Current Biology 18, 1078–1083, July 22, 2008 ©2008 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2008.06.061

Report

Bacterial Polysaccharides Suppress Induced Innate Immunity by Calcium Chelation

Shazia N. Aslam,[1] Mari-Anne Newman,[2] Gitte Erbs,[2] Kate L. Morrissey,[1] Delphine Chinchilla,[3] Thomas Boller,[3] Tina Tandrup Jensen,[2] Cristina De Castro,[4] Teresa Ierano,[4] Antonio Molinaro,[4] Robert W. Jackson,[1,5] Marc R. Knight,[6] and Richard M. Cooper[1,*]

[1]Department of Biology & Biochemistry
University of Bath
Bath BA2 7AY
UK

[2]Faculty of Life Sciences
Department of Plant Biology and Biotechnology
University of Copenhagen
Thorvaldsensvej 40,
1871 Frederiksberg
Denmark

[3]Zurich-Basel Plant Science Center
Botanical Institute
University of Basel
Hebelstrasse
4056 Basel
Switzerland

[4]Dipartimento di Chimica Organica e Biochimica
Università di Napoli
Complesso Universitario Monte SantAngelo
Via Cintia 4
80126 Napoli
Italy

[5]School of Biological Sciences
University of Reading
Whiteknights, Reading
Berks RG6 6AJ
UK

[6]Plant Stress Signalling Laboratory
Institute of Plant and Microbial Sciences
School of Biological and Biomedical Sciences
Durham University
South Road
Durham DH1 3LE
UK

Summary

Bacterial pathogens and symbionts must suppress or negate host innate immunity. However, pathogens release conserved oligomeric and polymeric molecules or MAMPs (Microbial Associated Molecular Patterns), which elicit host defenses [1–3]. Extracellular polysaccharides (EPSs) are key virulence factors in plant and animal pathogenesis, but their precise function in establishing basic compatibility remains unclear [4–7]. Here, we show that EPSs suppress MAMP-induced signaling in plants through their polyanionic nature [4] and consequent ability to chelate divalent calcium ions [8]. In plants, Ca^{2+} ion influx to the cytosol from the apoplast (where bacteria multiply [4, 5, 9]) is a prerequisite for activation of myriad defenses by MAMPs [10]. We show that EPSs from diverse plant and animal pathogens and symbionts bind calcium. EPS-defective mutants or pure MAMPs, such as the flagellin peptide flg22, elicit calcium influx, expression of host defense genes, and downstream resistance. Furthermore, EPSs, produced by wild-type strains or purified, suppress induced responses but do not block flg22-receptor binding in *Arabidopsis* cells. EPS production was confirmed in planta, and the amounts in bacterial biofilms greatly exceed those required for binding of apoplastic calcium. These data reveal a novel, fundamental role for bacterial EPS in disease establishment, encouraging novel control strategies.

*Correspondence: bssrmc@bath.ac.uk

Results and Discussion

Bacteria Release Defense Elicitors but Counter Host Responses

During host invasion, bacterial pathogens betray their presence by releasing various conserved molecules, which for the most part cannot be readily altered. The plant *Arabidopsis thaliana*, for example, is able to perceive flagellin (a constituent peptide flg22 is used here), the main peptide component of the motility organ, through the receptor FLS2; elongation factor EF-Tu (the peptide elf18 is used here), the most abundant bacterial protein, through the receptor EFR; and lipopolysaccharide (LPS), a glycolipid component of Gram-negative outer membranes, as well as peptidoglycan (PGN), an essential component of the cell envelope, through unknown receptors [1–3, 11]. Pathogens have evolved arsenals of type III-secreted protein effectors in order to counter MAMP-induced innate immunity, the first line of defense in vertebrates and one of the two major defense systems in plants [1, 2, 12, 13]. Effectors target defense components such as signaling pathways, programmed cell death, ubiquitination, and cytoskeleton and cell-wall reinforcement [1, 13], whereas plants have evolved to recognize effectors, directly or indirectly, via the products of resistance genes. The resulting typical phenotype is localized cell death or the hypersensitive response [1, 2, 13]. In addition to the effectors that are injected into the host cytoplasm, pathogens may also suppress defenses through secreted surface molecules. High molecular weight (0.5–2 MDa) bacterial extracellular polysaccharides (EPSs) have long been linked with pathogenicity and full virulence of bacteria infecting plants, invertebrates, and vertebrates [4–7, 14]. Evidence derives mainly from targeted disruptions in genes of EPS biosynthetic clusters such as *gum*, *alg*, and *ams* for xanthan, alginate, and amylovoran, respectively [5–7]. EPS-defective mutants activate host defenses more strongly than do their wild-type (WT) counterparts [5, 12, 15, 16]. EPSs may have other functions as well, including protection from host antimicrobial components and from environmental stresses such as desiccation and UV irradiation [5, 6].

Bacterial Polysaccharides are Polyanionic and Bind Cations

Comparing structures of many EPSs involved in virulence, we realized that all are polyanionic by the presence of uronic acids

(D-GlcA, D-ManA, D-GulA, and less frequently D-GalA) and, often, ketal-linked pyruvate and succinate. They, therefore, have the potential to bind or chelate cations. For example, alginate from *Pseudomonas aeruginosa*, xanthan from *Xanthomonas campestris*, and amylovoran from *Erwinia amylovora* strongly bind calcium [7, 8]. Models describing calcium chelation by alginate and xanthan reveal chain cooperativity and helix formation [8]; binding of Mg^{2+} is much weaker [17].

Calcium Signaling is Required for Defense Induction

Calcium functions as a second messenger in MAMP or pathogen perception. In plants, influx from the apoplastic pool $[Ca^{2+}]_{apo}$ to the cytosol $[Ca^{2+}]_{cyt}$, where Ca^{2+} levels are about four orders of magnitude lower, is a prerequisite for induced innate immunity [2, 10]. Associated defense responses include induction of reactive oxygen species (ROS), cell wall strengthening, upregulation of pathogenesis-related (PR) genes, and accumulation of antimicrobial low-molecular-weight phytoalexins [2, 10, 12, 18–21]. Calcium influx is also required for the hypersensitive response [19]. Because invading bacterial pathogens make intimate contact with plant cells in the apoplast, they have the ability to influence this critical calcium pool. We focus here on the role of EPS in plant-pathogen interactions and test the hypothesis that EPS suppresses host immunity by chelation of calcium.

EPS is Required by Wild-Type Bacteria for Virulence and Suppression of Calcium Signaling and Host Defenses

To study the role of EPSs in plant-pathogen interactions, we focused on WT *X. campestris* pv. *campestris* (*Xcc*) and an EPS-deficient mutant, but to demonstrate the broader importance of EPS in pathogenicity, we also carried out equivalent experiments with WT and corresponding EPS^{-} mutants of other pathogens. To ensure a rigorous test of our hypothesis, we examined a range of phenotypes associated with disease and resistance. First, in pathogenicity tests, *Xcc* multiplied in *A. thaliana* and caused chlorosis and later necrosis; the corresponding mutant (*Xcc* EPS^{-}) caused no visible symptoms, did not grow, and was undetectable within 3 dpi (Figure 1A), although WT and mutant grew equally well in vitro in NYGB (data not shown). Growth, spread, and symptoms with *Pseudomonas syringae* pv. tomato (*Pst*) in *Arabidopsis* (Figure S1A, available online) and *P. syringae* pv. *syringae* (*Pss*) in bean (*Phaseolus vulgaris*) [6] were also reduced in the EPS^{-} mutants. The contribution of EPS to virulence clearly varies, but the data support many studies on the requirement for EPS in pathogenicity, virulence, and symbiosis [4–7, 12, 14, 15, 21–28].

Aequorin-based measurements provide information about changes to cytosolic calcium concentrations and, thereby, about calcium influx [2, 10, 19]. Ca^{2+} ion influx in leaves of aequorin-transformed *Arabidopsis* was a single transient and greater after inoculation with the *Xcc* mutant than after inoculation with WT (Figure 1B), as was induction of the wound polymer callose by EPS^{-} mutants of *Xcc* [21] and *Pst* as compared with wild-types (Figure S1B). Generation of ROS was *ca*. 5-fold greater in *Arabidopsis* infiltrated with the *Xcc* mutant than in that infiltrated with WT (Figure 1C), and a similar pattern was given by *P. vulgaris* to *Pss* strains (Figure S1C).

Defense-related PR genes were monitored in *Arabidopsis* by real-time RT-PCR. Genes were chosen for their representation of different gene families and different signaling pathways, as well as for their diverse effects and kinetics; i.e., *PR1*, *PDF1.2*, *PAL*, *MPK3*, *GST* [20]. Generally, EPS-deficient mutants induced rapid and high gene transcription, in contrast to low

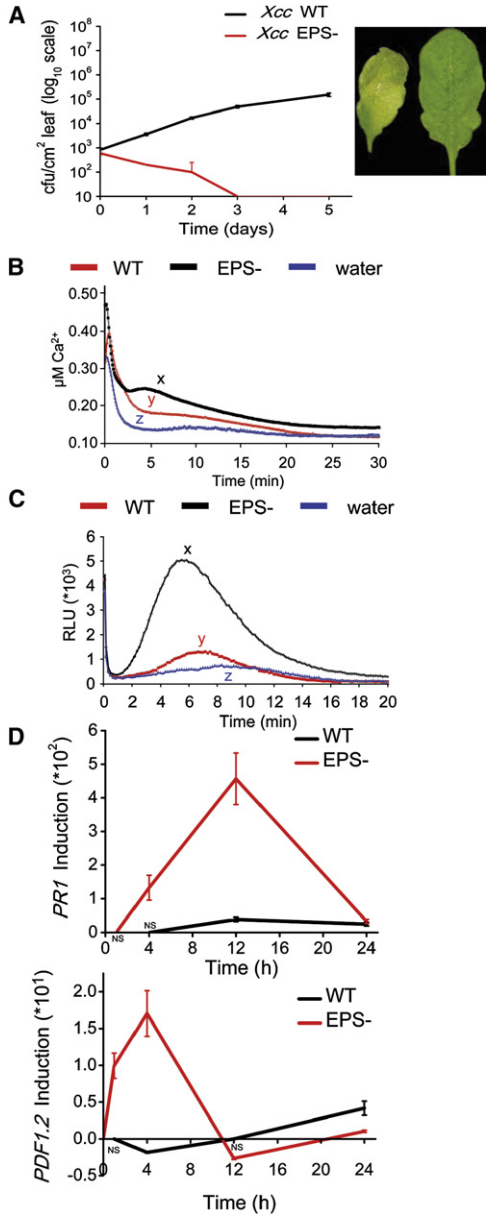

Figure 1. EPS is Required by Wild-Type Bacteria for Pathogenicity and Suppression of Host Defenses and Calcium Signaling

(A) Left: Bacterial-growth dynamics observed in *Arabidopsis* Col-0 over five days. Leaves were inoculated with 10^6 cfu/cm WT and EPS^{-} *X. campestris* pv. *campestris* (*Xcc*) (data shows mean of four replicates ± SD). Right: Symptoms observed in *Arabidopsis* leaves 5 days after inoculation with *Xcc* WT (left) and mutant (right).

(B) Cytosolic Ca^{2+} levels in *Arabidopsis*; *Xcc* WT and EPS^{-} infiltrated at 5×10^8 cfu/ml (mean of three replicates; ANOVA, $p < 0.0001$, $df = 2$). Significant differences (different letters) between samples were detected by Tukey-Kramer HSD.

(C) Oxidative burst (relative light units [RLU]) in *Arabidopsis* in response to inoculation with 5×10^8 cfu/ml *Xcc* WT and EPS^{-} mutant (mean of three replicates; ANOVA, $p < 0.0001$, $df = 2$). Significant differences (different letters) between samples were detected by Tukey-Kramer HSD.

(D) Expression levels of *PR1* and *PDF1.2* defense-related genes in response to inoculation with *Xcc* WT and EPS^{-} (5×10^6 cfu/ml) in *Arabidopsis* Col-0 leaves (mean of two replicates ± SE). Data are significant at $p < 0.001$ unless marked NS (not significant).

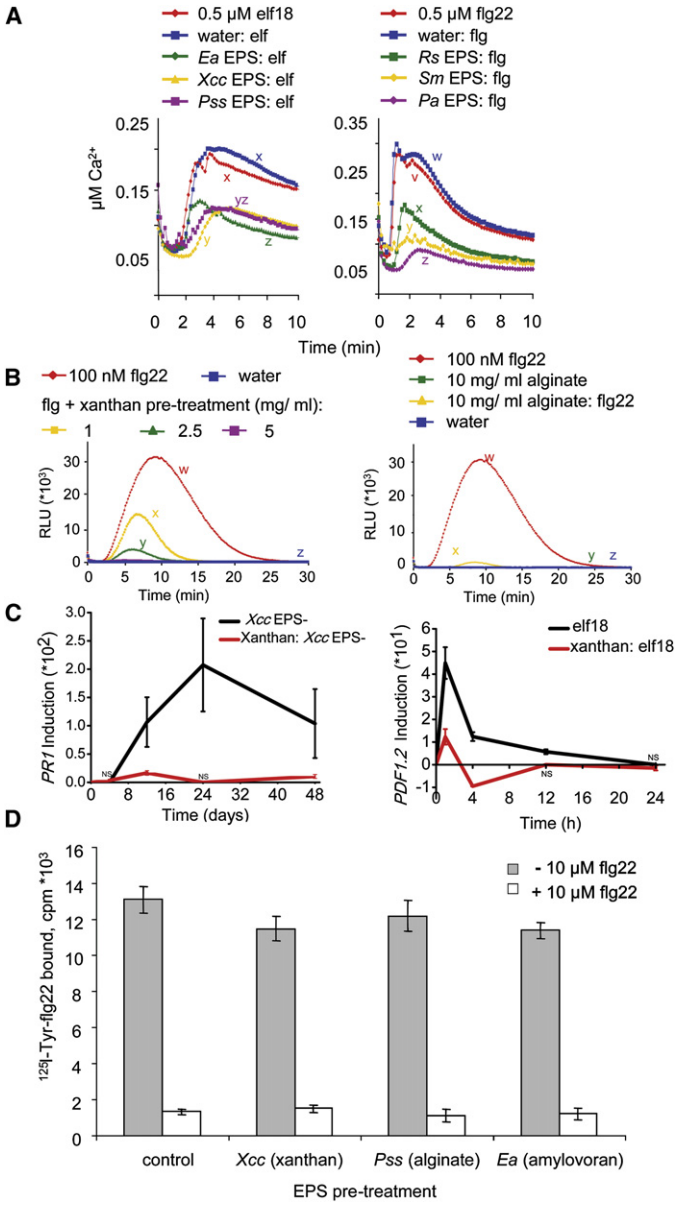

Figure 2. Pure EPSs Suppress Calcium Influx and Defenses Induced by EPS-Deficient Bacteria and by MAMPs but Do Not Block Receptor Binding of Flagellin

(A) Intracellular Ca^{2+} levels in response to flg22 or elf18 after preinfiltration with water or 10 mg/ml EPS from *X. campestris* pv. *campestris* (*Xcc*), *E. amylovora* (*Ea*), *P. syringae* pv. *syringae* (*Pss*), *R. solanacearum* (*Rs*), *S. meliloti* (Sm), *P. aeruginosa* (*Pa*) (mean of three replicates; ANOVA, $p < 0.0001$, df = 4, both graphs). Significant differences (different letters) between samples were detected by Tukey-Kramer HSD. All EPSs were tested against both MAMPs, with similar results; for clarity, only three of each combination are shown.

(B) Left: Oxidative burst (relative light units [RLU]) in response to flg22 after preinfiltration with various concentrations of xanthan in *Arabidopsis* (mean of three replicates; ANOVA, $p < 0.0001$, df = 4). Significant differences (different letters) between samples were detected by Tukey-Kramer HSD (df = 3, $p = 0.05$). Xanthan is suppressive at 1, 2.5, and 5 mg/ml, but xanthan levels of 7.5 and 10 mg/ml were not significantly different from water controls and so were omitted. Right: suppression of flg22-induced ROS by alginate (10 mg/ml).

(C) Left: Expression level of *PR1* in response to inoculation with *Xcc* EPS^{-} mutant in the presence or absence of xanthan. Right: Expression level of *PDF1.2* in response to treatment with elf18 in the presence or absence of xanthan (mean of two replicates ± SE). Data significant at $p < 0.001$ unless marked NS (not significant).

(D) *Arabidopsis* cells were incubated with 10 nM ^{125}I-Tyr-flg22 either alone (total binding; gray columns) or with an excess of 10 µM unlabelled flg22 (nonspecific binding; open columns). For determination of specific binding, nonspecific binding is subtracted from total binding (mean of three replicates ± SD; ANOVA, $p = 0.387$ [nonspecific binding data], $p = 0.050$ [total binding data]; df = 3). Also, the EPSs shown and those from *Rs* and *Sm* were used at 1 mg/ml and did not block binding.

Note 1. Xanthan at 1–10 mg/ml, whether free or in the calcium-saturated form, did not reduce diffusion of ^{125}I-Tyr-flg22, as determined by a dialysis method (data not shown).

Note 2. We included *Pss* because although *Pst* DC3000 is used widely as a model isolate, *Pst* did not produce significant EPS (alginate) in vitro, as has been previously reported for this pathovar [35]. However, alginate is produced in planta by *Pst* according to detection of EPS and the biosynthesis gene *algD* from infected plants [35, 36]. All alginate preparations used herein derive from *Pss*, which produces abundant alginate in vitro.

or late expression caused by WT isolates; this was the pattern observed with *PR1* and *PDF1.2* genes in response to *Xcc* (Figure 1D) and *GST*, *PAL* and *MPK3* in response to *Xcc* strains, and PR1 to *Pst* (Figure S7). Sometimes, downregulation of defense-gene expression by WT strains occurred (Figure 1D, Figure S7), as has been ascribed to the action of certain Type III effectors [1].

These results are consistent with the suppression of host responses by WT bacteria by virtue of EPS biosynthesis.

Pure EPSs Suppress Calcium Influx and Defenses Induced by EPS-Deficient Bacteria and by MAMPs

In order to further investigate the implied role of EPS, EPSs were purified from plant pathogens *Xcc*, *Pss*, *E. amylovora*, and *Ralstonia solanacearum*; from plant symbiont *Sinorhizobium meliloti*; from plant colonizer and opportunistic human pathogen *Pseudomonas aeruginosa* [25]; and from *Photorhabdus luminescens*, a symbiont of nematodes and pathogen of insects [29] and obtained from *Escherichia coli* (capsular polysaccharide K antigen) [22]. EPSs were analyzed (see Experimental Procedures, data not shown) and found to be equivalent in composition to published structures [4, 6–8, 15]. For determining whether EPSs prevented calcium signaling and defense induction, most of the EPSs were infiltrated into leaves, which were then exposed 15 min–1 hr later to EPS-deficient mutants and MAMPs. EPSs were generally not perceived as MAMPs (data not shown; see Conclusions). The EPS concentrations used (1–10 mg/ml) reflect levels found in planta, as discussed below. Calcium influx in response to flg22 or elf18 was suppressed to similar extents by purified EPSs (10 mg/ml) from plant, insect, and human pathogens and symbionts (Figure 2A). Levan, a neutral fructan produced from sucrose by some bacterial pathogens [4, 7], showed no significant suppressive activity (Figure S2).

ROS induction by flg22 was suppressed by xanthan in a concentration-dependent manner, with complete inhibition by ≥ 5 mg/ml (Figure 2B); elf18 elicitation was similarly suppressed by xanthan (data not shown), and alginate (10 mg/ml) reduced flg22-induced ROS by > 90% (Figure 2B). Likewise, coinfiltration of bean leaves with alginate (5 mg/ml) and the *Pss* EPS^{-} mutant resulted in low ROS levels equivalent to only those found in WT cells (Figure S1C). EPSs are well known for providing bacteria with some protection against ROS toxicity [e.g., 5, 6, 25]. The suppressive effect described here must be against

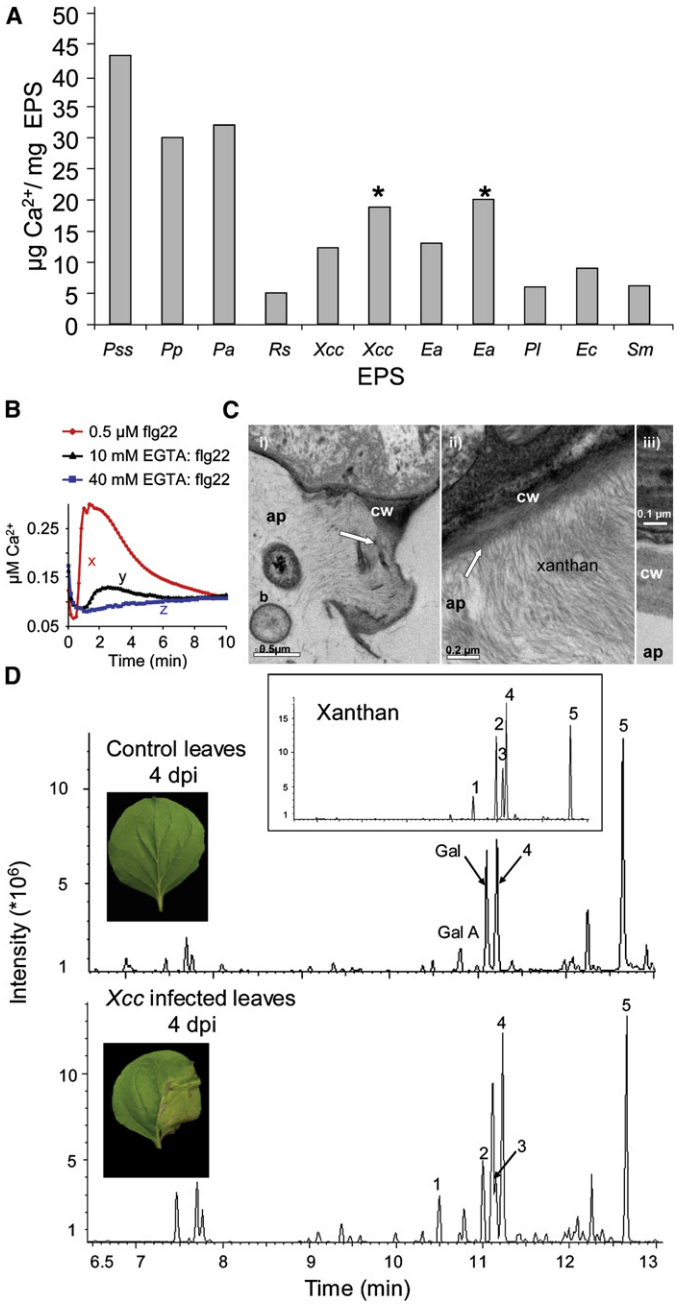

Figure 3. EPSs Bind Calcium and EPS Levels in Biofilms Can Potentially Deplete the Apoplastic Calcium Pool

(A) Calcium-binding potential by EPS from plant, insect, and human bacterial pathogens (used at 1 mg/ml and for *Xcc* and **Ea*, also at 10 mg/ml). Abbreviations: see Figure 2 legend, also *Pp* (*P. papulans*), *Pl* (*Photorhabdus luminescens*), *Ec* (*E. coli*) (mean of three replicates by AAS; ANOVA, $p < 0.0001$, df = 10; (error bars are too small to show because replicates were highly reproducible).

(B) Intracellular Ca^{2+} levels in response to treatment with flg22 after pretreatment with two concentrations of EGTA (mean of three replicates; ANOVA, $p < 0.0001$, df = 2). Significant differences (different letters) between samples were detected by Tukey-Kramer HSD ($p = 0.05$).

(C) Ultrastructure of *Arabidopsis* leaf intercellular spaces 5 dpi with *Xcc* (i) and 10 mg/ml pure xanthan 2 dpi (ii). Xanthan produced from bacteria (b) or infiltrated into apoplast (ap) of leaves appears to interact (arrows) with the cell wall (cw). (iii) Cell wall (cw) and apoplast (ap) from control, water-infiltrated leaf.

(D) GC-MS of pure xanthan (inset) and extracts from *Xcc*-inoculated 4 dpi and control leaves of *Nicotiana benthamiana*. Peaks are 1 = GlcA, 2 = Man, 3 = Man-Pyr, 4 = Glc, 5 = Inositol. Gal is a major peak in controls and infected tissue and slightly obscures Man-Pyr. Note that Man-Pyr is a characteristic component of xanthan [8]. Calculations for xanthan concentrations at 2, 4, and 6 dpi are based on a mean of two runs from two replicate extractions.

induction of ROS, because xanthan and alginate did not scavenge H_2O_2 from solution (unpublished data; see Experimental Procedures) as was suggested for EPS from *Burkholderia cenocepacia* [28].

EPS pretreatments also suppressed defense-gene induction in response to pathogens or to MAMPs. Xanthan (1 mg/ml) from *Xcc* reduced by ~90% the level of *PR1* transcription induced by *Xcc* mutant and reduced elf18-elicited *PDF1.2* expression by > 70% (Figure 2C). Induction of the wound polymer callose by flg22 and elf18 in *Arabidopsis* leaves was also markedly reduced by preinfiltration with xanthan (Figure S3). A related study reported suppression of host responses (callose formation) via 0.1 mg/ml of a partially purified xanthan (LPS was not removed) with a 24 hr interval between pretreatment and exposure to the *Xcc* EPS^- mutant [21], but this was found here to be entirely ineffective, presumably partly because calcium pools become replenished with time and because of the low amounts of EPS that were used.

Suppression by EPS Does Not Result from Interference with MAMP-Receptor Binding

We considered the possibility that defense suppression by EPSs results from physical blocking of MAMPs or of access to receptors, such as via pores in host cell walls. To check this, representative EPSs (used at 1 and 2.5 mg/ml) were examined for interference with binding of ^{125}I-Tyr-flg22 to its LRR-RLK receptor FLS2 on *Arabidopsis* cells [30]. There was no reduction in flg22 binding caused by any of the purified EPSs from diverse bacterial pathogens (Figure 2D). Thus, the suppressive effect must be exerted by prevention of perception, signaling, or both.

Diverse EPSs Bind Calcium Ions

All EPSs (1 mg/ml) bound from 5 to 43 µg Ca^{2+}/mg EPS (Figure 3A). Xanthan and amylovoran were also examined at a higher polymer concentration (10 mg/ml) when calcium binding increased by > 50%, presumably due to chain-chain cooperativity and ion chelation [8, 17]. A modified xanthan (lacking pyruvyl and acetyl groups) [8, 21] sequestered about 50% of the amount of calcium as compared with WT xanthan (Figure S4), and its suppression against flg22-induced Ca^{2+} influx was about half that of the WT polymer (data not shown). The calcium chelator EGTA ≥ 10 mM mimicked EPS suppression of flg22-induced Ca^{2+} influx, suggesting that EPSs function in this way and confirming the apoplastic origin of MAMP-induced calcium influx (Figure 3B). Likewise, $LaCl_3$, the calcium surrogate acting as a calcium-channel blocker, suppressed flg22-induced calcium influx at ≤ 1 µM (Figure S5).

EPS levels in Bacterial Biofilms and in Infected Hosts Can Potentially Deplete the Apoplastic Calcium Pool

In order for us to ascertain the calcium-binding potential of EPS present in bacterial biofilms, as are formed during

invasion of the host apoplast [4, 9, 12, 31], we first assessed amounts (w/v) of EPS in agar biofilms. GC-MS revealed xanthan from *Xcc* at 36 mg/ml, and the *Pss* biofilm comprised 24 mg/ml alginate. $[Ca^{2+}]_{apo}$ has been measured at ~50–150 μM [32]. From data in Figure 3A, the xanthan concentration found in agar biofilms would be theoretically capable of binding 648 μg/ml and alginate could bind 480 μg/ml calcium; this equates to > 10 mM Ca^{2+} and far exceeds that needed to impair local signaling.

Confirmation of EPS levels and timing in planta is also crucial for the understanding of their role. Amounts of EPSs reported from infected plants (from 1 mg/g fresh weight [23] to 30–50 mg/g dry weight [4, 24]) are likely to be considerable underestimates, because the apoplast may constitute only 10% of overall tissue volume [23]; also, EPSs can form gels with calcium and with some host-wall polymers [8]. Figure 3C shows pure xanthan infiltrated into *Arabidopsis* leaves, resembling the fibrillar biofilm matrix ultrastructurally visible in xanthomonad infections and, likewise, apparently interacting with host-wall fibrils. Interaction of xanthan with wall polymers has been noted by us before (*Xanthomonas axonopodis* pv. *manihotis* in cassava, unpublished data) and might reflect sequestration of Ca^{2+} ions, which perform a crosslinking structural function for the polygalacturonan matrix. Weakening of the wall could further contribute to host invasion.

We examined EPS production by *Xcc* in *Nicotiana benthamiana*, a pathosystem [21] that provides sufficient material for GC-MS analysis. After 2 dpi (water-soaking symptoms), 4 dpi (chlorosis), and 6 dpi (necrosis), xanthan levels from GC-MS were 14 (1), 19 (2), and 25 (6) mg/g as dry weight and fresh weight (bracketed), respectively; amounts were calculated from GC-MS runs as represented by Figure 3D. These concentrations of xanthan are sufficient for binding local $[Ca^{2+}]_{apo}$ and suppressing signaling. This result supports ultrastructural studies showing bacteria embedded in an EPS matrix from early infection [4] (e.g., after 2 hpi [9] and 8 hpi [31], and 18 hpi in this study), and concurs with the requirement for xanthan production in initial infection by *Xcc* [14]. Some evidence suggests that quorum sensing (QS) control of EPS biosynthesis results in relatively late appearance of these polymers [23, 33]. Alternative regulation of EPS biosynthesis, such as via surface contact (colanic-acid production by *E. coli*) and in response to reactive oxygen and desiccation stress (alginate from *P. syringae* and *P. aeruginosa*) [5, 6], might explain this apparent contradiction. Nevertheless, QS is likely to operate in the high populations ultrastructurally evident in early localized biofilms in planta.

Conclusions and Implications

Our results are consistent with the suppressive effects of bacterial EPSs on MAMP-elicited defenses exerted via apoplastic calcium sequestration and reduction of consequent signaling. Plant pathogenic bacteria proliferate adjacent to the host cell wall, which contains the apoplastic calcium pool. There, they produce EPS in amounts sufficient for depletion of the local free $[Ca^{2+}]_{apo.}$ The overall contribution of EPS to basic compatibility in host-pathogen interactions is likely to be additive to the rapidly produced type III effectors, of which some can suppress signaling mediated by multiple MAMPs [1, 13]. However, the novel and fundamental role revealed here for EPS underlines why most pathogens are dependent on EPS synthesis. Functions beyond mere protection have long been surmised [4, 8]. Evaluation of equivalent suppressive function for EPS of pathogens of animals requires suitable host-pathogen models, possibly as described for *Staphylococcus* spp. [27], but it currently remains a matter for speculation with respect to any role for calcium binding.

Clearly, EPSs are potential disease-control targets. One control strategy in the context of this study could involve depolymerases such as alginate, amylovoran, and xanthan lyases [8]. The resulting oligosaccharides would not only lose affinity for Ca^{2+} ions, which can be dependent on the 3D structure of the polymer, involving chain cooperation and chain length [8, 17], but the degradation products may also function as MAMPs. There are reports of eliciting activities of xanthan and alginate oligomers (e.g., [34]). Also, we have shown MAMP activity with the induction of ROS by hydrolysed *Pss* alginate in *Arabidopsis* cells (unpublished data). Intact, calcium-free EPSs have low eliciting activities, but calcium-saturated xanthan elicited *PR1, PDF1.2*, and *PAL* genes (Figure S6). Presumably, this form was no longer able to "self protect" by means of chelating calcium ions.

Supplemental Data

Supplemental data include supplemental experimental procedures and seven figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/14/1078/DC1/.

Acknowledgments

We acknowledge many colleagues for their provision of MAMPs, some EPSs, bacterial cultures, and seed (see Experimental Procedures). We thank Allan Downie (John Innes Centre, Norwich) and Ian S. Roberts (Manchester University) for early discussions. Research was mainly supported by a grant from The Leverhulme Trust to R.M.C. Electron microscopy was performed by Ursula Potter and Louise Haste (University of Bath).

Received: April 25, 2008
Revised: June 18, 2008
Accepted: June 19, 2008
Published online: July 17, 2008

References

1. He, P., Shan, L., and Sheen, J. (2007). Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. Cell. Microbiol. *9*, 1385–1396.
2. Erbs, G., Silipo, A., Aslam, S., De Castro, C., Liparoti, V., Flagiello, A., Pucci, P., Lanzetta, R., Parrilli, M., Molinaro, A., et al. (2008). Peptidoglycan and muropeptides from pathogens *Agrobacterium* and *Xanthomonas* elicit plant innate immunity: structure and activity. Chem. Biol. *15*, 438–448.
3. Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. Plant Cell *16*, 3496–3507.
4. Denny, T.P. (1995). Involvement of bacterial polysaccharides in plant pathogenesis. Annu. Rev. Phytopathol. *33*, 173–197.
5. Kemp, B.P., Horne, J., Bryant, A., and Cooper, R.M. (2004). *Xanthomonas axonopodis* pv. *manihotis gumD* gene is essential for EPS production and pathogenicity and enhances epiphytic survival on cassava (*Manihot esculenta*). Physiol. Mol. Plant Pathol. *64*, 209–218.
6. Yu, J., Penaloza-Vazquez, A., Chakrabarty, A.M., and Bender, C.L. (1999). Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. *syringae*. Mol. Microbiol. *33*, 712–720.
7. Bugert, P., and Geider, K. (1995). Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. Mol. Microbiol. *15*, 917–933.
8. Sutherland, I.W. (1994). Structure-function relationships in microbial exopolysaccharides. Biotechnol. Adv. *12*, 393–448.
9. Brown, I., Mansfield, J., Irlam, I., Conrads Strauch, J., and Bonas, U. (1993). Ultrastructure of interactions between *Xanthomonas campestris* pv. *vesicatoria* and pepper, including immunocytochemical localization

of extracellular polysaccharides and the AvrBs3 protein. Mol. Plant-Microbe Interact. *6*, 376–386.

10. Lecourieux, D., Raneva, R., and Pugin, A. (2006). Calcium in plant defence-signalling pathways. New Phytol. *171*, 249–269.
11. Bedini, E., De Castro, C., Erbs, G., Mangoni, L., Dow, J.M., Newman, M.-A., Parrilli, M., and Unverzagt, C. (2005). Structure-dependent modulation of a pathogen response in plants by synthetic O-antigen polysaccharides. J. Am. Chem. Soc. *127*, 2414–2416.
12. Keshavarzi, M., Soylu, S., Brown, I., Bonas, U., Nicole, M., Rossiter, J., and Mansfield, J. (2004). Basal defences induced in pepper by lipopolysaccharides are suppressed by *Xanthomonas campestris* pv. *vesicatoria*. Mol. Plant-Microbe Interact. *17*, 805–815.
13. Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. Nature *444*, 323–329.
14. Newman, M.A., Conrads-Strauch, J., Scofield, G., Daniels, M.J., and Dow, J.M. (1994). Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. Mol. Plant-Microbe Interact. *7*, 553–563.
15. Fraysse, N., Couderc, F., and Poinsot, V. (2003). Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. Eur. J. Biochem. *270*, 1365–1380.
16. Araud-Razou, I., Vasse, J., Montrozier, H., Etchebar, C., and Trigalet, A. (1998). Detection and visualization of the major acidic exopolysaccharide of *Ralstonia solanacearum* and its role in tomato root infection and vascular colonization. Eur. J. Plant Pathol. *104*, 795–809.
17. Lattner, D., Flemming, H.-C., and Mayer, C. (2003). ^{13}C-NMR study of the interactions of bacterial alginate with bivalent cations. Int. J. Biol. Macromol. *33*, 81–88.
18. Cooper, R.M., Resende, M.L.V., Flood, J., Rowan, M.G., Beale, M.H., and Potter, U. (1996). Detection and cellular localization of elemental sulphur in disease-resistant genotypes of *Theobroma cacao*. Nature *379*, 159–162.
19. Grant, M., Brown, I., Knight, M., Ainslie, A., and Mansfield, J. (2000). The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. *23*, 441–450.
20. Tsuda, K., Sato, M., Glazebrook, J., Cohen, J.D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. Plant J. *53*, 763–775.
21. Yun, M.H., Torres, P.S., El Oirdi, M., Rigano, L.A., Gonzalez-Lamothe, R., Marano, M.R., Castagnaro, A.P., Dankert, M.A., Bourab, K., and Vojnov, A.A. (2006). Xanthan induces plant susceptibility by suppressing callose deposition. Plant Physiol. *141*, 178–187.
22. McNulty, C., Thompson, J., Barrett, B., Lord, L., Andersen, C., and Roberts, I.S. (2006). The cell surface expression of group 2 capsular polysaccharides in *Escherichia coli*: the role of KpsD, RhsA and a multi-protein complex at the pole of the cell. Mol. Microbiol. *59*, 907–922.
23. McGarvey, J.A., Denny, T.P., and Schell, M.A. (1999). Spatial-temporal and quantitative analysis of growth and EPS1 production by *Ralstonia solanacearum* in resistant and susceptible tomato cultivars. Phytopathology *89*, 1233–1239.
24. Gross, M., and Rudolph, K. (1987). Demonstration of levan and alginate in bean plants (*Phaseolus vulgaris*) infected by *Pseudomonas syringae* pv. *phaseolicola*. J. Phytopathol. *120*, 9–19.
25. Yorgey, P., Rahme, L.G., Tan, M.W., and Ausubel, F.M. (2001). The roles of *mucD* and alginate in the virulence of *Pseudomonas aeruginosa* in plants, nematodes and mice. Mol. Microbiol. *41*, 1063–1076.
26. Cuccui, J., Easton, A., Chu, K.K., Bancroft, G.J., Oyston, P.C.F., Titball, R.W., and Wren, B.W. (2007). Development of signature-tagged mutagenesis in *Burkholderia pseudomallei* to identify genes important in survival and pathogenesis. Infect. Immun. *75*, 1186–1195.
27. Begun, J., Gaiani, J.M., Rohde, H., Mack, D., Calderwood, S.B., Ausubel, F.M., and Sifri, C.D. (2007). Staphylococcal biofilm exopolysaccharide protects against *Caenorhabditis elegans* immune defences. PLoS Pathog. *3*, e57.
28. Bylund, J., Burgess, L.A., Cescutti, P., Ernst, R.K., and Speert, D.P. (2006). Exopolysaccharides from *Burkholderia cenocepacia* inhibit neutrophil chemotaxis and scavenge reactive oxygen species. J. Biol. Chem. *281*, 2526–2532.
29. Ffrench-Constant, R.H., Waterfield, N., Burland, V., Perna, N.T., Daborn, P.J., Bowen, D., and Blattner, F.R. (2000). A genomic sample sequence of the entomopathogenic bacterium *Photorhabdus luminescens* W14: potential implications for virulence. Appl. Environ. Microbiol. *66*, 3310–3329.
30. Bauer, Z., Gomez-Gomez, L., Boller, T., and Felix, G. (2001). Sensitivity of different ecotypes and mutants of *Arabidopsis thaliana* toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. J. Biol. Chem. *276*, 45669–45676.
31. Cason, E.T., Richardson, P.E., Essenberg, M.K., Brinkerhoff, L.A., Johnson, W.M., and Venere, R.J. (1978). Ultrastructural cell-wall alterations in immune cotton leaves inoculated with *Xanthomonas malvacearum*. Phytopathology *68*, 1015–1021.
32. Felle, H.H., Hanstein, S., Steinmeyer, R., and Hedrich, R. (2000). Dynamics of ionic activities in the apoplast of the sub-stomatal cavity of intact *Vicia faba* leaves during stomatal closure evoked by ABA and darkness. Plant J. *24*, 297–304.
33. Vojnov, A.A., Slater, H., Daniels, M.J., and Dow, J.M. (2001). Expression of the *gum* operon directing xanthan biosynthesis in *Xanthomonas campestris* and its regulation *in planta*. Mol. Plant-Microbe Interact. *14*, 768–774.
34. Liu, H., Huang, C., Dong, W., Du, Y., Bai, X., and Li, X. (2005). Biodegradation of xanthan by newly isolated *Cellulomonas* sp. LX, releasing elicitor-active xantho-oligosaccharides-induced phytoalexin synthesis in soybean cotyledons. Process Biochem. *40*, 3701–3706.
35. Keith, R.C., Keith, L.M.W., Hernandez-Guzman, G., Uppalapati, S.R., and Bender, C.L. (2003). Alginate gene expression by *Pseudomonas syringae* pv. tomato DC3000 in host and non-host plants. Microbiology *149*, 1127–1138.
36. Fett, W.F., and Dunn, M.F. (1989). Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. Plant Physiol. *89*, 5–9.