The absence of D-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*

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In *Bacillus subtilis* the physiological consequences of depriving lipoteichoic acid and wall teichoic acid of D-alanine ester were analysed using insertional inactivation of the genes of the *dlt* operon. Mutant strains which lacked positively charged D-alanine ester in teichoic acids bound more positively charged cytochrome c than other strains. These mutant strains also showed enhanced autolysis and a higher susceptibility to methicillin, which was expressed as accelerated wall lysis, a faster loss of viability and a slower recovery in the postantibiotic phase. The effects of methicillin could be suppressed by simultaneous addition of magnesium ions at low concentrations. The degradation of whole bacteria by bone-marrow-derived macrophages was not influenced by the surface charge and alanylation of the teichoic acids had no protective effect.

Keywords: *Bacillus subtilis*, lipoteichoic acid, wall teichoic acid, autolysis, methicillin

INTRODUCTION

Lipoteichoic acids (LTAs) have been proposed to play an important role in the regulation of wall autolytic enzymes (Cleveland *et al.*, 1975; Höltje & Tomasz, 1975). However, the molecular basis of the regulation is unknown. The D-alanine ester substitution of LTA has repeatedly been suggested to be connected with the physiological role of LTA (Fischer, 1988; Lambert *et al.*, 1977). The positively charged D-alanyl ester residues apparently affect the binding of cations to cell-wall-linked teichoic acids (WTAs) by reducing the number of binding sites (Lambert *et al.*, 1975). Experiments with Mg²⁺-dependent enzymes located in the cytoplasmic membrane of *Bacillus licheniformis* demonstrated that this cation activated the enzymes, possibly via binding to WTA (Hughes *et al.*, 1973). On the other hand, Mg²⁺ may suppress the activity of wall autolytic enzymes, as has been shown with penicillin-treated staphylococci (Kersten & Wecke, 1993).

Since the discovery of the *dlt* operon in *Lactobacillus casei* (Heaton & Neuhaus, 1992, 1994; Neuhaus *et al.*, 1996; Debabov *et al.*, 1996) and *Bacillus subtilis* (Perego

et al., 1995), insertional inactivation of the genes encoding the proteins involved in alanylation of LTA and WTA allows investigation of the effect of the absence of positively charged D-alanine ester, which renders the polyglycerophosphate chains purely anionic. In *B. subtilis* four proteins are involved in the alanylation process (Perego *et al.*, 1995). The gene *dltC* encodes the D-alanine carrier protein (Dcp). The proposed functions of the proteins encoded by the other genes are briefly: *dltA*, ligation of D-alanine to Dcp via D-alanyl AMP; *dltB*, transport of activated D-alanine in a not yet defined form through the cytoplasmic membrane; *dltD*, transfer from the transport form to LTA and possibly WTA, unless WTA is alanylated by D-alanyl transfer from D-Ala-LTA as shown earlier with *Staphylococcus aureus* (Haas *et al.*, 1984; Koch *et al.*, 1985). The fifth gene of the *dlt* operon of *B. subtilis*, *dltE*, has no function in the D-alanylation process (Perego *et al.*, 1995). Accordingly, the mutant defective in this gene has normally alanylated WTA and LTA and it was therefore used in this study together with the wild-type strain JH642 as a control.

Recently we used insertional inactivations of the different genes of the *dlt* operon in *B. subtilis* and found that depriving LTA and WTA of D-alanine ester has no detectable effect on cell growth, basic metabolism, cellular content of phosphorus-containing compounds, ultrastructure, cell separation and formation of flagella

Abbreviations: LTA, lipoteichoic acid; WTA, wall teichoic acid.

(Wecke *et al.*, 1996). There was, however, a significantly enhanced autolysis of the cells in which LTA and WTA were lacking in D-alanine ester.

In the present study we extend the latter experiments and demonstrate an increase of the negative charge of the cell walls, accelerated autolysis and enhanced methicillin-induced lysis. D-Alanine ester thus provides limited protection of the bacterium against methicillin. However, it does not protect the bacterium against phagocytosis and degradation in macrophages.

METHODS

Materials. N-Acetyl-D-[1-¹⁴C]glucosamine (2.11 GBq mmol⁻¹) was purchased from Amersham, cytochrome *c* from Merck, Dulbecco's modified minimum essential medium (D-MEM) and HEPES buffer from Biochrom, glutaraldehyde and osmium tetroxide from Serva, LR white plastic resin from Science Services, methicillin (sodium salt) from Sigma, and Noble agar from Difco.

Bacterial strains and growth. *Bacillus subtilis* JH642 (wild-type) and the isogenic mutants listed below were prepared and characterized as described by Perego *et al.* (1995). The following mutants were used, with the insertionally inactivated genes given in parentheses (for the function of the proteins encoded by these genes; see Introduction): DLT65A (*dltA*), DLT72 (*dltB*), DLT77 (*dltC*), DLT74A (*dltD*), DLT76 (*dltE*). The D-alanine content [mol (mol phosphorus)⁻¹] of LTA was 0.44 in the wild-type JH642 and 0.49 in the mutant DLT76; in the WTA of both strains it was 0.09. In all other mutant strains no alanine was detected either in LTA or WTA. In all strains LTA was additionally substituted with N-acetyl-D-glucosamine [0.1–0.2 mol (mol phosphorus)⁻¹], and the WTA with D-glucosyl residues [0.65 ± 0.06 mol (mol phosphorus)⁻¹].

The bacteria were grown aerobically at 37 °C on a rotary shaker (150 r.p.m.) in a medium containing, l⁻¹: casein hydrolysate, 5 g; meat extract, 1.5 g; yeast extract, 1.5 g; glucose, 2 g (sterilized separately); K₂HPO₄, 3.68 g; KH₂PO₄, 1.32 g. The pH of this medium was adjusted to 7.4 by addition of 1 M NaOH. If not stated otherwise, overnight cultures were diluted with prewarmed medium to OD₅₇₈ 0.05 and incubated for approximately 2.5 h to obtain cells in the exponential growth phase.

Treatment of bacteria with cytochrome *c*. Stationary-phase bacteria of the different strains were suspended in 0.1 M HEPES buffer, pH 7.1, to OD₅₇₈ 1.0. Cytochrome *c* was added to give concentrations of 500 µg ml⁻¹ and 750 µg ml⁻¹. After incubation for 10 min at ambient temperature, the bacteria were removed by centrifugation (12000 r.p.m., 2 min). Binding of cytochrome *c* was expressed as the difference between the absorbance of the initial concentration (*A*_{526i}) and the absorbance in the supernatant after pelleting the bacteria (*A*_{526s}): $\Delta A_{526} = A_{526i} - A_{526s}$.

Electron microscopy. The bacteria were fixed overnight at 4 °C with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and, after washing with this buffer, partially postfixed with 1.5% (w/v) osmium tetroxide at room temperature for 1 h. The fixed samples were washed again and poststained with 1% (w/v) uranyl acetate for 1 h. The fixed and stained bacteria were dispersed in 2% (w/v) Noble agar. Small blocks of agar were dehydrated in ethanol and embedded in LR white plastic resin and polymerized at 60 °C for

48 h. Thin sections were cut with a Reichert OM U3 ultramicrotome and viewed under a Philips 400 electron microscope.

Microcalorimetry. An LKB 2107 flow-microcalorimeter was used as described by Krüger & Giesbrecht (1989). A flow rate of 40 ml h⁻¹ was maintained by a peristaltic pump. An instrumental baseline was established by pumping medium through the calorimeter before inoculating the bacteria. The incubation flask (100 ml) containing 60 ml bacterial suspension (OD₅₇₈ 0.05) was shaken (150 r.p.m.) at 37 °C. The bacterial suspension was pumped through the measuring coil (0.7 ml) of the microcalorimeter and the outflow was returned to the incubation flask. In parallel with microcalorimetric measurements, the OD₅₇₈ of the culture was continuously recorded in a flow-through photometer (Zeiss PM6) at a flow rate of 60 ml h⁻¹.

Degradation of whole bacteria by macrophages. Bone-marrow cells were isolated from the femur and tibia of 6–8-week-old female BALB/c mice (Zentralinstitut für Versuchstierzucht, Hannover, Germany). The cells were seeded on hydrophobic Petriperm dishes (Heraeus) and grown at 37 °C in an atmosphere containing 5% CO₂ on Dulbecco's modified minimum essential medium (D-MEM) containing 5% calf serum, 15% horse serum and 20% L-cell supernatant as colony-stimulating factor. The cells were re-fed on alternate days starting at day 5. The cultivation lasted 10–11 d. The cell walls of the bacteria used in this experiment were specifically labelled with N-acetyl-D-[1-¹⁴C]glucosamine. To 1.5 ml suspension of exponentially growing bacteria (OD₅₇₈ 0.2) N-acetyl-D-[1-¹⁴C]glucosamine, 92.5 kBq, in 100 µl water was added. The bacteria were grown at 34 °C on a shaker overnight to the stationary phase, pelleted by centrifugation, washed twice with cold growth medium and suspended in 1 ml PBS. The bacteria were opsonized with 1 ml 25% rabbit antiserum (diluted in D-MEM) for 30 min at room temperature. Then the bacteria were pelleted by centrifugation and washed twice in 2 ml PBS. After suspension aliquots of these bacteria (about 50 µl in 2 ml medium per dish) were added to the macrophages which adhered to the 'biofoil' of Petriperm dishes – approximately 5 × 10⁵ macrophages per dish (50 mm diameter) and about 100 bacteria per macrophage. After 30 min ingestion the macrophages were washed three times with D-MEM. After 3, 24, 48 and 72 h incubation the supernatant was withdrawn and replaced by 2 ml fresh D-MEM containing penicillin (100 i.u. ml⁻¹) and streptomycin (100 µg ml⁻¹) to prevent secondary infection. The ¹⁴C radio-label of the degraded material, released into the supernatant during the time intervals indicated, was measured using a β-counter from Packard. For each measurement, three dishes were run in parallel.

RESULTS

Growth of bacteria

Fig. 1 shows that the growth curves from the early exponential to the stationary phase are identical for the wild-type (JH642) and a mutant strain (DLT74A) that lacks alanine ester on LTA and WTA. The same is true for the concomitant decrease of the pH from 7.33 to 6.93. Over this pH range, D-alanine ester is relatively stable *in vitro* (Childs & Neuhaus, 1980; Fischer *et al.*, 1981) and *in vivo* the loss of alanine ester from LTA is compensated for by re-alanylation (Haas *et al.*, 1984; Koch *et al.*, 1985).

Fig. 1. Growth curve and concomitant decrease of pH for the wild-type strain JH642 (□) and the mutant DLT74A (▲).

Table 1. Binding of cytochrome *c* by *B. subtilis*

Strains JH642 and DLT76 contain D-alanine ester in LTA and WTA; strains DLT74A, DLT77, DLT72, DLT65A contain no alanine ester in these polymers. Binding of cytochrome *c* was expressed as ΔA_{526} , the difference between the absorbance of the solution containing the initial concentration of cytochrome *c* and that of the supernatant following incubation with the bacteria. Initial concentrations of cytochrome *c*: (a) 500 $\mu\text{g ml}^{-1}$ (38.5 μM), A_{526} 0.327; (b) 750 $\mu\text{g ml}^{-1}$ (57.7 μM), A_{526} 0.458.

Strain tested	ΔA_{526}	
	(a)	(b)
JH642	0.088	0.111
DLT76	0.061	0.086
DLT74A	0.137	0.181
DLT77	0.139	0.161
DLT72	0.147	0.171
DLT65A	0.191	0.213

Cellular content of LTA, WTA and other phosphorus-containing compounds

Irrespective of whether LTA and WTA was alanylated or alanine-free, the cells contained on average, (g dry mass) $^{-1}$: 1139 $\pm$ 85 μmol total phosphorus, 818 $\pm$ 42 μmol nucleic acid phosphorus, 346 $\pm$ 25 μmol WTA phosphorus, and 62 $\pm$ 4 μmol LTA phosphorus. Moreover, examination by electron microscopy indicated that the cell walls had the same thickness. Accordingly, the results presented below refer to the same amount of WTA and LTA, and therefore reflect the difference between purely anionic and partially alanylated polymers.

Detection of negative charges on bacterial surfaces by cytochrome *c*

Binding of the positively charged cytochrome *c* with its contrast-delivering iron atom served to identify the negative charges on the bacterial surfaces. Table 1

Fig. 2. Autolysis of bacteria in 0.1 M HEPES buffer, pH 7.1. Bacteria in the exponential phase of growth (OD_{578} 0.8) were harvested by centrifugation (7000 g, 4 °C, 10 min) washed twice with cold 0.1 M HEPES buffer, pH 7.1, suspended in this buffer to OD_{578} 1.6, and incubated at 32 °C with shaking (150 r.p.m.).

shows that the mutant strains of *B. subtilis* with no D-alanylation of LTA and WTA bound approximately twice as much cytochrome *c* as the wild-type JH642 and the mutant strain DLT76, which both contain D-alanylated LTA and WTA. These data demonstrate that the absence of D-alanylation of LTA and WTA led to increased availability of anionic binding sites.

Cell autolysis

The mutant strains of *B. subtilis* with alanine-free LTA offered the opportunity to analyse the influence of the D-alanyl groups on the activity of wall autolysins using isogenic cells. Exponentially growing bacteria were harvested and incubated in 0.1 M HEPES buffer at pH 7.1 at 32 °C. Autolysis was followed by measuring OD_{578} . As shown in Fig. 2, the mutant strain DLT77 autolysed at a higher rate than either strain with D-alanylated LTA and WTA (JH642, DLT76).

Sensitivity of different *B. subtilis* strains to methicillin

A sensitive indicator of metabolic disturbances is the metabolic heat production measured by flow microcalorimetry (Krüger & Giesbrecht, 1989). As shown in Fig. 3, there was no difference in the heat production profile of the wild-type strain JH642 and the mutant strain DLT74A. Under the influence of methicillin, the typical heat production profile was altered. While the heat output curve of the mutant strain declined to zero, the wild-type strain maintained a higher level of heat output.

The higher methicillin sensitivity of the mutant strain DLT74A was also reflected by the viable counts of the culture. Viability of the mutant strain declined faster than that of the wild-type strain JH642, resulting in a difference of two orders of magnitude after 2 h (Fig. 3).

Fig. 3. (a–d) Heat production (P , μW) in cultures of the wild-type JH642 and the mutant DLT74A in the absence and presence of methicillin ($0.35 \mu g \text{ ml}^{-1}$). Methicillin (meth) was added to exponentially growing bacteria ($OD_{578} 0.5$) at a heat output as indicated by the arrow. (e, f) Influence of methicillin ($0.3 \mu g \text{ ml}^{-1}$) on the viability (c.f.u. ml^{-1}) of exponentially growing cells. Methicillin was added at time zero. Samples were taken at the times indicated, diluted with growth medium, and aliquots ($92.2 \mu l$) were spread on agar plates using a spiral plater (Spiral Systems). Colonies were counted after incubation at $37^\circ C$ for 20 h.

Fig. 4. The postantibiotic effect was followed spectrometrically as described by Rescott *et al.* (1988). Bacteria in the exponential phase of growth ($OD_{578} 0.4$) were exposed to methicillin ($0.35 \mu g \text{ ml}^{-1}$) at $37^\circ C$, 1 h. The antibiotic was removed by centrifugation ($4000 g$, 5 min) and washing the bacteria three times with growth medium. The bacteria were then resuspended in pre-warmed growth medium and reincubated at $37^\circ C$ on a rotary shaker (150 r.p.m.). OD_{578} was measured at the times indicated.

The postantibiotic effect after treatment with methicillin

After short treatment of the bacteria with methicillin (1 h), the subsequent regrowth in antibiotic-free medium showed remarkable differences between the wild-type and the mutant strains (Fig. 4). The wild-type JH642 exhibited a decrease in optical density during the first hour. Then rapid growth followed. In the case of the two mutants with alanine-free LTA (DLT65A,

DLT74A) the period of decreasing optical density continued for more than 2 h and the rate of subsequent regrowth was, at least over the time period studied, approximately one-fifth of the recovery rate observed with the parent strain. Thus the postantibiotic effect also demonstrated that D-alanylation reduced sensitivity to methicillin.

Relief from methicillin effects by Mg^{2+} ions

Since Mg^{2+} ions were shown earlier to interfere with penicillin-induced lysis of *S. aureus* (Kersten & Wecke, 1993), we tested the effect of $MgCl_2$ on methicillin-induced lysis, heat production and viability of the parent strain JH642 and the mutant DLT74A. The results are summarized in Fig. 5. Compared with the addition of methicillin alone, the concomitant addition of $MgCl_2$ prevented autolysis in both the wild-type and the mutant strain. The lysis-protective effect of $MgCl_2$ was also reflected in the heat output curves (P , μW), which, compared with the curves in Fig. 3, suggest an approach to normal metabolism in both the parent and the mutant strain. Compared with Fig. 3, the effect of methicillin on the viability (c.f.u. ml^{-1}) was also strongly reduced by $MgCl_2$. The viability moderately increased in the wild-type strain and only slowly decreased in the mutant.

The protective effect of $MgCl_2$ could also be visualized by electron microscopy. Fig. 6(a) shows cells during normal growth and Fig. 6(b) the high degree of cell lysis caused by methicillin ($0.3 \mu g \text{ ml}^{-1}$). Fig. 6(c) shows the effect of simultaneous addition of methicillin ($0.3 \mu g \text{ ml}^{-1}$) and $MgCl_2$ (0.5 mM): the cells no longer lyse, they show irregular cell shape, irregular apposition of cell

Fig. 5. Effect of MgCl₂ (5.25 mM) on methicillin-induced lysis, heat production (P , μW) and viability (c.f.u. ml⁻¹). MgCl₂ was added simultaneously with methicillin (meth, 0.35 $\mu\text{g ml}^{-1}$). Experimental details were the same as for Fig. 3. For the effect of methicillin in the absence of MgCl₂ see Fig. 3.

wall material, variability of cell size, and the formation of mini-cells. At a tenfold higher magnification (Fig. 7) these alterations are seen in more detail. Mini-cells were identified by analysing the cell wall. If a definite wall could be detected, the thin section was cut from a rather central part of the cell. Caps or peripheral parts of the cell could be identified by the unclear border between cell wall and cytoplasm and the outside, respectively.

Phagocytosis of radiolabelled bacteria and cell wall degradation by macrophages

Ester-linked D-alanine residues are common substituents of both LTA and WTA in many Gram-positive bacteria. Among them are potential pathogens, such as enterococci, group A streptococci, *Listeria monocytogenes* and staphylococci. It is therefore an important question whether alanylation of LTA and WTA protects pathogens from phagocytosis and degradation by macrophages. The wild-type and the isogenic mutants of *B. subtilis* provided for the first time the opportunity to address this question. The cell walls of the wild-type JH642 and the isogenic mutant strains DLT65A and DLT74A were specifically labelled by growing the bacteria in the presence of *N*-acetyl-D-[1-¹⁴C]glucosamine. The labelled bacteria were incubated with bone-marrow-derived macrophages and the ¹⁴C radioactivity released into the supernatant served as a measure of phagocytosis and cell-wall lysis. The results, summarized in Table 2, show that over a time period of 72 h in which more than 60% of the radiolabel was released, there was no difference between the wild-type, containing D-alanine ester in LTA and WTA, and the two

mutant strains with no D-alanine ester in LTA or WTA. We may therefore conclude that the surface charge of the ingested bacteria plays no role in the degradation of cell walls within phagocytes.

DISCUSSION

There is considerable evidence that the charge of the cell wall is important for the activity of the cationic autolytic enzymes. Studies on isolated walls of *B. subtilis* indicated that negative charges are due to phosphate groups of teichoic acids and carboxyl groups of murein (Sonnenfeld *et al.*, 1985). Until now, however, it has not been known to what extent the positively charged D-alanine ester substituents of lipoteichoic acid and wall teichoic acid contribute to the net charge of the cell walls. Our recent measurements revealed for *B. subtilis* JH642 approximately 350 μmol WTA phosphorus and 60 μmol LTA phosphorus (g dry cells)⁻¹ and D-alanine/phosphorus molar ratios of 0.09 and 0.5, respectively (Perego *et al.*, 1995). Accordingly, lack of D-alanine ester, as shown here for the mutant strains, is expected to increase the net negative charge of WTA by 9% and that of LTA by 50%. In a recent electron microscopic study we tried to detect these charge differences by using cationized ferritin (molar mass > 5 $\times 10^5$ kDa) but found no difference between the parent and the mutant strains (Wecke *et al.*, 1996). In both cases iron-containing ferritin granules were visible on the outer surface of the cell. In the present study, we used cytochrome *c*, which is much smaller (13 kDa), and using a spectrophotometric procedure we found the amount of cytochrome *c* bound by the mutant strains was more than double that

Fig. 6. Ultrathin sections of cells of *B. subtilis* DLT77. Samples were taken (a) after normal growth; (b) 3 h after addition of methicillin ($0.3 \mu\text{g ml}^{-1}$); (c) 3 h after simultaneous addition of methicillin ($0.3 \mu\text{g ml}^{-1}$) and MgCl_2 (0.5 mM). Bars, $2 \mu\text{m}$.

of the wild-type at subsaturating cytochrome concentrations. Additionally, thin sections revealed heterogeneous distribution of the negative charges, in so far as the cylindrical part of the cell wall showed more cytochrome *c* label than the cross walls which had just separated (not shown). A similar difference has been previously reported and ascribed to differences in the level of teichoic acid (Graham & Beveridge, 1994; Mauël *et al.*, 1995).

The inhibitory action of negatively charged LTA on cationic autolysins is well established (Cleveland *et al.*, 1975, 1976; Suganaka *et al.*, 1979; Fischer *et al.*, 1981; Fischer, 1988). It is inversely related to the D-alanine ester content (Fischer *et al.*, 1981; Bierbaum & Sahl, 1991) and one would therefore expect autolytic activity to be lower in the mutant strains lacking D-alanine ester substitution. However, just the reverse is true: they show an increase of autolysis and methicillin-induced lysis. As pointed out previously (Wecke *et al.*, 1996), however, the earlier experiments were carried out with LTAs present as micelles, which have a high negative surface charge (Labischinski *et al.*, 1991). Accordingly, micelles may trap cationic autolysin on their surface and prevent its access to the cell-wall substrate. The situation in the living cell may be different because in the cytoplasmic membrane LTA molecules are separated by membrane lipids (Gutberlet *et al.*, 1991) and an increased negative charge may therefore increase the capacity to bind and retain the cationic autolysins. Support for this hypothesis comes from *in vitro* experiments in which deacylation of LTA or dilution of the charges by embedding LTA into Triton X-100 micelles abolished the inhibitory effect (Cleveland *et al.*, 1976; Fischer *et al.*, 1981). Further support is derived from studies on vancomycin-resistant *Enterococcus faecium* D366 mutants in which inducible and constitutively expressed resistance was associated with drastically decreased endogenous and β -lactam-induced lysis (Gutmann *et al.*, 1996). This phenomenon could be neither related to changes in the cell wall nor to alteration of autolysin. The only changed parameter was a doubling of the D-alanine ester content of LTA, which might have decreased the binding capacity for autolysins.

Consistent with this proposition are the observations that the *N*-acetylmuramyl-D-alanine amidase, a major autolytic enzyme of *B. subtilis*, is tightly bound to homologous teichoic acid, possibly by electrostatic interaction (Herbold & Glaser, 1975) and that mutants deficient in teichoic acid are usually also deficient in cell-wall-lytic enzymes (Chatterjee *et al.*, 1969; Boylan *et al.*, 1972; Fiedler & Glaser, 1973). Moreover, the induction of autolysis of *Staphylococcus simulans* by cationic group A lantibiotics was proposed to be caused by a competitive release of the cationic autolysins from negatively charged LTA and WTA (Bierbaum & Sahl, 1991).

The mechanism which triggers endogenous and β -lactam-induced lysis is not yet known. But when the autolytic enzymes are put into operation, the amount of enzyme available seems to decide the magnitude of the effect. Among the diverse effects evoked by methicillin, the primary event seems to be cell lysis (Fig. 6b), which reduces the number of living cells and leads to a breakdown of metabolic heat production (Fig. 3). It is further reflected in the postantibiotic period, during which the recovery of the less damaged alanine-containing cells is much faster than that of the mutant strain (Fig. 4). Confirmative support for this sequence of events comes from the observation that they can be diminished

Fig. 7. Ultrathin section of cells of *B. subtilis* DLT77 after synchronous treatment with methicillin ($0.3 \mu\text{g ml}^{-1}$) and MgCl_2 (0.5 mM) for 3 h. The arrow indicates a mini-cell. Bar, $0.2 \mu\text{m}$.

Table 2. Release of ^{14}C from macrophages after ingestion of wild-type and mutants whose cell walls had been labelled with *N*-acetyl-D-[^{14}C]glucosamine

Strain tested	Percentage of ^{14}C label released after:			
	3 h	24 h	48 h	72 h
JH642	29.8	48.4	60.3	69.5
DLT65A	30.9	46.1	59.6	68.9
DLT74A	27.1	44.3	54.9	67.2

or stopped synchronously by the addition of Mg^{2+} ions (Figs 5, 6c). The mode of action of Mg^{2+} ions in our experiments is not yet understood. It has been well established that the *N*-acetylmuramyl-L-alanine amidase of *B. subtilis* requires divalent metal ions for activity. Mg^{2+} ions at concentrations from 0.5 to 50 mM with a maximum at 20 mM have an activating effect (Foster, 1991; Herbold & Glaser, 1975; Rogers *et al.*, 1984), whereas the results of our experiments suggest a depressing effect on methicillin-induced lysis at Mg^{2+} concentrations as low as 0.5 mM and 2.5 mM (Figs 5, 6c).

The widespread occurrence of alanylated LTA and WTA among Gram-positive bacteria prompted us to study the effect of alanine substitution on the degradation of bacterial cells in macrophages. There was, however, no difference between the wild-type, whose

LTA and WTA contain D-alanine ester, and the mutant strains, in which these polymers lack D-alanine ester. This observation strongly suggests that alanylation is not a means to protect Gram-positive pathogens from degradation by macrophages in the mammalian host.

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