A Bioluminescence Assay Using *Nitrosomonas europaea* for Rapid and Sensitive Detection of Nitrification Inhibitors

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An expression vector for the luxAB genes, derived from *Vibrio harveyi*, was introduced into *Nitrosomonas europaea*. Although the recombinant strain produced bioluminescence due to the expression of the luxAB genes under normal growing conditions, the intensity of the light emission decreased immediately, in a time-and dose-dependent manner, with the addition of ammonia monoxygenase inhibitors, such as allylthiourea, phenol, and nitrapyrin. When whole cells were challenged with several nitrification inhibitors and toxic compounds, a close relationship was found between the change in the intensity of the light emission and the level of ammonia-oxidizing activity. The response of bioluminescence to the addition of allylthiourea was considerably faster than the change in the ammonia-oxidizing rate, measured as both the O$_2$ uptake and NO$_2^-$ production rates. The bioluminescence of cells inactivated by ammonia monoxygenase inhibitor was recovered rapidly by the addition of certain substrates for hydroxylamine oxidoreductase. These results suggested that the inhibition of bioluminescence was caused by the immediate decrease of reducing power in the cell due to the inactivation of ammonia monoxygenase, as well as by the destruction of other cellular metabolic pathways. We conclude that the assay system using luminous *Nitrosomonas* can be applied as a rapid and sensitive detection test for nitrification inhibitors, and it will be used to monitor the nitrification process in wastewater treatment plants.

The chemotrophic ammonia-oxidizing bacteria obtain their energy for growth by the oxidation of ammonia to nitrite (30). In *Nitrosomonas europaea*, ammonia is initially oxidized to hydroxylamine by ammonia monoxygenase (AMO) as follows: NH$_3$ + O$_2$ + 2H$^+$ + 2e$^-$ → NH$_2$OH + H$_2$O. The subsequent oxidation of hydroxylamine to nitrite is catalyzed by hydroxylamine oxidoreductase (HAO) as follows: NH$_2$OH + H$_2$O → NO$_2^-$ + 5H$^+$ + 4e$^-$. Two of the four electrons generated by the HAO-mediated second reaction are required to maintain steady-state AMO activity, and the remaining two electrons are used for ATP synthesis through a conventional electron transport chain (4, 10, 30).

In wastewater treatment plants and sewage disposal systems, ammonia-oxidizing bacteria play an important role in the removal of ammonia (19). Ammonia is oxidized to nitrite by nitrifying bacteria (ammonia-oxidizing bacteria and nitrite-oxidizing bacteria), and the resulting nitrate (nitrite) is then reduced to molecular nitrogen by denitrifying bacteria. The ammonia-removing process is known to be particularly susceptible to inhibition by certain chemical compounds at low concentrations, compared to the conventional process for removal of biochemical oxygen demand (19, 29). The largest cause of this phenomenon has been thought to be the inhibition of the AMO-mediated ammonia oxidation process, because several chemical compounds inhibit AMO activity at low concentrations compared with other enzymes (2, 9). Because AMO contains copper in its active center, metal binding compounds and chelating agents reversibly inhibit its activity (2, 9). For example, allylthiourea strongly inhibits AMO at very low concentrations (in the order of micromolar concentrations). Furthermore, AMO is able to oxidize several compounds, including sulfur, aliphatic, aromatic, and halogenated compounds, as alternative substrates instead of ammonia, and these compounds competitively inhibit AMO activity (2, 10, 11, 14, 26, 27). Because inhibition of nitrification causes serious problems for the effective treatment of wastewater, a rapid and sensitive method that can detect inhibition of ammonia oxidation is expected to be useful in monitoring the nitrification process in wastewater treatment plants.

The bacterial luciferase gene (luxAB), which encodes the two subunits of the luciferase enzyme, has been isolated from several luminous bacteria and used in several biological studies and applications (17, 24). The light-emitting reaction of luciferase is involved in the oxidation of reduced flavin mononucleotide (FMNH$_2$) and a long-chain fatty aldehyde in the presence of molecular oxygen. The reaction is as follows: FMNH$_2$ + RCHO + O$_2$ → FMN + RCOOH + H$_2$O + hv (490 nm), where R represents a long-chain alkyl group and h and v represent Planck’s constant and frequency, respectively. In recent years, bioluminescence by the bacterial luciferase system has been used for the evaluation of cell viability and the detection of toxic compounds, because toxic compounds destroy cellular metabolism and subsequently eliminate light production in vivo (5, 24, 31).

In the present study, we describe the application of the bacterial luciferase gene for the rapid and sensitive detection of nitrification inhibitors that inhibit ammonia-oxidizing bacteria. Although recombinant *N. europaea*, which carries an expression plasmid vector for the *Vibrio harveyi* luxAB genes, produced bioluminescence due to the expression of the luxAB genes, a loss of light emission was immediately observed with the addition of nitrification inhibitors at low concentrations. We demonstrated that the loss of light emission is caused by a decrease of reducing power in the cell due to the inhibition of AMO, as well as by the destruction of other cellular metabolic pathways.
**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** *N. europaea* IPO14208 (ATCC 19178) was grown aerobically at 30°C in P medium (2.5 g of (NH₄)₂SO₄, 0.7 g of KH₂PO₄, 13.5 g of NaH₂PO₄, 0.5 g of NaHCO₃, 100 mg of MgSO₄·7H₂O, 5 g of CaCl₂·2H₂O, and 1 g of Fe·EDTA per liter (pH 8.0)) in the dark (15). In cultivation using a 5-liter jar fermenter with a working volume of 3.5 liters (MD300-5L, B. E. Marubushi Co., Ltd., Tokyo, Japan), cells were grown in P medium in the dark (operating conditions: air flow, 0.5 vol/vol/min; agitation, 250 rpm; temperature, 30°C; pH 7.8, controlled by addition of 2 N NaOH). For the recombinant strain of *N. europaea*, kanamycin was added into the medium at a final concentration of 25 μg/ml. Cell growth was monitored at 600 nm by using a U-100 spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan) with cuvettes of a 50-mm light path.

**Nitrification inhibitors.** Nitrification inhibitors used in this study were purchased from Tokyo Kasei Industry Co., Ltd. (Tokyo, Japan) or Kishida Chemical Co., Ltd. (Osaka, Japan), except for nitrapyrin and formaldehyde. Nitrapyrin (2-chloro-6-(trichloromethyl)pyridine) was obtained from Dow Chemical Co. (Midland, Mich.). Formaldehyde was obtained as a 37% solution stabilized with 1% methanol for five years. Water-insoluble compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Mo.) as 2 mM (allyl sulfide, nitrapyrin, pentachlorophenol, and 8-quinolinol), 20 mM (picolinic acid), and 100 mM (2,4-dinitrophenol) solutions and then were dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Mo.) as 2 mM. These inhibitors were dissolved in P-medium (2 ml) and was preincubated for 10 min with agitation by using a stirrer magnet. Allylthiourea was added after the incubation, and the incubation was continued. A small aliquot of the reaction mixture was removed from the flask and used to measure bioluminescence and NO₂⁻ concentration.

**DNA manipulation.** The standard molecular genetic techniques used have been described previously (12). PCR was performed in a volume of 50 μl with a set of oligonucleotide primers (100 μM) and an Ex Taq DNA polymerase (Takara Syuzo Co., Ltd., Kyoto, Japan) under the following reaction conditions: 94°C for 0.5 min, 55°C for 1 min, and 72°C for 1 min (25 cycles). Insertion of plasmid into *N. europaea* was carried out by electroporation as described previously (12).

**Construction of plasmids.** pKLUX27 (12) was digested with BamHI and ligated with a BamHI-BglII-treated 2.2-kb fragment containing luxAB genes obtained by PCR amplification using 1 μg of *V. harveyi* ATCC 33843 chromosomal DNA as the template, with primers 5‘-CGGATCCCACAATATAGGAAATGTGAC-3’ and 5‘-CGGATCTTCTTATTACATCCAT-3’, corresponding to nucleotides 687 to 709 in the published luxA sequence (6) and 1063 to 1043 in the published luxB sequence (13), respectively. The resulting plasmid was named pKLUX27. A 0.35-kb fragment containing the promoter region of the luxA gene was obtained by PCR amplification using 1 μg of *N. europaea* chromosomal DNA as the template, with primers 5‘-CGGATCTTCTTATTACATCCAT-3’ and 5‘-CGGATCTTCTTATTACATCCAT-3’, corresponding to nucleotides 275 to 235 and 67 to 48, respectively, in the published sequence (21). These regions were digested with both BamHI and BglII and was ligated with BamHI-digested pKLUX27, yielding pHLUX20. The physical map of pHLUX20 is shown in Fig. 1. For all cloning experiments, Escherichia coli DH5α was used as the host strain. The nucleotide sequence of the 0.35-kb luxA promoter region was confirmed by the dideoxy chain termination method (20) with a BcaBEST sequencing kit from Takara Syuzo Co. There was a 6-base difference between the published and the observed sequence of the amplified fragment of the nonfunctional region of the promoter (C→T at position 74, C→A at 179, and GGGCC→AAGCG at 238). These substitutions might have been caused by in vitro random mutagenesis during PCR and/or cloning of an unpublished hao promoter region among the three copies of hao genes (3, 21).

**Bioluminescence measurement.** Bioluminescence was measured by using a Model 210 luminometer (Turner Design Co., Sunnyvale, Calif.). A standard polyethylene cuvette (8 by 50 mm) containing 2.5 μl of 10% (vol/vol) n-decyl aldehyde dissolved in ethanol was placed in the luminometer. The luminescent reaction was started by the injection of aliquots (100 μl) of the test samples. The relative light unit (RLU) was expressed as a “full integral value,” which means the average light output during 5 to 15 s after the start of the reaction. All measurements were performed at 25°C.

**Assay of inhibition of bioluminescence.** The culture broth of *N. europaea* (pHLUX20) was removed from the jar fermenter when the NO₂⁻ concentration in the culture broth was approximately 5 to 10 mM and was stored at room temperature in the dark for 15 to 30 min. An aliquot (0.95 ml) of the culture was placed in a test tube, and 50 μl of test sample was added. After the incubation at 25°C, bioluminescence was measured. If the test sample contained a high concentration of chemical compounds (more than approximately 10 mM), the pH of the test sample was adjusted to 7.8 by NaOH or HCl before use. The strength of inhibition of bioluminescence by the inhibitor was expressed as the LIC₉₀ (bioluminescence inhibitory concentration), defined as the concentration of inhibitor causing a 50% reduction in light output from that in the control reaction. The LIC₉₀ was calculated from graphed data obtained by dose-response experiments using twofold serial dilutions of the test sample.

**Assay of inhibition of ammonia-oxidizing activity.** Ammonia-oxidizing activity was measured as the NO₂⁻ production rate in whole *N. europaea* cells. *N. europaea* cells were harvested by filtration with a membrane filter (0.22-μm-pore-size cellulose-acetate filter unit; Corning, Inc., Corning, N.Y.) when the NO₂⁻ concentration of the culture broth was approximately 50 μM. The cells were washed and resuspended in cold 100 mM phosphate buffer (pH 7.8) at a final protein concentration of about 0.7 mg/ml. P medium (2 ml) was placed in a test tube and kept at 30°C. Aliquots (50 μl) of cell suspension were added to the test tube and preincubated for 10 min at 30°C with agitation in order to establish the steady-state NO₂⁻ production rate. A test sample of 100 μl was then added, and incubation was continued for 30 min. The NO₂⁻-producing reaction was stopped by the addition of 20 μl of 0.1 M alythioireua, and then the NO₂⁻ concentration of the reaction mixture was measured. The strength of inhibition of the ammonia-oxidizing activity by the inhibitor was expressed as the AIC₉₀ (ammonia oxidation inhibitory concentration), which was defined as the concentration of inhibitor causing a 50% reduction in NO₂⁻ production rate from that in the control reaction without inhibitor.

**Measurement of the O₂ uptake rate and the NO₂⁻ production rate.** *N. europaea* (pHLUX20) cells were harvested by filtration when the NO₂⁻ concentration of the culture broth was approximately 10 mM. The cells were washed and resuspended in cold 100 mM phosphate buffer (pH 7.8) at a final protein concentration of 2 mg/ml. A dissolved-oxygen (DO) electrode (GU-BMP; Lijima Electronics Co., Aichi, Japan) was mounted and sealed in a flask containing 64 ml of DO-saturated 100 mM phosphate buffer (pH 7.8) with 19 mM (NH₄)₂SO₄ at 25°C. A 500-μl aliquot of the cell suspension was injected into the flask and was preincubated for 10 min with agitation by using a stirrer magnet. Allythioireua was added after the preincubation, and the incubation was continued. A small aliquot of reaction mixture was removed from the flask and used to measure bioluminescence and NO₂⁻ concentration. The time-dependent change of DO concentration was monitored by DO meter (GU-BMP; Lijima Electronics Co.) with a pen chart recorder. All operations were carefully performed in a nitrogen-purged contamination- and air-free flask with air. The O₂ uptake rate (in micromoles per minute) was calculated by the change in DO concentration at 1-min intervals. The NO₂⁻ production rate (in micromoles per minute) was calculated by the change in NO₂⁻ concentration at 5-min intervals.

**Analytical methods.** Protein concentration was measured by using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) after the cells were solubilized in 0.1% sodium dodecyl sulfate for 10 min at 37°C (22). Bovine serum albumin was used as the standard. NO₂⁻ concentration was measured by a colorimetric assay (7).

**RESULTS**

**Expression of the luxAB genes in *N. europaea*.** Cultivation of *N. europaea* (pHLUX20) was performed by using a jar fermentor. Significant bioluminescence was observed, as shown in Fig. 2, indicating that the luxAB genes had been successfully expressed in *N. europaea*. The specific bioluminescence value was constant (about 8 to 10 RLU/ml/unit of optical density at 600 nm [OD₆₀₀]) up to a concentration of about 10 mM in the early- and mid-logarithmic phases but gradually declined in the late-logarithmic phase.

**Effect of AMO inhibitor on bioluminescence in *N. europaea*.** The effects of AMO inhibitors on bioluminescence in *N. europaea* (pHLUX20) were examined. Allythioireua is a potent and...
specific inhibitor for AMO due to the fact that it binds to copper in the active center of AMO (2, 9). When allylthiourea was added to the culture broth of *N. europaea* (pHLUX20), the intensity of the light emission was slightly decreased, as shown in Fig. 3A. The inhibition response was time and dose dependent. At a final concentration of 0.1 μM allylthiourea, the intensity of the light emission was reduced to 10% after 20 min of incubation, and only 2% of the light emission remained in the cell within 5 min at a concentration of 1 μM. Similar results were found when phenol or nitrapyrin was added to the culture. A low concentration of phenol may reversibly and competitively inhibit AMO activity, because it is oxidized by AMO as an alternative substrate in place of ammonia, resulting in hydroquinone (11). Nitrapyrin also inhibits AMO by acting as an alternative substrate. Moreover, the resulting oxidized compounds behave as protein-modifying agents that irreversibly inactivate not only AMO but also other proteins in the cell (26). In the presence of 100 μM phenol or 10 μM nitrapyrin, the intensity of the light emission declined to less than 5% of the initial value within 5 min, as shown in Fig. 3B and C. On the other hand, compounds noninhibitory for nitrification (9) did not affect bioluminescence. There was no significant loss of light emission in the presence of 5 mM DMSO, 1 mg of glycerol/ml, 1 mg of sodium acetate/ml, or 1 mg of bovine serum albumin/ml (final concentrations) (data not shown). These results indicated that AMO inhibitors strongly inhibited the bioluminescence of *N. europaea* (pHLUX20) at very low concentrations regardless of their mechanisms of inhibition of AMO.

**Bioluminescence reflects ammonia-oxidizing activity.** We attempted to clarify whether the intensity of bioluminescence reflected the inhibition of ammonia oxidation activity by various nitrification inhibitors and toxic compounds, including a HAO inhibitor (formaldehyde) and nonspecific inhibitors such as a heavy metal (HgCl₂) and respiratory inhibitors (Na₂S and NaN₃), as well as AMO inhibitors (9, 27). We also used 2,4-dinitrophenol and pentachlorophenol as uncouplers. In the strict sense, uncouplers inhibit not only ATP-dependent pyridine nucleotide reduction in *N. europaea* but also the AMO-mediated ammonia oxidation process (1, 9). To evaluate the effect of each inhibitor on both activities, we used measurement of LIC 50 and AIC 50. Figure 4 shows the dose-response curve of allylthiourea and the LIC 50 and AIC 50 obtained from the graphed data. The LIC 50 of allylthiourea were 0.54 and 0.15 μM when the incubation times were 1 and 5 min, respectively, and the AIC 50 was 0.06 μM when the incubation time was 30 min. As shown in Table 1, 16 compounds had various AIC 50. Although allylthiourea and thioacetamide were strong inhibitors of AMO and inhibited ammonia-oxidizing activity at concentrations in the order of 10⁻² μM, dicyandiamide and methanol were weak inhibitors and their AIC 50 were 4.5 and 32.6 mM. These compounds also decreased light emission. A strong correlation was found between the LIC 50
and AIC_{SO} over 6 orders of magnitude, as shown in Fig. 5. These results indicated that the change in light emission reflected the inhibitory effect on ammonia-oxidizing activity not only of AMO inhibitors but also of nonspecific and HAO inhibitors. Furthermore, it is interesting that the bioluminescence response indicated the presence of a low concentration of inhibitor after only a few minutes, while the NO_{2}⁻ production rate was similarly affected after 30 min of incubation.

Comparison of the ammonia-oxidizing rate and bioluminescence in the presence of inhibitor. *N. europaea* (pHLUX20) cells were incubated in a DO electrode-mounted flask. When the steady-state rate of O_{2} uptake activity was established, allylthiourea was added to the reaction mixture, and the NO_{2}⁻ concentration and bioluminescence were measured simultaneously. There was no significant difference between the changes in the O_{2} uptake rate and the NO_{2}⁻ production rate, but the change in bioluminescence was faster than those in both rates, as shown in Fig. 6. Within 5 min after the addition of 0.1 μM allylthiourea, the intensity of the light emission decreased to 30% of the initial value, but about 70% of the O_{2} uptake and NO_{2}⁻ production rates remained (Fig. 6A). After 15 min of incubation, we observed a decline in the O_{2} uptake and NO_{2}⁻ production rates to about 30% of each of the initial rates. These results confirmed that the response of bioluminescence to the inhibitor took place faster than the response of the ammonia-oxidizing rate, as measured by both the O_{2} uptake and NO_{2}⁻ production rates. A similar finding was also observed in the experiment using 0.5 μM allylthiourea (Fig. 6B).

**Effect of HAO substrates on bioluminescence.** The bacterial luminescence assay has been used for toxicity testing because toxic agents destroy the membrane, proteins, and several cellular metabolic pathways, resulting in the disappearance of light emission (5, 24, 31). However, in the assay described in this study, the disappearance of light emission was likely to depend on inhibition of AMO activity rather than on other cellular metabolic pathways, when AMO inhibitor was added. We suspected that the specific inhibition of AMO resulted in the disappearance of light emission because of the limitation of the reducing power in the cell, because FMNH₂ was necessary for the bacterial luciferase reaction and is generated by using the reducing power obtained from ammonia. To clarify this assumption, the effects of HAO substrates on light emission were examined. When intact cells were incubated with hydroxylamine or hydrazine, the intensity of bioluminescence increased by approximately 3 and 1.4 times the initial value, respectively (Table 2). Although allylthiourea-treated cells exhibited only 4.7% of the initial light emission value for intact cells, bioluminescence was immediately recovered by the addition of hydroxylamine or hydrazine. Interestingly, the recovered light emission was about 1.5 to 2 times stronger than the light emission produced by hydroxylamine- or hydrazine-utilizing intact cells. Similar results were also observed in phenol-treated cells with HAO substrates. These results indicate that the electron transfer pathway from HAO to luciferase is almost

**TABLE 1. LIC_{SO} and AIC_{SO} of several nitrification inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>LIC_{SO} (μM) with an incubation time of:</th>
<th>AIC_{SO} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Allylsulfide</td>
<td>1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Allylthiourea</td>
<td>0.54</td>
<td>0.15</td>
</tr>
<tr>
<td>Dicyandiamide</td>
<td>9,790</td>
<td>3,840</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>71.2</td>
<td>62.6</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1,680</td>
<td>1,110</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>2.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>4,440</td>
<td>3,230</td>
</tr>
<tr>
<td>NaN₃</td>
<td>2,330</td>
<td>460</td>
</tr>
<tr>
<td>Na₂S</td>
<td>47.5</td>
<td>10.3</td>
</tr>
<tr>
<td>Nitrapyrin</td>
<td>4.1</td>
<td>0.74</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>13.7</td>
<td>10.6</td>
</tr>
<tr>
<td>Phenol</td>
<td>19.8</td>
<td>18.0</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>199</td>
<td>91</td>
</tr>
<tr>
<td>8-Quinolinol</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>0.41</td>
<td>0.04</td>
</tr>
<tr>
<td>Thiosemicarbazide</td>
<td>3.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* LIC_{SO} and AIC_{SO} were calculated from graphed data of dose-response experiments as shown in Fig. 4.
* Determined after 30 min of incubation.
* Na₂S *· 9H₂O was used.
* Thiosemicarbazide hydrochloride was used.

**FIG. 4. Dose-response curve of allylthiourea and determination of the LIC_{SO} and AIC_{SO}.** The strength of inhibition of light emission in *N. europaea* (pHLUX20) was expressed as the LIC_{SO}, which was calculated from the graphed data as 0.54 and 0.15 μM when the reaction mixture was incubated for 1 (●) and 5 (▲) min, respectively. On the other hand, the strength of inhibition of ammonia-oxidizing activity in *N. europaea* was expressed as the AIC_{SO}, which was calculated as 0.06 μM for the 30-min incubation (○). Values are averages from three independent experiments.

**FIG. 5. Correlation between LIC_{SO} and AIC_{SO}**. Sixteen different nitrification inhibitors and toxic compounds were used. Data were plotted as shown in Table 1. LIC_{SO} were determined by incubations of 1 (○) and 5 (●) min.
independent of the presence of allylthiourea and phenol and that the decrease in light emission could be caused by the prevention of electron flow within the cell due to the inhibition of the AMO reaction.

DISCUSSION

The luxAB genes derived from V. harveyi were successfully expressed in N. europaea by transcriptional control of the promoter of the hao gene. Although there are only a few reports of the promoter sequence of N. europaea (3, 12, 18, 21), the hao promoter is one of the functionally well-characterized promoters. Because expression of the hao gene can be induced by the presence of ammonia (21), the hao promoter is thought to be suitable for the expression of foreign genes under normal growing conditions in N. europaea. Although the specific luciferase activity showed a constant ratio up to mid-logarithmic phase, it decreased in late-logarithmic phase. This phenomenon is not always caused by a decrease in the expression of luciferase, because the intensity of the light emission is dependent on the reducing power in the cell as described below, as well as on the production of luciferase.

In this study, we demonstrate that the change in light emission reflected the inhibitory effect on AMO activity of AMO inhibitors, as well as the destruction of other cellular metabolic pathways by nonspecific inhibitors. The decrease in light emission brought about by AMO inhibitors is thought to be caused by prevention of electron flow due to inhibition of the AMO reaction. In the bacterial luciferase reaction, FMNH₂ is generated from NADH or NADPH by a reaction catalyzed by NAD(P)H-FMN oxidoreductase, which is a ubiquitous enzyme found in several bacteria and is also present in N. europaea (17). If the quantities of luciferase and NAD(P)H-FMN oxidoreductase are constant in the cell, the intensity of bioluminescence depends on the concentration of NAD(P)H. In N. europaea, NAD(P)H is required for several enzyme reactions, for example, fixation of CO₂. Although there are some enzymes, such as glutamate dehydrogenase (28), which may reduce NAD(P)⁺ by the oxidation of organic compounds, NAD(P)H is thought to be produced mainly by the reducing power created by the oxidation of hydroxylamine from the HAO reaction (4, 10, 30). Therefore, when AMO is inhibited by a nitrification inhibitor, the series of electron transfer pathways initiated by ammonia oxidation is terminated, leading to a drastic decrease in NAD(P)H concentration. Thus, the degree of AMO inhibition can be measured by monitoring the intracellular NAD(P)H concentration by use of the in vivo bacterial luciferase reaction. The integration of electron transport is shown in Fig. 7.

As shown in Fig. 6, loss of the intensity of the light emission due to AMO inhibitors is a sensitive reaction compared with the decrease in the O₂ uptake and nitrite production rates. The reason for this difference is unclear. The reducing power generated from hydroxylamine by HAO is thought to pass through cytochrome c-554 to both cytochrome aa₃ oxidase and ubiquinone (10, 16). The reducing power of the resulting ubiquinol appears to be used for the reduction of NAD(P)⁺, as well as for the maintenance of the AMO reaction (10). However, the reduction of NAD(P)⁺ by ubiquinol is an energy-consuming process called “reverse electron transfer,” and only a few electrons are likely to be used for this reaction (1, 30). When the reducing power decreases, the remaining electrons may be predominantly transferred to the cytochrome aa₃ oxidase and the maintenance of the AMO reaction rather than NAD(P)⁺, resulting in a rapid decrease in the intensity of light emission.

<table>
<thead>
<tr>
<th>Cell condition</th>
<th>Conc of HAO substrate</th>
<th>Bioluminescence&lt;sup&gt;a&lt;/sup&gt; (RLU/ml)</th>
<th>Relative ratio&lt;sup&gt;b&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>None</td>
<td>30.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.2 mM hydroxylamine</td>
<td>89.2</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td>1 mM hydrazine</td>
<td>41.1</td>
<td>137</td>
</tr>
<tr>
<td>Allylthiourea treated</td>
<td>None</td>
<td>1.4</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>0.2 mM hydroxylamine</td>
<td>111.8</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td>1 mM hydrazine</td>
<td>89.9</td>
<td>299</td>
</tr>
<tr>
<td>Phenol treated</td>
<td>None</td>
<td>3.1</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>0.2 mM hydroxylamine</td>
<td>78.8</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>1 mM hydrazine</td>
<td>76.2</td>
<td>253</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inhibitor solution (10 μl) was added to 1 ml of N. europaea(pHLUX20) cell suspension (64 μg of protein/ml) in P medium. After incubation for 10 min at 25°C, HAO substrate solution (10 μl) was added. The luminescence of the mixture was measured after 1 min of incubation.

<sup>b</sup> Allylthiourea and phenol were used at final concentrations of 1 and 50 μM, respectively.

<sup>c</sup> Values are averages from three independent experiments.

<sup>d</sup> The intensity of light emission in intact cells without inhibitor is taken as 100%.
When intact cells were incubated with hydroxylamine or hydrazine, an increase in the intensity of light emission was observed. This result suggests that the supply of reducing power is insufficient for luciferase to exhibit maximum activity under ammonia-utilizing growth conditions. The bioluminescence of allylthiourea-treated cells was recovered by the addition of HAO substrates, the resulting ubiquinol (UQH₂) is thought to supply reducing power for the maintenance of the AMO reaction and the reduction of NAD(P)⁺. The remaining electrons from cytochrome c-554 may pass through cytochrome c-552 (c-552) to cytochrome a₃ oxidase (Cytox₃ oxidase). NAD(P)⁺ is reduced by reverse electron transfer, which needs energy supplied by the hydrolysis of ATP. The reducing power of the luciferase reaction must be obtained from FMNH₂, which is generated from the reduction of FMN in the NAD(P)H-FMN oxidoreductase reaction by using the reducing power of NAD(P)H. OM and IM, outer and inner membranes, respectively.

The operational maintenance of wastewater treatment plants to help ensure effective treatment. By the conventional methods, inhibition of ammonia oxidation has been judged by the measurement of the O₂ uptake rate or the NO₂⁻ production rate of ammonia-oxidizing bacteria (19, 23). However, measurement of these parameters requires several time-consuming and/or complicated steps. We propose that the luminous Nitrosomonas assay described in this paper is a more effective method for the detection of nitrification inhibitors, and for monitoring the nitrification process in wastewater treatment plants, because this bioluminescence reaction is more rapid and sensitive than conventional methods.

Several nitrification inhibitors are likely to be toxic to animals and other microorganisms. As mentioned above, the AMO activity of ammonia oxidizers is particularly susceptible to inhibition by a wide range of compounds at low concentrations. Categories of AMO inhibitors include halogenated compounds and oxidative phosphorylation inhibitors, and such compounds are toxic to several animals and microorganisms (2, 9, 10). Eckenfelder reported that nitrification inhibitors are generally related to biologically toxic compounds (8), as evidenced by the fact that the profile of the inhibition of Nitrosomonas by various chemical compounds correlates with the results of the Microtox test, a commercial toxicity-testing system based on the bioluminescence of Photobacterium phosphorum (5). In practice, a biosensor using a DO electrode with immobilized whole N. europaea cells has been applied to the on-line monitoring of drinking-water toxicity in Japanese water purification plants (25). We anticipate that the luminous Nitrosomonas system will be used not only for the monitoring of nitrification but also for monitoring of the toxicity of harmful materials in environmental samples and for evaluation of the safety of industrial products.

REFERENCES


