Specific host genes required for the killing of *Klebsiella* bacteria by phagocytes

Mohammed Benghezal,1 Marie-Odile Fauvarque,2 Régis Tournebize,3 Romain Froquet,4 Anna Marchetti,4 Evelyne Bergeret,2 Bernard Lardy,5 Gérard Klein,2 Philippe Sansonetti,3 Steve J. Charette4 and Pierre Cosson*2

1Athelas SA, Chemin des Aulx 18, 1228 Plan-les-Ouates, Geneva, Switzerland.
2Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, UMR5092, CEA/CNRS/UJF, 17 rue des Martyrs, 38054 Grenoble, Cedex 9, France.
3Institut Pasteur, Pathogénie Microbienne Moléculaire, 28, rue du Dr. Roux, 75724 Paris, Cedex 15, France.
4Université de Genève, Centre Médical Universitaire, Département de Physiologie Cellulaire et Métabolisme, 1 rue Michel Servet, CH-1211 Geneva 4, Switzerland.
5Groupe de Recherche et d'Etude du Phénomène Inflammatoire (GREPI)-(EA2938) Laboratoire d'Enzymologie, Centre Hospitalier Universitaire de Grenoble, France.

Summary

The amoeba *Dictyostelium discoideum* shares many traits with mammalian macrophages, in particular the ability to phagocytose and kill bacteria. In response, pathogenic bacteria use conserved mechanisms to fight amoebae and mammalian phagocytes. Here we developed an assay using *Dictyostelium* to monitor phagocyte–bacteria interactions. Genetic analysis revealed that the virulence of *Klebsiella pneumoniae* measured by this test is very similar to that observed in a mouse pneumonia model. Using this assay, two new host resistance genes (*PHG1* and *KIL1*) were identified and shown to be involved in intracellular killing of *K. pneumoniae* by phagocytes. Phg1 is a member of the 9TM family of proteins, and Kil1 is a sulphotransferase. The loss of *PHG1* resulted in *Dictyostelium* susceptibility to a small subset of bacterial species including *K. pneumoniae*. Remarkably, *Drosophila* mutants deficient for *PHG1* also exhibited a specific susceptibility to *K. pneumoniae* infections. Systematic analysis of several additional *Dictyostelium* mutants created a two-dimensional virulence array, where the complex interactions between host and bacteria are visualized.

Introduction

Bacterial infections are the result of complex interactions between invading bacteria and host defence mechanisms. Sophisticated genetic tools have led to the identification of many bacterial virulence genes essential for bacterial pathogenicity. Typically, a mutation in a virulence gene decreases the pathogenic potential of a bacterial strain. Alteration of host defence mechanisms in rare human genetic diseases or in transgenic animals can also alter the outcome of a bacterial infection, and can for instance account for specific susceptibility to subsets of usually harmless bacteria (Gallin, 1992). As phagocytic cells (neutrophils and macrophages) form the first line of defence of the organism against invading microorganisms, their role in antibacterial defence is certainly essential. Indeed, a few host genes specifically involved in the function of phagocytic cells have been linked to resistance to bacterial infections in mammals. This is notably the case of the Nramp1 protein, a cation transporter present in the membrane of the phagosomes, and which presumably influences the fate of intraphagosomal bacteria by influencing the ionic content of the phagosome (Forbes and Gros, 2001). In humans, mutations in NRAMP1 are associated with an increased susceptibility to bacterial infections, particularly mycobacteria. Similarly, mutations affecting genes encoding components of the NADPH oxidase complex cause susceptibility to bacterial diseases, in particular to *Staphylococcus aureus* or *Klebsiella pneumoniae* infections (Nathan and Shiloh, 2000; Reeves et al., 2002; Fang, 2004).

An extensive analysis of host defence mechanisms is limited by ethical and practical restrictions to animal experiments, and it is likely that our current knowledge of the genetic basis of host resistance to bacterial infections is far from complete. Consequently many investigators have been taking advantage of non-mammalian hosts such as insects (D’Argenio et al., 2001; Fauvarque et al., 2002), nematodes (Tan et al., 1999), plants (Rahme et al., 1997) and amoebae (Harb et al., 2000; Greub and Raoult, 2004) to study bacterial virulence. Many of these studies have shown that pathogenic bacteria use similar virulence
mechanisms when confronted with mammalian and non-mammalian hosts. In addition, the use of non-mammalian systems amenable to genetic analysis allowed the identification of new host factors necessary for resistance to pathogenic bacteria. In *Drosophila melanogaster*, this led notably to the identification of evolutionary conserved NF-kB-dependent signalling pathways inducing antimicrobial peptide synthesis by the fat body (Hoffmann, 2003).

Here we have used the amoeba *Dictyostelium discoideum* to identify two new host genes, *PHG1* and *KIL1*, involved in resistance to *K. pneumoniae*. Mutant *phg1* amoebae exhibit specific susceptibility to *K. pneumoniae*, caused by a specific inability to kill these bacteria intracellularly. Remarkably *Drosophila* *phg1* mutants also exhibit susceptibility to *K. pneumoniae* infections. This approach offers new possibilities to analyse complex host–pathogen relationships.

Results

*Mutant phg1 amoebae are susceptible to K. pneumoniae*

When plated on a lawn of non-pathogenic bacteria such as *Bacillus subtilis* or *K. pneumoniae* laboratory strains, *Dictyostelium* amoebae feed upon the bacteria, creating phagocytic plaques (Fig. 1A). This has been used previously to assess the virulence of *Pseudomonas aeruginosa* bacteria, as pathogenic *P. aeruginosa* strains do not allow the growth of *Dictyostelium* amoebae, while non-pathogenic strains do (Cosson *et al.*, 2002; Pukatzki *et al.*, 2002). To identify host genes involved in resistance to *K. pneumoniae*, we screened our laboratory collection of *Dictyostelium* mutants individually to identify a mutant susceptible to *K. pneumoniae*. The *phg1* mutant (Cornillon *et al.*, 2000) was incapable of growing on *K. pneumoniae*, while retaining the ability to grow on other bacterial substrates such as *B. subtilis* (Fig. 1B). This suggests that this *K. pneumoniae* strain has specific virulence traits, affecting susceptible *phg1* mutant cells. This situation is reminiscent of immunosuppressed mammalian hosts, which can be infected by normally harmless bacteria.

Fig. 1. Susceptibility of *phg1 Dictyostelium* mutant to *Klebsiella*. A. The ability of a *Dictyostelium* strain to grow on a bacterial lawn was assessed by plating the indicated bacteria and 100 *Dictyostelium* cells on SM-Agar. Scale bar: 1 cm. A phagocytosis plaque was observed after 5 days.

Klebsiella pneumoniae virulence genes are implicated in the *Dictyostelium–bacteria interaction*

In order to identify putative *K. pneumoniae* virulence genes, bacteria were mutagenized by random transposon insertion. Eight hundred bacterial mutants were tested individually, and six of them were permissive for growth of *phg1* amoebae (Fig. 2A). All six mutants grew as well as the parental wild-type *Klebsiella* strain (M. Benghezal, unpubl. data). The mutated genes were identified...
(Fig. 2B), and fell into several categories. The operons containing WaaQ (mutD), NagA (mutA) and wbbM (mutE) are implicated in the biosynthesis of the bacterial surface, notably the capsule and lipopolysaccharides (Vogler and Lengeler, 1989; Regue et al., 2001; Shankar-Sinha et al., 2004). The operons containing metB (mutC) and TrpC (mutF) are involved in amino-acid biosynthesis (methionine and tryptophan respectively). The detailed analysis of mutB would require further genetic dissection, as a number of genes with distinct functions might be affected, notably a DNA adenine methylase (transcriptional regulation) and TrpS (tryptophan biosynthesis). Interestingly not only a set of virulence genes to interact with their viability was measured in wild-type and phg1 cells. Wild-type Dictyostelium internalized and rapidly killed K. pneumoniae (Fig. 3C). Conversely phg1 mutant amoebae phagocytosed K. pneumoniae but failed to kill them efficiently (Fig. 3C). These persistent bacteria were resistant to the addition of gentamicin in the extracellular medium, confirming that they were intracellular (P. Cosson, unpubl. data). Interestingly, phg1 cells were still capable of killing B. subtilis normally (Fig. 3D). Remarkably, the three avirulent K. pneumoniae mutants involved in the biosynthesis of the bacterial cell surface (mutA, D and E) were much less resistant to killing by phg1 mutant amoebae (Fig. 3E) while K. pneumoniae mutants B, C and F were still resistant (Fig. 3E). This suggests that virulence of K. pneumoniae is caused in part by its resistance to intracellular killing but also by additional uncharacterized virulence mechanisms. Apparently, K. pneumoniae uses both types of virulence genes against Dictyostelium amoebae and mice.

Killing of Klebsiella bacteria by phg1 Dictyostelium

To identify new host genes involved in bacterial killing, we screened a cDNA expression library and isolated a phg1 suppressor plasmid encoding a new gene, KIL1. Overexpression of KIL1 in phg1 cells restored their ability to grow on K. pneumoniae (Fig. 4A), and to kill ingested K. pneumoniae (Fig. 4B). To investigate further the role of KIL1 in intracellular killing of bacteria, a knockout mutant strain harbouring a targeted disruption of KIL1 was constructed by homologous recombination. We observed that kil1 mutant cells were also defective for killing of K. pneumoniae, although the defect was slightly less pronounced than that observed in phg1 cells (Fig. 4B). On the contrary,
mutant kil1 cells were as efficient as wild-type cells for the killing of B. subtilis (P. Cosson, unpubl. data), indicating that Kil1 was specifically required for efficient killing of K. pneumoniae. Kil1 is highly homologous to human NDST1, a membrane-associated sulphotransferase implicated in the addition of sulphate to sugars, and the synthesis of sulphated proteins and proteoglycans (Hashimoto et al., 1992). Accordingly, Kil1 protein bound phosphoadenosine phosphate (PAP), a competitive inhibitor of sulphotransferases (Klaassen and Boles, 1997) (Fig. 4C), and no sulphated proteins could be detected in kil1 mutant cells (Fig. 4D).

As described above, phg1 mutant cells were initially shown to inefficiently phagocytose latex beads (phagocytosis: 3.9 ± 0.1% of wild type) (Cornillon et al., 2000). We observed that the overexpression of Kil1 did not complement the phagocytosis defect of phg1 (3.7 ± 0.2% of wild type). Similarly, mutant kil1 cells did not exhibit a defect for phagocytosis of latex beads (115 ± 22% of wild type). These results further suggest that the susceptibility of phg1 cells to K. pneumoniae is not related to their previously described phagocytosis defect.

A two-dimensional virulence array

To characterize more extensively the phenotype of phg1 mutants, we tested the ability of Dictyostelium to grow on a range of bacterial strains. Wild-type Dictyostelium was unable to grow on several pathogenic Gram-positive (S. aureus, Listeria monocytogenes, Enterococcus faecalis) as well as Gram-negative (uropathogenic Escherichia coli, Salmonella typhimurium, P. aeruginosa) bacteria (Fig. 5). However, Dictyostelium grew normally on avirulent variants of these strains (quorum-deficient P. aeruginosa or...
svrA S. aureus), as well as on non-pathogenic bacteria, thus providing a simple test of bacterial pathogenicity (Fig. 5). Phg1 mutant cells were specifically unable to grow on K. pneumoniae and one strain of E. coli (B/r strain) but grew on all other avirulent bacterial strains, further demonstrating the specificity of phg1 susceptibility.

A number of Dictyostelium mutants were also analysed. Mutants presenting a phagocytosis defect similar to that of phg1 (myoVII, phg2, talin) (Gebbie et al., 2004) were not susceptible to Klebsiella (Fig. 5), demonstrating that this type of phagocytosis defects did not per se affect host resistance to Klebsiella. Similarly noxA or noxA/noxB double mutants did not exhibit susceptibility to any avirulent bacterial strain tested. Interestingly, the bsg mutant described previously (Ratner and Newell, 1978) exhibited a distinct phenotype, being specifically unable to grow on the two Gram-positive strains tested (B. subtilis and avirulent S. aureus) (Fig. 5), while growth on all avirulent Gram-negative strains was normal. This demonstrates that distinct sets of host resistance genes are crucial in the confrontation with various groups of bacteria.

Drosophila phg1 mutants are susceptible to Klebsiella
Phg1 belongs to the family of TM9 proteins defined by a high degree of homology, and the presence of nine conserved transmembrane domains. The family includes many members in organisms ranging from yeast (three members) to Dictyostelium (three members), Drosophila (three members) and humans (four members), but the function of these proteins remains largely unknown (Benghezal et al., 2003). Note that in this study Phg1 designates the first characterized member of the family in Dictyostelium, sometimes referred to as Phg1a (Benghezal et al., 2003), and the Drosophila homologue of this gene.

The fact that Phg1 was implicated in the resistance of Dictyostelium to K. pneumoniae prompted us to test its role in Drosophila resistance to bacterial infections. For this, Drosophila phg1 null mutants were created by imprecise excision of a P transposable element. Homozygous phg1 mutants were fully viable and showed no visible phenotypic alterations. In particular, mutant larvae exhibited no detectable alteration of the fat body, the main source of antimicrobial peptides, and no significant

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Fig. 4. Kil1 is involved in intracellular killing of K. pneumoniae.
A. Kil1 overexpression restores the ability of phg1 mutant cells to grow on a lawn of K. pneumoniae.
B. As described in Fig. 3C, Dictyostelium cells (WT, phg1, phg1+KIL1 or kil1) and K. pneumoniae bacteria were mixed and the number of surviving bacteria was determined at the indicated times.
C. To assess the role of Kil1 in protein sulphation, Dictyostelium cell lysates (WT or kil1) were incubated with Sepharose-immobilized PAP. Bound proteins were separated on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and revealed using an antibody to Kil1.
D. To assess the role of Kil1 in protein sulphation, Dictyostelium cell lysates (WT or kil1) were separated on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and revealed with an antibody to Kil1.

Fig. 5. A host–pathogen two-dimensional virulence array. The ability of Dictyostelium mutants to grow on different bacterial strains was determined as described in Fig. 1. Growth of Dictyostelium is indicated by green, no growth by red. S. typhimurium; Ec UPEC, uropathogenic E. coli; Pa, P. aeruginosa; Pa-mut, P. aeruginosa rhlR-lasR mutant; Ec DH5α, E. coli (DH5α strain); Ec Br, E. coli B/r strain; Kp, K. pneumoniae; Kp-mut, K. pneumoniae mutants; Bs, B. subtilis; Sa, S. aureus; Sa-mut, S. aureus svrA mutant; Ef, E. faecalis; Lm, L. monocytogenes.
changes in the number or the morphology of haemocytes, the precursors of phagocytic cells (M.O. Fauvarque, unpubl. data). This indicated that the essential elements of the Drosophila immune system are still present in phg1 mutants.

In order to test the role of PHG1 in resistance to pathogenic bacteria, wild-type or mutant Drosophila were challenged with P. aeruginosa or K. pneumoniae, and their survival was analysed. Remarkably Drosophila phg1 mutants exhibited an increased susceptibility to K. pneumoniae, but not to P. aeruginosa (Fig. 6). More precisely, when challenged with a high dose of K. pneumoniae, only a small fraction of wild-type Drosophila died within 5 days (20%) versus 87% of infected phg1 mutant Drosophila (Fig. 6A). With a reduced infective dose of Klebsiella, no mortality was seen in wild-type flies, but a significant number (27%) of infected phg1 mutants died (Fig. 6B). Interestingly, when challenged with avirulent Klebsiella mutants described above (mutD), even phg1 mutants did not exhibit any mortality (Fig. 6C). Identical results were obtained with Klebsiella mutF (0% mortality within 5 days), and it was also observed that both mutants were less pathogenic than wild-type Klebsiella when a high infective dose was used (mortality at day 5: 32% for mutF, 47% for mutD, versus 87% for wild-type K. pneumoniae).

In contrast, wild-type and phg1 mutant flies died with very similar kinetics when infected with P. aeruginosa (Fig. 6D).

Discussion

**New host genes determining resistance to bacterial pathogens**

In this study we used Dictyostelium amoebae to investigate the complex interactions between phagocytic cells and bacteria. Bacterial genetics demonstrated that bacterial genes necessary for the virulence of K. pneumoniae in this system were also at play during the infection of a mammalian host. We thus used the Dictyostelium host to characterize further host genes implicated in resistance to K. pneumoniae. We identified two Dictyostelium genes specifically implicated in the interaction with Klebsiella, PHG1 and KIL1. Analysis of phg1 and kil1 mutants revealed that both genes are essential for efficient intracellular killing of K. pneumoniae following their phagocytosis by Dictyostelium. Both Phg1 and Kil1 have clear homologues in other eukaryotic systems, in particular in mammals. However, the function of TM9 proteins was only studied in Dictyostelium so far, and the function of Phg1 and Kil1 in the physiology of phagocytic cells has not been studied in mammalian systems. Our experiments provide the first clue that these gene products are involved in the function of phagocytic cells and in resistance to bacterial infections.

To test the relevance of our findings in another model system, we created a phg1 mutant in Drosophila and tested its susceptibility to K. pneumoniae as well as P. aeruginosa infections. Both humoral and cellular components participate to the very efficient immune system of Drosophila, allowing development of larvae in infested environments such as rotting fruits. The fat body ensures the synthesis of antimicrobial peptides in response to bacterial infections. Blood cells, or haemocytes, can differentiate into phagocytic plasmocytes ensuring bacterial engulfment (Meister and Lagueux, 2003) and resistance to bacterial infection (Elrod-Erickson et al., 2000; Avet-Rochex et al., 2005). These two elements of the immune system were present in mutant phg1 Drosophila. However, Drosophila phg1 mutants exhibited an increased susceptibility to Klebsiella infections. Although a detailed analysis of the function of the immune system in phg1 mutant Drosophila will be required to determine the exact cause of their susceptibility to Klebsiella, it can be hypothesized that this phenotype is linked to a defect in the function of the phagocytic cells in phg1 mutant Drosophila. This result indicates that Phg1 is implicated in resistance to pathogenic Klebsiella bacteria both in Dictyostelium amoebae and in Drosophila. Thus, host resistance genes identified in a Dictyostelium host are

![Fig. 6. Drosophila phg1 mutants exhibit an increased susceptibility to K. pneumoniae infections. Male Drosophila flies, either wild type (WT) or mutant (phg1), were infected with a high dose of WT K. pneumoniae (A), a low dose of WT K. pneumoniae (B), a low dose of mutD K. pneumoniae (C), or with WT P. aeruginosa (D).](image-url)
also implicated in resistance to bacterial infections in other eukaryotic hosts.

**Dual role of Phg1 in phagocytosis and intracellular killing of bacteria**

Phg1 is a member of the TM9 family of membrane proteins, previously implicated in the control of adhesion and phagocytosis in *Dictyostelium* (Cornillon et al., 2000; Benghezal et al., 2003). However, the susceptibility of *Dictyostelium* phg1 mutants to *K. pneumoniae* is clearly not the direct consequence of its phagocytosis defect, as phagocytosis of live *Klebsiella* is unaffected in these cells. Furthermore, several other mutants exhibiting phagocytosis defects similar to that of *phg1* (myoVII, talin, phg2) (Gebbie et al., 2004) were tested here and did not show susceptibility to *K. pneumoniae*, demonstrating that the *phg1* defect in phagocytosis is not sufficient *per se* to cause susceptibility to *K. pneumoniae*. The dual role of Phg1 in phagocytosis and resistance to *K. pneumoniae* is illustrated by the fact that *phg1* mutants overexpressing Kil1 kill *Klebsiella* bacteria efficiently, while they are still defective for phagocytosis of latex beads.

Together these results indicate that the loss of Phg1 causes two distinct phenotypes: first a specific loss of adhesion to certain substrates, and a concomitant-specific defect in phagocytosis of certain particles (e.g. latex beads, but not *K. pneumoniae*), and second a decrease in intracellular killing of *K. pneumoniae*.

**Putative role of Phg1 and Kil1 in intracellular killing**

Sequence analysis as well as biochemical characterization indicate that Kil1 is a sulphotransferase. Loss of a sulphotransferase may affect bacterial killing by perturbing intracellular transport. Indeed it has been shown that sulphated proteoglycans mediate the delivery of positively charged proteases to secretory granules in mouse mast cells (Forsberg et al., 1999; Humphries et al., 1999), as well as the delivery of lysozyme to lysosomes in human promonocytic U937 cells (Lemansky and Hasilik, 2001). In *Dictyostelium* cells, alterations in sulphation were also reported to modify the intracellular fate of mature lysosomal enzymes (Cardelli et al., 1990). Interestingly Phg1, in addition to its role in phagocytosis, also controls transport in the endocytic pathway in *Dictyostelium* (Benghezal et al., 2003). We hypothesize that Phg1 and Kil1 might both be involved in the targeting or activation of a subset of lytic enzymes in phagolysosomes. We are currently investigating the putative role of Phg1 and Kil1 in the biogenesis and function of endocytic compartments. We expect the alterations of the endocytic compartments to be rather specific, as our results demonstrate that these two host genes are specifically implicated in the killing of *K. pneumoniae* bacteria while being dispensable for the killing of other bacteria such as *B. subtilis*.

**A systematic analysis of host resistance genes**

The assay used in this study allows to test the susceptibility of a given *Dictyostelium* strain to various bacterial strains rapidly and with a high degree of reproducibility. Given the relative simplicity with which mutants can be created and analysed in *Dictyostelium*, we can now envisage a much more systematic analysis of host resistance genes. It is striking that *phg1* mutant cells characterized here exhibited a very narrow spectrum of susceptibility. Equally interesting is the fact that bsg mutants isolated previously show specific susceptibility to a distinct set of bacterial strains. Although the identity of the gene affected in this mutant is not known, this suggests a high degree of specificity in the interaction of hosts with various bacterial strains. Figure 5 can be seen as a simple virulence array where the complex relationships between the bacterial genome and the host genome become apparent, and a starting point for a more extensive analysis of host resistance genes.

**Experimental procedures**

**Cell culture and strains**

*Dictyostelium discoideum* cells were grown at 21°C in HL5 medium (Cornillon et al., 1998). Unless otherwise specified, *Dictyostelium* strains used in this study were all derived from the DH1-10 subclone (Cornillon et al., 2000) of the *D. discoideum* wild-type strain DH1 (Caterina et al., 1994). Strains mutated in PHG2, MYOVII or TALIN were described previously (Gebbie et al., 2004). The bsg strain (Ratner and Newell, 1978) was obtained from the American Type Culture Collection. The noxA, and the double noxA/noxB knockout mutants were derived from a wild-type JH10 strain that behaved like DH1-10 in our assays. Unless otherwise specified, the name PHG1 refers to the first characterized member of the TM9 family, sometimes called PHG1a (Cornillon et al., 2000; Benghezal et al., 2003).

Bacterial strains were *K. pneumoniae* laboratory strain and Kp52145 from the Collection of Institute Pasteur in Paris, S. typhimurium (ATCC 14028), *P. aeruginosa* strains PT5 and PT531 (Cosson et al., 2002), E. coli strains DH5a (Invitrogen), uropathogenic N2173-96 (Centre national des bactéries entéro-pathogènes, Bern, Switzerland) and B/r (Gerisch, 1959), *B. subtilis* strain 36.1 (Ratner and Newell, 1978), *S. aureus* RN6390, *E. faecalis* (ATCC 29212) and *L. monocytogenes* L028. A laboratory strain and strains PT5 and PT531 (Cosson et al., 2002), E. coli strains DH5a (Invitrogen), uropathogenic N2173-96 (Centre national des bactéries entéro-pathogènes, Bern, Switzerland) and B/r (Gerisch, 1959), *B. subtilis* strain 36.1 (Ratner and Newell, 1978), *S. aureus* RN6390, *E. faecalis* (ATCC 29212) and *L. monocytogenes* L028. *K. pneumoniae* strain expressing GFP was obtained by introducing the GFP-expressing plasmid pANT5 (Lee and Falkow, 1998) into our laboratory strain.

To test *Dictyostelium* growth on bacteria, 50 µl of overnight bacterial culture was plated on 2 ml of SM-Agar in one well of a 24-well plate, then 100 *Dictyostelium* cells were added to the bacterial lawn. Amoebal growth created phagocytic plaques after 4–7 days of incubation at 21°C.
Phagocytosis and killing of bacteria by Dictyostelium

To test the ability of Dictyostelium to ingest and kill live bacteria, 10⁶ bacteria from an overnight culture were mixed with 10⁶ Dictyostelium cells in 1 ml of phosphate buffer (2 mM Na₂HPO₄, 14.7 mM KH₂PO₄, pH 6.5) and incubated at 21°C with vigorous shaking. This high Dictyostelium:bacteria ratio (100:1) was chosen to ensure efficient phagocytosis of all bacteria. Other ratios (1:10, 1:1, 1:10) were also tested and yielded very similar results (P. Cosson, unpubl. data). At the indicated time, an aliquot of the suspension was collected, diluted in four volumes of ice-cold sucrose (40%), then diluted in 25 volumes of ice-cold phosphate buffer containing 0.5% saponin, before plating on an LB plate and incubating at 37°C. This procedure killed Dictyostelium cells while bacterial viability was unaffected. Bacterial colonies were counted after 16 h at 37°C. When indicated, the number of viable bacteria associated with Dictyostelium cells was determined by washing the cells twice with ice-cold HL5 medium before diluting in sucrose.

Immunofluorescence analysis was performed as described previously (Ravanel et al., 2001), as well as phagocytosis of latex beads (Cornillon et al., 2000). To measure phagocytosis of live Klebsiella, GFP-expressing K. pneumoniae were mixed with Dictyostelium at a ratio of 100:1, incubated at 21°C for various times, then washed with ice-cold HL5 medium containing 0.1% sodium azide before analysis in a Fluorescence Activated Cell Sorter.

Identification of KIL1, a high-copy suppressor of phg1

A suppressor screen was performed by transfecting a D. discoideum cdNA overexpression library in phg1a mutant cells and selecting transformants for their ability to grow on K. pneumoniae. Briefly, extrachromosomal replication of a Ddp2-ori-dependent cdNA library in phg1a mutant was obtained through genomic integration of the plasmid DIV1-REP carrying the REP gene and the PYR5-6 selection marker (Franke and Kessin, 1977). DIV1-REP was constructed by cloning the BglII/SacI DNA fragment sequencing the DIV1-REP strain and selection performed in HL5 containing 100 µg ml⁻¹ kanamycin. This yielded approxi- mately 15 000 primary transformants. Transfected cells were then applied on a lawn of K. pneumoniae and clones able to form plaques were recovered. Plasmids carrying a suppressor cdNA were purified from three independent Dictyostelium transformants as described (Robinson and Spudich, 2000), and sequenced. Two suppressor plasmids carried the coding sequence of PHG1 and one the coding sequence of KIL1.

To obtain KIL1 knockout cells, 5’ (position 1–613 of the coding sequence) and 3’ (position 977–1413) DNA fragments of KIL1 were cloned on both sides of a blasticidin selection cassette in pBluescript SK(-). The vector was digested to excise the knockout cassette and transfected into wild-type Dictyostelium. Blastici- din-resistant transformants were screened for KIL1 deletion by immunodetection using antibodies to KIL1 protein.

Characterization of KIL1 sulphotransferase activity

Cells (150 × 10⁶ cells) were grown in HL5 medium (3 × 10⁶ cells ml⁻¹), pelleted and lysed in 10 ml of Lysis buffer (20 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 2 mg ml⁻¹ aprotinin, 2 mg ml⁻¹ leupeptin, 100 mM phenylmethyl-sulphonyl fluoride) at 4°C for 1 h. After centrifugation at 4°C (10 000 g, 30 min), samples were incubated with PAP-agarose (Sigma, Saint-Louis, USA) for 2 h at 4°C. The resin was then washed three times with Lysis buffer and each sample was separated on a 9% polyacrylamide gel under non-reducing conditions. The proteins were then transferred to a nitrocellulose Protran BA 85 membrane (Schleicher and Schuell Bioscience, Dassel, Germany). The membrane was incubated sequentially with a rabbit antiserum directed against a GST-Kil1 fusion protein (amino acids 330–465), then a horseradish peroxidase-coupled goat antiserum to rabbit Ig (Bio-Rad Laboratories AG, Glattbrugg, Switzerland), and revealed by enhanced chemiluminescence (Amersham Biosciences, UK).

To detect sulphated cellular proteins, 2 × 10⁶ cells were resuspended in 10 µl of sample buffer. After migration in a 9% polyacrylamide gel and transfer to nitrocellulose, sulphated proteins were detected with a mouse monoclonal antibody 221-342-5 (Neuhaus et al., 1998) specific for proteins carrying a mannos 6-sulphate-containing epitope.

Mutagenesis of Klebsiella

Klebsiella pneumoniae transposon insertion mutants were obtained by electroporation of the pNKBOR plasposon carrying the mini-Tn10 transposon and selected on LB Petri dishes containing 50 µg ml⁻¹ kanamycin (Rossignol et al., 2001). Mutant strains were grown in LB at 37°C overnight in 96-well microplates, and their ability to sustain growth of Dictyostelium phg1 mutants was tested individually as described above. Bacterial mutants restoring the growth of phg1 mutants were selected. To identify the transposon insertion site in each bacterial mutant, genomic DNA was purified, digested with BglII, self-ligated, transformed into E. coli DH5αpir (Rossignol et al., 2001) and sequenced.

Mouse pneumonia model

A lethal pneumonia model was used to evaluate the virulence of Klebsiella strain Kp52145 and isogenic mutants. This protocol was approved by the Swiss federal veterinary office (authorization number 31.1.1083/2175/III). Female Balb/cJ mice (7–8 weeks old) were used. Bacteria were grown on a TSB Petri dish (cm1065, Oxoid, Basingstoke, UK) for 15 h at 37°C. They were then scraped from the Petri dish and resuspended in sterile 0.9% NaCl (5 × 10⁷ bacteria ml⁻¹). Mice (12 for each strain tested) were anaesthetized with ketamine (40 mg kg⁻¹) and xylazine (5 mg kg⁻¹), infected with 10⁶ bacteria by nasal instillation of 20 µl and tagged with a transponder for identification and body temperature measurement twice a day (Kort et al., 1998). A temperature below 34°C or a loss of more than 20% of body weight were found to be predictive of the death of the mice in the next 12 h. These criteria were used as humane endpoints for euthanasia.

Drosophila survival upon bacterial infections

Flies were grown on standard medium at 25°C. Drosophila phg1 mutant was generated by classical mobilization of a PlacW element ([lg2]k07245) inserted into the 5’ regulatory sequences of
phg1 (CG7364, http://flybase.bio.indiana.edu/) provided by the Bloomington stock centre.

For infection, 30 adult flies (5–10 days old) were pricked into the upper part of the thorax with a thin needle previously dipped into a bacterial solution. For P. aeruginosa infections, bacteria were grown to exponential phase [optical density at 600 nm (OD_{600}) = 0.8] and diluted in sterile saline buffer (final OD_{600} = 0.4) before use. In this condition, multiplicity of infection (moi), measured as described previously (Fauvarque et al., 2002), was approximately 30–50 bacteria per fly. In the case of K. pneumoniae infections, a pellet from overnight cultures was used to infect flies at either OD_{600} = 20 (low dose) or OD_{600} = 200 (high dose). In these conditions, moi corresponds, respectively, to 1000 (low dose) and 10 000 (high dose) bacteria per fly. Experiments were repeated at least three times with results identical to those presented.

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