An *Escherichia coli* Strain for Use in Nitrate Analysis

Richie H. Lowe* and Mike C. Gillespie

A method is described for nitrate determination which uses intact *Escherichia coli* as a source of nitrate reductase enzyme to reduce nitrate to nitrite. The nitrite is then allowed to react with the color developing reagent of equal amounts of 2% sulfanilamide in 3 *N* HCl and 0.1% naphthylethylenediamine dihydrochloride to yield a red color read at 540 nm. American Type Culture Collection Strain 23739, genotype K 12 Hfr (Hayes), when grown anaerobically does not possess nitrite reductase activity, but does have extremely active nitrate reductase that will utilize formate as an exogenous electron donor. The procedure is sensitive to 0.01 μg/ml of NO_3^-–N in the sample solution which is sufficient for ground and stream water pollution studies and works well either automated or manually. A single 1500-ml culture will produce enough bacteria for 1200–1600 analyses.

Recently, there has been a tremendous increase in investigations which require the measurement of nitrate ("Accumulation of Nitrate", 1972). These studies include nitrate excesses in animal feeds from high nitrogen fertilization, interactions of nitrate with other ions in relation to ion uptake and translocation, and nitrate pollution in surface and ground waters. For easier routine analyses of nitrate, a simple, rapid method is needed that is adaptable to a wide range of materials.

In 1967, we reported on a rapid automated method for determination of nitrate in plant and soil extracts which has since been used extensively in several laboratories (Lowe and Hamilton, 1967). This method is accurate, sensitive, highly specific, and has a wide range of application. Major criticism of the procedure has been the length of time necessary to produce the soybean nodule bacteria which was the source of the nitrate reductase enzyme, and the exacting environmental conditions necessary for growing soybeans with nodules having high nitrate reductase activity. *Rhizobium* grown in pure culture were unsuccessful because these organisms contain a nitrite reductase. Recently, McNamara et al. (1971) reported a modification of our method, whereby a partially purified nitrate reductase from *Escherichia coli* was used for reducing the nitrate to nitrite. However, the activity of their system was so low that they used incubation times of 4 hr. Such long incubations are completely unsatisfactory for the automated method and are undesirable for the manual procedure. Also, the preparation of the enzyme is tedious and time consuming. They reported that the reason for partial purification of the enzyme was to separate it from the nitrite reductase in the system.

This report describes the culture and use of a strain of *E. coli* that, when grown anaerobically, contains no nitrite reductase, but has an extremely active nitrate reductase that uses an exogenous supply of formate as electron donor.

MATERIALS AND METHODS

Culture of Bacteria. American Type Culture Collection Strain 23739, genotype K 12 Hfr (Hayes) (American Type Culture Collection, Rockville, Md.), was grown on a medium modified from that of Lester and DeMoss (1971). The modifications consisted of increasing the KNO_3 solution and the glucose level, and adding molybdate, selenite, casein hydrolysate, and nutrient broth (BBL Powder) to the medium as indicated below. The modified basal medium contained the following ingredients per liter: 40 ml of solution I (229 g of K_2HPO_4 and 25 g of $(NH_4)_2SO_4$ diluted to 1 l. with H_2O), 40 ml of solution II (2.5 g of $MgSO_4 \cdot 7H_2O$, 50 g of KH_2PO_4, 10 g of sodium citrate, and 0.025 g of ferrous ammonium sulfate diluted to 1 l. with H_2O), 10 ml of 10^{-3} *M* Na_2MoO_4, 0.1 ml of 10^{-3} *M* Na_2SeO_3, 50 ml of 2 *M* $KHCO_3$, 150 ml of 10% (w/v) KNO_3, 0.65 g of casein hydrolysate, 2.7 g of nutrient broth (BBL Powder), and 2% (w/v) D-glucose. The medium was made up in 1.5-l. batches in a 2-l. serum bottle and autoclaved for 20 min at 121° and 15 lb pressure. The sugar was autoclaved separately as a 30% (w/v) solution and added to the medium afterward.

The serum bottle was fitted with a rubber stopper having three outlet ports. One was used for N_2–CO_2 introduction, one for gas removal, and the other for culture sample removal. Small glass tubing was used in the gas-exchange ports, and small bore Teflon tubing attached to a stopcock was used for the sample-removal port.

Growth was carried out under anaerobic conditions at all times. A mixture of 95% N_2–5% CO_2 was bubbled vigorously through the medium for 0.5 hr before inoculation. Vigorous bubbling was continued throughout the growth cycle (250 ml/min flow rate). To assure oxygen removal and sterility, the gas was first passed through a reduced Methyl Viologen solution containing 200 ml of 0.1 *M* pH 7.2 potassium phosphate buffer, 0.2 g of Methyl Viologen, and 0.5 g of equal parts of $Na_2S_2O_4$ and $NaHCO_3$. Then it was passed through a 5 × 70 cm glass tube packed tightly with cotton and sterilized by autoclaving before each use. The culture was incubated at 37° in a water bath.

An inoculum grown aerobically for 14–16 hr in 50 ml of nutrient broth (BBL Powder) was used to initiate growth in the large culture. This inoculum culture was initiated by two small loop tips of bacteria from a slant. This culture was agitated slowly on a shaker and was warmed with a 250-W heat lamp placed 2–3 ft from the culture (approximately 35°). Stock cultures used for inoculation were maintained on nutrient agar slants and grown to an age of 18–24 hr at 37° before being refrigerated.

To check growth of the large culture, turbidity was monitored at 600 nm on a Gilford spectrophotometer at 1- to 2-hr intervals. The culture was grown to absorbance levels of 1.3 to 1.6 (5.5–6.5 hr).

Nitrate reductase activity was checked when the turbidity reached this level. A 10-ml sample of the culture suspension was diluted to 40 ml with 0.1 *M* pH 7.2 potassium phosphate buffer and spun down in a refrigerated centrifuge at 18,000*g* for 5 min. After centrifuging, the supernatant was poured off and the bacteria resuspended in 40 ml of the above buffer. We added 0.1 ml of the suspended bacteria to each of three tubes containing 1.7 ml of 0.1 *M* sodium formate and 0.2 ml of 0.1 *M* KNO_3. Then N_2 was vigorously bubbled through the solution in two of the tubes for 1 min, with the tubes being quickly stoppered under pressure after removal of the bubbling needle. The third tube was a control for residual nitrite and remained aerobic, with the

Agricultural Science Center, Agronomy Department, University of Kentucky, Lexington, Kentucky 40506.

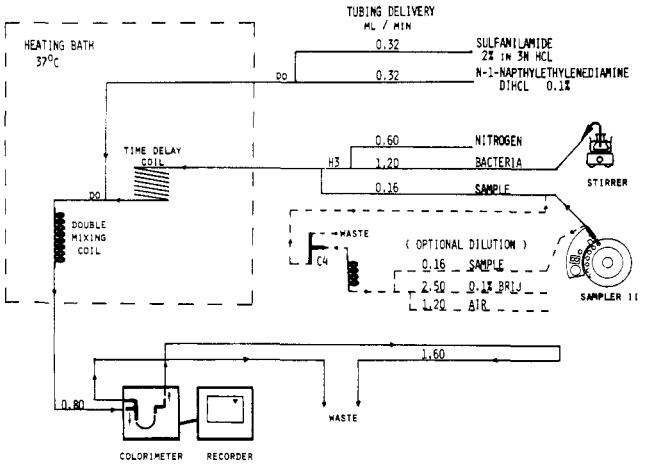

Figure 1. Flow diagram for nitrate autoanalyzer system.

reaction being stopped at zero time. The two anaerobic tubes were incubated for 10 min in a 37° water bath. The reaction was stopped by the addition of 5 ml each of 2% sulfanilamide in 3 *N* HCl and 0.1% *N*-1-naphthylethylenediamine dihydrochloride.

Color was read after 10 min on a Gilford spectrophotometer at 540 nm. The zero time reaction mixture was used as reference. Absorbances of 1.00 or greater indicated good culture activity and warranted culture harvesting. If low readings were encountered then the culture was discarded; however, this seldom happened and was usually traceable to human error.

Before harvest, the culture (still under N_2 aeration) was placed in an ice–water bath for 0.5 hr. At this phase in the growth cycle, rapid cooling prolonged enzyme activity and probably resulted in low mortality levels. After the culture was chilled, it was placed in 250-ml stainless centrifuge cups and spun down in a refrigerated centrifuge at 10,000*g* for 5 min. Further operations were carried out in an ice bath with no special aseptic conditions employed. After the supernatant was discarded, the bacteria were resuspended with a glass rod in about 50 ml of cold 0.05 *M* pH 7.2 potassium phosphate buffer containing 0.01 *M* sodium formate. All suspensions were combined into two centrifuge cups and diluted to about 200 ml with additional buffer. The suspension was recentrifuged as above, and the washing was repeated twice. Because the pellet was butyrous, we were careful to remix the cells thoroughly and rapidly into the buffer after each centrifugation.

After the final washing, the bacteria were taken up in about 50 ml of buffer, resuspended with a Ten Broeck Homogenizer, placed in a 1-l. flask, and diluted to a final buffer volume of 800 ml. The flask was fitted with two glass tubes to enable N_2 introduction, air removal, and stock solution drawoff. The tube used for N_2 introduction and stock solution drawoff extended to near the bottom of the flask, while the tube for air removal extended just through the stopper. Also, a stirring bar was added to aid in future mixing. Nitrogen was vigorously bubbled through the suspension for 5 min and the flask was stoppered under pressure. The stock solution was placed in an ice bath and refrigerated. Storing the cells in a flask of this design allows daily removal of the solution without disturbing the anaerobic state. After processing, all glassware was either rinsed in 70% ethanol or autoclaved before washing. The serum-culturing bottle had an additional acid rinse, and all spills were cleaned up with 70% ethanol. There is no known pathogenicity or virulence of this strain, but these measures are taken as standard sanitary procedure.

To determine the daily dilution factor for the stock suspension, we measured nitrate reductase activity by the method used during culture growth. A residual nitrite measurement was not necessary. The bacteria were diluted with potassium phosphate–formate buffer by the formula: dilution factor = 10 × (absorbance/0.5).

Table I. Recovery of Nitrate Added to Cured Burley Tobacco Samples

	ppm of NO_3^-–N[a]			
Sample	Analyzed	Added	Recovered	% recovery
1	23.46	10	33.83	101
2	16.51	10	26.57	100
3	9.4	10	19.56	100
4	13.24	10	23.50	101
5	14.64	10	24.50	99
6	8.11	10	18.09	100
7	12.37	10	22.32	99
8	19.25	10	29.69	101

[a] Average of three analyses.

Table II. Reproducibility of Replicate Samples of Cured Burley Tobacco

Replication no.	ppm of NO_3^-–N	Replication no.	ppm of NO_3^-–N
1	33.8	6	33.7
2	33.8	7	33.7
3	33.8	8	33.7
4	33.7	9	33.7
5	33.7		

Analytical System. A Technicon autoanalyzer was used to determine nitrate concentrations. The system design (Figure 1) was similar to that used by Lowe and Hamilton (1967). Under the new method the bacteria are active enough that the dialyzer can be eliminated if the sample is sufficiently clear and colorless. The use of a double mixing coil increased stability of the color reaction and promoted sharper peaks. The flow cell was 7.5 mm and the incubation bath was 37°. The sampling rate and wash ratio were the same.

Along with each set of 30–35 samples, a set of KNO_3 standards in 0.1% Brij (Atlas Powder Co., Wilmington, Del.) was run. The concentration was varied depending upon expected sample values and was always sufficient to cover the absorbance scale above the highest sample. Sample concentrations were read from the standard curve obtained from the desired range of nitrate concentrations. Samples high in nitrate were run using the optional dilution system illustrated in Figure 1 using standards in the range 0–80 μg/ml of NO_3^-–N. Medium levels of nitrate were determined with the standard autoanalyzer system and read against standards in the range 0–5 μg/ml of NO_3^-–N. For samples low in nitrate the concentrated stock suspension of bacteria was used with the sample and bacteria flow lines interchanged, and standards ranged from 0 to 0.5 μg/ml of NO_3^-–N.

The stock bacterial solution was diluted to the daily working suspension and placed in a 500-ml flask designed identically with the stock flask. This suspension was placed in an ice bath and kept under a nitrogen atmosphere during sample measurement. Strict anaerobiosis was necessary with the working suspension to avoid loss of activity during sample measurement. A magnetic stirring system maintained cellular suspension. A drop of 33% Brij 35 solution

added to the bacteria promoted a more uniform flow pattern.

For good sample duplication the automated system must be kept clean. Half-hour rinsing with 0.1% Brij solution kept coils clean for several days. Skewed peaks and erratic base lines, however, indicate a need for tubing changes and acid rinses for all coils. Sample preparation was the same as reported earlier (Lowe and Hamilton, 1967).

RESULTS AND DISCUSSION

This method is readily adaptable to a wide range of nitrate concentrations by making slight modifications in the autoanalyzer system as described above. Three levels of nitrate standards were measured (0–80, 0–5, and 0.–0.05 μg/ml of NO_3^-–N) and the curve of each was drawn. A linear curve resulted at each concentration and a wide range of sample types could be used. Tobacco tissue and other plant materials high in nitrate were measured by use of the higher concentration, soil at the middle range, and samples low in nitrate, such as stream or ground water, at the lower range. In fact, a measurement of as little as 0.01 ppm of nitrate/nitrogen was detected on the analyzer. Very low samples were measured by use of the concentrated stock suspension and then interchange of the sample and bacteria flow lines. Monitoring of such low levels of nitrate will probably find wide application in present and future pollution work.

The quantitativeness of the system was measured by adding a specific amount of nitrate to tobacco samples. Table I shows the recovery values for the different samples. Cured burley tobacco was used and four leaf positions of differing concentrations were taken for measurement. All recovery values were excellent with no more than ±1% difference between samples. These values show conclusively the quantitative specificity of the nitrate reductase enzyme. Even at high nitrate levels the enzyme responded effectively and gave continuous quantitative reductions.

To determine analyzer performance, a burley tobacco sample was replicated. The data of Table II shows the precision attainable with the autoanalyzer system. Replications differed no more than 0.1 ppm with a resulting error of only 0.4%. Thus, the data of Tables I and II show that reliable results can be achieved with the procedure because of the highly specific nature of the enzyme and the precision of the autoanalyzer. Few plants will be encountered that have more interfering compounds than tobacco. However, we have used the procedure with extracts of many plant materials without any complication.

The bacteria used in this method were easily cultured, and ordinary reagent grade chemicals were used in all medium preparations. With time, growth curves were relatively uniform from one harvest to the next. The extremely high enzyme levels can be duplicated if strict anaerobiosis is maintained. Oxygen severely limits high nitrate reductase levels and allows the formation of nitrite reductase; therefore, it should always be removed from the culture and stock suspensions. The entire culturing and harvesting process involves only 1 day and can be performed by any cautious technician. The harvest procedure is a critical phase of the process because temperature, resuspensions, and time control enzyme preservation. Under normal conditions, refrigerated stock suspensions have maintained activity up to 3 weeks and enough enzyme is available from a single culture to run 1200–1600 analyses.

LITERATURE CITED

"Accumulation of Nitrate", National Academy of Sciences, Washington, D.C., 1972.
Lester, R. L., DeMoss, J. A., *J. Bacteriol.* **105,** 1006 (1971).
Lowe, R. H., Hamilton, J. L., *J. Agric. Food Chem.* **15,** 359 (1967).
McNamara, A. L., Meeker, G. B., Shaw, P. D., Hageman, R. H., *J. Agric. Food Chem.* **19,** 229 (1971).

Received for review December 2, 1974. Accepted April 28, 1975. Contribution of the Agricultural Research Service, U.S. Department of Agriculture, and the Agricultural Experiment Station, University of Kentucky, Publication No. 74-3-36. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or University of Kentucky, and does not imply its approval to the exclusion of other products that may be suitable.

Structure–Activity Relationships of Some Antifungal Indoles

Wybo H. Dekker,[1] Hendrik A. Selling,* and Jan C. Overeem

In a series of 64 substituted indoles most members were found to possess antifungal activity against *Botrytis allii* and *Cladosporium cucumerinum*, while some were also active against *Penicillium italicum* and *Aspergillus niger*. Highest activities were found for 3-phenylindole and 3-(2-methylphenyl)indole. By regression analysis, the antifungal activities were found to correlate significantly with (1) a substituent constant π, derived from TLC R_f values, (2) the square of π, and (3) the NMR chemical shift of the NH proton in Me_2SO solution.

Although indoles are well known for their biological activity in medicinal chemistry and as plant growth regulators, antifungal activity has only been reported for 3-thiocyanatoindoles (Akerstrom et al., 1970, 1971). We now have found antifungal activity to be widespread among indole derivatives and in this paper we report the results obtained with 64 representatives. In addition, we give correlations between antifungal activity of the indoles with physicochemical properties by regression analysis, using the multiparameter approach according to Hansch (1971).

Institute for Organic Chemistry TNO (Organization for Applied Scientific Research in The Netherlands), Utrecht, The Netherlands.
[1] Present address: Chemisch Laboratorium RVO-TNO, Rijswijk, The Netherlands.

EXPERIMENTAL SECTION

Synthetic Methods. The following methods were employed for the synthesis of most of the indoles: (A) treatment of isatin or a substituted isatin with an appropriate Grignard reagent, followed by reduction of the intermediate dioxindole with $LiAlH_4$ (Bergman, 1971); (B) Fischer