NOTES

Inability of Pseudomonas stutzeri Denitrification Mutants with the Phenotype of Pseudomonas aeruginosa to Grow in Nitrous Oxide

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Pseudomonas aeruginosa PAO1 reduced nitrous oxide to dinitrogen but did not grow anaerobically in nitrous oxide. Two transposon insertion Nos" mutants of Pseudomonas stutzeri exhibited the P. aeruginosa phenotype. Growth yield studies demonstrated that nitrous oxide produced in vivo was productively respired, but nitrous oxide supplied exogenously was not. The defect may be in electron transport or in nitrous oxide uptake.

The ability to denitrify characterizes a heterogeneous group of bacteria of which Pseudomonas aeruginosa PAO1 is the best understood strain genetically (2). Unique among 25 denitrifying strains, it fails to grow anaerobically in nitrous oxide even though it slowly reduces nitrous oxide to dinitrogen (3, 4, 7). This report demonstrates that the phenotype can be mimicked by genetic mutants of Pseudomonas stutzeri, which normally grows vigorously in nitrous oxide (1, 8).

P. aeruginosa PAO1 grew rapidly aerobically (generation time [g] = 0.80 h) and anaerobically (with nitrate as the terminal electron acceptor, g = 1.4 h) in complex Luria-Bertani (LB) medium (4) at 37°C. P. stutzeri JM300 is a prototroph isolated from an anaerobic enrichment culture on succinate and nitrous oxide. It grew more slowly aerobically coupled with ATP generation. Growth yield studies were performed with the respiring Pseudomonas strains, wherein cell yield is proportional to net energy production when the terminal electron acceptor is limited.

Growth yield studies used a minimal succinate medium consisting of 40 mM Na2C4H4O4, 20 mM NH4Cl, 2.5 mM K2HPO4, 2.5 mM KH2PO4, 0.8 mM MgSO4 · 7H2O, 0.10% (vol/vol) trace mineral solution [250 mg of EDTA, 500 mg of FeSO4 · 7H2O, 154 mg of MnSO4 · H2O, 10 mg of CuSO4 · 5H2O, 24.5 mg of Co(NO3)2 · 6H2O, 17.7 mg of Na2B4O7 · 10H2O, water to 100 ml], and an electron acceptor as specified (pH 6.8). Cells were grown anaerobically in 100 mM sodium nitrate and washed, and about 2 × 108 were used to inoculate sealed 50-ml serum bottles containing 40 ml of medium supplemented with up to 20 mM sodium nitrate or one of the other electron acceptors. Sodium nitrite (up to 20 mM) was supplied in increments by syringe during incubation to avoid the toxic effects of high initial concentrations. Various final concentrations of oxygen and nitrous oxide up to 20 mM were injected into sealed vials containing cells in unsupplemented medium. We did not determine the yield from nitric oxide. Bottles were shaken at 30°C for 18 h. Dry weights were estimated from optical densities at 660 nm: 1.0 = 0.58 mg/ml from a standard curve. At least five concentrations of each electron acceptor were tested, with four replicates at each concentration. Cell yields were completely

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Electrons transferred/mol</th>
<th>P. stutzeri yield (g [dry wt])</th>
<th>P. aeruginosa yield (g [dry wt])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per mol of electron acceptor</td>
<td>Per electron pair</td>
</tr>
<tr>
<td>O2</td>
<td>4</td>
<td>9.6</td>
<td>14.8</td>
</tr>
<tr>
<td>NO3</td>
<td>5</td>
<td>12.6</td>
<td>5.0</td>
</tr>
<tr>
<td>NO2</td>
<td>3</td>
<td>8.0</td>
<td>5.3</td>
</tr>
<tr>
<td>N2O</td>
<td>2</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td>NO3 + C2H2</td>
<td>4</td>
<td>10.5</td>
<td>5.3</td>
</tr>
<tr>
<td>N2O + C2H2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* ND, Not determined.

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TABLE 2. Denitrification by P. stutzeri mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Generation time (h) with electron acceptor:</th>
<th>Resting cell N₂ production (nmol/min per mg [dry wt]) with electron acceptor:</th>
<th>Cell yield (g [dry wt/molI] with electron acceptor:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂</td>
<td>NO₂⁻</td>
<td>NO₃⁻</td>
</tr>
<tr>
<td>JM354</td>
<td>1.25</td>
<td>2.51</td>
<td>2.81</td>
</tr>
<tr>
<td>JM423</td>
<td>1.38</td>
<td>2.48</td>
<td>2.56</td>
</tr>
<tr>
<td>JM425</td>
<td>1.43</td>
<td>2.49</td>
<td>2.74</td>
</tr>
</tbody>
</table>

proportional to concentration (linear regression coefficients = 0.98 ± 0.01).

The cell yield of P. stutzeri depended on the electron acceptor (Table 1). It was highest with oxygen and decreased anaerobically, with the nitrogen oxides representing successive denitrification intermediates. The yields on nitrate, nitrite, and nitrous oxide corresponded to the ratio 5.0:3.2:2.0, closely reflecting the total nitrogen valence of the three electron acceptors. Thus, the three reductive steps were equivalent (i.e., nitrous oxide reduction supplied as much energy for net cell production as did the reduction of nitrate to nitrite or of nitrite to nitrous oxide). Acetylene, which blocks nitrous oxide reduction (9), abolished nitrous oxide-dependent growth of P. stutzeri (Table 1). Cell yield from nitrate with acetylene decreased by almost 20%, as expected for the transfer of four, rather than five, electrons per mole of nitrate reduced; the yield per electron pair was unchanged. Nitrous oxide accumulated under these conditions, so acetylene was inhibiting nitrous oxide reduction rather than uncoupling it.

In P. aeruginosa, also, oxygen respiration was more efficient than anaerobic nitrate respiration (Table 1). Growth yields on nitrite were not determined, because the later steps of denitrification in P. aeruginosa are differentially inhibited by nitrite (4). If nitrous oxide reduction in P. aeruginosa is unproductive, one would expect a 20% decrease in cell yield from nitrate, compared with that of P. stutzeri, and would not expect acetylene to have any effect. Yield from nitrate was the same in both species, and acetylene decreased that yield to the same extent (Table 1). P. aeruginosa must derive energy from this denitrification step.

Lack of growth in exogenous nitrous oxide was apparently not due to any inhibitory effect at the concentrations supplied. When growth-limiting concentrations of nitrate were supplemented with 25 mM nitrous oxide, there was no decrease or increase in cell yield, although dinitrogen gas production increased, consistent with accelerated nitrous oxide reduction (data not shown).

We began a study of the physiological genetics of denitrification by using mutants of P. stutzeri primarily. Plasmid pAS8 rep-l::Tn7, carried in Escherichia coli, is effective in mutagenesis because it transfers to many other gram-negative species without stably replicating in them; transposition of Tn7 to the chromosome of recipients is detectable through the antibiotic resistances conferred (6). We used P. stutzeri JM354 (i.e., rifampin-resistant JM300) as a recipient and selected for aerobic growth on LB agar containing 20 μg of rifampin and 100 μg of streptomycin per ml; colonies arose at a frequency of 10⁻² per recipient. Putative mutants were scored for absence of the plasmid by testing sensitivity to kanamycin (20 μg/ml) and resistance to bacteriophage φPRD-1 (5). A number of auxotrophs and several mutants defective in denitrification were isolated. Two of the latter were unable to form colonies on LB agar plates incubated anaerobically in nitrous oxide but could grow anaerobically on nitrate or nitrite. We termed this class Nos⁺ and designated the two strains JM423 and JM425.

For growth and denitrification studies, these strains were grown in LB broth with limiting nitrate and nitrite and used to inoculate unsupplemented LB broth in sealed 70-ml serum bottles. After repeated evacuation and regassing with helium, solutions of sodium nitrate, sodium nitrite, or nitrous oxide were injected, the latter as a saturated solution (45 mM) in LB broth held at 9°C to increase gas solubility. The bottles were then incubated unshaken at 37°C for 24 to 48 h. Samples of headspace gas or culture broth were removed at intervals by syringe for assay of denitrification intermediates (4). The two mutant strains grew as well as did parental strain JM354 in oxygen, nitrate, or nitrite (Table 2), although slightly more slowly than did wild-type JM300. Dinitrogen production by washed and concentrated resting cells grown on 33 mM nitrate, 36 mM nitrite, or 18 mM nitrous oxide was compared (Table 2). The rates varied with the electron acceptor and from strain to strain; nevertheless, the mutants readily reduced all three acceptors. Nitrous oxide did not accumulate during reduction of nitrate or nitrite in any of the strains.

Finally, we determined the anaerobic growth yield of cultures on limited nitrite with or without acetylene (Table 2). Higher yields than those shown in Table 1 resulted from incubation at a higher temperature in nutrient-rich LB medium. The yield of strain JM354 was proportional to nitrite concentrations up to 32 mM and was almost identical to that of JM300 under these conditions (23.0 g [dry weight]/mol). Acetylene decreased the growth yield by about one-third as expected for transfer of two, rather than three, electrons (Table 2). Mutant yields were slightly depressed compared with those of JM354, but the effects of acetylene were comparable. Thus, the mutants derived as much ATP from growth on nitrite as did JM354, consistent with nitrous oxide reduction still being coupled with oxidative phosphorylation.

Each of the Nos⁻ mutants of P. stutzeri was phenotypically similar to wild-type P. aeruginosa PAO1. Each mutation was a single-step event caused by Tn7; when Nos⁺ P. stutzeri was transformed by the portion of the chromosome carrying the transposon, it became Nos⁻ and streptomycin resistant simultaneously (data not shown). Possibly, a similar Nos⁻ defect arose in an ancestral strain of P. aeruginosa that grew in nitrous oxide. The function of the affected gene is not obvious. It is not a structural or regulatory gene for nitrous oxide reductase, or activity would have been absent. Since a primary feature of the phenotype is the distinction between exogenously supplied nitrous oxide and nitrous oxide generated internally by nitrite reduction, the affected gene product may be a protein involved in either the uptake of nitrous oxide or in the coupling of its reduction to productive ATP generation. Mutants that can reduce, but not grow in, nitrous oxide have recently been isolated from among Pseudomonas perfectomarina (10).
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LITERATURE CITED
ERRATA

Effects of Low-Temperature Acclimation and Oxygen Stress on Tocopherol Production in *Euglena gracilis* Z

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Volume 50, no. 6, p. 1406 and 1407, Fig. 1 and 2: figures are incorrectly placed and should be transposed to match their legends.

Inability of *Pseudomonas stutzeri* Denitrification Mutants with the Phenotype of *Pseudomonas aeruginosa* to Grow in Nitrous Oxide

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Volume 50, no. 5, p. 1301, Table 1, column 3, line 1: "9.6" should read "29.6."