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A bacteriolytic agent that detects and kills *Bacillus anthracis*

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The dormant and durable spore form of *Bacillus anthracis* is an ideal biological weapon of mass destruction[1,2]. Once inhaled, spores are transported by alveolar macrophages to lymph nodes surrounding the lungs, where they germinate; subsequent vegetative expansion causes an overwhelming flood of bacteria and toxins into the blood, killing up to 99% of untreated victims. Natural and genetically engineered antibiotic-resistant bacilli amplify the threat of spores being used as weapons, and heighten the need for improved treatments and spore-detection methods after an intentional release. We exploited the inherent binding specificity and lytic action of bacteriophage enzymes called lysins for the rapid detection and killing of *B. anthracis*. Here we show that the PlyG lysin, isolated from the γ phage of *B. anthracis*, specifically kills *B. anthracis* isolates and other members of the *B. anthracis* 'cluster' of bacilli *in vitro* and *in vivo*. Both vegetative cells and germinating spores are susceptible. The lytic specificity of PlyG was also exploited as part of a rapid method for the identification of *B. anthracis*. We conclude that PlyG is a tool for the treatment and detection of *B. anthracis*.

Bacteriophage lysins are lytic agents used by double-stranded DNA (dsDNA) phages to coordinate bacterial host lysis with completion of viral assembly[3,4]. Late in infection, lysin translocates from the cytoplasm into the cell-wall matrix, where it rapidly hydrolyses covalent bonds essential for peptidoglycan integrity, causing bacterial lysis and concomitant release of progeny phages. Lysin family members are often chimaeric proteins, consisting of a usually well-conserved catalytic domain fused to a largely divergent specificity or binding domain[4,5]. High-affinity binding (the affinity constant $K_A = 3–6 \times 10^8$, similar to affinity-matured antibodies) is directed towards species- or strain-specific cell-wall carbohydrates that are often essential for viability, thus implying that intrinsic lysin resistance could be rare. Lysins have not previously been investigated as a means for bacterial control, although we have demonstrated efficient 'lysis from without' of pathogenic streptococci in the mouse nasopharynx using purified streptococcal phage lysins[6,7]. On the basis of these findings, we thought that lysins might be able to deliver a rapid and specific lethal action to any particular bacterial pathogen, not just streptococci. The main requirement for developing lysins, as such, is a dsDNA phage specific for the pathogen of interest to serve as a lysin source. We investigated this possibility with a dsDNA phage specific for the biowarfare agent *B. anthracis*.

The dsDNA phages of *B. anthracis* form a homogeneous family[8]. We chose the γ phage as a lysin source because of its specificity for *B. anthracis* and its usage by the US Centers for Disease Control and Prevention (CDC, Atlanta, Georgia) for identification of this pathogen[2,9]. It is known that γ phages infect most *B. anthracis* isolates, including some rare *Bacillus cereus* strains that could represent *B. anthracis* cured of its virulence plasmid or an environmental reservoir of potential progenitors[10]. Isolates of RSVF1 (streptomycin-resistant *B. cereus* strain 4342 from the American Type Culture Collection, ATCC) and *B. anthracis* are monomorphic at multiple allozyme loci, and therefore are part of the same highly related cluster of isolates within the *B. cereus* lineage[11]. Consistent with this, we found that RSVF1 was sensitive to the γ phage (Table 1) and displayed several other features typical of *B. anthracis*: matt colony morphology, filamentous structure, lack of motility, and characteristic sequences in the hypervariable *vrrA* locus (data not shown). For these reasons, RSVF1 is an appropriate representative of the γ-phage-sensitive *B. anthracis* cluster of *B. cereus* for use in this study.

An expression library of γ phage proteins was screened for agents capable of lysing RSVF1 'from without'. Each lytic clone identified contained a 702-base-pair (bp) γ open reading frame (ORF) encoding a product that is homologous to *N*-acetylmuramoyl-L-alanine amidases (amidases), a class of phage lysins (Fig. 1a). The homology was restricted to the catalytic amino-terminal halves, and absent in the cell-wall-specific carboxy-terminal binding domains[4,5]. Recombinant γ lysin (called PlyG, for phage lysin γ) was purified to homogeneity by column chromatography (Fig. 1b). Gel filtration confirmed a predicted relative molecular mass of about 27,000 (M_r 27K), and suggested that PlyG acts as a monomer and is not proteolytically processed.

To evaluate activity and specificity, 0.5 U of PlyG was initially added in drops to bacterial lawns of RSVF1 (Fig. 1c) and isolates of *B. anthracis* gathered worldwide as well as other bacilli from the *B. cereus* lineage (*B. cereus* and *Bacillus thuringiensis*) and other Gram-positive genera (Table 1). RSVF1 was the only *B. cereus* strain found to be as sensitive to PlyG killing (and sensitive to the γ phage) as the diverse set of *B. anthracis* isolates (Table 1). *B. cereus* ATCC 10987, a strain closely related to *B. anthracis*[11], was slightly susceptible to

Table 1 **Susceptibility to γ phage infection and purified PlyG activity**

Isolate or strain*	γ phage titre (PFU ml^{-1})†	γ lysin activity‡	Strain source
B. anthracis§			
Vollum	10^8–10^{10}	+++	L. W. Mayer
Ames	10^8–10^{10}	+++	L. W. Mayer
A1.a/10 (USA)	10^8–10^{10}	+++	L. W. Mayer
A1.b/23 (Turkey)	10^8–10^{10}	+++	L. W. Mayer
A2/29 (Pakistan)	10^8–10^{10}	+++	L. W. Mayer
A3.a/34 (Korea)	10^8–10^{10}	+++	L. W. Mayer
A3.b/57 (China)	10^8–10^{10}	+++	L. W. Mayer
A4/69 (Pakistan)	10^8–10^{10}	+++	L. W. Mayer
B/80 (France)	10^8–10^{10}	+++	L. W. Mayer
ΔSterne	10^8–10^{10}	+++	A. L. Turetsky
VNR1Δ1	10^8–10^{10}	+++	A. L. Turetsky
ΔAmes	10^8–10^{10}	+++	A. L. Turetsky
NNR1Δ1	10^8–10^{10}	+++	A. L. Turetsky
ΔNH1	10^8–10^{10}	+++	A. L. Turetsky
B. cereus			
RSVF1	2.2×10^{10}	+++	ATCC
ATCC 10987	<10	+/−	ATCC
ATCC 27348	<10	−	A. Aronson
T	<10	−	A. Aronson
ATCC 14579	<10	−	A. Keynan
ATCC 15816	<10	−	ATCC
RTS134	<10	−	A. Keynan
RSVF2	<10	−	A. Keynan
ATCC 13472	<10	−	H.-W. Ackerman
S154-2 HER1414	<10	−	H.-W. Ackerman
B. thuringiensis			
T24	<10	−	H.-W. Ackerman
ATCC 33679	<10	−	A. Aronson
ATCC 35866	<10	−	R. McNall

*Additional bacteria were also tested and were resistant to the γ phage and lysin. These include *B. subtilis*, *B. megaterium*, *B. stearothermophilis*, *B. pumilus*, *B. brevis*, *B. licheniformis*, *Brevibacillus laterosporus*, *Sporosarcina ureae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*.

†Titres indicate the maximum plaque-forming units per ml of donor phage stock obtained with the indicated bacterium.

‡Designations refer to the degree of bacterial lawn clearance mediated by purified lysin and are as follows: +++, complete clearance at the site of lysin application; +/−, partial clearance observable only at the centre of lysin application; −, no clearance.

§Numerical designations for *B. anthracis* strains represent distinct genotypes by MLVA (multiple-locus variable number of tandem repeat analysis)[30]. The geographical region of isolation for some *B. anthracis* strains is indicated.

PlyG, but all other strains examined were resistant. A more sensitive test of PlyG-mediated killing was evaluated in buffer containing 20 U of PlyG. After 15 min, RSVF1 was nearly sterilized (Fig. 2a), whereas the viability of ATCC 10987 was reduced about 100-fold. Other strains examined were largely resistant, even after a 3-h incubation. PlyG can clearly direct a potent and specific lethal action to the *B. anthracis* cluster, exhibiting a substrate specificity that closely matches the γ phage host range. Moreover, the capsulated state of several *B. anthracis* strains examined indicated that the capsule does not block access of PlyG to the cell wall.

We found that PlyG, like most lysins, is a highly active enzyme. The addition of 2 U of PlyG to 1.0×10^4 RSVF1 caused a pronounced release of intracellular ATP within 10 s (measured as light emitted by luciferin/luciferase) (Fig. 2b). This rapid lytic effect was not observed with other isolates tested, suggesting that the ATP release assay is a rapid diagnostic tool for γ-sensitive bacilli. In a separate kinetic analysis of RSVF1 viability after PlyG treatment, as little as 2 U added to 1 ml of log-phase cells effected a 17,000-fold decrease within 20 s, and near sterilization at 2 min (Fig. 2c). The loss of bacterial absorbance lagged a few minutes behind the loss in viability, implying that PlyG does not cause immediate cell-wall degradation. To visualize the lytic effect, we used phase-contrast microscopy of PlyG-treated RSVF1. The normally filamentous RSVF1 (Fig. 3a) rapidly converts to short rod- and minicell-like forms 30 s after exposure (Fig. 3b); nearly complete loss of cytoplasmic material occurs by 15 min, leaving 'ghost' cells (Fig. 3c). Transmission electron micrographs of the rod form reveals cytoplasmic membrane extrusions from distinct regions of cell-wall hydrolysis at polar and septal positions (Fig. 3d), and the formation of ghost-like forms (Fig. 3e).

We next tested the effect of PlyG on RSVF1 using a mouse model of infection. The intraperitoneal (i.p.) injection of some *B. cereus* isolates can induce a rapidly fatal illness[12,13]. We injected about 1.0×10^6 RSVF1 cells and observed a characteristic progression of symptoms in BALB/c mice, leading to death in 5 h or less (Fig. 4). At death, many mice exhibited severe oedema at the inoculation site, and haemorrhaging through the eyes and mouth. When PlyG (50 U in 0.5 ml) was injected i.p. 15 min after infection, a pronounced therapeutic effect was observed: 13 of 19 mice (68.4%) recovered fully, and the remaining animals survived 6–21 h. When 150 U of PlyG were used in 0.5 ml, a similar rate of recovery was observed (76.9%), possibly owing to the convoluted nature of the peritoneum and the inability of the enzyme to reach some bacteria concealed within peritoneal folds. No toxicity was detected upon administration of PlyG alone. PlyG does, therefore, rapidly kill γ-sensitive bacteria in an infected animal.

To evaluate the therapeutic potential of PlyG, we looked for resistance to its binding or catalytic activity. Repeated exposure of RSVF1 to low or high PlyG concentrations on either agar plates or in liquid cultures did not identify spontaneously resistant mutants (a frequency of $< 5.0 \times 10^{-9}$), although we readily identified mutants resistant to novobiocin and streptomycin (Table 2) using a similar strategy. We then subjected RSVF1 to mutagenesis with methanesulphonic acid ethyl ester (EMS) and observed roughly 1,000-fold and 10,000-fold increases in novobiocin and streptomycin resist-

Figure 1 Characterization of PlyG. **a**, Sequence alignment of the γ lysin with *B. cereus* and *B. subtilis* phage amidases (TP21 and ϕ105, respectively) and *B. cereus* and *B. subtilis* prophage amidases (CwlA and XlyA, respectively). Dark shading represents sequence identity and lighter shading represents similarity. **b**, SDS–polyacrylamide gel electrophoresis of purified PlyG stained with Coomassie blue. The molecular mass was estimated with Kaleidoscope (Bio-Rad) standards that are not shown. **c**, An RSVF1 lawn on a BHI plate treated with 0.5 U of purified PlyG. The clearing zone is 18.0 mm in diameter.

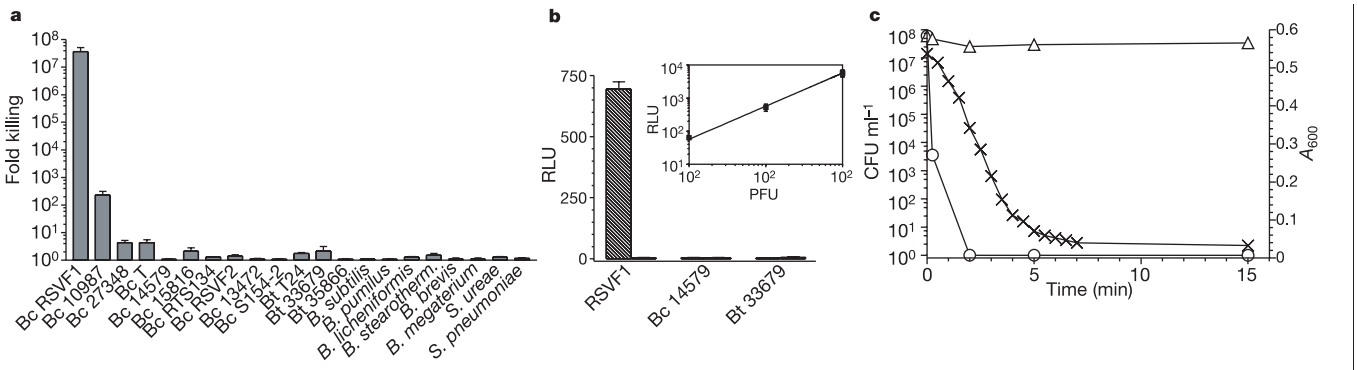

Figure 2 The specific and rapid killing action of PlyG. Error bars indicate the s.d. from 3–5 independent experiments. **a**, We treated 1 ml of log-phase cultures with either PlyG (20 U) or phosphate buffer for 15 min. Fold killing is the number of viable bacteria after buffer treatment divided by the number after PlyG treatment. 'Bc' and 'Bt' indicate *B. cereus* and *B. thuringiensis*, respectively. **b**, ATP released from 1.0×10^4 vegetative bacteria after treatment with 2 U of PlyG. Luciferin/luciferase consumes ATP and the resulting signal is relative light units (RLU, an arbitrary value). The inset shows a correlation between RSVF1 numbers and RLU ($r^2 = 0.848$), using total ATP release in the presence of a strong detergent mix. **c**, Time course of RSVF1 killing in 1-ml cultures treated with buffer (triangles) or 2 U of PlyG (circles). Colony-forming units per ml are shown with the corresponding absorbance value (A_{600}; crosses).

ance, respectively. Despite the enhanced mutation rate, no PlyG-resistant derivatives were found. In addition, we have found that RSVF1 derivatives spontaneously resistant to γ phage remain sensitive to the action of PlyG (data not shown). Intrinsic PlyG resistance is therefore unlikely, supporting a theory that lysins target essential cell-wall molecules to ensure release of progeny phages[14].

We next examined the ability of PlyG to degrade germinating spores. In the dormant state, spore peptidoglycan, or cortex, is protected from lysozymes and amidases by a proteinaceous coat[15–17]; however, within 10 min of inducing germination, coat porosity increases[18]. We therefore reasoned that the subjacent peptidoglycan may be rendered susceptible to PlyG after the induction of germination. To evaluate this, we induced 1-ml aliquots of about 10^8 heat-activated spores from RSVF1, closely related *B. cereus* (ATCC 14579) and *B. thuringiensis* (ATCC 33679) strains, and *Bacillus subtilis* to germinate for 5 min with L-alanine (or treated for 5 min with D-alanine, a germination inhibitor). The suspensions were treated with PlyG (10 U) for 5 min, washed and the resulting viabilities were determined (Fig. 5a). The D-alanine-treated spores were resistant to PlyG, suggesting that the enzyme is not being carried by spores onto agar plates for determinations of colony-forming units. Among the L-alanine-treated spores, only RSVF1 was sensitive after the induction of germination, showing a 7,500-fold decrease in viability. A bacteriocidal action, therefore, occurs rapidly after the induction of germination, probably when PlyG can access the cortex.

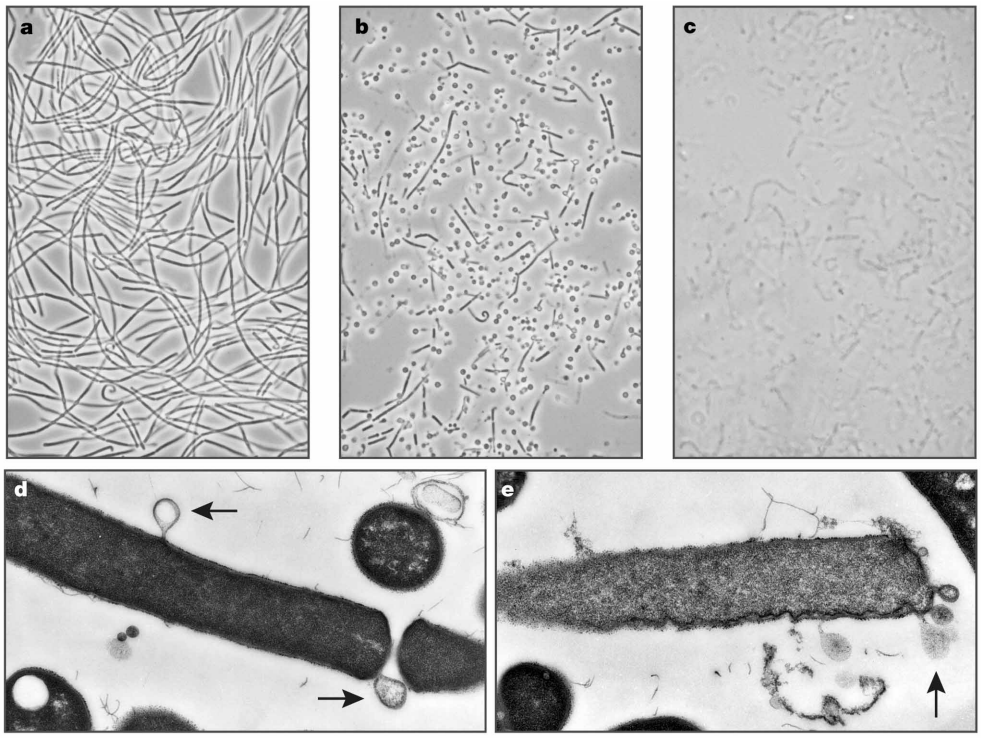

Figure 3 PlyG causes profound morphological changes in, and ultimately lysis of, RSVF1. **a**–**c**, We examined morphology by phase-contrast microscopy before PlyG treatment (**a**) and at 1 min (**b**) and 15 min (**c**) after treatment (with 5 U). **d**, **e**, Thin-section transmission electron micrographs (45,000 ×) 1 min (**d**) and 10 min (**e**) after treatment (with 5 U of PlyG). The arrows indicate membrane protrusions, and remnants thereof, pressing through zones of peptidoglycan degradation.

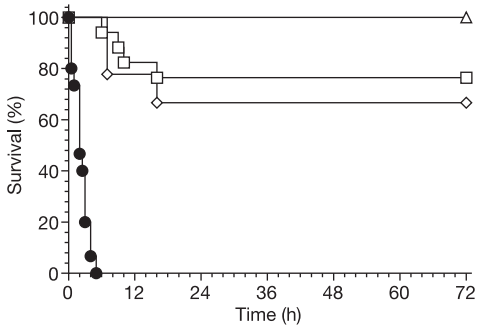

Figure 4 Survival of PlyG-treated BALB/c mice infected with RSVF1. Mice were injected i.p. with $\sim 1.0 \times 10^6$ RSVF1 colony-forming units and treated after 15 min with either phosphate buffer (circles, $n = 15$), 50 U PlyG (diamonds, $n = 19$), or 150 U PlyG (squares, $n = 13$). As a control for toxicity, mice were injected with 50 U PlyG (triangles, $n = 5$) alone. The experiment was terminated after 72 h. Administration of 50 U and 150 U to the infected mice was significantly protective compared with the buffer control ($P < 0.0001$). The median survival time for buffer-treated mice was 2 h.

Table 2 **Intrinsic PlyG resistance in wild-type and mutagenized RSVF1**

Treatment	RSVF1		RSVF1 + EMS	
	Resistance	Frequency	Resistance	Frequency
Novobiocin 3.5 μg ml^{-1}	Yes	2.4×10^{-9}	Yes	2.1×10^{-6}
Streptomycin 150 μg ml^{-1}	Yes	4.0×10^{-10}	Yes	4.3×10^{-6}
PlyG 20 U	No	$<5.0 \times 10^{-9}$	No	$<5.0 \times 10^{-9}$
PlyG 0.5–50 U	No	$<5.0 \times 10^{-9}$	No	$<5.0 \times 10^{-9}$
PlyG 20 U*	No	$<5.0 \times 10^{-9}$	No	$<5.0 \times 10^{-9}$

Descriptions of each treatment are provided in the Methods. PlyG 20 U and PlyG 0.5–50 U refer to the amounts used over a 4-day period. PlyG 20 U* refers to the amount present in a 24-h culture, before plating.

The ability of PlyG to kill germinating spores was exploited to develop a rapid and specific system for detecting γ-sensitive spores using a hand-held luminometer. Spores were immobilized on filters and incubated in consecutive 5-min rounds with germinant and PlyG (2 U); ATP release from degrading spores was then measured as light emitted in the presence of luciferin/luciferase. Using 2.5×10^3 spores, we detected a PlyG-mediated signal only with germinating RSVF1 spores (Fig. 5b), demonstrating both the recognition specificity of PlyG and its rapid lytic action. This signal was detected 10 min after germinant addition and only 5 min after PlyG was added. With mixed spore preparations, only a combination containing RSVF1 yielded a light signal (Fig. 5c); spore cortex fragments released from the germinating spores of related *Bacillus* isolates in the mix do not competitively inhibit PlyG. The sensitivity of this system was demonstrated using as few as about 100 RSVF1 spores, which yielded a signal 60 min after the addition of PlyG (Fig. 5d). No signal was detected in the presence of other germinating spore types, and was, therefore, specific to the γ-sensitive spores. This sensitivity, combined with the specificity, rapidity and portable nature of our detection method, suggests applications in monitoring domestic and battlefield use of *B. anthracis*.

In this study, we identified the first lysin (to our knowledge) from a *B. anthracis* bacteriophage and demonstrated its potent lytic effect on *B. anthracis* and other members of the *B. anthracis* cluster of *B. cereus*. Our findings encourage the continued development of PlyG as a means to prevent or treat anthrax and as a tool to detect vegetative or spore forms of *B. anthracis*. In the face of new and emerging bacterial pathogens, the widespread distribution of antibiotic resistance, and the real threat of bioterrorism and biowarfare, new adjuncts or alternatives to antibiotics, such as lysins, are imperative. In addition to their rapid and specific lytic activities, lysins are attractive compounds for development for several reasons. First, the biochemical pathways leading to the species- and serovar-specific cell-wall antigens bound by lysins are largely new or underused targets in current antimicrobial therapies for Gram-positive bacteria. Second, the modular design of lysins, with their independent functional domains, makes them ideal for domain-swapping studies in which bacterial specificities and catalytic activities are improved or adapted for use with other pathogens[4]. Third, because the catalytic and binding targets of lysins are largely essential for viability, lysin resistance should be rare. Our inability to detect resistance to PlyG certainly suggests that its peptidoglycan catalytic target and carbohydrate-binding sites cannot be easily modified in the bacillus to prevent lysin action. Finally, we must realize that lysins are highly evolved enzymes, modified and improved for high activity and specificity over the millennia. Considering the staggering global population of dsDNA phages (estimated to be more than 10^{30})[19], lysins are already one of the most ubiquitous and successful antimicrobial agents on Earth. Bacteriophages, and their corresponding lytic activities, represent an enormous untapped pool of agents with which to control human pathogens. □

Methods

Strains

Most strains were grown at 30 °C in Luria broth (LB) or brain–heart infusion broth (BHI). *B. anthracis* was grown on sheep blood agar (trypticase soy base) with or without a CO_2

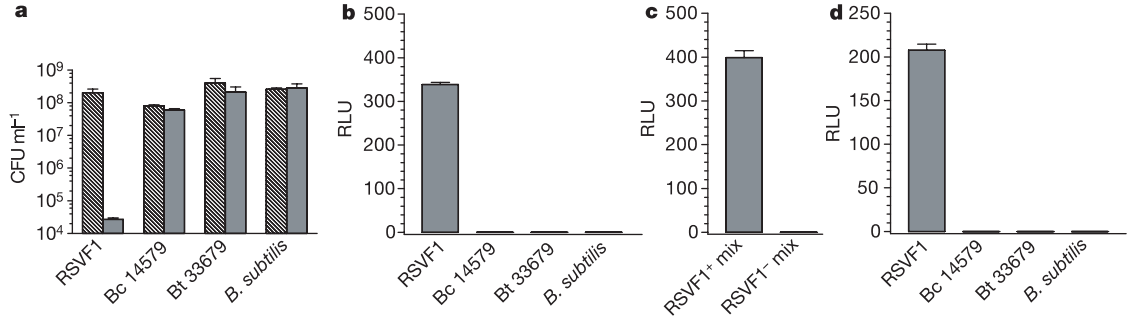

Figure 5 PlyG-mediated spore killing and detection. Error bars indicate s.d. from 3–5 independent determinations. **a**, The effect of PlyG on spore viability. Spores were incubated with D-alanine (hatched bars) or L-alanine (grey bars) and treated with PlyG (10 U). The resulting viability is shown. **b**, Specific detection of 2.5×10^3 germinating spores. ATP released 5 min after PlyG treatment (2 U) generates the indicated light signal. **c**, Detection of RSVF1 in a spore mixture. Mixtures containing 2.5×10^3 spores each of *B. cereus* (Bc) 14579, *B. thuringiensis* (Bt) 33679 and *B. subtilis* with (RSVF1$^+$) or without (RSVF1$^-$) RSVF1 were induced to germinate; the light release 5 min after PlyG treatment (2 U) is indicated. **d**, One hundred spores of the indicated types was induced to germinate; the light release 60 min after addition of 2 U PlyG is shown.

atmosphere. Work with *E. coli* XL1-Blue (Stratagene) and *B. anthracis* was performed at 37 °C, whereas *B. stearothermophilis* was handled at 55 °C. RSVF1, except for the absence of virulence plasmids, is nearly indistinguishable from *B. anthracis*[20–22]. The *vrrA* locus and motility of RSVF1 were analysed as described[23,24]. The susceptibility of *B. anthracis* to PlyG was examined by L. W. Mayer (CDC) and Abraham L. Turetsky (Aberdeen Proving Grounds, Maryland).

Virus manipulations

The γ phage was obtained from H.-W. Ackermann. Susceptibilities were initially tested using drops of ~10^7 phages applied to fresh lawns of the indicated strains. A phage stock containing 2.2×10^{10} plaque-forming units (PFU) per ml was prepared on RSVF1 as described[6], and used for the titre determinations in Table 1.

Identification of γ lysin

We partially digested 5-μg aliquots of γ DNA with *Tsp*509I and cloned 0.5–3.0-kilobase fragments into the arabinose-inducible expression vector pBAD24 (ref. 25). The library was transformed into *E. coli* and screened for lysin activity in a manner derived from another protocol[26]. The expression library was replica plated on to glass plates with LB containing 0.25% L-arabinose and 100 μg ml^{-1} ampicillin. After overnight incubation at 37 °C, the plates were inverted over lids containing 5 ml of chloroform to permeabilize the library. After 5 min, the library was overlain with 3 ml of 0.75% LB soft agar containing 300 μl of a tenfold-concentrated log-phase culture of RSVF1 and incubated for 24 h. Clear lytic zones appeared at the periphery of lysin-encoding library members.

Protein purification

PlyG was induced from XLl-Blue/pBAD24::*plyG* with 0.25% L-arabinose in overnight LB cultures. Cells were washed, resuspended in 50 mM Tris buffer at pH 8.0, and lysed with chloroform to yield crude PlyG. PlyG, which passed through a HiTrap Q Sepharose XL column (Amersham Bioscience), bound to a Mono S HR 5/5 column (Amersham Biosciences) and was eluted in a linear gradient containing 1 M NaCl. Analysis of the N-terminal protein sequence confirmed the identity of PlyG.

In vitro lysin activity

Activity was examined in several ways. A Spectramax Plus 384 spectrophotometer (Molecular Devices) followed the drop in absorbance at 600 nm (A_{600}) of log-phase RSVF1 incubated for 15 min at 37 °C with PlyG. Enzyme activity in units was determined as described[7], using lysis of exponentially growing RSVF1 with serial dilutions of PlyG. Fortuitously, 1 U corresponds to 1 μg of PlyG. For the liquid killing assay, 1.0 ml of log-phase cells was treated with the indicated amounts of PlyG at 37 °C and, at the specific time points, samples were washed to remove lysin and plated for enumeration. The ATP release assay was performed using the PROFILE-1 reagent kit and model 3550i microluminometer (New Horizons Diagnostics). Use of this system, and detailed validation studies, have been described[27]. In brief, filter-immobilized vegetative bacteria were treated with 2 U of PlyG in 150 μl for 2 min; a luciferin/luciferase reagent (50 μl) was then added and light release was assayed in a 10-s integration. Relative light units (RLU) produced by RSVF1 were consistently 10–20% of total releasable light, determined using a detergent mix that releases all intracellular ATP. Total RLU was similar for each sample. The correlation between RSVF1 concentration and RLU was performed as described looking at total ATP release[27].

Mutagenesis and screening for PlyG resistance

RSVF1 was treated with EMS as described[28]. Mutagenized cells were incubated with 20 U PlyG for 30 min at 37 °C, washed, and either plated or incubated overnight in BHI liquid. Colonies arising from plated cells were evaluated spectrophotometrically for resistance to 20 U of PlyG. For overnight BHI cultures, log-phase cells were established and treated with PlyG as above; this sequence was repeated over 4 d using either 20 U PlyG at each treatment or increasing amounts of PlyG (0.5–50 U).

Spontaneous lysin resistance was examined as described[6]. Spontaneous and EMS-induced mutation frequencies were estimated by determining resistance to 150 μg ml^{-1} streptomycin or to 3.5 μg ml^{-1} novobiocin.

Microsocopy

We treated 1 ml of log-phase RSVF1 with PIyG (5 U) for the indicated periods. For phase-contrast microscopy, cells were viewed with an Eclipse E400 microscope (Nikon). For electron microscopy, cells were examined as described[6].

In vivo lysin activity

Female BALB/c mice 4–8 weeks old were infected as previously described[12,13]. We i.p. injected 0.1-ml aliquots of 1.0×10^6 log-phase RSVF1 in 50 mM potassium phosphate buffer at pH 7.4. After 15 min, 0.5 ml of buffer or PlyG in buffer were injected i.p. Injections of PlyG alone (no bacteria) were also performed to assess toxicity. Mice were then monitored for 3–4 d.

Spore preparation and germination

Samples containing 95–99% spores were prepared as described[29]. Aliquots of 2.0×10^8 spores were heat activated at 65 °C for 5 min and suspended in 1.0 ml tryptic soy broth (TSB) with 100 mM L-alanine or D-alanine for 5 min at 37 °C. Samples were then treated with 10 U of PlyG for 5 min, washed three times (to remove residual PlyG), and plated for counting. TSB with L-alanine induced >99% germination, whereas D-alanine blocked germination.

For spore detection, we modified the germinating spore-killing protocol for use with the model 3550i microluminometer. Filter-immobilized, heat-activated spores were treated with 100 μl TSB and 100 mM L-alanine for 5 min, and 150 μl PlyG (2 U) for a further 5 min. Luciferin/luciferase (50 μl) was added and RLU were determined at the indicated time point. When PlyG or the germinant was omitted, no light signal was detected. When PlyG was replaced with a strong detergent mix, and total ATP was released, similar RLU were observed for equivalent numbers of each germinating spore type.

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Competing interests statement

The authors declare that they have no competing financial interests.

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A saponin-detoxifying enzyme mediates suppression of plant defences

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Plant disease resistance can be conferred by constitutive features such as structural barriers or preformed antimicrobial secondary metabolites. Additional defence mechanisms are activated in response to pathogen attack and include localized cell death (the hypersensitive response)[1,2]. Pathogens use different strategies to counter constitutive and induced plant defences, including degradation of preformed antimicrobial compounds[3] and the production of molecules that suppress induced plant defences[4–6]. Here we present evidence for a two-component process in which a fungal pathogen subverts the preformed antimicrobial compounds of its host and uses them to interfere with induced defence responses. Antimicrobial saponins are first hydrolysed by a fungal saponin-detoxifying enzyme. The degradation product of this hydrolysis then suppresses induced defence responses by interfering with fundamental signal transduction processes leading to disease resistance.

Many solanaceous plants produce glycosylated steroidal and/or steroidal alkaloid saponins[7]. In *Lycopersicon* species such as tomato (*L. esculentum*), the main saponin is the steroidal glycoalkaloid α-tomatine, which has potent antifungal activity[7,8]. The tomato leaf spot fungus *Septoria lycopersici* produces tomatinase, an extracellular enzyme that hydrolyses glucose from α-tomatine to give β_2-tomatine, which is substantially less inhibitory to fungal growth[9–15]. Targeted mutation of the tomatinase gene results in loss of ability to degrade α-tomatine and enhanced sensitivity to α-tomatine[12,16]. Tomatinase-deficient mutants of *S. lycopersici* are not obviously compromised in their ability to cause disease on tomato leaves but trigger enhanced cell death and elevated expression of plant defence genes in the early stages of infection[12]. These effects may be due to subtle differences in growth of the mutant and wild-type strains during infection. Alternatively, they may be an indication that tomatinase interferes with mechanisms of disease resistance in plants[12]. This led us to investigate whether *S. lycopersici* can infect and cause disease on another solanaceous species, *Nicotiana benthamiana*. Virus-induced gene silencing (VIGS) methodology has been developed for *N. benthamiana*, which makes this species an attractive model for further analysis[17].

We inoculated three strains of *S. lycopersici* (NEV, 16R and NY) onto *N. benthamiana* leaves. All three strains caused spreading disease lesions and extensive tissue damage (shown for NEV in Fig. 1). The fungus infected the leaves through the stomata (Fig. 1g) and grew intercellularly in the mesophyll tissue, forming V-shaped branches around the plant cells as it does in tomato (ref. 12 and Fig. 1h, i). By contrast, tomatinase-deficient mutants of NEV failed to cause disease on *N. benthamiana* leaves (Fig. 1c, f). Microscopic analysis showed intense trypan blue staining of the guard cells of challenged leaves (Fig. 1j) and evidence of localized cell death in the mesophyll tissue at the infection site (Fig. 1k). This cell death was first apparent 2 d after inoculation, which is consistent with the hypersensitive response.

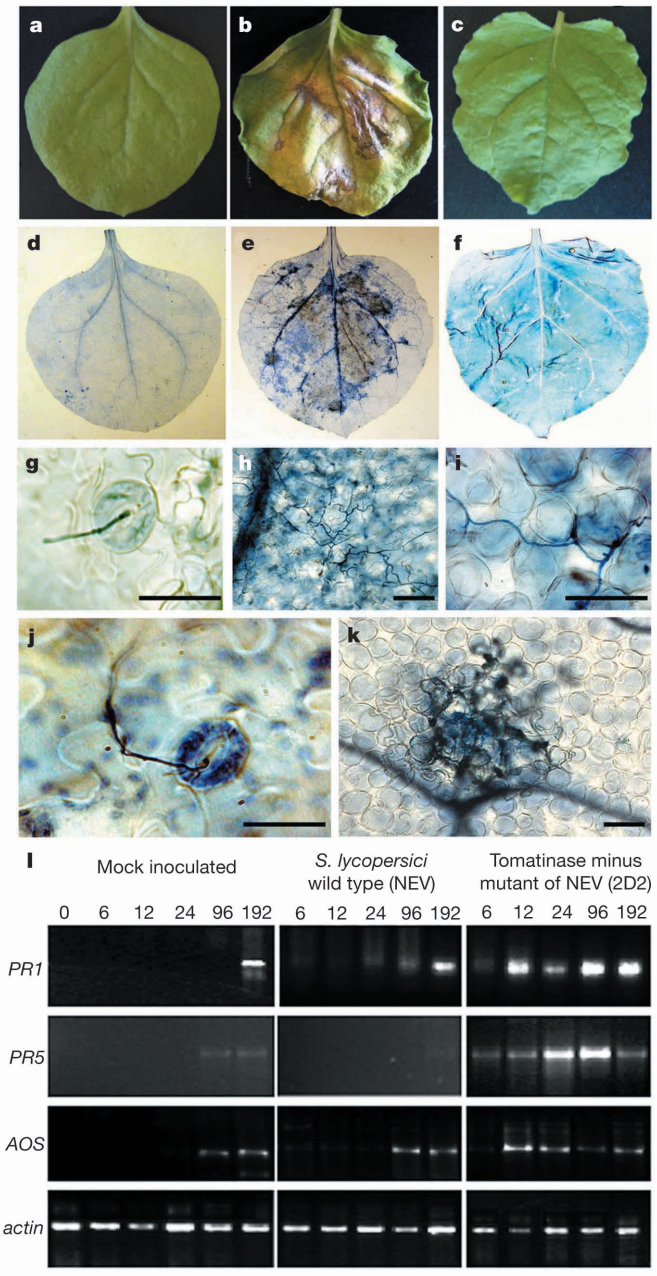

Figure 1 Infection of *N. benthamiana* by *S. lycopersici* strains. **a**–**c**, Leaves from *N. benthamiana* either mock inoculated (**a**) or inoculated with the wild-type *S. lycopersici* strain NEV (**b**) or the 2D2 tomatinase-deficient mutant of NEV (**c**). **d**–**f**, Leaves equivalent to those shown in **a**–**c** stained with lactophenol and trypan blue (**d**, mock inoculated; **e**, NEV wild-type strain; **f**, 2D2). **g**–**k**, Microscopic analysis of infection by the NEV wild-type strain (**g**–**i**) and 2D2 (**j**, **k**). Scale bars, 40 μm.
l, Induction of defence-related gene expression by wild-type and tomatinase-deficient strains of *S. lycopersici*. RNA was extracted from *N. benthamiana* leaves that had been either mock inoculated or inoculated with spores of the wild-type *S. lycopersici* strain NEV or the tomatinase-deficient mutant of NEV, 2D2. Expression of the defence-related genes *PR1*, *PR5* and *AOS*, and *actin* as a control was analysed by RT–PCR. Results were reproduced in three independent experiments. Numbers along the top of the panel indicate time (h) after inoculation.