Comparison of Denitrification by Pseudomonas stutzeri, Pseudomonas aeruginosa, and Paracoccus denitrificans

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Received 29 July 1982/Accepted 29 December 1982

A comparison was made of denitrification by Pseudomonas stutzeri, Pseudomonas aeruginosa, and Paracoccus denitrificans. Although all three organisms reduced nitrate to dinitrogen gas, they did so at different rates and accumulated different kinds and amounts of intermediates. Their rates of anaerobic growth on nitrate varied about 1.5-fold; concomitant gas production varied more than 8-fold. Cell yields from nitrate varied threefold. Rates of gas production by resting cells incubated with nitrate, nitrite, or nitrous oxide varied 2-, 6-, and 15-fold, respectively, among the three species. The composition of the gas produced also varied markedly: Pseudomonas stutzeri produced only dinitrogen; Pseudomonas aeruginosa and Paracoccus denitrificans produced nitrous oxide as well; and under certain conditions Pseudomonas aeruginosa produced even more nitrous oxide than dinitrogen. Pseudomonas stutzeri and Paracoccus denitrificans rapidly reduced nitrate, nitrite, and nitrous oxide and were able to grow anaerobically when any of these nitrogen oxides were present in the medium. Pseudomonas aeruginosa reduced these oxides slowly and was unable to grow anaerobically at the expense of nitrous oxide. Furthermore, nitric and nitrous oxide reduction by Pseudomonas aeruginosa were exceptionally sensitive to inhibition by nitrite. Thus, although it has been well studied physiologically and genetically, Pseudomonas aeruginosa may not be the best species for studying the later steps of the denitrification pathway.

Denitrification is the anaerobic reduction of a fixed nitrogen oxide, coupled with respiratory generation of ATP and release of a gas. The process has been the topic of several recent reviews (6, 15, 24, 25, 27, 38). Complete denitrification is thought to involve the sequential reduction of nitrate, nitrite, nitric oxide, and nitrous oxide to dinitrogen gas. A variety of incomplete denitrification pathways also exist: some denitrifying bacteria reduce both nitrate and nitrite; others reduce only nitrite. Some produce only dinitrogen; some produce a mixture of dinitrogen and nitrous oxide; others produce only nitrous oxide. Even within a single species, Pseudomonas fluorescens, the biotypes differ in the end product of the pathway (11). In a species of Vibrio, only nitrite and nitrous oxide are reduced (41). Such diversity, in a sense a natural set of mutant phenotypes, has contributed to our understanding of the intermediates of denitrification and the significance of each reductive step. However, it is becoming increasingly evident that significant differences exist in the rate and regulation of each step even among organisms that possess the complete pathway (i.e., reduction of nitrate to dinitrogen). Knowledge about such differences may help in choosing the appropriate organism for particular denitrification studies. Pseudomonas aeruginosa has been extensively studied genetically and therefore is often considered a favorable organism for use in studies on denitrification that involve the analysis of mutant strains. However, it may not be completely desirable. We undertook this study to compare several aspects of denitrification in Pseudomonas aeruginosa with two other organisms capable of complete denitrification, Paracoccus denitrificans and Pseudomonas stutzeri. The latter appeared to be better suited for our physiological genetic studies concentrating on nitrous oxide reduction.

MATERIALS AND METHODS

Materials. Nitrous oxide and a mixture of 10% methane–90% helium were products of Matheson Scientific, Inc., Rutherford, N.J. Nitric oxide, helium, and dinitrogen were obtained from Liquid Carbonic, Chicago, Ill. Cleve's acid (o-naphthylamine-7-sulfonic acid) was from Polysciences, Inc., Warrington, Pa.

Media. The medium used routinely was modified Luria-Bertani (LB) broth (5), consisting of (per liter): 10 g of tryptone, 5.0 g of yeast extract, 5.0 g of NaCl, 1.0 g of glucose, 10 ml of 1 M Tris-hydrochloride (pH 7.5), 1.0 ml of 1 M MgSO$_4$·7H$_2$O, and water. For
solid medium, 20 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) was added.

Measurement of denitrification intermediates. Nitrite was measured by a modification of the method of Nicholas and Nason (23). Samples were mixed vigorously into 4 ml of reagent containing 0.25% sulfuric acid, 0.005% Cleve’s acid, and 15% acetic acid, and the absorbance at 540 nm was measured 30 min later. Gas samples were analyzed with a Perkin-Elmer Sigma 4 gas chromatograph equipped with a thermal conductivity detector. Samples were injected onto a column (1/8 in. by 18 ft [ca. 0.476 by 550 cm]) of Porapak Q at 40°C and eluted with helium at a flow rate of 32 ml/min. Retention times (in seconds) of the gases of interest were N2 (173), O2 (183), NO (196), CH4 (280), CO2 (530), N2O (700), and C2H2 (958). Quantitation was aided by a Perkin Elmer M-2 calculating integrator standardized with known gas mixtures.

Organisms. *Pseudomonas stutzeri* JM300 was a natural soil isolate, obtained from B. A. Bryan and C. C. Delwiche of this university, characterized as previously described (4a). *Pseudomonas aeruginosa* PAO1 was provided by T. C. Hollocher, Brandeis University. *Paracoccus denitrificans* ATCC 19367 was obtained from M. K. Firestone, University of California, Berkeley.

Growth of cultures. The stock cultures were maintained aerobically on LB agar. Cells were routinely grown anaerobically at 37°C in 70-ml stationary serum vials completely filled with LB broth supplemented with sodium nitrate. Anaerobic cultures to be assayed for gas production as well as growth rate were incubated in vials with a 20-ml head space, filled with 90% helium–10% methane; the latter was used as an internal standard to normalize measurements of gas volume. Samples of culture or gas were removed by syringe. Alternatively, growth rates alone were measured on anaerobic cultures incubated in half-filled Erlenmeyer flasks modified by the addition of a nepholometer tube side arm and fitted for gas sparging. The flasks were made anaerobic by being sparged with helium or with nitrous oxide when appropriate. Turbidity was measured in a Klett-Summerson photoelectric colorimeter equipped with a red filter. Dry cell mass was determined by standard methods (10).

Assay of denitrification. Cells grown anaerobically to stationary phase on limiting nitrate were harvested by centrifugation at 20°C for 10 min at 5,000 × g, washed in fresh LB broth, recentrifuged, and resuspended in LB broth to a cell concentration of about 10^11 ml^{-1}. Then 0.3 ml of the suspension was transferred to LB broth supplemented with 40 mM sodium succinate in a 9-ml serum vial, sealed with a rubber septum. The headspace was evacuated and regassed with 10% methane in helium, and a solution of electron acceptor in LB broth was injected to initiate the reaction. The final volume of the cell suspension was 3 ml. Nitrous oxide, as well as nitrate and nitrite, was added as a solution (i.e., nitrous oxide-saturated LB broth) to avoid a lag. The vials were agitated at 150 rpm on a rotary shaker at 24°C. Gas samples (100 μl) were withdrawn from the headspace with a Hamilton gastight syringe; samples for analysis of nitrite were...
withdrawn with a Hamilton liquid syringe. The rate of denitrification was proportional to the concentration of cells in suspension. Each vial was sampled at least five times over a period of 2 to 4 h.

Nitrate reductase activity was measured in vitro with crude extracts prepared by harvesting cells by centrifugation, suspending them in 50 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM MgSO4, and 1 mg of DNase per ml, and breaking them in a French pressure cell, all at 4°C. The production of nitrite was estimated in the presence of dithionite-reduced benzylviologen as electron donor by a modification of the method of Lowe and Evans (18). The reaction mixture contained, in a final volume of 2 ml, 80 μmol of potassium phosphate buffer (pH 7.4), 40 μmol of NaN30, 30 μmol of benzyl viologen, and cell extract. After several minutes of equilibration at 30°C, the reaction was initiated by adding 0.2 ml of a freshly prepared solution of 46 mM sodium dithionite in 0.1 M sodium bicarbonate. Samples were removed at various times for the measurement of nitrite. Reaction rates were proportional to time and protein concentration, the latter measured by the method of Lowry et al. (19).

One unit of activity was defined as the amount of enzyme that catalyzed the production of 1 mmol of NO3− min−1.

RESULTS

Denitrification of nitrate. Measurements were made during anaerobic growth on nitrate to compare the denitrification properties of Pseudomonas stutzeri, Pseudomonas aeruginosa, and Paracoccus denitrificans. Cell yield reflected the net energy gain, and the amount and composition of the gases evolved reflected the relative rates of intermediate steps of the pathway. The results (Fig. 1 and Table 1) indicated that there were slight differences in growth rate among the three organisms but dramatic differences in gas production and cell yield from limiting nitrate. In all cases N2 was produced during the growth phase and continued to be evolved as the cultures entered stationary phase (Fig. 1). The pattern of accumulation of nitrogen oxide intermediates varied markedly among the three species. Nitrite accumulated transiently in growing cultures of all three species; it was highest with Pseudomonas stutzeri (Fig. 1), but it disappeared from the medium late in the growth phase. A small amount of nitrous oxide appeared as Paracoccus cells entered stationary phase (Fig. 1C). Larger amounts were produced by Pseudomonas aeruginosa at about the same growth phase (Fig. 1B). Pseudomonas stutzeri never produced detectable extracellular N2O.

The rates of gas production by early-stationary-phase cells are shown in Table 1. Pseudomonas aeruginosa was by far the most active, but a large proportion of the gas produced was nitrous oxide (Fig. 1). Pseudomonas stutzeri and Paracoccus denitrificans produced N2 exclusively, but the former produced it at a significantly higher rate.

The two pseudomonads produced similar cell yields from denitrification of nitrate (Table 1). However, the yield of Pseudomonas stutzeri cells per mole of nitrate was proportional to the concentration of nitrate up to 40 mM, whereas the yield of Pseudomonas aeruginosa cells was proportional only up to about 25 mM NaNO3 (data not shown). The cell yield of Paracoccus denitrificans was two to three times that of the other cultures and was proportional to nitrate concentration up to 40 mM (data not shown).

The assay for dissimilatory nitrate reductase activity provided an in vitro index of the ability of the cells to reduce nitrate. Since an artificial electron donor was employed, the assay might not necessarily be expected to reflect in vivo rates, but in fact it did. The specific activities of nitrate reductase in cell extracts of Pseudomonas stutzeri and Paracoccus denitrificans were both higher than in extracts of Pseudomonas aeruginosa cells (Table 1), and growing cells of the latter species excreted less nitrite (Fig. 1).
doubling time (90 min) of Pseudomonas stutzeri cells in LB broth continuously sparged with nitrous oxide was even shorter than its doubling time in the same medium with nitrate (114 min), but longer than in the same medium sparged with air (55 min).

Production of gases by resting cell suspensions. We also compared the rates of gas evolution from various nitrogen oxides by concentrated suspensions of washed cells grown on nitrate. The rate of gas production from nitrate by Pseudomonas stutzeri and Paracoccus denitrificans cells (Table 2) was similar to that by growing cells (Table 1). N₂ was the only gas released from either 33 or 100 mM nitrate. Neither nitrite, nitric oxide, nor nitrous oxide accumulated during nitrate reduction. The rate of gas production from nitrate by resting cells of Pseudomonas aeruginosa (Table 2) was much lower than by growing cells (Table 1). Unlike growing cells that produced mainly nitrous oxide, resting cells produced only N₂.

The three species metabolized nitrite quite differently. Both pseudomonads produced gas much more slowly from nitrite than from nitrate (Table 2). However, Paracoccus denitrificans produced gas from both electron acceptors at about the same rate. Like growing cultures, resting cells of both Pseudomonas stutzeri and Paracoccus denitrificans produced primarily N₂, with a trace of nitrous oxide accumulating from the latter. With 100 mM sodium nitrite, gas production was further inhibited, and nitrous oxide constituted the major portion (70%) of the product from both species (data not shown). Pseudomonas aeruginosa differed dramatically: the predominant product with 36 mM nitrite was nitric oxide, and only traces of N₂ and nitrous oxide were present (Table 2). Since the reduction of nitric oxide appeared to be almost completely inhibited by 36 mM nitrite, the effect of higher concentrations was not examined. Lower concentrations differentially inhibited the reduction of NO and N₂O (Fig. 2). Cells supplied with 6 mM nitrite produced nitric oxide transiently in addition to nitrous oxide and N₂. Total gas production was 35 nmol of N min⁻¹ mg (dry wt)⁻¹. At a nitrite concentration of 12 mM, total gas production decreased, the accumulation of nitric oxide was higher and persisted longer, and production of both N₂ and nitrous oxide was depressed (Fig. 2B). These trends continued in the presence of 18 mM nitrite (data not shown), and with 36 mM nitrite only nitric oxide was present even after several hours of incubation (Fig. 2C). The evolution of gas by Pseudomonas stutzeri cells incubated with 36 mM nitrite is shown (Fig. 2D) for comparison, and that by Paracoccus denitrificans cells was similar, as mentioned above.

Pseudomonas stutzeri and Paracoccus denitrificans both vigorously reduced nitrous oxide to N₂ (Table 2), but the rate of reduction by Pseudomonas aeruginosa was an order of magnitude slower. Furthermore, gas production by the other two species from nitrous oxide was markedly faster than it was from either nitrate or nitrite; this was not true for Pseudomonas aeruginosa. Exogenous nitrous oxide was not toxic to this organism, because its growth on nitrate was not inhibited by 1.0 atm of nitrous oxide (data not shown). Rather, the slow rate of reduction of N₂O and the failure of cells growing on nitrate to reduce it completely to N₂ (Fig. 1) must reflect a defect in the ability of this species to metabolize nitrous oxide.

**DISCUSSION**

Bacteria that carry out complete denitrification differ fundamentally in a number of ways. For the three species in this study, the ability to denitrify serves the same function, allowing anaerobic growth of these facultative aerobes, but the growth rates, cell yields, ability to utilize exogenous intermediates, and relative rates of various steps of the pathway varied greatly. Pseudomonas aeruginosa grew most rapidly (doubling time, 1.3 h) and released the largest

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**TABLE 2. Rates of denitrification by anaerobic resting-cell suspensions**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gas production (nmol of N min⁻¹ mg [dry wt]⁻¹) from electron acceptor at indicated concn (mM):</th>
<th>Nitrato</th>
<th>Nitrate</th>
<th>Nitrous oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>100</td>
<td>2.5</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>90.2b</td>
<td>96.4b</td>
<td>58.0b</td>
<td>77.6b</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>45.2b</td>
<td>44.0b</td>
<td>9.4c</td>
<td>7.6b</td>
</tr>
<tr>
<td>Paracoccus denitrificans</td>
<td>34.2b</td>
<td>64.4b</td>
<td>58.8d</td>
<td>92.4b</td>
</tr>
</tbody>
</table>

a The sum of volatile nitrogen oxides. Values are the average of measurements made during incubation for two or more samples.

b Entirely N₂.

c Predominantly NO, with 9% N₂O and 11% N₂ after 5 h.
d Predominantly N₂, with 6 to 17% N₂O. There was no NO.
amount of gas; however, it had the lowest cell yields (28 g/mol of nitrate) and produced the highest proportion of nitrous oxide. In contrast, *Pseudomonas stutzeri* and *Paracoccus denitrificans* grew more slowly (doubling times, 1.9 and 1.5 h, respectively) and released gas more slowly, but the gas produced was nearly all N₂ and the cell yields were markedly higher (34 and 78 g/mol of nitrate, respectively). These species apparently realized a significantly greater net ATP yield from denitrification; *Paracoccus denitrificans* cells derived more than twice as much ATP per electron transported to a nitrogen oxide acceptor as did cells of *Pseudomonas aeruginosa*.

The patterns of accumulation of intermediates also differed. Presumably these intermediates are excreted into the medium when a subsequent step in the pathway is rate limiting, as suggested recently by the kinetic study of Betlach and Tiedje (3). We found that growing cells of *Pseudomonas stutzeri* and *Paracoccus denitrificans* excreted nitrite; their high nitrate reductase activity in vitro suggested that nitrite reduction was rate limiting. In contrast, only a third as much nitrite accumulated in the medium of the *Pseudomonas aeruginosa* culture, and its nitrate reductase activity was fivefold lower.

The relative rates of various steps of denitrification in the three representative denitrifiers were also compared by measuring gas evolution in resting cells incubated with nitrate or intermediate electron acceptors; again the three species had different patterns (Table 2). *Pseudomonas stutzeri* reduced nitrate, nitrite, and nitrous oxide completely to N₂ at the highest rates. *Paracoccus denitrificans* behaved similarly, although some nitrous oxide was excreted when cells were incubated with 36 mM nitrite. *Pseudomonas aeruginosa* was strikingly different; it reduced each of the nitrogen oxides much more slowly. The reduction rates for nitrite and nitrous oxide were one-fourth and one-fifteenth those of the other species, respectively. Surprisingly, nitric oxide accumulated as the major product of nitrite reduction. Only with low concentrations of nitrite were nitrous oxide and N₂ produced. Possibly the reductases constituting the latter part of the denitrification pathway in *Pseudomonas aeruginosa* are differentially inhibited by nitrite. Payne (26) discussed earlier studies that also concluded that nitrite plays a dominant role in determining the composition of the gaseous products of denitrification. We found that concentrations of nitrite greater than 15 mM markedly inhibited anaerobic growth of *Pseudomonas aeruginosa* (data not shown), consistent with reports that 10 mM nitrite inhibited respiration, active transport, and oxidative phosphorylation in this organism (40). This concentration of nitrite was also reported to inhibit the growth of *Paracoccus denitrificans* (39); however, we found that the resting cells of this organism were much less sensitive to nitrite than were those of *Pseudomonas aeruginosa*.

Recently, a group of denitrifiers was compared for their apparent affinity for the uptake of nitrate (R. M. Edwards and J. M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N48, p. 181, and personal communication). In that report *Pseudomonas stutzeri* JM300 compared very favorably with the others. Its apparent Kₘ for nitrate was 5.7 ± 2.4 µM, and its Vₘₐₓ was 1.56 µM min⁻¹; the comparable values for *Pseudomonas fluorescens*, for example, were 6.0 ± 1.8 µM and 1.60 µM min⁻¹, respectively. Such data suggest that *Pseudomonas stutzeri* may compete effectively in low-nitrate natural environments.

A number of denitrifiers have been shown to grow on nitrous oxide (Table 3). Indeed, this capability is probably limited to denitrifying bacteria, but it is not common to all of them. Several species otherwise capable of denitrification cannot grow on nitrous oxide. Most of these produce only nitrous oxide from nitrate or nitrite denitrification and thus apparently lack the last step in the pathway. However, *Pseudomonas aeruginosa* is unique. It can produce N₂ and reduce exogenous nitrous oxide, but it is unable to grow with nitrous oxide as the sole oxidant (4, 37). The biochemical basis of this phenomenon is particularly obscure because, as Bryan and
TABLE 3. Classification of denitrifying bacteria by their metabolism of nitrous oxide

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrous oxide utilizing*</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Pseudomonas perfectomarinus</em></td>
<td>24</td>
</tr>
<tr>
<td><em>Pseudomonas denitrificans</em></td>
<td>20</td>
</tr>
<tr>
<td><em>Pseudomonas lemoineii</em></td>
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</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>11</td>
</tr>
<tr>
<td><em>Pseudomonas chlororaphis</em></td>
<td></td>
</tr>
<tr>
<td>B561*</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas pickettii</em></td>
<td>9</td>
</tr>
<tr>
<td><em>Paracoccus denitrificans</em></td>
<td>31</td>
</tr>
<tr>
<td><em>Paracoccus halodenitrificans</em></td>
<td>M. R. Betlach and L. Hochstein*</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>30</td>
</tr>
<tr>
<td>Agrobacterium radiobacter</td>
<td>30</td>
</tr>
<tr>
<td>Bacillus azotoformans</td>
<td>28</td>
</tr>
<tr>
<td>Bacillus starothermophilus</td>
<td>8</td>
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<tr>
<td>Flavobacterium sp.</td>
<td>29</td>
</tr>
<tr>
<td>Alcaligenes faecalis</td>
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</tr>
<tr>
<td>Vibrio succinogenes*</td>
<td>41</td>
</tr>
<tr>
<td>Azospirillum brasilense*</td>
<td>21, 22</td>
</tr>
<tr>
<td>Thiobacillus denitrificans</td>
<td>14</td>
</tr>
<tr>
<td>Nitrous oxide nonutilizing*</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td></td>
</tr>
<tr>
<td>PJ188*</td>
<td>11</td>
</tr>
<tr>
<td><em>Pseudomonas chlororaphis</em></td>
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<tr>
<td><em>Pseudomonas aureofaciens</em></td>
<td>7</td>
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<tr>
<td><em>Azospirillum lipoferum</em></td>
<td>21, 22</td>
</tr>
<tr>
<td><em>Azospirillum iersonii</em></td>
<td>E. J. Hansen*</td>
</tr>
<tr>
<td><em>Corynebacterium nephridi</em></td>
<td>12, 34</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>4, 37</td>
</tr>
</tbody>
</table>

* Can grow with nitrous oxide as sole electron acceptor and produce dinitrogen gas from nitrate or nitrite.
* Reclassified as *Alcaligenes* (2).
* N₂ produced from NO₃⁻; N₂O growth not established.
* Now assigned to *Pseudomonas fluorescens* bio-type D (36).
* Personal communication.
* An exception: N₂ is produced from N₂O but not from NO₃⁻ or NO₂⁻.
* Formerly *Spirillum lipoferum* (17).
* Cannot grow on nitrous oxide. All of these species produce only nitrous oxide from nitrate or nitrite except *Pseudomonas aeruginosa*, which also produces dinitrogen gas.
* Now assigned to *Pseudomonas fluorescens* bio-type E (36).
* Now provisionally classified as *Achromobacter* (27).

Delwiche showed, acetylene inhibition of nitrous oxide reduction decreased the cell yield from nitrate of *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* to the same extent (B. A. Bryan, Ph.D. thesis, University of California, Davis, 1980).

We initiated this comparative study as an aid in choosing an organism suitable for use in physiological genetic investigations on denitrification. Our aim was to select an organism that was typical of those carrying out complete denitrification, easily manipulated in culture, and capable of genetic exchange. Even from the comparison of only three organisms, it was clear that the level of variation among their patterns of denitrification was too great for any of them to be considered typical. *Pseudomonas aeruginosa* might still be considered desirable because it has a well-characterized genetic system (13, 35), but its inability to grow anaerobically with exogenous N₂O argues persuasively against it. Mutants blocked in the last step of the pathway would be difficult to select or score. We chose *Pseudomonas stutzeri* for our further studies. It denitrifies actively, is easy to manipulate in culture for physiological and genetic experiments, and, as we found recently, has an effective natural transformation system for the exchange of genetic information between cells (4a).

ACKNOWLEDGMENTS

This work was supported in part by National Science Foundation grant AER77-07301 and by U.S. Department of Agriculture grant 59-2063-1-1657-0. We thank JoAnne J. Rosen for able technical assistance.

LITERATURE CITED


