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Accumulation of Pyrimidine Intermediate Orotate Decreases Virulence Factor Production in *Pseudomonas aeruginosa*

Abdurahman Niazy[1] · Lee E. Hughes[1]

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Abstract The impact of orotate accumulation in the medically important bacterium *Pseudomonas aeruginosa* was studied by deleting *pyrE,* the gene encoding orotate phosphoribosyltransferase and responsible for converting orotate into orotate monophosphate within the de novo pyrimidine synthesis pathway. The *pyrE* mutant accumulated orotate and exhibited decreased production of hemolysin, casein protease, and elastase. Feeding orotate at a concentration of 51.25 μM to the wild type, PAO1, likewise decreased production of these factors except for hemolysin, which was not affected. A significant increase in the pigments pyocyanin and pyoverdin was also observed. Pyocyanin increase in the *pyrE* mutant was heightened when the mutant was supplemented with orotate. Although pyoverdin production in the wild-type PAO1 was unaffected by orotate supplementation, a decrease in the mutant's production was observed when supplemented with orotate. These results indicate a significant reduction in virulence factor production in the *pyrE* mutant and reduction in some virulence factors in the wild type when supplemented with orotate.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that can cause severe infections in immune-compromised individuals such as patients with cystic fibrosis [24] and is also an important cause of nosocomial infections [4]. This bacterium is also notorious for its ability to resist multiple antibiotic families, making it a challenging infection to treat [12]. The ability to resist multiple types of antibiotics contributes to prolonged hospital stays and increased treatment costs [11]. *P. aeruginosa* also produces a plethora of virulence factors that contribute to the severity of infections [20].

Given the medical importance of *P. aeruginosa*, further study is necessary to understand the mechanism by which this organism causes disease. This includes understanding the role that pyrimidine synthesis, important in providing nucleotides for nucleic acid synthesis, plays in virulence factor production. Pyrimidine synthesis in bacteria has been extensively studied [1, 6, 13, 14]. The enzymes of the first three dedicated steps of the pyrimidine pathway (aspartate transcarbamylase encoded by *pyrB,* dihydroorotase encoded by *pyrC*, and dihydroorotate dehydrogenase encoded by *pyrD*) catalyze the production of orotate from carbamoyl phosphate and aspartate. The next step in the pathway is the production of orotate monophosphate (OMP) from orotate and phosphoribosyl pyrophosphate (PRPP) by orotate phosphoribosyltransferase. The gene encoding this enzyme, *pyrE*, is the subject of this work. The final step of the pyrimidine de novo pathway is the decarboxylation of OMP to uracil monophosphate by the product of the *pyrF* gene, OMP decarboxylase.

Mutations in the *pyrE* and *pyrF* genes lead to accumulation and secretion of orotate in bacteria [26]. A similar phenotype is seen in humans exhibiting orotic aciduria [25]. Brichta [2] first described a novel connection between pyrimidine synthesis and virulence factor production in 2003. A closer look at the effect of orotate on virulence factor production was undertaken here by comparing the impact of orotate accumulation in a $pyrE^{-}$ mutant to the wild-type PAO1.

✉ Lee E. Hughes
lhughes@unt.edu

[1] Department of Biological Sciences, University of North Texas, Denton, USA

Materials and Methods

Strains

The strains utilized in this work are listed in Table 1.

Construction of the *pyrE* Mutant

A *pyrE* mutant was constructed utilizing the technique described by Choi and Schweizer [3] using the Gateway® technology from Life Technologies™. A 74-bp segment of the 642 bp *pyrE,* extending from bp336 to bp411, was replaced by a gentamicin resistance cassette. The gentamicin resistance cassette was utilized for final screening of the mutant. The modified *pyrE* was amplified from the mutant with PCR and sequenced for confirmation.

PyrE and PyrF Assays

The enzymatic activities of both PyrE and PyrF were measured using methods described by Beckwith et al. [1] and Schwartz and Neuhard [21, 22]. Cells were grown in *Pseudomonas* minimal media (PsMM) [15] to late log phase, reaching optical density ($OD_{600\ nm}$) of 0.9 A. Cells were lysed by sonication using a Branson 200 Cell Disruptor for 1 min intervals to 6 min total with power amplitude at setting 5, and a cell-free extract was produced by centrifugation at 10 °C for 25 min at 1876.9×*g* using a Sorvall RT600 with an H1000B rotor. The extract was dialyzed against 2 mM β-mercaptoethanol, 20 μM $ZnSO_4$, 50 mM Tris–HCl, pH 8.0 at 4 °C for 18 h, and then kept at 4 °C until use, typically within 24 h. Reaction buffers contained 100 mM Tris/HCl buffer at pH 8.6, 6 mM $MgCl_2$ for both enzymes. In addition, 0.25 mM orotate and 0.6 mM PRPP were added as substrates for PyrE or 0.2 mM OMP included for PyrF assays. 1 mg/ml protein from cell extracts was used for both reactions using a Bradford assay standard curve. Incubation was at 30 °C for 5 min before the addition of substrates. Reactions were monitored every 5 min for 15 min. PyrE activity was measured at $\lambda_{295\ nm}$ and PyrF at $\lambda_{290\ nm}$. A change equal to 3.67 absorbance units is equal to 1 mM increase in OMP for PyrE, while a change equal to 1.38 absorbance units is equal to 1 mM decrease in OMP for PyrF.

Casein Protease

Casein protease activity was assessed for *pyrE*$^-$, and wild-type PAO1 cells were grown in 5 ml peptone tryptic soy broth (PTSB) for 24 h. Cells were collected by centrifugation at 1876.9×*g* using a Sorvall RT600 centrifuge with a H1000B rotor. The supernatant was filter-sterilized using a 0.45-μM filter. Reaction solution contained 0.05 M Tris, pH 7.5, and 0.5 mM $CaCl_2$ containing 0.3 % azocasein from Sigma-Aldrich™ [7]. The reaction contained 50 μl filtered supernatant in a 1-ml reaction solution. The reaction was incubated at 37 °C for 15 min and terminated with 0.5 ml of 10 % trichloroacetic acid. Samples were then centrifuged, and absorbance of the supernatant was determined using a spectrophotometer at $\lambda_{400\ nm}$.

Elastase Production

Elastase production was determined using the elastin congo red assay [16]. Supernatants were prepared as for casein protease measurements. The reaction solution contained 0.1 M Tris, pH 7.2, and 1 mM $CaCl_2$. The reaction contained 50 μl of supernatant in 1 ml reaction solution with 20 mg of elastin congo red (ECR) from Sigma-Aldrich™. The reaction mixture was incubated for 18 h at 37 °C. The mixture was placed on ice, and EDTA was added to 0.12 mM. After a 10 min incubation, the sample was centrifuged to remove remaining ECR, and the absorbance was measured at $\lambda_{495\ nm}$.

Hemolysin Production

TSA plates supplemented with 5 % sheep blood were used to test for the production of hemolysin. Using a sterile toothpick, a culture samples from 24 h colonies were patched onto blood agar plates, which were then incubated for 48 h at 37 °C. Orotate-supplemented plates contained 51.25 μM orotate.

Orotate Accumulation and Secretion

Cells were grown at 37 °C in PsMM broth to $OD_{600\ nm}$ 0.6 A. Cells were then removed by centrifugation to yield final cell pellets and supernatants. For orotate secretion, uninoculated medium with known concentration of orotate

Table 1 Strains used in this study

Strain	Genotype	Source
PAO1	Wild-type *Pseudomonas aeruginosa*	ATCC
pyrE$^-$	PAO1 *pyrE*Δ, GM^r	This study
E. coli	*recA1 hsdR17 endA1 supE44 thi-1 relA1 gyrA96* Δ	BRL, 1986
DH5α	*(argF-lacZYA)U169* φ*80lacZ* Δ *M15*	

was used as reference to create an orotate standard curve at $\lambda_{278\ nm}$. Uninoculated medium was used as blank and media for both mutant and wild-type *P. aeruginosa* samples. Cells were sonicated in breaking buffer (2 mM β-mercaptoethanol, 20 μM $ZnSO_4$, 50 mM Tris–HCl, pH 8.0, and 20 % glycerol) without orotate supplement, and cell debris was removed by centrifugation. Orotate was measured in the supernatant at $\lambda_{278\ nm}$ [17].

Pyocyanin Production Assay

The method adapted from Essar et al. [5] was used for this assay. 30 ml of King's A [8] broth was used in a 125-ml flask. Bacteria were grown in King's A broth for 20 h in triplicate samples at 37 °C with shaking. To determine cell density, absorbance reading was measured at $\lambda_{600\ nm}$ for each flask. Five ml of the sample was transferred to a 15-ml conical centrifuge tube and centrifuged for 25 min at 1876.9×*g* using a H1000B rotor on a Sorval TR 6000B centrifuge. The supernatant was then filtered through a 0.45-μm filter into another 15 ml conical centrifuge tube. To precipitate organic matter, 3 ml of chloroform was added, and the mixture was vortexed 10 times for 2 s. The mixture was then centrifuged for 7 min at 1876.9×*g* using the same conditions mentioned above. The top layer was removed using a 5-ml pipet, 1.5 ml of 0.2 N HCL was added, and the mixture was vortexed 10 times for 2 s. The sample was centrifuged again at 1876.9×*g* for 7 min. The top pink layer was collected, and the absorbance was measured at λ_{520}.

Pyoverdin Production Assay

50 ml of King's B broth [8] in a 125-ml flask was inoculated with freshly grown cells. The broth was incubated for 12 h with shaking at 37 °C. After 12 h, cell density was measured at $\lambda_{600\ nm}$. 1 ml of culture was centrifuged at 10053×*g* in a Speed Fuge® HSC10 K microcentrifuge using a HSR-24 rotor. The absorbance of the supernatant was measured at $\lambda_{405\ nm}$. The results were recorded as 12-h $\lambda_{405\ nm}/\lambda_{600\ nm}$ [22].

Results

PyrE/PyrF Activities and Orotate Accumulation

A complete loss of PyrE activity was observed in the mutant. As expected, the resultant absence of *pyrF* inducer (OMP) eliminated expression of this gene, too. It was found that the PAO1 had 1.96 μM orotate in cell extracts, while the *pyrE*$^-$ had 3.36 μM. This represented a 58 % increase in the mutant strain. Wild-type PsMM cultures had no detectable orotate in the medium, while medium from *pyrE*$^-$ cultures contained 0.403 μM of orotate.

Elastase and Casein Protease Assays

pyrE$^-$ cultures exhibited a sharp decrease in elastase production as compared to PAO1, with an average reading of 0.035 A at $\lambda_{495\ nm}/\lambda_{600\ nm}$, which compared to 0.102 A for the wild type. Elastase production in the wild-type fed orotate decreased to an average reading of 0.037 A, which is a similar level to that of the mutant (Fig. 1). No casein protease was measurable for the *pyrE*$^-$ cells. Casein protease production by the PAO1 showed a 64 % reduction when cells were supplemented with orotate, falling from an average reading at $\lambda_{495}/\lambda_{600}$ for non-supplemented wild type of 0.0797 to 0.0512 A for orotate-supplemented (at 51.25 μM) wild type (Fig. 2).

Hemolysis

The wild type showed β-hemolysis both with and without orotate supplementation. The *pyrE*$^-$ exhibited α-hemolysis. However, when fed orotate, the *pyrE*$^-$ lost its hemolysis completely (γ-hemolysis) (Fig. 3).

Pyocyanin Production

Pyocyanin production increased significantly by the *pyrE*$^-$ compared to PAO1. PAO1 produced 4.2 μM, while there was an increase in pyocyanin production to 12.34 μM. When feeding the *pyrE*$^-$ orotate at 51.25 μM, it was observed that there was an additional increase in pyocyanin production to 15.1 μM, while PAO1 increased to 4.97 μM (Fig. 4).

Pyoverdin Production

The *pyrE*$^-$ produced almost double the amount of pyoverdin produced by PAO1 when measured at 12 h of

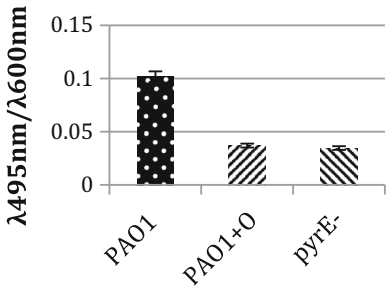

Fig. 1 Elastase production in PAO1 without any supplement, with orotate supplement, and the *pyrE*$^-$. Elastase production was measured in PTSB medium after 24 h of incubation at 37 °C and measured at $\lambda_{495\ nm}/\lambda_{600\ nm}$

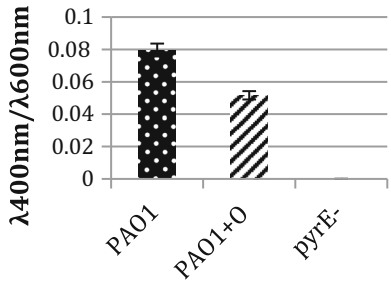

Fig. 2 Casein protease production in PAO1 without any supplement, with orotate supplement, and the *pyrE*$^{-}$. Casein protease production was measured in PTSB medium after 24 h of incubation at 37 °C and measured at $\lambda_{400\ nm}/\lambda_{600\ nm}$

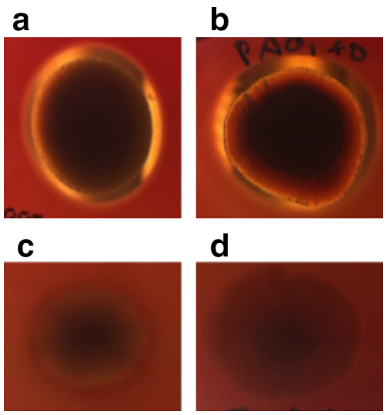

Fig. 3 Hemolysis in the presence and absence of orotate for both PAO1 and mutant. **a** β-hemolysis for PAO1 without any supplement. **b** β-hemolysis of PAO1 with orotate supplement. **c** α-hemolysis of the *pyrE*$^{-}$ without any supplement. **d** γ-hemolysis of *pyrE*$^{-}$ in the presence of orotate

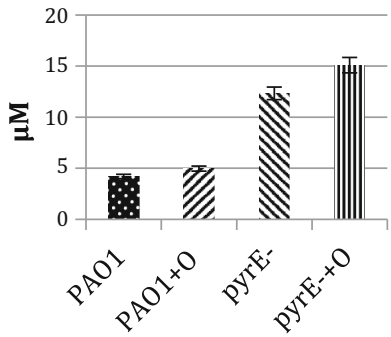

Fig. 4 Increase in pyocyanin production in *pyrE*$^{-}$ compared to PAO1 and increase in pyocyanin production when PAO1 and *pyrE*$^{-}$ were fed orotate at 51.25 μM after 20-h incubation in King's A medium

incubation. When adding orotate to the media at a concentration of 51.25 μM, PAO1 did not show any increase in pyoverdin production, while the *pyrE*$^{-}$ dropped to levels similar to that of PAO1 (Fig. 5).

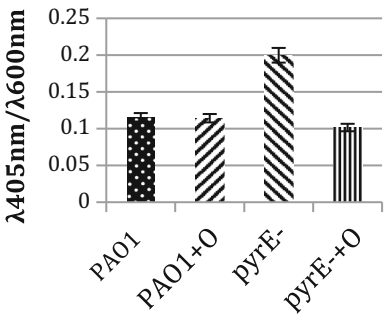

Fig. 5 Pyoverdin production of PAO1 compared to the *pyrE*$^{-}$ in the presence of 51.25 μM orotate and in the absence of it after 12 h of incubation in King's B medium

Discussion

Previous studies have shown that uracil increases quorum sensing activity in *P. aeruginosa* and, as a result, increases virulence factor production [23]. Because uracil increases quorum sensing activity, it was expected that orotate would act likewise given the similarity in structure. Surprisingly, the opposite was found as orotate supplementation or overproduction decreased the production of the virulence factors casein protease, elastase, and hemolysin. Brichta [2] found a connection between pyrimidine synthesis and virulence factor production through construction of a double knockout on *pyrC* that led to decreased virulence factor production. In comparison, we find that knocking out *pyrC* led to decreased production of hemolysin, casein protease, elastase, pyoverdin, and pyocyanin. Casein protease was decreased in the *pyrC*$^{-}$ unlike the *pyrE*$^{-}$ where casein protease activity was completely absent. Pigment production in the *pyrC*$^{-}$ showed decreased pyoverdin and pyocyanin production, while the *pyrE*$^{-}$ showed an increase in these pigments. The pyoverdin increase is possibly due to the accumulation of dihydroorotate which is proposed to be a part of the pyoverdin structure [10]. What is unclear is why the *pyrE*$^{-}$ showed a sharp increase in pyocyanin production. When feeding wild-type PAO1 orotate at 51.25 μM, pyocyanin increased slightly as also seen when the mutant is fed orotate. With dihydroorotate being structurally a part of pyoverdin, it is possible that orotate may also be involved in the structure of pyocyanin or it may be a stimulator of the pyocyanin synthesis pathway. There is a similar pattern of increase in both pyocyanin and pyoverdin levels, indicating that orotate may be involved in the production of both. Further studies could explore these relationships, including utilizing transcriptomics or ^{14}C labeled orotate.

Even though it was shown in a previous study by Santiago and West [19] that pyoverdin also increased slightly in *Pseudomonas putida pyrC* mutants, for the *pyrE* mutant in the same study, a much higher increase in pyoverdin

production was seen. This may indicate that dihydroorotate is not absolutely essential for pyoverdin production in *P. putida*. It is also observed in *P. aeruginosa* that *pyrC* double knockouts showed a sharp drop in pyoverdin production which differs from the results observed in *P. putida* [2]. Supplementation of media with orotate clearly decreased virulence factor production in the wild-type strain of *P. aeruginosa*, and the *pyrE*$^{-}$ strain also showed a significant decrease in virulence factors, which is likely due to the accumulation of orotate in these cells. The decreases in virulence factors seen in this study could also be due to disruption of the quorum sensing network by orotate.

The reduction in virulence factor production as a result of disrupting the pyrimidine synthesis pathway could provide a target with implications for the control of *P. aeruginosa* infections. Finding approaches which attack the *P. aeruginosa* pyrimidine synthesis pathway without harming eukaryotic cell pyrimidine synthesis would be a significant step in this direction. However, due to the similarities between the eukaryotic pyrimidine synthesis pathway and the bacterial pathway, it might be difficult to achieve that goal. Similar strategies, such as 5-flourouracil used as a treatment for skin cancer [9], would be a starting point for consideration. Other pyrimidine analog might be utilized to inhibit the pyrimidine pathway in bacteria in concert with antibiotics. This might especially be useful when bacteria form complex biofilms inside the body where they are protected from phagocytosis and traditional antibiotics. It would also be intriguing to take a look at the effect of external application of orotate to subjects infected with *P. aeruginosa*. Exogenous feeding of orotate could reduce disease symptoms and help to combat the infection.

Further study on the increased production of pyocyanin could also have commercial implications. In 2005, Rabaey et al. [18] showed that pyocyanin increased electron transfer in biofuel cells. They found that introduction of mutants that were deficient in pyocyanin production resulted in reduction of power output to 5 %. Output increased up to 50 % when pyocyanin was added. The addition of pyocyanin also enhanced power output by other bacteria within the biofuel cell. Additional studies on *Pseudomonas* strains that are known to produce high amounts of pyocyanin could determine if knocking out *pyrE* might further increase pyocyanin production. If so, higher pyocyanin production in *P. aeruginosa* could contribute to improved biofuel cell output.

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References

1. Beckwith JR, Pardee AB, Austrian R, Jacob F (1962) Coordination of the synthesis of enzymes in the pyrimidine pathway of *E. coli*. J Mol Biol 5:618–634
2. Brichta DM (2003) Construction of a *Pseudomonas aeruginosa* dihydroorotase mutant and the discovery of a novel link between pyrimidine biosynthetic intermediates and the ability to produce virulence factors. Ph.D. Dissertation, University of North Texas
3. Choi KH, Schweizer HP (2005) An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. BMC Microbiol 5:30
4. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP (1999) Nosocomial blood infection in United States hospitals: a three-year analysis. Clin Infect Dis 29:239–244
5. Essar DW, Eberly L, Hadero A, Crawford IP (1990) Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. J Bacteriol 172:884–900
6. Evans DR, Guy HI (2004) Mammalian pyrimidine biosynthesis: afresh insight into an ancient pathway. J Biol Chem 279: 30335–33038
7. Kessler E, Safrin M, Olson JC, Ohman DE (1993) Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. J Biol Chem 268:7503–7508
8. King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of pyocanin and fluorescin. J Lab Clin Med 44:301–307
9. Love WE, Bernhard JD, Bordeaux JS (2009) Topical imiquimod or fluorouracil therapy for basal and squamous cell carcinoma: asystematic review. Arch Dermatol 145:1431–1438
10. Maksimova NP, Blazhevich OV, Fomichev IuK (1993) The role of pyrimidines in the biosynthesis of the fluorescing pigment pyoverdin Pm in *Pseudomonas putida* M. Mol Gen Mikrobiol Virusol 5:22–26
11. Mauldin PD, Salgado CD, Hansen IS, Durup DT, Bosso JA (2009) Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant gram-negative bacteria. Antimicrob Agents Chemother 54:109–115
12. Morita Y, Tomida J, Kawamura Y (2013) Responses of *Pseudomonas aeruginosa* to antimicrobials. Front Microbiol 4:422
13. Neuhard J, Kelln RA, Stauning E (1986) Cloning and structural characterization of the *Salmonella typhimurium pyrC* gene encoding dihydroorotase. Eur J Biochem 157:335–342
14. O'Donovan GA, Neuhard J (1970) Pyrimidine metabolism in microorganisms. Bacteriol Rev 34:278–343
15. Ornston LN, Stanier RY (1966) The conversion of catechol and protocatechuate to beta-ketoadipate by *Pseudomonas aeruginosa*. J Biol Chem 241:3776–3786
16. Pearson JP, Pesci EC, Iglewski BH (1997) Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol 179: 5756–5767
17. Peters GJ, Laurensse E, Leyva A, Pinedo HM (1987) A sensitive, nonradiometric assay for dihydroorotic acid dehydrogenase using anion-exchange high-performance liquid chromatography. Anal Biochem 15:32–38
18. Rabaey K, Boon N, Höftee M, Verstraete W (2005) Microbial phenazine production enhances electron transfer in biofuel cells. Environ Sci Technol 39:3401–3408
19. Santiago MF, West TP (2002) Control of pyrimidine formation in *Pseudomonas putida* ATCC 17536. Can J Microbiol 48: 1076–1081

20. Schuek KN, Breidenstein E, Hancock RE (2012) *Pseudomonas aeruginosa*: a persistent pathogen in cycstic fibrosis and hospital-associated infections. In: Dougherty TJ, Pucci MJ (eds) Antibiotic discovery and development, 1st edn. Springer, New York, pp 679–715
21. Schwartz M, Neuhard J (1975) Control of expression of the *pyr* genes in *Salmonella typhimurium*: effects of variations in uridine and cytidine nucleotide pools. J Bacteriol 121:814–822
22. Stintzi A, Barnes C, Xu J, Raymond KN (2000) Microbial iron transport via a siderophore shuttle: a membrane ion transport paradigm. Proc Natl Acad Sci USA 97:10691–10696
23. Ueda A, Attila C, Whiteley M, Wood KT (2009) Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist. Microb Biotechnol 2:62–74
24. Wilson R, Dowling RB (1998) Lung infections. 3. *Pseudomonas aeruginosa* and other related species. Thorax 53:213–219
25. Winkler JK, Suttle DP (1988) Analysis of UMP synthase Gene and mRNA structure in hereditary orotic aciduria fibroblasts. Am J Hum Genet 43:86–94
26. Womack JE, O'Donovan GA (1978) Orotic acid excretion in some wild-type strains of *Escherichia coli* K-12. J Bacteriol 136:825–827