INFECTION AND IMMUNITY, July 2002, p. 3973–3977 Vol. 70, No. 7
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.7.3973–3977.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

Activation of the *Pseudomonas aeruginosa* Type III Secretion System Requires an Intact Pyruvate Dehydrogenase *aceAB* Operon

Denis Dacheux,[1,2] Olivier Epaulard,[1,3] Arjan de Groot,[1] Benoit Guery,[4] Rozen Leberre,[4] Ina Attree,[1] Benoit Polack,[3] and Bertrand Toussaint[1,5]*

Département de Biologie Moléculaire et Structurale, BBSI, UMR 5092 CNRS, CEA-Grenoble,[1] and GREPI EA 2938 MENRT[3] and DBPC/Enzymologie,[5] CHU-Grenoble, Grenoble, Université de Bordeaux, Paris,[2] and Réanimation Médicale et Maladies Infectieuses, CH Dron, Tourcoing,[4] France

Received 23 January 2002/Returned for modification 21 March 2002/Accepted 15 April 2002

***Pseudomonas aeruginosa* clinical cystic fibrosis isolate CHA was mutagenized with Tn*5*Tc to identify new genes involved in type III secretion system (TTSS)-dependent cytotoxicity toward human polymorphonuclear neutrophils. Among 25 mutants affected in TTSS function, 14 contained the insertion at different positions in the *aceAB* operon encoding the PDH-E1 and -E2 subunits of pyruvate dehydrogenase. In PDH mutants, no transcriptional activation of TTSS genes in response to calcium depletion occurred. Expression in *trans* of ExsA restored TTSS function and cytotoxicity.**

Pseudomonas aeruginosa is a major gram-negative opportunistic pathogen responsible for both acute and chronic infections. Chronic *P. aeruginosa* colonization of the airways of cystic fibrosis (CF) patients and subsequent acute infections are the leading cause of morbidity in CF. The adaptation of *P. aeruginosa* to the environment of CF lungs is accompanied by the synthesis of diverse virulence factors comprising various exoproteins and mucoid exopolysaccharides (11). One of the reasons for the secretion of virulence factors is to allow the bacteria to avoid the host defense mechanism, the main line of which comprises the bactericidal activity of polymorphonuclear neutrophils (PMNs). The type III secretion system (TTSS) is a recently identified virulence determinant of *P. aeruginosa* (19). It encodes on the order of 20 proteins, including (i) components of a secretory apparatus, (ii) components of machinery devoted to the direct translocation of effectors into the host cell cytoplasm, and (iii) four effectors, ExoS, -T, -U, and -Y, thought to alter normal host cell processes (10). It has recently been shown that some CF isolates of *P. aeruginosa* are able to resist the bactericidal activity of PMNs and to induce rapid TTSS-dependent oncotic cell death of both PMNs and macrophages (1–4, 15). The first step of the activation of the expression of TTSS genes in *P. aeruginosa* is the upregulation of the transcription of the *exsA* gene (part of the *exsCBA* operon) in response to different stimuli such as calcium depletion in vitro or target cell contact in vivo (9). In a previous study we showed that expression of ExsA in *trans* was sufficient to activate in vitro secretion and ex vivo cytotoxicity toward phagocytes in noncytotoxic CF isolates (2). Here we used large-scale genetic screening to identify new genes required for cytotoxicity.

The bacterial strains and plasmids used in this study are listed in Table 1. The parental CHA strain has previously been characterized as cytotoxic and able to induce rapid TTSS-dependent oncosis of PMNs and J774 macrophages (1–4). This strain produces the type III effectors ExoS and ExoT but not ExoU because of the absence of the *exoU* gene in the CHA genome (1). Transposon mutants of the cytotoxic *P. aeruginosa* CHA strain were generated by bacterial conjugation. A conjugation-proficient suicide plasmid, pUTTn5-Tet^r was introduced into the *P. aeruginosa* strain CHA by triparental mating (8 h at 37°C) using the helper plasmid pRK2013. Mating mixtures were recovered and resuspended in 10 ml of sterile 10 mM $MgSO_4$ before plating on Vogel-Bonner minimal medium (17) agar plates containing tetracycline (100 μg ml^{-1}) to counterselect against *Escherichia coli* donor strains. Growth on Vogel-Bonner minimal medium allowed us to eliminate auxotrophic mutants, which may appear noncytotoxic. Southern blot analysis of chromosomal DNA isolated from randomly picked mutants was performed to verify that most of the obtained mutants resulted from independent transposition events rather than from replication of siblings. Only a single and different *Pst*I restriction fragment from each random mutant hybridized to a probe derived from PCR labeling of the tetracycline gene, amplified using primers 5′TAATGCGGTAGTTTATCACAG and 5′ACTGGCGATGCTGTCGGAATG (GenBank accession number X67018), indicating that only a single transposon insertion event occurred in each mutant. For the cytotoxicity assay, the human PMNs were obtained as described previously (1). The assay conditions for *P. aeruginosa* cytotoxicity on PMNs were adapted from previously reported conditions (1) for 96-well microplates. In each well, 10^6 PMNs were infected with 10^7 bacteria. For each experiment a positive control corresponding to the parental cytotoxic CHA strain and a negative control using its ExsA isogenic, noncytotoxic CHA-D1 mutant were added (1). Cytotoxicity was quantified spectrophotometrically by measuring the release of lactate dehydrogenase (LDH) (1, 4) in the infection medium 3 h after addition of bacteria. The PMNs released less than 10% LDH in uninfected conditions whereas infections with the CHA strain resulted in an eightfold increase in LDH release. The screening of 5,070 mutants on PMNs allowed us to select 53 mutants yielding less than 30% of the cytotoxicity of CHA. These experiments were

* Corresponding author. Mailing address: DBPC/Enzymologie, CHU-Grenoble BP217, 38043 Grenoble cedex, France. Phone: 33.4.76.76.54.83. Fax: 33.4.76.76.56.08. E-mail: btoussaint@chu-grenoble.fr.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties or genotype[a]	Source or reference
Strains		
P. aeruginosa		
CHA	Mucoid CF isolate, cytotoxic	5
CHA-D1	*exsA*::Gm mutant of CHA, noncytotoxic	1
E. coli DH5α	Φ80d*lacZ*ΔM15 F^- *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169	Eurogentec
Plasmids		
pUC18	Ap^r, cloning vector	New England Biolabs
PRK2013	Km^r, mobilizing plasmid helper	8
pUTTn5-Tet^r	Tet^r, containing Tn*5* transposon	6
pIApC	Cb^r Ap^r, containing *gfpmut3* gene under p*exsC* promoter	3
pAG710	Cb^r Ap^r, containing *aceB* gene under constitutive promoter	This work; reference 1
pDD2	pUCP20-derived plasmid containing *exsA* gene under a constitutive promoter	3

[a] Ap^r, ampicillin resistance; Tet^r, tetracycline resistance; Km^r, kanamycin resistance; Cb^r, carbenicillin resistance.

performed in triplicate at least three times with several different PMN preparations.

Each noncytotoxic mutant was checked for the functionality of TTSS by examining the protein secretion profile of the culture supernatant with or without induction of the secretion system by calcium depletion (19). Among the several proteins secreted when the CHA strain is grown under calcium-depleted conditions, four major proteins have been shown to be characteristic of TTSS: ExoS, ExoT, PopB, and PopD (1, 4) (Fig. 1A). Twenty-five of the mutants were found to be unable to give a secreted protein profile similar to that of CHA (Fig. 1 and Table 2). The mutants affected in TTSS function were genetically characterized by sequencing the Tn*5* transposon insertion site. Briefly, *P. aeruginosa* chromosomal DNA was isolated, digested with the restriction enzyme *Pst*I, and then cloned in pUC18. Plasmids from the selected colonies that grew on 10-μg ml^{-1} tetracycline Luria-Bertani (LB) medium were sequenced (Genome Express, Grenoble, France) with a primer hybridizing the 3′ end of the transposon (5′GCCGGA TCCGCCGGTAGAC) (6). Comparison of the nucleotide sequences with the data bank using the Blast 2.0 program (www.ncbi.nlm.nih.gov/blast) and the finished genomic sequence of *P. aeruginosa* (16) revealed possible functions for all the genes harboring transposon insertions (Table 2). The number of occurrences for each of the inactivated genes is indicated. For mutants 12, 21, 22, 23, 26, 43, and 46, ExoS and ExoT bands were completely absent from the protein profile (Fig. 1A). However, among these mutants, only 12 and 22 had an insertion in a known TTSS gene, *exsD* and *pscL*, respectively. We cannot exclude that Tn*5* insertions have a polar effect on downstream genes, but the results presented here suggest the involvement of new genes in TTSS functioning. We will describe only two of them (*aceA* and *aceB*) further in this work and will perform complementation with them. Mutant 37, inactivated in *pcrV*, secreted ExoS and ExoT but failed to secrete PopB and PopD. Mutant 43 had transposon insertion in the *ppX*

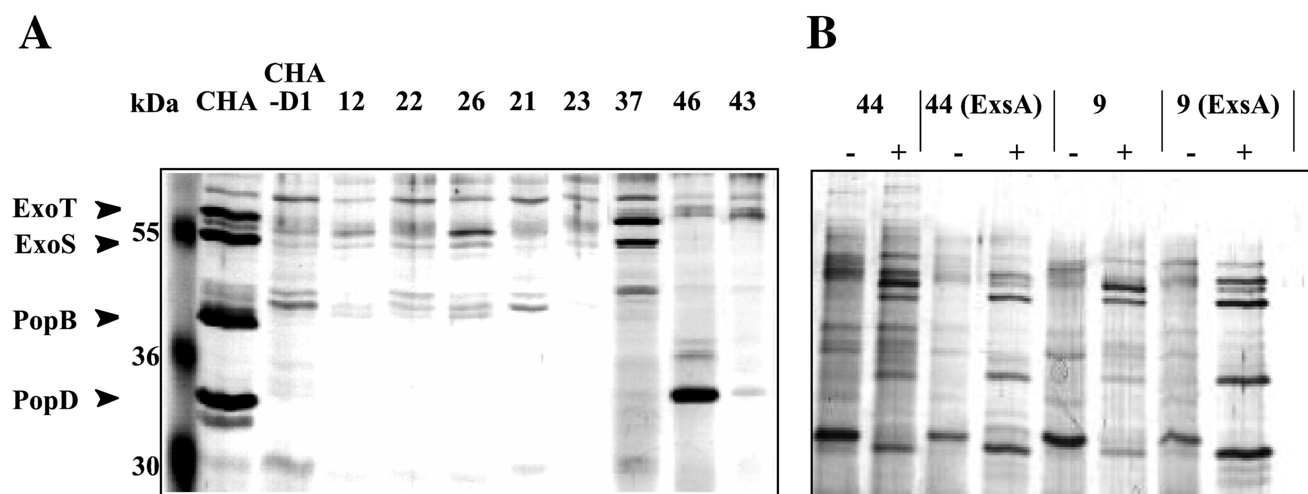

FIG. 1. Analysis of TTSS function by electrophoretic analysis of *P. aeruginosa* proteins secreted into extracellular media under TTSS-inducing calcium depletion conditions (5 mM EGTA, 20 mM $MgCl_2$). Secreted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by silver staining. In each lane, the same amount of supernatants collected from culture grown to the same optical density at 600 nm was used. (A) Profile obtained with CHA, CHA-D1, and noncytotoxic mutants cultured in inducing conditions. (B) Profile obtained either in noninducing (−) or inducing (+) conditions with mutant 44 (*aceA*) and mutant 9 (*aceB*). (ExsA), the strain contained the pDD2 plasmid expressing ExsA. The CHA wild type was used as a control for both panels.

TABLE 2. TTSS-deficient noncytotoxic mutants

Mutant	Gene(s)	GenBank accession no.	Cytotoxicity (% of CHA)[a]	No. of gene occurence	TTSS function[b]
12	*exsD*	PA1714	3	1	−
21	*phoA*	PA3296	3	1	−
22	*pscL*	PA1725	3	1	−
23	*rpoN*	PA4462	2	1	−
26	*mreC*	PA4480	8	1	−
43	*ppx*	PA5241	10	1	−
36, 37	*pcrV*	PA1706	8	2	−(PopB/D)
27, 32, 46	*dsbA*	PA5489	2	3	−
24, 25, 44, 52	*aceA*	PA5015	1	4	Small amount
9, 11, 13, 39, 42, 45, 47, 50, 53, 54	*aceB*	PA5016	3	10	Small amount
44 (pDD2)	*aceA*, with constitutive *exsA*		80		+
9 (pDD2)	*aceB*, with constitutive *exsA*		80		+
9 (pAG710)	*aceB*, complemented *aceB*		70		+

[a] For further details, see the text. Values are means of three experiments.
[b] +, TTSS function is present; −, no TTSS function.

gene encoding an exopolyphosphatase. In *P. aeruginosa*, the genes encoding polyphosphate kinase and exopolyphosphate phosphatase, which are involved in polyphosphate synthesis and degradation, respectively, are contiguous and convergently transcribed (20). Mutant 43 (*ppX*) presents a clear defect in the secretion of type III effectors (Fig. 1). The intracellular level of inorganic polyphosphate is regulated through the action of the exopolyphosphate kinase and phosphatase. Many studies have been performed to characterize the effects of inactivation of polyphosphate kinase, which is involved not only in the twitching motility phenotype, but also in biofilm development, quorum sensing, and the virulence of *P. aeruginosa* (13). The work described above suggests that polyphosphate phosphatase could also be involved in TTSS functioning. Mutant 46 is inactivated in the *dsbA* gene encoding a periplasmic thiol/disulfide oxidoreductase known to have pleiotropic effects in *E. coli* and to affect the periplasmic maturation of TTSS proteins in *Shigella flexneri* (7). It has to be noticed that the main protein band visible on the profile of mutant 46 is not PopD: the band actually migrates below the level of PopD and was not seen in any of the experiments done with the different *dsbA* mutants. The mutants in *aceB* (pyruvate dehydrogenase [PDH]-E2 subunit, dihydrolipoamide acetyltransferase) and *aceA* (PDH-E1 subunit, PDH) have a secretion profile containing the four characteristic proteins but in much lower amounts (Fig. 1). These PDH mutants correspond to the majority (14 of 25) of the isolated TTSS-deficient mutants. The transposon insertion sites of the *aceA* or *aceB* mutants determined by sequencing were located at different positions throughout the *aceAB* operon, indicating the absence of a true hot spot of transposition. We focused on two representatives of these mutants (9 and 44) in order to analyze why an insertion in the *aceAB* operon results in a defect in TTSS-dependent cytotoxicity.

We previously showed that expression of ExsA in *trans* confers TTSS-dependent cytotoxicity on noncytotoxic *P. aeruginosa* CF isolates (3). We transformed mutant PDH-E1 and -E2 with the plasmid pDD2 carrying the constitutively expressed *exsA* gene (3). The cytotoxicity level of the PDH mutants containing pDD2 reached 80% (±11%) of the level of the wild-type CHA strain (Table 2). Furthermore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of supernatants of PDH mutants and PDH mutants containing pDD2 confirmed a partial restoration of the TTSS function (Fig. 1B). Taken together, these results suggest that in the PDH mutants *exsA* expression is not induced, which does not allow the TTSS to function efficiently. To verify this hypothesis we performed transcriptional analysis in PDH mutants containing the plasmid pIApC, which harbors a transcriptional fusion of the *exsCBA* promoter (pC) with the *gfp* gene (3). Strains were cultured under noninduced and TTSS-inducing conditions (5 mM EGTA, 20 mM $MgCl_2$) and fluorescence was measured as described previously (3). Both the *aceA* and *aceB* mutants were unable to activate transcription from the *exsCBA* promoter in response to calcium depletion (Fig. 2). Previous studies concerning PDH mutants of *P. aeruginosa* have shown that

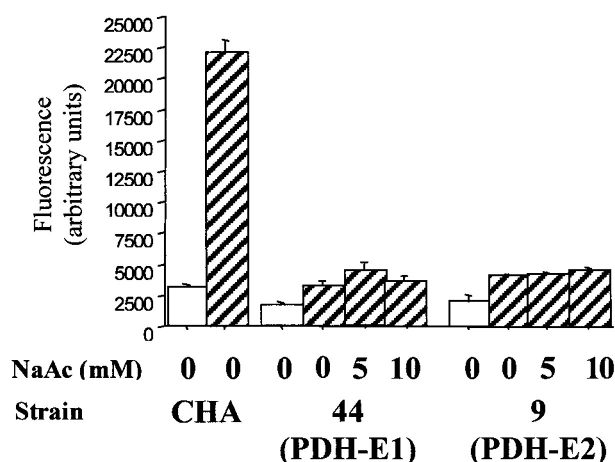

FIG. 2. GFP fluorescence in CHA and PDH mutants transformed with plasmid pIApC grown in LB medium (white bars) or in LB medium supplemented with 5 mM EGTA and 20 mM $MgCl_2$ (inducing conditions) (hatched bars). The final concentration of sodium acetate in the culture medium is indicated. Error bars represent the standard deviations. Data are from at least three independent experiments.

they require 5 mM acetate for normal aerobic growth (12). We checked the effect of supplementation with 5 and 10 mM sodium acetate, pH 7.2, in LB culture on the level of expression of the *exsCBA* operon and cytotoxicity. Although able to grow as fast as the CHA strain when supplemented with 5 mM acetate (data not shown), PDH mutants were still unable to activate transcription of the *exsCBA* operon upon calcium limitation (Fig. 2). Furthermore, cytotoxicity was not improved by acetate supplementation (data not shown). Although we cannot exclude that the mutation in the *aceAB* operon could lead to a more global effect on cellular physiology, the results obtained with growth on acetate suggest that the metabolic effect of PDH inactivation is not responsible for the observed phenotype. In order to eliminate polar effects of the transposon mutation we performed complementation of the PDH-E2 mutants with the *aceB* gene isolated from CHA genomic DNA. Plasmid pAG710 was obtained by amplifying *aceB* using oligos ABX1 (5′-TATA**TCTAGA**GCGGTGGTGGCACGTAA) and ABX2 (GAGA**TCTAGA**TTCGCCGAGCCGTCAC) (*Xba*I sites are shown in bold). The PCR contained genomic DNA from CHA as template and we used *Pfu* polymerase. The obtained 1.8-kb fragment was digested with *Xba*I and cloned in the proper orientation behind the X12 promoter in pIAX12 (1). Plasmid pAG710 was introduced in PDH-E2 mutants by electroporation. The restoration of both cytotoxicity toward PMNs to 70% (±9%) of the level of the wild-type CHA strain (Table 2) and the in vitro TTSS secretion was noticed, allowing us to eliminate a possible polar effect of the Tn*5*Tc insertion on downstream genes. No complementation of the *aceA* mutant was noticed with the *aceB* genes (data not shown).

In view of our results, we investigated the virulence of the PDH mutants 9 and 44 in a rat model of pneumonia. Ten Sprague-Dawley rats (280 to 300 g) were infected with either the wild-type CHA strain, a CHA-D1 ExsA-deficient mutant (1), PDH mutant 44 (*aceA*), or PDH mutant 9 (*aceB*). Rats were briefly anesthetized with ethanol ether. The neck was washed extensively with 70°C ethanol, and an anterior median incision was performed to locate the trachea with forceps. A bacterial inoculum of 2×10^9 bacteria/kg of body weight reconstituted in 0.5 ml/kg in isotonic saline was injected through a needle inserted in the trachea. The skin was sutured up and the rats were returned to their cages. Surviving rats were counted for each mutant at 72 h postinfection. Whereas infection with CHA led to 100% lethality in 72 h, no rat death occurred when infection was performed with the ExsA or PDH mutants.

We showed here that screening of CHA transposon mutants for the loss of cytotoxicity towards PMNs allowed us to identify new genes involved in TTSS function. Most of the transposon insertions were found in the *aceAB* operon encoding the PDH-E1 and -E2 subunits. In these mutants there is no activation of the transcription of the TTSS regulatory operon *exsCBA*. The cytotoxicity and the TTSS function are restored by complementation with *exsA* or *aceB*. Furthermore, PDH mutants are totally avirulent in a rat model of acute pneumonia. Previous work showed that the E1 or E2 subunits of PDH could play a role in the regulation of gene expression. In *Azotobacter vinelandii*, the E1 subunit of the PDH is a transcription activator of the NADPH:ferredoxin reductase in response to an oxidative stress provoked by superoxide anions (14). The authors of that report also stated that the PDH-E1 subunits of *P. aeruginosa* and *A. vinelandii* share 77% amino acid identity and that the *A. vinelandii* E1 sequence contains a putative helix-turn-helix motif also present in the *P. aeruginosa* E1 sequence. *P. aeruginosa* is likely to be submitted to oxidative stress during the interaction with the PMN; therefore, a transcriptional activation by the PDH-E1 subunit could take place to determine an aggressive reaction towards the source of the oxidative species. In *Bacillus thuringiensis*, the PDH-E2 acts as a transcription activator for coupling postexponential catabolism changes to the synthesis of the protoxin virulence factors (18). Work is under investigation to characterize the *P. aeruginosa* signaling pathway that uses the PDH-E1 and/or -E2 subunit to activate TTSS gene expression.

This work was supported by grant 98033 from the Association Française de Lutte contre la Mucoviscidose (AFLM) and by grants from DGA (DSP/STTC).

We thank F. Morel (Laboratoire d'Enzymologie, CHU-Grenoble) for whole blood facility and student exchange, A. Colbeau for helpful discussions, and J. Chabert for technical assistance.

REFERENCES

1. **Dacheux, D., I. Attree, C. Schneider, and B. Toussaint.** 1999. Cell death of human polymorphonuclear neutrophils induced by a *Pseudomonas aeruginosa* cystic fibrosis isolate requires a functional type III secretion system. Infect. Immun. **67:**6164–6167.
2. **Dacheux, D., I. Attree, and B. Toussaint.** 2001. Expression of ExsA in *trans* confers type III secretion system-dependent cytotoxicity on noncytotoxic *Pseudomonas aeruginosa* cystic fibrosis isolates. Infect. Immun. **69:**538–542.
3. **Dacheux, D., J. Goure, J. Chabert, Y. Usson, and I. Attree.** 2001. Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages. Mol. Microbiol. **40:**76–85.
4. **Dacheux, D., B. Toussaint, M. Richard, G. Brochier, J. Croize, and I. Attree.** 2000. *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. Infect. Immun. **68:**2916–2924.
5. **Delic-Attree, I., B. Toussaint, A. Froger, J. C. Willison, and P. M. Vignais.** 1996. Isolation of an IHF-deficient mutant of a *Pseudomonas aeruginosa* mucoid isolate and evaluation of the role of IHF in *algD* gene expression. Microbiology **142:**2785–2793.
6. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
7. **Fabianek, R. A., H. Hennecke, and L. Thony-Meyer.** 2000. Periplasmic protein thiol:disulfide oxidoreductases of *Escherichia coli*. FEMS Microbiol. Rev. **24:**303–316.
8. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
9. **Frank, D. W.** 1997. The *exoenzyme S* regulon of *Pseudomonas aeruginosa.* Mol. Microbiol. **26:**621–629.
10. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62:**379–433.
11. **Hutchison, M. L., and J. R. Govan.** 1999. Pathogenicity of microbes associated with cystic fibrosis. Microbes Infect. **1:**1005–1014.
12. **Jeyaseelan, K., and J. R. Guest.** 1980. Isolation and properties of pyruvate dehydrogenase complex mutants of *Pseudomonas aeruginosa* PAO. J. Gen. Microbiol. **120:**385–392.
13. **Rashid, M. H., and A. Kornberg.** 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **97:**4885–4890.
14. **Regnstrom, K., S. Sauge-Merle, K. Chen, and B. K. Burgess.** 1999. In *Azotobacter vinelandii*, the E1 subunit of the pyruvate dehydrogenase complex binds *fpr* promoter region DNA and ferredoxin I. Proc. Natl. Acad. Sci. USA **96:**12389–12393.
15. **Sawa, T., T. L. Yahr, M. Ohara, K. Kurahashi, M. A. Gropper, J. P. Wiener-Kronish, and D. W. Frank.** 1999. Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. Nat. Med. **5:**392–398.
16. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. 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, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, and I. T. Paulsen. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959–964.
17. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase in *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
18. **Walter, T., and A. Aronson.** 1999. Specific binding of the E2 subunit of pyruvate dehydrogenase to the upstream region of *Bacillus thuringiensis* protoxin genes. J. Biol. Chem. **274:**7901–7906.
19. **Yahr, T. L., J. Goranson, and D. W. Frank.** 1996. Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. Mol. Microbiol. **22:**991–1003.
20. **Zago, A., S. Chugani, and A. M. Chakrabarty.** 1999. Cloning and characterization of polyphosphate kinase and exopolyphosphatase genes from *Pseudomonas aeruginosa* 8830. Appl. Environ. Microbiol. **65:**2065–2071.

Editor: E. I. Tuomanen