JOURNAL OF BACTERIOLOGY, July 1997, p. 4631–4634 Vol. 179, No. 14
0021-9193/97/$04.00+0

Identification and Characterization of *xcpR* Encoding a Subunit of the General Secretory Pathway Necessary for Dodecane Degradation in *Acinetobacter calcoaceticus* ADP1

STEPHAN PARCHE, WALTER GEIßDÖRFER, AND WOLFGANG HILLEN*

Lehrstuhl für Mikrobiologie, Institut für Mikrobiologie, Biochemie und Genetik der Friedrich-Alexander-Universität Erlangen-Nürnberg, 91058 Erlangen, Germany

Received 21 February 1997/Accepted 7 May 1997

A mutant of *Acinetobacter calcoaceticus* ADP1 unable to grow on alkanes was complemented for growth on hexadecane with a DNA fragment encoding a protein with homology to XcpR, a subunit of the general secretion pathway for exoproteins in *Pseudomonas aeruginosa*. Insertional inactivation of *xcpR* in *A. calcoaceticus* ADP1 by transcriptional fusion to *lacZ* abolishes secretion of lipase and esterase and leads to lack of growth on dodecane and slower growth on hexadecane. We, therefore, propose the participation of a secreted protein in alkane degradation.

Acinetobacter calcoaceticus ADP1, also called BD413 (18, 28), is able to grow on *n*-alkanes as sole carbon and energy sources via the ω-hydroxylation pathway (2). Candidates (n = 2,650) able to grow on minimal medium with lauric acid were obtained by ethyl methanesulfonate mutagenesis and screened for the inability to grow on dodecane, which yielded 27 candidates with a negative phenotype on solid agar plates (11). Mutant WH365 is also unable to grow on hexadecane and fails to secrete lipase and esterase, in contrast to the wild type (20–22). Extracellular lipase of *A. calcoaceticus* ADP1, which shows activity towards triglycerides and phospholipids, was monitored on indicator plates with NB medium (Difco) containing 2% (vol/vol) egg yolk (Oxoid), whereas esterase activity was determined on NB medium with 1% (vol/vol) Tween 80. This Tween esterase is supposed to be secreted, but the corresponding gene has not been identified and nothing is known about the biological importance of this activity.

Partially *Sau*3A-digested total DNA from *A. calcoaceticus* ADP1 was inserted into *Bam*HI-cleaved pWH1274 carrying an origin of replication for *Escherichia coli* and *A. calcoaceticus* (16). The resulting gene library was transformed into WH365 by electroporation, and transformants were selected for growth on Luria broth (LB) plates with ampicillin (300 mg/liter). Replica plating on minimal medium with metal 44 (8), hexadecane delivered through the gas phase from a droplet on a filter disk in the lid of the plate, and ampicillin (200 mg/liter) yielded seven candidates. After passage of the plasmids through *E. coli* DH5α (13) and retransformation into WH365, one plasmid, called pWH1590, conferred the ability to grow with hexadecane as the sole carbon source. It contains a 15-kb insertion of contiguous chromosomal DNA, as confirmed by Southern hybridization (data not shown). pWH1590 was digested with *Hin*dIII, resulting in nine fragments. Eight of them were ligated with *Hin*dIII-restricted pBluescript SKII+ (Stratagene), yielding pWH1591 to -98. pWH1597 transformed WH365 to grow on hexadecane and to secrete lipase and esterase. The 2.8-kb insert was sequenced on both strands. It contains three open reading frames (ORFs) (Fig. 1), each of which is preceded by a putative ribosome binding site, as judged by sequence comparison with other *Acinetobacter* genes (38). Transformation of plasmids obtained from a nested-deletion reaction of pWH1597 mapped the complementing fragment within ORF3 (Fig. 1).

ORF1 encodes a protein of 145 amino acids (aa) with similarities to several multidrug resistance regulators of the MarR family (25); e.g., 23% of its residues are identical to those of MexR from *Pseudomonas aeruginosa* (SwissProt accession no. P52003). The 143-aa protein encoded by ORF2 has 30% identity to the osmotically inducible protein OsmC from *E. coli* (12). ORF3 encodes a protein of 479 aa, called XcpR, with homology to members of the general secretory pathway protein E (GspE) family (Table 1). These cytoplasmic proteins are necessary for protein secretion and assembly of type IV pili in gram-negative bacteria and for DNA uptake by competent *Bacillus subtilis* cells (15). Proteins with homology to type IV prepilin and to a type IV pilus assembly factor are also involved

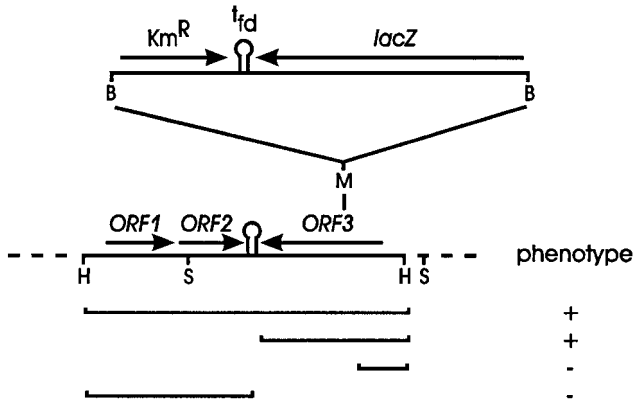

FIG. 1. Sequence analysis of the 2,785-bp insert of pWH1597 revealed three ORFs and a putative transcriptional terminator. Plasmids from a nested-deletion reaction mixture used for complementation studies are shown as bars in the lower part. Phenotypes: +, growth on hexadecane and protein secretion; −, no growth on hexadecane and no protein secretion observed. The upper part shows the genetic situation with WH399. The cassette from pKOK6.1 contains a promoterless *lacZ* gene, preceded by stop codons in all three ORFs, a transcriptional terminator sequence from the filamentous phage fd (t_{fd}), and a Km^r gene. Restriction sites: B, *Bam*HI; H, *Hin*dIII; M, *Msc*I; S, *Sac*II.

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Institut für Mikrobiologie, Biochemie und Genetik der Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstrasse 5, 91058 Erlangen, Germany. Phone: 49 (9131) 858081. Fax: 49 (9131) 858082. E-mail: whillen@biologie.uni-erlangen.de.

TABLE 1. Comparison of XcpR from *A. calcoaceticus* ADP1 to proteins in the SwissProt database (incomplete list)

Homologous protein	Size (aa)	Organism	% Identical aa	Function	Reference
XcpR	502	*Pseudomonas aeruginosa*	68	Protein secretion	4
ExeE	501	*Aeromonas hydrophila*	61	Protein secretion	17
PulE	497	*Klebsiella oxytoca*	58	Protein secretion	29
XcpR	482	*Pseudomonas putida*	54	Protein secretion	6
PilB	566	*Pseudomonas aeruginosa*	47	Type IV pilus assembly	27
ComG1	356	*Bacillus subtilis*	42	DNA uptake	1

in the competence of *A. calcoaceticus* ADP1 (3, 9). Figure 2 shows a sequence alignment of XcpR from *A. calcoaceticus* with some of the homologous proteins and indicates the conserved sequence motifs. Walker box A (34) is typically found in nucleotide binding proteins like XcpR from *P. aeruginosa* (37). Two aspartate-rich boxes (TXEDPXE and RXXPDXXXXGEI/MRD) distinguish members of the GspE family from other nucleotide binding proteins (30). Most GspE proteins also contain two cysteine pairs, each separated by 2 aa (Fig. 2). We propose on the basis of this sequence analysis and the phenotype of the inactivated mutant (see below) that *xcpR* encodes the corresponding component of the general secretory pathway for proteins in *Acinetobacter.*

We inactivated *xcpR* on the *A. calcoaceticus* ADP1 chromo-

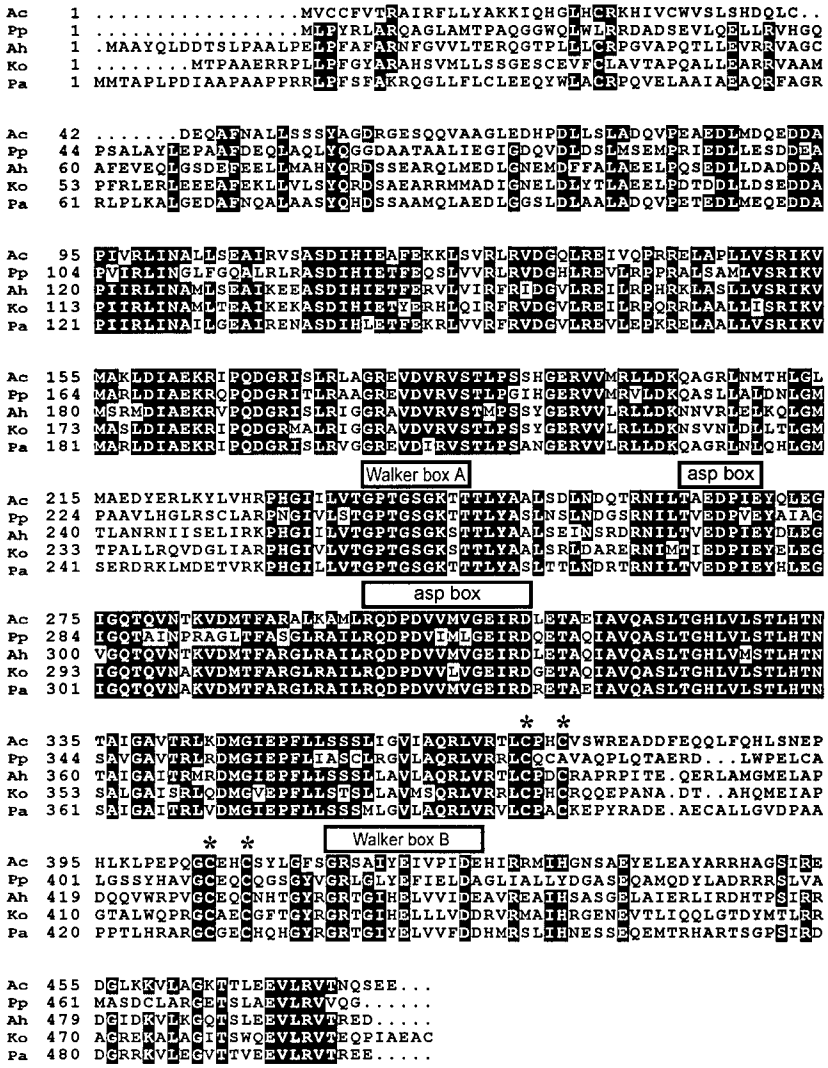

FIG. 2. Alignment of XcpR from *A. calcoaceticus* with four related proteins involved in protein secretion. Amino acids are given in the one-letter code. White letters indicate that at least 4 of the 5 compared residues are identical. Motifs are indicated by boxes above the alignment. The conserved cysteine residues are marked by asterisks. Dots indicate gaps introduced to improve alignments. Abbreviations: Ac, XcpR from *A. calcoaceticus*; Pp, XcpR from *P. putida*; Ah, ExeE from *Aeromonas hydrophila*; Ko, PulE from *K. oxytoca*; Pa, XcpR from *P. aeruginosa; asp, aspartate.*

TABLE 2. Total β-galactosidase activity of WH399

Time of growth (h)	β-Galactosidase activity of WH399 (Miller units)[a] in:			
	LB		MM plus succinate	
	With hd	Without hd	With hd	Without hd
2	12 ± 1	17 ± 5	8 ± 2	13 ± 1
4	11 ± 4	20 ± 16	9 ± 2	11 ± 1
5	15 ± 1	16 ± 1	5 ± 3	18 ± 0
6.5	20 ± 1	22 ± 1	22 ± 2	24 ± 1
9	27 ± 1	28 ± 1	29 ± 1	31 ± 1
12	34 ± 1	36 ± 2	31 ± 5	36 ± 1

[a] β-Galactosidase activities were determined from three independent cultures according to the method of Miller (26). MM, Minimal medium containing metal 44; hd, hexadecane.

some by insertion of the *lacZ* Km^r cassette from pKOK6.1 (23). The 4.7-kb *Bam*HI fragment was ligated into the *Msc*I site of *xcpR*, resulting in a *xcpR*::*lacZ* transcriptional fusion on pWH1605 (Fig. 1). pWH1605 was digested with *Sac*II, and the resulting fragment was transformed to *A. calcoaceticus* ADP1. Recombinants were selected on LB plates with kanamycin (10 mg/liter), and correct integration was confirmed by Southern hybridization. The resulting strain, called WH399, contains the genetic arrangement shown in Fig. 1. WH399 is unable to secrete lipase and esterase, as determined with indicator plates. WH399 shows impaired growth on solid medium with hexadecane as the sole carbon source and no growth on dodecane. In liquid mineral medium with hexadecane as the sole carbon source, WH399 grows to an optical density at 600 nm of 0.32 within 5 days, whereas ADP1 grows to an optical density at 600 nm of 0.76. These results establish clearly that XcpR is necessary for protein secretion and for growth on dodecane and that it supports growth on hexadecane. WH399 is as efficiently transformable via natural competence with supercoiled plasmid pWH1274 as the wild type, indicating that disruption of *xcpR* does not affect DNA uptake (data not shown). Therefore, we propose the presence of two distinct type IV pilus-related systems for protein secretion and DNA uptake in *A. calcoaceticus* ADP1.

WH399 and the wild type were grown in LB with and without hexadecane and in minimal medium containing succinate in the presence and absence of hexadecane. β-Galactosidase activities expressed from the single copy *xcpR*::*lacZ* fusion shown in Table 2 indicate constitutive expression of *xcpR* at a low level.

Secreted proteins must cross two membranes in gram-negative bacteria. In the main terminal branch of the general secretory pathway, secretion occurs in a two-step process, which is well conserved among gram-negative bacteria (31). In *P. aeruginosa*, proteins are translocated across the outer membrane via the Xcp system, which consists of the products of at least 12 *xcp* genes. Most of them are localized in the cytoplasmic membrane, some span the periplasmic space, and one forms an energized pore through the outer membrane. The *xcpR* gene from *P. aeruginosa* is localized in an operon where it is followed by *xcpS* (4). This arrangement is also found in the corresponding genes from *Pseudomonas putida* (*xcpRS*) (11), *Klebsiella oxytoca* (*pulEF*) (29), *E. coli* K-12 (*gspEF*) (10), *Erwinia chrysanthemi* (*outEF*) (24), *Aeromonas hydrophila* (*exeEF*) (17), *Vibrio cholerae* (*epsEF*) (EMBL accession no. L33796), and *Xanthomonas campestris* (*xpsEF*) (7). *xcpR* from *A. calcoaceticus* ADP1 has a different genetic arrangement, as it is followed by a putative transcriptional terminator, and no coding region with homology to known *xcp* genes was found within 500 bp upstream of *xcpR*.

We conclude from these results that a secreted protein must be involved in the degradation of dodecane and contribute substantially to hexadecane utilization. The nature of that protein is not clear. A lipase mutant of *A. calcoaceticus* ADP1, AAC320 (22), is able to grow on hexadecane and dodecane. This finding shows that lipase activity is not essential for alkane degradation as supposed by Breuil et al. (5), who found lipase activity in each of the tested 25 bacteria capable of growing on hexadecane. Alkanes have only limited solubility in aqueous media. Since the known enzymes involved in alkane oxidation are cell bound, either bacteria must be in direct contact with the organic phase or they must be able to pseudosolubilize the substrate. *A. calcoaceticus* RAG-1 secretes a surfactant consisting of about 15 to 20% protein in addition to carbohydrates (35). The release of RAG-1–emulsan from the cell surface is mediated by a secreted esterase (32). It may be speculated that a secreted esterase is also involved in the production of an emulsifier, like the one produced at high levels in *A. calcoaceticus* BD413 growing on ethanol (19). Stimulation of growth on *n*-hexadecane by a secreted protein with emulsifying activity has also been reported for *P. aeruginosa* (14). An adherence factor enabling *Acinetobacter* to bind to alkane droplets has been suggested (33) and may be necessary on the outside of the cell for efficient alkane utilization. It is also possible that type IV pilus-related systems may not only promote protein secretion but also constitute a more general transport system for various macromolecules (reviewed in reference 36) or help the cells to overcome membrane stress resulting from alkane dissolution.

Nucleotide sequence accession number. The EMBL accession number for the nucleotide sequence of *xcpR* from *A. calcoaceticus* ADP1 is Y09102.

We thank A. Ratajczak for critically reading the manuscript. pKOK6.1 was kindly provided by W. Lotz.

This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

1. **Albano, M., R. Breitling, and D. A. Dubnau.** 1989. Nucleotide sequence and genetic organization of the *Bacillus subtilis comG* operon. J. Bacteriol. **171:** 5386–5404.
2. **Asperger, O., and H.-P. Kleber.** 1991. Metabolism of alkanes by *Acinetobacter*, p. 323–351. *In* K. J. Towner, E. Bergogne-Berezin, and C. A. Fewson (ed.), The biology of *Acinetobacter*. Plenum Press, New York, N.Y.
3. **Averhoff, B.** Personal communication.
4. **Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazdunski, and J. Tommassen.** 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol. Microbiol. **6:**1121–1131.
5. **Breuil, C., D. B. Shindler, J. S. Sijher, and D. J. Kushner.** 1978. Stimulation of lipase production during bacterial growth on alkanes. J. Bacteriol. **133:** 601–606.
6. **de Groot, A., J.-J. Krijger, A. Filloux, and J. Tommassen.** 1996. Characterization of type II protein secretion (*xcp*) genes in the plant growth-stimulating *Pseudomonas putida*, strain WCS358. Mol. Gen. Genet. **250:**491–504.
7. **Dums, F., J. M. Dow, and M. J. Daniels.** 1991. Structural characterization of protein secretion genes of the bacterial phytopathogen *Xanthomonas campestris* pathovar *campestris*: relatedness to secretion systems of other gram-negative bacteria. Mol. Gen. Genet. **229:**357–364.
8. **Ehrt, S., F. Schirmer, and W. Hillen.** 1995. Genetic organization, nucleotide sequence and regulation of expression of genes encoding phenol hydroxylase and catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCIB8250. Mol. Microbiol. **18:**13–20.
9. **Eickernjäger, S., U. Drotschmann, D. Porstendörfer, M. Madkour, and B. Averhoff.** Identification of a gene, *comC*, encoding a novel competence factor with dual function in natural transformation and biogenesis of thin fimbriae in *Acinetobacter calcoaceticus*. Submitted for publication.
10. **Francetic, O., and A. P. Pugsley.** 1996. The cryptic general secretory pathway (*gsp*) operon of *Escherichia coli* K-12 encodes functional proteins. J. Bacteriol. **178:**3544–3549.

11. **Geissdörfer, W., S. C. Frosch, G. Haspel, S. Ehrt, and W. Hillen.** 1995. Two genes encoding proteins with similarities to rubredoxin and rubredoxin reductase are required for conversion of dodecane to lauric acid in *Acinetobacter calcoaceticus* ADP1. Microbiology **141:**1425–1432.
12. **Gutierrez, C., and J. C. Devedjian.** 1991. Osmotic induction of gene *osmC* expression in *Escherichia coli* K12. J. Mol. Biol. **220:**959–973.
13. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
14. **Hardegger, M., A. K. Koch, U. A. Ochsner, A. Fiechter, and J. Reiser.** 1994. Cloning and heterologous expression of a gene encoding an alkane-induced extracellular protein involved in alkane assimilation from *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **60:**3679–3687.
15. **Hobbs, M., and J. S. Mattick.** 1993. Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. **10:**233–243.
16. **Hunger, M., R. Schmucker, V. Kishan, and W. Hillen.** 1990. Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. Gene **87:**45–51.
17. **Jiang, B., and S. P. Howard.** 1992. The *Aeromonas hydrophila exeE* gene, required both for protein secretion and normal outer membrane biogenesis, is a member of a general secretion pathway. Mol. Microbiol. **6:**1351–1361.
18. **Juni, E., and A. Janik.** 1969. Transformation of *Acinetobacter calcoaceticus* (*Bacterium anitratum*). J. Bacteriol. **98:**281–288.
19. **Kaplan, N., Z. Zosim, and E. Rosenberg.** 1987. Reconstitution of emulsifying activity of *Acinetobacter calcoaceticus* BD4 emulsan by using pure polysaccharide and protein. Appl. Environ. Microbiol. **53:**440–446.
20. **Kok, R. G.** 1995. Lipolytic enzymes in *Acinetobacter calcoaceticus*. Ph.D. thesis. University of Amsterdam, Amsterdam, The Netherlands.
21. **Kok, R. G., J. J. van Thor, I. M. Nugteren-Roodzant, M. B. Brouwer, M. R. Egmont, C. B. Nudel, B. Vosman, and K. J. Hellingwerf.** 1995. Characterization of the extracellular lipase, LipA, of *Acinetobacter calcoaceticus* BD413 and sequence analysis of the cloned structural gene. Mol. Microbiol. **15:**803–818.
22. **Kok, R. G., J. J. van Thor, I. M. Nugteren-Roodzant, B. Vosman, and K. J. Hellingwerf.** 1995. Characterization of lipase-deficient mutants of *Acinetobacter calcoaceticus* BD413: identification of a periplasmic lipase chaperone essential for the production of extracellular lipase. J. Bacteriol. **177:**3295–3307.
23. **Kokotek, W., and W. Lotz.** 1989. Construction of a *lacZ*-kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. Gene **84:**467–471.
24. **Lindeberg, M., and A. Collmer.** 1992. Analysis of eight *out* genes in a cluster required for pectic enzyme secretion by *Erwinia chrysanthemi*: sequence comparison with secretion genes from other gram-negative bacteria. J. Bacteriol. **174:**7385–7397.
25. **Martin, R. G., K. W. Jair, R. E. Wolf, and J. L. Rosner.** 1996. Autoactivation of the *marRAB* multiple antibiotic resistance operon by the MarA transcriptional activator in *Escherichia coli*. J. Bacteriol. **178:**2216–2223.
26. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Nunn, D., S. Bergman, and S. Lory.** 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. **172:**2911–2919.
28. **Patel, N. R., S. Mazumdar, and L. N. Ornston.** 1975. β-Ketoadipate enol-lactone hydrolases I and II from *Acinetobacter calcoaceticus*. J. Biol. Chem. **250:**6567–6577.
29. **Possot, O., C. d'Enfert, I. Reyss, and A. P. Pugsley.** 1992. Pullulanase secretion in *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. Mol. Microbiol. **6:**95–105.
30. **Possot, O., and A. P. Pugsley.** 1994. Molecular characterization of PulE, a protein required for pullulanase secretion. Mol. Microbiol. **12:**287–299.
31. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
32. **Reddy, P. G., R. Allon, M. Mevarech, S. Mendelovitz, Y. Sato, and D. L. Gutnick.** 1989. Cloning and expression in *Escherichia coli* of an esterase-coding gene from the oil-degrading bacterium *Acinetobacter calcoaceticus* RAG-1. Gene **76:**145–152.
33. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. **148:**51–57.
34. **Saraste, M., P. R. Sibbald, and A. Wittinghofer.** 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15:**430–434.
35. **Shabtai, Y., and D. L. Gutnick.** 1985. Exocellular esterase and emulsan release from the cell surface of *Acinetobacter calcoaceticus*. J. Bacteriol. **161:**1176–1181.
36. **Tommassen, J., A. Filloux, M. Bally, M. Murgier, and A. Lazdunski.** 1992. Protein secretion in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **103:** 73–90.
37. **Turner, L. R., J. C. Lara, D. N. Nunn, and S. Lory.** 1993. Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J. Bacteriol. **175:**4962–4969.
38. **White, P. J., I. S. Hunter, and C. A. Fewson.** 1991. Codon usage in *Acinetobacter* structural genes, p. 251–257. *In* K. J. Towner, E. Bergogne-Berezin, and C. A. Fewson (ed.), The biology of *Acinetobacter*. Plenum Press, New York, N.Y.