A NosA-specific Bacteriophage Can Be Used to Select Denitrification-defective Mutants of *Pseudomonas stutzeri*

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ϕ PS5, a double-stranded DNA bacteriophage of *Pseudomonas stutzeri* JM604 that adsorbs specifically to the outer-membrane protein NosA, was isolated from stagnant irrigation ditch water. Mutant strains that do not produce NosA are resistant to ϕ PS5 and cannot grow anaerobically with N_2O as the sole electron acceptor. ϕ PS5 did not adsorb to *nosA* mutants and adsorption to the wild-type strain was reduced when cells were grown with a high concentration of copper, a condition that represses the synthesis of NosA. The isolation of spontaneous ϕ PS5-resistant mutants yielded strains that were clearly defective in growth on N_2O at about a 10% incidence. About half of these strains could respire N_2O when supplied with a high concentration of exogenous copper.

INTRODUCTION

Biological denitrification is the reduction of nitrate or nitrite to gaseous products, either nitrous oxide (N_2O) or dinitrogen (N_2). In *Pseudomonas stutzeri* JM300, the final step in the pathway, the reduction of N_2O to N_2 , requires NosA, a 65 000- M_r copper-containing protein located in the outer membrane (Mokhele *et al.*, 1987; Lee *et al.*, 1989). Some *nosA* strains were isolated using frameshift mutagenesis (ICR-191; Polysciences Inc.) and counterselection for an inability to grow anaerobically with N_2O as the only electron acceptor (Mokhele *et al.*, 1987); i.e. these mutants selected as being denitrification-defective lacked the outer-membrane protein NosA. In order to explore further the relationship between defects in NosA and denitrification, we sought a method of selecting directly for mutations in *nosA* using phage resistance. Outer-membrane proteins act as phage receptors in *Escherichia coli* (Datta *et al.*, 1977) and *Salmonella typhimurium* (Nurminen *et al.*, 1976). Although detectable levels of NosA are present in the outer membrane of *P. stutzeri* only when cultures are grown anaerobically in an environment with a low concentration of copper, it seemed possible that NosA might be a receptor for some phage. Such a phage could be used to isolate phage-resistant, and thereby NosA-defective, mutants. Here we report the isolation of a NosA-specific phage and its use in selecting phage-resistant mutants defective in N_2O respiration (Nos⁻).

METHODS

Bacteria and media. The *Pseudomonas* strains used in this study are listed in Table 1. LT medium was modified Luria-Bertani broth (Carlson *et al.*, 1983) supplemented with trace minerals solution lacking added copper (1.0 ml l⁻¹) (Mokhele *et al.*, 1987); by atomic absorption spectroscopy, this medium was found to contain 1.0 μ M-copper. LT-nitrate medium was LT containing 40 mM- $NaNO_3$. Plate and overlay media were solidified with 1.5% (w/v) and 0.75% (w/v) agar, respectively. The overlay medium was LT-nitrate supplemented with $CaCl_2$ (10 mM) and $MgCl_2$ (10 mM). LT with $CaCl_2$ (10 mM) and $MgCl_2$ (10 mM) (LTCM) was used to dilute phage suspensions. Phosphate-buffered saline (pH 7.2) contained 1.27 g Na_2HPO_4 , 0.41 g KH_2PO_4 and 7.36 g $NaCl$ l⁻¹.

Growth of bacteria. Host bacteria were grown overnight in LT-nitrate. Unless indicated otherwise, all incubations were stationary at 30 °C and all plate cultures were incubated anaerobically in an H_2/CO_2 atmosphere (Oxoid) for 24 h.

Table 1. *Bacterial strains and ϕ PS5 host range*

Species	Strain	Genotype or phenotype	Source or reference	Lysis pattern*
<i>Pseudomonas aeruginosa</i>	PAO1	Wild-type	T. C. Hollocher, Branders Univ., Waltham, MA	—
<i>Pseudomonas alcaligenes</i>	12815			—
<i>Pseudomonas mendocina</i>	25413			—
<i>Pseudomonas pseudoalcaligenes</i>	17440			—
<i>Pseudomonas stutzeri</i>	JM299			—
	JM299	Rough	B. A. Bryan & C. C. Delwiche, Univ. of California, Davis	—
	JM300	Smooth variant	Carlson <i>et al.</i> (1983)	+
	JM604	<i>nal-7</i>	Mokhele <i>et al.</i> (1987)	+
	JM753	<i>nal-7 nosA14</i>		T
	JM757	<i>nal-7 nosA16</i>		T
	JM764	<i>nal-7 nosA19</i>		—
	JM766	<i>nal-7 nosA21</i>		—
	JM769	<i>nal-7 nosA22</i>		—
	JM790	<i>nal-7 nosA35</i>		—
	11607	Wild-type	ATCC	—
	17587			—
	17682			—
	17832			—
	ZoBell			—
			Döhler <i>et al.</i> (1987)	—

* +, Approximately 5000 clear plaques (semiconfluent lysis); T, 100–200 very turbid plaques; —, no plaques.

Isolation of phage. The method was based on those of Rao & Labzoffsky (1969) and Primrose & Day (1977). Samples (400 ml) of stagnant irrigation ditch water collected in Yolo County, California were incubated at room temperature and inoculated each day with 20 ml of an overnight culture of JM604. After 4 d samples were taken; cells and large particulate matter were removed by centrifugation (6000 g, 15 min). The supernatant was further clarified by filtration through an AP20 prefilter (Millipore) that had been washed with Tween 80 (0.1%, w/v) and water to prevent phage adsorption. The filtrate was then passed through a 47 mm HA membrane filter with 0.45 μ m pores (Millipore). The adsorbed phages were eluted from the filter by shaking (60 r.p.m.) for 1 h in 4.0 ml of 5 $\times$ nutrient broth (Difco) in 0.05 M-bicarbonate buffer (pH 9.0) (Hill *et al.*, 1972). Chloroform was added to the phage-containing broth and plaques of different size and morphology were purified (Adams, 1959).

Titration of phage. Phage preparations were assayed by the agar overlay technique (Adams, 1959).

Preparation of high-titre lysates. Lysates were prepared by the method of Gliesche *et al.* (1988). The lysates were centrifuged (5000 g, 15 min) to remove whole cells and debris, and stored over chloroform at 4 °C. Phage stocks routinely contained approximately 10^{10} p.f.u. ml⁻¹.

Electron microscopy. ϕ PS5 was sedimented (110000 g, 3 h), washed, and resuspended in phosphate-buffered saline. One drop of the concentrated phage suspension (about 5×10^{11} p.f.u. ml⁻¹) was applied to a Formvar-coated copper grid; after 3 min, excess sample was removed with a Kimwipe. Similarly, the phage particles were negatively stained with 2% (w/v) ammonium molybdate and washed with distilled water. Specimens were observed in a Zeiss EM109 electron microscope.

Host range. Different phage isolates were screened for infecting JM604 by spotting 1 μ l of a preparation containing 10^8 to 10^{10} p.f.u. ml⁻¹ onto an overlay containing JM604 and examining for lysis following incubation. The host range of ϕ PS5 was determined by plating in an overlay the strain to be tested with the lowest ϕ PS5 dilution that gave semiconfluent lysis (approximately 5000 plaques per plate) on JM604.

Fluorescent staining of nucleic acid. The acridine orange fluorescent staining method of Bradley (1966) was used to determine the type and strandedness of the ϕ PS5 nucleic acid. The double-stranded DNA coliphage λ gt11 (Promega) was employed as a control. The phage suspensions were incubated with 30 μ g DNAase I and RNAase A ml⁻¹ at 37 °C for 1 h. Following two cycles of high-(110000 g, 3 h) and low-speed (5000 g, 15 min) centrifugation, the phage particles were resuspended in phosphate-buffered saline to give preparations containing approximately 10^{12} p.f.u. ml⁻¹. Droplets were dried onto microscope slides for staining and other treatments.

Molybdc acid was used after the Na₂HPO₄ soak. The results were confirmed by testing the sensitivities to DNAase and RNAase.

One-step growth experiment. The method of Eisenstark (1967) was used. ϕ PS5 was added to JM604 at an m.o.i. of 0.1. Phage antiserum was not used and withdrawn samples were treated with chloroform rather than being centrifuged.

Adsorption kinetics. An overnight culture was diluted 1:50 in LT-nitrate and incubated to early stationary phase. Cultures were harvested by centrifugation (6000 g, 15 min) and suspended in LTCM containing 200 μ g chloramphenicol ml⁻¹ to give a cell density of 10⁹ c.f.u. ml⁻¹. The chloramphenicol was included in the adsorption mixture to prevent NosA induction and phage synthesis; it did not interfere with adsorption. ϕ PS5 was added at an m.o.i. of 0.1 and the infected cultures were agitated (60 r.p.m.) at 30 °C. Samples were removed at intervals, diluted 1:100 in cold LTCM containing chloroform, and assayed for unadsorbed phage.

Isolation of spontaneous ϕ PS5-resistant mutants. JM604 and ϕ PS5 were combined at an m.o.i. of 10–20 and incubated for 10 min to allow adsorption. The mixture was plated and incubated for 48 h. The resulting colonies were purified aerobically on LT plates.

RESULTS

Isolation and characterization of a NosA-specific phage

Since *P. stutzeri* is found in soil and water (Palleroni *et al.*, 1970), stagnant irrigation ditch water seemed to be a probable source of phages that infect it. Ten water samples were enriched for *P. stutzeri* phages by inoculation with a broth culture of JM604. Out of 53 phages collected, one isolate, ϕ PS5, formed plaques on JM604, but not on *nosA* derivatives of JM604 (JM753, JM757, JM764, JM766, JM769 and JM790) (Table 1). This suggested that NosA acted as a receptor for ϕ PS5 and that the property of phage resistance resulted from a defect in NosA. Two-dimensional gel electrophoresis had shown that these *nosA* strains did not produce detectable quantities of NosA (Mokhele *et al.*, 1987).

Fluorescent staining with acridine orange indicated that ϕ PS5 contained double-stranded DNA, because the diagnostic green colour did not fade when treated with molybdc acid, and was eliminated by DNAase, but not RNAase, digestion.

The host range of ϕ PS5 was quite limited (Table 1). As expected, it lysed JM604, the strain used for enrichment. JM300, the parent of JM604, was also lysed. Yet the wild-type rough strain, JM299, from which JM300 was derived was immune to infection. Apparently, the large quantities of polysaccharide surrounding the JM299 cells prevented phage adsorption. ϕ PS5 did not infect five other *P. stutzeri* strains, notably strain ZoBell, the organism employed by W. G. Zumft and coworkers (Matsubara *et al.*, 1982; Coyle *et al.*, 1985; Riester *et al.*, 1989). Recent experiments showed that NosA antiserum does not react with extracts from strain ZoBell (Lee *et al.*, 1989). ϕ PS5 did not lyse any of the other four *Pseudomonas* species that belong to the same DNA homology group as *P. stutzeri* (Palleroni *et al.*, 1973).

ϕ PS5 formed clear, small (1 mm) plaques. The particle morphology of ϕ PS5 resembled that of a Bradley type C phage (Bradley, 1967) (Fig. 1). ϕ PS5 had a hexagonal head approximately 40 nm in diameter and a short noncontractile tail.

One-step growth experiments with ϕ PS5 using early stationary phase cells of JM604 indicated a latent period of about 30 min followed by a burst of three phages per cell; then the phage titre continued to rise without reaching a plateau. The low burst size is not unusual for *Pseudomonas* phages (Sutton, 1966; Kelln & Warren, 1971).

Adsorption experiments

To support the hypothesis that ϕ PS5 adsorbs to NosA, we sought to show that ϕ PS5 does not adsorb to cells lacking the NosA receptor and that NosA-repressing conditions reduce ϕ PS5 adsorption to wild-type cells (Fig. 2). Very few, if any ϕ PS5 particles adsorbed to the *nosA* strain JM769, nor to another *nosA* mutant JM753 (data not shown). Thus, the inability of ϕ PS5 to form normal plaques on *nosA* mutants (Table 1) is a consequence of ϕ PS5 being unable to adsorb to these strains, probably because NosA is the phage receptor. If so, strains grown in the presence of elevated concentrations (i.e. greater than 10 μ M) of exogenous copper, which represses the synthesis of NosA (Lee *et al.*, 1989), might be expected to be poor phage-adsorbers. Indeed, the phage adsorption rate (Adams, 1959) was maximum for JM604 cells grown with low copper

Fig. 1. Electron micrograph of ϕ PS5 stained with ammonium molybdate. Bar, 10 nm.

(1.0 μ M) (Fig. 2), and 35% lower (for the first 10 min of infection) for cells grown with a high copper concentration (200 μ M). This reduced level of adsorption is not sufficient to interfere with plaque formation; a high copper concentration (400 μ M) in solid media did not decrease the number or size of plaques formed. ϕ PS5 adsorption to aerobically grown cells could not be tested easily, because of the difficulty of maintaining fully aerobic conditions in full-grown cultures, the growth phase in which anaerobically grown cultures were tested, because they contain maximum levels of NosA.

Isolation and characterization of ϕ PS5-resistant mutants defective in N_2O respiration

Chemical mutagens were not used because the frequency of spontaneous ϕ PS5 resistance was high (10^{-6}). Following purification to remove contaminating ϕ PS5-sensitive (and presumably Nos⁺) cells, 500 putative ϕ PS5-resistant isolates were scored for anaerobic growth with N_2O on LT plates containing either low (1.4 μ M) or high (300 μ M) copper. Exogenous copper is essential for N_2O respiration (Matsubara *et al.*, 1982) because N_2O reductase contains copper (Coyle *et al.*, 1985). Thus, the copper concentration of the LT-nitrate medium was enriched slightly (to 1.4 μ M) to improve N_2O -dependent growth. Approximately 10% of the ϕ PS5-resistant strains were clearly defective in growth with N_2O (Nos⁻). We presume that the remaining (90%) ϕ PS5-

Fig. 2. Adsorption of ϕ PS5 to JM604 (■) and JM769 (▲) grown with a low concentration of copper ($1.0\ \mu\text{M}$), and to JM604 (□) grown with a high concentration of copper ($200\ \mu\text{M}$).

Table 2. Phenotypic characterization of ϕ PS5-resistant mutants defective in N_2O respiration

Strain or mutant class	Growth with N_2O *		No. of isolates
	Low CuSO_4 ($1.4\ \mu\text{M}$)	High CuSO_4 ($300\ \mu\text{M}$)	
JM604 (wild-type)	+	+	
JM753 (<i>nosA</i>)	—	—	
I	—	—	15
II	—	+	6
III	w	w	11
IV	w	+	19

* —, No growth; +, good growth; w, weak growth.

resistant clones had defects in *NosA* that impaired receptor function but did not alter the *NosA* activity sufficiently to prevent growth on N_2O . The ϕ PS5-resistant, *Nos*⁻ mutants fell into four phenotypic classes (Table 2). In a low copper medium ($1.4\ \mu\text{M}$), classes I and II did not grow with N_2O ; classes III and IV grew weakly. When the copper concentration was increased to $300\ \mu\text{M}$, classes II and IV grew like the wild-type. High copper was not remedial for frameshift *nosA* mutants (e.g. JM753).

DISCUSSION

Ten *nosA* mutants were isolated previously using frameshift mutagenesis (Mokhele *et al.*, 1987). These strains lacked *NosA* and produced an inactive, copper-free N_2O reductase. These results suggested that *NosA* was necessary for copper to enter N_2O reductase. Yet concentrations of exogenous copper up to toxic levels did not permit these *nosA* strains to grow anaerobically with N_2O as the sole electron acceptor. We concluded that *NosA* did not function exclusively to take copper into the cell; rather, it participated in the insertion of copper into N_2O reductase. But, since frameshift mutations can exert polarity effects on downstream genes, it is possible that other proteins not expressed in frameshift mutants may participate in the pathway through which exogenous copper is inserted into N_2O reductase. Some of these proteins might be required only with low concentrations of exogenous copper; others might be required at all concentrations. This suspicion was strengthened when we recently found that *NosA* is a channel-forming protein (Lee *et al.*, 1989). *NosA* may function as a copper channel when the level of exogenous copper is low (i.e. less than $10\ \mu\text{M}$). When the copper concentration is high,

copper may enter the cell by a NosA-independent route and may be inserted into N₂O reductase by a different protein. Thus, it became important to be able to select directly for *nosA* mutants and to determine their effect on N₂O reductase activity at various concentrations of exogenous copper. We chose to select for NosA-defective strains by isolating strains that were resistant to a NosA-specific phage. Such a phage, ϕ PS5, was isolated from stagnant irrigation ditch water. Curiously, this phage can infect its host only when it is growing under the special conditions that allow NosA to be expressed – anaerobiosis and a low concentration of exogenous copper.

The host range of ϕ PS5 is limited to *P. stutzeri* JM300 and its derivatives, including JM604. The particle morphology was similar to that of the *Pseudomonas* phage gh-1 (Liss *et al.*, 1981).

The properties of mutant strains that are resistant to ϕ PS5 seem to support the hypothesis that other proteins are needed to insert copper into N₂O reductase. Out of 51 phage-resistant strains that were defective in N₂O reduction (Table 2), about half (classes II and IV) regained their ability to reduce N₂O when high concentrations of exogenous copper were made available to them. We are now investigating other proteins in the copper pathway.

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