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Detection of Nitrate/Nitrite Bioavailability in Wastewater Using a *luxCDABE*-Based *Klebsiella oxytoca* Bioluminescent Bioreporter

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Abstract In the present study, we have constructed a bioluminescent bioreporter for the assessment of nitrate/nitrite bioavailability in wastewater. Specifically, an approximately 500-bp DNA fragment containing a nitrate/nitrite-activated *nasR*-like promoter (regulating expression of genes encoding nitrite reductase in the genus *Klebsiella*) was fused upstream of the *Vibrio fischeri luxCDABE* gene cassette in a modified mini-Tn5 vector. Characterization of this strain, designated W6-1, yielded dose-dependent increased bioluminescence coincident with increased nitrate, nitrite, and ammonium added to the growth medium from 1 to 11 ppm. Bioluminescence in response to nitrogen species addition was light dependent up to 10, 7, and 8 ppm with nitrate, nitrite, and ammonium, respectively. This response was linear in the range from 1 to 8 ppm for nitrate (R^2=0.98), 1 to 6 ppm for nitrite (R^2=0.99), and 1 to 7 ppm for ammonium (R^2=0.99). A significant bioluminescent response was also recorded when strain W6-1 was incubated with slurries from aged, nitrate/nitrite contaminated wastewater. Thus, bioreporter strain W6-1 can be used to elucidate factors that constrain the use of nitrate/nitrite in wastewaters.

Keywords: *Klebsiella oxytoca*, bioluminescence, bioreporter, *lux*, *nasR*, nitrate, nitrite, ammonium, bioavailability

Nitrogen removal in wastewater treatment is traditionally achieved by degradation of organic compounds followed by nitrification of liberated ammonium (NH_4^+) to nitrate (NO_3^-) that is in turn reduced (denitrification) to free nitrogen (N_2), with oftentimes transient accumulation of nitrite (NO_2^-). Surveillance of ammonium, nitrate, and nitrite levels are important, since concentration fluctuations can be predictive of ensuing treatment plant process upsets such as sludge bulking, and elevated concentrations can significantly influence the receiving water quality [29]. Free form ammonia (NH_3) is acutely toxic to aquatic life and creates large oxygen demands that promote high algal productivity (eutrophication) and subsequent alterations in ecosystem balances [9, 29]. Nitrates similarly contribute to eutrophication and are believed to be linked to human health effects, especially in infants and pregnant women, although this remains controversial [4, 17, 30]. Effluent nitrite compounds are toxic to aquatic life [11], increase chlorine demand [31], and contribute to greenhouse gases *via* nitrous oxide emission [31]. As with nitrate, the human health effects related to nitrite ingestion, typically gastric cancers, are inconclusive [4, 33].

Because of the various influences that nitrate, nitrite, and ammonia have, their monitoring becomes an important consideration in proper wastewater treatment management. Although analytical methods such as gas and high-performance liquid chromatographies or mass and atomic absorption spectrometries can be used, the complexity and cost involved are obstructive. In addition, they fail to provide data as to the bioavailability of the pollutant, its effects on living systems, or its potential synergistic/antagonistic behavior in mixtures. A recently developed approach for the quantification of nutrient availability in freshwater environments uses cyanobacterial whole-cell bioluminescent bioreporters [6, 7, 18]. These bioreporters provide data on the capacity of the biota to acquire and assimilate these nutrients. Recombinant bioluminescent cyanobacterial strains have been successfully applied in monitoring iron [14, 15] and phosphate [16] availability in freshwater.

In the present study, we constructed a *Klebsiella oxytoca* strain W6-1 bioluminescent bioreporter to assess nitrate, nitrite, and ammonium bioavailability in wastewater environments. The construct employs a promoter analogous to the nitrate/nitrite reductase gene *nasR* fused to the *Vibrio fischeri luxCDABE* gene cassette. It is known that *Klebsiella* spp., members of the family *Enterobacteriaceae*, can use nitrate

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and nitrite as sole nitrogen sources during aerobic growth. Nitrate and nitrite are reduced to ammonium by assimilatory nitrate and nitrite reductases, respectively [21, 32]. *K. oxytoca* can assimilate nitrate and nitrite by using enzymes encoded by the *nasFEDCBA* operon. Expression of this operon is controlled by general nitrogen regulation (Ntr) *via* the NtrC transcriptional activator and by pathway-specific nitrate and nitrite induction *via* the NasR transcriptional antiterminator [8, 21, 23].

The constructed W6-1 bioreporter strain has advantages over previously constructed *luxAB*-based cyanobacterial whole-cell bioluminescent bioreporters [6, 7, 18]. By using the complete *luxCDABE* cassette rather than solely *luxAB*, the bioreporter is fully autonomous in its bioluminescent response, requiring no addition of substrate or otherwise light-activating manipulations. The bioluminescent signal is also short-lived, thus allowing for repetitive sampling under dynamic conditions. Similar bioreporters have recently been shown to be compatible with emerging signal detection technologies, such as integrated circuits capable of processing and communicating signal input [1, 24, 25].

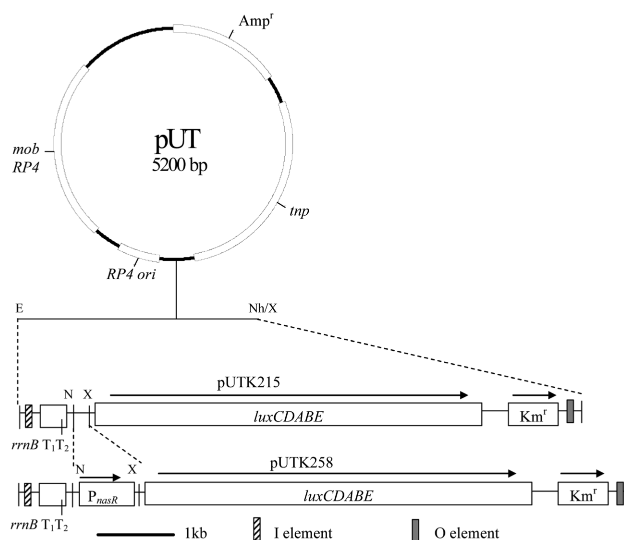

Fig. 1. Genetic constructs used for construction of the W6-1 bioluminescent bioreporter.

For descriptions of the suicide vectors pUT and pUTK215, see de Lorenzo *et al.* [12] and Applegate *et al.* [5] respectively. pUTK258 contains the *nasR*-like promoter isolated from strain DF4 ligated to the *V. fischeri luxCDABE* cassette. E, EcoRI; X, XbaI; Nh, NheI; Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

MATERIALS AND METHODS

Isolation and Characterization of the Nitrite/Nitrate Assimilating Strain W6

Klebsiella oxytoca strain W6 was isolated from activated sludge from the West-Alexandria wastewater treatment plant, Egypt. Genetic and molecular characterization of this strain has been previously reported [2].

Construction of a Bioluminescent Bioreporter for Nitrite/Nitrate

An approximate 0.5 kb *nasR*-like promoter segment was amplified from strain W6 using two primers specific for the *nasR* promoter of *K. oxytoca* M5aI (GenBank Accession No. L27824) [32]. The first primer, GCGGCCGCTTTCAG-CTGGCATTGT-3', targeted the region from 3–20 bp of the *nasR* sequence and contained an introduced unique NotI site on its 5' end (underlined). The second primer, TCTAGAAACCAGTCGACCACCTCAG-3', was targeted to the region from 523–542 bp and contained an introduced unique XbaI site on its 5' end (underlined). PCR was performed using the following protocol: initial 3 min denaturation at 94°C; 30 cycles in which the denaturing, annealing, and extension times and temperatures were 94°C for 30 s, 52°C for 30 s, and 72°C for 1.5 min, respectively; and a final extension cycle at 72°C for 5 min. The resulting PCR product was cloned into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) to form the plasmid W-6P, which was digested with NotI and XbaI. This produced a 0.5 kb *nasR*-like promoter fragment that was then ligated into a promoterless *luxCDABE* cassette from *V. fischeri* carried on a modified mini-Tn5 vector pUTK215 [5] that had previously been digested with NotI and XbaI (Fig. 1). The ligation mix was transformed into electrocompetent *Escherichia coli* SV17 (λ*pir*) and resulting transformants selected on Luria-Bertani (LB) plates containing 50 mg kanamycin/L. *E. coli* SV17 (W-6P) was then mated with strain W6 and selected on LB plates containing ampicillin and kanamycin at 50 mg/l each. Colonies present after overnight incubation at 30°C were replicated onto LB plates containing 100 mM nitrite ($NaNO_2$), nitrate ($NaNO_3$), or ammonium (NH_4Cl) and examined for light production visible by the naked eye. One colony, designated W6-1, was chosen for further analysis.

Bioluminescent Assays

Bioluminescent assays using the W6-1 bioreporter were performed essentially as described by Abd-El-Haleem *et al.* [3]. Strain W6-1 was grown overnight at 30°C in yeast extract-peptone-glucose (YEPG) medium (in g/l; yeast extract, 0.2; polypeptone, 2.0; glucose, 1.0; pH 7.0). The next day, cultures were diluted 1:10 in YEPG medium and incubated with shaking at 30°C to an optical density of 0.56 at 546 nm. The culture was washed two times and then resuspended in two volumes of a nitrogen-free medium (NFM) containing (in g/l) 2.75 g of K_2HPO_4, 2.25 g of KH_2PO_4, 0.2 g of $MgSO_4$, 0.1 g of NaCl, 0.02 g of $FeCl_3 \cdot 6H_2O$, and 0.01 g of $CaCl_2$, pH 7.0. Two ml of the culture was then added to 20-ml scintillation vials containing 2.0 ml of NFM supplemented with nitrite, nitrate, or ammonium-

saturated NFM at final concentrations ranging from 0 to 11 ppm. Aliquots of 200 µl were removed from each scintillation vial and transferred to black 96-well microtiter plates (Nunc, Rochester, NY, U.S.A.) to produce 3 replications of each dilution. Wells were covered with transparent plate sealer (Topseal-A, Perkin-Elmer, Boston, MA, U.S.A.) and placed in a microtiter plate luminometer (Lumistar Galaxy, BMG, Germany). Bioluminescence, as counts per second (CPS), was monitored at regular time intervals at room temperature with shaking (1 s duration with shaking speed set at high).

Substrate Specificity

To determine substrate specificity of the W6-1 bioreporter, microtiter plate assays were performed essentially as described above using NFM supplemented with sodium nitrate, sodium nitrite, and ammonium chloride as sole sources of nitrogen (6 ppm each). In addition, various classes of organic chemicals (6 ppm each) including sulfanilamide, 4-nitrophenol, amidosulfate, and dinitrosalicylic acid were tested. Non-nitrogenous compounds such as phenylhydrazinum chloride, salicylic acid, pentachlorophenol, acetone, phenol, catechol, methanol, naphthalene, and chromium sulfate were also examined.

Bioluminescent Assay in Nitrogen-Contaminated Water

To assess the response of strain W6-1 under more environmentally relevant conditions and examine its ability to sense nitrate/nitrite in real wastewater samples, bioluminescent assays were performed using aged nitrogen-contaminated water obtained from the inlet and the outlet of the Burg-Elarab sewage and industrial wastewater treatment plant, Alexandria, Egypt. This wastewater treatment plant uses a biological oxidation pond system. Two ml of each water sample was filtered through 0.2-µm syringe filters and then added to 2 ml of a W6-1 culture resuspended in NFM. W6-1 cells were also added to sterile dH_2O void of nitrogen contaminants (negative control) as well as to NFM containing 6 ppm nitrite or nitrate (positive control). Aliquots of 200 µl were removed from each scintillation vial and transferred to black 96-well microtiter plates to produce 3 replications of each dilution. Wells were covered with transparent plate sealer and placed in the Lumistar Galaxy microtiter plate luminometer for luminescence detection at room temperature. The concentrations of nitrate, nitrite, ammonium, and other water quality indicators in the water samples were determined using standard analytical methods [10].

Effect of Heavy Metals, and Aromatic and Polyaromatic Hydrocarbons on Cellular Bioluminescence of Strain W6-1

For each bioassay, the effect of each individual metal of Fe, Cu, Ni, Co, Cr, Hg, and Pb on cellular bioluminescence of strain W6-1 in the presence of 6 ppm nitrite was determined from three independent replicates. Standard solutions of chlorides of heavy metals were prepared in HPLC-grade sterile water. Heavy metals were used at a concentration of 20 ppm. Stock solutions were prepared at concentrations at which no precipitation was observed over 72 h at room temperature. If a precipitate was observed in the medium over the experimental period, the assayed concentration was not considered for further investigation. All manipulations were conducted under controlled conditions, avoiding metal and microbial contamination and using disposable polystyrene or high-density polyethylene. The final experimental metal concentrations were obtained by dilution of the corresponding stock solutions. All hydrocarbons (phenol, 2-nitrophenol, 5-chlorophenol, dimethylphenol, catechol, anthrathene, and naphthalene) were of analytical grade and were used without further purification. The metals and organic substances were obtained from Sigma (St. Louis, MO, U.S.A.) and ACS certified.

For the bioassay, 100 µl of each metal sample was added to 100 µl of freshly grown bacterial suspension (previously pelleted by centrifugation, washed twice with sterile dH_2O, and resuspended in NFM containing 6 ppm nitrite) and placed in a microtiter plate well. For each treatment, a positive control (containing only 6 ppm nitrite) was prepared that was identical to the metal stock but without the metal salt. A negative control (bacterial cells in NFM without addition of nitrite or metals) was also prepared. Duration of exposure to the examined metal was the same for all metals, ranging from zero (just before addition of the metal in the medium) until the maximum amount of light produced by the positive control was observed.

RESULTS

Bioluminescent Response Kinetics of the W6-1 Bioreporter

The W6 *nasR*-like promoter was ligated to the *luxCDABE* cassette to form the bioluminescent bioreporter W6-1, which was exposed to varying concentrations of nitrate, nitrite, and ammonium (0–11 ppm) to determine overall response kinetics. Resulting bioluminescent responses are plotted in Fig. 2. Typical Michaelis-Menten kinetics were observed. Bioluminescence induction occurred at nitrate, nitrite, and ammonium concentrations as high as 10, 7, and 8 ppm, respectively, after which toxicity effects led to a rapid decrease in optical density and consequent decrease in bioluminescence. For all examined nitrogen species, the lower limit of detection within the tested range was determined to be 1 ppm. Linearity of the bioluminescent response was observed at nitrate, nitrite, and ammonium concentrations ranging from 1 to 8 (R^2=0.98), 1 to 6 (R^2=

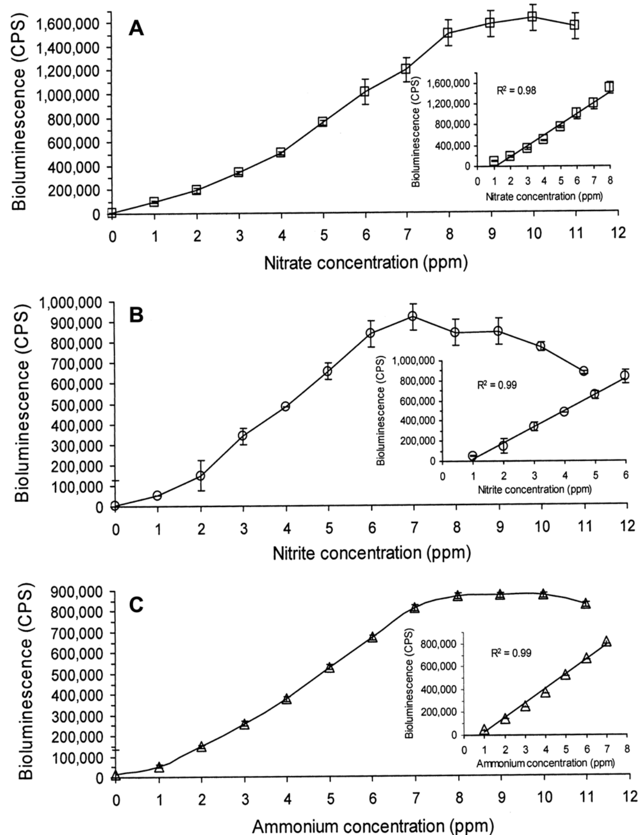

Fig. 2. Bioluminescent response of the W6-1 bioreporter to nitrate (**A**), nitrite (**B**), and ammonium (**C**) and the parental W6 *Klebsiella* strain (control) to various concentrations of nitrate, nitrite, and ammonium.

Inset shows linearity of the curve occurring at 1 to 8, 1 to 6, and 1 to 7 ppm nitrate, nitrite, and ammonium, respectively. CPS, bioluminescent counts s^{-1}. Error bars represent standard error of the mean (n=3).

0.99), and 1 to 8 ppm (R^2=0.99), respectively. At nitrate, nitrite, and ammonium concentrations within this range, bioluminescence was typically initiated at 50, 100, and 50 min,

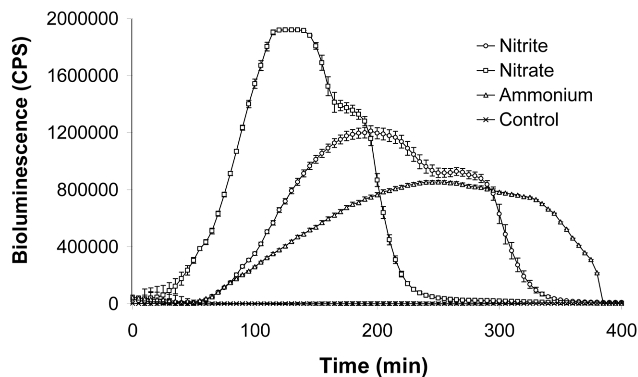

Fig. 3. Bioluminescent response of strain W6-1 in nitrogen-free medium (NFM) containing nitrate, nitrite, or ammonium at 6 ppm and NFM as a control.

Error bars calculated as for Fig. 2.

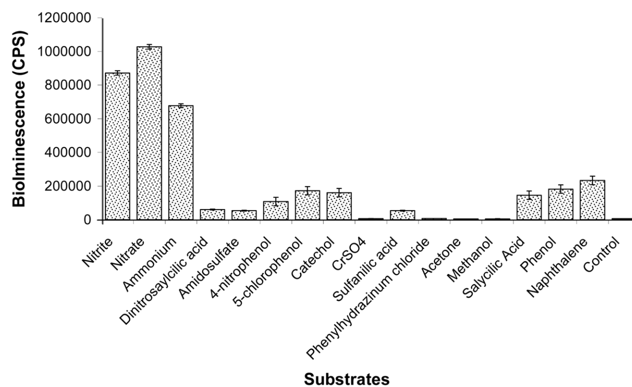

Fig. 4. Bioluminescence induction of the W6-1 bioreporter to nitrate, nitrite, ammonium and some organic chemicals containing or not containing nitrogen.

Graphs shown are representative exposures of each chemical at concentrations of approximately 6 ppm. Error bars calculated as for Fig. 2.

respectively, after exposure and increased exponentially thereafter up to approximately 130 min with nitrite and ammonium and 260 min with nitrate whereupon maximum bioluminescence occurred at an average of 1,900,000, 1,200,000, and 850,000±59,000 counts/s (CPS), respectively (Fig. 3). Bioluminescence rapidly decreased beyond 130 and 260 min owing to cell death, as determined by reductions in optical density.

Substrate Specificity of the W6-1 Bioreporter

To establish substrate specificity, the W6-1 bioreporter was exposed to sodium nitrate, sodium nitrite, and ammonium chloride as sole sources of nitrogen (Fig. 4). In addition, various classes of organic chemicals containing or not containing nitrogen were tested. Exposures to organic chemicals containing or not containing nitrogen yielded only background levels of bioluminescence (<20,000 CPS) (Fig. 4). However, exposure to the three examined nitrogen species (nitrate, nitrite, and ammonium) produced bioluminescence values varied in the following sequence: nitrate>nitrite>ammonium.

Nitrogen Bioavailability in Wastewater Samples

To examine the applicability of the developed W6-1 bioreporter strain for monitoring the bioavailability of nitrogen in real water samples, wastewater samples were collected on 15 August 2004 from the inlet and outlet of the sewage and industrial wastewater treatment plants at New Burg-Elarab City, Alexandria, Egypt (both sewage and industrial wastewater treatment plants are based on a stabilization pond system). The concentrations of total nitrogen, nitrate, nitrite, ammonium, and other water quality indicators in the collected water samples are presented in Table 1. As shown in Figs. 5A and 5B, light output emitted by the *Klebsiella* W6-1 sensor strain correlated well with the concentrations of total nitrogen, nitrate, and nitrite in

Table 1. Concentrations of water quality indicators in the inlet and outlet of both sewage and industrial wastewater treatment plants at New Burg-Elarab City.

Water quality indicators	Measuring unit	Wastewater treatment plants			
		Sewage		Industrial	
		Inlet	Outlet	Inlet	Outlet
pH	Value	7.7	8.81	6.98	7.17
BOD	mg/l	810	330	660	180
COD	mg/l	1441	979	1718	716
TSS	mg/l	150	100	200	75
Fats & oils	mg/l	110	50	180	20
Total nitrogen	mg/l	11.6	4.24	10.6	3.36
Nitrate-N	mg/l	10.82	2.89	8.04	1.65
Nitrite-N	μg/l	80	50	105	20
Ammonium-N	mg/l	1.21	1.02	2.24	1.12
Total phosphate	mg/l	2.94	4.83	2.37	5.1
Cyanide	mg/l	0.0	0.0	0.0	0.0
Phenol	mg/l	0.171	0.024	0.23	0.10
Total bacterial count	CFU/ml	2285	342	500	32

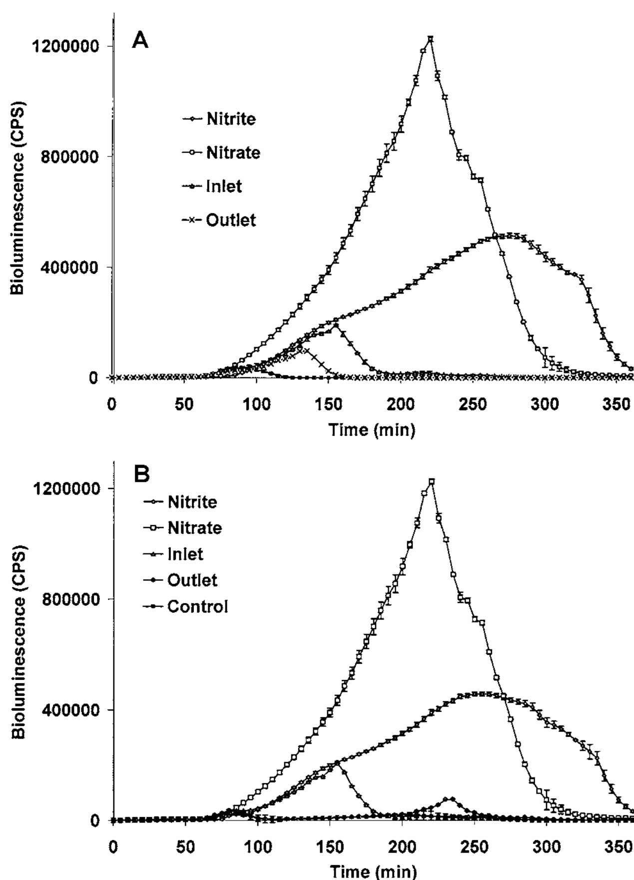

Fig. 5. Bioluminescent response of strain W6-1 to an aged nitrogen-contaminated sewage wastewater (**A**) and industrial wastewater (**B**) collected from the inlet and outlet collection tanks from industrial and sewage wastewater treatment plants at New Burg-Elarab City, Alexandria, Egypt.

Control refers to MSM containing the bioreporter without addition of substrates (negative control). Error bars calculated as for Fig. 2.

the inlet and the outlet of the two wastewater treatment plants.

Effect of Heavy Metals and Aromatic and Polyaromatic Hydrocarbons on the Expression of the *nasR* Promoter by Strain W6-1

If water is contaminated with nitrogen, it may include many other kinds of pollutants as well. Therefore, we monitored the specific response of the W6-1 bioreporter to different toxicants such as heavy metals, and aromatic and polyaromatic hydrocarbons. As shown in Fig. 6A, in the presence of 6 ppm nitrite, exposure to the heavy metals Cr, Hg, Cu, Pb, Fe, and Co produced bioluminescence that did not significantly differ from that of the negative control. However, upon exposure to Ni, bioluminescence significantly increased by about 25% that of the positive control (only nitrite). Furthermore, as demonstrated in Fig. 6B, the effect of various aromatic and polyaromatic hydrocarbons on the bioluminescent response of strain W6-1 in the presence of 6 ppm nitrite indicates that catechol, 5-chlorophenol, and

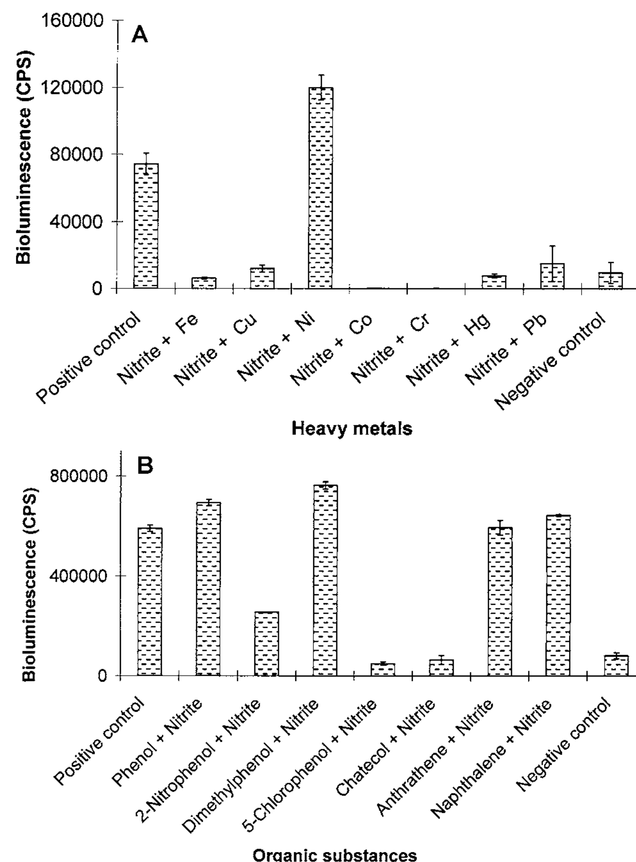

Fig. 6. Effect of heavy metals (**A**) and aromatic and polyaromatic hydrocarbons (**B**) on the expression of the *nasR* gene in strain W6-1.

Along with each experiment, a positive control (containing only 6 ppm nitrite) and a negative control (bacterial cells in NFM without addition of nitrite, metals, or organic substrates) were run. Error bars calculated as for Fig. 2.

4-nitrophenol proved to be more effective inhibitors of bioluminescence than phenol, dimethylphenol, anthrathene, and naphthalene, respectively.

DISCUSSION

In this paper, we describe a novel bioreporter capable of assessing the nitrate/nitrite assimilatory capacity of industrial and sewage wastewater treatment plants. Data reported here indicate that the W6-1 bioreporter can be used to yield a signal of suitable sensitivity and reproducibility from which bioavailable nitrate/nitrite and ammonium can be quantified. The strain yields nitrate/nitrite-responsive induction of bioluminescence due to the action of a *nasR*-like promoter that fused to a *V. fischeri luxCDABE* cassette. The onset of luminescence and nitrate/nitrite uptake is tightly coupled, thus is the intensity of the luminescent signal can be viewed as a measure of nitrate assimilation. Light emission by this strain was dependent upon the increase in nitrogen concentrations. The linear response range of the emitted luminescence was 1 to 8, 1 to 6, and 1 to 7 ppm for the nitrogen species nitrate, nitrite, and ammonium, respectively.

Recently, several studies demonstrated that the NasR protein is a nitrate/nitrite-specific positive regulator of *nasF* operon expression in the genus *Klebsiella* [21, 32]. They hypothesized that the NasR protein mediates antitermination by binding to the *nasF* leader transcript. DNA sequencing of the putative *nasR* gene described here exhibited 65% similarity to *Klebsiella oxytoca* nitrate regulatory protein (AC: Q48468), 63% similarity to *Klebsiella pneumoniae* nitrate regulatory protein (AC: AAA25101), and 65% similarity to *Klebsiella pneumoniae* nitrate regulatory protein (AC: AAA25101.2). To determine whether *nasR* gene expression is autoregulated, we examined the effects of nitrate, nitrite, and ammonium on the expression of the *nasR* gene. The gene expression was quantified by the amount of the bioluminescence produced by strain W6-1. As shown in Fig. 3, nitrate caused approximately two-fold increase in *nasR* expression over nitrite and about two- and half-fold increase over ammonium.

The same finding was revealed previously by Wu *et al.* [33] for F(*nasR-lacZ*) expression in various *nas* mutants. However, they did not explain the reason of this phenomenon. On the other hand, we find this result to be of almost logic, especially when we take into account that in *Klebsiella,* nitrate is reduced to ammonium through two steps; the first is the reduction of nitrate to nitrite, followed by the second step where nitrite is reduced to ammonium by assimilatory nitrate and nitrite reductases. However, nitrite is reduced to ammonium in only one step by nitrite reductase. This hypothesis is also supported by examining Fig. 3, where the induction of the *nasR* gene was increased dramatically after 60 min with nitrate and 120 min with nitrite and ammonium, respectively. However, a significant decrease in the bioluminescence was shown after 130 and 260 min of incubation for nitrate, and nitrite and ammonium, respectively.

For ammonium, we think that the bioreporter W6-1 is not induced by ammonium ion itself. However, the occurred induction may be due to the nitrite produced from the oxidation process of ammonium by the bioreporter W6-1. Classically, ammonia is oxidized to nitrite by autotrophic ammonia oxidizers. However, many heterotrophic bacteria can also oxidize ammonia to nitrite [28]. In this process, the nitrite produced by ammonia oxidation is reduced to nitric oxide, nitrous oxide, and possibly to nitrogen gas. Goss and Bender [16] reported that *Klebsiella*, like *E. coli* and other enteric bacteria, can also use a large number of organic and inorganic compounds as their sole source of nitrogen for growth. However, the enzymes encoding this diverse metabolic potential cannot be expressed when the preferred nitrogen source is available [27]. On the other hand, the enzymes required for ammonia assimilation can be expressed only when ammonia is present in the medium [16, 19]. Other investigators have shown that oxidation enzymes (such as oxygenases) exhibit nonspecific activity to methane and ammonium [20].

As shown in Fig. 4, the specificity of the W6-1 bioreporter provides a means for determining the bioavailability of nitrogen species in a broader spectrum response to nitrate, nitrite, and ammonium, which may be very useful properties for the measurement of total nitrogen bioavailability. Moreover, the data presented in Fig. 5 show a good correlation between bioluminescence output by strain W6-1 and the concentrations of both nitrite and nitrate in the inlet and the outlet of the two examined wastewater treatment plants. The lower bioluminescence levels in the aged nitrogen-contaminated water (Fig. 5), as compared with those observed in the aqueous samples (Fig. 3), were somewhat expected based on similar results obtained by Abd-El-Haleem *et al.* [3] using a bioluminescent bioreporter for the detection of phenol in soil. The decreased bioluminescence is likely the result of reduced bioavailability due to adsorption and partitioning as well as scattering and/or absorption of bioluminescent signals by the very fine suspended solid particles in collected water samples.

The effect of heavy metals, and aromatic and polyaromatic hydrocarbons on the expression of the *nasR* gene was also examined (Fig. 6). Overall, the W6-1 strain affords a direct method for determining the role of both chemical and physical factors in regulating nitrogen removal in biological wastewater treatment plants. In addition, the properties of the *Klebsiella oxytoca* strain W6-1 differ in many respects from three cyanobacterial nitrogen bioreporters previously described [15, 18, 22].

These bioreporters are *luxAB* fusions employing the *Synechocystis* sp. PCC6803 *nblA* [22], *Synechococcus* sp.

PCC7942 *glnA* promoters [15], and *Synechocystis* sp. PCC6803 *nirA* promoter [18], controlling the genes encoding a phycobilisome degradation regulator, glutamine synthetase, and nitrite reductase, respectively. However, with the exception of the third strain, the bioluminescent response was induced upon nitrogen deficiency, not during nitrogen use as described in this paper. Secondly, the responses of the *Synechococcus* sp. P*glnA* and *Synechocystis* sp. P*nblA* and *Synechocystis* sp. PCC6803 *nirA* bioreporters were considerably slower, yielding dose-dependent luminescence on the order of 15, 25, and 3 h [15, 18, 22]. Additionally, the *glnA* strain yielded dose-dependent responses to a wide variety of N species ranging from nitrate, nitrite, and ammonium [15], *nblA* expression was responsive to nitrate and ammonium [22], and *nirA* was expressed with nitrate and nitrite.

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