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Artificial Control of Nitrate Respiration through the *lac* Promoter Permits the Assessment of Oxygen-Mediated Posttranslational Regulation of the *nar* Operon in *Pseudomonas aeruginosa*[▽]

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In this study, oxygen and nitrate regulation of transcription and subsequent protein expression of the unique *narK1K2GHJI* respiratory operon of *Pseudomonas aeruginosa* were investigated. Under the control of P_{LAC}, *P. aeruginosa* was able to transcribe *nar* and subsequently express methyl viologen-linked nitrate reductase activity under aerobic conditions without nitrate. Modulation of P_{LAC} through the LacI repressor enabled us to assess both transcriptional and posttranslational regulation by oxygen during physiological whole-cell nitrate reduction.

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium capable of growth and/or survival anaerobically through arginine catabolism (36), pyruvate fermentation (6), or denitrification in the presence of nitrogen oxides (37). The latter process allows this organism to persist in soil as part of the global nitrogen cycle. Additionally, denitrification has been implicated in infections by this opportunistic pathogen in the airways of cystic fibrosis patients (9, 18, 35).

During anaerobic growth of *Escherichia coli*, the Fnr protein is responsible for activation of the synthesis of anaerobic respiratory enzymes such as nitrate reductase (28, 31). In addition, the presence of external nitrate induces the transcription of the nitrate reductase operon through the dual two-component regulatory systems of *narX-narL* (31, 32) and *narQ-narP* (3, 21, 22). Parallel studies of *P. aeruginosa* have resulted in the characterization of a unique *nar* operon (24, 27) regulated by the proteins Anr and Dnr (40) as well as *narX* and *narL* (24). However, a *narQ* homologue has not been identified (30, 34).

Posttranslationally, oxygen also has the capacity to inhibit denitrification immediately at the level of nitrate uptake and nitrite excretion (10, 11, 33) as well as through the diversion of electron flow to oxygen in *E. coli* and in *Paracoccus denitrificans* (4, 33). Despite these studies, an experimental method for the measurement of posttranslational regulation by oxygen has been lacking.

In the present study, the effects of oxygen and nitrate on the expression of the *narK1K2GHJI* operon (27) were examined during aerobic or anaerobic growth with and without nitrate. In addition, a P_{LAC} element was inserted upstream of the respiratory nitrate reductase genes (*narK2GHJI*) of *P. aeruginosa* to overcome transcriptional regulation of the *nar* operon by oxygen and nitrate. The levels of transcription, respiratory nitrate reductase activity, and whole-cell physiological reduction of nitrate to nitrite were measured under both aerobic and anaerobic conditions, thus allowing quantitative assessment of posttranslational regulation by oxygen.

The bacterial strains and plasmids used in this study are listed in Table 1. All bacteria were grown at 37°C from single-colony isolates or overnight cultures in Luria-Bertani (LB) broth (Fisher Scientific, Pittsburgh, PA). The medium was supplemented with 1% (wt/vol) KNO_3 (LB-NO_3) when indicated. Aerobic cultures were set up as 50-ml volumes of LB or LB-NO_3 in a 500-ml Erlenmeyer flask by inoculating 500 μl of cells and shaking at 250 rpm to an optical density at 600 nm of 0.3 to 0.4. Cultures used to measure anaerobic transcription of *narG* and to conduct in vitro anaerobic respiratory nitrate reductase assays were first grown aerobically to an optical density at 600 nm of 0.3 to 0.4, after which time they were shifted to complete anaerobic conditions (BBL Anaerobic GasPak System) for 3 h. For anaerobic growth cultures and nitrite excretion studies, 1 ml of mid-log-phase cells was inoculated in 100-ml volumes of LB-NO_3 and incubated with magnetic stirring in a 125-ml Erlenmeyer flask with rubber stoppers equipped with ports for sample withdrawal and one-way gas release valves. To ensure complete anaerobiosis of the system, the medium was supplemented with 2% (wt/vol) Oxyrase (Oxyrase, Inc., Mansfield, OH) and flushed with argon. The antibiotics (BioWorld, Dublin, OH) used for *E. coli* were as follows: ampicillin (100 μg/ml), gentamicin (15 μg/ml), and tetracycline (25 μg/ml). Gentamicin (300 μg/ml) and carbenicillin (500 μg/ml) were used for *P. aeruginosa*.

For the construction of strain JVC, a 1.5-kb SmaI digest containing Gmxlac was blunt ended into the NotI site of pEX18Ap::*narK1K2*. Single-copy, chromosomal gene disruptions were created using a gene replacement technique previously described (25, 29). *loxP* excision of the gentamicin resistance marker was conducted by transformation with pCRE into a *P. aeruginosa* strain with a *narK1*-Gmxlac disruption. Several passages of growth in LB containing 500 μg/ml carbenicillin were followed by selection for the loss of growth in LB containing 300 μg/ml gentamicin. The pCRE plasmid was cured from the strain through one passage in LB, followed by

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TABLE 1. Strains, plasmids, and oligonucleotide primers

Strain, plasmid, or primer	Relevant genotype, description or sequence (5′ to 3′)[a]	Source or reference
Strains		
E. coli		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lacU169*(φ80*lacZ*Δ*M15*)	Gibco[e]
TOP10F′	F′ [*lacI*^{q} Tn*10*(Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen[e]
SM10	Km^r; mobilizer strain	29
P. aeruginosa		
PAO1	Wild type	C. Manoil
JVC	PAO1 with *narK1*-P_{lac}	This study
Plasmids		
pUCP18	Ap^r; broad-host-range cloning vector	26
pUCGM*lox*	Gm^r; Gm^r cassette flanked by *lox* sequences	20
pUCGMxlac	Gm^r; P_{LAC} promoter ligated within the ClaI and SacII sites of pUCGM*lox*	This study
pGem-lacI^q	Ap^r; 1-kb PCR fragment containing *lacI*^{q} from TOP10F′ in pGEM-T Easy vector (Promega)	This study
pREP	Ap^r; ligation of a blunt-ended 1-kb EcoRI fragment containing *lacI*^{q} from pGem-lacI^q into the blunt-ended EcoRI and SapI sites of pUCP18	This study
pFLP2	Ap^r; Flp vector containing *sacB* and *flp* (Flp recombinase)	12
pCM157	Tc^r; source of *cre* recombinase	15
pCR2.1-cre	Ap^r; Km^r; 1.3-kb PCR fragment containing *cre* recombinase in pCR2.1 (Invitrogen)	This study
pCRE	Ap^r; 1.3-kb EcoRI fragment of pCR2.1-cre ligated into an EcoRI digest of pFLP2	This study
pEX18Ap::*narK1K2*	Ap^r; 2.7-kb EcoRI fragment containing *narK1* and *narK2* ligated into the EcoRI site of pEX18Ap	27
pEXJVC	Ap^r; Gm^r; 1.5-kb SmaI fragment from pUCGMxlac blunt ended into the NotI site of pEX18Ap::*narK1K2*	This study
Primer/template[b]		
Plac/Pucp18 (+)	TGTATCGATTCGCCACCTCTGACTT	This study
Plac/Pucp18 (−)	CTCCGCGGCGTAATCATGGTCATAG	This study
lacI^q/Top 10F′ DNA (+)	GTGCAAAGCTCTTCGCGGTAT	This study
lacI^q/Top 10F′ DNA (−)	CGCGAATTCACATTAATTGCGTTG	This study
cre/pCM157 (+)	GAATTCGCAAACCGCCTCTC	This study
cre/pCM157 (−)	CCAGTGAATTCTTACTAATCGCCATC	This study
narK1K2 (+)[c]	CCTGTCACTACCTCCAAAG	27
narK1K2 (−)[c]	AGAAGCTGATATTGGACATG	27
narG (+)[d]	ACGACCTCAACACCTCCGAC	This study
narG (−)[d]	GATCTCCCAGTCGCTCTTGG	This study
rpoD (+)[d]	GGGCGAAGAAGGAAATGGTC	23
rpoD (−)[d]	CAGGTGGCGTAGGTGGAGAA	23

[a] Km^r, Ap^r, Gm^r, and Tc^r indicate resistance to kanamycin, ampicillin, gentamicin, and tetracycline, respectively. Underlined nucleotides were modified to accommodate a restriction site.
[b] Template DNA used with the corresponding primer is indicated; plus and minus symbols indicate forward and reverse primers, respectively.
[c] Used for mutant confirmation.
[d] Used for real-time PCR.
[e] Located in Carlsbad, CA.

growth in LB containing 5% sucrose and selection for carbenicillin sensitivity. Constructs were confirmed by PCR using primers listed in Table 1.

JVC was constructed by inserting the Gmxlac cassette within *narK1* for two reasons. First, it has been shown for *P. aeruginosa* that while *narK1K2GHJI* is transcribed as an operon, only NarK2 is required for respiratory nitrate reduction (27). Second, we did not want to interfere with physiologically regulated anaerobic transcription of the *nar* promoter since expression levels manipulated through a *lac* promoter could be either limiting or overexpressed and, thus, will not reflect physiological responses. Using the same reasoning, transcriptional termination sequences were not placed upstream of P_{LAC} in the pUCGmxlac vector. Thus, the transcriptional activity of *narK2GHJI* would be under the control of both the *nar* promoter and the P_{LAC} element during anaerobic conditions and be under the control of solely P_{LAC} during aerobic conditions.

Transcription of *nar* under aerobic and anaerobic conditions in JVC (*narK1*-P_{LAC}). To test the ability of P_{LAC} to aerobically transcribe *narK2GHJI*, the mRNA levels of *narG* in PAO1 and JVC were measured through quantitative reverse transcription-PCR in LB with or without nitrate in aerobic and anaerobic conditions. The results further corroborated the previous experimental proof that in *P. aeruginosa*, *narK1* and *narK2* were contained in the *nar* operon (27) and demonstrated that the presence of nitrate alone was not sufficient for the transcription of *narG* in wild-type PAO1 and that anaerobic conditions were required (Table 2). In contrast, JVC transcribed *narG* aerobically in both the presence and the absence

TABLE 2. Transcriptional levels of *narG* in *P. aeruginosa* PAO1 and JVC

Strain[a]	Relative fold increase under indicated conditions[b]		
	Aerobic		Anaerobic
	LB	LB + 1% nitrate	LB + 1% nitrate
PAO1	1	1.1 ± 0.1	31.3 ± 2.2
PAO1 + pREP	ND[c]	1.5 ± 0.9	ND
JVC	23.4 ± 6.0	15.5 ± 3.8	55.5 ± 4.4
JVC + pREP	ND	6.2 ± 0.9	ND

[a] The PAO1 and JVC strains were grown in LB with or without 1% (wt/vol) KNO_3 under aerobic and anaerobic conditions as described in the text.
[b] The difference (*n*-fold) was calculated using the Pfaffl model (19), setting PAO1 in LB aerobically as 1. Values shown are means ± standard errors as calculated from three independent cell suspensions. PCR was accomplished by a 3-min denaturation step at 95°C, followed by 40 cycles of 30 s at 95°C, 45 s at 60°C, and 45 s at 72°C.
[c] ND, not determined.

of nitrate (15.5 ± 3.8 and 23.4 ± 6.0 relative severalfold increases, respectively). The level of *narG* transcription anaerobically was significantly higher for both PAO1 and JVC since the operon was under the physiological control of the *nar* promoter. Finally, we manipulated the transcription of *narG* by transforming a plasmid containing *lacI*^{q} (pREP) into JVC. As expected, the addition of pREP significantly reduced aerobic *narG* transcriptional levels in JVC (Table 2).

The addition of P_{LAC} upstream of *narK2GHJI* enabled us to constitutively transcribe the operon in both the presence and the absence of oxygen and nitrate and to modulate aerobic transcriptional activity by utilizing pREP.

Respiratory nitrate reductase activity. To determine whether the same trends are observed at the enzyme level, methyl viologen-linked respiratory nitrate reductase activity was measured (14) in cell extracts of strains grown in LB with or without nitrate aerobically and anaerobically (Table 3). The results indicated that enzyme activity was detected only in wild-type PAO1 under anaerobic conditions in the presence of nitrate (Table 3), confirming the *narG* transcriptional activities measured in Table 2 and the requirement of both nitrate and anaerobiosis for expression of the respiratory nitrate reductase. *P. aeruginosa* also contains a periplasmic nitrate reductase (38), but we did not detect any enzyme activity for *P. aeruginosa* under aerobic conditions (17, 27). Nitrate reductase activity in JVC was consistently detected even in the absence of nitrate and under aerobic conditions due to the expression of *narGHJI* through P_{LAC} (Table 3). Eighty percent of the specific activity detected aerobically without nitrate was retained within the insoluble fraction of the crude extract, indicating that the aerobic nitrate reductase measured was predominantly membrane bound (data not shown). Anaerobically, the nitrate reductase activity of JVC was approximately twice the level observed in wild-type PAO1 (1,120 ± 150 and 560 ± 77 nmol nitrite min^{-1} mg^{-1} protein, respectively [Table 3]). We also determined the ability of pREP to inhibit nitrate reductase activity and found both aerobic and anaerobic nitrate reductase activities to be significantly reduced in JVC with pREP (Table 3). Although pREP did not completely diminish anaerobic nitrate reductase activities, the level of inhibition was sufficient to significantly hinder anaerobic growth (data not shown).

TABLE 3. Nitrate reductase activities of *P. aeruginosa* PAO1 and JVC

Strain[a]	Nitrate reductase activity (nmol nitrite min^{-1} mg^{-1} protein) under indicated conditions[b]			
	Aerobic		Anaerobic	
	LB	LB + 1% nitrate	LB	LB + 1% nitrate
PAO1	<1	<1	<1	560 ± 77
PAO1 + pREP	<1	<1	<1	548 ± 64
JVC	222 ± 27	210 ± 9	367 ± 66	1,120 ± 150
JVC + pREP	23 ± 7	28 ± 12	36 ± 13	218 ± 7

[a] PAO1 and JVC were grown in LB with or without 1% (wt/vol) KNO_3 under aerobic and anaerobic conditions as described in the text.
[b] Nitrate reductase activities were determined in cell extracts by using reduced methyl viologen as the electron donor. Values shown are means ± standard errors as calculated from three independent cell suspensions.

TABLE 4. Nitrite excretion levels of *P. aeruginosa* PAO1 and JVC

Strain[a]	Nitrite excretion (nmol nitrite μg^{-1} protein)[b]	
	Aerobic	Anaerobic
PAO1	<0.1	131.1 ± 5.6
PAO1 + pREP	<0.1	128.0 ± 9.9
JVC	1.5 ± 0.1	123.6 ± 4.7
JVC + pREP	0.5 ± 0.1	9.4 ± 0.5

[a] PAO1 and JVC were grown aerobically and anaerobically in LB with 1% (wt/vol) KNO_3 as described in the text.
[b] Nitrite levels were determined as described in the text. Values shown are means ± standard errors as calculated from three independent measurements.

Posttranslational oxygen regulation of nitrate reduction. Through quantitative reverse transcription-PCR and methyl viologen-linked respiratory nitrate reductase activity, we have shown that oxygen inhibition of transcription and translation of the *nar* operon was overcome by modulating transcription of *narK2GHJI* through P_{LAC}. The use of pREP also allowed us to express nitrate reductase activity anaerobically in JVC over a wide range from 218 ± 7 to 1,120 ± 150 nmol nitrite min^{-1} mg^{-1} protein. More importantly, the levels of enzyme activity in JVC with pREP under anaerobic conditions are similar to those observed aerobically in JVC alone (218 ± 7 and 210 ± 9 nmol nitrite min^{-1} mg^{-1} protein, respectively). These expression levels allowed us to measure whole-cell nitrate reduction to nitrite aerobically and anaerobically in strains with comparable nitrate reductase activities and thus explore posttranslational regulation by oxygen (Table 3). We examined JVC for physiological whole-cell nitrate reduction (non-methyl viologen driven) to nitrite indirectly by measuring the levels of nitrite in the growth medium. Specifically, the supernatant was collected by centrifugation (10,000 × *g*, 4°C, 10 min) and measured for nitrite (16). The disappearance of nitrite was not considered since previous studies have shown that the presence of nitrate inhibits the reduction of nitrite and that its subsequent secretion by whole cells is stoichiometrically correlated to the disappearance of nitrate (5, 13). The JVC strain excreted nitrite when grown aerobically (1.5 ± 1 nmol nitrite μg^{-1} protein [Table 4]) while the levels of aerobic nitrite excretion in wild-type PAO1 remained undetectable. During anaerobic growth, the JVC strain containing pREP excreted nitrite at a rate approximately sixfold higher than the levels observed under aerobic conditions in the same strain without pREP (9.4 ±

0.5 and 1.5 ± 0.1 nmol nitrite μg^{-1} protein, respectively [Table 4]). Although similar nitrate reductase activities were observed in aerobic JVC and anaerobic JVC with pREP (Table 3), the levels of nitrite excretion were significantly lower under aerobic conditions. The P_{LAC} insertion enabled JVC to aerobically transcribe *nar* and produce functional wild-type levels of respiratory nitrate reductase activity under aerobic conditions, thus clearly demonstrating the physiological significance of posttranslational regulation by oxygen at the level of nitrate transport and/or diversion of electron flow (4, 10, 11).

The results in this investigation are consistent with those of past studies of transcriptional regulation of *nar* (1, 2, 8, 27, 39–41) that were recently challenged by a DNA microarray-based study (7) which reported no significant changes in *narG* transcription aerobically versus anaerobically in *P. aeruginosa* grown on complex medium supplemented with nitrate. The authors of that recent study concluded that nitrate alone was sufficient for *nar* expression in both aerobic and anaerobic environments.

Finally, the modification of pUCGM*lox* allowed us to modulate the expression of the *narK2GHJI* operon for physiological studies which would otherwise be difficult to perform by utilizing plasmid expression vectors currently available for *P. aeruginosa*. With the combination of pREP and P_{LAC}, we were able to conditionally express nitrate reductase activities chromosomally. Intermediate levels of nitrate reductase activities can even be obtained using IPTG (isopropyl-β-D-thiogalactopyranoside) (data not shown). Therefore, the use of this technique enables the investigator to integrate a regulatory element(s) and modulate gene expression of operons and multiple open reading frames to further take advantage of the available *P. aeruginosa* genome database (34).

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REFERENCES

1. **Arai, H., Y. Igarashi, and T. Kodama.** 1995. Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel Crp/Fnr-related transcriptional regulator, Dnr, in addition to Anr. FEBS Lett. **371:**73–76.
2. **Arai, H., T. Kodama, and Y. Igarashi.** 1997. Cascade regulation of the two CBP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. Mol. Microbiol. **25:**1141–1148.
3. **Chiang, R. C., R. Cavicchioli, and R. P. Gunsalus.** 1992. Identification and characterization of *narQ*, a second nitrate sensor for nitrate-dependent gene regulation in *Escherichia coli*. Mol. Microbiol. **6:**1919–1923.
4. **Denis, K. S., F. M. Dias, and J. J. Rowe.** 1990. Oxygen regulation of nitrate transport by diversion of electron flow in *Escherichia coli*. J. Biol. Chem. **265:**18095–18097.
5. **Dias, F. M., R. M. Ventullo, and J. J. Rowe.** 1990. Regulation and energization of nitrate transport in a halophilic *Pseudomonas stutzeri*. Biochem. Biophys. Res. Commun. **166:**424–430.
6. **Eschbach, M., K. Schreiber, K. Trunk, J. Buer, D. Jahn, and M. Schobert.** 2004. Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. J. Bacteriol. **186:**4596–4604.
7. **Filiatrault, M. J., V. E. Wagner, D. Bushnell, C. G. Haidaris, B. H. Iglewski, and L. Passador.** 2005. Effect of anaerobiosis and nitrate on gene expression in *Pseudomonas aeruginosa*. Infect. Immun. **73:**3674–3772.
8. **Galimand, M., M. Gamper, A. Zimmermann, and D. Haas.** 1991. Positive Fnr-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. J. Bacteriol. **173:**1598–1606.
9. **Hassett, D. J., J. Cuppoletti, B. Trapnell, S. V. Lymar, J. J. Rowe, S. S. Yoon, G. M. Hilliard, K. Parvatiyar, M. C. Kamani, D. J. Wozniak, S. H. Hwang, T. R. McDermott, and U. A. Ochsner.** 2002. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv. Drug Delivery Rev. **54:**1425–1443.
10. **Hernandez, D., F. M. Dias, and J. J. Rowe.** 1991. Nitrate transport and its regulation by O_2 in *Pseudomonas aeruginosa*. Arch. Biochem. Biophys. **286:**159–163.
11. **Hernandez, D., and J. J. Rowe.** 1987. Oxygen regulation of nitrate uptake in denitrifying *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **53:**745–750.
12. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
13. **Körner, H., and W. G. Zumft.** 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl. Environ. Microbiol. **55:**1670–1676.
14. **MacGregor, C. H., C. A. Schnaitman, and D. E. Normansell.** 1974. Purification and properties of nitrate reductase from *Escherichia coli* K12. J. Biol. Chem. **249:**5321–5327.
15. **Marx, C. J., and M. E. Lidstrom.** 2002. Broad-host-range *cre-lox* system for antibiotic marker recycling in gram-negative bacteria. BioTechniques **33:**1062–1067.
16. **Nicholas, D. J., and A. Nason.** 1954. Mechanism of action of nitrate reductase from *Neurospora*. J. Biol. Chem. **211:**183–197.
17. **Noriega, C., D. J. Hassett, and J. J. Rowe.** 2005. The *mobA* gene is required for assimilatory and respiratory nitrate reduction but not xanthine dehydrogenase activity in *Pseudomonas aeruginosa*. Curr. Microbiol. **51:**419–424.
18. **Palmer, K. L., S. A. Brown, and M. Whiteley.** 2007. Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum. J. Bacteriol. **189:**4449–4455.
19. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.
20. **Quénée, L., D. Lamotte, and B. Polack.** 2005. Combined *sacB*-based negative selection and *cre-lox* antibiotic marker recycling for efficient gene deletion in *Pseudomonas aeruginosa*. BioTechniques **38:**63–67.
21. **Rabin, R. S., and V. Stewart.** 1993. Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. J. Bacteriol. **175:**3259–3268.
22. **Rabin, R. S., and V. Stewart.** 1992. Either of two functionally redundant sensor proteins, NarX and NarQ, is sufficient for nitrate regulation in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **89:**8419–8423.
23. **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.
24. **Schreiber, K., R. Krieger, B. Benkert, M. Eschbach, H. Arai, M. Schobert, and D. Jahn.** 2007. The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. J. Bacteriol. **189:**4310–4314.
25. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195–1204.
26. **Schweizer, H. P.** 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene **97:**109–112.
27. **Sharma, V., C. E. Noriega, and J. J. Rowe.** 2006. Involvement of NarK1 and NarK2 proteins in transport of nitrate and nitrite in the denitrifying bacterium *Pseudomonas aeruginosa* PAO1. Appl. Environ. Microbiol. **72:**695–701.
28. **Shaw, D. J., and J. R. Guest.** 1981. Molecular cloning of the *fnr* gene of *Escherichia coli* K12. Mol. Gen. Genet. **181:**95–100.
29. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad-host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
30. **Stewart, V.** 2003. Biochemical Society Special Lecture. Nitrate- and nitrite-responsive sensors NarX and NarQ of proteobacteria. Biochem. Soc. Trans. **31:**1–10.
31. **Stewart, V.** 1982. Requirement of Fnr and *narL* functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151:**1320–1325.
32. **Stewart, V., and J. Parales.** 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **170:**1589–1597.
33. **Stouthamer, A. H.** 1991. Metabolic regulation including anaerobic metabolism in *Paracoccus denitrificans*. J. Bioenerg. Biomembr. **23:**163–185.
34. **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, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959–964.
35. **Van Alst, N. E., K. F. Picardo, B. H. Iglewski, and C. G. Haidaris.** 2007. Nitrate sensing and metabolism modulates motility, biofilm formation, and virulence in *Pseudomonas aeruginosa*. Infect. Immun. **75:**3780–3790.

36. **Vander Wauven, C., A. Pierard, M. Kley-Raymann, and D. Haas.** 1984. *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. J. Bacteriol. **160:**928–934.
37. **van Hartingsveldt, J., and A. H. Stouthamer.** 1973. Mapping and characterization of mutants of *Pseudomonas aeruginosa* affected in nitrate respiration in aerobic or anaerobic growth. J. Gen. Microbiol. **74:**97–106.
38. **Vollack, K. U., J. Xie, E. Hartig, U. Romling, and W. G. Zumft.** 1998. Localization of denitrification genes on the chromosomal map of *Pseudomonas aeruginosa*. Microbiology **144:**441–448.
39. **Ye, R. W., D. Haas, J. O. Ka, V. Krishnapillai, A. Zimmermann, C. Baird, and J. M. Tiedje.** 1995. Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. J. Bacteriol. **177:**3606–3609.
40. **Zimmermann, A., C. Reimmann, M. Galimand, and D. Haas.** 1991. Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. Mol. Microbiol. **5:**1483–1490.
41. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.