JOURNAL OF BACTERIOLOGY, Oct. 1987, p. 4577–4580
0021-9193/87/104577-04$02.00/0

Vol. 169, No. 10

Physical Mapping of Transposon Tn*5* Insertions Defines a Gene Cluster Functional in Nitrous Oxide Respiration by *Pseudomonas stutzeri*

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Received 30 March 1987/Accepted 24 June 1987

By transposon Tn*5* mutagenesis, 19 strains of *Pseudomonas stutzeri* were acquired that had defects in nitrous oxide respiration (Nos$^-$ phenotype). A physical map of the mutants showed nearly random Tn*5* insertions into genomic DNA within a single region ca. 8 kilobases long. Mutants were characterized immunochemically, enzymatically, and chemically. Several functions related to the synthesis and regulation of nitrous oxide reductase were associated with this DNA region, indicating that in *P. stutzeri* part of the genetic information necessary to respire nitrous oxide is clustered.

Denitrification is the major biological process for nitrous oxide evolution and reduction (11). N_2O interacts with a copper protein as the terminal oxidoreductase of a respiratory chain (5) and thus may be used by a bacterium as an electron acceptor for respiration and to sustain growth. The ability to do so is limited by various factors; among them is an apparent instability of the genetic information for N_2O utilization in the denitrifying bacteria (10), and sometimes the restricted ability of a bacterium to utilize exogenous N_2O though being a denitrifier (4). Another prerequisite for N_2O respiration is the initiation and continuing expression of the N_2O-reducing system during the transition from aerobic respiration to denitrification. No information addressing these problems on a genetic level is currently available.

We have previously adapted a transposon Tn*5* mobilization system (15) for insertional mutagenesis of *Pseudomonas perfectomarina* (16) to approach these questions. Tn*5* was chosen because of its low insertional specificity and because transposons leave the mutagenized DNA region amenable to identification. Nineteen Tn*5* insertions were physically mapped and found to be clustered within two contiguous *Eco*RI fragments. Immunochemical, enzymatic, and chemical analysis of these mutants suggested the presence of several functions for N_2O utilization within this region. These functions were closely related to the expression and biosynthesis of N_2O reductase.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. perfectomarina* ATCC 14405, used in this study, was recently transferred as strain ZoBell to *Pseudomonas stutzeri* (7). The isolation of Tn*5* insertion mutants from this bacterium has been described (16). Mutants with the Nos$^-$ phenotype were designated MK401, MK402, and so forth and correspond to strains previously labeled KD1, KD2, etc. Other bacterial strains used were *Escherichia coli* HB101 (3) and *E. coli* BHB2688 and BHB2690 (9). As cloning vectors we used plasmid pBR325 (2) and the cosmid vector pJA1, which is a derivative of pBR322 with two *cos* sites (12).

Media and growth conditions. For recombinant DNA techniques, *E. coli* and *P. stutzeri* were grown in LB medium containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. For solid medium 1.5% agar (Difco Laboratories, Detroit, Mich.) was added. Cultures were incubated for 16 h at 37°C on a rotary shaker (150 rpm). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml. For biochemical assays *P. stutzeri* was grown with asparagine in a synthetic medium (16).

Isolation of DNA and *Mbo*I digestion. Genomic, high-M_r DNA from *P. stutzeri* Tn*5* mutants was isolated with minor modifications of a published method (14). Cells of a 5-ml overnight culture of *P. stutzeri* (LB medium with kanamycin) were washed once with cold 1 M NaCl and once with cold 10 mM Tris hydrochloride, pH 8.0 (150 mM in NaCl). The cells were suspended in 2 ml of TE buffer (10 mM Tris hydrochloride, pH 8.0, 25 mM EDTA). After addition of 200 μl of a lysozyme solution (2 mg of lysozyme per ml of TE buffer), the suspension was incubated for 15 min at 37°C, and 240 μl of TE buffer containing 10% Sarkosyl and proteinase K (1 mg/ml) was added. The mixture was incubated for 60 min at 37°C. The lysed cells were extracted three times with an equal volume each of phenol (saturated with 100 mM Tris hydrochloride, pH 8.0) and chloroform-isoamyl alcohol (24:1, vol/vol), and once with an equal volume of the chloroform-isoamyl alcohol mixture. The aqueous phase was made 0.3 M in ammonium acetate before 0.54 volume of isopropanol was added. After 30 min of incubation at room temperature, precipitated DNA was recovered by centrifugation. The DNA pellet was washed once with 70% ethanol and dissolved in the cold overnight in 150 μl of Tris hydrochloride, pH 8.0, 0.5 mM in EDTA and 10 mM in NaCl.

Isolated DNA was digested partially with restriction endonuclease *Mbo*I. About 20 to 30 μg of DNA was digested with various dilutions of the enzyme for 60 min at 37°C in a volume of 150 μl. The extent of digestion was monitored by electrophoresis on a 0.3% agarose gel. DNA digestion with 0.04 U of *Mbo*I gave the maximal fluorescence intensity of DNA fragments in the desired size range of 30 to 50 kilobases (kb) on an ethidium bromide-stained gel. Digestion was stopped by one cycle of the above extraction procedure with phenol and chloroform-isoamyl alcohol.

DNA was recovered by ethanol precipitation, dissolved at a concentration of about 200 μg/ml, and dephosphorylated for 30 min at 37°C with calf intestinal alkaline phosphatase (0.02 U/μg of DNA). After a second addition of the enzyme,

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TABLE 1. Characteristics of Tn*5*-induced mutants of *P. stutzeri* with Nos$^-$ phenotype[a]

Strain(s)	Group	Activity[b]		Immunoassay[c]	M_r-70,000 polypeptide[d]	Copper assay[e]
		In vivo	In vitro			
Wild type		+	+	+	+	+
MK408, MK411, MK415, MK419	IIa	−	−	−	−	−
MK403, MK406, MK409, MK413, MK418	IIb	−	−	Tr	ND[f]	ND
MK410, MK414	III	−	(+)	(+)	(+)	(+)
MK401, MK402, MK404, MK405, MK407, MK412, MK416, MK417	I	−	−	+	+	−

[a] Representative numerical data for the wild-type assays denoted by + are given in reference 16. For other strains symbols indicate from 0% [−] to 5 to 15% [(+)] of the wild-type measurements.
[b] In vivo, asparagine-dependent N_2O reduction assayed by gas chromatography; in vitro, N_2O-dependent oxidation of photochemically reduced benzyl viologen.
[c] Electroimmunoassay of cell extracts for N_2O reductase.
[d] SDS gradient polyacrylamide gel electrophoresis.
[e] Atomic absorption spectroscopy of fractions from gel filtration.
[f] ND, Below detection limit.

incubation was continued for another 30 min. Phosphatase was removed by extraction with the phenol-chloroform procedure. The dephosphorylated DNA was recovered by ethanol precipitation and used for cloning in the cosmid vector.

Cosmid cloning. The vector pJA1 was cut with *Hpa*I, dephosphorylated, and digested with *Bam*HI by standard procedures (13). This yielded two vector arms with phosphorylated *Bam*HI cloning sites. From 2 to 3 μg of insert DNA was mixed with 3 μg of vector arms. Ligation with T4 DNA ligase (1.2 U) in a volume of 20 μl was carried out overnight at 12°C. The in vitro packaging mixture was prepared from *E. coli* BHB2688 and BHB2690 (9). Four microliters of ligated DNA and 1 μl of 40 mM ATP were added to 20 μl of frozen packaging mixture. After incubation of 37°C for 60 min, 2 μl of 100 mM $MgCl_2$ and 1 μl of DNase (2 mg/ml) were added. Incubation was continued for 30 min. The reaction mixture was then diluted with 0.5 ml of TM buffer (50 mM Tris hydrochloride, pH 7.5, 10 mM in $MgSO_4$) and stored at 4°C over chloroform. For infection, *E. coli* HB101 was grown to late exponential phase in LB medium containing 0.4% maltose. The host bacteria (0.1 ml) were mixed with the same volume of TM buffer and 1 to 2 volumes of packed cosmids. After it had stood for 30 min at 30°C, 2 ml of LB medium was added, and incubation was continued under shaking for another 60 min at 37°C. The mixture was centrifuged, suspended in 0.1 ml of LB medium, and plated on LB agar containing ampicillin and kanamycin.

Subcloning procedure. Cosmid DNA was prepared by alkaline lysis (1) from a 2-ml overnight culture of transduced *E. coli* HB101. The DNA was completely digested with *Eco*RI. The digest was ligated at 12°C overnight with T4 DNA ligase to *Eco*RI-cleaved, dephosphorylated plasmid pBR325. The ligation mixture was used to transform *E. coli* HB101. Preparation of competent cells and transformation were done as described (6). Transformants were selected on LB plates supplemented with tetracycline and kanamycin. Dephosphorylation of DNA with calf intestinal alkaline phosphatase and ligation with T4 DNA ligase followed standard procedures (13).

Mapping of Tn*5* insertion sites. Plasmid DNA from transformants was prepared by alkaline lysis (1) and used for restriction enzyme digestions (13). Restriction maps were constructed by analysis of single- and double-digestion patterns of plasmids. Restriction endonuclease digests of DNA were analyzed on 1 to 2% agarose gels. λ DNA cleaved with *Hin*dIII or *Hin*dIII and *Eco*RI was used as a size standard.

Biochemical characterization of mutants. Mutants were tested in vivo for N_2O reduction by gas chromatography (8). The in vitro assay was based on photochemically reduced benzyl viologen as an electron donor (5). Material cross-reacting with anti-N_2O reductase rabbit antiserum was detected by double immunodiffusion. Quantitation of this material was done by electroimmunoassay. Size information for the cross-reacting material was obtained by sodium dodecyl sulfate (SDS) gradient electrophoresis after gel filtration of crude extracts on Sephacryl S-200. The conditions for these assays were given previously (16). Copper was determined by atomic absorption spectroscopy.

Biochemicals. Restriction endonucleases were purchased from Bethesda Research Laboratories (Eggenstein, Federal Republic of Germany [FRG]), Boehringer (Mannheim, FRG), and Pharmacia (Freiburg, FRG). T4 DNA ligase was obtained from GIBCO, and calf intestinal alkaline phosphatase was from Boehringer.

RESULTS AND DISCUSSION

Characterization of mutants. By Tn*5* mutagenesis, 19 mutants from 11 independent experiments were obtained (16). Mutants were selected for Km^r as an indicator of Tn*5* transposition and loss of growth under N_2O (Nos$^-$ phenotype). The methods to characterize these mutants have been described (16). This characterization was now extended to all mutants listed in Table 1, allowing better differentiation than had been possible previously. The assays were designed to detect N_2O-reducing activity of cell extracts, indicative of mutants with putative defects in electron donation, and to detect the presence and chemical nature of immunochemically cross-reacting material with an N_2O reductase-specific antiserum. If such material was found, it was tested for the M_r-70,000 subunit of the enzyme and for prosthetic copper.

In Table 1 are listed the Nos$^-$ mutants according to their common traits. A group of mutants, comprising strains MK408, MK411, MK415, and MK419, was negative in all tests and had apparently lost the ability to synthesize the copper enzyme. A second group of mutants, MK403, MK406, MK409, MK413, and MK418, showed identical characteristics except that the electroimmunoassay revealed traces of cross-reacting material. Because of its very low content, no further analysis was made. Two mutants, MK410 and MK414, expressed a low amount of enzyme with activity close to the detection limit. The largest group of

mutants, consisting of mutants MK401, MK402, MK404, MK405, MK407, MK412, MK416, and MK417, contained an amount of immunodetectable enzyme equal to that of *P. stutzeri* wild type. Metal analysis of these mutant proteins revealed the absence of prosthetic copper or only a low content. For instance, the enzyme isolated from mutants MK402 and MK407 (by the procedure described in reference 5), which were grown under the conditions of the wild type in a copper-supplemented medium, contained only about 2 atoms of Cu per unit of mass.

Although these biochemical phenotypes allow preliminary assignments of presumptive gene functions, they are equivocal, particularly with respect to mutants expressing no or only traces of the enzyme. This leaves it open whether regulatory functions or the structural gene of the enzyme is affected. Also, little can be deduced at this stage about the likely number of those functions. The site of Tn*5* insertion in Nos$^-$ mutants was therefore physically mapped.

Mapping Tn*5* insertion sites. Three strategies were used for cloning the mutagenized *Pseudomonas* DNA into *E. coli*: direct cloning of *Eco*RI-digested DNA into pBR325, construction of prime factors with plasmid R68.45, and cloning into the cosmid vector pJA1. The latter strategy proved best and was applied to all mutants. The procedure is outlined in Fig. 1. In each case high-M_r genomic DNA from a Tn*5* mutant strain of *P. stutzeri* was isolated. It was partially digested with *Mbo*I to a fragment length of about 35 to 50 kb and ligated with cosmid vector arms as described in the Materials and Methods section. Hybrid molecules were assembled in vitro into phage particles and used to infect *E. coli* HB101. Colonies were selected for Ap^r, mediated by the vector pJA1, and for Km^r, mediated by Tn*5*.

For mapping of Tn*5* insertion sites, *Eco*RI fragments of the cosmids were subcloned into the *Eco*RI site of vector pBR325. *E. coli* HB101 was used for transformation. Transformants that were Tc^r Ap^r, mediated by vector pBR325, and Km^r, mediated by Tn*5*, were isolated. Tn*5* insertion sites were mapped by single and double digestions with the restriction endonucleases indicated in Fig. 2A. Initially, from mutants MK403 and MK413 distinct *Eco*RI fragments, carrying Tn*5* and about 11 kb each, were obtained. These two fragments were shown to be contiguous by an overlapping *Hin*dIII fragment. A cosmid clone of mutant MK416 was digested with *Hin*dIII and subcloned into vector pBR325. One clone, pNS600, of several isolated subclones contained an insert of about 12 kb. Comparison of cleavage patterns with the restriction endonucleases *Cla*I, *Pst*I, *Sal*I, and *Sma*I proved that it overlapped the two *Eco*RI fragments.

The Tn*5* insertions of the other mutants were located without exception within these two fragments, thus defining a single region of Nos-specific functions related to the phenotypes listed in Table 1. The sites of Tn*5* insertion covered about 8 kb of the two *Eco*RI fragments, showing nearly random spacing and no bias towards the orientation of Tn*5*. The insertions in mutants MK405 and MK417 and in MK410 and MK414 mapped adjacent for each mutant pair but were still distinguishable, positioning the mutations in MK405 and MK410 closer to the *Kpn*I restriction site. The Tn*5* insertion sites for mutants MK406 and MK409 were indistinguishable and may represent identical sites.

A physical map of the *nos* region is shown in Fig. 2B. For precise mapping, the *Eco*RI-*Hin*dIII fragment from coordinates 0 to 2.5 and the *Hin*dIII fragment from coordinates 2.5 to 6.7 were each subcloned into plasmid pBR325 and mapped with *Cla*I, *Pst*I, and *Sal*I. Outside the *nos* region

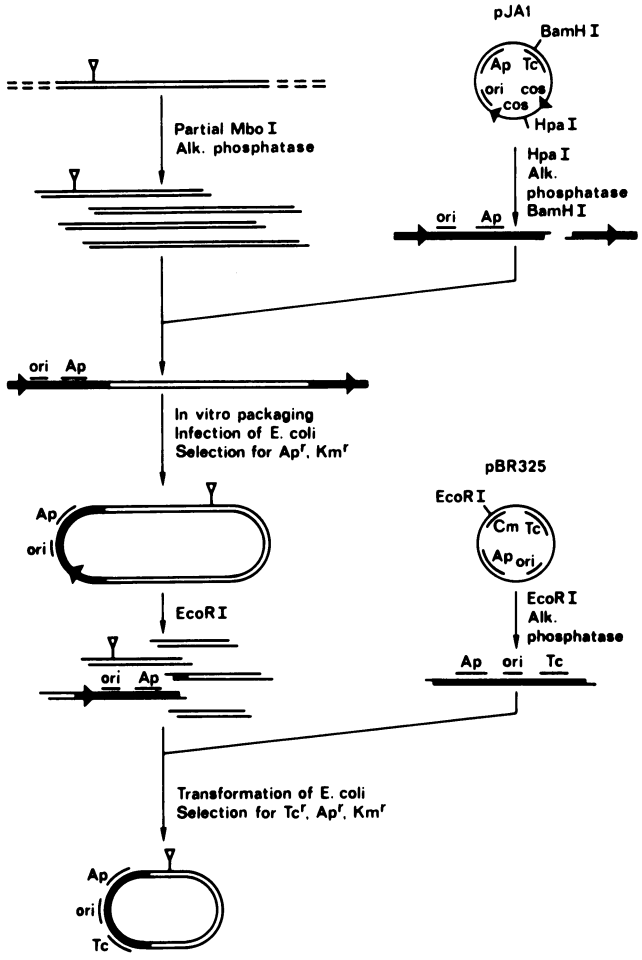

FIG. 1. Procedure for cloning and subcloning Tn*5*-mutagenized DNA from *P. stutzeri*. For details, see the text. Alk., Alkaline.

defined by the Tn*5* insertions, these sites were not mapped completely. No cleavage sites for *Bam*HI and *Hpa*I and only a single site for *Kpn*I were found within the overall region of the two contiguous *Eco*RI fragments.

Conclusions. By mapping the locations of 19 Tn*5* insertions to the genomic DNA of *P. stutzeri*, we identified a region of about 8 kb which codes for several essential functions for N_2O utilization. These functions were lost by Tn*5* insertion, resulting in a Nos$^-$ phenotype. The phenotypes distinguished thus far (Table 1), include lack of synthesis of the structural protein of N_2O reductase (mutant group IIa; group numbering according to the notation given in reference 16), lack or strong impairment of copper chromophore synthesis or insertion into the apoenzyme (mutant group I), and loss of enzyme expression (mutant groups IIb and III). These biochemically distinguishable groups of mutants all carried mutations that mapped within the 8 kb DNA region but were clustered in separate areas. Mutant group IIa was differentiated as a separate cluster from groups IIb and III, which have similar phenotypes. Grouping of mutants according to their biochemical characteristics and the location of their Tn*5* insertion site is complementary. The interpretation suggested by the two sets of data is the existence of at least three functions within the *nos* region, one of which constitutes the structural gene for N_2O reductase. One-fourth of the DNA region identified by the Tn*5* insertions would be

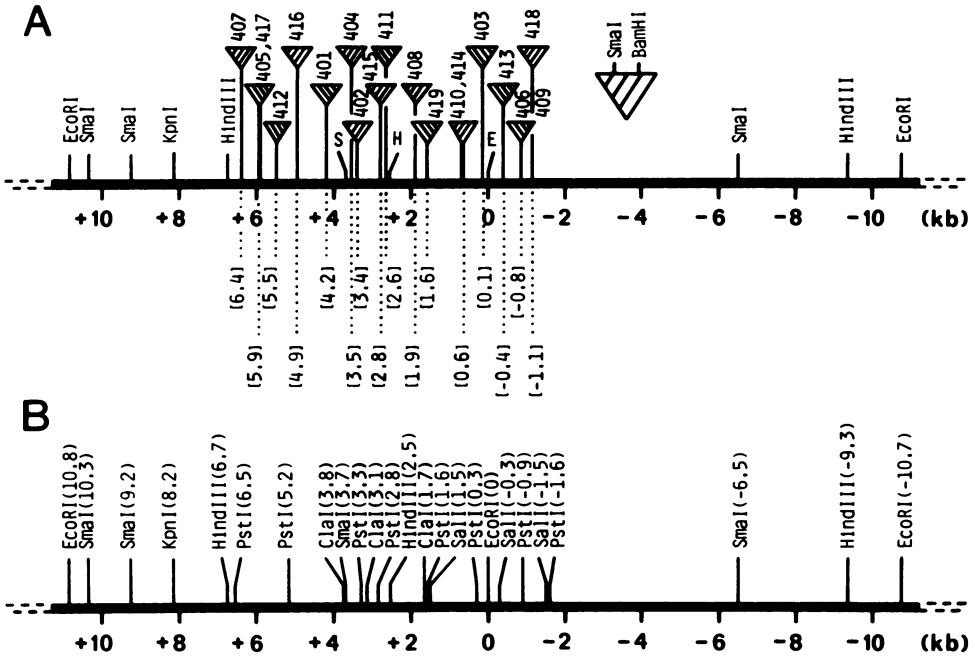

FIG. 2. (A) Physical map of Tn*5* insertions causing the Nos^- phenotype. (B) Restriction map of the *nos* coding region. Mapping of the Tn*5* insertions (represented by triangles) was done with respect to the restriction endonucleases indicated. The orientation of Tn*5* is shown with respect to its *Sma*I and *Bam*HI restriction sites and by the direction of hatching of the triangles. The numbers above the triangles indicate mutant strains, with the coordinates of Tn*5* insertions given in brackets below the black bar. All coordinates are given relative to the *Eco*RI site within the *nos* cluster. Abbreviations E, *Eco*RI; H, *Hin*dIII; S, *Sma*I. Recognition sites in panel B for *Pst*I, *Cla*I, and *Sal*I are shown only within the *nos* region.

required to accommodate the structural gene for N_2O reductase, leaving most of the *nos* region available for the other functions. Further analysis is under way to define these functions.

ACKNOWLEDGMENTS

We thank A. Schwickerath for excellent technical assistance.

We thank the Deutsche Forschungsgemeinschaft for financial support.

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