The Effect of Antibiotics on Synthesis of Mucopeptide and Teichoic Acid by *Pediococcus cerevisiae* and by a Substrain that Requires Methicillin for Growth

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SUMMARY

Mucopeptide synthesis by *Pediococcus cerevisiae* ATCC 8081 and *P. cerevisiae* CRD (a methicillin-requiring substrain) was measured by incorporation of radioactivity from ^{14}C -labelled-alanine, -glutamate, -lysine and -aspartate into the mucopeptide fraction of organisms incubated in a solution inadequate to support growth. Teichoic acid synthesis was measured by incorporation of $[^{32}\text{P}]\text{H}_3\text{PO}_4$ into a hot trichloroacetic acid soluble fraction from isolated walls.

Mucopeptide synthesis by both strains (as measured by incorporation of $[1\text{-}^{14}\text{C}]\text{glutamic acid}$) was unaffected by chloramphenicol but was sensitive to benzylpenicillin. The incorporation was resistant to methicillin in strain CRD (2 mg/ml gave 50 % inhibition) but not in parent organisms (120 $\mu\text{g/ml}$ gave 50 % inhibition).

Strain CRD incorporated twenty times less $[^{32}\text{P}]\text{H}_3\text{PO}_4$ into teichoic acid than did the parent strain. With either strain, benzylpenicillin (25 $\mu\text{g/ml}$) and D-cycloserine (200 $\mu\text{g/ml}$) inhibited this synthesis, but methicillin (500 and 1000 $\mu\text{g/ml}$) did not.

Electron micrographs of strain CRD incubated for four hours with methicillin showed large deposits of wall material around septa; these were not seen in parent organisms.

INTRODUCTION

Turbidity studies and chemical measurements showed that *Pediococcus cerevisiae* and *P. cerevisiae* CRD (a methicillin-requiring substrain) synthesized mucopeptide when incubated in a 'wall synthesis' solution that was inadequate for growth (Wilkinson & White, 1969). To observe the effects of antibiotics on this process more closely, especially at early stages, incorporation of radioactively labelled precursors has now been used to measure mucopeptide and teichoic acid synthesis. Walls of parent organisms contain, as well as mucopeptide, 35 % (w/w) of teichoic acid, whereas walls of strain CRD have only 16 % (w/w) teichoic acid (White, 1968*b*). The effects of some inhibitors of mucopeptide synthesis on teichoic acid synthesis have also been examined. A preliminary report of some of these results has been made (Wilkinson & White, 1971).

METHODS

Organisms. The origins and methods of maintenance of *Pediococcus cerevisiae* strain 8081 and substrain CRD are described by White (1968*a*).

Media. All media were made in distilled water and sterilized by autoclaving at 121 °C for 15 min. The partly defined medium was that of White (1968*a*).

Peptone+yeast extract+glucose medium (PYG) contained (g/l): Oxoid peptone, 20; Difco yeast extract, 10; and glucose, 20; the pH value was adjusted to 6.5. Peptone+yeast

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extract + glucose + acetate medium (PYGAc) was PYG plus sodium acetate trihydrate (20 g/l). The pH value was adjusted to 6.5.

Conditions of growth. One litre of PYGAc medium (in a 2 l conical flask) was inoculated with 10 ml of an overnight culture of organisms (strain 8081) grown in partly defined medium. Strain CRD was grown by inoculating 1 l of PYGAc + methicillin (100 µg/ml) with 1 ml of a 24 h culture grown in partly defined medium + methicillin (100 µg/ml). The flasks were incubated standing at 37 °C until the mid-exponential phase of growth was reached. This was after parent organisms had been incubated for about 8 h and had attained a reading of 1.2 to 1.8 (in $\frac{1}{4}$ in diameter tubes) in the EEL colorimeter with neutral density no. 1.0 filter (Evans Electroselenium Co. Ltd, Halstead, Essex) against a zero of distilled water. Strain CRD was grown for 26 to 30 h before harvesting. The organisms were harvested by centrifuging at 5000 g for 5 min at 2 °C and washed once in distilled water.

Solutions for study of wall synthesis. Solution W100 contained (g/l): KH_2PO_4 (adjusted to pH 6.5 with 5 N-NaOH), 13.6; glucose, 1.8; $(\text{NH}_4)_2\text{SO}_4$, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; L-lysine, 0.002; L-aspartate, 0.002; L-glutamate, 0.002; L-alanine, 0.004. Solution WT (for studies of teichoic acid synthesis) was solution W100 with most of the phosphate buffer replaced by 0.1 M-maleate-NaOH buffer, pH 6.5, and only a substrate amount of KH_2PO_4 (2 µg/ml) included.

Isolation of walls and mucopeptide. Mucopeptide was isolated by the method of Park & Hancock (1960) modified by including a wash in water after treatment with hot trichloroacetic acid, TCA (otherwise the trypsin was inactivated) and by raising the concentration of trypsin to 100 µg/ml. The mucopeptide residue was finally washed in water to remove trypsin and hydrolysis products. Walls were prepared as described by White (1968*b*) except that the organisms were broken at 2 °C in a Mickle (1948) tissue disintegrator.

Measurement of mucopeptide synthesis. Washed organisms were suspended in a volume of water such that when 10 ml of this suspension was added to 50 ml of solution W100 (at $1.2 \times$ strength, pre-incubated at 37 °C in a conical flask) the final bacterial concentration was 0.3 to 0.4 mg dry wt/ml. One µCi of the ^{14}C -labelled amino acid was included in solution W100 (60 ml final volume in a 100 ml flask). The $E_{610\text{nm}}$ of the suspension was measured after dilution (1 ml + 5 ml of water) at the beginning of the experiment; duplicate 5 ml samples of suspension were taken at intervals during the incubation and mixed with 1 ml of 30 % (w/v) TCA at 2 °C in a test tube. The tubes were stored at 2 °C before the mucopeptide was isolated as described above and its radioactivity measured after resuspension in scintillation fluid.

Measurement of teichoic acid synthesis. Organisms were grown, harvested, washed and resuspended in solution WT (0.3 to 0.4 mg dry wt of organisms/ml). The suspension (420 ml in a 1 l flask) plus 50 µCi of $^{32}\text{P}[\text{H}_3\text{PO}_4]$ was incubated at 37 °C. Samples (100 or 200 ml) were taken at intervals, centrifuged, washed and stored overnight as a pad at -15 °C. Walls were prepared and samples (0.1 ml) of the wall after suspension in water (2 ml) were removed for estimation of radioactivity. The $E_{610\text{nm}}$ of the suspension of walls was measured and their weight assessed by comparison with a calibration curve. The walls were centrifuged (15000 g for 15 min) and resuspended in 2 ml TCA (10 %, w/v) then heated at 60 °C for 90 min (Boylan & Ensign, 1968). After removing the solid residue by centrifuging, samples (0.1 ml) of the extracted walls that had been resuspended in water (2 ml) were also counted. The phosphorus content of the TCA extract was measured as described below.

Measurement of radioactivity. All samples were counted in a Nuclear-Chicago coincidence liquid scintillation spectrometer (model 6801, Nuclear-Chicago Corporation, Des Plaines, Illinois, U.S.A.). With ^{14}C , efficiency of counting was calculated using the channels ratio

method (Nuclear-Chicago Liquid Scintillation Manual, 1965). An efficiency of 55 % for ^{32}P -counting was determined by counting 0.1 μCi of $[\text{}^{32}\text{P}]\text{H}_3\text{PO}_4$ and assuming the information supplied on the strength of the isotope to be correct.

The scintillation fluid was N.E. 220 (Nuclear Enterprises Ltd, Sighthill, Edinburgh 11) or an equivalent fluid prepared in the laboratory by adding 7 g of 2,5-diphenyloxazole, 0.3 g of *p*-bis-2-(5-phenyloxazolyl)-benzene and 100 g of naphthalene to 1 l of dioxan and stirring for several hours.

Autoradiography. Chromatograms were put in contact with Kodirex X-ray film (Kodak Ltd) for five weeks before development. Amino acids were located by ninhydrin treatment of the chromatogram and the positions of fogged areas of film were compared.

Estimation of phosphorus. The samples for phosphorus determination contained 10 % (w/v) TCA, and were ashed to convert organic P to inorganic P before estimation, as described by Leloir & Gardini (1957). A calibration curve was prepared with 1.0 mM-glucose 6-phosphate di-sodium salt in 10 % (w/v) TCA.

Electron microscopy. The following procedures were carried out by Mrs M. Haynes of the Biochemistry Department, University of Sheffield.

Organisms were washed in water before being fixed in a 2.5 % (v/v) solution of glutaraldehyde in 0.1 M-phosphate buffer, pH 7.2, for 2 h at 2 °C. They were transferred to a 7.5 % (w/v) solution of sucrose in 0.1 M-phosphate buffer, pH 7.2, for overnight washing at 2 °C and were then post-fixed in 1 % (w/v) OsO_4 for 0.5 h at room temperature. The organisms were dehydrated, after washing in water for 5 min, by passing through the following concentrations of ethanol in water (each v/v): 50 %, 75 %, 95 %, 100 % (twice), and dry absolute ethanol; they remained in each solution for 10 min. The organisms were embedded in dry absolute ethanol+epon (1 + 1, v/v) for 2 h and then transferred to fresh epon at 60 °C for 48 h. The capsules were removed with boiling water and the blocks trimmed with a razor blade. Sections 60 to 90 nm thick were cut with an ultramicrotome and stained with lead citrate (Reynolds, 1963) before being examined in an A.E.I. EM6B electron microscope (Associated Electrical Industries Ltd, 1 Stanhope Gate, London W. 1).

Chemicals. Radioisotopes were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. D-Cycloserine was obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A. Benzylpenicillin and methicillin were from Beecham Research Laboratories, Betchworth, Surrey. Chloramphenicol was a gift (to Dr B. A. Fry) from Parke Davis Co., Hounslow, Middlesex.

RESULTS

*The effects of benzylpenicillin, methicillin and other antibiotics on the incorporation of amino acids into the mucopeptides of *Pediococcus cerevisiae* and *P. cerevisiae* CRD*

Hancock & Park (1958) and Mandelstam & Rogers (1959) have shown that the incorporation of labelled amino acids into the mucopeptide of *Staphylococcus aureus* was sensitive to benzylpenicillin but resistant to chloramphenicol. In *Pediococcus cerevisiae* 8081 incorporation of $[\text{}^{14}\text{C}]\text{glutamic acid}$ was almost entirely abolished by benzylpenicillin (25 $\mu\text{g/ml}$); incorporation in the presence of chloramphenicol (100 $\mu\text{g/ml}$) was indistinguishable from incorporation in the absence of any antibiotic, while methicillin (200 $\mu\text{g/ml}$) caused substantial inhibition (Fig. 1*a*). Strain CRD was sensitive to benzylpenicillin but less sensitive to methicillin (Fig. 1*b*) than the parent strain. The amounts of $[\text{}^{14}\text{C}]\text{glutamic acid}$ incorporated by strain CRD (25 nmol/mg dry wt) and parent (12 nmol/mg dry wt) are typical values. The concentration of methicillin required to inhibit mucopeptide synthesis in parent and strain CRD grown in the presence and absence of methicillin were measured (Table 1). With the

Fig. 1. Effect of chloramphenicol, methicillin and benzylpenicillin on the incorporation of [^{14}C]DL-glutamic acid into the mucopeptide of (a) parent strain, (b) strain CRD. The organisms were grown in (a) PYGAc medium, or (b) PYG medium, harvested, washed and resuspended (0.3 to 0.4 mg dry wt/ml) in solution W100 containing 1 μCi of [^{14}C]DL-glutamic acid. —○—, No antibiotic; —△—, chloramphenicol (100 $\mu\text{g}/\text{ml}$); —□—, benzylpenicillin (25 $\mu\text{g}/\text{ml}$); and —●—, methicillin (200 $\mu\text{g}/\text{ml}$). The flasks were incubated standing at 37 °C and samples were taken at intervals for isolation of mucopeptide and subsequent measurement of radioactivity.

Table 1. *Inhibition of incorporation of [^{14}C]glutamic acid into mucopeptides by benzylpenicillin and methicillin*

Exponential phase organisms were harvested from the media indicated, washed and resuspended (0.3 to 0.4 mg dry wt/ml) in solution W100 in flasks containing various concentrations of penicillins. The flasks were incubated standing at 37 °C and samples were taken at intervals for isolation of mucopeptide and measurement of its radioactivity.

		Inhibition (%) after 3 h of:		
		CRD		
	Concentration ($\mu\text{g}/\text{ml}$)	Parent grown in PYGAc	Grown in PYG	Grown in PYGAc + methicillin (100 $\mu\text{g}/\text{ml}$)
Benzylpenicillin	2.5	48	—	—
	25	96	71	—
Methicillin	50	34	—	—
	100	—	10	34
	200	56	21	—
	250	72	—	—
	500	87	31	40
	1000	94	37	50
	3000	—	56	56

parent strain, relatively low concentrations of methicillin were inhibitory: an effect of the drug was seen after 30 min with 50 $\mu\text{g}/\text{ml}$, after 20 min with 250 $\mu\text{g}/\text{ml}$, in less than 10 min with 500 $\mu\text{g}/\text{ml}$ or 1 mg/ml. Mucopeptide synthesis in strain CRD (grown in PYG medium without methicillin) was resistant to methicillin; 3 mg/ml caused only about 50 % inhibition and 500 $\mu\text{g}/\text{ml}$ only began to have a noticeable effect after about 50 min. When strain CRD was grown in PYGAc medium in the presence of methicillin the incorporation of [^{14}C]-

Fig. 2. Effect of methicillin on the incorporation of [$1\text{-}^{14}\text{C}$]DL-glutamic acid into the mucopeptide of strain CRD previously grown in the presence of antibiotic, i.e. in PYGAc medium plus methicillin ($100\text{ }\mu\text{g/ml}$). A series of flasks with different antibiotic concentrations was set up as in Fig. 1. —○—, No antibiotic; —△—, + methicillin ($100\text{ }\mu\text{g/ml}$); —□—, + methicillin ($500\text{ }\mu\text{g/ml}$); —●—, + methicillin (1 mg/ml); —▲—, + methicillin (3 mg/ml).

Table 2. *The incorporation of ^{14}C -labelled amino acids into the mucopeptides of parent and strain CRD*

Parent organisms were grown in PYGAc and strain CRD grown in PYG. Five flasks were set up for each strain, each containing $1\text{ }\mu\text{Ci}$ of the ^{14}C -labelled amino acid indicated. The flasks were incubated at $37\text{ }^{\circ}\text{C}$. Cold carrier L-phenylalanine ($10\text{ }\mu\text{g/ml}$) was included in the [^{14}C]phenylalanine containing flask.

	nmol Amino acid incorporated/mg dry wt of organisms in $2\frac{1}{2}\text{ h}$	
	Parent	Strain CRD
[U- ^{14}C]L-aspartic acid	10	12
[1- ^{14}C]DL-glutamic acid	10	18
[U- ^{14}C]L-lysine	22	19
[U- ^{14}C]L-alanine	38	19
[U- ^{14}C]L-phenylalanine	0.01	0.07

glutamic acid in 3 h was less than half that of organisms grown in PYG medium without methicillin (Fig. 2). Mucopeptide synthesis in CRD organisms grown with methicillin present was still highly resistant to methicillin, although not as resistant as that of organisms grown in the absence of the drug. Growth with methicillin may facilitate the uptake of methicillin or expose methicillin binding sites, or may partly saturate a sensitive enzyme system.

The four component amino acids were incorporated into mucopeptide but phenylalanine, which is absent from the mucopeptide, was not, indicating that the method for isolation gives a mucopeptide uncontaminated with protein (Table 2). From the analysis of mucopeptide, one mole of glutamate, one of lysine and one of aspartate might be expected to be incorporated for every two moles of alanine. The parent strain incorporated alanine in large amounts although not in twice the molar amounts of all the other amino acids. There was a high incorporation of lysine in both strains, and strain CRD incorporated relatively

Table 3. *Effect of methicillin on the incorporation of [³²P]phosphate into the hot TCA soluble fraction of the walls of Pediococcus cerevisiae and Pediococcus cerevisiae CRD*

Parent organisms were grown in PYGA_c, and strain CRD grown in PYG. Washed organisms were incubated in solution WT at 37 °C. Samples (100 ml) were taken at intervals and walls isolated and treated as described in Methods.

Strain	10 ⁻⁴ × d.p.m. in hot TCA extract/μmol P in extract					
	Parent			CRD		
Methicillin (μg/ml)	0	200	500	0	200	1000
Time (h) $\left\{ \begin{array}{l} 0 \\ 1 \\ 2 \\ 3 \end{array} \right.$	1.1 27.0 73.9 101.0	0.96 32.8 82.6 114.0	3.7 28.1 62.8 82.8	0.22 1.39 3.58 9.44	0.87 1.30 4.21 8.22	0.91 2.01 4.73 10.0

more glutamate than the parent. This pattern of incorporation may be affected by the level of unlabelled pool amino acids since Holden (1962) showed generally high levels of endogenous glutamate and low levels of lysine in a number of bacterial species. Aspartic acid was incorporated in substantial quantities, even though it was not required to produce a turbidity increment (Wilkinson & White, 1969).

Autoradiography of the hydrolysed mucopeptides showed only one fogged area of film corresponding to the radioactively labelled amino acid supplied.

Teichoic acid synthesis by parent and strain CRD. These were compared (Table 3). The results are expressed as specific activities because the amounts of walls obtained varied. Typically, about 1.7 and 0.3 μmol of P were extracted from 1 mg of walls from parent and strain CRD respectively. This difference in P content between parent and strain CRD is rather more than that found in previous experiments using partially defined media (White, 1968*b*). Possibly the difference in P content of the walls of the organisms is more marked when they are grown on crude media. [³²P] was incorporated into hot TCA extractable material during the 3 h incubation period though the time course of incorporation was not linear (Table 3). The most striking feature was that strain CRD incorporated about 20 times less ³²P than did parent organisms. Approximately 10 nmol of phosphate were incorporated (from solution WT) by 1 mg of parent organism in 3 h whereas only 0.5 nmol of phosphate was incorporated/mg of strain CRD in the same time. These values may be compared with the incorporation of a typical amino acid (from solution W100) into the mucopeptide, e.g. 12 and 25 nmol of [¹⁴C]glutamic acid incorporated into the mucopeptides of parent and strain CRD respectively/mg of organism in 3 h. There was no loss of phosphorus from the walls of either strain as the ratio of phosphorus to weight of wall remained relatively constant.

D-Cycloserine (200 μg/ml) inhibited ³²P incorporation by 85 % in parent organisms, and by 67 % in strain CRD. Benzylpenicillin (25 μg/ml) inhibited incorporation by parent strain by 56 % and strain CRD by 29 %. Both these inhibitors had less effect on teichoic acid synthesis than on mucopeptide synthesis at the same concentrations. It was surprising to find that methicillin, at concentrations high enough to give substantial inhibition of mucopeptide synthesis, had little effect on ³²P incorporation by parent or strain CRD (Table 3).

Effect of methicillin on septum formation. Electron microphotographs of organisms incubated in solution W100 for 4 h and organisms suspended in solution W100 and immediately processed (zero time control) showed wall and membrane and ingrowing septa (Fig. 3*a, b, d* and *e*). When methicillin (1 mg/ml) was present during incubation of strain CRD large deposits of wall material accumulated (Fig. 3*f*). This concentration of methicillin was

Fig. 3. Parent organism (grown in PYGAc) incubated for (a) 0 h and (b) 4 h at 37 °C in solution W100; bar = 200 nm. (b), plus methicillin (50 µg/ml); bar = 100 nm. Strain CRD organisms (grown in PYG) incubated for (d) 0 h (bar = 500 nm) and (e) 4 h (bar = 200 nm) at 37 °C in solution W100; (f) As (e) plus methicillin (1 mg/ml); bar = 200 nm.

chosen because it gives about the same percentage inhibition of glutamate incorporation as does 50 $\mu\text{g/ml}$ in the parent strain (Table 1). No such deposits could be seen in the parent strain incubated in the presence of 50 μg of methicillin/ml (Fig. 3c).

DISCUSSION

Chemical measurements have shown previously that the mucopeptide content of organisms incubated in solution W (i.e. W 100 with concentrations of amino acids raised 100-fold) increases without protein synthesis (Wilkinson & White, 1969). Mucopeptide synthesis has now been measured by incorporation of radioactivity from ^{14}C -labelled amino acids. This incorporation seems to represent mucopeptide synthesis because all the four amino acids found in the mucopeptide were incorporated in substantial amounts, whereas phenylalanine, an amino acid not found in the wall, was hardly incorporated at all.

The incorporation of $[^{14}\text{C}]$ glutamic acid into mucopeptide was inhibited by benzylpenicillin and methicillin but not by chloramphenicol, as was expected. About 5 μg of benzylpenicillin/ml and 120 μg of methicillin/ml inhibited mucopeptide synthesis by 50 % in the parent strain. These are about ten times the minimum inhibitory concentrations measured using small inocula (White, 1968a). This may be due to new sites for penicillin fixation becoming available on the membrane (Rogers, 1970), or to appreciable mucopeptide synthesis by washed suspensions of organisms before an inhibitory concentration of penicillin has accumulated in the organisms.

The most striking difference between mucopeptide synthesis by parent and strain CRD was the resistance of the process to methicillin in strain CRD, yet the organism was only slightly less susceptible to benzylpenicillin than the parent organisms.

Teichoic acid synthesis has been measured by incorporation of $\text{H}_3[^{32}\text{P}]\text{O}_4$ into a fraction of isolated walls that is soluble in hot trichloroacetic acid (as is teichoic acid). Teichoic acid is the major if not the only phosphate-containing polymer in the walls of *Pediococcus cerevisiae* (White, 1968b). The low incorporation of $[^{32}\text{P}]\text{H}_3\text{PO}_4$ by strain CRD implies that it has a less active teichoic acid synthesizing system and may explain the lower proportion of this polymer in the walls of this organism. Mucopeptide and teichoic acid are synthesized co-ordinately (Rogers, 1970; Boylen & Ensign, 1968) and the inhibition of teichoic acid synthesis by benzylpenicillin and cycloserine is in accord with this. It is difficult to explain why methicillin (in high concentrations) does not inhibit teichoic acid synthesis but it may be a further instance of differences between methicillin and other penicillins (Rogers & Jeljaszewicz, 1961; Izaki, Matsushashi & Strominger, 1966).

If the parent organism contains very roughly 500 nmol of mucopeptide monomer (mol. wt about 1000) per mg dry wt of walls (White, 1968b) and 50 nmol of mucopeptide monomer is synthesized per mg of wall in 150 min (Table 2; wall is about 20% of dry wt of organisms) then approximately 10 % thickening of the wall during the incubation would be expected. This is difficult to detect on the electron micrographs. They do however provide evidence that new wall was not laid down all in one place. Few mesosomes were seen in electron micrographs of *Pediococcus cerevisiae* although a 'wavy' membrane connecting newly formed septa was often seen. The significance of this structure is not known, it may represent a kind of mesosome structure. Electron micrographs of strain CRD incubated with methicillin revealed large deposits of wall material around forming septa. Similar deposits were not seen in parent organisms incubated with methicillin. Large depositions of wall material induced by penicillin at ends and septal sites have been observed in two Gram-positive rods (Fitz-James & Hancock, 1965; Highton & Hobbs, 1971).

Strain CRD shows certain features similar to some penicillin resistant mutants of *Staphylococcus aureus* described by Park, Griffith & Stevenson (1971). It did not produce penicillinase, it contained more mucopeptide per organism than its parent strain (White, 1968*b*) and it formed mucopeptide at double the rate of the parent strain. It bound penicillin as well as the parent strain (Widdowson & White, 1971). Park *et al.* (1971) have suggested that the mucopeptide transpeptidase in the septal region of their mutants is prevented from reacting with penicillin, perhaps by sealing off or being flooded with substrate, rather than modification of the penicillin sensitive transpeptidase. Mucopeptide synthesis by strain CRD (previously grown in PYG) was only inhibited 37% by methicillin (1 mg/ml) (Table 1). The electron micrographs show that the antibiotic inhibited synthesis of mucopeptide except at the septum. This implies an insensitivity of the transpeptidase in the septal region towards methicillin.

It is possible that strain CRD has defective autolytic enzymes causing mucopeptide synthesis to be rapid. In the absence of methicillin this would lead to wall-encased organisms. The presence of methicillin may be required to slow mucopeptide synthesis to enable insertion of new pieces of wall material. Many penicillin derivatives can support the growth of strain CRD (White, 1968*a*) but methicillin is best, possibly because of its lack of effect on teichoic acid synthesis. There is slight evidence for less active lytic enzymes in strain CRD (Wilkinson & White, unpublished). A number of workers have observed a correlation between teichoic acid defects and susceptibility to lysis. The specific autolytic activity of a *Staphylococcus aureus* mutant lacking teichoic acid was lower than the wild type (Chatterjee, Mirelman, Singer & Park, 1969). Strains of *Pneumococcus* with the choline component of the teichoic acid replaced by ethanolamine became resistant to autolysin and tolerant to penicillin and cycloserine (Tomasz, Albine & Zanati, 1970). The walls of a teichoic acid deficient mutant of *Bacillus subtilis* autolysed less readily than the parent strain (Van Heijenoort *et al.* 1971). Strain CRD walls contain less teichoic acid than the parent strain (White, 1968*b*) and the organisms have now been found to synthesize teichoic acid less quickly than the parent strain. These observations are consistent with strain CRD having less active autolytic enzymes.

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