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PAPER

Detection of *Staphylococcus aureus* cell walls by enzyme-linked immunoassay using antibodies prepared from a semi-synthetic peptidoglycan precursor

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The peptidoglycan layer of *Staphylococcus aureus* contains a $(Gly)_5$ cross-link which is not found in other bacteria, and which could be used to develop a specific immunoassay for detection of *S. aureus* in MRSA infections. A semi-synthetic route was used to prepare the *S. aureus* peptidoglycan precursor UDPMurNAc-L-Ala-γ-D-Glu-L-Lys$(Gly)_5$-D-Ala-D-Ala, which was covalently attached to carrier protein bovine serum albumin *via* the UDP nucleotide. Serum raised using this antigen showed specificity for chemically immobilised peptidoglycan monomer containing $(Gly)_5$, using an ELISA immunoassay. ELISA assays using 0.1 or 1.0 μg samples of cell walls prepared from two MRSA strains and one penicillin-sensitive *S. aureus* strain, and from three other bacteria, showed the highest response against cell walls containing $(Gly)_5$, with a particularly high response against cell walls from one MRSA strain. Competition assays to investigate antibody selectivity demonstrated that the antibody response could be most effectively antagonised using ligands containing $(Gly)_5$. These data demonstrate that it is possible to generate antibodies with high affinity and selectivity for the $(Gly)_5$ containing monomer in *S. aureus* peptidoglycan, that could be used to develop an immunoassay for *S. aureus*.

Introduction

Hospital-acquired infections involving methicillin-resistant *Staphylococcus aureus* (MRSA) cause a range of complications, and risk of death, to hospitalised patients in many countries across the world, and the incidence of community-acquired MRSA infections has increased dramatically since the mid-1990s.[1] Screening of patients for MRSA at hospital admission is carried out at many hospitals, and has been shown to lead to decreased risk of MRSA bloodstream infections.[2] Methods for detection of infectious bacteria traditionally involve microbiological tests, immunochemical methods, PCR-based methods, or DNA screening methods.[3] There is considerable interest in the development of novel microfluidic platforms for detection of infectious bacteria including MRSA,[4,5] and recent approaches include a chip-based approach for antibiotic susceptibility analysis,[6] and the use of an anti-staphylococcal bacteriophage.[7] Screening methods in current clinical practice involve either microbiological tests,[2] which can take 48–72 h, or PCR-based methods, which require 2–3 h for a diagnostic test.[8] PCR-based methods are usually based upon detection of the *mecA* gene,[8] encoding a low-affinity penicillin binding protein PBP2′, which is the major genetic determinant for methicillin resistance in MRSA strains.[9,10]

Antibody-based diagnostic tests, which are used to detect human chorionic gonadotropin (hCG) in pregnancy tests,[11] offer the advantage of an immediate diagnostic result, and high sensitivity and selectivity. Enzyme-linked immunoassays are also used in the screening for *Chlamydia trachomatis* lower genital tract infection,[12] where they show comparable accuracy and efficacy to a PCR-based assay.[13] In this study, we wished to investigate whether differences in the peptidoglycan cell wall of *S. aureus* could be used to develop an antibody-based assay for MRSA strains.

The peptidoglycan layer of bacterial cell walls contains a polysaccharide of MurNAc-GlcNAc disaccharide units, cross-linked *via* a pentapeptide sidechain of general structure L-Ala-γ-D-Glu-X-D-Ala-D-Ala.[14,15] In Gram-negative bacteria, X is commonly *meso*-diaminopimelic acid, which forms a direct cross-link with D-alanine at position 4 of a second peptide strand. Gram-positive bacteria usually have lysine at position 3, to which is often attached a short peptide cross-link, which shows significant variation in structure, between bacterial classes, and within classes. *S. aureus* is unusual in containing a Gly_5 cross-link between lysine of one cross-linking peptide chain, and D-Ala of the second peptide chain,[15,16] as shown in Fig. 1. Other staphylococcal strains contain smaller numbers of glycine units in the cross-link, while other Gram-positive bacteria generally contain either no cross-link, or dipeptides such as Ala-Ala or Ser-Ala (found in *Streptococcus pneumoniae*), or a single residue of D-Asp

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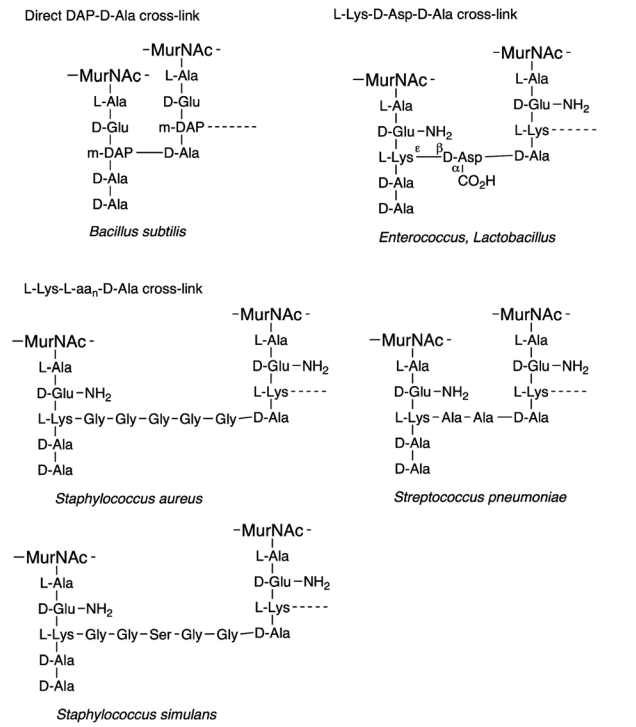

Fig. 1 Peptidoglycan cell wall structures found in Gram-positive bacteria. The α-carboxyl group of D-Asp in *E. faecium* is partly amidated.

(found in *Enterococcus faecium*).[15] A small number of micrococci and streptococci of doubtful taxonomic position are also reported to contain peptidoglycan types similar to staphylococci, but in general the staphylococci are unusual in containing polyglycine cross-links.[15] The variability of the peptidoglycan cross-link has been used as a means of bacterial classification and taxonomy.[15]

The presence of the Gly_5 cross-link could in principle be used to raise anti-Gly_5 antibodies selective for staphylococcal cell walls. In a previous study, Seidl and Schleifer have raised antibodies to the Gly_5 pentapeptide-albumin conjugate, and have found that these antibodies show selectivity in their recognition of staphylococcal cell walls.[17] However, the antigen used in the latter study represents only a part of the peptidoglycan cell wall structure. We have recently developed methods for the enzymatic synthesis of the UDPMurNAc-pentapeptide precursor, containing either L-lysine or *meso*-diaminopimelic acid at position 3,[18] which can be used either to incorporate unnatural amino acids,[19] or to attach, using aqueous solution peptide coupling, amino acid or peptide units selectively to position 3 of this precursor.[20] In this paper, we apply these methods to prepare the UDPMurNAc-decapeptide peptidoglycan precursor found in *S. aureus*, and we report the detection of the staphylococcal peptidoglycan monomer and staphylococcal cell walls using antibodies raised to this antigen.

Results

Preparation of UDPMurNAc-decapeptide antigen

UDPMurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala **1** was prepared enzymatically by the BaCWAN cell wall synthesis facility, in 10–20 mg quantities. Commercially available $H_2NGly_5CO_2H$ was protected with an N-terminal Fmoc group, by treatment with Fmoc-OSu, in 70% yield. $FmocNHGly_5CO_2H$ was activated by EDC and N-hydroxysuccinimide at pH 5.0, forming the intermediate N-hydroxysuccinimide ester, and then coupled at pH 10.0 to **1**, forming UDPMurNAc-L-Ala-γ-D-Glu-L-Lys(ε-$Gly_5NHFmoc$)-D-Ala-D-Ala (HPLC retention time 23.9 min). The Fmoc protecting group was then removed by treatment with piperidine, to give UDPMurNAc-L-Ala-γ-D-Glu-L-Lys(ε-Gly_5NH_2)-D-Ala-D-Ala (**2**, HPLC retention time 7.8 min), which was characterised by electrospray mass spectrometry (*m*/*z* 1433.1 for $[M - H]^-$, calculated 1433.2). The structures are shown in Fig. 2.

UDPMurNAc-decapeptide **2** was then immobilised onto chicken egg lysozyme and bovine serum albumin (BSA) as a carrier proteins. Although the MurNAc-pp-uridine glycosyl phosphate linkage could be readily cleaved under acidic conditions to give decapeptide-MurNAc-OH, attempts to link the glycosidic position to surface lysine groups on both proteins were unsuccessful. However, oxidative cleavage of the uridine

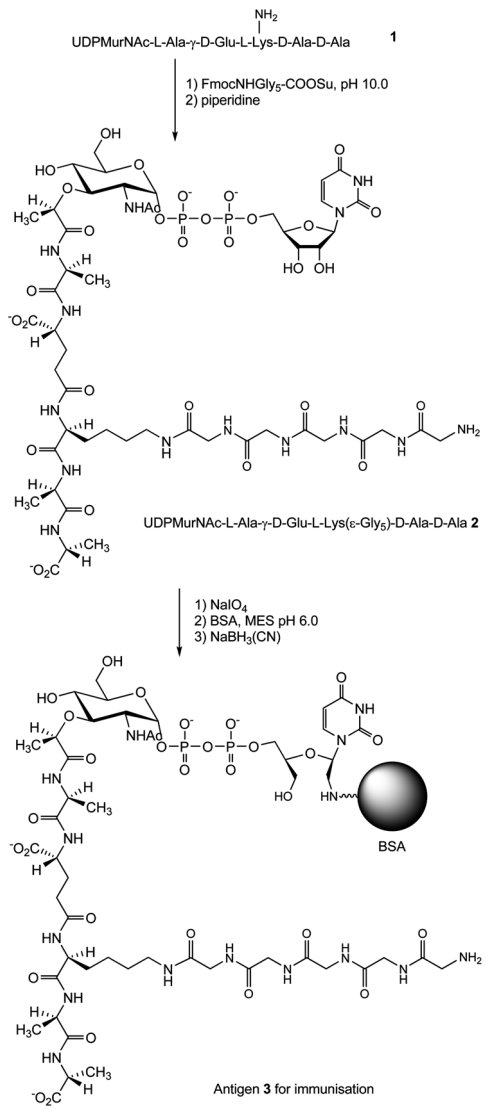

Fig. 2 Chemical synthesis of UDPMurNAc-decapeptide antigen.

ribofuranose ring with sodium periodate formed a reactive dialdehyde, which could be coupled with lysozyme in 50 mM MES buffer at pH 5.5 or 6.0, in the presence of sodium cyanoborohydride, to yield a 1 : 1 conjugate as observed by SDS-PAGE (see Fig. 3).

The same method was used to prepare a UDPMurNAc-decapeptide:BSA conjugate **3**, which was isolated *via* dialysis. Analysis by SDS-PAGE showed a mixture of ligand:BSA conjugates in the range 66–85 kDa, due to the presence of multiple surface lysine residues in BSA, and the presence of some higher conjugates containing a BSA dimer (data not shown). Antibodies to this antigen were raised in sheep, and sera isolated after 6, 10, and 14 weeks.

Affinity of sera for antigen monomer

The affinity of the three sera for the antigen monomer were assessed by ELISA assay. Samples of UDPMurNAc-decapeptide:BSA conjugate **3** were adsorbed onto microtitre plates at loadings of 1 μg and 0.1 μg (both gave satisfactory results). Samples of UDPMurNAc-decapeptide **2**, UDPMurNAc-pentapeptide **1**, and UDPMurNAc-tripeptide **4** were also chemically immobilised onto microtitre plates containing amino functionalised surfaces, using the same periodate cleavage method used above, as shown in Fig. 4A. Plates were treated with serum samples at 1 : 200, 1 : 2,000, and 1 : 20,000 dilutions, washed,

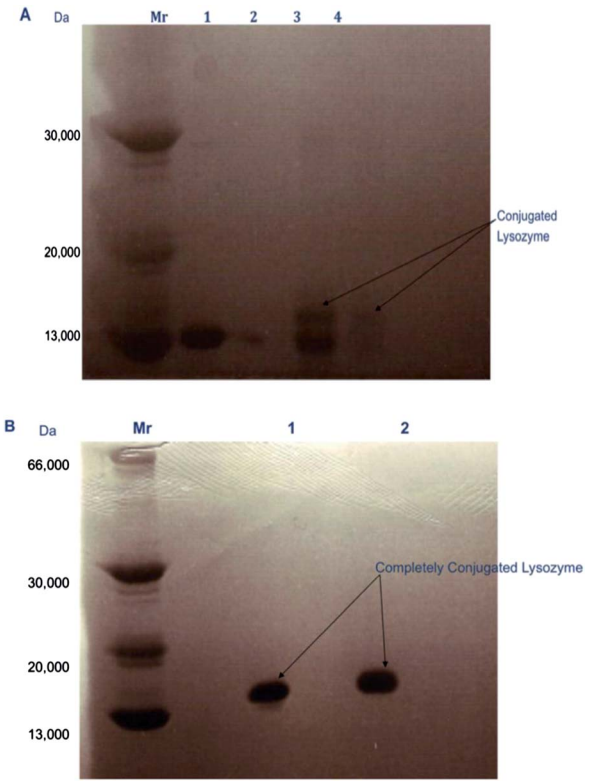

Fig. 3 12% SDS-PAGE gel analysis of lysozyme conjugation with UDPMurNAc-decapeptide after (A) 12 h and (B) 36 h. A. M_r, molecular weight markers; lane 1, lysozyme standard; lane 3, lysozyme conjugation reaction at pH 5.5; lane 4, lysozyme conjugation reaction at pH 6.0. B. M_r, molecular weight markers; lane 1, lysozyme conjugation reaction at pH 5.5; lane 2, lysozyme conjugation reaction at pH 6.0.

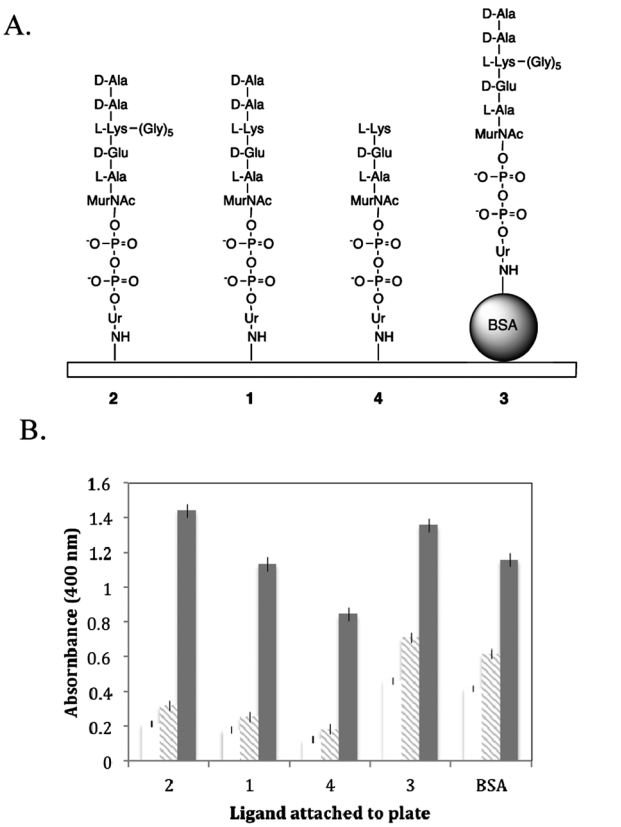

Fig. 4 ELISA data (B) for serum 1 (6 weeks, white), serum 2 (10 weeks, shaded), and serum 3 (14 weeks, grey) for ligands **1–4**, at 1 μg dose, whose structures are shown in part A. Serum was added at 1 : 200 dilution, as described in Experimental Section.

and then treated with anti-sheep IgG-alkaline phosphatase, and binding activity was detected using p-nitrophenyl phosphate.

The ELISA data for the three sera are shown in Fig. 4B. The size of the signal increases from serum 1 (6 weeks) to serum 2 (10 weeks), and markedly to serum 3 (14 weeks), and a higher response was observed at higher dose of ligand. In each case, the response for **2** is greater than that for **1**, which in turn is greater than that for **4**, indicating the presence of antibodies able to recognise the Gly_5 pentapeptide, and the D-Ala-D-Ala dipeptide. Similarly, the signal for the UDPMurNAc-decapeptide:BSA conjugate **3** is 15–25% greater than for BSA alone.

Detection of *Staphylococcus aureus* cell walls

In order to test whether the antibody sera were able to recognise cell walls from *S. aureus*, bacterial cell walls were prepared using a literature method[21] from two methicillin-resistant *S. aureus* strains (MRSA 1049 and MRSA 1050), one penicillin-sensitive *Staphylococcus aureus* strain (strain 1254), *S. simulans* strain 2441, *Streptococcus pneumoniae* strain 298, and *Escherichia coli*. Consistent yields of cell walls were obtained (14–20 mg per 200 ml culture), similar to material prepared previously, which had been used for NMR spectroscopic analysis,[21] and we could not detect the presence of any capsular or non-peptidoglycan polysaccharide in the cell wall samples. Cell walls were adsorbed onto microtitre plates at 1 μg and 0.1 μg doses; sera were applied

at 1 : 200, 1 : 2,000, and 1 : 20,000 dilutions; and ELISA assays carried out as described in the Experimental section.

Signals were observed for each of the cell wall samples, at 1 μg and 0.1 μg doses, as shown in Table 1, with higher response observed at higher cell wall dose, and lower response at higher serial dilutions. In each case, the strongest signal was observed for MRSA strain 1050; followed by *S. aureus* 1254, MRSA 1049 and *S. simulans* 2441; then *S. pneumoniae* 298; then *E. coli*. Even at 0.1 μg dose, a response of >0.2 absorbance units was observed for MRSA 1050. The results indicate a selective, high affinity response for cell walls containing the Gly_5 cross-link, with a particularly high response observed for one of the two MRSA strains.

Competitive ELISA assays were then carried out, in order to examine the specificity of the antibody response. Cell walls were adsorbed onto microtitre plates at 1 μg and 0.1 μg doses. Samples of competitive ligands **1**, **2**, and **4** were mixed at 1 μg ml^{-1} concentration with sera (at 1 : 200, 1 : 2,000, and 1 : 20,000 dilutions), and incubated for 1 h, before applying to the plates; ELISA assays were then carried out as before. The results for the 1 : 200 dilution of bleed 3 are shown in Fig. 5. In each case, competition was observed, in the order: UDPMurNAc-decapeptide (**2**) > UDPMurNAc-pentapeptide (**1**) > UDPMurNAc-tripeptide (**4**). The results indicate that much of the specificity of the antibody response is due to interaction with the Gly_5 chain, and the D-Ala-D-Ala terminus of the pentapeptide chain.

Conclusions

Using a previously developed synthetic procedure,[20] we have prepared the UDPMurNAc-decapeptide peptidoglycan precursor of *S. aureus*, which has been conjugated to BSA. This antigen has been used to raise a sheep polyclonal antibody response, that shows binding specificity for chemically immobilised UDPMurNAc-decapeptide monomer (Fig. 4), and shows binding specificity for bacterial cell walls containing the Gly_5 cross-link (Table 1). Of the cell walls tested, the three *S. aureus* strains all contain the Gly_5 cross-link, and show the highest antibody response. The peptidoglycan structures of other staphylococcal strains such as *S. simulans* also contain pentaglycine cross-links, but contains 0.5–1 equivalent of Ser in place of Gly,[15] which would explain the slightly lower antibody response against *S. simulans* cell walls. The peptidoglycan of *S. pneumoniae* is based upon the same pentapeptide stem peptide, but contains either no cross-link, or an Ala-Ala or Ser-Ala dipeptide, consistent with the lower antibody response observed, while *E. coli* peptidoglycan contains a direct cross-link.

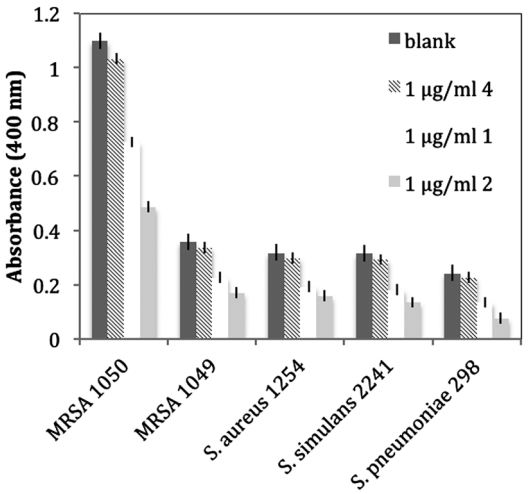

Fig. 5 Competitive cell wall ELISA data for 1 μg dose of cell walls from *S. aureus* MRSA 1050, MRSA 1049, strain 1254, *S. simulans* strain 2441, and *S. pneumoniae* strain 298, at a 1 : 200 dilution of serum from bleed 3, in competition with 1 μg ml^{-1} **4** (diagonal shading), **1** (unfilled), and **2** (light grey).

The competitive ELISA data shown in Fig. 5 shows a reduction in signal in the order: UDPMurNAc-decapeptide **2** > UDPMurNAc-pentapeptide **1** > UDPMurNAc-tripeptide **4**. This is the same order of binding affinity observed using chemically immobilised ligands (Fig. 4), and together these data demonstrate that there are antibodies present that show selectivity for the Gly_5 cross-link, and indicate that the terminal D-Ala-D-Ala dipeptide on the pentapeptide stem is also responsible for binding specificity. We note that the amount of D-Ala-D-Ala dipeptide present in cell wall compositions will vary considerably, due to the action of D,D-carboxypeptidases.[14] Some level of competition is also observed for the *S. pneumoniae* cell walls, indicating that there are antibodies present that are able to recognise the pentapeptide stem and a dipeptide cross-link, which are antagonised to a lesser extent by the same ligands. Antibodies raised previously against a $(Gly)_5$-albumin conjugate also showed specificity towards cell walls containing Gly_5 cross-links,[17] but somewhat lower sensitivity: in the earlier study, 10–80 μg antigen was required for a strong response,[17] whereas the ELISA assay developed here is sensitive to 1 or 0.1 μg antigen.

The observation that a much stronger response was obtained against cell walls from MRSA strain 1050 is particularly interesting. The peptidoglycan of all three *S. aureus* strains is biosynthesised from the same monomer unit,[14] and yet there is

Table 1 ELISA data for serum assayed against bacterial cell walls at 0.1 μg and 1 μg dose. Minimum Inhibitory Concentration (MIC) for ampicillin for each strain is also listed

Strain	Cell wall peptide cross-link	MIC (μg ml^{-1})	ELISA assay data @ 0.1 μg cell walls	ELISA assay data @ 1.0 μg cell walls
S. aureus MRSA 1050	-Gly_5-	64	0.225 ± 0.01	0.994 ± 0.02
S. aureus MRSA 1049	-Gly_5-	16	0.061 ± 0.005	0.667 ± 0.01
S. aureus 1254	-Gly_5-	0.25	0.067 ± 0.005	0.707 ± 0.03
S. simulans 2241	-Gly_4Ser-	0.125	0.038 ± 0.004	0.587 ± 0.01
S. pneumoniae 298	-Ala-Ala-	ND	0.023 ± 0.002	0.480 ± 0.02
Escherichia coli	direct	ND	0.004 ± 0.002	0.346 ± 0.02

clearly a difference in the antibody response. MRSA strain 1050 shows a higher level of penicillin resistance (MIC 64 μg ml^{-1}) than MRSA 1049 (MIC 16 μg ml^{-1}), therefore it is possible that there are differences in the three-dimensional structure of the polymerised cell wall in MRSA 1050, compared with that of MRSA 1049 and *S. aureus* 1254, that are recognised by antibodies present in the sera. There are literature reports of increased cell wall thickening in some MRSA strains that show associated resistance to vancomycin[22] and other antibiotics,[23] therefore, there may be changes in the three-dimensional structure of the peptidoglycan cell wall in such strains.

In summary, these observations indicate that it is feasible to generate antibodies that can bind selectively to the peptidoglycan structure found in *S. aureus* cell walls, and that there may be differences between the cell wall architecture of MRSA *vs.* penicillin-sensitive *S. aureus* strains, that could be used to develop an immunoassay for detection of MRSA strains.

Experimental section

Bacterial strains

Staphylococcus aureus strains 1254 (ampicillin MIC 0.25 μg ml^{-1}), MRSA 1049 (ampicillin MIC 16 μg ml^{-1}), MRSA 1050 (ampicillin MIC 64 μg ml^{-1}), *Staphylococcus simulans* strain 2441 (ampicillin MIC 0.125 μg ml^{-1}) and *Streptococcus pneumoniae* strain 298 were from the strain collection of Prof. C. Dowson (Department of Life Sciences, University of Warwick).

Preparation of FmocGly$_5$-OH

H_2N-Gly$_5$-OH (25 mg, Bachem) was dissolved in 20 ml water followed by the addition of $NaHCO_3$ (17.5 mg). The resulting solution was cooled to 4 °C. A cold solution of Fmoc-OSu (27.5 mg) in 20 ml acetonitrile was added drop wise. The resulting mixture was stirred at 4 °C for 1 h and was allowed to warm to room temperature overnight. Acetonitrile was removed *in vacuo*. Excess water was added to the mixture. The mixture was extracted with dichloromethane (2 × 100 ml) and concentrated *in vacuo* to yield the final product, which was a white powder (30 mg, 68%). The product was not purified further and was stored at −20 °C. A sample was analysed by electrospray mass spectrometry (*m/z* 524.1, −ve ion) to confirm the molecular weight of the final compound. FmocNH-Gly$_5$-OH was analysed by HPLC (see gradient below), and gave a retention time of 29.5 min.

Preparation of UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(ε-Gly$_5$)-D-Ala-D-Ala (2)

Fmoc-pentaglycine (12.6 mg, 24 μmol) was dissolved in 2 ml acetonitrile followed by the addition of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (17.2 mg, 110 μmol), N-hydroxysuccinimide (6.9mg, 60 μmol) and N-(ethylsulfite)-morphiline (1.9 mg, 10 μmol). The pH was adjusted to 5.0. The reaction mixture was incubated at room temperature for 20 min. After the incubation, 100 μl of 20 mM UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (**1**, prepared by the BaCWAN synthesis facility[24]) in 500 mM sodium bicarbonate at pH 10.0 was added to the mixture. The suspension was stirred for 20 min and was allowed to react overnight at room temperature. After the overnight incubation, 100 μl of 20% piperidine was added to the reaction mixture and was further incubated for 30 min. The reaction mixture was diluted by the addition of 18 ml of water, and the solution was filtered with a nitrocellulose syringe filter (0.20 μm pore size). The final product was freeze-dried and stored at −20 °C, prior to purification.

The UDP-MurNAc-decapeptide was purified using reverse-phase HPLC, using a Phenomenex semi-prep C_{18} column. The dried sample was resuspended in 3 ml of water, and 300 μl aliquots were injected on each run. The solvents used for the purification were water (A) and acetonitrile (B). The detection method used was UV-detection and the wavelength at which the samples were detected was 262 nm. The flow rate used was 2.5 ml min^{-1}, and the gradient used was as follows: from 0–70% B (0–20 min); 70–100% B (20–25 min); 100% B (25–30 min). UDP-MurNAc-decapeptide had a retention time of 7.8 min.

The purified UDP-MurNAc-decapeptide from each run was pooled together and the acetonitrile was removed *in vacuo*. The solution was freeze-dried to yield the final product, which was a white powder (1.5 mg, 60% yield). The final product was stored at −20 °C. A sample was analysed by electrospray mass spectrometry (*m/z* 1433.1 (M-H)$^-$, −ve ion) to confirm the molecular weight of the final compound.

Attachment to carrier protein & immunisation

UDP-MurNAc-decapeptide (30 μl, 10 mM) was treated with sodium periodate (30 μl, 30 mM) and incubated for 2 h at 25 °C. The mixture was diluted by the addition of water (240 μl), and was stored at −20 °C. A single conjugation reaction of ring-cleaved material was performed with bovine serum albumin (BSA) in the presence of a pH 6.0 buffer. The incubation contained: 400 μl BSA (10 mg ml^{-1}); 400 μl 0.2 M MES buffer (pH 6.0); and 2.2 ml cleaved UDP-MurNAc-decapeptide (1 mM). The reaction mixture was incubated for 30 min at 37 °C, followed by the addition of sodium cyanoborohydride (200 μl, 80 mM). The reaction mixture was left to react for 2 days, and was stored at 4 °C. The conjugated sample of BSA (1.5 ml) was transferred into dialysis membrane (12–14 kDa molecular weight cut off) and was dialysed in 500 ml of phosphate buffered saline solution (PBS), which contained 4 g NaCl, 0.1 g KCl, 0.72 g Na_2HPO_4, and 0.12 g KH_2PO_4. The pH of the buffer was adjusted to pH 7.4 by the addition of dilute HCl. The sample was dialysed overnight at 4 °C, and was stored at the same temperature.

Immunisation of one mixed-hybrid vigour Mule sheep was carried out according to the following schedule: 0.3 mg protein in complete Freund's adjuvant (prebleed taken); at 4 weeks, booster with 0.1 mg protein in incomplete Freund's; at 6 weeks, Sample Bleed 1 taken (2–4 ml); at 8 weeks, booster with 0.1 mg protein in incomplete Freund's; at 10 weeks, production Bleed 2 taken (400 ml); at 12 weeks, booster with 0.1 mg protein in incomplete Freund's; at 14 weeks, production Bleed 3 taken (400 ml).

ELISA assay against ligand monomer

The UDP-MurNAc-peptide samples were treated with sodium periodate to cleave the ribose ring (as explained above) before being plated on to the 96-well plates. The UDP-MurNAc-peptide

samples (100 μl volume) were plated on amino-functionalised plates (NUNC-immuno plates), whereas all the other samples were plated on MaxiSorp plates (NUNC-immuno plates). The plates were incubated at room temperature for 3–5 h followed by an overnight incubation at 4 °C, before being used for the ELISA assays.

The whole assay was carried out at room temperature. The plates were washed with a phosphate buffered saline tween solution (PBST), which contained 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and 2 ml Tween 20, in 1 litre of water, by emptying the contents of the plates and washing them twice with about 200 μl of the PBST buffer. The washes were carried out to remove any unbound material. Milk marvel (0.2% in PBST) 100 μl was added to each well to block non-specific binding. The plates were incubated for 1 h at room temperature, followed by a PBST buffer washing step (as above). Dilutions (1 in 200, 1 in 2,000, and, 1 in 20,000) of serum samples (containing primary antibodies) were prepared by diluting the original serum sample in the PBST buffer and applied on to the appropriate wells (100 μl in each well). For the negative controls, PBST buffer (100 μl) was added instead of serum samples. The plates were incubated again for 1 h at room temperature, followed by a PBST buffer washing step. A 1 in 30,000 dilution sample of the secondary antibody-enzyme complex of donkey anti-sheep IgG antibody fused to alkaline phosphatase (Sigma) was prepared by diluting 3 μl of the antibody-enzyme complex in 90 ml of PBST buffer, and 100 μl of this sample was added to each well. The plates were incubated again for 1 h at room temperature, followed by a PBST buffer washing step. A substrate solution of p-nitrophenyl-phosphate (1 mg ml^{-1} in PBST buffer) was prepared. The substrate was added to the plates 50 μl in each well) and incubated for 15–30 min at room temperature. After 15–30 min incubation the absorbance values were measured for the all the samples at 400 nm using a TECAN GENios (Jencons-PLS) plate reader.

Preparation of bacterial cell walls

The procedure is based on the method of Sharif *et al.*[21] Staphylococcal and streptococcal strains were grown (in a Category 3 microbiological facility) in brain heart infusion media (200 ml) at 37 °C to OD_{600} 0.8; *Escherichia coli* and micrococcal strains were grown in ZY media (200 ml) at 37 °C to OD_{600} 0.6. After reaching this optical density, the strains were boiled at 100 °C for 1 h. The cells were then harvested by centrifugation at 10,000 *g* for 10 min at 4 °C. The cells were rinsed with 30 ml of an ice-cold solution of 40 mM triethanolamine hydrochloride (pH 7.0). Cell pellets were then resuspended in 5 ml of the same buffer, followed by rapid freezing and lyophilisation. Lyophilised cells were resuspended in 10 ml of 25 mM potassium phosphate solution (pH 7.0) and boiled for 30 min, then placed on an ice bath for 2 min. The cell solution was then placed in a BioSpec Bead Beater with beads of a 0.5 mm diameter, followed by ten 1 min cycles of cell disruption at 4 °C, separated by 1 min cooling periods at 0 °C. The glass beads were separated from the broken cells with a coarse sintered glass funnel (20 μm). The cell solution was then centrifuged at 10,000 *g* for 1 h at 4 °C to remove the cell debris.

The crude sample of cell walls was resuspended in 5 ml of 10 mM triethanolamine hydrochloride buffer (pH 7.0), and this solution was added dropwise to 10 ml of boiling 4% sodium dodecyl sulphate (SDS) with stirring. The mixture was boiled for 30 min, and then allowed to cool to room temperature, and incubated overnight. The solution was then centrifuged at 38,000 *g* for 1 h. The cell pellet was washed 6 times with 10 ml of 10 mM triethanolamine hydrochloride buffer to remove the SDS. The pellet was treated with DNAse I (0.5 mg), trypsin (1.6 mg) and α-chymotrypsin (1.6 mg) in 6 ml of 10 mM Tris-buffer (pH 8.2) at 37 °C, and shaken at 150 rpm for 16 h. The solution was then centrifuged at 38,000 *g* for 1 h at 20 °C, followed by 6 washes with 6 × 10 ml of 10 mM triethanolamine hydrochloride buffer. The cell pellet was resuspended in 5 ml of 10 mM triethanolamine hydrochloride buffer, followed by rapid freezing and lyophilisation to provide pure cell wall. This procedure yielded 14–20 mg of cell walls for each of the staphylococcal strains studied, from an initial 200 ml culture, and 9 and 11 mg respectively for *S. pneumoniae* 298 and *Escherichia coli*. Treatment of a small sample of cell wall with lysozyme, followed by analysis by thin layer chromatography (silica plates, 88% $CHCl_3$/48% MeOH/10% H_2O/1% NH_3) revealed the disappearance of the R_f 0.0 spot corresponding to peptidoglycan, indicating the absence of other polysaccharides in the samples.

ELISA assays against bacterial cell walls

All the three bleeds (bleed 1, 2 and 3) and the pre-immune bleed were screened against samples of bacterial cell walls from all the six different strains. Cell wall (1 μg or 0.1 μg) was adsorbed on to the walls of MaxiSorp 96-well plates. Serum was added at three different dilutions (1 in 200, 1 in 2,000 and 1 in 20,000 of the original sample). The assay was carried out as described for the ELISA assay above.

For competitive ELISA assays, the bacterial cell walls were adsorbed on to the surface of the plates, and samples of **4**, **2**, and **1** (1 μg ml^{-1}) were incubated with the serum samples (bleeds 2 and 3 and pre-immune bleed @ 1 in 200 dilution) for 1 h before being added to the plates. The rest of the assay was performed as described above. After performing the assay, the absorbance values were measured for all the samples at 400 nm. Two different amounts of the cell walls were adsorbed (1 μg to 0.1 μg). A control for each cell wall sample was carried out, in which the serum samples were directly applied on to the plates.

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24 For details of the BaCWAN synthesis facility, see: http://www2.warwick.ac.uk/fac/sci/lifesci/people/droper/bacwan/synthesis/.

