Two-component regulatory systems are a common element regulating the response of bacteria to their environment (13). These systems involve signal transduction by phosphorelay from a sensor histidine kinase protein, which often spans the cytoplasmic membrane, to a cognate response regulator of transcription of several genes. Analysis of the recently published Pseudomonas aeruginosa PAO1 genome sequence identified 63 different genes whose products are homologous to previously identified sensors in P. aeruginosa and other bacteria, although for the majority of these sensors, the sensed stimulus, the regulator, and the responding genes remain unknown (13). One well-characterized example is PhoQ that, with its cognate regulator PhoP, senses the divalent cation content of the environment and responds in low-concentration Mg\(^{2+}\) regulator PhoP, senses the divalent cation content of the environment and responds in low-concentration Mg\(^{2+}\) medium by upregulating transcription of the oprH-phoP-phoQ and other operons and increasing resistance to polymyxin (8) and certain cationic antimicrobial peptides and aminoglycosides (7). Knockout mutants of the phoQ gene demonstrate attenuated virulence in a neutrophil mouse model.

We have been participating in a multidisciplinary Pathogenomics project (http://www.pathogenomics.bc.ca) that includes an investigation of the evolution of virulence factors. During this project we identified, and now report here, unusually high similarity between a Candida albicans virulence factor, NIK1, and two Streptomyces coelicolor genes identified from a genome project (2, 17, 11) (Fig. 1). This Candida gene, containing a sensor histidine kinase gene, shares 61% identity (74% similarity) with the Streptomyces gene, and a homolog is also present in the pathogenic fungus Fusarium solani (Fig. 1). This finding is notable as histidine kinases are relatively uncommon in eukaryotes and the origin of these particular fungal histidine kinases was unknown. This similarity between the Streptomyces and fungal genes now suggests that these fungi may have obtained this gene by horizontal gene transfer from bacteria. To further investigate this, and to identify the closest homologs of these fungal genes, we performed a phylogenetic analysis of known or putative histidine kinases (Fig. 1 and data not shown). The fungal histidine kinases, all from the Ascomycota, clustered together with high confidence with the Streptomyces homologs in all trees constructed (i.e., multiple trees were constructed using different numbers of genes from the extended histidine kinase family and using both neighbor-joining and maximum parsimony methods from PHYLIP). If these genes reflected organism phylogeny, we would expect the genes of gram-positive Streptomyces to be more related to those of their gram-negative relatives (proposed orthologous group in Fig. 1) than to those of the fungi. However, this is not the case, and so the most parsimonious explanation is that these fungal histidine kinases were obtained by horizontal gene transfer from an ancestor of the Streptomyces bacteria.

Although the fungal/Streptomyces clade was distinct, it was also found to be part of a larger group of experimentally studied histidine kinases (Fig. 1), of which all members investigated to date for their role in pathogenicity have been shown to be virulence factors (Fig. 1, bold). Like the C. albicans NIK1 sensor (14), some members of these histidine kinases have been demonstrated to be novel tripartite (two transmitter and one receiver domains) sensor proteins (10). The closest relative to these fungal/Streptomyces genes among the 63 predicted sensors in P. aeruginosa PAO1 was the product of a gene, gacS, also called lemA. This sensor is known, in related type 1 Pseudomonadaceae, to be a part of the two-component regulatory system GacS-GacA, which regulates such factors as production of proteases, pectate lyase, alginate exopolysaccharide, and siderophores, as well as swelling and virulence towards plants (e.g., see references 4, 5, and 13). In P. aeruginosa, the GacA response regulator has been well studied and has been shown to be involved in several of the above phenomena (12), and although these genes are separated on the genome by nearly 2 Mbp, it has been assumed that GacS (also called LemA) (P. aeruginosa PAO1 genome identification number, PA0928; 15) is the cognate sensor, due to its relatively high similarity (62% identity, 72% similarity) to the Pseudomonas syringae GacS.
(LemA) protein and the lack of linkage between GacS and GacA orthologs in other organisms (10). A recent study identified a
P. aeruginosa homolog of P. syringae lemA that, when knocked out, attenuated virulence in a Caenorhabditis elegans model of
P. aeruginosa virulence (however, no sequence was associated with the publication to confirm identification of the
gene) (16). Furthermore, the authors demonstrated a decrease of 3 orders of magnitude in the growth of this
lemA mutant on Arabidopsis thaliana leaves and preliminary evidence for a role in virulence in mice (i.e., change in lethality detected at an
infectious dose of 10^7 organisms).

As stated above, GacS is the closest homolog in P. aeruginosa of the C. albicans virulence factor NIK1, as well as the
other fungal histidine kinases shown in Fig. 1. In addition, it appears to be the ortholog (i.e., diverged only due to speciation
of these organisms) of the Streptomyces genes, as well as the other Pseudomonas species homologs shown in Fig. 1 and
some enteric genes (BarA, ExpS; Fig. 1), since the phylogeny of these genes matches organism phylogeny. This proposed
orthologous group of virulence-associated sensor histidine
kinases may therefore have similar sensing functions (since
orthologs tend not to diverge in function, compared with para-
logs which are genes that diverge after a gene duplication
event). Five other putative, unstudied P. aeruginosa sensors
were more distantly related to this group but were still part of
an apparent subfamily (data not shown), whereas the known
P. aeruginosa virulence factor, the sensor kinase PhoQ, had
only 24% identity and 39% similarity over a smaller region
and appeared to be an outgroup. Therefore, we examined here
whether GacS had a significant role in virulence in mice and in
certain virulence-related characteristics and compared this to
PhoQ, using knockouts of both genes for comparison.

A knockout mutation in the gacS gene (PAK-gacS) was
produced in wild-type (WT) strain P. aeruginosa PAK (PAK-
WT) by insertional mutagenesis using a mini-Tn5 transposon system. The transposon insertion element contained a tetracycline resistance marker and the *Escherichia coli* *rrnB* transcription terminator. The vector containing the transposon was delivered into strain PAK by conjugal transfer from *E. coli* strain S17.1λpir. A library of PAK transposon mutants was isolated by subsequent selection on Luria-Bertani agar containing 100 μg of tetracycline/ml. Mutant colonies were picked at random and propagated, and their genomic insertion sites were determined by a semi-random PCR strategy (10). The insertion sites were mapped on the completed *P. aeruginosa* genome. *gacS* mutants were chosen for the present study.

Virulence was assessed as the number of bacteria constituting a 50% lethal dose (LD50) in a mouse infection model in which B6D2 mice were made neutropenic with three doses of cyclophosphamide (3). *P. aeruginosa* PAK-WT or PAK-*gacS* (infectious dose, 8, 80, 800, 8,000, 8 × 10^3, 8 × 10^4, or 8 × 10^5 organisms/mouse) was injected into the peritoneum of groups of seven or eight mice (one group per infectious dose) on two separate occasions. Results were remarkably consistent, with LD50 of 10 ± 1 organisms/mouse recorded for PAK-WT and 7,500 ± 100 organisms/mouse for PAK-*gacS*, a 750-fold increase in LD50 (significantly different at a P value of <0.05 by Fisher’s exact test). We previously demonstrated that a *phoQ* mutant (strain H854; *phoQ::xylE-Gm*) demonstrated a 100-fold increase in LD50 in the neutropenic mouse model compared to its wild-type PA01 strain H103 (8).

We examined two virulence-related phenotypes of these *P. aeruginosa* strains, as we felt it important to examine how GacS and PhoQ had evolved in terms of differences or overlaps in virulence function. Swarming, the outward movement of *P. aeruginosa* from an inoculation site on low-agar medium (6), has been demonstrated to depend on the rhl quorum sensing system, type IV pili, and flagella (6). In *P. syringae*, swarming is GacS dependent (4). To test swarming, we inoculated each strain onto brain heart infusion medium (Difco Labs) solidified with 0.5% Bacto-agar. The PAK-*gacS* mutant was substantially less able to swarm than its parent PAK-WT, with the average size of the swarming colony after an 18-h incubation at 37°C being 76 ± 1 mm, compared with 178 ± 8 mm for the wild type (Fig. 2). The *phoQ* mutant H854 showed a similar decrease in ability to swarm, with an average colony size of 90 ± 7 mm compared with 201 ± 11 mm for its parent strain H103. In the latter case, it appeared that this down-regulation of swarming ability in the PhoQ null mutant involved the cognate regulator PhoP, since PhoP null strain H851 (*phoP::xylE-Gm*) was a superswarmer, with a colony size of 518 ± 20 mm under these conditions (Fig. 2).

We also examined lipase production in stationary phase cultures (optical density at 600 nm [OD600] = 2.5) using the substrate para-nitrophenyl palmitate. The extent of lipase production varied substantially over four trials with both the parent strains PAK-WT (415 ± 198 nmol of *p*-nitrophenol produced/ml of culture/min; Table 1) and H103 (84 ± 17 nmol/ml/min), as revealed by the large standard error values. However, when expressed as a percentage of the value observed for the controls, PAK-*gacS* expressed only 30% ± 9% of the lipase observed in the supernatants of PAK-WT, while H854 (*phoQ::xylE-Gm*) expressed only 16% ± 2% of the lipase observed with its parent strain, H103. Such an alteration in lipase activity was previously observed for mutants with disruptions in *gacA*, the proposed cognate response regulator of gacS (12).

To observe if these two sensors, GacS and PhoQ, were completely overlapping in the genes they regulated, we examined antibiotic susceptibility (Table 1). We previously published the observation that the *phoQ* mutant H854 was four- to eightfold more resistant to polymyxin B and aminoglycosides like amikacin and streptomycin and two- to fourfold more resistant to the cationic antimicrobial peptides CP28 and CP29 (7, 8), consistent with the known ability of PhoP-PhoQ to regulate susceptibility to cationic antibiotics in other species. Other classes of antibiotics were unaffected. In contrast, testing here of the susceptibility of PAK-*gacS* to 16 different antimicrobials by the broth microdilution assay (1) revealed no change in susceptibility to polymyxin B or the cationic peptide CP11CN or CP28. Similarly, there was no significant change (i.e., less than a twofold decrease) in the MICs of the quinolones nalidixic acid, ciprofloxacin, fleroxacin, and norfloxacin, of the macrolide erythromycin, and of the β-lactams carbenicillin, cefepime, cephaloridine, and ceftazidime. On the other hand, for PAK-*gacS* a fourfold decrease in the MIC of (i.e., supersusceptibility to) gentamicin and a twofold decrease in the MIC of amikacin were observed, in contrast to the two-
fourfold increases in MICs of aminoglycosides for the *phoQ* mutant H854, compared to their respective parent strains. PAK-*gacS* also demonstrated an eightfold decrease in the MIC of chloramphenicol. While we were unable to explain why the *gacS* mutant is more susceptible to these agents, we were able to clearly demonstrate independent regulation of antibiotic susceptibility by these two sensors.

Histidine kinases are relatively rare in eukaryotes (compared with the prevalence of eukaryotic serine/threonine/tyrosine kinases or compared with the prevalence of histidine kinases in bacteria); however, we now report a possible explanation for the presence of some particular histidine kinases in fungi: horizontal gene transfer from an ancestor of *Streptomyces*. Putative histidine kinases in *Arabidopsis* leaves and the slime mold *Dictyostelium* have also been recently identified; however, the level of similarity between these plant and slime mold genes and any bacterial homologs is much lower than that between the fungal and *Streptomyces* genes which we report here. Whether these plant and slime mold histidine kinases are the result of lateral transfer from yet-unknown bacteria or have other evolutionary origins will remain unclear until better sampling of histidine kinases from diverse lineages is made. The proposed orthologous bacterial histidine kinases (including *P. aeruginosa* GacS) and related fungal histidine kinases that we show in Fig. 1 share notable commonalities, particularly with regard to their role in virulence and the presence of the orthologous proteins in such a wide range of bacteria, indicating that this virulence factor’s function has ancient origins. Presumably, these genes manifest their effects by regulating one or more virulence determinants, such as lipase, proteases, toxin, and siderophore production, as well as swarming behavior and invasion. (Only a subset of these virulence determinants in any given species has been demonstrated. Notably, the GacS-associated phenotypes such as swarming are similar for different species which have different genes downstream of *gacS*, so polar effects of GacS mutants on downstream genes are unlikely a cause of such phenotypes.) Interestingly, GacS and PhoQ, the latter of which is not a member of this subfamily, do appear to have some overlapping effects that impact on virulence. We felt it was of interest to examine the phenotypes of GacS and PhoQ, as they represent two evolutionarily divergent histidine kinases with an apparent role in pathogenicity. PhoQ has been implicated in regulation of susceptibility to polycationic antibiotics and cationic antimicrobial peptides that are agents of innate immunity (7, 8). These primary phenotypes were evidently not shared by GacS, as our results indicate, and the *gacS* knockout mutant appeared not to influence expression of *phoQ* since it did not upregulate the outer membrane protein OprH, which is the product of the first gene in the oprH-*phoP-*phoQ operon. However, we could demonstrate other distinct properties of *phoQ* and *gacS* knockouts: they had in common defects in virulence, lipase production, and swarming ability. We propose that these sensors have evolved to have overlapping functions that permit regulation of virulence factors under different growth conditions since, e.g., PhoQ is known to be regulated by divalent cations. Presumably, GacS senses a different environmental signal that is relevant in detecting or maintaining host infection and which may also be the signal detected by other members of the proposed orthologous group that includes GacS. Determining exactly what property or compound makes up this signal now becomes extremely significant, as it will have relevance for a wide range of bacterial and fungal pathogens.

Funding support from a CIHR special genomics grant and from the Peter Wall Institute for Advanced Studies to R.E.W.H. and F.S.L.B. is gratefully acknowledged.

We thank Hong Yan for help in analysis of virulence of the *P. aeruginosa* GacS mutant.

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