Zool. (London) **177**, 179 (1975).
6. S. D. Twiss, P. P. Pomeroy, S. S. Anderson, *ibid.* **233**, 683 (1994).
7. W. Amos, S. S. Twiss, P. P. Pomeroy, S. S. Anderson, *Proc. R. Soc. London Ser. B* **252**, 199 (1993).
8. W. N. Bonner, *Oceanogr. Mar. Biol. Annu. Rev.* **10**, 461 (1972).
9. H. R. Hewer, *Proc. Zool. Soc. London* **142**, 593 (1964).
10. J. C. Coulson and G. Hickling, *J. Anim. Ecol.* **33**, 485 (1964).
11. A. R. Hiby, C. D. Duck, D. Thompson, *NERC News* (January 1993), p. 20.
12. P. P. Pomeroy, S. S. Anderson, S. D. Twiss, B. J. McConnell, *J. Zool. (London)* **233**, 429 (1994).
13. For any female, the probability of a pair of her pups being full sibs (F) relates to the probability of paternity by all n possible males as follows:

$$F = \sum_{i=1}^{n} P_{i1}P_{i2}$$

where P_{i1} and P_{i2} are the probabilities of the i^{th} male fathering pups 1 and 2, respectively. If P_{i1} and P_{i2} differ principally by the probability that the male stops reproducing between the two conceptions, $P_{i1}P_{i2}$ can be rewritten as

$$P_i^2\left(1 - \frac{a}{r_i}\right),$$

where a is the time interval separating the two pups and r_i is the reproductive longevity of the i^{th} male. Substituting mean values for a and r, F = 0.3 and rearranging gives:

$$\frac{0.3\,\bar{r}}{(\bar{r} - \bar{a})} = \sum_{i=1}^{n} p_i^2$$

For our data set, $\bar{a}$ = 1.53. Male reproductive longevity is unknown. Bonner (*8*) suggests that 90% of males die by age 15 and that none live beyond 20. Yet, 25 branded males (~30%) were resighted in a single season 7 years later. Because these males were mature at branding, and few males reappear every year, we suggest that r = 10 is a reasonable value. Substituting and solving for P_i, we can identify two extremes. If a single male is likely, all others being highly unlikely, he will father 59% of the female's pups during his lifetime. If all fathers are equally likely, there must be 2.86 males who between them father 100% of her pups.
14. Sampling is limited by lack of opportunity and the need to minimize disturbance.
15. Estrus is taken as 14 days post-parturition [I. L. Boyd, *J. Reprod. Fertil.* **69**, 157 (1983)]. The mean between-season shift of 2.54 days (±0.3 SE) was observed for 59 females in our data set. Pupping is initially late, becoming progressively earlier with age (*12*).
16. Paternity data are from (*7*). Seventy branded males were tested against 120 pups that included an estimated 21 pairs of full sibs. Branded males gained 29 paternities but only two pairs of full sibs: $\chi^2 = 7.56$, $P < 0.01$.
17. The mean probability of shared paternity, P_s, among a sample of pups can be estimated from the difference between the population gene identity (G, see legend to Fig. 1) and the gene identity calculated for paternal alleles among pups, G_p, as follows:

$$G_p = G(1 - P_s) + \frac{P_s(1 + G)}{2}$$

and, by rearrangement:

$$P_s = \frac{2(G_p - G)}{(1 - G)}$$

Within-season values of G_p were calculated for 84 pups born to branded females, half-alleles being assigned in cases in which the paternal allele was ambiguous (1986, n = 16, 12.5 alleles, G_p = 0.063; 1987, n = 25, 18 alleles, G_p = 0.053; 1988, n = 18, 15.5 alleles, G_p = 0.022; and 1989, n = 24, 20 alleles, G_p = 0.035), giving a weighted mean of 0.043 and an overall P_s of 0.023.
18. C. F. Summers, R. W. Burton, S. S. Anderson, *J. Zool. (London)* **175**, 439 (1975).
19. J. R. Baker, *ibid.* **203**, 23 (1984); B. J. Le Boeuf, in *Behaviour of Pinnipeds*, D. Renouf, Ed. (Chapman & Hall, London, 1991), pp. 45–65; _____ and K. T. Briggs, *Mammalia* **41**, 167 (1977).
20. S. D. Twiss, thesis, University of Glasgow (1991).
21. D. J. Boness, S. S. Anderson, C. R. Cox, *Can. J. Zool.* **60**, 2270 (1982).
22. We thank P. Clapham, T. Clutton-Brock, G. Cooper, C. Duck, M. Fedak, J. Harwood, and J. Pemberton for comments on the manuscript; J. A. Barrett for statistical advice; I. A. van Pijlen for cloning locus *HgMS-A5*; and many colleagues for help during fieldwork. S.T. was supported by a National Environment Research Council (NERC) CASE studentship; B.A. was supported by NERC and the Royal Society.

16 November 1994; accepted 5 May 1995

Common Virulence Factors for Bacterial Pathogenicity in Plants and Animals

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A *Pseudomonas aeruginosa* strain (UCBPP-PA14) is infectious both in an *Arabidopsis thaliana* leaf infiltration model and in a mouse full-thickness skin burn model. UCBPP-PA14 exhibits ecotype specificity for *Arabidopsis*, causing a range of symptoms from none to severe in four different ecotypes. In the mouse model, UCBPP-PA14 is as lethal as other well-studied *P. aeruginosa* strains. Mutations in the UCBPP-PA14 *toxA, plcS, and gacA* genes resulted in a significant reduction in pathogenicity in both hosts, indicating that these genes encode virulence factors required for the full expression of pathogenicity in both plants and animals.

Bacterial pathogens comprise a large and diverse group of species capable of infecting both animals and plants. Most of these pathogens cause disease in a single or limited number of host species. The interactions between bacterial and host factors that limit host range and determine resistance or susceptibility are not fully understood.

Despite the vast evolutionary gulf between plants and animals, two types of observations suggest that some of the underlying mechanisms of bacterial pathogenesis may be similar in the two kingdoms. First, bacterial proteins involved in the export of proteinaceous virulence factors have been shown to be conserved between plant and mammalian pathogens (*1*). Second, for some bacterial species, including *Pseudomonas cepacia* (*2*), *Pseudomonas aeruginosa* (*3*, *4*), and *Erwinia* spp. (*5*), specific strains have been reported to be either plant or animal pathogens.

Reports indicating similarities between plant and animal pathogens prompted us to search for a strain of *P. aeruginosa* that was capable of eliciting disease in both a well-defined plant pathogenesis model and a well-defined animal pathogenesis model. We chose *P. aeruginosa* for these studies because it is a serious opportunistic pathogen in immunocompromised human patients (*6*) and because individual clinical isolates have been reported to cause disease in plants (*3*). Given such a "dual" animal-plant pathogen, it would be interesting from an evolutionary perspective to determine which, if any, bacterial virulence factors were involved in both plant and animal pathogenesis.

A collection of 75 *P. aeruginosa* strains (*7*), of which 30 were human isolates, were screened for their ability to cause disease on leaves of at least four different *Arabidopsis thaliana* ecotypes (*8*, *9*) (land races or wild accessions). We reasoned that a *P. aeruginosa* pathogen that exhibited ecotype specificity on *Arabidopsis* would most likely be a true plant pathogen, rather than a strain that has no capacity to be a plant pathogen under natural settings but infects plants as a consequence of the artificial environment created in the laboratory (*10*).

Most of the 75 *P. aeruginosa* strains that were screened elicited no symptoms in *Arabidopsis* leaves. Several strains elicited weak to moderate soft-rot symptoms. However, two strains, UCBPP-PA14, a human isolate, and UCBPP-PA29, a plant isolate, caused severe soft-rot symptoms in some, but not all, of the ecotypes tested, a result

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typical of a highly virulent *Arabidopsis* bacterial pathogen such as *Pseudomonas syringae* (*8*) (Table 1). The severe symptoms elicited by UCBPP-PA14 were characterized by a water-soaked reaction zone and chlorosis, resulting in complete maceration and collapse of the leaf 4 to 5 days after infection.

The degree of proliferation of strains UCBPP-PA14 and UCBPP-PA29 in *Arabidopsis* leaves was correlated with the severity of disease symptoms (*11*) (Fig. 1, A and B, and Table 1). In each case, reduced bacterial counts in leaves were associated with less severe symptom development (*12*). Moreover, the growth profile of UCBPP-PA14 and UCBPP-PA29 in the susceptible ecotype Llagostera (Ll) was essentially identical to that of the *Arabidopsis* bacterial pathogen *P. syringae* pv. *maculicola* strain ES4326 (*8*) (Fig. 1A).

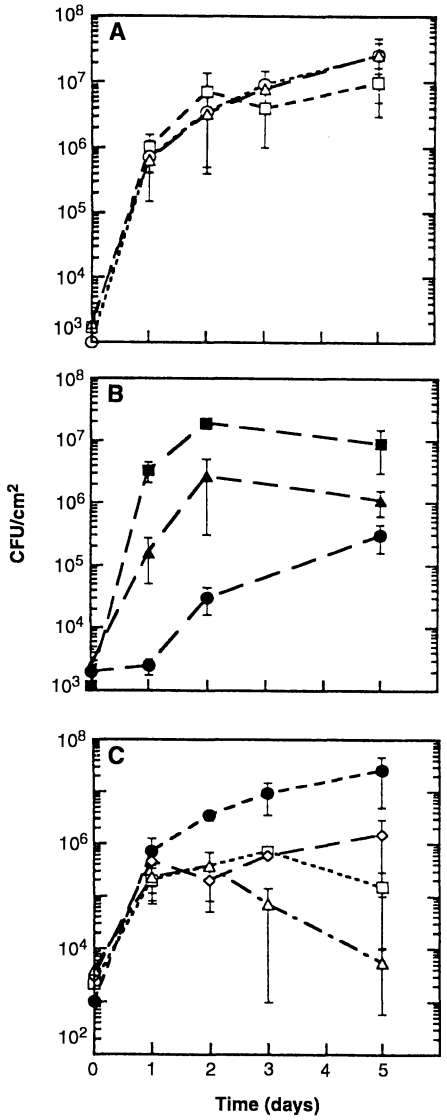

Fig. 1. Growth of *P. syringae* and *P. aeruginosa* in *Arabidopsis* leaves. (**A**) Growth of *P. syringae* pv. *maculicola* strain ES4326 (□) and *P. aeruginosa* strains UCBPP-PA14 (○) and UCBPP-PA29 (△) in ecotype Llagostera. (**B**) Growth of *P. aeruginosa* strain UCBPP-PA14 in three *Arabidopsis* ecotypes: Columbia (■), Argentat (●), and Bensheim (▲). (**C**) Growth of *P. aeruginosa* strain UCBPP-14 (●) and isogenic *plcS* (□), *toxA* (◇), and *gacA* (△) mutants in ecotype Llagostera. Bacterial counts in *Arabidopsis* leaves were done as described (*8*). Means of four samples ± SD are shown. Three independent experiments gave similar results. Incubation conditions were as described in Table 1.

The pathogenicity of strains UCBPP-PA14 and UCBPP-PA29 was measured in a mouse model in which a full-thickness thermal burn injury involving 5% of the body surface area was fashioned on an outstretched area of abdominal skin (*13*). In this model, the damaged epidermis and dermis undergo coagulation necrosis, but the underlying rectus abdomini (RA) muscles are not injured (*13*). A *P. aeruginosa* inoculum is injected intradermally into the midline crease of the burn eschar (*13, 14*). The bacteria proliferate in the burn wound, and some strains may invade the normal underlying RA muscles. The number of bacteria found in the RA muscles underlying and adjacent to the burn after 24 hours gives a sensitive and quantitative measure of local invasiveness. Some strains may also invade the vasculature and lymphatics, resulting in the widespread dissemination of bacteria throughout the host. Mortality is an indicator of systemic invasiveness.

Strains UCBPP-PA14 and UCBPP-PA29 proliferated and invaded the RA muscles as well as the *P. aeruginosa* human isolates PO37 (*13*), PAO1 (*15*), and PAK (*16*) (Table 2). In addition, strain UCBPP-PA14 caused a high level of mortality (≥77%) in this model (Table 3). Importantly, heat-killed cells caused no mortality, suggesting that mortality is not a simple consequence of endotoxin-induced shock.

Strain UCBPP-PA14 was selected for additional studies because it was locally invasive and was significantly lethal in an established mouse model and because the severity of symptoms that it elicited in different *Arabidopsis* ecotypes was directly correlated with the extent of growth in *Arabidopsis* leaves. Moreover, the level of virulence of UCBPP-PA14 in the mouse and *Arabidopsis* models was comparable to that of well-studied plant (*P. syringae*) and animal (*P. aeruginosa*) pathogens. Specifically, we sought to determine whether there are common virulence determinants in strain UCBPP-PA14 required for pathogenicity in both hosts. We used a marker exchange procedure to generate UCBPP-PA14 mutants carrying insertion mutations in three different genes, two known to be virulence determinants for *P. aeruginosa* in animal hosts and one known to be a virulence determinant for phytopathogenic bacteria in plant hosts. The two animal virulence-related genes of *P. aeruginosa* were *plcS* and *toxA*, encoding the exported proteins phospholipase S (*17*) and exotoxin A (*18*), respectively. Phospholipase C preferentially degrades phospholipids of eukaryotic cells (*17*) and exotoxin A inhibits protein syn-

Table 1. Growth of *P. aeruginosa* strains UCBPP-PA14 and UCBPP-PA29 on different *Arabidopsis* ecotypes and disease symptoms elicited.

Arabidopsis ecotype*	*P. aeruginosa* UCBPP-PA14		*P. aeruginosa* UCBPP-PA29	
	CFU/cm² leaf area†	Symptoms‡	CFU/cm² leaf area†	Symptoms
Ll	$2.6 \times 10^7 \pm 2.0 \times 10^7$	Severe	$2.7 \times 10^7 \pm 1.3 \times 10^7$	Severe
Col	$9.0 \times 10^6 \pm 6.0 \times 10^6$	Severe	$6.0 \times 10^5 \pm 3.0 \times 10^5$	Weak
Ag	$3.0 \times 10^5 \pm 1.4 \times 10^5$	None	$1.5 \times 10^5 \pm 9.0 \times 10^4$	None
Be	$1.1 \times 10^6 \pm 4.9 \times 10^5$	Moderate	$4.5 \times 10^5 \pm 2.0 \times 10^5$	None

**Arabidopsis* land races: Ll, Llagostera; Col, Columbia; Ag, Argentat; and Be, Bensheim. †Means of four samples ± SD of maximum bacterial counts obtained at 5 days after infection of 10^3 cells. ‡Symptoms were elicited 5 days after injection. None, no symptoms; weak, localized weak water-soaking and chlorosis of tissue circumscribing the injection site; moderate, moderate water-soaking and chlorosis with most of the tissue softened around the inoculation site; severe, severe soft-rotting of the entire inoculated leaf characterized by a water-soaked reaction zone and chlorosis circumscribing the injection site at 2 to 3 days after injection. The soft-rot symptoms pervaded the leaf at 4 to 5 days after injection.

Table 2. Proliferation of *P. aeruginosa* strains in a mouse full-thickness skin burn model.

P. aeruginosa strain	Mean titer ± SD in biopsies underneath burn*	Mean titer ± SD in biopsies adjacent to burn
UCBPP-PA14	$20.0 \times 10^7 \pm 9.0 \times 10^7$	$6.0 \times 10^7 \pm 2.1 \times 10^7$
UCBPP-PA29	$36.0 \times 10^7 \pm 10.0 \times 10^7$	$8.2 \times 10^7 \pm 2.0 \times 10^7$
PO37	$30.0 \times 10^7 \pm 11.0 \times 10^7$	$5.8 \times 10^7 \pm 1.0 \times 10^7$
PAK	$18.0 \times 10^7 \pm 9.1 \times 10^7$	$6.0 \times 10^7 \pm 1.2 \times 10^7$
PA01	$31.0 \times 10^7 \pm 10.0 \times 10^7$	$4.0 \times 10^7 \pm 1.8 \times 10^7$

*Mice were injected with ~5×10^3 cells. No viable bacterial cells were retrieved from the underlying rectus abdominus muscle immediately after bacterial injection or in sham-injured animals in other studies.

thesis by ribosylating elongation factor 2 (*18*). The plant pathogen virulence determinant was *gacA*, first identified as a global regulator of excreted antifungal factors in the nonpathogenic soil bacterium *Pseudomonas fluorescens* (*19*, *20*). In the phytopathogen *P. syringae* pv. *syringae*, *gacA* appears to serve as a transcriptional regulator of genes that encode extracellular products involved in pathogenicity (*21*).

The UCBPP-PA14 homologs of *plcS* and *toxA* were identified in a genomic cosmid library of strain UCBPP-PA14 (*22*) with cloned DNA fragments corresponding to the *plcS* and *toxA* genes of *P. aeruginosa* strain PAK as hybridization probes (*23*, *24*). The UCBPP-PA14 homolog of *gacA* was identified in the same cosmid library with a polymerase chain reaction–amplified product corresponding to a conserved region of the *P. fluorescens gacA* gene (*19*, *25*). All three genes were subcloned and mutagenized by the insertion of a cassette encoding gentamicin resistance (*26*). The mutated genes were transferred to the UCBPP-PA14 genome by means of standard marker exchange techniques (*27*).

The effects of the *plcS*, *toxA*, and three independently constructed *gacA* mutations on the pathogenicity of UCBPP-PA14 in the *Arabidopsis* model were tested by infiltrating the mutant strains into *Arabidopsis* ecotype Ll. Unlike wild-type UCBPP-PA14, none of the mutants caused maceration and collapse of the leaf. Specifically, the isogenic *toxA* mutant caused attenuated soft-rot and chlorosis symptoms without the accompanying maceration of the affected tissue characteristic of UCBPP-PA14. The *plcS* and *gacA* mutants elicited even weaker symptoms, causing only chlorosis. Consistent with the attenuated symptoms, the growth of the *toxA*, *plcS*, and *gacA* mutants after 5 days was less than the growth of the wild-type by a factor of approximately 10, 10^2, and 5×10^3, respectively (Fig. 1C).

In the mouse model, mice that were burned and infected with either *plcS* or *toxA* mutants exhibited significantly lower mortality (40% with both mutants) as compared to infection with the wild-type strain (77%) (Table 3) (*28*). None of the three independent *gacA* mutants caused mortality (Table 3).

Table 3. Lethality of *P. aeruginosa* strains in a mouse full-thickness skin burn model.

P. aeruginosa strain	Mortality ratio of mice at 10 days after burn and infection*
UCBPP-PA14	17/22
UCBPP-PA14-heat killed†	0/5
UCBPP-PA14 *plcS*	6/15
UCBPP-PA14 *toxA*	6/15
UCBPP-PA14 *gacA*	0/10‡
UCBPP-PA29	1/16

*Mice were injected with ~5×10^3 cells. †Cells were boiled for 10 min. ‡Three independently constructed *gacA* mutants gave the same result.

The pathogenicity of the UCBPP-PA14 mutants in both plants and mice was restored to either wild-type levels or near wild-type levels when the *toxA* mutant was complemented with a plasmid carrying only the *toxA* gene, when the *plcS* mutant was complemented with a plasmid carrying the *plcSR* operon, and when the mutated *gacA* gene was replaced with a 2.4-kb DNA fragment containing the wild-type gene (*29*).

The above results demonstrate that mutations in the *plcS*, *toxA*, and *gacA* genes result in diminished pathogenicity in both *Arabidopsis* and mice (*30*). One of these genes, *gacA*, is active at the regulatory level, suggesting that mechanisms for regulation of virulence factors may be conserved between plant and animal pathogens. The *plcS* and *toxA* gene products are specific virulence determinants that attack membranes and inhibit protein synthesis, respectively, in animal cells (*17*,*18*). Although there is no direct evidence that they play the same roles in plant cells, the fact that the UCBPP-PA14 *plcS* and *toxA* mutants caused less severe disease in *Arabidopsis* than the wild-type strain is consistent with the possibility that the *plcS* and *toxA* gene products play a similar role in plant and animal pathogenesis.

The "dual" animal-plant pathogen system described in this report has both fundamental and practical ramifications. From an evolutionary perspective, our results suggest that the molecular basis of *P. aeruginosa*–based pathogenesis may be similar in plants and animals. At a practical level, the dual pathogen system can be used to circumvent certain inherent limitations that an animal model imposes on the identification and study of *P. aeruginosa* virulence factors. *Pseudomonas aeruginosa* genes first identified as encoding virulence factors required for pathogenesis in plants can subsequently be tested in the mouse full-thickness skin burn model to identify virulence factors that are required for both plant and animal pathogenesis. Insights gained from this dual animal-plant pathogen system may help to elucidate the molecular basis underlying host specificity of bacterial pathogens.

REFERENCES AND NOTES

1. S. Fenselau, I. Balbo, U. Bonas, *Mol. Plant-Microbe Interact.* **5**, 390 (1992); C. L. Gough, S. Genin, C. Zischek, C. A. Boucher, *ibid.*, p. 384; H. C. Huang, S. Y. He, D. W. Bauer, A. Collmer, *J. Bacteriol.* **174**, 6878 (1992); H. C. Huang *et al.*, *Mol. Plant-Microbe Interact.* **6**, 515 (1993).
2. W. H. Burkholder, *Phytopathology* **40**, 115 (1950).
3. J. J. Cho, M. N. Schroth, S. D. Kominos, S. K. Green, *ibid.* **65**, 425 (1974); M. N. Schroth, J. J. Cho, S. K. Green, S. D. Kominos, in Pseudomonas aeruginosa: Ecological Aspects and Patient Colonization, V. M. Young, Ed. (Raven, New York, 1977), pp. 1–29.
4. S. D. Kominos, C. E. Copeland, B. Grosiak, B. Postic, *Appl. Microbiol.* **24**, 567 (1972); R. P. Elrod and A. C. Braun, *J. Bacteriol.* **46**, 633 (1942).
5. B. R. Meyers, E. Bottone, S. Z. Hirshman, S. S. Schneierson, *Ann. Intern. Med.* **76**, 9 (1972).
6. G. P. Bodey, R. Bolivar, V. Fainstein, L. Jadeja, *Rev. Infect. Dis.* **5**, 279 (1983).
7. The UCBPP *P. aeruginosa* strains, a collection of 30 human, 20 soil, and 25 plant isolates, were obtained from M. Schroth, University of California at Berkeley.
8. X. Dong, M. Mindrinos, K. R. Davis, F. M. Ausubel, *Plant Cell* **3**, 61 (1991); K. R. Davis, E. Schott, F. M. Ausubel, *Mol. Plant-Microbe Interact.* **4**, 477 (1991).
9. *Pseudomonas aeruginosa* strains were grown in Luria broth medium at 37°C, washed twice in 10 mM $MgSO_4$, and resuspended at an absorbance at 600 nm (A_{600}) of 0.2 in 10 mM $MgSO_4$, diluted 1:100, and injected into leaves of 6-week-old *Arabidopsis* plants [corresponding to 10^3 colony-forming units (CFU) per square centimeter of leaf area] as described for infiltration of *P. syringae* (*8*). The plants were kept in a growth chamber during the course of the experiment at 28° to 30°C and in an atmosphere of 90 to 100% relative humidity. Symptoms and growth were monitored daily for 5 days. Control plants inoculated with 10 mM $MgSO_4$ showed no symptoms during the course of the experiments.
10. Infecting several *Arabidopsis* ecotypes increased the likelihood of identifying a plant pathogenic isolate, because plant pathogens, including *Arabidopsis* pathogens, typically exhibit a high degree of specificity for the host cultivar or ecotype [I. Crute *et al.*, in *Arabidopsis*, E. Meyerowitz and C. Somerville, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994), pp. 705–747].
11. Leaf intercellular fluid–containing bacteria were harvested and bacterial counts were determined as described for *P. syringae* (*8*). Four different samples were taken, with two leaf disks per sample.
12. The ecotype specificity of UCBPP-PA14 and UCBPP-PA29 parallels what happens when a series of isolates of a well-studied plant pathogen such as *P. syringae* are screened for pathogenicity on a series of *Arabidopsis* ecotypes [T. Debener, H. Lehnackers, M. Arnold, J. L. Dangl, *Plant J.* **1**, 289 (1991)]. This type of host specificity is usually not directly related to the presence or absence of basic virulence factors, but rather to interactions between so-called avirulence genes in the pathogen and cognate resistance genes in the host [S. R. Long and B. J. Staskawicz, *Cell* **73**, 921 (1993)].
13. E. J. Stevens *et al.*, *J. Burn Care Rehabil.* **15**, 232 (1994).
14. Animal study protocols were reviewed by the subcommittee on animal studies of the Massachusetts General Hospital. Six-week-old male CD-1 mice (Charles River Animal Farms, Wilmington, MA), weighing between 25 to 30 g, were used in all experiments according to the animal model described in (*13*). In both quantitation and mortality studies, immediately after the burn, mice were injected with 5×10^3 cells, and the number of animals that died of sepsis was monitored each day for 10 days. Mice that were burned but not injected with UCBPP-PA14 exhibited no mortality.
15. B. W. Holloway, *J. Gen. Microbiol.* **13**, 572 (1955).
16. K. S. Ishimoto and S. Lory, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1954 (1989).
17. R. M. Ostroff and M. L. Vasil, *J. Bacteriol.* **169**, 4597 (1987); R. M. Ostroff, B. Wretlind, M. L. Vasil, *Infect. Immun.* **57**, 1369 (1989).
18. D. E. Ohman, J. C. Sadoff, B. H. Iglewski, *Infect. Immun.* **28**, 899 (1980); B. H. Iglewski and D. Kabat, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2284 (1975); R. M. Berka and M. L. Vasil, *J. Bacteriol.* **152**, 239 (1982).
19. J. Laville *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1562 (1992).
20. T. D. Gaffney *et al.*, *Mol. Plant-Microbe Interact.* **7**, 455 (1994).
21. J. J. Rich, T. G. Kinscherf, T. Kitten, D. K. Willis, *J. Bacteriol.* **176**, 7468 (1994).
22. The genomic library of strain UCBPP-PA14 was pre-

pared in the cosmid cloning vector pJSR1, which was constructed by ligating a 1.6-kb Bgl II fragment containing the bacteriophage lambda *cos* site from pHC79 [B. Hohn and J. Collins, *Gene* **11**, 291 (1980)] into the Bgl II site of pRR54 [R. C. Roberts, R. Burioni, D. R. Helinski, *J. Bacteriol.* **172**, 6204 (1990)].
23. A 1.7-kb Bam HI fragment isolated from plasmid pMS150 containing the *toxA* gene (*24*) and a 3.0-kb Bam HI–Pst I fragment from plasmid pSL2 containing the *plcS* gene [S. Lory and P. C. Tai, *Gene* **22**, 95 (1983)] were used to probe the UCBPP-PA14 genomic library in pJSR1 (*22*).
24. S. Lory, M. S. Strom, K. Johnson, *J. Bacteriol.* **170**, 714 (1988).
25. The oligonucleotides 5′-GCTAGTAGTCGATGACC-3′ and 5′-GTGGCATCAACCATGC-3′ were designed on the basis of the sequence of the *gacA* gene (*19*) and were used to amplify a 625-base pair (bp) product containing the *gacA* gene of *P. fluorescens*, which in turn was used to probe the UCBPP-PA14 genomic library in pJSR1 (*22*).
26. A 3.6-kb Bgl II–Bam HI fragment containing the *plcS* gene was subcloned into the Bam HI site of pBR322 and then mutagenized by insertion of a gentamicin-resistance gene cassette into the Xho I site of the *plcS* gene to construct pLGR201. The gentamicin-resistance gene cassette is a 1.8-kb Bam HI fragment from plasmid pH1JI [R. A. Rubin, *Plasmid* **18**, 84 (1987)] provided by S. Lory. The Xho I site is located approximately in the middle of the *plcS*-coding region (*17*). A 1.6-kb Bam HI fragment containing the *toxA* gene was subcloned into pBR322 and then mutagenized by introduction of the gentamicin cassette into the Bgl II site of the *toxA* gene to construct plasmid pLGR202. The Bgl II site is located near the middle of the *toxA*-coding region (*24*). A 2.4-kb Hind III–Eco RI fragment containing the *gacA* gene was subcloned into pBR322 and then mutagenized by insertion of the gentamicin cassette into the Bam HI site of *gacA* to construct the plasmid pLGR203. Approximately 300 bp of the presumptive *gacA* gene was sequenced (from the Bam HI site) and was found to exhibit 89% identity at the nucleotide level to the *P. fluorescens gacA* gene (*19*). The Bam HI site is located near the middle of the *P. aeruginosa gacA* gene-coding region.
27. Plasmids pLGR201, pLGR202, and pLGR203 were used for gene replacement of *plcS*, *toxA*, and *gacA* genes, respectively, as described [L. G. Rahme, M. N. Mindrinos, N. J. Panopoulos, *J. Bacteriol.* **170**, 575 (1991)], by first selecting for resistance to gentamicin (30 μg/ml) and then screening for sensitivity to carbenicillin (300 μg/ml). Three independent *gacA* mutants were constructed. The structures of all of the resulting marker exchange mutations were verified by DNA blot analysis. The UCBPP-PA14 *plcS* mutant exhibited reduced hemolytic activity on blood agar plates (*17*) and the UCBPP-PA14 *gacA* mutant exhibited sensitivity to ultraviolet (UV) radiation (*21*) and did not produce extracellular cyanide (*19*). None of these three mutations had any detectable effect on either the growth rate or the final density of the bacteria in vitro compared to the wild type in either rich or minimal media.
28. Statistical significance for mortality data was determined by the χ^2 test with Yates'correction. Differences between groups were considered statistically significant at $P \leq 0.05$. All the mutants were significantly different from the wild type (*plcS* and *toxA*, $P \leq 0.05$; *gacA*, $P \leq 0.00005$).
29. A 6.1-kb Bam HI fragment containing the *plcSR* operon of strain UCBPP-PA14 was subcloned into the Bam HI site of plasmid pRR54 and used for genetic complementation studies. A 2.4-kb Eco RI–Eco RV fragment containing only the *toxA* gene from *P. aeruginosa* strain PAK was cloned in the Sma I site of pRR54 and used for complementation studies. An 8.7-kb DNA fragment containing the *gacA-uvrC* operon of strain UCBPP-PA14 was cloned in the polylinker of pRR54 and used for complementation studies. In addition, a 2.4-kb Hind III–Eco RI fragment containing the UCBPP-PA14 *gacA* gene was cloned into pBR322 and used to replace the *gacA* gene interrupted by the gentamicin cassette with a wild-type copy of *gacA*. In the mouse model, the lethality elicited by the complemented *plcS* and *toxA* mutants and the reconstructed *gacA*$^+$ strain was not statistically different from that of the UCBPP-PA14 wild type. The complemented *gacA* mutant was UV-resistant, synthesized cyanide, and elicited approximately 50% lethality in the mouse model as compared to 0% for the *gacA* mutant. In the plant model, the complemented *plcS* and *toxA* mutants and the reconstructed *gacA*$^+$ strain elicited disease symptoms similar to those of wild-type UCBPP-PA14, the reconstructed *gacA*$^+$ strain grew to wild-type levels, the final population level of the complemented *gacA* mutant was approximately 1.5 orders of magnitude less than that of the wild type, and the complemented *toxA* and *plcS* mutants grew to a level that was approximately 0.5 order of magnitude less than that of the wild type. The partial complementation observed in some of these experiments may be due to gene dosage effects.
30. The *toxA* gene is not known to be part of an operon. Because the *plcS* gene is situated in an operon with *plcR*, a gene that regulates *plcS* expression, and the *gacA* gene is situated in an operon with *uvrC*, the reduced pathogenicity phenotypes of the *plcS* and *gacA* mutants may be due to polar effects on the downstream *plcR* or *uvrC* genes.
31. We thank N. Panopoulos for helpful suggestions at the initial stage of this work; M. Schroth, S. Kominos, S. Lory, and J. Goldberg for the *P. aeruginosa* strains and plasmids; and S. Calderwood for helpful advice. Supported by a grant from Shriners Hospitals for Crippled Children and by a grant from Hoechst AG to Massachusetts General Hospital.

23 December 1994; accepted 24 May 1995

Reversal of Raf-1 Activation by Purified and Membrane-Associated Protein Phosphatases

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The Raf-1 protein kinase participates in transduction of mitogenic signals, but its mechanisms of activation are incompletely understood. Treatment of human Raf-1 purified from insect Sf9 cells co-expressing c-H-Ras and Src(Y527F) (in which phenylalanine replaces tyrosine at residue 527) with either serine-threonine or tyrosine phosphatases resulted in enzymatic inactivation of Raf-1. Inactivation of purified Raf-1 was blocked by addition of either the 14-3-3ζ protein or heat shock protein 90. Loading of plasma membranes from transformed cells with guanosine triphosphate (GTP) resulted in inactivation of endogenous or exogenous Raf-1; inactivation was blocked by inclusion of protein phosphatase inhibitors. These results suggest the existence of protein phosphatases in the cell membrane that are regulated by GTP and are responsible for Raf-1 inactivation.

The proto-oncogene product Raf-1 is a serine-threonine protein kinase that functions in one or more protein kinase cascades important for mitogenic signaling (*1*). Raf-1 phosphorylates and activates mitogen-activated protein (MAP) kinase kinase (MKK, also known as MEK), the specific activator of MAP kinase. MAP kinase in turn phosphorylates several regulatory proteins (*2*) in the cytoplasm and nucleus to alter the program of transcription and translation required for mitogenesis.

The mechanism of Raf-1 activation is unclear. Activation of Raf-1 in vivo occurs at the plasma membrane and is dependent on association with the guanosine triphosphate (GTP)–bound form of Ras (*1*). Artificial targeting of Raf-1 to the plasma membrane in vivo bypasses Ras dependence and results in an enhancement of Raf-1 enzymatic activity (*3*). Raf-1 is also activated in vitro by purified plasma membranes from transformed cells in an adenosine triphosphate (ATP)–dependent manner (*4*). However, the association of purified Raf-1 and Ras proteins in the presence of ATP in vitro is not sufficient for Raf-1 activation, suggesting that an ATP-dependent mechanism such as phosphorylation exists for Raf-1 activation at the plasma membrane.

Raf-1 is phosphorylated in quiescent cells, and its phosphorylation increases at several sites in cells stimulated with mitogens (*5–7*). Threonine-268, serines-43, -259, -499, and -621 (*5*), and tyrosines-340 and -341 have been identified as phosphorylation sites in Raf-1 (*6*). Mitogenic stimulation of fibroblasts with platelet-derived growth factor or phorbol esters increases phosphorylation of Ser^{259} (*5*) and Ser^{499} (*6*), respectively. Mutation of Ser^{259} or Ser^{499} to Ala does not inhibit the activation of Raf-1 in insect Sf9 cells by Ras and Src(Y527F) (*5*); the effects of these mutations on activation by phorbol esters are unclear (*7*). Mutation of Ser^{621}, which is phosphorylated in quiescent fibroblasts and in Sf9 cells expressing Raf-1 alone, results in a protein that cannot be activated (*5*). Furthermore, mutation of Tyr^{340} and Tyr^{341}, the major sites of tyrosine phosphorylation, to Phe also results in a Raf-1 pro-

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