Vaccine development is a priority for global health due to the growing multidrug resistance in bacteria. D-glutamate synthesis is essential for bacterial cell wall formation. Here we present a strategy for generating effective bacterial whole-cell vaccines auxotrophic for D-glutamate. We apply this strategy to generate D-glutamate auxotrophic vaccines for three major pathogens, Acinetobacter baumannii, Pseudomonas aeruginosa and Staphylococcus aureus. These bacterial vaccines show virulence attenuation and self-limited growth in mice, and elicit functional and cross-reactive antibodies, and cellular immunity. These responses correlate with protection against acute lethal infection with other strains of the same species, including multidrug resistant, virulent and/or high-risk clones such as A. baumannii AbH12O-A2 and Ab307-0294, P. aeruginosa PA14, and community-acquired methicillin-resistant S. aureus USA300LAC. This approach can potentially be applied for the development of live-attenuated vaccines for virtually any other bacterial pathogens, and does not require the identification of virulence determinants, which are often pathogen-specific.
The emergence of multidrug-resistant (MDR) bacteria such as MDR Acinetobacter baumannii, MDR Pseudomonas aeruginosa and methicillin-resistant Staphylococcus aureus (MRSA), and the lack of licensed vaccines against these pathogens, have resulted in significantly increased mortality rates with limited or no options for therapeutic interventions\(^1\)-\(^3\). Due to this threat to public health, a call for greater consideration for the role of vaccines and prevention to combat antibiotic-resistant bacteria was recently made by the National Vaccine Advisory Committee\(^9\). Bacterial live vaccines can be highly efficient because they are harmless versions of pathogenic bacteria and mimic the natural infection. They have been used extensively for decades to prevent respiratory or enteric pathogens in humans\(^10\)-\(^12\), and the most frequent infections of herd animals\(^13\)-\(^20\). Due to this threat to public health, a call for greater consideration for the role of vaccines and prevention to combat antibiotic-resistant bacteria was recently made by the National Vaccine Advisory Committee\(^9\). Bacterial live vaccines can be highly efficient because they are harmless versions of pathogenic bacteria and mimic the natural infection. They have been used extensively for decades to prevent respiratory or enteric pathogens in humans\(^10\)-\(^12\), and the most frequent infections of herd animals\(^13\)-\(^20\). Traditionally, attenuation of virulence was achieved through natural selection by multiple passages of the microorganism on growth medium, random mutagenesis or genetic modification of target genes to induce virulence attenuation. Considering that there are no universal gene targets for bacterial attenuation, the latter approach usually involves basic knowledge of molecular mechanisms of pathogenicity for each bacterial species or strain.

D-glutamate (D-Glu) is an essential component of bacterial peptidoglycan and is found in the cell wall of virtually all bacteria\(^21\)-\(^24\). Earlier work suggests that glutamate racemase (MurI; EC 5.1.1.3), enzyme that converts L-glutamate to D-Glu for the synthesis of peptidoglycan, can be targeted for antibiotic development\(^25\),\(^26\). However, none of the previous work proposed the usefulness of MurI mutants as live bacterial vaccines. In contrast, here we present a platform for the generation of effective live-attenuated bacterial vaccines composed of D-Glu auxotrophic strains, which can be obtained through the inactivation of the gene (or genes)-encoding MurI and also the gene (or genes) coding for the D-amino acid transaminase (Dat) capable of catalysing the interconversion of D-alanine to D-Glu (Fig. 1). More importantly, this strategy has potential application to any bacterial pathogen.

In response to the challenge for combating antibiotic-resistant pathogens, we genetically manipulated these bacteria to obtain D-Glu auxotrophic strains, creating mutant derivatives with in-frame deletions in MurI or MurI plus Dat-coding genes, on A. baumannii ATCC 17978, P. aeruginosa PAO1 and S. aureus 132 and demonstrated their reliable efficacy as live vaccines in a mouse model of acute lethal infection.

### Results

**Characterization of D-glutamate auxotrophic strains.** Analysis of their genome sequences revealed two murI genes in A. baumannii strain ATCC 17978 (hereafter ‘Ab’) (A1S_0380/murI1 and A1S_3398/murI2)\(^27\), a single-murI gene in P. aeruginosa strain PAO1 (hereafter ‘Pa’) (PA4662/murI)\(^28\) and a single-murI gene and a single-dat gene\(^29\) in S. aureus strain 132 (hereafter ‘Sa’). All these genes were targeted for unmarked in-frame deletion. The resulting Pa \(\Delta murI\) mutant showed an absolute requirement of exogenous D-Glu for growth. Likewise, the double-mutants Ab \(\Delta murI\Delta dat\) and Sa \(\Delta murI\Delta dat\) also required exogenous D-Glu for growth. However, the single-mutants grew normally without the addition of D-Glu (Fig. 2a). Using real-time PCR with quantitative reverse transcription (qRT–PCR) (Fig. 2b), we confirmed the absence of murI and dat genes mRNA in these mutant strains. This means that for those bacteria not possessing the Dat enzyme, the inactivation of a single- or multiple-MurI enzymes may be required for production of a D-Glu auxotrophic strain, as in the cases of Pa and Ab (MurI\(^-\) mutants). In other cases, the inactivation of genes coding for MurI protein as well as those coding for Dat seems to be required (MurI\(^-\)Dat\(^-\) mutants), as in the case of Sa.

To assess the effects of D-Glu deprivation on cellular growth of the wild-type and D-Glu auxotrophic strains, we collected bacteria grown in media supplemented with different concentrations of D-Glu and performed a morphological analysis by scanning electron microscopy (SEM) (Fig. 2c). Ab \(\Delta murI\Delta murI\Delta dat\) showed growth impairment when the exogenous supply of D-Glu decreased, until complete loss of replication. Both Ab \(\Delta murI\Delta murI\Delta dat\) and Sa \(\Delta murI\Delta dat\) strains exhibited an altered pattern of cell division when D-Glu was supplied at 0.1 mM. The Ab \(\Delta murI\Delta murI\Delta dat\) mutant showed filamentous aggregates consisting of three or

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**Figure 1** | **D-Glu auxotrophy is achieved by inactivation of D-Glu producing enzymes.** (a) Sequence of metabolic processes culminating in the formation of D-Glu and the incorporation in the bacterial peptidoglycan. (b) Cell wall structure of a gram-negative bacterium (non-depicted lipopolysaccharides and proteins). (c) Cell wall structure of a gram-positive bacterium (non-depicted teichoic acids and proteins). D-Ala, D-alanine; DAP, Diaminopimelic acid; D-Glu, D-glutamate; PG, peptidoglycan; OM, outer membrane; IM, inner membrane; M, cytoplasmic membrane.
more units of cells with atypical binary fission while Sa Δmur1 Δdat displayed large clusters of irregular cells due to impaired cell separation (Supplementary Fig. 1). A mass of presumptive protoplasts was observed in the vicinity of the intact cell forms of the mutant strains. With 1.25 mM D-Glu for A. baumannii and 1.5 mM D-Glu for S. aureus (Fig. 2c) a greater cell density was

Figure 2 | Characterization of D-Glu auxotrophic. (a) Growth and viability of A. baumannii ATCC 17978 (Ab), P. aeruginosa PAO1 (Pa), S. aureus 132 (Sa) and derived mutant strains (mean ± s.e.m.). (b) Comparative expression levels by qRT–PCR of murI in Ab (normalized relative to gyrB), murI in Pa (normalized relative to proC), murI and dat in Sa (normalized relative to gyrB) and derived mutant strains (mean ± s.d.). *P < 0.05. (c) SEM of Ab, Pa, Sa and derived D-Glu auxotrophic strains in the presence of different D-Glu concentrations.
seen with respect to the preceding condition but with many visible protoplast-like structures. Also, though lower in number, some cells had an appearance similar to their wild-type morphology. Finally, when D-Glu was supplemented at 10 or 20 mM, both morphology and cell density of the mutant strains were indistinguishable from the corresponding wild-type homologues.

Transmission electron microscopy (TEM) showed that the cell walls of the three mutant strains experienced progressive destruction when kept in the absence of D-Glu. We observed cells with altered conformations that had lost their semi-rigid structure, several ruptures and displacement of membranes, lysis and extrusion of the intracellular content (Fig. 3). Thus, we propose the following mechanism for the lysis of D-Glu auxotrophic bacteria (Fig. 3d). First, blockage of peptidoglycan biosynthesis leaves the protoplasm surrounded only by the inner membrane (gram-negative) or cell membrane (gram-positive bacteria), which renders this cell body susceptible to the variations in the osmolarity of the medium. Ultimately, lysis occurs through osmotic pressure, leaving traces of the cytoplasmic membranes that can form aggregates, and internal cellular content. These phenomena may occur either simultaneously or sequentially.

To be safe and suitable as a genetically modified organism (GMO) vaccine, an inherent property of these organisms must be that the attenuation mutations are not reversible. To check the irreversibility of the nutritional, the three mutant strains were grown with D-Glu and their viability evaluated for 8 days on agar plates with and without D-Glu. In the hypothetical case of a phenotype reversion, similar bacterial counts would be expected in plates over time. In contrast, we observed higher bacterial counts in supplemented plates at the initial stage of incubation and on subsequent days than on standard medium (Supplementary Fig. 2a), indicating that these strains remain auxotrophic for D-Glu over time.

**Figure 3 | D-Glu auxotrophy produces cell wall degeneration and bacterial lysis.** (a–c) Different atypical morphologies, progressive degeneration of the cell wall, membrane-bursting events and lysis of D-Glu auxotrophic strains after being kept in the absence of D-Glu (red dotted arrows). Micrographs were taken with a TEM at different scales. (a) *A. baumannii* ATCC 17978 (Ab) and Ab Δmurl1 Δmurl2. (b) *P. aeruginosa* PAO1 (Pa) and Pa Δmurl. (c) *S. aureus* 132 (Sa) and Sa Δmurl Δdat. (d) Proposed schematic mechanism of cell wall degeneration and bacterial lysis of (1) gram-negative and (2) gram-positive bacteria auxotrophic for D-Glu.
We also evaluated immune responses in BALB/c mice administered different doses of the three mutant strains (Supplementary Fig. 6). Significant levels of IgM and total IgG were present in all immunized mice at the end of the immunization schedules (P<0.05, Student’s t-test). All tested α-Ab vaccine doses above ~2x10^6 CFU elicited significant levels of IgG1, IgG2a, IgG2b and IgG3 subtypes. Although 6x10^6 CFU of α-Pa vaccine was sufficient to induce IgM and IgG titres substantially, total IgG titres were higher when using a dose of 2x10^7 CFU. For α-Sa vaccine, only doses of 7.5x10^7 and 1.5x10^8 CFU raised significantly all IgG subtypes (P<0.05, Student’s t-test); while using 2.5x10^7 CFU elicited IgG1 and IgG3 subtypes only.

Adverse reactions can potentially occur upon vaccine administration. In those cases, vaccines need to be reformulated without their immunogenic properties being negatively affected. To test the ability of D-Glu auxotrophic vaccines to trigger humoral responses at low-dose formulation, a single immunization with 4x10^6 CFU of α-Ab vaccine was used in BALB/c mice (Fig. 4c). Despite the low dose used, significant levels of antigen-specific IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 were detected in the sera of mice (P<0.05, Student’s t-test), 123 days and 1 year after the immunization. Also, IgM titres were higher at day 7 but markedly reduced on day 123. In contrast, IgG1, IgG2b and IgG3 titres increased significantly between days 7 and 123 (P<0.05, one-way ANOVA followed by Bonferroni’s post hoc test); total IgG levels were maintained until 1 year after immunization, indicating a biphasic response and long-term antibody memory.

A key feature of any single-strain vaccine is its ability to generate antibodies reactive against heterologous strains. IgG antibodies elicited with α-Ab vaccine and obtained on days 21, 42, 98 and 292 were cross-reactive with A. baumannii ATCC 19606, MDR-AbH120-A2 and encapsulated-Ab307-0294 strains (Fig. 4d). IgG antibodies obtained on days 21–25 after two α-Pa immunizations were cross-reactive with P. aeruginosa PA21-ST175, PA14, PA51441321, PA12142, LES400, LES431, PA28562 and PA51442390. At days 42, 98 and 292, these IgG’s were cross-reactive with the previous strains plus P. aeruginosa PA_ST235. Also, IgG’s obtained with three α-Pa immunizations were cross-reactive against all the P. aeruginosa strains tested (Supplementary Fig. 7). Note, P. aeruginosa PA28562 and PA51442390 present the mucoid phenotype associated with chronic infections from cystic fibrosis patients. IgG antibodies elicited with α-Sa vaccine and obtained on days 21, 42, 98 and 301 were cross-reactive with S. aureus FPR3757 and MW2 strains of the clinically relevant CA-MRSA high-risk clones USA300 and USA400, respectively; with Newman and Sa07997; as well as with S. aureus strains of animal origin, RF122 (bovine), ED133 (ovine) and ED98 (poultry). These data indicate that vaccination with D-Glu auxotrophic strains elicits both early and long-term antibody memory against parental and heterologous unrelated strains, suggesting these vaccine candidates would be suitable to induce ample protection.

In addition, vaccination with α-Sa strain elicited IgG cross-reactivity with a variety of coagulase-negative (CoN) Staphylococcus species (Supplementary Fig. 8), supporting that α-Sa vaccine could be considered for broader applications, such as the prevention of device-associated health care-associated infections recognized as the most clinical relevant CoN-staphylococcal infections.

**Activation of cell-mediated immunity.** To explore the nature of T-cell responses generated by the vaccine strains, we measured antigen-specific IFN-γ-, IL-4- and IL-17-secreting splenocytes of BALB/c mice after ex vivo restimulation, using ELISpot. These
Figure 4 | Vaccination with D-Glu auxotrophic strains elicits early and long-term antibody memory against parental and heterologous unrelated strains. (a) Antibody titres against A. baumannii ATCC 17978 (n = 5–13), P. aeruginosa PA01 (n = 4–10) and S. aureus 132 Δspa (n = 5–10) in vaccinated and control mice after one or two injections with ATCC 17978 ΔmurI ΔmurI2 (α-Ab vaccine) (6 × 10^7 CFU), PA01 ΔmurI (α-Pa vaccine) (2 × 10^7 CFU) and 132 ΔmurI Δdat (α-Sa vaccine) (3 × 10^7 CFU), respectively, or saline. (b) Antibody titres against PAO1 in vaccinated and control mice (n = 8) after three intramuscular (IM) injections with α-Pa vaccine (2 × 10^7 CFU), or saline, respectively. (c) Antibody titres against ATCC 17978 in vaccinated and control mice (n = 5–8) after α-Ab vaccine (4 × 10^7 CFU), or saline administration, respectively. (d) IgG titres against different A. baumannii (n = 6–10), P. aeruginosa (n = 5–10) and S. aureus (n = 7–10) strains in vaccinated and control mice after two injections with α-Ab (6 × 10^7 CFU), α-Pa (2 × 10^7 CFU) and α-Sa (3 × 10^7 CFU) vaccines, respectively, or saline. (a–d) S, saline; D, day. *P<0.05 (Student’s t-test), compared with saline group. #P<0.05, compared with the preceding condition (one-way ANOVA followed by Bonferroni’s post hoc test).
cytokines were selected as markers of Th1, Th2 and Th17 T-cell subsets, respectively. All three mutant strains induced an increase in the number of IL-17-secreting splenocytes, and this was the predominant T-cell subset triggered by the α-Sa vaccine (Fig. 5). A significant number of IFN-γ-secreting splenocytes was stimulated by α-Sa vaccine (P < 0.05, Student’s t-test), while an increment—although not statistically significant—was observed with α-Ab and α-Pa vaccines. IL-4-producing splenocytes were stimulated in response to α-Ab and α-Pa strains, but not to α-Sa vaccine. These data indicate that active immunization with α-Ab and α-Pa vaccines triggers a consistent Th2 immune response while α-Sa vaccine elicits a Th1 response.

Protective immunity against acute infection. Antibody and cell-mediated immunity can only be predictors for protective immune responses. To verify whether D-Glu auxotrophic strains confer protection, we assessed their effectiveness using an acute systemic infection model in mice (Figs 6–8). First, the effect of vaccination was determined by measuring bacterial loads in multiple tissues from vaccinated and control mice after infection with the vaccine wild-type homologues. Vaccination with D-Glu auxotrophic strains resulted in all cases in a significant reduction in tissue bacterial loads (104–106-fold reductions, P < 0.05, Mann–Whitney U test) compared to those observed in control mice (Figs 6a,7a and 8a). Moreover, the α-Sa vaccine prevented mice from severe body weight loss. Further, we determined mice survival after challenge with parental and heterologous strains. A two-dose vaccination schedule with the α-Ab strain protected mice from challenge with the wild-type strain A. baumannii ATCC 17978 on day 21, whereas all control mice died within 24 h (Fig. 6b). Similarly, survival rates of vaccinated mice were 100 and 86% after challenge with A. baumannii AbH12O-A2 on day 21, and Ab307-0294 on day 22, respectively. Mice challenged with P. aeruginosa PAO1 on day 25 presented 87.5 and 100% survival after vaccination with 2 × 10^7 CFU and 2 × 10^8 CFU of α-Pa vaccine, respectively. In this case, all control mice died within 15 h (Fig. 7b). Similar results were obtained when challenging mice with the mucoid strain P. aeruginosa PA28562. Using the intramuscular route (Fig. 7c), vaccinated mice challenged with PAO1 on day 49 presented 88.9% survival whereas all control mice died. Mice vaccinated with 3 × 10^7 CFU of α-Sa vaccine were completely protected from challenge with strain S. aureus 132 on day 21, whereas 81.3% of control mice died within 11 days (Fig. 8b). All vaccinated mice recovered their pre-infection body weight within 85 h. In addition, control mice that survived until day 53 (n = 3) presented high post-challenge bacterial loads in tissues (4.33, 6.66 and 6.96 log_{10} CFU g^-1 in livers; 0, 6.71 and 7.41 log_{10} CFU g^-1 in the left kidneys) in contrast to vaccinated mice (0 log_{10} CFU g^-1 in liver and left kidneys; n = 3, randomly selected). Moreover, multiple infectious abscesses were evident on the tissues of controls; but not detected on vaccinated mice (Fig. 8b). When challenging mice on day 21 with S. aureus FRP3757-USA300-LAC, RF122 and ED98 strains, we observed 100% survival, whereas all control mice died within 15 h (Fig. 8c). All these vaccinated mice recovered their initial body weight. We next assessed the protective efficacy of D-Glu auxotrophs in C57BL/6 mice. When challenged with the wild-type strains (ATCC 17978, PAO1 or 132), survival rates were 100% for vaccinated versus 0% for control mice (Figs 6b,7b and 8c); and when α-Pa vaccine was administered intramuscular, survival was 100% for vaccinated versus 25% for control mice, after PAO1 infection (Fig. 7c).

To demonstrate mid- and long-term vaccine efficacy, challenges with parental and heterologous strains were carried out one and three months after the last immunization (days 42–43 and 98, respectively). As illustrated (Figs 6c,7c,d and 8d,e), survival rates were higher than 88.9% in all vaccinated groups; and in case of S. aureus, α-Sa-vaccinated mice began to recover their pre-infection body weight 30 h after the challenge (Fig. 8d,e). Conversely, all control mice infected with A. baumannii ATCC 17978, ATCC 19060 or AbH12O-A2 (Fig. 6c), or P. aeruginosa PAO1 or PA14 (Fig. 7d) died within 14 h, whereas 90%, 89% and 55% mortality rates were observed after challenge with S. aureus 132, Sa07997 and FRP3757-USA300LAC respectively (Fig. 8d,e). In addition, control mice that overcame the acute phase of S. aureus FRP3757-USA300LAC infection (n = 4) presented high bacterial loads in tissues on day 146 (0, 2.57, 3.19 and 1.24 log_{10} CFU g^-1 in livers; 7.61, 7.85, 7.12 and 6.29 log_{10} CFU g^-1 in the left kidneys; 0, 3.23, 3.78 and 7.26 log_{10} CFU g^-1 in heart) in contrast to vaccinated mice (0 log_{10} CFU g^-1 in liver, left kidneys and heart, n = 5 randomly

Figure 5 | Vaccination with D-Glu auxotrophic strains triggers cytokine-secreting T-cells. (a) Number of spot-forming cells per 4 × 10^5 splenocytes collected at day 82 from mice vaccinated twice with A. baumannii ATCC 17978 ΔmurI ΔmurII (α-Ab vaccine) (6 × 10^7 CFU) (n = 6) and control mice (n = 7) after being restimulated ex vivo with α-Ab vaccine (4 × 10^5 CFU). (b) Number of spot-forming cells per 8 × 10^5 splenocytes collected at day 68 from mice vaccinated twice with P. aeruginosa PAO1 ΔmurI (α-Pa strain) (2 × 10^7 CFU) (n = 6) and control mice (n = 6) after being restimulated ex vivo with α-Pa vaccine (8 × 10^4 CFU). (c) Number of spot-forming cells per 8 × 10^5 splenocytes collected at day 50 from mice vaccinated twice with S. aureus 132 ΔmurI ΔmurII (α-Sa vaccine) (3 × 10^7 CFU) (n = 6) and control mice (n = 6) after being restimulated ex vivo with α-Sa vaccine (3 × 10^5 CFU). (a–c) S, saline; V, vaccinated. *P < 0.05 (Student’s t-test), compared with saline control mice (mean ± s.e.m.).
selected). Again, multiple infectious abscesses were evident in the tissues of controls (kidneys mainly), but not detected on vaccinated mice (Fig. 8e). Overall, these results demonstrate that vaccination with the D-Glu auxotrophic strains provides mid- and long-term protection against systemic infections.

To address whether D-Glu auxotrophic strains have any benefit as vaccines over inactivated bacteria, we compared their effectiveness with heat-killed and formalin-inactivated wild-type strains, using the previous systemic infection model in mice (Figs 7e and 8f). A conventional two-dose vaccination schedule consisting of a sole dose of α-Aβ vaccine (4 × 10^7 CFU) on day 43 (n = 10) and Ab307-0294 (4 × 10^8 CFU) on day 22 (n = 7–8).

### Figure 6 | Immunization with A. baumannii D-Glu auxotroph (α-Ab vaccine) provides effective protection against diverse bacteria.

(a) Protective effect against bacterial dissemination after acute infection. Bacterial loads in tissues from vaccinated (6 × 10^7 CFU of α-Ab strain) and control mice after challenge with A. baumannii ATCC 17978 (3 × 10^8 CFU) (n = 10; after 12 h). *(P < 0.05) (Mann–Whitney U-test). (b) Early vaccine protection against infection. Mice survival after α-Ab vaccine (6 × 10^7 CFU) or saline administration, and challenge with ATCC 17978 on day 21 (3 × 10^8 CFU, n = 11–13 for BALB/c; and 6 × 10^8 CFU, n = 8 for C57BL/6 mice), AbH12O-A2 (4 × 10^7 CFU) on day 21 (n = 9) and Ab307-0294 (6 × 10^6 CFU) on day 22 (n = 7–8). (c) Long-term vaccine protection against infection. Mice survival after α-Ab vaccine (6 × 10^7 CFU) or saline administration, and challenge with ATCC 17978 (3 × 10^8 CFU) on day 98 (n = 10), AbH12O-A2 (4 × 10^7 CFU) on day 98 (n = 10) and ATCC 19606 (1 × 10^8 CFU) on day 43 (n = 8).

### Functional and protective vaccine antisera

In high-risk situations, the use of passive immunization may be beneficial. Those antibodies formed with α-Ab, α-Pa and α-SA strains (α-Ab, α-Pa and α-SA sera) can be obtained from the host and transferred into another recipient where they can provide immediate passive immunity. Since antibodies measured by ELISA often do not correlate with protection, we assessed the functionality of α-Ab, α-Pa and α-SA sera by *in vitro* opsonophagocytic and killing activity (OPA/OPKA) assays, since these are generally more predictive. As illustrated in Fig. 9a, there was effective killing of α-Ab serum in the presence of murine macrophages against A. baumannii ATCC 17978, ATCC 19606, AbH12O-A2 and Ab307-0294 strains, whether minimal killing was observed with naive serum. In the absence of macrophages, α-Ab serum presented direct bactericidal activity against ATCC 17978, ATCC 19606 and AbH12O-A2 strains. There was effective killing of α-Pa serum in the presence of human PMNs against *P. aeruginosa* PAO1, PA21_ST175, PA14 and PA28562 strains, whether minimal killing was observed with naive serum. In the absence of cells, α-Pa serum also presented direct bactericidal activity against PAO1, PA21_ST175 and PA14 strains. Finally, significant opsonophagocytic activity of α-SA serum was observed against *S. aureus* 132, FRP3757-USA300LAC, MW2, NEWMAN, Sa07997, ED133 and ED98 strains, compared to naive serum in the presence of murine macrophages (P < 0.05, Student’s *t*-test). However, no bactericidal effect was detected in the absence of phagocytic cells.

Even though the generation of functional antibodies may be necessary to induce protective immunity, it could not be sufficient for complete protection against infection by certain pathogens and/or strains. In this sense, we determined if vaccine antisera formed with the D-Glu auxotrophs could be used to...
Figure 7 | Immunization with *P. aeruginosa* D-Glu auxotroph (α-Pa vaccine) provides effective protection against diverse bacteria. (a) Protective effect against bacterial dissemination after acute infection. Bacterial loads in tissues from vaccinated (2 × 10⁷ CFU of α-Pa strain) and control mice after challenge with *P. aeruginosa* PAO1 (2 × 10⁷ CFU) (n = 8; after 10 h). *P < 0.05 (Mann–Whitney U-test). (b) Early vaccine protection against infection. Mice survival after vaccination with α-Pa strain (2 × 10⁶ and 2 × 10⁷ CFU), or saline administration and challenge with PAO1 on day 25 (2 × 10⁷ CFU, n = 8 for BALB/c), on day 28 (4 × 10⁷ CFU, n = 4 for C57BL/6 mice) and PA28562 (4 × 10⁷ CFU) on day 25 (n = 8). (c) Different routes of immunization and vaccine protection. Mice survival after IM vaccination with α-Pa strain (2 × 10⁷ CFU) or saline administration and challenge with PAO1 (6 × 10⁷ CFU, n = 8 for BALB/c and 2 × 10⁷ CFU, n = 8 for C57BL/6) and PA14 (4 × 10⁷ CFU) (n = 7–8). Mice survival after intranasal (IN) vaccination with α-Pa strain (2 × 10⁷ CFU) or saline administration and challenge with PAO1 (5 × 10⁶ CFU) (n = 9). (d) Long-term vaccine protection against infection. Mice survival after vaccination with α-Pa strain (2 × 10⁷ CFU) or saline administration and challenge with PAO1 (2 × 10⁷ CFU) on day 98 (n = 9–10) and PA14 on day 98 (4 × 10⁶ CFU) (n = 10). (e) Superior vaccine protection conferred by *P. aeruginosa* D-Glu auxotroph. Mice survival after vaccination with α-Pa strain (2 × 10⁷ CFU), formalin-inactivated PAO1 (2 × 10⁷ CFU) or saline administration and challenge with PA14 (8 × 10⁶ CFU) (n = 9). Mice survival after vaccination with α-Pa strain (2 × 10⁷ CFU), formalin-inactivated PAO1 (2 × 10⁷ CFU), heat-killed PAO1 (2 × 10⁷ CFU) or saline administration and challenge with PAO1 (4 × 10⁷ CFU) (n = 8). (a–e) S, saline; I, administered the formalin-inactivated vaccine; H–K, administered the heat-killed vaccine; V, vaccinated. (b–e) *P < 0.05 (log-rank test), compared with saline group. (e) #P < 0.05 (log-rank test), compared with mice administered the formalin-inactivated vaccine. **P < 0.05, compared with mice administered the heat-killed vaccine.
Figure 8 | Immunization with S. aureus D-Glu auxotroph (α-Sa vaccine) provides effective protection against diverse bacteria. (a) Protective effect against bacterial dissemination and body weight loss after acute infection. Bacterial loads in tissues and percentage of body weight loss (mean ± s.e.m.) from vaccinated (3 × 10^7 CFU of α-Sa strain) and control mice after challenge with S. aureus 132 (3 × 10^7 CFU with 3% of mucin; n = 14, pooled data set from two independent experiments; after 45h). (b) Early vaccine protection against infection, body weight change and infectious abscesses. BALB/c mice survival and percentage of body weight loss (mean ± s.e.m.) after vaccination with α-Sa strain (3 × 10^7 CFU) or saline administration and challenge with S. aureus 132 (3 × 10^7 CFU with 3% of mucin) (n = 16–17, pooled data set from two independent experiments). Images show livers collected at day 53 from random saline and vaccinated mice. (c) Early vaccine protection against infection and body weight change. Mice survival and percentage of body weight loss (mean ± s.e.m.) after vaccination with α-Sa strain (3 × 10^7 CFU) or saline administration and challenge with S. aureus 132 (3 × 10^7 CFU with 3% of mucin; n = 14, pooled data set from two independent experiments). Images show livers collected at day 53 from random saline and vaccinated mice. (d) Long-term vaccine protection against infection and body weight change. Mice survival and percentage of body weight loss (mean ± s.e.m.) after vaccination with α-Sa strain (3 × 10^7 CFU) or saline administration and challenge with S. aureus 132 (3 × 10^7 CFU with 3% of mucin) on day 98 (n = 10) and Sa07997 (1.3 × 10^7 CFU with 3% of mucin) on day 42 (n = 9). (e) Long-term vaccine protection against infection, body weight change and infectious abscesses. Mice survival and percentage of body weight loss (mean ± s.e.m.) after vaccination with α-Sa strain (3 × 10^7 CFU) or saline administration and challenge with S. aureus 132 (3 × 10^7 CFU with 3% of mucin) on day 98 (n = 10) and Sa07997 (1.3 × 10^7 CFU with 3% of mucin) on day 42 (n = 9). (f) Superior vaccine protection conferred by S. aureus D-Glu auxotroph survival rates. Mice survival after vaccination with α-Sa strain (2 × 10^7 CFU), formalin-inactivated 132 (2 × 10^7 CFU), heat-killed 132 (2 × 10^7 CFU) or saline administration and challenge with FPR3757-USA300LAC (2.7 × 10^7 CFU with 3% of mucin) (n = 6–8). (a–e) S, saline; I, administered the formalin-inactivated vaccine; H–K, administered the heat-killed vaccine; V, vaccinated. (a) *P < 0.05 (Mann-Whitney U-test). (b–e) *P < 0.05 (log-rank test), compared with saline group.
Figure 9 | Immunization with D-Glu auxotrophs generates functional and cross-reactive vaccine antisera. (a) In vitro OPA and OPKA assays. Per cent killing of A. baumannii ATCC 17978 (4 × 10^8 CFU), ATCC 19606 (6 × 10^4 CFU), AbH12O-A2 (6 × 10^8 CFU) and Ab307-0294 (4 × 10^4 CFU) strains, by α-Ab serum (or naive serum) in the absence or presence of murine RAW 264.7 cells (1.6 × 10^5). Per cent killing of P. aeruginosa PAO1 (4 × 10^6 CFU), PA21_ST175 (3 × 10^4 CFU), PA14 (4 × 10^4 CFU) and PA28562 (1 × 10^4 CFU) strains, relative to naive serum. α-Pa serum (or naive serum) in the absence or presence of human PMNs. Per cent opsonophagocytosis of S. aureus 132 (1.2 × 10^9 CFU), FPR3757 (2.7 × 10^6 CFU), MW2 (1.8 × 10^4 CFU), NEWMAN (1.5 × 10^6 CFU), Sa07997 (3.6 × 10^4 CFU), ED133 (4 × 10^4 CFU) and ED98 (7 × 10^4 CFU) strains by α-Sa serum (or naive serum) in the absence or presence of murine RAW 264.7 cells (1.6 × 10^5). *P < 0.05 (Student’s t-test), compared with naive serum or the absence of RAW/PMN cells. (b) In vivo passive immunizations performed with BALB/c mice administered α-Ab, α-Pa, α-Sa or naive sera and challenged with ATCC 17978 (4 × 10^8 CFU) (n = 8), AbH12O-A2 (1.4 × 10^8 CFU) (n = 8), PAO1 (2 × 10^7 CFU) (n = 8), PA14 (2 × 10^7 CFU) (n = 8), 132 (7.5 × 10^7 CFU) (n = 6), FPR3757 (1.5 × 10^7 CFU) (n = 6) and NEWMAN (5.3 × 10^7 CFU) (n = 7) strains, 3.5–4 h after serum administration. *P < 0.05 (log-rank test), compared with naive serum.

with α-Pa serum and challenged with P. aeruginosa PAO1 and PA14 strains. Survival for controls receiving naive serum was 56% and 25%, respectively. Mice administered α-Sa serum and challenged with S. aureus 132 and FRP3757-USA300LAC strains also showed 100% survival while 67% of controls receiving naive serum succumbed to infection in both assays. The passive transfer of α-Sa serum resulted in a significant survival rate of mice (43%) (P < 0.05, log-rank test), compared to mice receiving naive serum (0%), after challenge with NEWMAN strain.

Overall, these results indicate that vaccine antisera can effectively enhance bacterial clearance and offer protection against lethal infections caused by a global spectrum of heterologous and relevant strains of these pathogens.
Safety profiles of D-Glu auxotroph vaccines. Finally, the attenuation should be carried out in such a way that does not allow a persistent carrier state of the vaccine\(^3\). This can be achieved by the auxotrophic strain’s ability to colonize and multiply in the host over a limited period being eliminated without causing the disease. This was observed for the three auxotrophic strains when injected intravenously and IP to mice, as they were completely cleared from blood (Supplementary Fig. 9a). In addition, the IP administration of D-Glu auxotrophic strains did not affect mice body weight significantly, as no differences were observed between vaccinated and saline (vehicle) control mice (Supplementary Fig. 9b).

In summary, these observations support that D-Glu auxotrophs present good safety profiles for parenteral administrations.

Discussion

Having a whole-cell vaccine that contains all the antigenic determinants to induce protective immunity, including those that are only expressed in vivo, without the risk of clinical infection and with minimal possibilities of adverse reactions, is the paradigm for effective and safe immunizations. Some of these aspects can be afforded by live-attenuated vaccines obtained by natural selection or genetic engineering of bacterial strains. However, they require particular attention regarding their safety as they may propagate and be released into the environment by the vaccinees\(^3\). Furthermore, obtaining virulence attenuation is usually impeded by the laborious identification of rational targets in each particular bacterium.

Although D-amino acids are increasingly recognized as physiologically functional molecules in mammals, the amounts of free D-Glu are trace substances in most cases\(^3\). By abrogating D-Glu synthesis, we took advantage of the highly-conserved function of MurI and the strict requirement of sufficient D-Glu pool levels to impair bacterial replication in the mammal host. Indeed, A. baumannii, P. aeruginosa and S. aureus D-Glu auxotrophs are incapable of proliferating in the mouse due to the blockage of the cell wall synthesis being attenuated in comparison with the parental strains. These GMOs triggered appropriate cellular immune responses and the production of specific and cross-reactive antibodies to several clonally unrelated epidemic, MDR and highly virulent strains. These results correlated with protection against acute lethal infections mainly using a two-dose immunization schedule and the IP route, although intramuscular and intranasal vaccinations were also effective. D-Glu auxotrophs also conferred superior vaccine efficacy with respect to formalin-inactivated or heat-killed wild-type bacteria. In addition, these vaccine strains did not appear to represent a risk for causing disease and spreading to non-target recipients, considering their rapid elimination from the blood and their limited persistence in non-optimal conditions, more likely to occur in the hypothetical case of an accidental environmental release. Altogether, these attributes constitute advantages of D-Glu auxotrophic vaccines over live-attenuated or inactivated. Other auxotrophic bacterial strains carrying mutations in aromatic amino acid and/or purine biosynthetic pathways have also been described as potential live vaccine candidates\(^3\). Generally, these auxotrophs have a limited ability to undergo replication in the host with survival duration ranging from days to weeks, which is higher than the observed persistence for D-Glu auxotrophs. Moreover, their level of attenuation and their ability to confer efficacy will rely on the bacteria, the enzymatic step disrupted and the animal model utilized.

MDR or pandrug-resistant A. baumannii strains are responsible of an escalating number of infections\(^4\) and therefore, prophylactic vaccination represents an option for the future\(^4\). So far, several preclinical experimental vaccines have been described\(^8,43\). Particularly, inactivated whole cells can elicit better vaccine coverage, but the high endotoxin levels (LPS) complicate their use in humans. Although LPS-deficient inactivated whole cells could be a good vaccine\(^4\), the lack of the immunostimulatory LPS may impair immunogenicity. In contrast, Ab MurI\(^-\) seems not to pose a risk for endotoxin accumulation in view of the rapid elimination from the blood of mice and the absence of adverse effects. Little is known regarding the pathogenesis and the immune responses against A. baumannii disease. Ab MurI\(^-\) was found to elicit high levels of opsonic, bactericidal and cross-reactive antibodies, probably due to the presentation of whole-cell antigens. Considering the cellular immunity, a significant increment in IL-4- and IL-17-producing splenocytes was obtained, which is consistent with a Th2/Th17 response.

Despite the research efforts, there is no vaccine against P. aeruginosa licensed yet. Effective immunotherapies or vaccines have long been sought as alternatives to prevent P. aeruginosa infections\(^3,45,46\). The closest strategy of vaccine design included the introduction of mutations into the aroA gene for the creation of live-attenuated strains\(^37,39,40,47\). These mutants are unable to synthesize aromatic amino acids and cannot efficiently acquire them from the host, surviving at detectable levels in the lungs up to 3-4 days following administration\(^37\). In our study, Pa MurI\(^-\) was shown to be eliminated from blood until 10 h after intravenous administration suggesting a lesser risk of side effects. As P. aeruginosa is an extracellular pathogen, a strong opsonophagocytic antibody response is generally predictive for protection, as observed in our work. However, T-cell responses can also mediate protective immunity\(^48\). Pa MurI\(^-\) triggered a substantial increment in IL-4-producing splenocytes, consistent with a Th2 immune response. IL-4 is known to enhance pulmonary clearance of P. aeruginosa in mice\(^49\). More importantly, Pa MurI\(^-\) mediated a significant increase in the number of IL-17-secreting splenocytes, essential in clearance of acute pulmonary P. aeruginosa infection\(^50\).

S. aureus is a leading cause of healthcare- and community-acquired infections worldwide, affecting virtually all human and animal tissues\(^4\). Despite continuous efforts, active immunizations have been largely unsuccessful in human clinical trials\(^2,51–54\). Potential reasons underlying these vaccine failures have been identified\(^55–58\). Apparently, there is only one whole-cell vaccine, composed by an inactivated SA75 strain, in the pipeline to prevent human infections\(^52,59\). Although it completed a phase I trial successfully, it appears to be no longer under active development. Moreover, only antibody responses were measured, without determining their functionality or measuring T-cell responses. In turn, auxotrophic aroA mutants have been tested in a preclinical intramammary mouse model to target S. aureus-mastitis in dairy cattle\(^38\), but seems not to be suitable for safe immunizations others than intramammary. Indeed, 57% of mice succumbed after the intravenous injection of 2 × 10\(^7\) CFU of the aroA mutant. In contrast, we showed that an equivalent dose of Sa MurI\(^-\) Dat\(^-\) was completely cleared from the mouse blood without causing disease. The immune protection of S. aureus vaccines is a matter of some debate. The current consensus establishes that cell-mediated immunity (Th1/Th17) and neutrophil activation may be required for effective protection, while antibodies may play a supportive role for opsonization and/or neutralization of virulence factors\(^51,56,57\). In this sense, live-attenuated vaccines may offer an advantage over inactivated formulations, such as SA75 vaccine, considering they elicit effective cellular immune responses. Indeed, Sa MurI\(^-\) Dat\(^-\) immunization triggered not only functional and cross-reactive antibodies but an increase in...
the number of IFN-γ- and IL-17-secreting splenocytes that correlated with long-term protection. As previously noted, Th1 effector cytokines such as IFN-γ may play a crucial role in the eradication of S. aureus, while Th17/IL-17 pathway may collaborate in promoting neutrophil recruitment, and was demonstrated to be essential for combating S. aureus sepsis and pneumonia in mice.

Although with substantial differences among the three targeted pathogens, the D-Glu auxotrophic vaccines tested here in mice conferred long-term survival (up to three months) after immunization against lethal systemic infections. The protection observed could be derived from the combination of opsonic antibodies as well as concomitant T-cell responses, with IL-17 cytokine playing a relevant role, the common features observed amongst the three vaccine prototypes.

For all the above reasons, we believe that our D-Glu auxotrophs are promising candidates to be developed as whole-cell vaccines against the high-priority pathogens A. baumannii, P. aeruginosa and S. aureus, and that our approach for bacterial attenuation can potentially be applied to many other gram-negative and gram-positive pathogens for their potential use as prophylactics for human or animal health.

Methods

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Supplementary Table 1. We selected A. baumannii ATCC 17978, P. aeruginosa PAO1 and S. aureus 1322 for genetic manipulation to generate the indicated mutant strains. S. aureus 1322 is an MRSA strain isolated from a patient at the Microbiology Department of the Clínica Universitaria de Navarra (Pamplona, Spain). This strain can produce alternative biofilm matrix depending amongst the three vaccine prototypes.

A. baumannii, P. aeruginosa and S. aureus, that our approach for bacterial attenuation can potentially be applied to many other gram-negative and gram-positive pathogens for their potential use as prophylactics for human or animal health.

Construction of unmarked deletion mutants. We used in vitro methods to construct mutant alleles, designated ΔmurI and ΔmurII in A. baumannii ATCC 17978, ΔmurI in P. aeruginosa PAO1, ΔmurI and Δdat in S. aureus 1322 with in-frame deletions corresponding to murI1, murI2, murI3 and dat genes, respectively. These mutant alleles were replaced, either singly or together, by the corresponding wild-type alleles used previously described allelic exchange systems with the pMo130, pEX18Gm and pMAD plasmids in A. baumannii, P. aeruginosa and S. aureus, respectively. Primers used are listed in Supplementary Table 2. For A. baumannii, fragments of about 1 kb corresponding to the upstream and downstream regions of murI1 and murI2 were amplified by PCR with the combination of primers UP_murI1(NotI)/murI1(BamHI), DOWN_murI1(BamHI)/DOWN_murI1(SphI), UP_murI2(NotI)/murI2(BamHI) and DOWN_murI2(BamHI)/DOWN_murI2(SphI).

The upstream fragments obtained were digested with NotI and BamHI restriction enzymes; downstream fragments were digested with BamHI and SphI. Digested products (ligated with PCR products) resulting from the previously described allelic exchange systems with the pMo130, pEX18Gm and pMAD plasmids were confirmed by sequencing and independently transformed by electroporation into E. coli TG1 and DC10b, respectively. For the allelic exchange of pMAD_UP/DOWN_murI with the murI allele, the recombinant plasmid was transformed in S. aureus RN4220 and in S. aureus 1322 by electroporation. For the allelic exchange of pMAD_UP/DOWN_dat with the dat allele, the recombinant plasmid was directly introduced into S. aureus 1322 from E. coli DC10b. Homologous recombination experiments were performed as follows: one blue erythromycin-resistant colony of S. aureus containing pMAD_UP/DOWN_murI or pMAD_UP/DOWN_dat was grown in TSB with erythromycin at 30 °C for 2 h and then, at 45.5 °C, a non-permissive temperature for pMAD replication. Resulting cultures were streaked and light blue erythromycin-resistant colonies, representing the first crossover event, were selected at 45.5 °C on TSB plates with erythromycin and X-Gal. Several of these colonies were grown in TSB without antibiotic at 30 °C for 18 h, and then plated on the same agar medium. The resulting white colonies were confirmed by PCR using the combination of plasmids pEX18Gm, pMAD_UP/DOWN_murI and pMAD_UP/DOWN_dat with the murI3 and murI2 alleles, respectively. pMAD_UP/DOWN_dat plasmid was also introduced by electroporation in the 132 ΔmurI mutant. Transformant bright blue erythromycin-resistant colonies were firstly grown in TSB at 30 °C and then, at 45.5 °C to obtain colonies resulting from the first crossover. Several of these colonies were transferred to TSB with 10 mM D-Glu, incubated at 30 °C for 18 h and then plated on the same agar medium. The resulting white colonies were picked and inoculated in plates at comparable locations on TSB agar plates with and without 10 mM D-Glu. Presumptive colonies with the ΔmurI1 ΔmurI2 double-mutant genotype that grew only on D-Glu containing plates, were analysed by PCR using the combination of primers EXTfw_murI2/INTfw_murI2 to confirm the second crossover event, produced by the allelic exchange of plasmid pMo130_UP/DOWN_murI2 with the murI2 allele. For P. aeruginosa, fragments of about 1 kb in length corresponding to the upstream and downstream regions of the murI1 gene were amplified by PCR with the combination of primers UP_murI1(HindIII)/UP_murI1(NotI) and DOWN_murI1(NotI)/DOWN_murI1(XbaI). The upstream fragment obtained was digested with HindIII and NotI restriction enzymes; the downstream fragment was digested with NotI and XbaI. Digested products were ligated into pEX18Gm, which was previously linearized with HindIII and XbaI and the recombinant plasmid pEX18Gm_UP/DOWN_murI1 was transformed in E. coli S17-1 by electroporation. S17-1 transformants were selected on LB containing gentamycin and were analysed by PCR to confirm the presence of the recombinant plasmid, which was then introduced in P. aeruginosa PA01 by electroporation. P. aeruginosa gentamycin-resistant colonies, representing the first crossover event, were grown on LB containing 15% sucrose and 10 mM D-Glu for 6 h, and then plated on the same agar medium. The resulting colonies were picked from agar plates containing 10 mM D-Glu and inoculated in patches at comparable locations on LB agar plates with and without 10 mM D-Glu. Presumptive colonies with the ΔmurI1 ΔmurI2 double-mutant genotype that grew only on D-Glu containing plates, were analysed by PCR using the combination of primers EXTfw_murI2/INTfw_murI2 to confirm the second crossover event, produced by the allelic exchange of plasmid pMo130_UP/DOWN_murI2 with the murI2 allele. For A. baumannii, the D-Glu auxotrophic vaccines tested here in mice conferred long-term survival (up to three months) after immunization against lethal systemic infections. The protection observed could be derived from the combination of opsonic antibodies as well as concomitant T-cell responses, with IL-17 cytokine playing a relevant role, the common features observed amongst the three vaccine prototypes.

Transformant bright yellow and kanamycin-resistant colonies, were then grown on LB containing 15% sucrose and supplemented with 10 mM D-Glu for 6 h, and then plated on the same agar medium. The resulting white colonies (after spraying with pyrocatechol) were picked from agar plates containing 10 mM D-Glu and inoculated in patches at comparable locations on LB agar plates with and without 10 mM D-Glu. Presumptive colonies with the ΔmurI1 ΔmurI2 double-mutant genotype that grew only on D-Glu containing plates, were analysed by PCR using the combination of primers EXTfw_murI2/INTfw_murI2 to confirm the second crossover event resulting in ΔmurI1 ΔmurI2 genotypes, produced by the allelic exchange of plasmids pMo130_UP/DOWN_murI1 with the murI1 and murI2 alleles, respectively. The pMo130_UP/DOWN_murI2 plasmid was also introduced in the ATCC 17978 ΔmurI1 mutant previously obtained, by electroporation.
System instructions, Bio-Rad Laboratories (Madrid, Spain) or protocols described elsewhere.29

Growth and viability curves. To determine the growth and viability of A. baumannii ATCC 17978 (Ab), P. aeruginosa PA01 (Pa) and derived mutant strains, these bacterial cultures were cultured overnight at 37 °C and 180 r.p.m. in 5 ml LB supplemented with 10 mM D-Glu. Bacterial cultures were centrifuged (4,000 g, 15 min) and the pellets were washed twice with LB. After pellet suspension in 5 ml LB, 1 ml was used to inoculate 100 ml of TSB supplemented with 20 mM D-Glu and 10 mM D-Glu, respectively. All the cultures were incubated at 37 °C under agitation (180–210 r.p.m.) for up to 8 days. Samples from these cultures were taken at the beginning of the incubation period and at days 1, 2, 3, 4 and 8, washed twice and plated on LB or TSB agar with 0 and 10 mM D-Glu. Agar plates were incubated at 37 °C for 4 days. Cultures were made in triplicate.

Water osmolality assay. To determine the viability of Ab, Ab ΔmurI1 ΔmurI2, Pa, Pa ΔmurI, Sa and Sa ΔmurI Adat in water, these bacterial strains were cultured overnight at 37 °C in LB or TSB agar with (mutant strains) and without (wild-type strains) 10 mM D-Glu, adjusted to 0.5 McFarland in water and left incubating at 37 °C under agitation (180 r.p.m.) for the time necessary to observe the loss of viability of cells. For A. baumannii strains, daily samples of culture were taken until day 2, next samples were taken twice a week until day 26 and thereafter, once a week until day 40 for the determination of CFU counts in LB agar (wild-type strain) and LB agar with 10 mM D-Glu (Ab ΔmurI1 ΔmurI2 mutant strain). For P. aeruginosa strains, daily samples of culture were taken until day 3, next samples were taken twice a week until day 48. Finally, samples were taken at least once every 2 weeks until day 157 for the determination of CFU counts in LB agar (wild-type strain) and LB agar supplemented with 10 mM D-Glu (Pa ΔmurI mutant strain). For S. aureus, daily samples of culture were taken until day 5 for the determination of CFU counts in TSB agar (wild-type strain) and TSB agar supplemented with 10 mM D-Glu (Sa ΔmurI Adat mutant strain). Cultures were made in triplicate.

Desiccation assay. To determine the viability of Sa and Sa ΔmurI Adat after being kept under drought stress, these bacterial strains were cultured at 37 °C under agitation (180 r.p.m.) in TSB with 20 mM D-Glu. Bacterial cultures (OD600nm = 0.5–0.6) were centrifuged (3,900 g, 20 min), washed once and serially diluted in TSB. Drops of 5 μl from undiluted, 10−1 and 10−2 diluted cultures were then spotted on sterile cellulose filters (0.45 μm pore size) and sequentially placed on TSB agar with D-Glu 10 mM at day 0 (control) and after keeping for 4, 5, 6, 11, 18, 25 and 31 days at room temperature (desiccation without agar). Agar plates were incubated at 37 °C for 16 h. All cultures were made in triplicate.

Animal experiments. All mice were maintained in the specific pathogen-free facility at the Centro Tecnológico de Formación de la Xerencia de Xestión Integrada A Coruña (CTF-XXIAC), Servicio Galego de Saúde. All experiments were done with the approval of and in accordance with regulatory guidelines and institutional ethical committee guidelines of the Complutense University (Madrid, Spain). Male and female mice were used for first time procedures between the ages of 6 and 8 weeks. BALB/c mice were bred in our facility at the Centro Tecnológico de Formación de la Xestión Integrada A Coruña (CTF-XXIAC), Servicio Galego de Saúde. All experiments were done with the approval of and in accordance with regulatory guidelines and institutional ethical committee guidelines of the Complutense University (Madrid, Spain). Male and female mice were used for first time procedures between the ages of 6 and 8 weeks. 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cooled in ice. Exceptionally for *P. aeruginosa* PA28586, we used a 250 l µl volume for infection. All challenges were made using the IP route of administration. To pre-
pare immunized mice, *Staphylococcus aureus* strain Newman (ATCC 29213) were cultured in TSB or TSB with 20 mM D-Glu at 37 °C and 210 r.p.m. until OD600nm was 0.7. Bacterial cultures were then centrifuged (3,900 g, 20 min), washed twice with sterile NaCl 0.9% and suspended in the same solution. For *S. aureus* infections, we pre-
pared inocula with 3% of mucin (as described above). Both for immunizations and infections, immunized mice were enriched with the protein A binding moiety of *S. aureus* and control (n = 6–7) mice, and exposed ex vivo to *α*-Ab (4 × 10^5 CFU), *α*-Pa (10 × 10^5 CFU) and *α*-Sa (3 × 10^5 CFU) vaccines during 43 h at 37 °C, 5% CO2. Mice spleens were aseptically removed after the second vaccine or saline administration, mechanically disrupted and washed in saline by centrifugation at 450 for 10 min at room temperature. The suspension of spleens was resuspended in a Neutrophil haemocytometer and adjusted to 4 × 10^5 or 8 × 10^5 cells ml^-1 in RPMI 1640 medium containing 10% FBS. Next, 100 µl were transferred to a 96-
well ELISA plates and reconstituted with 100 µl of vaccine strain, 100 µl of 1 ×
cocktail (positive control) or 100 µl RPMI + 10% FBS. The number of the spot-forming units was visually determined using images obtained with a dissection microscope (Nikon SMZ-745, Nikon Corporation). Spot frequency in stimulated wells was corrected by subtracting background signal from wells with cells plus media alone.

All findings of the study are available in the article and its Supplementary Information.

**ELISPOT assay.** IFN-γ, IL-4 and IL-17 producing splenocytes were quantified by ELISPOT analysis (R&D Systems, Minneapolis, Minnesota) with splenocytes obtained from vaccinated mice on days 49, 63 and 84; sera obtained was pooled as before and IP adoptive transfer of either 250 µl serum or naive serum was made in naive mice (6–7/group) 3.5 h before the challenge. To generate naïve and *α*-Sa serum (1:81,920) or naive serum (1:160) was diluted and plated in duplicate onto LB plates for CFU determination to calculate the killing activity.

**Opsonophagocytic and killing assays.** OPKAs were assessed using *S. aureus* and *A. baumannii* as test organisms. To generate *S. aureus* and *A. baumannii* macrophages with a *3* day exposure to 100 µM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in DMEM plus 10% FBS, harvested after scraping (Cell scraper 179693, Nalgene Nunc International), counted in a Neu-

**Statistics.** Means were compared by using Student’s *t*-test for hypothesis testing to compare individual conditions and corresponding control groups. Mann–Whitney *U*-test was applied if data set failed the Kolmogorov–Smirnov normality test. The one-way analysis of variance (ANOVA) was used for multiple comparisons, followed by Bonferroni’s post hoc test. Survival data were compared using the log-rank test. Differences were considered significant when *P* < 0.05. *P* < 0.05 compared with control group. *P* < 0.05 compared with the preceding group represented. All statistical analyses were performed using Prism 6.0 (GraphPad Prism, GraphPad Software, Inc.).

**References.** The authors declare that all the relevant data supporting the findings of the study are available in the article and its Supplementary Information files, or from the corresponding author upon request.

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Author contributions

M.P.C., P.G., A.B. and G.B. conceived and designed the experiments. M.P.C., P.G., A.B., C.R., A.P. and M.M. performed the experiments. M.P.C., P.G. and G.B. analysed data. M.P.C., P.G. and G.B. wrote the paper. All authors read and approved the final manuscript.

Additional information

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