

Bacteria grown under K^+ limitation (medium 1) and for 16 h in batch culture (nutrient broth) were collected by centrifugation and suspended in 0.02 M Tris · HCl buffer, pH 7.8, so that a 1 in 20 dilution had $E_{550\,nm} = 0.5$. Samples (0.5 ml) of each

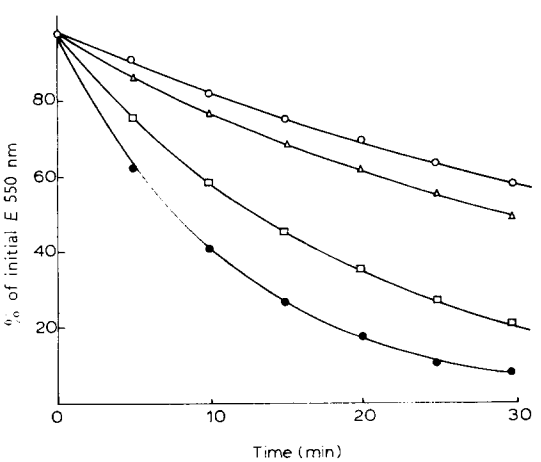

Fig. 1. Hydrolysis of cell walls with *Myxobacter* AL-1 enzyme. ○ — ○, walls of strain H grown in batch culture; △ — △, walls of nutritional mutant growth in batch culture; □ — □, walls of nutritional mutant grown in K^+ limiting conditions with additional amino acids (medium 2); ● — ●, walls of nutritional mutant grown in K^+-limiting (medium 1) conditions.

suspension were mixed with equal volumes of the same buffer containing 1 M sucrose and *Flavobacter* L-11 enzyme (3.0 mg/ml). The suspensions were incubated at 37 °C and portions (0.1 ml) were removed at intervals and tested for osmotic stability by measurement of their absorbance at 550 nm after dilution to 1.0 ml with buffer. After incubation for 30 and 60 min the absorbances of the diluted batch grown cells fell to 87 and 81 %, respectively, of the original whereas after such incubation the absorbances of the K^+-limited cells fell to 40 and 30 %, respectively, of the original value.

DISCUSSION

The "trained" derivative of *S. aureus* H differed from the parent strain in that it was able to grow in batch and continuous culture in media in which the only amino acids were arginine, cysteine, glutamic acid and proline. A recent study of the genetic basis of the multiple nutritional requirements of *Lactobacillus casei* has indicated [12] that in many cases these are due to small lesions rather than to large deletions affecting the genes involved in each biosynthetic pathway. A similar genetic basis for the amino acid requirements of *S. aureus* would permit the isolation of mutants, or revertants, having simplified nutritional requirements as described in this paper.

Walls of the bacteria grown under potassium limitation contained ribitol teichoic acid and the amino compounds typical of staphylococcal peptidoglycan although the proportions of glucosamine and of glycine were less than those present in bacteria grown in batch culture in rich nutrient medium. The ratio (2.1 : 1) of glycine to glutamic acid in the peptidoglycan fraction of the walls was much less than that (4.8 : 1) in the peptidoglycan fraction of the walls of bacteria grown in nutrient broth and 36 % of the lysine residues had free ε-amino groups. This finding is similar to that of Schleifer [9] who reported that batch growth of *S. aureus* in media containing only low levels of glycine resulted in a diminution in the extent of cross-linking in the peptidoglycan. Addition of glycine, alanine and lysine to the potassium-limited medium led to incorporation of an increased proportion of glycine in the peptidoglycan and to more extensive cross-linking. The diminished proportion of glucosamine present in the walls of potassium-limited bacteria is explained by the finding that approx. 45 % of the ribitol units did not possess *N*-acetylglucosaminyl substituents. The presence of ribitol units lacking such substituents has not previously been observed in any wild type strain of *S. aureus* and such units were not present in the nutritional mutant grown in nutrient broth. When the partially substituted teichoic acid was mixed with antiserum active against strain H, the teichoic acid which was precipitated contained no unsubstituted ribitol units. The teichoic acid in walls of the potassium-limited bacteria thus consists of chains in which every ribitol unit bears an *N*-acetylglucosaminyl substituent together with chains in which such substituents are absent.

Bacteria were also grown under phosphate limitation in media containing reduced concentrations of phosphate and of glutamic acid. However, these bacteria still contained wall teichoic acid. *S. aureus* H has been shown to produce teichuronic acid rather than wall teichoic acid when grown under phosphate limitation in media containing tryptone [6] and it is possible that the continued incorporation of wall teichoic acid during growth of the nutritional mutant under phosphate limitation may be a consequence of the growth conditions employed in this study rather than a

reflection of a genetic difference concerning the regulation of teichoic acid synthesis. Since the *N*-acetylglucosaminyl substituents on the teichoic acid are required for adsorption of bacteriophage 52a [24] it seemed possible that the walls of the potassium -limited bacteria might, for this reason or because of alterations in the organisation of the wall resulting from the reduced cross-linking in the peptidoglycan, have a reduced ability to absorp the bacteriophage when compared with walls of bacteria grown in nutrient broth. Walls of both potassium-limited and phosphate-limited bacteria were found to differ from those of the bacteria grown in nutrient broth in their ability to absorb bacteriophages. However, the differences were fairly small and it is clear that the alterations in composition of the wall resulting from growth under the various conditions described have not led to alterations in the structural arrangement of the wall sufficient to abolish the bacterium-bacteriophage recognition and adsorption process. The reduced cross-linking in the peptidoglycan of the potassium limited bacteria is, however, accompanied by an increased susceptibility of the walls to enzymic dissolution.

ACKNOWLEDGEMENTS

We thank the Medical Research Council for financial support and Mr K. Glassey for his technical assistance.

REFERENCES

1 Ghuysen, J. M. (1968) Bacteriol. Rev. 32, 425–464
2 Tipper, D. J. and Berman, M. F. (1969) Biochemistry 8, 2183–2192
3 Schleifer, K. H. and Kandler, O. (1972) Bacteriol. Rev. 36, 407–477
4 Baddiley, J., Buchanan, J. G., RajBhandary, U. L. and Sanderson, A. R. (1962) Biochem. J. 82, 439–448
5 Sanderson, A. R., Strominger, J. L. and Nathenson, S. G. (1962) J. Biol. Chem. 237, 3603–3613
6 Ellwood, D. C. and Tempest, D. W. (1972) Adv. Microbiol. Physiol. 7, 83–117
7 Korman, R. Z. (1966) J. Bacteriol. 92, 762–768
8 Browder, H. P., Tavormina, P. A. and Zygmuut, W. A. (1968) J. Bacteriol. 96, 1452–1453
9 Schleifer, K. H. (1969) Proc. Soc. Gen. Microbiol. XIV
10 Archibald, A. R., Baddiley, J. and Heptinstall, S. (1973) Biochim. Biophys. Acta 291, 629–634
11 Ellwood, D. C. and Tempest, D. W. (1969) Proc. Soc. Gen. Microbiol. XV
12 Jarvis, A. W., Lawrence, R. C. and Pritchard, G. G. (1975) J. Gen. Microbiol. 86, 75–87
13 Gladstone, G. P. (1937) Br. J. Exp. Pathol. 18, 322–333
14 Mah, R. A., Fung, D. Y. C. and Morse, S. A. (1967) Appl. Microbiol. 15, 866–870
15 Miller, R. D. and Fung, D. Y. C. (1973) Appl. Microbiol. 25, 800–806
16 Wingard, M., Matsueda, G. and Woolfe, R. S. (1972) J. Bacteriol. 112, 940–949
17 Kato, K., Kotani, S., Matsubara, T., Koyani, J., Hashimoto, S., Chimori, M. and Kagekawa, I. (1962) Biken J. 5, 155–179
18 Kato, K. and Strominger, J. L. (1968) Biochemistry 7, 2754–2761
19 Archibald, A. R., Heckels, J. E. and Baddiley, J. (1973) Nat. New Biol. 241, 29–31
20 Ghuysen, J. M., Tipper, D. J. and Strominger, J. L. (1969) Methods in Enzymology, Vol. VIII pp. 685–699, Academic Press, New York
21 Archibald, A. R. (1971) Methods in Carbohydrate Chemistry, Vol. VI, pp. 162–172
22 Archibald, A. R. and Coapes, H. E. (1972) J. Gen. Microbiol. 73, 581–585
23 Lovett, P. S. and Shockman, G. D. (1970) J. Virol. 6, 125–134
24 Chatterjee, A. N. (1969) J. Bacteriol. 98, 519–527
25 Herbert, D., Phipps, P. J. and Tempest, D. W. (1965) Lab. Pract. 14, 1150–1161