Effect of Mixed Culture on *Nitrosomonas europaea* Simulated by Uptake and Utilization of Pyruvate

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ABSTRACT

CLARK, CONNIE (University of Minnesota, Minneapolis), AND E. L. SCHMIDT. Effect of mixed culture on *Nitrosomonas europaea* simulated by uptake and utilization of pyruvate. J. Bacteriol. 91:367-373. 1966.—Contamination of a 5-liter culture of *Nitrosomonas europaea* resulted in enhanced nitrite formation as compared with pure cultures. The dominant contaminant, a yellow-pigmented, heterotrophic bacterium, was isolated and studied both in pure culture and in deliberate mixture with *N. europaea*. Growth of *N. europaea* was stimulated in mixed cultures with the heterotrophic isolate to varying degrees, apparently depending on the age and density of autotrophic inocula. Mixed culture stimulation of *N. europaea* was evidenced only by a shortened lag phase, since post-lag phase growth of the autotroph was equivalent in both pure and mixed cultures. The heterotrophic component had a nutritional requirement for amino acids when grown on simple media; presumably, in the mixed culture system, the heterotroph was provided with amino acids synthesized by the autotroph. The mixed culture effect was reproduced in all essential features by the addition of sodium pyruvate to the nitrification medium. Pure cultures of *N. europaea* were shown to incorporate sodium pyruvate-2- C^{14} during growth. Labeled carbon was widely distributed in cell fractions. The more pronounced and consistent pyruvate effect was observed on old inocula of *N. europaea*.

Isolation of nitrifying bacteria in pure culture justifiably is recognized as a difficult matter: contaminants build up rapidly and reach high population densities under conditions designed to favor the chemoautotrophic nitrifiers, especially in the case of *Nitrosomonas*. The corollary problem, that of maintaining as pure cultures those once obtained, is also well known, and large-scale cultures are particularly susceptible to contamination. It is an interesting consequence of mixed culture situations that nitrification reactions frequently are observed to proceed more rapidly in the presence of heterotrophic microorganisms than in pure culture (3).

Organic metabolites formed by the chemosynthetic bacteria could readily account for any extensive development of heterotrophs in inorganic nitrification media, but the benefits of mixed culture to the autotroph are less obvious. The oft suggested possibility that organic compounds formed by the heterotrophs may stimulate growth of associated autotrophic nitrifiers is without experimental support. Use of organic

materials supplied exogenously was reported for *Nitrobacter* (1) while the present paper was in preparation. Previously, the better-characterized instances of organic matter stimulation of nitrifying bacteria have been attributed to effects other than organic nutrition (2, 4, 5).

The present report is concerned with a stimulatory effect on nitrite formation first observed as the result of contamination of a culture of the chemoautotrophic nitrifier *Nitrosomonas europaea*. Study of the stimulation led to an examination of the effect of a number of organic compounds on the growth of the autotroph in pure culture. One organic compound is shown both to stimulate and to participate in the growth of *N. europaea*.

MATERIALS AND METHODS

The *N. europaea* culture used in these studies was obtained from D. Pramer, Rutgers University. The culture medium was composed of: Na_2HPO_4 , 13.5 g; KH_2PO_4 , 0.7 g; $(\text{NH}_4)_2\text{SO}_4$, 2.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 18.4 mg; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 14.4 mg;

NaHCO_3 , 0.5 g; and deionized water, 1 liter. After autoclaving for 20 min at 121 C, approximately 3 g of neutral decolorizing charcoal, Norit A, was added to remove traces of organic matter and the colloidal opalescent precipitate present after autoclaving. Filtration the next morning, followed by autoclaving, resulted in a sterile, clear medium with a pH of 7.4 to 7.6.

From 5 to 15% of each autotrophic culture was used as inoculum in one or more organic media to monitor the purity of the cultures. If no growth occurred after 7 days of static incubation at 25 to 30 C, the autotrophic culture was considered to be free from heterotrophic contaminants. The organic media used consisted of (w/v): (i) 0.3% beef extract and 0.5% peptone; (ii) 0.3% beef extract, 0.5% peptone, 1.0% glucose, and 0.3% yeast extract; (iii) 0.3% beef extract, 0.5% peptone, and 1.0% Proteose Peptone (Difco).

Nitrite-nitrogen determinations were made by use of sulfanilic acid and α -naphthylamine reagents. Color intensity was determined in the Klett-Summerson colorimeter with the use of a 540-m μ filter.

Sodium pyruvate was obtained from Sigma Chemical Co., St. Louis, Mo., and the labeled sodium pyruvate ($\text{CH}_3\text{C}^{14}\text{OOCNa}$) was from New England Nuclear Corp., Boston, Mass. A sample of 1.5 g of unlabeled sodium pyruvate was washed and analyzed for trace elements on a model GE XRD5 X-ray fluorescent spectrometer, with the use of a polyethylene planchet and a platinum target. Zinc with a maximal concentration of less than 10 ppm was the only metal indicated in a spectrum which also included copper, nickel, cobalt, iron, manganese, vanadium, and titanium.

Radioactivity of whole cells was measured in Packard Tri-carb liquid scintillation spectrometer system (model 314EX). The scintillator fluid consisted of: dioxane, 2,913 ml; naphthalene, 375 g; 2,5-diphenyloxazole, 22.5 g; 1,4-bis-2(5-phenyloxazolyl)-benzene, 2.19 g; and methanol, 200 ml.

Cells were fractionated by the method of Schneider, Hogeboom, and Ross (6). Radioactivity of cell fractions was measured by use of a scaler (model 181B) and a gas-flow counter (model D47; Nuclear-Chicago Corp., Des Plaines, Ill.) at 1,350 v, with a gas mixture of 98.7% helium and 1.3% butane. Samples were plated on glass planchets, and counts were corrected to infinite thinness. The protein fraction was hydrolyzed for 22 hr at 110 C in 6 N HCl under nitrogen gas. The HCl was evaporated; the residue was suspended and evaporated twice in water and was then suspended in 0.2 ml of water for spotting on Whatman no. 1 paper. Two-dimensional ascending chromatography was used for amino acid separation with the solvent systems: glacial acetic acid–water–*n*-butanol (1:1.66:4, v/v) and water–pyridine–methanol (1:10:40, v/v).

RESULTS

Attempts to grow pure cultures of *N. europaea* in aerated 7-liter fermentors frequently failed when the culture became contaminated and had

to be discarded. During one fermentor run, a marked increase in turbidity, accompanied by an abrupt rise in nitrite concentration, appeared suddenly during the 10- to 14-day incubation period. Culture development in this case was in sharp contrast to the faint growth encountered in all previous work with the fermentors. A yellow pigmented, heterotrophic bacterium that appeared to be the dominant contaminant was isolated from this fermentor culture, and was designated as isolate "H-y."

The obvious turbidity which developed rather suddenly in this instance after prolonged incubation suggested that the heterotrophic contaminants were dependent on some growth of *Nitrosomonas* for organic substrates, whereas the sharp increase in nitrite concentration which occurred at the same time indicated much-enhanced growth of *Nitrosomonas*. The effects observed in the chance contamination were repeated in a fermentor culture inoculated with *N. europaea* in deliberate mixture with washed cells of the heterotrophic isolate H-y and were compared with a duplicate pure culture of the autotroph. Some results of the experiment are shown in Table 1. By the 24th day, the nitrite of the mixed culture was more than 50 times that of the pure culture, with a heterotroph population of about 2×10^5 per milliliter (Table 1). In subsequent incubation, the activity of *Nitrosomonas* increased in the pure culture but not in the mixed culture, whereas the H-y isolate not only died rapidly but apparently lysed, as evidenced by a clearing of the culture.

Enhanced growth of *N. europaea* in the presence of the heterotroph was also demonstrated in small-volume (100 ml) shaken culture, and was shown to coincide with the death of the heterotrophic cells (Fig. 1). The pure culture of *Nitrosomonas* again exhibited a very long lag phase, probably due to the small inoculum (1 ml of a lag phase culture). Replicate inocula, however, augmented with a relatively high concentration of washed H-y cells, developed rapidly, and maximal levels of nitrite were

TABLE 1. Nitrite formation by *Nitrosomonas europaea* in pure culture and in mixture with heterotroph isolate H-y, in 5-liter cultures

Incubation days	NO ₂ -N/ml		Count of H-y cells in mixed culture
	<i>N. europaea</i> μg	<i>N. europaea</i> + H-y μg	
24	5	270	$2 \times 10^5/\text{ml}$
29	50	220	<1/0.1 ml
32	125	205	<1/0.1 ml

FIG. 1. Development of *Nitrosomonas europaea* in pure culture and in the presence of a large inoculum of bacterium *H-y*. Shaken flask culture; data are averages of four replications. Symbols: $\square$, *N. europaea* plus *H-y*; $\triangle$, *N. europaea* pure culture; $\circ$, *H-y* cells per ml.

reached before the control culture emerged from lag phase. Plate counts of the heterotroph in the mixed culture showed clearly that exponential development of the nitrifier occurred during an abrupt decline in the numbers of viable *H-y* cells, further suggesting that lytic products of the heterotroph may have benefited *Nitrosomonas*. At the termination of the experiment (34 days), more than 10% of each control culture inoculated into the test broths gave no indication of contamination; inoculation with 2% of the mixed culture showed that the *H-y* component had died out completely in three of the four replicate cultures.

Heterotroph *H-y*. Examination of the heterotrophic isolate *H-y* showed it to be a gram-negative to gram-variable ovoid rod that produced golden yellow, highly mucoid colonies on solid media. No acid or gas was produced from glucose, lactose, maltose, or mannitol. The medium used for cultivation of *Nitrosomonas* did not support growth of *H-y* in pure culture, nor did the aseptic addition of 0.05 to 1.0% glucose render the medium suitable for growth.

Examination of the nitrogen requirements of

H-y disclosed that the glucose-inorganic nitrification medium supported good growth if the ammonium nitrogen was augmented with certain amino acids. Threonine, proline, and arginine added in combination allowed nearly maximal growth in 24 hr. Comparable but slower growth was obtained with the addition of only proline. Further experiments showed that L-proline would serve as sole carbon and nitrogen source when added to the basal salts of the nitrification medium. Good growth was obtained but no nitrite was formed when *H-y* was grown in the glucose-proline medium with nitrate or ammonium present. Only limited growth with no indication of nitrite metabolism was observed with nitrite added to the glucose-proline medium at the maximal concentration found in mixed culture.

It appeared that the accumulation of nitrite in mixed systems was due to autotrophic proliferation alone. Moreover, the relatively simple growth requirements of the heterotroph, *H-y*, apparently were met by organic substrates provided by the growth of the autotroph in the mixed culture system.

Autotrophic inoculum. In subsequent experiments, the effect of the *H-y* heterotroph on *Nitrosomonas* was not consistent, and the presence or absence of a mixed culture effect similar to that shown in Fig. 1 appeared to depend on the nature of the autotrophic inoculum. In all instances, the deliberate mixture of *H-y* and *N. europaea* resulted in a relatively short lag period of 4 to 8 days for nitrite formation, but in some experiments the development of the pure culture control closely paralleled that of the mixed culture. The effect of density of *N. europaea* inoculum was examined in shaken culture with several dilutions of autotrophic inoculum used alone or in combination with a constant inoculum of *H-y* cells. A 14-day *N. europaea* culture in late log phase was used for preparation of the inoculum. Data from this experiment (Fig. 2) show that the length of the lag phase of the pure cultures decreased with increasing density of the inoculum. In the presence of a constant inoculum of *H-y*, cultures inoculated with the lower number of autotrophic cells developed at a rate which approximated those pure culture systems which received a 10-fold greater autotrophic inoculum. All comparisons in Fig. 2 indicate a mixed culture effect at each of the ratios used, with no instance of the pure culture paralleling that of the mixture; perhaps a larger autotrophic inoculum or an earlier log-phase inoculum was necessary to eliminate the mixed culture effect as noted on some other occasions.

Observations were consistent with the view

FIG. 2. Effect of varying dilutions of *Nitrosomonas europaea* inoculum in pure culture and in mixture with a constant inoculum of H-y cells. Shaken flask culture; data are averages of three replications. Solid lines, mixed cultures; broken lines, pure cultures of *N. europaea*; Δ , 10^0 dilution of *N. europaea*; $\circ$, 10^{-1} dilution; $\square$, 10^{-2} dilution.

that the heterotroph resulted in enhanced growth of *N. europaea* when the autotrophic inoculum was comprised of very young or relatively old cells. It seemed probable that such cells in mixed culture were provided with one or more organic metabolites, and consequently reached their full synthetic potential more rapidly than in pure culture conditions which required autotrophic synthesis of the metabolite. The mixed culture effect was one of stimulation to the autotroph rather than merely the fulfillment of a medium deficiency because, once the pure culture emerged from lag phase, the rate and extent of nitrite formation were almost equivalent to that of *N. europaea* in the mixed culture system. Further work on the nature of the stimulation by H-y was interrupted when it was found that the same effect could be reproduced by addition of an

organic compound, sodium pyruvate, to the medium.

Response of *Nitrosomonas* to sodium pyruvate. Aseptic addition of either autoclaved or filter-sterilized sodium pyruvate to the inorganic medium of *Nitrosomonas* resulted in a stimulatory effect on nitrite formation by *N. europaea* under conditions carefully monitored to insure that pure cultures were maintained. Data typical of the pyruvate effect are presented in Fig. 3. Inorganic medium augmented in this case with filter-sterilized pyruvate at a final concentration of 5 μ moles/ml reduced the lag phase of the *N. europaea* from 7 to 4 days. The effect of the pyruvate was similar to the mixed culture effect, not only in the marked reduction of the lag phase but also in the lack of any appreciable effect on post-lag phase growth.

The pyruvate effect further mimicked the effect of heterotroph H-y with regard to a certain lack of consistency. Not all inocula responded to pyruvate by evidencing a shortened lag phase, and the more pronounced effects seemed to be associated with the old age of the autotrophic culture used as inoculum. Age of inoculum as a variable in the pyruvate effect was investigated, with the results presented in Fig. 4. In the experiment, a single static culture of *N. europaea*

FIG. 3. Growth of *Nitrosomonas europaea* as measured by nitrite formation with and without filter-sterilized sodium pyruvate added to inorganic medium at the rate of 5 μ moles/ml. Shaken flask cultures. Symbols: Δ , pyruvate added; $\circ$, control, no pyruvate.

FIG. 4. Effect of progressive aging of *Nitrosomonas europaea* inoculum on response to sodium pyruvate at $4.5 \mu\text{moles/ml}$. Shaken flask cultures; data are averages of three replications. Symbols: $\circ$, pyruvate added; $\triangle$, control, no pyruvate. Same mother culture used as inoculum after growing: (a) 16 days; (b) 30 days; (c) 44 days; (d) 57 days.

was maintained as the source of inoculum, and 1.0-ml portions were transferred at varying intervals into 100-ml volumes of fresh medium with and without pyruvate. Inocula from the 16- and the 44-day-old culture responded almost identically, with a pronounced pyruvate effect, whereas the inoculum of intermediate age (30-day) unaccountably initiated growth more promptly and showed little response to pyruvate. The inoculum taken from the 57-day-old culture showed a more extended lag phase even with pyruvate present, but the pyruvate effect in the case of this very old inoculum was very marked. In cultures with the 57-day-old inoculum, the nitrite-nitrogen level with pyruvate was 12 times that of the controls at the 18th day of incubation.

Uptake of pyruvate in growing cultures. Experiments with the 57-day inoculum included the introduction of sodium pyruvate- 2-C^{14} to allow observation of incorporation of organic carbon. After 18 days of incubation in shaken culture, portions of the culture suspension and washed cells from a 40-ml portion were analyzed for C^{14} activity. The results given in Table 2 show that 0.86% of the added radioactivity was incorporated into cell material.

The distribution of labeled carbon in cell fractions was examined in cells taken from a replicate culture in which 0.68% of added label was incorporated. Data in Table 3 show that a little over half of the radioactivity taken up by the cells remained in the residue (protein) after fractionation. Samples of the supernatant fluid were found to lose 97.88% of their radioactivity after treatment with 1 N HCl , suggesting that over this extended growth period (18 days) the greatest part of the labeled carbon of sodium pyruvate was returned to the medium as carbonate after utilization by the autotroph.

The cell residue remaining after the perchloric acid and ether-ethyl alcohol extractions was hydrolyzed, spotted for amino acid chromatog-

TABLE 2. Uptake of C^{14} by cultures of *Nitrosomonas europaea* grown in shaken culture for 18 days in inorganic medium containing sodium pyruvate- 2-C^{14}

Sample	Total count/min after 18 days of incubation	Per cent radioactivity
Culture suspension . . .	1.7×10^7	100
Supernatant fluid	1.6×10^7	96.53
Washed cells	1.48×10^5	0.86

TABLE 3. Distribution of C^{14} in fractions obtained from 1.4 mg (dry weight) of *Nitrosomonas europaea* cells grown in shaken culture for 18 days in the presence of sodium pyruvate- 2-C^{14}

Sample	Total count/min after 18 days of incubation	Per cent radioactivity	Per cent of total uptake in cell fractions
Washed cells	2.04×10^5	0.680	100
Cold perchloric acid extract . . .	12,340	0.041	6
Ether-ethyl alcohol extract . . .	58,800	0.191	29
Hot perchloric acid extract . . .	25,700	0.085	13
Cell residue	106,800	0.354	52

FIG. 5. Autoradiograph of chromatogram of *Nitrosomonas europaea* protein hydrolysate. Cells grown in the presence of sodium pyruvate-2- C^{14} and 100 $\mu\text{g/ml}$ of unlabeled sodium pyruvate.

raphy, and a radioautograph was prepared after chromatographic separation. Figure 5 shows the rather general distribution of the label in the amino acids of the hydrolysate.

DISCUSSION

Enhancement of the growth of *N. europaea* which occurred under certain conditions when the autotroph was grown in mixed culture with a heterotrophic bacterium was simulated in its essential features by the presence of pyruvate in the medium. Whether the stimulatory effect of the heterotroph was in fact due to the release of pyruvate was not determined, but it seems highly probable that some organic metabolites of heterotrophic origin may be assimilated by *Nitrosomonas* in mixed culture environments. The nature of such organic metabolites, the circumstances of assimilation, and the extent to which they may be used by the nitrifier are of marked interest, both to study of the metabolism of the autotroph and to study of its development in natural environments.

Nutritional requirements of the heterotroph

H-y appeared to be surprisingly complex. As the dominant bacterium present in the contaminated system that was first observed to stimulate nitrification, it was expected that H-y would be a versatile opportunist, capable of using nonspecifically the dilute organic materials provided by autotrophic growth. It was found instead to have a requirement for amino acids and was observed to grow rapidly only when the glucose-inorganic nitrification medium was supplemented with several amino acids. Presumably, the nitrifier had supplied the required amino acids in amounts adequate to allow the H-y to compete favorably with the other heterotrophs present initially. This particular system of isolate H-y and *N. europaea* affords indirect evidence that specific interactions to the mutual benefit of both autotrophic and heterotrophic components may be a feature of mixed culture nitrification. Similar interactions may account for observations by both Stapp (7) and Gundersen (3) that certain bacteria seemed to be present consistently in enrichment cultures of nitrifiers.

The results of experiments with labeled pyruvate show that some organic carbon was assimilated by *N. europaea* and was incorporated into a wide range of cell constituents. Since such cultures would be particularly susceptible to contamination, special precautions were taken to insure that all cultures used for analysis remained free from contaminants; in addition to the routine test broths, pyruvate cultures were tested in the inorganic nitrification medium augmented with dilute glucose, yeast extract, or casein hydrolysate. During the preparation of this report, a paper by Delwiche and Finstein (1) appeared, demonstrating the incorporation of labeled glutamic acid into growing cultures of a *Nitrobacter* sp. and of acetate and glycine into resting suspensions of the same organism. They found that acetate, especially, stimulated growing cultures of *Nitrobacter*, but neither acetate nor other organic amendments tried allowed any growth or nitrification in the absence of carbon dioxide. It seems clear now that both *Nitrosomonas* and *Nitrobacter*, generally regarded as among the most strictly autotrophic of bacteria, may incorporate various organic compounds and, under some conditions at least, show enhanced growth due to the metabolism of certain organic compounds provided in the growth medium.

The features of the pyruvate effect on *N. europaea* are of special interest. Pyruvate did not result in a marked growth response in all instances, for in a few experiments control cultures progressed either at the same rate or only

slightly slower than pyruvate-treated cultures. However, consistent and pronounced response to pyruvate could be attained when old inocula were used. Inocula from cultures still in log or late log phase varied in response from good (Fig. 4a) to slight (Fig. 4b), perhaps as a function of inoculum density. Growth response was mainly a matter of reduction of the lag phase, and once the corresponding control (inorganic) cultures entered log-phase growth their rate of nitrite formation appeared to be essentially equivalent to that of pyruvate-treated cultures. It seems possible that old cells, in particular, may be deficient in certain synthetic systems, which are reconstituted more efficiently from pyruvate carbon than from carbon dioxide.

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