Bacterial Discrimination by Dictyostelid Amoebae Reveals the Complexity of Ancient Interspecies Interactions

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Summary

Background: Amoebae and bacteria interact within predator-prey and host-pathogen relationships, but the general response of amoeba to bacteria is not well understood. The amoeba Dictyostelium discoideum feeds on, and is colonized by, diverse bacterial species, including Gram-positive [Gram(+)] and Gram-negative [Gram(−)] bacteria, two major groups of bacteria that differ in structure and macromolecular composition.

Results: Transcriptional profiling of D. discoideum revealed sets of genes whose expression is enriched in amoebae interacting with different species of bacteria, including sets that appear specific to amoebae interacting with Gram(+) or with Gram(−) bacteria. In a genetic screen utilizing the growth of mutant amoebae on a variety of bacteria as a phenotypic readout, we identified amoebal genes that are only required for growth on Gram(+) bacteria, including one that encodes the cell-surface protein gp130, as well as several genes that are only required for growth on Gram(−) bacteria, including one that encodes a putative lysozyme, AlyL. These genes are required for parts of the transcriptional response of wild-type amoebae, and this allowed their classification into potential response pathways.

Conclusions: We have defined genes that are critical for amoebal survival during feeding on Gram(+), or Gram(−), bacteria that we propose form part of a regulatory network that allows D. discoideum to elicit specific cellular responses to different species of bacteria in order to optimize survival.

Introduction

The social amoeba D. discoideum inhabits the forest soil and feeds on diverse species of bacteria [1, 2]. As a model eukaryote and proficient phagocyte, D. discoideum has proven to be useful for studying aspects of host-pathogen interactions [3–5] and has been used to identify and study bacterial virulence factors [5–8]. It has also been suggested that amoebae serve as environmental reservoir for certain human pathogens [9]. Recent studies have focused on specific amoeba-bacterium interactions, but Dictyostelium amoebae reside in soil environments that are inhabited by thousands of bacterial species [10]. It should be informative to investigate how the amoebae cope with such diversity and to determine how they elaborate physiological responses to different bacteria for feeding and defense. A detailed understanding of the amoebal response should enrich our understanding of the interactions between amoebae and bacteria and may reveal novel antibacterial strategies in eukaryotes.

Antibacterial responses in plants and animals have a number of similarities, especially in the recognition of microbial-associated molecular patterns, or MAMPs [11]. For example, the TIR (Toll/interleukin-1 receptor) domain is often present in MAMP receptors in plants and animals that are involved in microbial recognition. The globular TIR protein domain is an adaptor that signals through protein-protein interactions and is thought to play a role in the specificity of antimicrobial responses [12]. The recent discovery of the function of the TIR domain protein TirA in D. discoideum raises the general question of whether amoebae discriminate between different bacteria as well [13, 14]. Transcriptional profiling of D. discoideum exposed to a variety of bacterial species has revealed the differential accumulation of specific sets of gene transcripts, suggesting that the amoebae discriminate between different bacteria [15–17].

One way in which D. discoideum amoebae might handle the diversity of bacterial species in the soil would be to activate specific response pathways for different classes of bacteria. The largest natural grouping of bacteria, the Gram-positive [Gram(+)], and Gram-negative [Gram(−)] species, is based on physiological differences that are of particular relevance to bacterial discrimination systems. The cytoplasmic membrane of Gram(+) bacteria is surrounded by a thick outer cell wall of peptidoglycan strands that are crosslinked by short peptides and containing teichoic acid, which is absent in the Gram(−) bacteria [18, 19]. Gram(−) bacteria have a thin peptidoglycan layer that is surrounded by an outer membrane containing lipoproteins and lipopolysaccharide (LPS) in the outer leaflet, which are absent in the Gram(+) bacteria. There is also some genetic evidence that D. discoideum amoebae discriminate between Gram(+) and Gram(−) bacteria. Mutations in several uncharacterized D. discoideum genes preclude growth on Bacillus subtilis but allow normal growth on K. pneumoniae [20, 21], whereas mutations in the phg1a gene impair growth on K. pneumoniae but not on B. subtilis [22].

We have undertaken a general approach to investigate the response of amoebae to bacteria by exploring the genetic control of D. discoideum growth on different species of bacteria. Our results suggest that D. discoideum amoebae respond in a highly specific manner to different species of bacteria and also deploy general response systems for dealing with Gram(+) bacteria that are distinct from those deployed to deal with Gram(−) bacteria.
Results

Distinct *D. discoideum* Transcriptional Responses to Different Bacteria

Transcriptional profiling is a reliable method for detecting differential physiological responses in *D. discoideum* [23]. To test whether these amoebae can respond differentially to different bacteria, we grew them on two species of Gram(−) bacteria, *K. pneumoniae* and *Pseudomonas aeruginosa*, and two species of Gram(+) bacteria, *Staphylococcus aureus* and *B. subtilis*. We then measured the steady-state levels of messenger RNAs (mRNAs) within the amoebae by RNA sequencing (RNA-seq) and obtained transcriptional profiles that include data on ~10,000 genes (Table S1 available online). We chose to examine the physiological status of amoebae growing exponentially on bacteria to avoid confounding dynamic changes in gene expression resulting from the initial contact with bacteria so that we could rely on the transcriptional profiles as robust indicators of diverse gene regulatory events. Hierarchical clustering of the profiles of growth on the two Gram(+) bacterial species tested are most similar to each other compared with the profiles on the two Gram(−) bacteria (Figure 1A). These results support the hypothesis that the amoebae have differential responses to the two types of bacteria, although transcriptomes for amoebae growing on several more Gram(+) and Gram(−) bacterial species would have to be compared in order to determine whether this is a consistent trend.

Differences in the complete profiles were due to specific transcriptional changes observed during growth on individual species of bacteria. For each growth condition, we were able to classify a subset of genes whose mRNA levels were elevated compared to growth on the other three species tested, suggesting that each of the bacteria elicit a specific transcriptional response (Figure 1B and Table S1). The cohort of 780 genes that were differentially expressed during *D. discoideum* growth on *K. pneumoniae* was particularly striking. Although many of these genes likely encode enzymes needed to utilize *K. pneumoniae* as a food source and represent metabolic adaptation, some may encode specific signaling proteins involved in the detection of *K. pneumoniae* or antimicrobial proteins. Indeed, three genes within this cohort, *aplM*, *aplN*, and *aplQ*, encode amoebapore proteins that are known to destabilize bacterial cytoplasmic membranes (Table S1) [24]. Additional transcriptional responses observed in *D. discoideum* growing on *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* are described in detail in Table S1.

We also explored whether we could define transcriptional responses in *D. discoideum* that were specific to Gram(−) or Gram(+) bacteria. We identified 50 genes that were preferentially expressed on both Gram(−) species tested and 68
genes that were preferentially expressed on both Gram(+) species (Figure 1B and Table S1). We used Gene Ontology (GO) annotation of the genes that express the Gram( +) and Gram(−)-enriched transcripts to provide insight into the physiology of the amoebae growing on different groups of bacteria (Figure 1D) [25]. The Gram(+) -enriched gene transcripts were most commonly annotated under the “metabolism” descriptor and included a number of putative hydrolyses that are likely to be involved in peptidoglycan cell wall degradation—the amoeba lysozymes (alyA, alyB, alyC, and alyD), glycosidases and endopeptidases (Table S1). On the other hand, the Gram(−)-enriched expression of alyL, a gene encoding an amoeba lysozyme-like protein AlyL [26], suggests that it may be involved in the degradation of the Gram(−) cell walls (see below). The Gram(+) -enriched set also includes the cysteine protease gene, cprF, and a polykette synthase gene, stlA (Table S1) [27, 28]. Genes of unknown function represent a substantial proportion of the Gram(+) and the Gram(−) profiles and include sets of genes predicted to encode proteins with signal peptides that may represent novel secreted antimicrobial peptides. These analyses support the idea that D. discoideum responds differently to Gram(+) and Gram(−) bacteria.

To begin to confirm the regulatory responses underlying the transcriptional profiles, we constructed expression vectors consisting of the green fluorescent protein (GFP) coding sequence placed under the control of species-specific or group-specific gene promoters and introduced them into wild-type D. discoideum cells. We found that D. discoideum cells expressing GFP under the control of the hydR1 or hydR2 promoters fluoresced when exposed to Gram(+) bacteria, but not on any of the Gram(−) bacteria we have tested (Figures 1E and S1A). Amoebae expressing GFP under the control of the ctnC promoter fluoresced when feeding on bacteria of the Enterobacteriaceae family, such as K. pneumoniae, but not on P. aeruginosa or any Gram(+) bacterium that we tested (Figures 1E and S1B). The actin15 gene is not differentially expressed during growth on bacteria, and, accordingly, the act15/GFP construct was expressed on all bacteria tested (Figure 1E). These results indicate that the increased abundance of mRNAs quantified by RNA-seq are the result of differential promoter regulation, at least for these promoters, suggesting the involvement of a signal transduction cascade in their induction.

**Genetic Evidence for Discrimination between Gram(+) and Gram(−) Bacteria**

The existence of mutations that restrict amoebal growth on one type of bacteria but not on the other type would provide independent support in favor of bacterial discrimination. We isolated such mutants by screening the growth of 10,000 mutated D. discoideum strains on Gram(−) K. pneumoniae bacteria and on Gram(+) B. subtilis bacteria (Table S2). D. discoideum mutants that grew well on one bacterial species but poorly on the other bacterial species were the most interesting to us because we expected they might reveal key elements of bacterial discrimination (Table 1). We first examined the growth of these two classes of mutants on additional species of bacteria to test the generality of their phenotype. Mutations in nagB1, gpi, or swp1 exhibited growth defects on each of the five Gram(+) bacterial species we tested but not on Gram(−) bacteria (Figures 2A and 2B). Dictyostelium strains carrying mutations in cikB, spc3, alyL, or the insertion at u1334 grew poorly on the seven Gram(−) bacterial species but grew well on the five Gram(+) bacterial species tested (Figures 2 and 2A). We also examined a previously characterized mutant of gp130 that we hypothesized might be a receptor for Gram(+) bacteria [30, 31]. Interestingly, the gp130-null mutant exhibited a growth defect on lawns of Gram(+) but not on Gram(−) bacteria (Figures 2A and 2B). Thus, we found several genes that are required for growth on every Gram(+) bacterial species we tested but are dispensable for growth on every Gram(−) species we tested, and we found several genes with the inverse phenotype. These findings suggest the existence of distinct pathways that allow D. discoideum to grow on each type of bacteria.

For an amoeba any bacterium can be food, a dangerous pathogen, or both. To begin to test whether the genes we identified are required for feeding on bacteria or for defense against them, we measured the ability of the mutant strains to grow on dead bacteria. We found that each of the Gram(−)-growth-defective mutants grew well on each of the seven Gram(−) species we tested (Figures 2 and S2A). Dictyostelium strains carrying mutations in cikB, spc3, alyL, or the insertion at u1334 grew poorly on the seven Gram(−) bacterial species but grew well on the five Gram(+) bacterial species tested (Figures 2 and 2A). We also examined a previously characterized mutant of gp130 that we hypothesized might be a receptor for Gram(+) bacteria [30, 31]. Interestingly, the gp130-null mutant exhibited a growth defect on lawns of Gram(+) but not on Gram(−) bacteria (Figures 2A and 2B). Thus, we found several genes that are required for growth on every Gram(+) bacterial species we tested but are dispensable for growth on every Gram(−) species we tested, and we found several genes with the inverse phenotype. These findings suggest the existence of distinct pathways that allow D. discoideum to grow on each type of bacteria.

### Table 1. Mutant Strains Used in This Study

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Relevant Gene</th>
<th>Growth Phenotype</th>
<th>Annotation*</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>AX4</td>
<td>-</td>
<td>wild-type</td>
<td>parental strain</td>
<td>[29]</td>
</tr>
<tr>
<td>2F5</td>
<td>gp130</td>
<td>Gram(+) defective</td>
<td>gpi30 adhesion protein</td>
<td>[30]</td>
</tr>
<tr>
<td>AK1321</td>
<td>swp1</td>
<td>Gram(+) defective</td>
<td>oligosaccharide transferase subunit</td>
<td>this work</td>
</tr>
<tr>
<td>AK1333</td>
<td>gpi</td>
<td>Gram(+) defective</td>
<td>glucose phosphate isomerase</td>
<td>this work</td>
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<tr>
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<td>Gram(+) defective</td>
<td>glucose phosphate isomerase</td>
<td>this work</td>
</tr>
<tr>
<td>AK1372</td>
<td>nagB1</td>
<td>Gram(+) defective</td>
<td>glucosamine-6-phosphate deaminase</td>
<td>this work</td>
</tr>
<tr>
<td>TirA-KO</td>
<td>tirA</td>
<td>Gram(+) defective</td>
<td>TIR-domain-containing protein</td>
<td>[13]</td>
</tr>
<tr>
<td>AK1338</td>
<td>cikB</td>
<td>Gram(+) defective</td>
<td>CDC7-related protein</td>
<td>this work</td>
</tr>
<tr>
<td>AK1346</td>
<td>spc3</td>
<td>Gram(+) defective</td>
<td>signal peptidase</td>
<td>this work</td>
</tr>
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<td>alyL</td>
<td>Gram(+) defective</td>
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<td>Gram(+) defective</td>
<td>insertion between two genes, DDB_G0295477 and DDB_G0271574</td>
<td>this work</td>
</tr>
</tbody>
</table>

All strains are available from the Dictyostelium Stock center (http://www.dictybase.org/).

*Adapted from http://www.dictybase.org/ [32].
**D. discoideum**

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Gram(+) bacteria or 1% live Gram(−) bacteria (Figure 2B). This indicates that dead Gram(+) bacteria can be utilized as a food source and suggests that the amoebae must be exposed to live bacteria in order to feed on the dead Gram(+) bacteria. Mixing of live Gram(+) bacteria did not induce feeding on dead Gram(+) bacteria by the Gram(+) growth-defective mutants. However, we were able to induce the Gram(+) growth-defective mutants to feed on live, or heat-killed, Gram(+) bacteria by adding 50%, or even as little as 1%, live *K. pneumoniae* (Figure 2B). The use of Gram(−) bacteria to stimulate these mutants to feed on Gram(+) bacteria indicates that the Gram(+) growth defective mutants are not nutritional auxotrophs for feeding on Gram(+) bacteria and suggests that the recognition of live bacteria by the amoebae is required for efficient feeding.

**Transcriptional Phenotyping of the Gram(+) and Gram(−) Growth-Defective Mutants**

To explore potential functions of the genes identified in growth-defective mutants, we examined expression profiles of a limited set of differentially expressed genes. We isolated total RNA from mutant amoebae and carried out reverse transcription followed by quantitative RT-PCR (qRT-PCR) analyses with primers against selected Gram(+) enriched and Gram(−) enriched transcripts, as well as two *K. pneumoniae* specific transcripts. The transcriptional profiles of the Gram(+) growth-defective mutants growing on Gram(−) bacteria were similar to the wild-type *D. discoideum* profile (Figure 3, clade 1, and Figure S3). However, when exposed to Gram(+) bacteria, the transcriptional profiles of these mutants were similar to one another and different from the wild-type (Figure 3, clade 4, and Figure S3). Within this group, the nagB1+ and gpi− mutants exhibited wild-type levels of gp130 and DDB_G0267848 transcripts and lower levels of alyD, DDB_G0293366, DDB_G0274181, and DDB_G0270922. The swp1+ mutant, on the other hand, expressed near wild-type levels of the putative peptidoglycan-degrading enzyme genes but had significantly lower expression of gp130 and DDB_G0267848. Hierarchical clustering (Figures 3 and S4) and multidimensional scaling (MDS, Figure S5) indicate that the nagB1+ and gpi− mutant transcriptional profiles are most similar to each other and distinct from the swp1+ mutant profile, suggesting that nagB1 and gpi are components of one pathway that is required for growth on Gram(+) bacteria.

Transcriptional profiles of the Gram(−) growth-defective mutants exposed to Gram(+) bacteria showed lower levels of most of the Gram(−)-enriched genes that we tested (Figure 3, clade 2), whereas their profiles during growth on Gram(+) bacteria were similar to those of the wild-type (Figure 3, clades 3 and 5, and Figures S3–S5). TirA is a known component of the amoebal response to Gram(+) bacteria [13, 14]. We included the tirA mutant in our analysis and found that its transcriptional profile on Gram(−) bacteria was similar to that of the Gram(−) growth-defective mutants (Figure 3, clade 2, and Figures S3–S5). Two Gram(−)-enriched genes showed higher transcript levels or little change: cprF, a cysteine proteinase gene, and uduB, a gene of unknown function. The spc3 and DDB_G0293366 genes but had significantly lower expression of gp130 and DDB_G0267848. Hierarchical clustering (Figures 3 and S4) and multidimensional scaling (MDS, Figure S5) indicate that the nagB1+ and gpi− mutant transcriptional profiles are most similar to each other and distinct from the swp1+ mutant profile, suggesting that nagB1 and gpi are components of one pathway that is required for growth on Gram(+) bacteria.

**Figure 2**. *D. discoideum* Mutants with Growth Defects on Gram(+) or Gram(−) Bacteria

(A) Each spot represents a coculture of about 500 *D. discoideum* cells and a thick bacterial culture spotted on buffered agar, imaged after 4 days of incubation. The image (top view) was assembled from 12 images of 12 experiments conducted under similar conditions. All *D. discoideum* strains were tested on the same day with the same culture of a given bacterium. Rows represent different Gram(−) or Gram(+) bacterial species as indicated on the left, and columns represent different wild-type or mutant amoebae as indicated on the top. The left-most column contains no amoebae. Wild-type strains were tested on the same day with the same culture of a given bacterium.

(B) *D. discoideum* growth on heat-killed *S. aureus*. Rows represent wild-type or nagB1+ mutant amoeba as indicated on the left, and columns represent different bacterial species or a mixture of bacterial species (live or heat-killed) in different mass ratios as indicated (HK, heat killed; S.a., *S. aureus*; K.p., *K. pneumoniae*). Each spot is a coculture of about 500 Gram(+) bacteria or 1% live Gram(−) bacteria (Figure 2B). This indicates that dead Gram(+) bacteria can be utilized as a food source and suggests that the amoebae must be exposed to live bacteria in order to feed on the dead Gram(+) bacteria. Mixing of live Gram(+) bacteria did not induce feeding on dead Gram(+) bacteria by the Gram(+) growth-defective mutants. However, we were able to induce the Gram(+) growth-defective mutants to feed on live, or heat-killed, Gram(+) bacteria by adding 50%, or even as little as 1%, live *K. pneumoniae* (Figure 2B). The use of Gram(−) bacteria to stimulate these mutants to feed on Gram(+) bacteria indicates that the Gram(+) growth defective mutants are not nutritional auxotrophs for feeding on Gram(+) bacteria and suggests that the recognition of live bacteria by the amoebae is required for efficient feeding.

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D. discoideum cells and a thick bacterial culture spotted on buffered agar and imaged after 4 days of incubation. The bacteria appear as tan areas within the spots. Intermediate levels of feeding are indicated by partial clearing of the tan bacterial lawn. Similar results were obtained for the other Gram(+) growth-defective mutants gp130 and swp1 (unpublished data). See also Figure S2 and Tables S2 and S3.
alyL mutant profiles were the most similar (Figure 3, clade 2). Spc3 is a signal peptidase subunit and AlyL has a signal peptide and likely resides in the lysosome or is otherwise secreted. The absence of Spc3 may alter signal peptidase function in a way that affects the biogenesis of AlyL, resulting in the similar transcriptional profiles of these two mutants.

To explore the pathway(s) by which the Gram(–)-enriched genes are induced, we examined the transcriptional profiles of the mutants after exposure to heat-killed K. pneumoniae. As described in the previous section, growth of the Gram(–)-defective mutants on live K. pneumoniae is defective, but their growth rates on heat-killed K. pneumoniae are comparable to that of the wild-type. Hierarchical clustering and MDS analyses indicate that the transcriptional profiles of the Gram(–)-growth-defective mutants exposed to live K. pneumoniae are similar to their transcriptional profiles on dead K. pneumoniae and are also similar to the wild-type profile on dead K. pneumoniae (Figure 3, clade 2, and Figure S3–S5). Interestingly, the wild-type amoebae did not induce the expression of six out of ten Gram(–)-enriched gene transcripts when exposed to dead K. pneumoniae and exhibited higher levels of the cprF and uduB transcript, similar to the expression profile of Gram(–)-defective mutants on live K. pneumoniae (Figure 3, clade 2, and Figures S3–S5). These results suggest that the physiological changes that are specific for growth on live Gram(–) bacteria are not required for feeding on dead Gram(–) bacteria. They also suggest that the predicted lysozyme, AlyL, is not needed to digest Gram(–) bacteria, but instead is needed to kill them, as lysozymes are known to do in other eukaryotes [33]. These data support the idea that the genes identified in our genetic screen are key elements of a bacterial discrimination network in D. discoideum that result in specific responses to either Gram(+) or Gram(–) bacteria.

The Role of N-Linked Glycosylation in the Response to Gram(+) Bacteria

The transcriptional responses of D. discoideum mutants to Gram(+) bacteria suggest a pathway requiring swp1 and a...
distinct pathway involving **gpi** and **nagB1**. The **swp1** gene encodes a subunit of the oligosaccharyl transferase protein complex that catalyzes asparagine (N-linked) glycosylation of proteins [34]. We hypothesized that the **swp1** gene is important in the biogenesis of glycoproteins that are required for growth on Gram(+) bacteria, such as Gp130 [31]. To test this possibility, we treated wild-type *D. discoideum* with tunicamycin, which blocks N-linked glycosylation in eukaryotes by inhibiting the charging of dolichol phosphate with N-acetyl-glucosamine [35]. Wild-type amoebae treated with tunicamycin displayed growth defects on Gram(+) bacteria, but not on the Gram(−) bacteria *K. pneumoniae* (Figure 4A), phenocopying the **swp1** mutant. Tunicamycin-treated wild-type cells were also similar to the **swp1** mutant in their transcriptional profile (Figure 3, clades 3 and 4). Closer examination revealed that they expressed the Gram(+) enriched hydrolase genes normally but had similar low levels of **gp130** and **DDB_G0267848** transcripts (Figure 4B). This finding suggests that N-linked glycosylation is required for growth on Gram(+) bacteria but is dispensable for growth on Gram(−) bacteria.

**The Role of a Glucose Metabolite in the Response to Gram(+) Bacteria**

The **gpi** gene encodes phosphoglucoisomerase, which interconverts glucose-6-phosphate and fructose-6-phosphate, whereas the **nagB1** gene product is predicted to catalyze the conversion of glucosamine-6-phosphate into pentose, through glucose-6-phosphate and the pentose phosphate shunt. *D. discoideum* may rely more on this pathway when growing on Gram(+) bacteria compared to when they are growing on Gram(−) bacteria due to the abundance of hexose monomers.
produced during the breakdown of thick Gram(+) bacterial cell walls. Supporting this notion, we observed increased expression of nagB1 during amoebal growth on Gram(+) bacteria compared to Gram(−) bacteria (Figure 1 and Table S1). Thus, we hypothesize that glucose-6-phosphate, or a metabolite of glucose-6-phosphate, signals the presence of Gram(+) bacteria (Figure 5A). If true, wild-type D. discoideum growing on Gram(+) bacteria would have higher glucose-6-phosphate levels than cells growing on Gram(−) bacteria, and the nagB1− and gpi− mutants would have lower glucose-6-phosphate levels. To test this, we grew wild-type (AX4) and nagB1 mutant amoebae on K. pneumoniae, S. aureus, and B. subtilis, and measured glucose-6-phosphate levels and the expression of Gram(+) enriched genes. Consistent with our hypothesis, the glucose-6-phosphate levels in the wild-type amoebae did correlate directly with growth on Gram(+) bacteria and with the mRNA levels of the Gram(+) enriched hydrolase genes, whereas glucose-6-phosphate levels and Gram(+) gene expression were significantly reduced in the nagB1 mutant (e.g., Figures 5B and 5C).

To test whether glucose-6-phosphate (or a metabolite) might serve as an internal cue we added glucose to the growth media to elevate glucose-6-phosphate levels within the amoebae and tested growth and gene expression. We found that glucose partially rescued the growth of the nagB1− and gpi− mutants on Gram(+) bacteria but had no effect on growth of the wild-type (Figures 4C and S6). Glucose treatment did not rescue the growth of the swp1− mutant (Figures 4C and S6), indicating that glucose rescue is specific to the gpi/nagB1 pathway. The addition of 2-deoxy-glucose, which cannot be processed by the glycolytic enzymes [36], did not rescue the growth of the nagB1− or gpi− mutants on Gram(+) bacteria (Figure S6). The transcriptional profiles of the nagB1− and gpi− mutants growing on B. subtilis with glucose resembled the wild-type profile (Figure 3, clade 5, and Figures 4D and S3) more than that of the untreated mutants (Figure 3, clade 4). More specifically, the treatment increased the abundance of the hydrolase transcripts to near wild-type levels, and more modest increases in expression were observed in the mutant mixed with S. aureus (Figure 3, clade 4, and Figures 4D and S3). These data support a model whereby a glucose metabolite signals the presence of Gram(+) bacteria.

Discussion

Amoebae feed on a variety of bacteria and are subject to numerous pathogenic threats, so a regulated response to varying microbiota would be critical for amoebal defense and optimal feeding [3, 37]. It is known that D. discoideum amoeba induce characteristic physiologic changes when engulfing and degrading food bacteria, particularly when the bacteria have pathogenic potential [3, 37]. The transcriptional profiles that we report here expand earlier studies demonstrating differential transcriptional responses to bacteria [3, 15–17], reveal the specificity and extent of the physiologic differences in amoebae growing on different bacteria, and are suggestive of adaptive responses by the amoebae that are highly regulated.

Our overarching hypothesis is that a bacterial response network exists in D. discoideum that begins with detection of bacterial elicitors and ends with a differentiated amoebal response that is critical for survival. Included in our hypothesis is the notion that the amoebae discriminate between Gram(+) and Gram(−) bacteria. Our evidence for this is based on the
**D. discoideum** mutants unable to grow on live Gram(−) bacteria do not induce these genes but are able to grow on dead Gram(−) bacteria nonetheless, and dead bacteria do not induce the expression of these genes in wild-type cells. Furthermore, we found that live (and not dead) Gram(−) bacteria can induce amoebae to feed on dead Gram(+) bacteria. These findings reinforce the notion that the differential response we observe is at least in part an amoebal defense response to live bacteria and not simply a set of responses that optimize the digestion of bacteria.

Our work has provided new insights into the interaction of amoebae and bacteria by showing that amoebae use distinct pathways to discriminate between different types of bacteria and by identifying components of the regulatory network that allow amoebae to recognize specific groups of bacteria, including potential receptors and signaling molecules that directly facilitate recognition (Figure 6). Phagocytosis, killing by superoxide radicals, and enzymatic digestion of bacteria are well-known examples of universal eukaryotic defense mechanisms [3, 4, 37, 39], but there are likely others that have yet to be uncovered. The Amoebozoa are a monophyletic group of eukaryotes that arose in evolution soon after the divergence of the plants and animals, about a billion years ago [27, 40]. A closer examination of bacterial recognition by amoebae and characterization of the responses that recognition engenders should inform strategies for subverting bacterial pathogenesis and may also provide useful insight into the origin of innate immune function in plants and animals.

**Experimental Procedures**

**Growth of D. discoideum**

We used Dictyostelium discoideum laboratory strain AX4 [29] for all growth experiments. *D. discoideum* was recovered on *K. pneumoniae* bacterial lawn on Sussman’s Medium agar and was then grown axenically on H5S medium and maintained at mid-log phase (2.5 × 10⁵ cells/ml) [41] before subsequent experiments.

(0.2 cm in diameter) into the agar plate and removing the agar disk along with the surface contents. The plug was transferred to 0.33–1 ml of buffer (volume determined empirically to get a count of 20–200 amoebae in each 0.1 ml aliquot; the mixture was diluted further if cell count exceeded 200) in a 2 ml Eppendorf tube, vortexed, and shaken vigorously until the amoebae cells were dissociated completely from the agar and the bacteria, and cells were then counted in a hemocytometer.

For growth curves of *D. discoideum* on HL5 axenic medium, we inoculated 5 × 10⁴ cells/ml into a shaking culture of HL5 media and counted cells on a hemocytometer.

**RNA Extraction for RNA-Seq**

Wild-type or mutant *D. discoideum* cells (1–3 × 10⁷) were mixed with 2 ml thick bacterial culture as mentioned above (slow growing mutants were mixed at higher density) and spread evenly on a 10 cm Petri plate of buffered agar. After 14–16 hr, amoebae and bacteria were harvested and resuspended in ice-cold mSor buffer to wash away bacteria by low centrifugation (200 × g at 4 °C). Supernatant with bacteria was discarded. This procedure was repeated two to four times until no visible bacteria were detected in supernatant. Washed amoebae cells (2.5 × 10⁷) were immediately lysed in TRIZOL reagent, and total RNA was prepared according to the manufacturer’s protocol (Invitrogen by Life Technologies).

**cDNA Library Preparation, RNA-Seq Data, and Mapping**

To prepare complementary DNA (cDNA) libraries, we processed 20 μg total RNA through one round of poly-A selection, RNA fragmentation, and first-strand and second-strand cDNA synthesis, carried out according to Parikh et al. [42]. Two biological replicates were analyzed for each condition. We sequenced the cDNA libraries (read length = 35 bases) on a high-throughput Illumina Genome Analyzer II using the manufacturer’s recommended pipeline (versions 1.2 and 1.3). The resulting FASTQ files were mapped using the short-read alignment software bowtie (version 0.12.7, 64 bit) [43] allowing only for single hits (–m 1) and trimming unmapped reads up to 10 bp iteratively by 2 bp. The mapping procedure was similar to that explained in Parikh et al. [42], and the data are maintained on the PIPAx server (http://pipa.biolab.si). Raw abundance level of a transcript is defined as the sum of all the reads that uniquely map to that transcript. In order to compare transcript abundance between different bacterial growth conditions, we normalized raw abundance values to account for differences in total number of mapable reads obtained with each RNA-seq run and the differences in mapable gene length as described in Parikh et al. [42].

**Data Visualization**

We generated the heat maps in Figure 1 using the heatmap.2 function from the gplots package in R [44]. To allow comparisons between gene expression profiles with different abundances, we normalized them to have a mean of 0 and a standard deviation of 1. The resulting Z scores are used to color the heat map. To calculate similarity between transcriptomes of *D. discoideum* grown on different bacteria (Figure S1A), we performed hierarchical clustering (R function hclust) on the expression vectors from each growth condition consisting of all the genes and visualized the results as a dendrogram. The expression vectors consist of normalized mRNA abundance levels averaged between the two replicates. We used Spearman’s correlation (SC) to calculate the distance (D = 1 − SC) and complete linkage as the clustering criterion. Two objects (individual transcriptomes, or joints) are joined by means of a horizontal line if these objects are more similar to one another than any other object in the data. The vertical distance between objects is inversely proportional to the similarity between them. The horizontal distances in the dendrogram are meaningless. The pie charts were
generated via annotation categorization with GO annotations used as a reference framework (R function pie).

Differential Expression from RNA-Seq Data
baySeq was used to perform differential expression analysis and genes with false discovery rates (FDRs) of 0.2 or lesser were considered to be differentially expressed [45]. We calculated the fold change of a gene on a bacterial species as the log2 ratio of averaged normalized mRNA abundance on that bacterial species to the maximum of the averaged scaled mRNA abundance on the other bacterial species. Genes that were differentially expressed and upregulated in D. discoideum cells grown on one bacterial species were categorized as species-specific genes. Genes that were differentially expressed between D. discoideum cells grown on Gram(+) and those grown on Gram(-) bacteria were categorized as group-enriched genes. The genes that were present in both group-enriched and species-enriched categories were removed from the set of species-enriched genes.

Genetic Screen
To generate random D. discoideum mutants, we performed restriction enzyme-mediated integration (REMI) on AX4 cells [46]. The following day, each transformation was diluted into two 96-well plates and kept under bacterial selection. We monitored clonal growth of D. discoideum mutants in each well (no more than one colony formation per well for clonal growth). Clonally growing mutants were consolidated into 96-well plates and maintained at mid-log phase density (total HL5 volume in each well was 100 μl). Using a multi-channel pipette, we mixed 5 μl from D. discoideum mutant culture from each well with 120 μl overnight bacterial culture of K. pneumoniae (K.p.) or B. subtilis (B.s.) bacteria in nutrient media and spotted each mixture on 9 x 9 inch nutrient agar plate (made from 2% Bacto agar in nutrient media broth), and each row had 12 different D. discoideum mutants mixed with K.p. and next to it another row of the same 12 mutants mixed with B.s. Each spot contained 150-200 amoebae cells. Growth phenotype of each D. discoideum mutant was monitored over 5 days on K.p. or B.s. (Figure S7). Selected mutants were tested for their growth phenotype on other Gram(+) or Gram(-) bacterial species with spot assays on buffered and nutrient media agar, and a subset of mutants were assayed for growth rates on different bacteria.

Identification of Insertion Sites and Recreating Mutants
To identify plasmid insertion sites, we carried out plasmid rescue using Clal, BclI, KpnI, Ncol, NdeI, and BamHI restriction enzymes and sequencing of D. discoideum cells grown on one bacterial species to the maximum of the averaged scaled mRNA abundance (14–16 hr; see above for growth of amoebae on bacteria on buffered agar). Samples were centrifuged at 10,000 × g for 10 min at 4 °C. Supernatant was spun down in 10 kDa molecular weight cutoff filters to remove enzymes that utilize glucose-6-phosphate as a substrate. Flow through was processed for quantification of glucose-6-phosphate concentration with the Glucose-6-Phosphate Assay Kit according to manufacturer’s recommended protocol (BioVision, Milpitas, CA; catalog number K657-100). We measured optical density at 450 nm with an ASYS UVM 340 microplate reader (Hitech, Austria).

Repertoire Gene Plasmid Construction
We cloned each gene’s promoter (350–700 bp fragment immediately upstream of gene start codon) into pPT165 plasmid [48] using the Gateway cloning system (Invitrogen). This positioned the promoters immediately upstream of the GFP coding sequence, allowing for differential control of GFP expression in D. discoideum cells. The ctnC promoter fragment that we used was the 557 bp immediately upstream of the start codon, the DDB_G0293366 (hydr1) promoter was 364 bp, the DDB_G0274181 (hydr2) promoter was 700 bp, and the act15 promoter was 393 bp.

We transformed 10 μg plasmid into 5 × 10^6 wild-type AX4 cells using electroporation in H-50 buffer [49]. Transformed cells were maintained in H5L media supplemented with 20 μg/ml concentration of G418 (Geneticin). Transformed amoebae were mixed with different bacterial species and spotted on buffered agar; see above for the spot assay on buffered agar. One to two days later, fluorescent amoebae were visualized under a fluorescence microscope or under the Dark Reader spot lamp (Clare Chemical Research, SL95).

Accession Numbers
The RNA-seq data were reported to the NCBI Gene Expression Omnibus ( GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The accession number for the data reported here is GSE46386.

Supplemental Information
Supplemental Information includes seven figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.04.034.

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