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

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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.

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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-κB-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*,

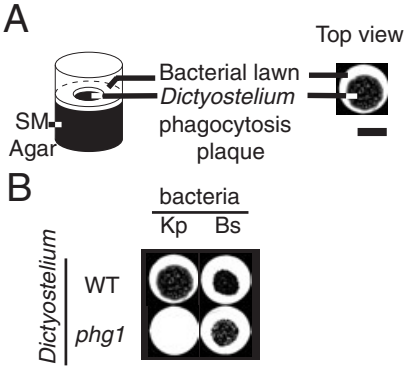

Fig. 1. Susceptibility of *phg1 Dictyostelium* mutant to *Klebsiella*.
A. The ability of a *Dictyostelium* strain to grow on a bacterial lawn was assessed by plating the indicated bacteria and 100 *Dictyostelium* cells on SM-Agar. Scale bar: 1 cm. A phagocytosis plaque was observed after 5 days.
B. Knockout *phg1* mutant cells exhibit a specific inability to grow on *K. pneumoniae* (Kp), but no growth defect on a lawn of *B. subtilis* (Bs).

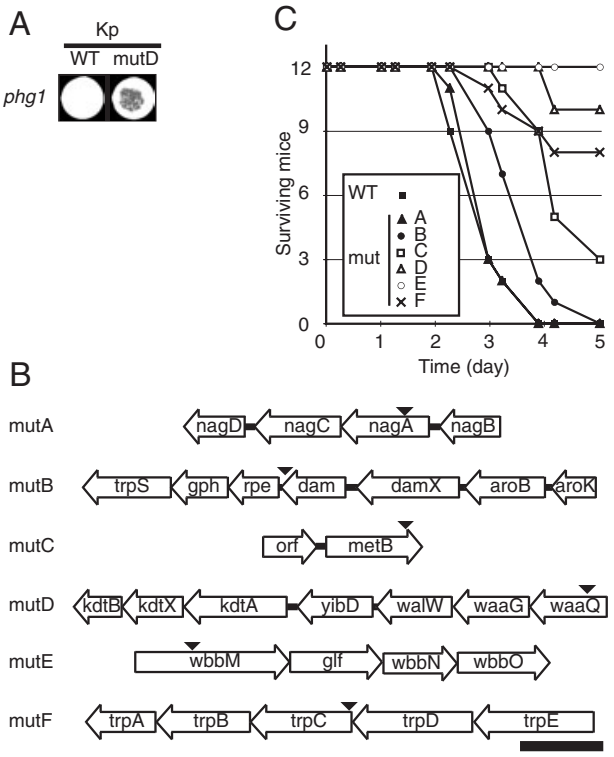

Fig. 2. *Klebsiella pneumoniae* virulence genes account for *phg1* susceptibility.
A. *K. pneumoniae* (Kp) mutants permissive for the growth of *phg1* mutant cells were isolated. Growth of *phg1 Dictyostelium* on *K. pneumoniae* mutant D is shown.
B. For each avirulent mutant the complete structure of the operon is indicated, as well as the transposon insertion site (arrowhead). Bar: 1 kb.
C. A lethal mouse pneumonia infection model was used to assess the virulence of each *Klebsiella* mutant strain, after transposition into a highly virulent strain (Kp52145). Survival of mice was plotted as a function of time following intranasal infection. Wild-type and mutA *Klebsiella* strains killed mice within 4 days, but death was delayed or abolished in other mutants.

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.

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 several of these operons have previously been implicated in the virulence of various bacteria in mammalian hosts, in particular WaaQ (Regue *et al*., 2001), WbbM (Shankar-Sinha *et al*., 2004), TrpD (Mei *et al*., 1997; Smith *et al*., 2001) and the DNA adenine methylase (Low *et al*., 2001).

In order to directly test the effect of these mutations on *K. pneumoniae* virulence in a mammalian host, each mutation was transposed in the pathogenic *Klebsiella* Kp52145 strain, and the virulence of the resulting strains assessed in a mouse pneumonia model (Nassif *et al*., 1989). In this system, bacterial clearance in the lungs has been shown to depend largely on alveolar macrophages, suggesting that resistance to phagocytes is a crucial component in the development of *Klebsiella* infections in the lungs (Cheung *et al*., 2000). Mice infected with wild-type Kp52145 *Klebsiella* became severely ill, and died within 3–4 days (Fig. 2C). Survival curves showed that five out of the six mutants tested displayed a decreased virulence, ranging from a mild attenuation (mutB) to a complete loss of virulence (mutE). Thus *K. pneumoniae* use largely similar sets of virulence genes to interact with *Dictyostelium* and with mice.

Klebsiella pneumoniae *bacteria are resistant to killing by* phg1 Dictyostelium

The *Dictyostelium phg1* mutant was initially shown to be defective for the phagocytosis of hydrophilic latex beads, due to a specific adhesion defect (Cornillon *et al.,* 2000). A phagocytosis defect could in principle account for *phg1* inability to grow upon *K. pneumoniae*. However this hypothesis was refuted by measuring the ability of *phg1* mutants to phagocytose live *Klebsiella* expressing green fluorescent protein (GFP). Indeed wild-type and *phg1* cells internalized bacteria at the same rate over the first 30 min (Fig. 3A). In wild-type cells a rapid decrease of the internal fluorescence followed this initial accumulation phase, reflecting the degradation of ingested bacteria. In *phg1* mutant cells the internal fluorescence increased continuously and reached a plateau (Fig. 3A), suggesting that bacteria survived intracellularly in *phg1* mutant cells. Indeed a huge number of fluorescent bacteria were seen in *phg1* endosomes (Ravanel *et al.,* 2001), while fluorescent bacteria were rarely observed in wild-type cells (Fig. 3B).

To precisely investigate the fate of ingested bacteria, 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 *Klebsiella* 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.

Kil1 is necessary for intracellular killing of Klebsiella *by* Dictyostelium

The production of superoxide by NADPH oxidase has been implicated in bacterial killing by mammalian phagocytic cells and its absence in humans causes chronic granulomatous disease (Nathan and Shiloh, 2000; Fang, 2004). In vegetative *Dictyostelium*, only one NADPH oxidase (NoxA) is expressed, while the two others (NoxB, NoxC) are not (Lardy *et al*., 2005). *Dictyostelium* strains harbouring a targeted disruption of *NOXA* (*noxA*) grew normally on *K. pneumoniae* or *B. subtilis* and retained the ability to efficiently kill both bacteria (Fig. 3F; P. Cosson, unpubl. data). Thus, in *Dictyostelium*, the production of superoxide by NADPH oxidase is not essential to kill these bacteria. Other cellular factors such as lysosomal enzymes may play crucial roles in bacterial killing, as proposed recently for neutrophils (Reeves *et al*., 2002).

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,

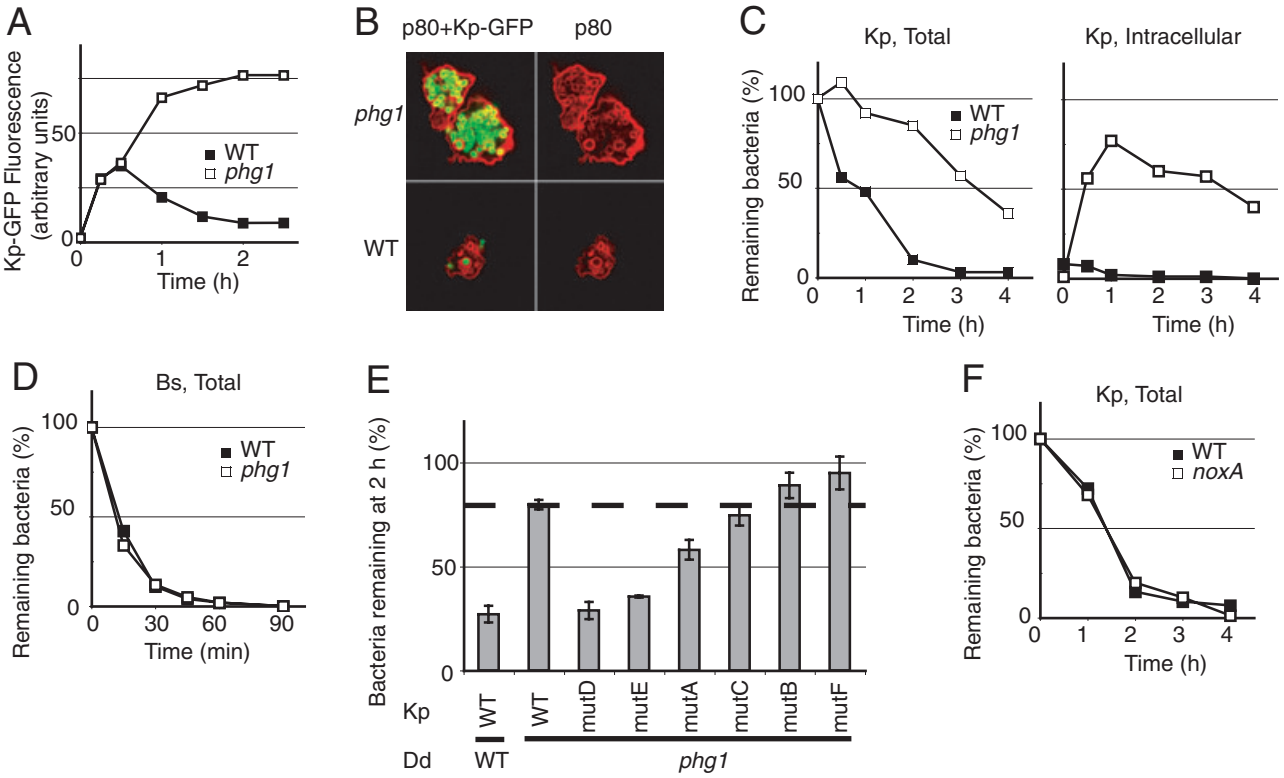

Fig. 3. The *phg1* mutant is specifically defective for intracellular killing of *Klebsiella*.
A. *Dictyostelium* cells and *K. pneumoniae* expressing GFP (Kp-GFP) were mixed, and cell-associated fluorescence levels measured by FACS at the indicated times.
B. Cells incubated for 90 min with Kp-GFP bacteria were fixed, permeabilized, labelled with an antibody to p80 (red), a marker of the cell surface and of endocytic compartments (Ravanel *et al*., 2001), before observation by confocal microscopy. Many bacteria accumulated in enlarged *phg1* mutant cells compared with wild-type (WT) cells.
C. *Dictyostelium* cells (WT or *phg1*) and *K. pneumoniae* bacteria were mixed. At the indicated times, the total number of surviving bacteria was determined (Total, left) as well as the number of viable bacteria associated to *Dictyostelium* cells (Intracellular, right).
D. *Dictyostelium* cells (WT or *phg1*) and *B. subtilis* bacteria were mixed, and the total number of surviving bacteria was determined at the indicated times.
E. *Dictyostelium* cells (WT or *phg1*) and *K. pneumoniae* bacteria (WT or mutant) were mixed, and the total number of surviving bacteria was determined after 2 h. Each bar indicates the average and SEM of at least four independent experiments. Dd, *Dictyostelium discoideum*.
F. *Dictyostelium* cells (WT or *noxA*) and *K. pneumoniae* bacteria were mixed, and the total number of surviving bacteria was determined at the indicated times.

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

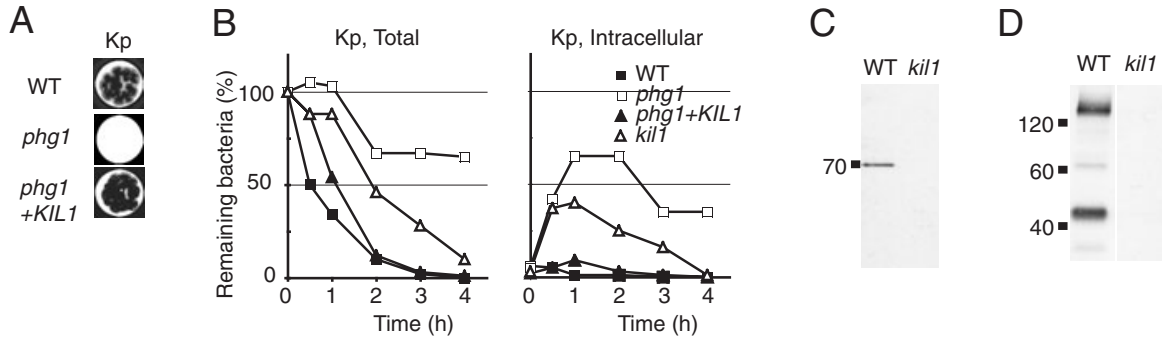

Fig. 4. Kil1 is involved in intracellular killing of *K. pneumoniae*.
A. Kil1 overexpression restores the ability of *phg1* mutant cells to grow on a lawn of *K. pneumoniae*.
B. As described in Fig. 3C, *Dictyostelium* cells (WT, *phg1*, *phg1*+*KIL1* or *kil1*) and *K. pneumoniae* bacteria were mixed and the number of surviving bacteria was determined at the indicated times.
C. To assess the ability of Kil1 to bind PAP, a competitive inhibitor of sulphotransferases, *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 *Dictyostelium* sulphated proteins. Molecular weights (kDa) are indicated. Kil1 corresponds exactly to the predicted coding sequence DDB0189417 of the *Dictyostelium* genome (http://dictybase.org).

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

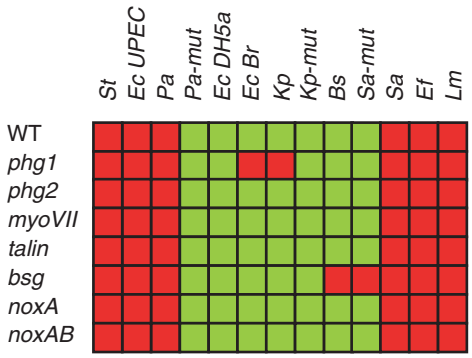

Fig. 5. A host–pathogen two-dimensional virulence array. The ability of *Dictyostelium* mutants to grow on different bacterial strains was determined as described in Fig. 1. Growth of *Dictyostelium* is indicated by green, no growth by red. *St*, *S. typhimurium*; *Ec* UPEC, uropathogenic *E. coli*; *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).

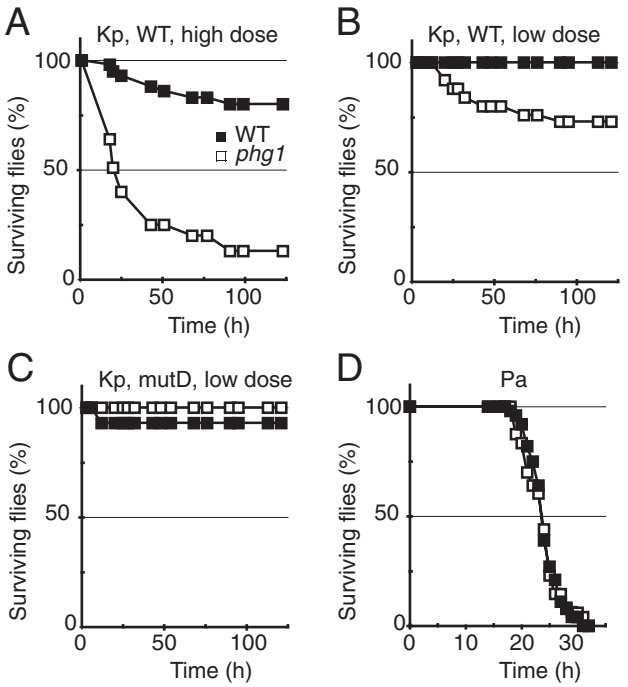

Fig. 6. *Drosophila phg1* mutants exhibit an increased susceptibility to *K. pneumoniae* infections. Male *Drosophila* flies, either wild type (WT) or mutant (*phg1*), were infected with a high dose of WT *K. pneumoniae* (A), a low dose of WT *K. pneumoniae* (B), a low dose of mutD *K. pneumoniae* (C), or with WT *P. aeruginosa* (D).

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 plasmatocytes 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

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 DH5α (Invitrogen), uropathogenic N2173-96 (Centre national des bactéries entéropathogènes, 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 *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, 10^4 bacteria from an overnight culture were mixed with 10^6 *Dictyostelium* cells in 1 ml of phosphate buffer (2 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 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, 10:1) 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 *phg1* 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 carrying the *REP* gene from pREP (Slade *et al*., 1990) into the DIV1 plasmid digested with SacI/BamHI. Then the cDNA library (Robinson and Spudich, 2000) was transformed by electroporation into the *phg1a* + *REP* strain and selection performed in HL5 medium containing 10 μg ml^{-1} geneticin. This yielded approximately 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*. Blasticidin-resistant transformants were screened for *KIL1* deletion by immunodetection using antibodies to Kil1 protein.

Characterization of Kil1 sulphotransferase activity

Cells (150×10^6 cells) were grown in HL5 medium (3×10^6 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 phenylmethylsulphonyl 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, 2×10^5 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 mannose 6-sulphate-containing epitope.

Mutagenesis of Klebsiella

Klebsiella pneumoniae transposon insertion mutants were obtained by electroporation of the pNKBOR plasposon carrying the mini-Tn*10* 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 (5×10^7 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 10^6 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 [*l(2)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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