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The Posttranscriptional Regulator RsmA Plays a Role in the Interaction between *Pseudomonas aeruginosa* and Human Airway Epithelial Cells by Positively Regulating the Type III Secretion System

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Posttranscriptional regulation of certain virulence-related genes in *Pseudomonas aeruginosa* is brought about by RsmA, a small RNA-binding protein. During interaction with airway epithelial cells, RsmA promoted actin depolymerization, cytotoxicity, and anti-internalization of *P. aeruginosa* by positively regulating the virulence-associated type III secretion system.

Pseudomonas aeruginosa is an opportunistic gram-negative bacillus that causes a variety of serious infections in immunocompromised patients and cystic fibrosis sufferers. Colonization is brought about by the production of virulence factors, including structural and extracellular proteins, secondary metabolites, and the type III secretion system (TTSS). *P. aeruginosa* tightly regulates the production of virulence determinants through numerous interlinked systems, including quorum sensing, alternative sigma factors, the GacS/GacA/RsmZ regulatory network and the posttranscriptional regulator RsmA. RsmA is a small RNA-binding protein that plays an important role in the posttranscriptional regulation of a number of virulence-related genes in *P. aeruginosa* (4, 17, 27). Because of the impact that RsmA has on virulence factor production, we sought to investigate the consequences of RsmA mutation for the interaction between *P. aeruginosa* and human airway epithelial cells in a cell culture model.

The *rsmA* mutant fails to induce actin depolymerization and cytotoxicity. The 16HBE14o$^-$-S normal human bronchial epithelial cell line, which becomes fully differentiated and forms tight junctions (21, 28), was maintained in minimum essential medium (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G-418. For all infection experiments, PAO1 (19), the *rsmA* mutant (27), and Δ3TOX (PAO1 lacking the effector proteins ExoS, ExoT, and ExoY) (30) were cultured aerobically for 16 to 18 h in the cell culture medium without antibiotics (infection medium) at 37°C. Following washing in phosphate-buffered saline, bacterial densities were adjusted so as to infect fully confluent cell monolayers at the indicated multiplicities of infection (MOIs) in infection medium. In this study, infected airway epithelial cells underwent cell rounding and detachment from the tissue culture flask in response to infection with PAO1 but not the *rsmA* mutant (data not shown). Previous studies have indicated that translocation of the effector proteins ExoS, -T, and -Y modulates the host cell cytoskeleton, inducing a rounded phenotype, via actin depolymerization (7, 10, 11). At 3 hours postinfection (starting MOI of 50:1), actin microfilaments, visualized by phalloidin staining and fluorescence microscopy, demonstrated PAO1-induced actin rearrangements, reduced cell-cell contact, and cellular retraction (Fig. 1A). In contrast to the PAO1-induced phenotype, infection with the *rsmA* mutant or Δ3TOX resulted in a phenotype similar to that observed in uninfected control cells, where there was no evidence of actin reorganization (Fig. 1A).

Cytotoxicity of airway epithelial cells was measured by quantifying the release of lactate dehydrogenase (LDH) into culture supernatants, using an LDH cytotoxicity detection kit (Roche) according to manufacturer's instructions. PAO1 induced cytotoxicity of airway epithelial cells to a greater extent than the *rsmA* mutant or Δ3TOX at 9 h postinfection, using a starting MOI of 50:1 (Fig. 1B).

The *rsmA* mutant displays increased invasion of airway epithelial cells. In order to ensure that the failure of the *rsmA* mutant to induce actin depolymerization was not simply due to its inability to interact with epithelial cells, the number of adhered and invasive bacteria at 3 h postinfection was quantified as described previously (3), with modifications. Due to noticeable rounding and detachment of epithelial cells in response to PAO1 infection, adhered bacteria were enumerated by subtracting the number of extracellular and invasive bacteria from the total bacterial count. Adhesion assays indicated no significant difference in adherence of the *rsmA* mutant compared to PAO1 at five of the six MOIs investigated ($P > 0.05$, Student's t test) (data not shown). In contrast, significantly increased invasion of epithelial cells by the *rsmA* mutant, relative to PAO1, was observed at four of the six MOIs tested ($P = 0.007$, 0.008, 0.040, and 0.023 at MOIs of 10:1, 50:1, 100:1, and 500:1, respectively) (Fig. 2). Increased bacterial uptake by epithelial cells has previously been described for strains attenuated in production of the TTSS (16), and internalization of *P. aeruginosa* by epithelial cells and macrophages has been shown to be reduced by specific translocation of the effector proteins ExoS, -T, and -Y (7, 10, 11, 14).

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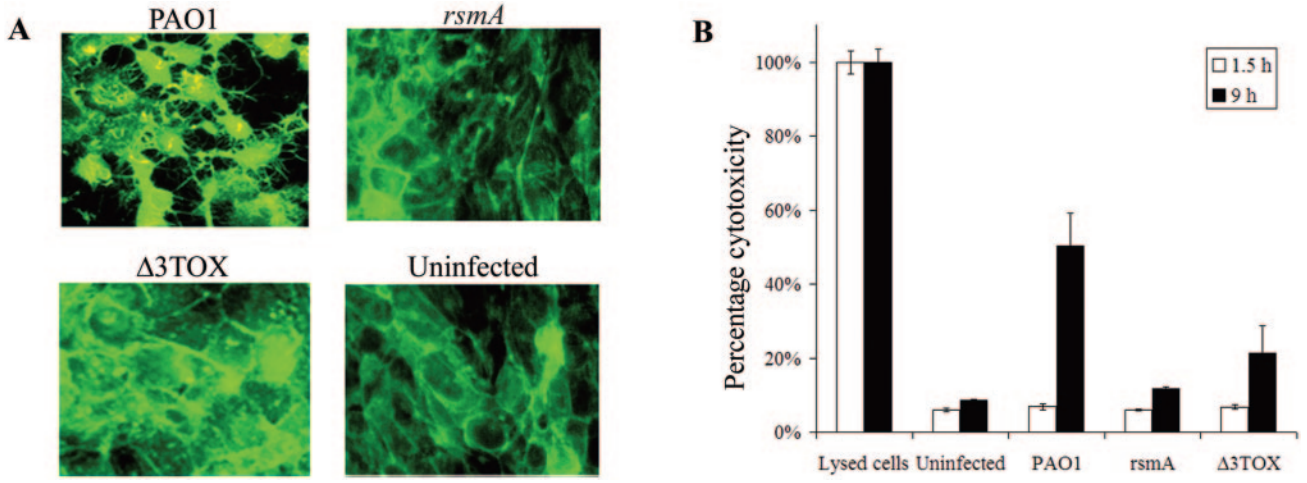

FIG. 1. Actin staining and cytotoxicity of airway epithelial cells in response to *P. aeruginosa* infection. (A) Epithelial cells were uninfected or exposed to PAO1, the *rsmA* mutant, or Δ3TOX at an MOI of 50:1. At 3 hours postinfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.1% Triton X-100, and stained with Cy2-labeled phalloidin for 40 min at room temperature, and actin microfilaments were captured with an Olympus IX-70 at a magnification of ×600 and processed using Adobe Photoshop 6.0. (B) The release of LDH into culture supernatants was measured using an LDH cytotoxicity detection kit (Roche). Cytotoxicity is expressed as a percentage of the total amount of LDH released from cells treated with 1% Triton X-100 (lysed cells). LDH release was measured 1.5 h and 9 h postinfection with a starting MOI of 50:1. Error bars represent the SDs for triplicate experiments.

The *rsmA* mutant is defective in the production of key effector and translocation proteins and shows decreased expression of regulators of the TTSS. Bacterial growth in Luria-Bertani (LB) broth supplemented with 10 mM nitrilotriacetic acid (13) induced secretion of type III proteins in PAO1 (data not shown). Secreted protein samples were prepared as described previously (15), normalized to 15 μg protein/lane, and subjected to sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes and probed with rabbit antisera against ExoS, ExoT, PopB, PopD, and PcrV, followed by secondary goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Dakocytomation), and developed with chemiluminescent substrate (Bio-Rad). Under nitrilotriacetic acid-inducing conditions, the *rsmA* mutant failed to secrete detectable amounts of ExoS or ExoT (effector proteins) or of PopB, PopD, or PcrV (essential for pore formation). In contrast, ExoS, ExoT, PopB, PopD, and PcrV were secreted by PAO1 (Fig. 3). The ability of the *rsmA* mutant to influence actin depolymerization, cytotoxicity, and invasion may be due to a failure or defect in the secretion of key effector and translocation proteins of the TTSS or, indeed, to altered expression of regulators of the TTSS.

Recently, work in this laboratory has shown that *vfr* transcription is reduced in the *rsmA* mutant relative to PAO1 in LB broth (5). In our current study, the expression of several known regulators of the TTSS, namely, *retS* (12, 22, 32), *exsA* (9), (20), *exsC* (8), *exsD* (25), *cyaB* (31), and *vfr* (31), was analyzed in PAO1 and the *rsmA* mutant during interaction with epithelial cells. At 3 hours postinfection (starting MOI of 50:1), epithelial

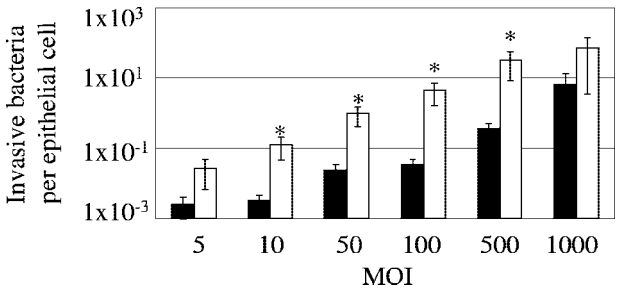

FIG. 2. Comparative study showing the ability of *P. aeruginosa* PAO1 (black bars) and the *rsmA* mutant (white bars) to invade airway epithelial cells. Results are presented as the mean number of bacteria/epithelial cell ± SD. Data from triplicate samples from two biological replicate experiments are presented. *, $P \leq 0.05$ for PAO1 versus the *rsmA* mutant (Student's *t* test).

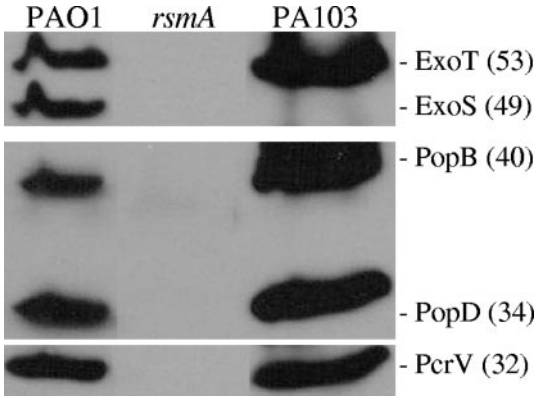

FIG. 3. Immunoblotting of type III proteins. Secreted proteins (15 μg) were prepared as described in Materials and Methods and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were probed with antisera against ExoS, ExoT, PopB, PopD, and PcrV. Detected proteins and molecular masses are indicated on the right. *P. aeruginosa* PA103 was used as a control, as it does not express ExoS. Representative data from two independent experiments are shown.

cells were lysed using 0.1% Triton X-100 to release intracellular bacteria and total bacterial cell pellets were collected for RNA extraction. Bacterial samples cultured for 3 h in infection medium without epithelial cells were used as controls. Total RNA was isolated using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. For experiments involving *N*-butanoyl-L-homoserine lactone (C_4-HSL), epithelial cells were infected with PAO1, with or without C_4-HSL, at a final concentration of 10 μM and bacterial RNA was isolated as described above. RNA samples were reverse transcribed using random hexamers (Promega). Real-time reverse transcription-PCR was performed in a LightCycler apparatus using LC Faststart reaction mix SYBR green I (Roche). All test genes were normalized to the housekeeping gene *proC* (5, 29) (primer sequences are available upon request). A change of fivefold or greater was used as a cutoff to identify differential gene expression. Basal transcription levels were measured when PAO1 and the *rsmA* mutant were grown in infection medium in the absence of epithelial cells. Transcript levels were also measured in PAO1 and the *rsmA* mutant following infection of epithelial cells (inducing conditions). Under basal conditions, the *rsmA* mutant displayed significantly lower expression of *exsA*, *exsC*, *cyaB*, and *vfr* but not *exsD* or *retS* relative to PAO1, as follows (mean fold change ± standard deviation [SD] in the *rsmA* mutant compared to PAO1): *exsA*, −8.45 ± 4.18; *exsC*, −20.51 ± 1.67; *cyaB*, −5.26 ± 1.95; *vfr*, −12.58 ± 1.68; *exsD*, −1.81 ± 0.66; and *retS*, −4.69 ± 3.33. This trend in gene expression was also observed following bacterial interaction with epithelial cells. However, the fold change difference in gene expression between PAO1 and the *rsmA* mutant was more pronounced for all the transcripts examined, as follows: *exsA*, −15.10 ± 2.05; *exsC*, −36.51 ± 16.83; *cyaB*, −10.52 ± 3.30; and *vfr*, −19.80 ± 3.61. In addition to this, under these conditions a significant fold change difference was observed in the expression of *retS* (−5.94 ± 2.22) and *exsD* (−31.06 ± 3.67) in the *rsmA* mutant compared to PAO1. Thus, during the interaction of *P. aeruginosa* with epithelial cells, a lack of RsmA resulted in decreased gene expression of five positive regulators (RetS, ExsA, ExsC, CyaB, and Vfr) and one negative regulator (ExsD) of the TTSS.

There is evidence indicating that neither a *lasR* nor an *rhlR* mutation has an effect on the production of ExoS, ExoT, or PcrV under inducing conditions in PAO1 (24). However, in a recent study, addition of C_4-HSL was shown to negatively effect the transcription of the *exoS*, *exoT*, and *exoY* genes and the *exsD*, *pcrG*, and *popN* operons in *P. aeruginosa* (2). It has been shown that the *rsmA* mutant produced higher levels of C_4-HSL than PAO1 (27). However, RsmA affects the transcription of *exsA*, *exsC*, *cyaB*, *vfr*, and *rtsM* independent of increased C_4-HSL concentration (data not shown).

The *rsmA* mutant displays increased antibiotic resistance. Previously published data have indicated a relationship between the TTSS and the expression of multidrug efflux (Mex) pumps in *P. aeruginosa*, whereby overexpression of MexCD-OprJ or MexEF-OprN resulted in decreased expression of the TTSS regulon (24). Separately, a MexAB-OprM mutant showed reduced invasion of MDCK epithelial cells, suggesting that the MexAB-OprM system may have an effect on the TTSS, as evidenced by the effect of MexAB-OprM on the invasion capacity of *P. aeruginosa* (18). Recently in this laboratory, increased expression of the *mexEF-oprN* operon in the *rsmA* mutant has been shown (5). To further investigate the role of RsmA in antibiotic resistance, bacterial strains were grown in a grid-like format on LB agar, transferred to LB agar with or without antibiotics, and incubated at 37°C for 24 h, and the minimal bactericidal concentration was recorded. The results indicated that compared to PAO1, the *rsmA* mutant had increased resistance to amikacin, nalidixic acid, and trimethoprim (2-fold in each case) and ceftazidime (128-fold) (known substrates of MexEF-OprN), as well as the aminoglycoside gentamicin (4-fold), a specific substrate of MexXY-OprM. Levels of resistance to chloramphenicol and tetracycline were comparable, and the *rsmA* mutant showed decreased resistance to polymyxin B and colistin (twofold in each case). Overexpression of the MexAB-OprM system has been shown to lead to increased secretion of 3-oxo-C_{12}-HSL (26). However the increased level of C_4-HSL observed in the *rsmA* mutant is unlikely to be due to overexpression of MexEF-OprN, as C_4-HSL is thought to diffuse freely across the bacterial membrane (26). In this study, we confirm the role of RsmA in antibiotic resistance, thus identifying RsmA as a possible global regulator involved in regulating cross talk between antibiotic resistance and the virulence-associated TTSS.

Goodman and coworkers have shown that RetS has a positive effect on the TTSS (12), and they hypothesized that this occurred through the GacS/GacA/RsmZ regulatory pathway and its subsequent influence on RsmA. We have now clearly identified RsmA as an additional player in the positive control of the TTSS in *P. aeruginosa*. Furthermore, RsmA was shown to have a positive effect on the transcription of *retS* during infection of epithelial cells. This effect is most likely to be indirect, as no difference in the stability of the *retS* transcript between PAO1 and the *rsmA* mutant was observed (data not shown).

Furthermore, this study showed that RsmA increased the transcription of *cyaB* and *vfr*, neither of which is significantly altered at the transcriptional level by a *retS* mutation (12, 22). Therefore, RsmA controls important regulators of the TTSS, some of which are not transcriptionally regulated by RetS.

P. aeruginosa RsmA is a homolog of *Escherichia coli* CsrA. In *Salmonella enterica* serovar Typhimurium, a CsrA homolog has been shown to be a positive regulator of invasion genes and components of the TTSS (1, 23). In contrast to this, the overexpression of RsmA in *Erwinia carotovora* leads to repression of genes encoding structural and secreted proteins of the TTSS (6). Thus, the mechanism of RsmA-mediated regulation of the TTSS in *P. aeruginosa*, a model organism for both plant and animal pathogens, warrants further study.

RsmA is involved in actin depolymerization and cytotoxicity of epithelial cells and in anti-internalization of *P. aeruginosa*. RsmA achieves these effects by positively regulating the virulence-associated TTSS. RsmA negatively affects the expression of MexEF-OprN and possibly additional Mex pumps, as indicated by antibiotic resistance profiling. However, whether RsmA-mediated regulation of the TTSS is dependent on Mex pumps or an alternative mechanism under RsmA control is currently unknown. RsmA is known to regulate pathogenicity determinants such as motility, biofilm formation, HSL and secondary metabolite production, and iron acquisition (4, 17, 27) and now also the TTSS and antibiotic resistance. Mecha-

nisms aimed at reducing the production of RsmA may aid in diminishing the expression of selected virulence factors, a number of which are important in the initial stages of infection.

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REFERENCES

1. **Altier, C., M. Suyemoto, and S. D. Lawhon.** 2000. Regulation of *Salmonella enterica* serovar Typhimurium invasion genes by *csrA*. Infect. Immun. **68:** 6790–6797.
2. **Bleves, S., C. Soscia, P. Nogueira-Orlandi, A. Lazdunski, and A. Filloux.** 2005. Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **187:**3898–3902.
3. **Burns, J. L., M. Jonas, E. Y. Chi, D. K. Clark, A. Berger, and A. Griffith.** 1996. Invasion of respiratory epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia*. Infect. Immun. **64:**4054–4059.
4. **Burrowes, E., A. Abbas, A. O'Neill, C. Adams, and F. O'Gara.** 2005. Characterisation of the regulatory RNA RsmB from Pseudomonas aeruginosa PAO1. Res. Microbiol. **156:**7–16.
5. **Burrowes, E., C. Baysse, C. Adams, and F. O' Gara.** 2006. Influence of the regulatory protein RsmA on cellular functions in Pseudomonas aeruginosa PAO1 as revealed by transcriptome analysis. Microbiology **152:**405–418.
6. **Chatterjee, A., Y. Cui, and A. K. Chatterjee.** 2002. Regulation of Erwinia carotovora hrpL(Ecc) (sigma-L(Ecc)), which encodes an extracytoplasmic function subfamily of sigma factor required for expression of the HRP regulon. Mol. Plant-Microbe Interact. **15:**971–980.
7. **Cowell, B. A., D. J. Evans, and S. M. Fleiszig.** 2005. Actin cytoskeleton disruption by ExoY and its effects on Pseudomonas aeruginosa invasion. FEMS Microbiol. Lett. **250:**71–76.
8. **Dasgupta, N., G. L. Lykken, M. C. Wolfgang, and T. L. Yahr.** 2004. A novel anti-anti-activator mechanism regulates expression of the Pseudomonas aeruginosa type III secretion system. Mol. Microbiol. **53:**297–308.
9. **Frank, D. W.** 1997. The exoenzyme S regulon of Pseudomonas aeruginosa. Mol. Microbiol. **26:**621–629.
10. **Garrity-Ryan, L., B. Kazmierczak, R. Kowal, J. Comolli, A. Hauser, and J. N. Engel.** 2000. The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages. Infect. Immun. **68:**7100–7113.
11. **Geiser, T. K., B. I. Kazmierczak, L. K. Garrity-Ryan, M. A. Matthay, and J. N. Engel.** 2001. Pseudomonas aeruginosa ExoT inhibits in vitro lung epithelial wound repair. Cell Microbiol. **3:**223–236.
12. **Goodman, A. L., B. Kulasekara, A. Rietsch, D. Boyd, R. S. Smith, and S. Lory.** 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in Pseudomonas aeruginosa. Dev. Cell **7:**745–754.
13. **Goranson, J., A. K. Hovey, and D. W. Frank.** 1997. Functional analysis of *exsC* and *exsB* in regulation of exoenzyme S production by *Pseudomonas aeruginosa*. J. Bacteriol. **179:**1646–1654.
14. **Ha, U., and S. Jin.** 2001. Growth phase-dependent invasion of *Pseudomonas aeruginosa* and its survival within HeLa cells. Infect. Immun. **69:**4398–4406.
15. **Hauser, A. R., E. Cobb, M. Bodi, D. Mariscal, J. Valles, J. N. Engel, and J. Rello.** 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa. Crit. Care Med. **30:**521–528.
16. **Hauser, A. R., S. Fleiszig, P. J. Kang, K. Mostov, and J. N. Engel.** 1998. Defects in type III secretion correlate with internalization of *Pseudomonas aeruginosa* by epithelial cells. Infect. Immun. **66:**1413–1420.
17. **Heurlier, K., F. Williams, S. Heeb, C. Dormond, G. Pessi, D. Singer, M. Camara, P. Williams, and D. Haas.** 2004. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **186:** 2936–2945.
18. **Hirakata, Y., R. Srikumar, K. Poole, N. Gotoh, T. Suematsu, S. Kohno, S. Kamihira, R. E. Hancock, and D. P. Speert.** 2002. Multidrug efflux systems play an important role in the invasiveness of Pseudomonas aeruginosa. J. Exp. Med. **196:**109–118.
19. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
20. **Hovey, A. K., and D. W. Frank.** 1995. Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon. J. Bacteriol. **177:**4427–4436.
21. **Kube, D., U. Sontich, D. Fletcher, and P. B. Davis.** 2001. Proinflammatory cytokine responses to P. aeruginosa infection in human airway epithelial cell lines. Am. J. Physiol. Lung Cell Mol. Physiol. **280:**L493–502.
22. **Laskowski, M. A., E. Osborn, and B. I. Kazmierczak.** 2004. A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in Pseudomonas aeruginosa. Mol. Microbiol. **54:**1090–1103.
23. **Lawhon, S. D., J. G. Frye, M. Suyemoto, S. Porwollik, M. McClelland, and C. Altier.** 2003. Global regulation by CsrA in Salmonella typhimurium. Mol. Microbiol. **48:**1633–1645.
24. **Linares, J. F., J. A. Lopez, E. Camafeita, J. P. Albar, F. Rojo, and J. L. Martinez.** 2005. Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in *Pseudomonas aeruginosa*. J. Bacteriol. **187:**1384–1391.
25. **McCaw, M. L., G. L. Lykken, P. K. Singh, and T. L. Yahr.** 2002. ExsD is a negative regulator of the Pseudomonas aeruginosa type III secretion regulon. Mol. Microbiol. **46:**1123–1133.
26. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J. Bacteriol. **181:**1203–1210.
27. **Pessi, G., F. Williams, Z. Hindle, K. Heurlier, M. T. Holden, M. Camara, D. Haas, and P. Williams.** 2001. The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. J. Bacteriol. **183:**6676–6683.
28. **Rajan, S., G. Cacalano, R. Bryan, A. J. Ratner, C. U. Sontich, A. van Heerckeren, P. Davis, and A. Prince.** 2000. Pseudomonas aeruginosa induction of apoptosis in respiratory epithelial cells: analysis of the effects of cystic fibrosis transmembrane conductance regulator dysfunction and bacterial virulence factors. Am. J. Respir. Cell Mol. Biol. **23:**304–312.
29. **Savli, H., A. Karadenizli, F. Kolayli, S. Gundes, U. Ozbek, and H. Vahaboglu.** 2003. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of Pseudomonas aeruginosa by real-time quantitative RT-PCR. J. Med. Microbiol. **52:**403–408.
30. **Vance, R. E., A. Rietsch, and J. J. Mekalanos.** 2005. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of *Pseudomonas aeruginosa* PAO1 in vivo. Infect. Immun. **73:**1706–1713.
31. **Wolfgang, M. C., V. T. Lee, M. E. Gilmore, and S. Lory.** 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev. Cell **4:**253–263.
32. **Zolfaghar, I., A. A. Angus, P. J. Kang, A. To, D. J. Evans, and S. M. Fleiszig.** 2005. Mutation of retS, encoding a putative hybrid two-component regulatory protein in Pseudomonas aeruginosa, attenuates multiple virulence mechanisms. Microbes Infect. **7:**1305–1316.

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