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Identification of *Pseudomonas aeruginosa* Genes Involved in Virulence and Anaerobic Growth

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Pseudomonas aeruginosa **is a gram-negative, opportunistic pathogen and a significant cause of acute and chronic infections in patients with compromised host defenses. Evidence suggests that within infections *P. aeruginosa* encounters oxygen limitation and exists in microbial aggregates known as biofilms. However, there is little information that describes genes involved in anaerobic growth of *P. aeruginosa* and their association with virulence of this pathogen. To identify genes required for anaerobic growth, random transposon (Tn) mutagenesis was used to screen for mutants that demonstrated the inability to grow anaerobically using nitrate as a terminal electron acceptor. Of approximately 35,000 mutants screened, 57 mutants were found to exhibit no growth anaerobically using nitrate. Identification of the genes disrupted by the Tn revealed 24 distinct loci required for anaerobic growth on nitrate, including several genes not previously associated with anaerobic growth of *P. aeruginosa*. Several of these mutants were capable of growing anaerobically using nitrite and/or arginine, while five mutants were unable to grow anaerobically under any of the conditions tested. Three mutants were markedly attenuated in virulence in the lettuce model of *P. aeruginosa* infection. These studies have identified novel genes important for anaerobic growth and demonstrate that anaerobic metabolism influences virulence of *P. aeruginosa*.**

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen that is responsible for numerous acute and chronic infections and presents a considerable problem for patients with severe burns, cystic fibrosis (CF), and other immunocompromising states (24). *P. aeruginosa* is the most common pathogen infecting CF patients and is the leading cause of morbidity and mortality in these individuals (25). Once acquired, it is difficult to eradicate *P. aeruginosa* due to several factors previously shown to contribute to the chronic infection observed with *P. aeruginosa* lung infections. The organism has an intrinsic ability to resist a variety of antimicrobial agents, making successful treatment very difficult. Furthermore, it has been demonstrated that within the infected host, the bacteria reside in biofilms, which are also highly resistant to antibiotic treatment (22, 41). For these reasons, identifying new targets for therapy has become exceedingly important.

In nature, *P. aeruginosa* frequently inhabits environments where anaerobic niches develop, such as water-logged soils, bogs, and sediments. Also, in their natural habitats, microorganisms, including *P. aeruginosa*, often grow as biofilms, which have been shown to contain oxygen gradients and anaerobic pockets (8, 50). In addition to other well-studied stresses encountered in the host (i.e., nutrient deprivation, changes in pH, and iron limitation), there is mounting evidence that *P. aeruginosa* encounters oxygen-limited environments in vivo which develop during the infections themselves or as a result of biofilm development (15, 48). Anaerobic and microaerobic conditions have been described for a number of *P. aeruginosa* infections, including various forms of otitis, wound injuries, and pulmonary infections, such as those seen in individuals with CF (5, 6, 24, 30, 36). In chronic CF lung infections, it has been shown that *P. aeruginosa* grows in low-oxygen environments present within the mucus plugs found in the lower airways of CF patients (48). As neutrophils infiltrate and mucus secretion increases, the metabolically active bacteria and CF epithelial cells consume the available oxygen and the environment quickly becomes anaerobic (48). The organisms become mucoid by secreting the exopolysaccharide alginate, which further restricts the diffusion of oxygen and contributes to the formation of microaerobic or anaerobic environments (16, 18). Furthermore, during a number of infections, *P. aeruginosa* resides in biofilms (9), where bacteria at the base and the center of the biofilm are encased by an anaerobic environment (50). It has been suggested that *Pseudomonas* prefers this oxygen-limited environment, displaying enhanced biofilm formation under anaerobic conditions (51).

When oxygen is not available, alternative external electron acceptors, including nitrate, nitrite, or nitrous oxide, can be utilized by *P. aeruginosa* (16). In the absence of nitrate or nitrite, arginine can be catabolized by substrate-level phosphorylation and serve as an energy source for anaerobic growth (28, 45). More recently it was shown that *P. aeruginosa* can use pyruvate to sustain long-term survival; however, pyruvate fermentation cannot support growth of the bacteria under hypoxic conditions (11). The major regulator controlling the physiological switch between aerobic and anaerobic growth conditions in *Pseudomonas* is the transcriptional regulator ANR (anaerobic regulation of arginine catabolism and nitrate reduction), which is an orthologue of the *Escherichia coli* qumarate and nitrate reductase regulator (FNR) (39). Anaerobic growth of *P.*

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aeruginosa on nitrate or arginine requires ANR, as mutants defective in ANR cannot grow anaerobically with nitrate or arginine (52). ANR has been demonstrated to influence expression of a limited number of genes in *P. aeruginosa* that are involved in arginine fermentation, cyanogenesis, and denitrification. More recently another transcriptional regulator, termed DNR (PA0527), was shown to control expression of several denitrification genes (2).

Although the denitrification pathways used during anaerobic growth have been well studied, research investigating the ability of *P. aeruginosa* to survive under strict anaerobic conditions is very limited, and only recently has this topic been explored more closely (7, 18, 21, 37, 51). While our recent microarray studies indicate that a large number of *P. aeruginosa* genes encoding proteins of unknown function are differentially expressed as a result of anaerobiosis, these studies do not indicate whether these genes are required for anaerobic growth (12). Thus, in this study, we used transposon (Tn) mutagenesis to identify *P. aeruginosa* genes essential for anaerobic growth on nitrate. The mutants were examined for the ability to grow anaerobically on the alternative electron acceptor nitrite and arginine. To determine if the genes are required for virulence, we also tested the mutants for their ability to cause disease in a plant model of *P. aeruginosa* infection.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* strain PAO1 was maintained at 37°C on NY (2.5% nutrient broth and 0.5% yeast extract) agar plates supplemented with 100 mM KNO_3. Procedures for growing *P. aeruginosa* anaerobically are described elsewhere (46). Briefly, medium was prepared by dissolving dry ingredients into H_2O which had been boiled to remove oxygen and then sparging the solution with N_2 gas prior to autoclaving. Prior to inoculation, agar plates were prereduced by incubation in an anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2; Coy Laboratories Inc., Ann Arbor, MI) for 24 h. For anaerobic growth screening using various electron acceptors, Luria agar base medium was used with the electron acceptors KNO_3 and KNO_2 at 100 mM and 10 mM final concentrations, respectively, and arginine at a concentration of 40 mM (17). For broth studies, aerobic overnight cultures were used to inoculate 200-ml portions of medium in 500-ml Erlenmeyer flasks to obtain a starting optical density at 600 nm (OD_{600}) of 0.05, and the resulting cultures were grown aerobically at 37°C with rotary shaking at 300 rpm. For anaerobic growth studies, aerobic overnight cultures were subcultured into 200 ml of prereduced NY containing KNO_3 in 250-ml culture bottles, agitated with a magnetic stir bar, and grown in an anaerobic chamber. The bacterial cultures were monitored spectrophotometrically (OD_{600}) at 1-h intervals for growth curve analysis. For growth analyses in minimal medium, strains which were grown overnight in LB medium or LB medium containing tetracycline were used to inoculate M9 medium (38) and growth was monitored spectrophotometrically (OD_{600}) at 1-h intervals. *Escherichia coli* DH5α was grown on LB agar or in LB broth. When required, the medium was supplemented with ampicillin at 50 μg/ml for *E. coli*, carbenicillin (Cb) at 200 μg/ml for *P. aeruginosa*, or tetracycline at 12 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*.

Construction of *P. aeruginosa* Tn mutants. *P. aeruginosa* strain PAO1 was subjected to insertional mutagenesis with the EZ:Tn tetracycline kit (Epicenter Technologies, Madison, WI) according to the manufacturer's instructions, and clones harboring the Tn were selected on NY medium supplemented with 100 mM KNO_3 and 100 μg/ml of tetracycline (NY-$KNO_3$$Tet_{100}$). Tetracycline-resistant colonies were replica plated onto fresh NY-$KNO_3$$Tet_{100}$ plates and incubated at 37°C in the presence or absence of oxygen for 48 h to identify clones incapable of anaerobic growth. All mutants that were unable to grow anaerobically but able to grow aerobically were chosen and retested for their inability to grow anaerobically at least three independent times.

Southern Blot analysis. Southern blotting was carried out by standard methods (38). Briefly, chromosomal DNA isolated from the anaerobic-growth-deficient mutants was digested with either EcoRI or XhoI and electrophoretically separated on 0.8% agarose gels. DNA was transferred to Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by capillary blotting overnight and fixed by UV cross-linking. A DNA fragment corresponding to the tetracycline resistance gene was biotinylated using the Psoralen-biotin kit (Ambion, Austin, TX) and used as a probe. Detection was performed with the BrightStar BioDetect kit (Ambion).

Determination of the Tn insertion site. (i) Complementation of Tn mutants. Competent cells of the Tn mutants were prepared and transformed with the *P. aeruginosa* plasmid DNA library which was constructed in pUCP18 (40; M. J. Filiatrault, unpublished data). Cells were plated on NY-$KNO_3$$Tet_{100}$ supplemented with Cb (200 μg/ml) and incubated anaerobically. After 24 to 48 h of incubation, colonies which appeared to grow anaerobically were isolated, and reinoculated onto NY-$KNO_3$$Tet_{100}$ plates supplemented with Cb, and grown aerobically, and the plasmids were isolated (QIAGEN, Valencia, CA). Following reconfirmation of the ability of each plasmid to complement the anaerobic-growth defect, the plasmids were reisolated and sequenced. PCR using gene-specific primers and chromosomal DNA from the Tn mutant was then used to determine the Tn insertion site.

(ii) Inverse PCR. DNA sequences adjacent to the site of Tn insertion were amplified using an inverse PCR procedure (27) with the following modifications. Briefly, EcoRI-digested mutant genomic DNA was self-ligated and a portion of this ligation mixture used as a template for an inverse PCR with Tn-specific primers provided in the EZ:Tn tetracycline kit. (Tn For, 5′-GGGTGCGCATG ATGGTGTAGAGT-3′; Tn Rev, 5′-TAAATTGACTGAAATCTAGAAATA-3′). PCR was carried out using the GC-Rich kit (Roche, Indianapolis, IN) using the following conditions: denaturation at 95°C for 3 min, annealing at 55°C for 30 s, and elongation at 72°C for 2 to 4 min for a total of 35 cycles. Amplicons were separated by agarose gel electrophoresis and purified using the QIAGEN gel extraction kit (QIAGEN). DNA sequences of PCR products were obtained by using automated DNA sequencing (University of Rochester Sequencing Facility, Rochester, N.Y.) and primers to the Tn (described above).

(iii) Cloning of the Tn. Fragments from EcoRI-digested mutant chromosomal DNA were ligated into EcoRI-digested pUCP18, and the ligation mixture was used to transform *E. coli* DH5α. Plasmids from transformants selected on LB containing tetracycline (15 μg/ml) were isolated, and their inserts were sequenced.

Genomic sequence analysis. DNA sequences were obtained by using automated DNA sequencing (Functional Genomics Center, University of Rochester, Rochester, N.Y.). The sequences obtained were compared with the *P. aeruginosa* PAO1 genomic sequence (www.pseudomonas.com) by using BLAST analysis to identify Tn insertion sites.

Lettuce leaf model of infection. The protocol for using romaine lettuce leaves in a model of *P. aeruginosa* infection was performed as described previously (32) with the noted modifications. Dole romaine hearts were purchased commercially, and infected leaves were incubated at 37°C in small plastic containers containing moistened Whatman paper (10 mM $MgSO_4$). *P. aeruginosa* strains were grown aerobically overnight at 37°C in LB broth or LB broth containing tetracycline (100 μg/ml) when appropriate, washed twice with 10 mM $MgSO_4$, and diluted in sterile $MgSO_4$ to a bacterial density of 1×10^8 CFU/ml. Lettuce leaves were prepared by washing with distilled H_2O and 0.1% bleach. Lettuce mid-ribs were inoculated with 10 μl of bacterial suspension at a density of 1×10^8 CFU/ml (corresponding to $\sim 1 \times 10^6$ bacteria) as previously described (32) and placed in plastic containers containing Whatman paper moistened with 10 mM $MgSO_4$. Lettuce was incubated at 37°C, and symptoms were monitored daily over the course of 5 days. As a control, lettuce leaves were inoculated with 10 mM $MgSO_4$. A separate lettuce leaf was used for each mutant. The experiments were repeated a minimum of three times on independent days.

RESULTS

Construction and screening of *Pseudomonas aeruginosa* Tn mutants deficient in anaerobic growth on nitrate. To identify factors important for anaerobic growth, Tn mutants of *P. aeruginosa* were constructed and clones that demonstrated the inability to grow anaerobically after 48 h using nitrate as a terminal electron acceptor were selected for further study (see Materials and Methods). Southern analysis confirmed randomness of the insertions into the PAO1 genome and verified that

TABLE 1. *P. aeruginosa* genes essential for anaerobic growth on nitrate

ORF[a]	No. of mutants[b]	Gene name	Description[c]
PA0520	3	*nirQ*	Regulatory protein NirQ
PA0523	2	*norC*	Nitric oxide reductase subunit C
PA0524	5	*norB*	Nitric oxide reductase subunit B
PA0525	4	*norD*	Probable dinitrification protein NorD
PA1006	2		Conserved hypothetical protein
PA1544	7	*anr*	Transcriptional regulator Anr
PA1546	6	*hemN*	Oxygen-independent coproporphyrinogen III oxidase
PA1850	1		Probable transcriptional regulator
PA2637	1	*nuoA*	NADH dehydrogenase I chain A
PA2638	2	*nuoB*	NADH dehydrogenase I chain B
PA2639	2	*nuoD*	NADH dehydrogenase I chain C,D
PA2641	1	*nuoF*	NADH dehydrogenase I chain F
PA2642	4	*nuoH*	NADH dehydrogenase I chain G
PA2644	2	*nuoI*	NADH dehydrogenase I chain I
PA2645	1	*nuoJ*	NADH dehydrogenase I chain J
PA2646	1	*nuoK*	NADH dehydrogenase I chain K
PA2648	5	*nuoM*	NADH dehydrogenase I chain M
PA2649	1	*nuoN*	NADH dehydrogenase I chain N
PA3164	1	*aroA*	3-Phosphoshikimate 1-carboxyvinyltransferase prephenate dehydrogenase
PA3912	1		Conserved hypothetical protein
PA3913	1		Probable protease
PA3918	1	*moaC*	Molybdopterin biosynthetic protein C
PA4049	1		Hypothetical protein
PA5497	2		Hypothetical protein

[a] *Pseudomonas* genome database designation (www.pseudomonas.com).
[b] Number of independent mutants isolated with insertion in designated gene.
[c] Descriptions were taken from the *Pseudomonas* genome database (www.pseudomonas.com).

a single copy of the Tn was present in each mutant (data not shown). Although PAO1 displayed anaerobic growth after 24 h, incubation was carried out for 48 h to minimize the selection of mutants which may exhibit slower growth. Of approximately 35,000 Tn mutants screened, 57 mutants failed to grow anaerobically with nitrate yet retained the ability to grow in the presence of oxygen.

Identification of genes essential for anaerobic growth of *P. aeruginosa* on nitrate. Due to the difficulty encountered when directly sequencing chromosomal DNA from the Tn mutants, the insertion sites of the Tn in the 57 mutants were determined by inverse PCR, genetic complementation, or direct subcloning as described in Materials and Methods. The sequences obtained from each of these methods were compared with the *P. aeruginosa* PAO1 genomic sequence (www.pseudomonas.com).

Among the 57 mutants, 24 distinct open reading frames (ORFs) were disrupted (Table 1). While many of the genes identified encode proteins previously shown to be required for anaerobic growth (e.g., *anr* [52], *nirQ* [19], and *norCB* [10]), unique genes (PA1006, PA3912, PA3913, PA4049, and PA5497) which have not been previously associated with anaerobic growth were also identified. *norCB* have not been previously reported to be required for anaerobic growth of *P. aeruginosa*; however, studies by Yoon et al. showed poor anaerobic biofilm development with this mutant and suggested that this phenotype might be due to poor anaerobic growth (51). Also, PA0525 (putative *norD*), previously referred to as "ORF6," has been reported to be required for anaerobic growth in the presence of nitrate, but this was referred to as "unpublished data" (1). In contrast to a previous report for *P. aeruginosa* (35), our screen identified *hemN*, a gene involved in heme biosynthesis in *P. aeruginosa*, which is in agreement with the requirement for this enzyme for anaerobic growth in other organisms (23, 44).

Characterization of the Tn mutants. Both aerobic and anaerobic growth curves in liquid media were generated for each of the Tn mutants and compared to the growth of the wild-type strain PAO1. Since there were numerous insertions in the *nuo* operon, we randomly chose several representative strains to evaluate. Due to the inability of the mutants to grow anaerobically, an aerobically grown culture was used as a source of inoculum. Representative growth curves (Fig. 1) illustrate that aerobic growth was comparable between all of the mutants and the wild type. As expected, a majority of the Tn mutants failed to exhibit anaerobic growth after 10 h (Fig. 1B), with no growth evident even after 24 h (data not shown). However, one Tn mutant (disrupted in PA3912) demonstrated slight anaerobic growth, albeit significantly less than that of the wild type (Fig. 1B).

Since *P. aeruginosa* can also use nitrite as a terminal electron acceptor and arginine for energy, we examined the ability of the mutants to use these substrates for anaerobic growth (Table 2). Only three mutants (those with disruptions in PA1006, PA1850, and PA3918) demonstrated anaerobic growth on nitrite and arginine. This is consistent with the role of PA3918 (*moeC*) in nitrate respiration and suggests a role for PA1006 and PA1850 in nitrate respiration. Sixteen mutants were able to grow anaerobically using arginine but not nitrate or nitrite, suggesting a role for these genes in denitrification and not arginine catabolism. The Tn mutants disrupted in *anr*, *hemN*, PA3912, PA3913, and PA5497 were unable to grow anaerobically using any of the substrates tested. These data are consistent with previous reports on growth characteristics of the *anr* mutant (52) and indicate a general requirement for *hemN*, PA3912, PA3913, and PA5497 in anaerobic growth and not exclusively in denitrification.

To determine if the Tn insertions were lethal under anaerobic conditions, mutants were tested for their ability to be rescued from anaerobic growth after the designated incubation time for each electron acceptor or arginine. All of the Tn mutants displayed visible growth when incubated aerobically, implying that all of the mutations were static rather than bactericidal (Table 2). However, the mutant with a disruption in *hemN* required 48 h to recover from exposure to anaerobic conditions, whereas all other mutants displayed aerobic growth within 24 h, suggesting that the *hemN* mutation was more detrimental than the others.

Anr, HemN, and PA1850 are required for full infection in the lettuce leaf. While there have been numerous reports implicating genes involved in denitrification in the virulence of several bacteria, these studies have not been extended to *P. aeruginosa* (3, 4, 13, 14, 20, 47). Plants have been used as a fast, inexpensive, high-throughput screening tool to identify putative virulence determinants (32). Although no direct measurements of oxygen levels in plant-associated infections or biofilms have been reported, the biofilms on leaves or roots of plants are likely to experience conditions similar to those found in laboratory biofilms (29) and hence

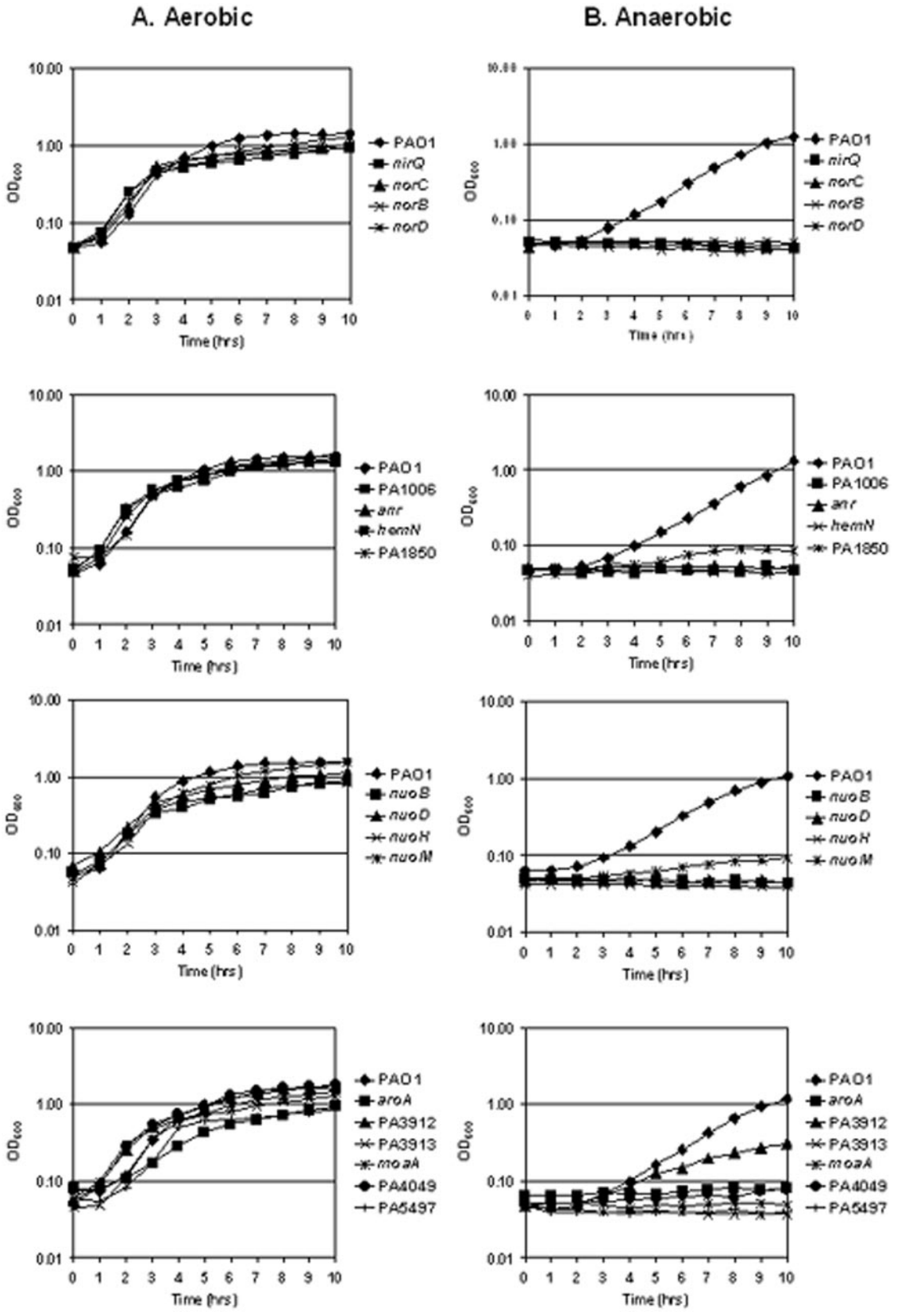

FIG. 1. Representative growth curves of wild-type PAO1 and Tn mutants grown aerobically (A) or anaerobically (B).

TABLE 2. Growth of Tn mutants on various electron acceptors and arginine

ORF[a]	Gene name	Growth on:					
		KNO_3[b]		KNO_2[c]		Arginine[d]	
		Anaerobic	Rescue[e]	Anaerobic	Rescue	Anaerobic	Rescue
PA0520	*nirQ*	−	+	−	+	+	+
PA0523	*norC*	−	+	−	+	+	+
PA0524	*norB*	−	+	−	+	+	+
PA0525	*norD*	−	+	−	+	+	+
PA1006		−	+	+	+	+	+
PA1544	*anr*	−	+	−	+	−	+
PA1546	*hemN*	−	+	−	+	−	+
PA1850		−	+	+	+	+	+
PA2637	*nuoA*	−	+	−	+	+	+
PA2638	*nuoB*	−	+	−	+	+	+
PA2639	*nuoD*	−	+	−	+	+	+
PA2641	*nuoF*	−	+	−	+	+	+
PA2642	*nuoH*	−	+	−	+	+	+
PA2644	*nuoI*	−	+	−	+	+	+
PA2645	*nuoJ*	−	+	−	+	+	+
PA2646	*nuoK*	−	+	−	+	+	+
PA2648	*nuoM*	−	+	−	+	+	+
PA2649	*nuoN*	−	+	−	+	+	+
PA3164	*aroA*	−	+	−	+	+	+
PA3912		−	+	−	+	−	+
PA3913		−	+	−	+	−	+
PA3918	*moaC*	−	+	+	+	+	+
PA4049		−	+	−	+	+	+
PA5497		−	+	−	+	−	+

[a] *Pseudomonas* genome database designation (www.pseudomonas.com).

[b] Anaerobic growth of mutants on KNO_3 was determined by plating on LB plates containing 100 mM KNO_3 and incubating under anaerobic conditions for 48 h. The wild-type strain PAO1 grows on this medium after 24 h. +, growth; −, no growth.

[c] Anaerobic growth of mutants on KNO_2 was determined by plating strains on LB plates containing 10 mM KNO_2 and incubating under anaerobic conditions for 72 h. Growth of the wild-type strain PAO1 appears after 72 h. +, growth; −, no growth.

[d] Anaerobic growth of mutants on arginine was determined by plating on LB plates containing 40 mM L-arginine and incubating under anaerobic conditions for 5 days. Growth of the wild-type strain PAO1 appears after 5 days. +, growth; −, no growth.

[e] After anaerobic incubation with either nitrate, nitrite, or arginine, plates were then transferred to aerobic growth conditions at 37°C for a period of 24 h to determine if growth could be restored. +, growth aerobically; −, no growth aerobically.

TABLE 3. Virulence of PAO1 and Tn mutants in the lettuce leaf infection model of *P. aeruginosa*

Strain or ORF[a]	Gene	Description[b]	Virulence in lettuce[c]
PAO1			Severe
PA0520	*nirQ*	Regulatory protein NirQ	Moderate
PA0523	*norC*	Nitric oxide reductase subunit C	Moderate
PA0524	*norB*	Nitric oxide reductase subunit B	Moderate
PA0525	*norD*	Probable dinitrification protein NorD	Moderate
PA1006		Conserved hypothetical protein	Moderate
PA1544	*anr*	Transcriptional regulator Anr	Weak
PA1546	*hemN*	Oxygen-independent coproporphyrinogen III oxidase	Weak
PA1850		Probable transcriptional regulator	Weak
PA2637	*nuoA*	NADH dehydrogenase I chain A	Moderate
PA2638	*nuoB*	NADH dehydrogenase I chain B	Moderate
PA2639	*nuoD*	NADH dehydrogenase I chain C,D	Moderate
PA2641	*nuoF*	NADH dehydrogenase I chain F	Severe
PA2642	*nuoH*	NADH dehydrogenase I chain G	Moderate
PA2644	*nuoI*	NADH dehydrogenase I chain I	Moderate
PA2645	*nuoJ*	NADH dehydrogenase I chain J	Moderate
PA2646	*nuoK*	NADH dehydrogenase I chain K	Moderate
PA2648	*nuoM*	NADH dehydrogenase I chain M	Moderate
PA2649	*nuoN*	NADH dehydrogenase I chain N	Severe
PA3164	*aroA*	3-Phosphoshikimate 1-carboxyvinyltransferase prephenate dehydrogenase	Moderate
PA3912		Conserved hypothetical protein	Severe[d]
PA3913		Probable protease	Severe
PA3918	*moaC*	Molybdopterin biosynthetic protein C	Moderate
PA4049		Hypothetical protein	Severe
PA5497		Hypothetical protein	Moderate

[a] *Pseudomonas* genome database designation (www.pseudomonas.com). ORF represents the ORF that was disrupted by the transposon in the Tn mutant.

[b] Descriptions were taken from the *Pseudomonas* genome database (www.pseudomonas.com).

[c] Virulence was assayed in the lettuce model of infection and symptoms recorded after 5 days postinoculation. None, no signs of infection; weak, localized water soaking and chlorosis of tissue at injection site; moderate, moderate water soaking and chlorosis of tissue around injection site; severe, soft rotting of the entire leaf characterized by a water-soaked reaction zone and chlorosis around the injection site. PAO1 Iglewski is "severe" on this scale. Symptoms reported represent observations from three independent experiments.

[d] Severe maceration of the leaf was observed after only 3 days, and therefore this mutant was considered to be "hypervirulent."

may develop anaerobic pockets. To determine if disruptions in genes required for anaerobic growth on nitrate would influence virulence of *P. aeruginosa*, the Tn mutants were tested for their ability to cause infection in a lettuce leaf model. As summarized in Table 3, most of the Tn mutants elicited moderate disease symptoms on the lettuce mid-ribs compared to the wild-type strain PAO1. The Tn mutants with disruptions in *anr*, *hemN*, and PA1850 displayed a significantly reduced infection potential in the lettuce leaf (Table 3 and Fig. 2), whereas severe maceration of the leaf was observed with the mutant disrupted in PA3912 (data not shown).

To exclude the possibility that the reduced virulence observed with the Tn mutants with disruptions in *anr*, *hemN*, and PA1850 was not due to growth defects, the ability of these mutants to grow in vitro in a minimal medium was tested. The Tn mutants with disruptions in *anr*, *hemN*, and PA1850 grew similarly to the wild-type strain PAO1 (Fig. 3), suggesting that the reduced virulence in the lettuce is not simply due to growth defects.

DISCUSSION

Bacterial pathogens are exposed to various environments during infection. The elucidation of those genes essential to growth and survival in appropriate surroundings, such as oxygen limitation, not only will provide better insights into the anaerobic metabolism of *P. aeruginosa* but also may help to identify potential targets as possible candidates for antimicrobial therapy. Through the use of random Tn mutagenesis, we have identified genes required for anaerobic growth of *P. aeruginosa* with the terminal electron acceptor nitrate. Although 35,000 mutants were screened, a number which theoretically represents reasonable coverage of the genome, we recognize that there were some preferences of the Tn within certain regions of the chromosome (*nuo* operon), and it is likely that additional genes remain to be identified. By further evaluating the ability of the mutants to grow anaerobically on nitrite and arginine, we were able to identify genes required for anaerobic growth on all substrates tested, demonstrating that our approach was successful in identifying genes generally required for anaerobic growth. We also acknowledge that essen-

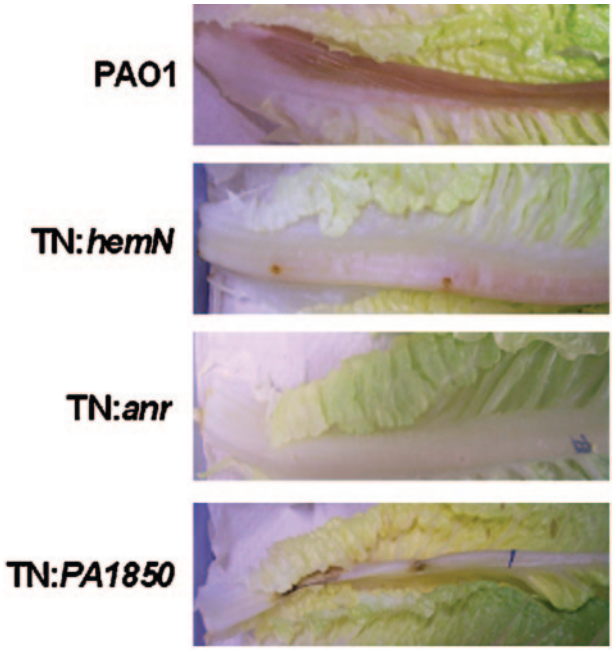

FIG. 2. Infection of lettuce mid-ribs. Photographs of lettuce mid-ribs after 5 days infection with 1×10^6 bacteria are shown. Infection by PAO1 shows necrosis and tissue maceration. The *hemN*, *anr*, and PA1850 mutants show weak signs of infection.

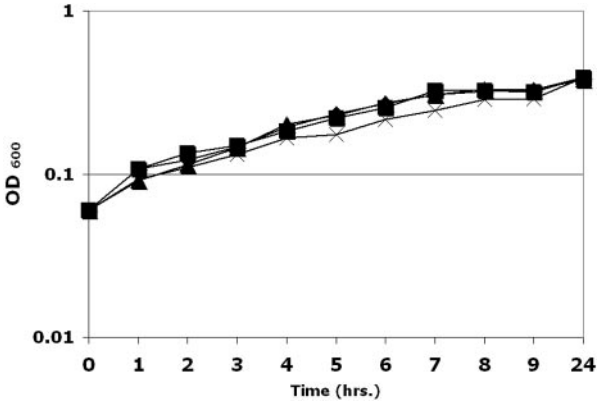

FIG. 3. Representative growth curves of wild-type PAO1 (◆), Tn:PA1850 (■), Tn:*anr* (▲), and Tn:*hemN* (×) grown aerobically in M9 minimal medium.

tial genes for both aerobic and anaerobic growth would have been eliminated based on the approach used. Although this is beyond the scope of our study, an alternative approach using DNA microarrays was used to identify genes conditionally essential for aerobic and anaerobic growth for *E. coli* (42). That study identified 319 genes potentially essential for both aerobic and anaerobic growth. However, a high ratio of false positives was seen, and the requirement of these genes for both aerobic and anaerobic growth was not confirmed. No homologues of the genes we identified as required for anaerobic growth of *P. aeruginosa* were found in this previous study.

Our studies identified genes previously associated with anaerobic growth of *P. aeruginosa*. Our screen revealed that the genes required for the NADH dehydrogenase (NDH) complex I are required for anaerobic growth on nitrate. This is not surprising, since NDH complex I is reported to be coupled to the denitrification pathway (34) and the bacteria contain a second NDH (*ndh*) which is fully capable of supplying electrons to the respiratory chain during aerobic growth.

The coproporphyrinogen III oxidases catalyze the last step in heme biosynthesis, which is the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX (35). For this step in heme biosynthesis, most bacteria possess at least two different coproporphyrinogen III oxidases, one which is an oxygen-dependent enzyme (HemF) for aerobic conditions and one which is oxygen independent (HemN) for anaerobic conditions. We found that *hemN* is required for anaerobic growth for *P. aeruginosa*. In addition, the Tn mutants disrupted in *hemN* were unable to grow on nitrite and arginine, suggesting that the inability of these mutants to grow anaerobically can be correlated to the absence of oxygen and not the utilization of a specific terminal electron acceptor or arginine. However, our results are in contrast to previous studies which reported that mutation of *hemN* or *hemF* did not abolish aerobic or anaerobic growth (35). This appears to be somewhat contradictory in that the authors of the previous study found that expression of both *hemF* and *hemN* was induced approximately 20-fold during anaerobic growth. In several other bacteria, *hemN* has been found to be essential for anaerobic growth on nitrate and nitrite and *hemF* is required for aerobic production of heme (23). We isolated several mutants with disruptions in *hemN*, supporting a role for *hemN* in anaerobic growth of *P. aeruginosa*. Polar effects can be excluded, since there is not a gene located downstream of *hemN*, further supporting a role for *hemN* in anaerobic growth of *P. aeruginosa*.

Importantly, our studies successfully identified several novel genes that currently have no known function and have not been previously associated with anaerobic growth of *P. aeruginosa*. One of the ORFs that we found to be required for anaerobic growth is PA1006. This ORF is classified as encoding a conserved protein with no known function. However, BLAST analysis revealed that this predicted protein contained a conserved domain homologous to disulfide bond redox regulators. Tn mutants with disruptions in PA1006 were able to grow anaerobically using nitrite and arginine. These data provide further insight as to a possible role for the protein in denitrification or, specifically, the utilization of nitrate.

Another cluster of genes found to be required for anaerobic growth on nitrate consisted of PA3913 and PA3912. PA3913 and PA3912 encode probable proteases (peptidase family U32 signature) and are 78% similar to a putative collagenase of *E. coli*. Since these genes are located in a potential operon with PA3911, it is possible that the Tn insertions had polar effects. PA3911 encodes a conserved hypothetical protein with homology to putative lipid carrier proteins belonging to the SCP-2 sterol transfer family. Additional studies are in progress to determine if all three genes of this operon are involved in anaerobic growth. Interestingly, these genes are located directly downstream of genes involved in molybdenum cofactor biosynthesis (PA3914 to PA3918). The fact that these mutants were unable to grow on nitrite and arginine suggests that the inability of these mutants to grow anaerobically can be corre-

lated to the absence of oxygen and not the utilization of a specific terminal electron acceptor or arginine.

PA4049 encodes a hypothetical protein with homology to bacterial ABC-type periplasmic transport systems, which are a diverse group of periplasmic transport receptors for lysine/arginine/ornithine, glutamine, histidine, sulfate, phosphate, molybdate, and methanol. Also, the insertion in this gene may have had a downstream effect on PA4048, which appears to be in an operon with PA4049. Further studies are needed to confirm the role of PA4049 in anaerobic growth of *P. aeruginosa*.

PA1850 encodes a probable transcriptional regulator containing an amidase domain and an AraC-type DNA-binding helix-turn-helix domain. The AraC family contains highly versatile regulators which have been shown to regulate a number of bacterial functions, including sugar catabolism, responses to stress, and virulence factor expression (26). We are currently investigating what genes may be under control of this putative regulator. These studies will likely identify additional genes required for anaerobic growth on nitrate.

PA5497 was previously classified as a hypothetical protein but has been recently reported to encode the class II ribonucleotide reductase (43). Based on transcriptional studies and enzyme activities, those authors suggested that the two-component class II ribonucleotide reductase in *P. aeruginosa* is used primarily for DNA repair and/or possibly DNA replication at low oxygen tension. Our data show that this enzyme is required for anaerobic growth of *P. aeruginosa*.

We have recently determined the transcriptional response of *P. aeruginosa* to the presence of the electron acceptor nitrate and anaerobiosis (12). Very few of the novel genes we found to be required for anaerobic growth on nitrate demonstrate transcriptional regulation by oxygen or nitrate (12). PA1006, PA1850, and PA4049 are not differentially expressed in response to the presence of nitrate or anaerobiosis, suggesting that these transcripts are constitutively expressed between aerobic and anaerobic conditions, that they may be regulated posttranscriptionally, or that the microarray analyses were not sensitive enough to detect changes of these transcripts (12). Three of the five genes which encode conserved hypothetical proteins demonstrated differential gene expression in our previous experiments. Transcripts for PA3913 and PA3912 were promoted by nitrate but not by anaerobiosis, and the PA5497 transcript was promoted by anaerobiosis. In silico analysis shows putative DNA binding sequences for Anr in the putative promoter regions of PA3913 and PA5497 (data not shown), suggesting that these genes may be regulated by Anr. In a recent proteomic study (49), the proteins encoded by PA3913 and PA5497 were reported to be upregulated under anaerobiois, further demonstrating a role of these genes in anaerobic metabolism.

There is precedence for linking genes involved in anaerobic respiration to virulence of bacteria other than *P. aeruginosa* (3, 4, 13, 14, 20, 47). We chose to use the lettuce leaf model of infection to efficiently screen the mutants described to determine if they displayed reduced virulence. Plants have been used as an in vivo pathogenesis model for the identification of virulence factors of the human opportunistic pathogen *P. aeruginosa* (32), and there are supporting data that demonstrate that the virulence mechanisms between plant and animal models are conserved (31). Many of the mutants showed at least a slight decrease in virulence in the lettuce model (Table 3). Interestingly, the most dramatic decreases in virulence were observed with the mutants containing disruptions in *anr*, PA1850, and *hemN*. These genes have not previously been reported to be involved in virulence of *P. aeruginosa*. It is interesting that two of the genes encode known (Anr) or putative (PA1850) transcriptional regulators. Since not all of the genes required for anaerobic growth on all substrates tested (nitrate, nitrite, or arginine) were required for virulence in the lettuce model (PA3912, PA3913, and PA5497), this suggests that the lettuce model may not involve strict anaerobic growth of this pathogen or that in vivo *P. aeruginosa* can utilize alternative pathways for anaerobic growth. These data also suggest alternative explanations for the reduced virulence in the *anr*, *hemN*, and PA1850 mutants. One possibility is that these genes may directly or indirectly regulate or influence expression of other genes which affect virulence. In fact, it has been suggested that *anr* represses components of the aerobic respiratory pathway, such as the terminal cytochrome oxidases, while activating a number of genes under low-oxygen and anaerobic conditions (33). Therefore, it is likely that *anr*, as well as PA1850, may regulate genes that have yet to be identified which are involved in virulence.

One of the mutants, with a disruption in PA3912, demonstrated a "hypervirulent" phenotype; however, a mutation in PA3913 (the first gene of the putative gene cluster of PA3913 to -11) did not affect virulence. Although we have no direct evidence, one possible explanation for this result is that disruption of PA3912 results in an increase in expression of PA3913, which encodes a putative protease. Other proteases have been shown to be involved in virulence, lending support to this hypothesis. Further study of this gene cluster is under way to determine the function and regulation of the genes in this cluster. It will be interesting to determine if these genes are required for virulence in other models of *P. aeruginosa* infection, since virulence determinants have been shown to be conserved between plants and animals (31).

While this study identified mutants with disruptions in some genes previously associated with anaerobic growth, it also links several novel genes and the anaerobic physiology of *P. aeruginosa*. We are currently investigating the roles of these genes in the anaerobic growth of *P. aeruginosa* as well as their effects on virulence of this important pathogen. Further transcriptional characterization of the identified genes is under way to determine their inclusion in possible operons and if surrounding genes may also be required for anaerobic growth. Also, these mutants will be more thoroughly characterized using additional virulence models of *P. aeruginosa* pathogenesis. Our current and future studies of these genes will extend our knowledge of the anaerobic physiology of *P. aeruginosa* and the relationship to pathogenesis.

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