INFECTION AND IMMUNITY, Aug. 2001, p. 5207–5211 Vol. 69, No. 8
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.8.5207–5211.2001

Evolutionary Relationships among Virulence-Associated Histidine Kinases

FIONA S. L. BRINKMAN,[1] EMMA L. A. MACFARLANE,[1] PAUL WARRENER,[2] AND ROBERT E. W. HANCOCK[1]*

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada,[1] and Pathogenesis Corporation, Seattle, Washington 98119[2]

Received 11 January 2001/Returned for modification 10 April 2001/Accepted 11 May 2001

A strong relationship between virulence-associated sensor histidine kinases of fungi and those in *Streptomyces coelicolor* was observed, and phylogenetic analysis suggested that bacterium-to-eukaryote horizontal gene transfer had occurred between ancestors of these organisms. Phylogenetic analysis also identified a group of histidine kinases orthologous to the *Streptomyces* proteins that includes *Pseudomonas aeruginosa* GacS. We provide evidence that GacS is important for swarming motility, lipase production, and virulence in mice and had evolved to have partial functional overlaps with PhoQ, a less-related virulence-associated histidine kinase.

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+} 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 neutropenic 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 pathogencity 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 swarming 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. aerguinosa* 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

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Blvd., Vancouver, BC V6T 1Z3, Canada. Phone: (604) 822-2682. Fax: (604) 822-6041. E-mail: bob@cmdr.ubc.ca.

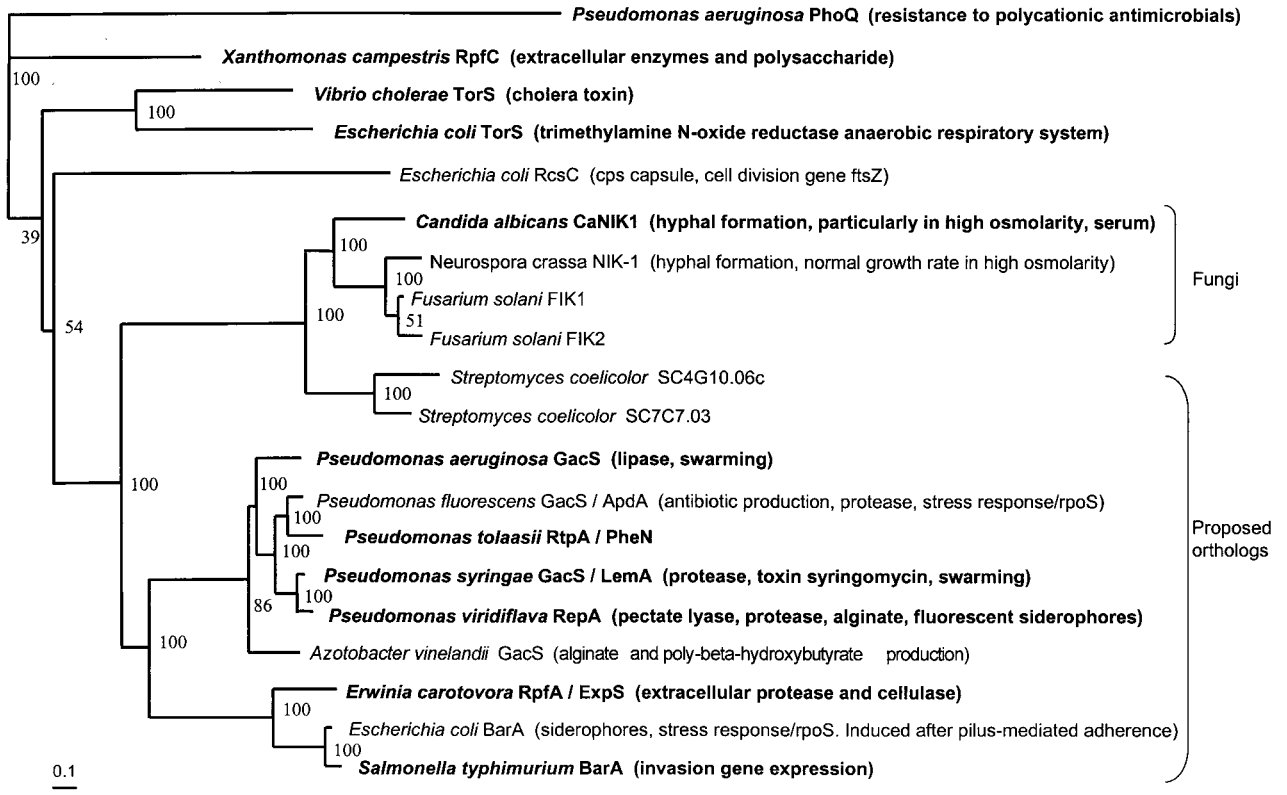

FIG. 1. Phylogenetic analysis of a selection of experimentally studied bacterial histidine kinases that are related to a group of fungal genes (one exception: the bacterial sequences from *Streptomyces* are from a published sequence from a genome project in progress and not yet experimentally studied). The bacterial proteins shown in this tree include a cluster of proteins most related to the fungal homologs (proposed orthologs) and selected experimentally studied proteins from a subfamily of histidine kinases that help illustrate the orthologous group. The tree was rooted by a more divergent member of the extended sensor histidine kinase family, *P. aeruginosa* PhoQ. The tree was constructed using the neighbor-joining method of PHYLIP (for methodology, see the unpublished PHYLIP documentation that is freely available: http://www.ibb.waw.pl/docs/PHYLIPdoc/main.html). Horizontal branch lengths in the tree correspond to the degree of relatedness of each protein. The number of times out of 100 that a given node of the tree was present in 100 bootstrapped replicates of the analysis is also indicated at each node to provide a statistical evaluation of the branching order in the tree. Brackets contain a description of phenotypes affected by disruption or overexpression of each gene, including changes in compounds secreted. Those proteins that were investigated and then were shown to play a role in virulence are in bold (i.e., all those investigated for their role in pathogenicity to date that have been shown to be virulence factors). We propose that the proteins shown here from *Pseudomonas, Azotobacter,* and *Streptomyces*, as well as the enteric ExpS and BarA proteins, are orthologous (i.e., diverged due to organism speciation), while the fungal genes are xenologous (obtained through horizontal gene transfer from an ancestor of the *Streptomyces* to an ancestor of these fungi and then subsequently diverged). Analysis using iPSORT (http://HypothesisCreator.net/iPSORT/) for the prediction of mitochondrial targeting sequences indicated that the fungal histidine kinases do not function in the mitochondria and they are apparently not encoded in the mitochondrial genome and thus are not similar to bacterial kinases due to the bacterial origin of mitochondria.

(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 10^5 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 paralogs 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-Tn*5* 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 S17.1λ*pir*. 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 (LD_{50}) 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, 8×10^4, 8×10^5, or 8×10^6 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 LD_{50}s of 10 ± 1 organisms/mouse recorded for PAK-WT and $7{,}500 \pm 100$ organisms/mouse for PAK-*gacS*, a 750-fold increase in LD_{50} (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^r) demonstrated a 100-fold increase in LD_{50} 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^r) 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 [OD_{600}] = 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 $30\% \pm 9\%$ of the lipase observed in the supernatants of PAK-WT, while H854 (*phoQ*::*xylE*-Gm^r) expressed only $16\% \pm 2\%$ 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).

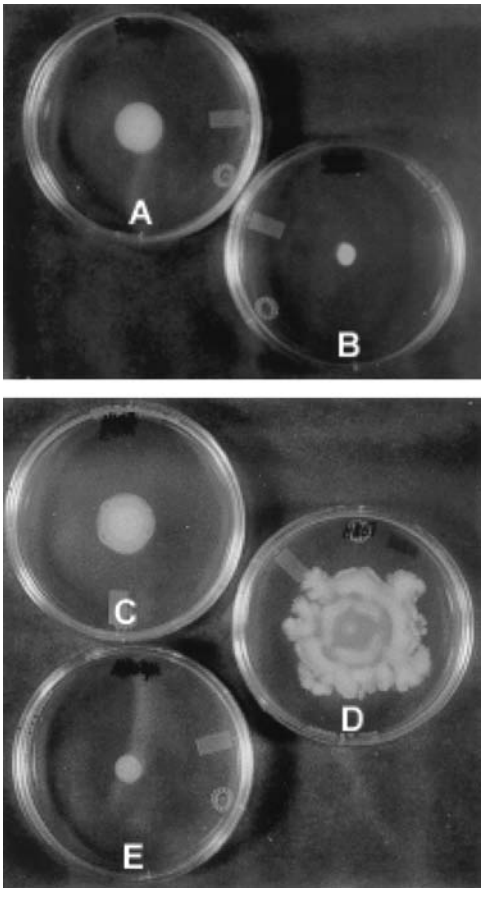

FIG. 2. Swarming behavior of *P. aeruginosa* cells with disrupted *gacS*, *phoP*, or *phoQ* putative two-component regulator genes. (A) Wild-type strain PAK; (B) PAK-*gacS* mutant; (C) wild-type strain H103; (D) H851 *phoP* mutant; (E) H854 *phoQ* mutant.

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 ceftazadime. 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- to

TABLE 1. Phenotype of the *gacS* mutant

Strain	MIC (μg/ml) of drug[a]										Lipase production[b]	Zone of swarming (mm) (±SE)	LD_{50} (no. of organisms/mouse) (±SE)
	GM	AK	CM	CFP	IMI	PX	CIP	NOR	NAL	CEMA			
PAK-WT	1	0.5	32	8	1	0.25	0.03	0.06	32	4	415 ± 198	178 ± 8	10 ± 1
PAK-*gacS*	0.25	0.25	4	2	1	0.25	0.03	0.06	32	4	124 ± 37	76 ± 1	7,500 ± 100

[a] Results are the median of three experiments. Abbreviations: GM, gentamicin; AK, amikacin; CM, chloramphenicol; CFP, cefpirome; IMI, imipenem; PX, polymyxin B; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CEMA, a cationic antimicrobial peptide.
[b] Expressed as specific activity in nanomoles of *p*-nitrophenol produced per milliliter of culture per minute at a culture OD_{650} of 2.

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-unstudied 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.

REFERENCES

1. **Amsterdam, D.** 1996. Susceptibility testing of antimicrobials in liquid media, p. 52–111. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams and Wilkins, Baltimore, Md.
2. **Calera, J. A., and R. Calderone.** 1999. Histidine kinase, two-component signal transduction proteins of *Candida albicans* and the pathogenesis of candidosis. Mycoses **42**(Suppl. 2)**:**49–53.
3. **Gough, M., R. E. W. Hancock, and N. M. Kelly.** 1996. Anti-endotoxic potential of cationic peptide antimicrobials. Infect. Immun. **64:**4922–4927.
4. **Kinscherf, T. G., and D. K. Willis.** 1999. Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone biosynthetic gene *ahlI*. J. Bacteriol. **181:**4133–4136.
5. **Kitten, T., T. G. Kinscherf, J. L. McEvoy, and D. K. Willis.** 1998. A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. **28:**917–929.
6. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. Pechere.** 2000. Swarming in *Pseudomonas aeruginosa* is dependent on cell-to-cell signalling and requires flagella and pili. J. Bacteriol. **182:**5990–5996.
7. **Macfarlane, E. L. A., A. Kwasnicka, and R. E. W. Hancock.** 2000. Role of *Pseudomonas aeruginosa* in resistance to antimicrobial peptides and aminoglycosides. Microbiology **146:**2543–2554.
8. **Macfarlane, E. L. A., A. Kwasnicka, M. M. Ochs, and R. E. W. Hancock.** 1999. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol. Microbiol. **34:**305–316.
9. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
10. **Pernestig, A., O. Melefors, and D. Georgellis.** 2001. Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli*. J. Biol. Chem. **276:**225–231.
11. **Redenbach, M., H. M. Kieser, D. Denapaite, A. Eichner, J. Cullum, H. Kinashi, and D. A. Hopwood.** 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol. Microbiol. **21:**77–96.
12. **Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino, A. Lazdunski, and D. Haas.** 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol. Microbiol. **24:**309–319.

13. **Rodrigue, A., Y. Quentin, A. Lazdunski, V. Méjean, and M. Foglino.** 2000. Two component systems in *Pseudomonas aeruginosa*. Why so many? Trends Microbiol. **8:**498–504.
14. **Smkantha, T., L. Tsai, K. Daniels, L. Enger, K. Highley, and D. R. Soll.** 1998. The two component hybrid kinase regulator Ca NIK1 of *Candida albicans*. Microbiology **144:**2715–2719.
15. **Stover, K. C., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, R. Lim, K. Smith, D. Spencer, G. K.-S. Wong, Z. Wu, I. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa*: an opportunistic pathogen. Nature **406:**959–964.
16. **Tan, M. W., L. G. Rahme, J. A. Sternberg, R. G. Tompkins, and F. M. Ausubel.** 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proc. Natl. Acad. Sci. USA **96:**2408–2413.
17. **Yamada-Okabe, T., T. Mio, N. Ono, Y. Kashima, M. Matsui, M. Amsawa, and H. Yamada-Okabe.** 1999. Roles of three histidine kinase genes in hyphal development and virulence of the pathogenic fungus *Candida albicans*. J. Bacteriol. **181:**7243–7247.

Editor: J. T. Barbieri