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Transduction of the Nitrogen-Fixation Genes in *Klebsiella pneumoniae*

(mutants/P1 phage/histidine operon/nitrogenase)

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ABSTRACT The bacteriophage P1 infects and functions as a generalized transducing phage for nitrogen-fixing strains of the coliform bacterium *Klebsiella pneumoniae.* Bacterial mutants (nif^-) unable to grow on molecular nitrogen as a nitrogen source were found to be deficient in nitrogenase activity as assayed by the conversion of acetylene to ethylene. These mutants regained normal nitrogenase activity and the ability to grow on N_2 after transduction with lysates of P1 phage prepared from wild-type bacteria. Transductional analysis with P1 revealed that several *nif* genes are located on the genetic linkage map of *Klebsiella* near the histidine operon.

During the last decade, work in the field of biological nitrogen fixation has been focused primarily on the enzymology of the process. The enzymatic conversion of nitrogen to ammonia has been studied in greatest detail in two bacterial species—*Clostridium* and *Azotobacter* (1). Unfortunately, neither of these bacteria is a good prospect for the study of the genetic basis of nitrogen fixation; neither has been studied genetically, and no method of genetic transfer has been reported for either of them. Furthermore, the complexity of the symbiotic relationship between the root-nodule bacteria and leguminous plants makes it difficult to exploit the genetic transformation that has been reported for these bacteria (2).

We are investigating the genetic basis of nitrogen fixation by applying the genetic techniques developed with *Escherichia coli* to its nitrogen-fixing relative, *Klebsiella pneumoniae* (3, 4). We report here the genetic transfer of the nitrogen fixation genes by transduction with the bacteriophage P1.

MATERIALS AND METHODS

Media. All bacterial strains and mutants were stored on nutrient agar, adjusted to pH 7, containing (per liter): 10 g Bacto tryptone; 1 g yeast extract; 1 g glucose; 8 g NaCl; 15 g Bacto agar. Bacteria were grown under N_2 on the minimal (nitrogen-free) medium of Yoch and Pengra (5) with the NaCl and sucrose concentrations reduced to 2 g/liter and 15 g/liter, respectively. The Colab ionagar used to make minimal agar contributed about 0.3 μg/mg of nitrogenous compounds. For ammonia-supported gowth, the minimal medium was supplemented with 0.5 g of $(NH_4)_2SO_4$ per liter. L broth (LC broth (6) minus $CaCl_2$) was supplemented with 2×10^{-3} M $CaCl_2$, 25 μg/ml of thymidine, and 0.1% glucose to make LCTG broth and agar for phage growth and assay.

Abbreviation: LCTG medium, L broth supplemented with $CaCl_2$, thymidine, and glucose (see *Methods*).

In keeping with the current genetic nomenclature, the abbreviation *nif* is suggested for the *ni*trogen-*f*ixation genes. Mutants are listed as *nif-1*, *nif-2*, ...

Bacterial Strain. Klebsiella pneumonia M5A1, used for most of the experiments reported here, was kindly furnished by P. W. Wilson.

Isolation of Mutants. Nutritional auxotrophs of *K. pneumoniae* M5A1 were prepared by the nitrosoguanidine procedure described by Adelberg, Mandel, and Chen (7), followed by replica plating to suitable agar. Mutants unable to use N_2 as their sole source of nitrogen (nif^-) were also selected from nitrosoguanidine-treated cultures by the penicillin enrichment technique, as modified by Roth (8). Since nitrogen fixation is an anaerobic process in *Klebsiella,* the penicillin selection was done in flanks bubbled with N_2. The treated cells were diluted and plated on minimal medium supplemented with 15 μg/ml of $(NH_4)_2SO_4$ and incubated at 30°C in desiccators filled with N_2. A circular piece of filter paper was pressed into the lid of each Petri dish to prevent excessive moisture accumulation and dripping from the lid. The plates were incubated for 3–4 days to allow colony formation. Small, light-colored colonies (see *Results*) were streaked on nitrogen-free and ammonia-containing minimal agar. Nif^- mutants isolated in this way were stored on nutrient agar and checked periodically for nif^+ revertants.

Phage Lysates. Phage P1 kc, obtained from Beverly Wolf, was grown, assayed, and stored by methods similar to those of Wolf, Newman, and Glaser (9). For growth of the phage, bacteria were grown in L broth to 10^8 cells/ml. Thymidine, glucose, and $CaCl_2$ were then added. The culture was infected with P1 at a multiplicity of 1–2 and vigorously shaken for 3–5 hr at 37°C until it cleared. These phage lysates were shaken with chloroform, centrifuged at 10,000 $\times$ *g* for 5 min, and stored over chloroform.

Phage lysates were assayed as follows: exponentially growing bacteria were suspended in 0.01 M $MgCl_2$–0.005 M $CaCl_2$, at a final cell concentration of 1–2 $\times$ 10^8 cells/ml. Phage samples, diluted in 1% Bacto tryptone–0.01 M $MgCl_2$ (pH 7), were mixed with an equal volume of these bacteria and incubated for 10 min at 0°C, and then for 20 min at 37°C. The phage and bacteria plus liquid LCTG soft agar (0.6%) were poured over LCTG agar and incubated overnight at 37°C. Lysates typically contained 1–5 $\times$ 10^9 plaque-forming units per ml.

P1 Transductions. Transductions were performed according to the method of Wolf *et al.* (9). Treated cells (0.1 ml/plate) were spread with 1 drop of 0.5 M sodium citrate on suitable agar and incubated at 30°C. When nif^+ transductants were selected, the plates were incubated under N_2 for 4–5 days.

Extracts. Bacterial cultures were grown in 20-liter carboys containing minimal medium and bubbled with N_2. All strains

were provided with 50 μg/ml of L-aspartate to induce nitrogenase if the enzyme was present (5). Cells were harvested with a Sharples centrifuge, and the frozen cell paste was crushed with a Hughes press. The broken cells were suspended in deoxygenated water (containing about 10 μg/ml of pancreatic DNase (Calbiochem) to hydrolyze DNA) to give a final protein concentration of about 40 mg/ml. After incubation for 20 min at 37°C in an argon-filled flask, the cell debris was removed by centrifugation at 30,000 × *g* for 20 min, and the extracts were stored anaerobically in serum bottles at −20°C.

Nitrogenase Assay. The dithionite–acetylene assay (10) was used to measure nitrogenase activity of extracts. Ethylene production was monitored with a Varian–Aerograph model 1400 gas chromatograph, equipped with a peak-height integrator. Whole-cell assays for nitrogenase were performed in 5-ml flasks containing 1.0 ml of cell culture and the appropriate gas mixture.

RESULTS

Nitrogen-fixing strains of *Klebsiella pneumoniae* and *nif*⁻ auxotrophs

Nitrogen-fixing strains of *Klebsiella* have been isolated from a wide range of habitats, including leaf nodules of tropical plants (11), plant and soil sources (3), and the human intestine (12). Unusually large numbers occur in the intestines of New Guinea natives, where they may fix some nitrogen in the gut (12). *K. pneumoniae* is essentially identical to *Aerobacter aerogenes*, and is also related to the common colon bacterium, *E. coli*. In fact, it has a chromosomal map similar to that of *E. coli* and *Salmonella typhimurium* (13, 14), though there are several distinguishing characteristics of *K. pneumoniae*, such as the higher G + C content of its DNA (60% compared to 50% for *E. coli*) (4). The bacterial and genetic techniques developed for use with *E. coli* and *Salmonella typhimurium* can be used with *K. pneumoniae*, thus making feasible the application of biochemical genetics to the study of nitrogen fixation.

We have taken advantage of unique differences in colony size and pigmentation to isolate many *nif*$^-$ mutants that produce small, light colonies on minimal agar (Fig. 1); addition of small amounts of ammonium sulfate to the agar allowed the colonies to develop to visible size. Most of these *nif*$^-$ mutants do not show nitrogenase activity in extracts of whole cells, as measured by the sensitive acetylene assay (Table 1). Mutant *nif-41*, which has a low level of nitrogenase activity, grows slowly on minimal medium under N_2. The growth rate (both aerobic and anaerobic) of the mutants is the same as that of the wild-type strain on ammonia-supplemented media. A few mutants appear to produce defective nitrogenases; for example, extracts of mutant *nif-95* were able to form an active nitrogenase complex (15) when supplemented with purified iron-sulfide protein from the wild-type strain (16). Purified iron-molybdeum component did not complement the *nif-95* extract, which indicates that this mutant produced active iron-molybdeum protein, but was lacking the iron-sulfide protein. A biochemical analysis of these mutants will be presented in a later publication. In summary, the *nif*$^-$ mutants of *K. pneumoniae* appear to be similar to *nif*$^-$ mutants described for *Azotobacter vinelandii* (17).

A transducing phage

Although *K. pneumoniae* is related to *E. coli*, genetic transfer has not been extensively studied in this organism. The genetic systems previously reported for *Klebsiella*—conjugation (13) and transduction by a *Klebsiella*-specific phage (18)—may be helpful in future studies. However, we found that neither the sexually fertile *K. pneumoniae* strains nor the *K. aerogenes* strain sensitive to the specific transducing phage were able to fix nitrogen. Our nitrogen-fixing strains were not fertile and were not sensitive to the new phage. The reported transfer of episomes between *Klebsiella* and both *E. coli* and *Shigella* apparently involved the episomes only, without transfer of the bacterial chromosome (19, 20). Thus, none of the reported genetic systems was suitable for a study of

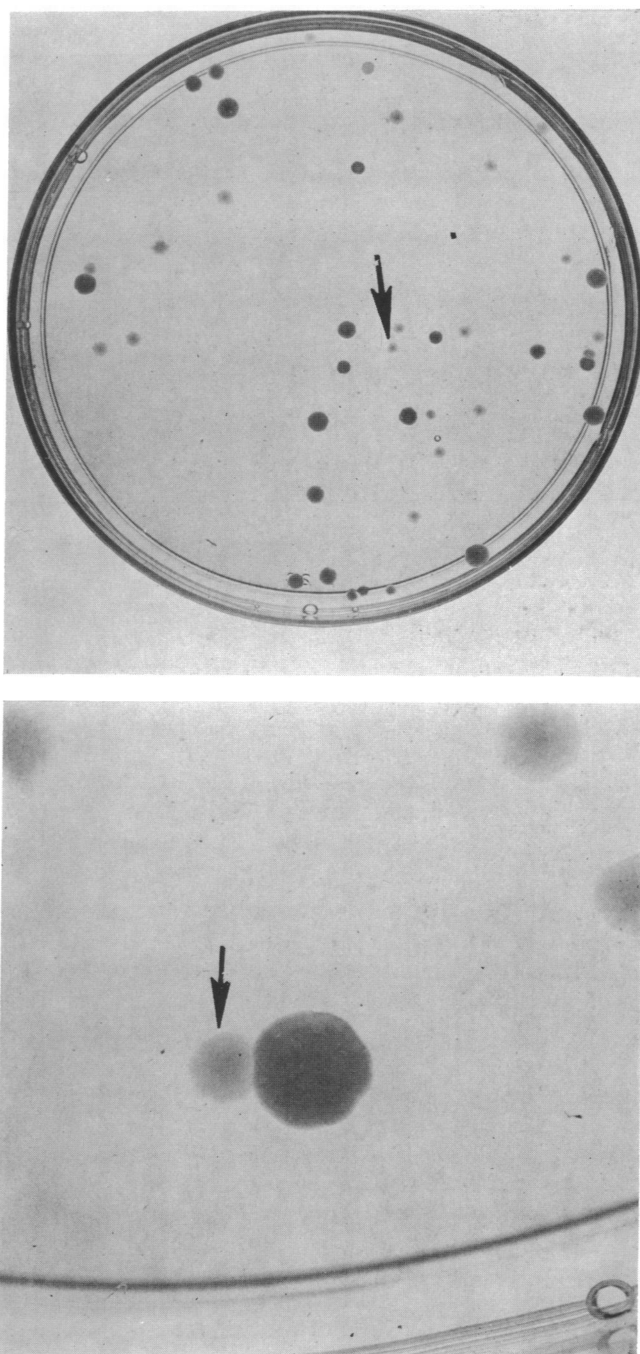

FIG. 1. Colonies of wild type and nitrogen-fixation mutants (*nif*$^-$) of *Klebsiella pneumoniae* selected by the penicillin technique. Dark colonies are able to fix nitrogen, while the small light colonies (*arrows*) grow at the expense of a small amount of $(NH_4)_2SO_4$ (15 μg/ml) added to the Petri dishes.

TABLE 1. *Properties of nitrogen-fixation mutants (nif⁻) of Klebsiella pneumoniae M5A1*

Bacterial mutant	Reversion frequency ($\times 10^9$) ($nif^- \rightarrow nif^+$)	Anaerobic utilization of nitrate* and chlorate† resistance		Nitrogenase activity (nmol of ethylene formed per min)	
		NO_3^-	ClO_3^-	Cells (1 ml)	Extracts (1 mg)‡
K. pneumoniae					
Wild type	–	+	(S)ensitive	5.0	41
nif-41	7.7	+	S	0.31	0.60
nif-88	18.4	+/–	(R)esistant	<0.01	<0.01
nif-94	7.4	+	S	<0.01	<0.01
nif-95	14.2	+	S	<0.01	<0.01
nif-104	3.4	+	S	<0.01	<0.01
nif-105	1.5	+	R	<0.01	<0.01
nif-23	2.5	–	R	<0.01	<0.01
nif-30	0.57	+	S	<0.01	—

* Nitrate plates contained minimal medium plus 0.05% $NaNO_3$.
† Chlorate plates contained 1% $KClO_3$ in L Broth.
‡ *Nif⁻* cultures were grown in minimal medium supplemented with 50 μg/ml of L-aspartate, to a cell density of about 0.2 A_{550} unit/ml.

nitrogen fixation. Various new *K. pneumoniae* phage isolates were tested for transduction with negative results. However, we found that several *nif⁺* strains were sensitive to the generalized transducing phage P1, active against *E. coli* and *Shigella. K. pneumoniae* M5Al is readily infected by Pl. Furthermore, Pl is a generalized transducing phage for this nitrogen-fixing strain. The frequency of transduction of various amino acid and vitamin markers (Table 2) is comparable to the values reported for *E. coli* (*21*).

Transduction of the nitrogen-fixation (*nif*) genes

Mutants unable to fix nitrogen (*nif⁻*) were converted to *nif⁺* by transduction with P1 that had been grown on wild-type bacteria. Transductant colonies appeared at a frequency of 1–4 $\times$ 10^{-5} nitrogen-fixing colonies per infective phage particle (Table 3) and had the dark colony-coloration and nitrogenase activity characteristic of the wild-type donor.

Transductional crosses between the *nif⁻* mutants were undertaken to investigate the number of genes affected and their relative locations on the bacterial chromosome. Earlier work with transducing phages (21–23) indicated that: (*a*) genetic markers separated by large physical distances on the chromosome (unlinked genes) give transductants at a frequency comparable to that obtained with a wild-type donor, (*b*) conversely, closely linked markers give low frequencies of transductants, and (*c*) P1 transducing particles can transfer

TABLE 2. *P1-mediated transfer of the genetic material of Klebsiella pneumoniae M5A1*

Nutritional defect of recipient strain*	Spontaneous revertants per 10^9 cells	Transductant colonies produced per 10^6 infectious phage (wild-type strain as donor)
Leu⁻	17.1	61
Pro⁻	6.4	63
Trp⁻	2.2	97
Thi⁻†	4.8	38

* Derived from *K. pneumoniae* M5A1.
† Thi, thiamine.

a portion of the bacterial chromosome corresponding to about 100 genes.

Table 3 shows representative data from a series of two-point transductional crosses. The recipient *nif⁻* mutants were infected by P1 that had been grown on the donor bacteria, and the number of *nif⁺* transductant colonies was recorded. Several of these crosses (e.g., *nif-88* $\times$ *nif-95*) gave high frequencies of transductants, indicating that these mutations are in distant genes. All the other mutants that

TABLE 3. *Transductional crosses of nif⁻ mutants: nif⁺ transductant colonies per 10^7 infectious phage*

Recipient	Donor							
	nif-95	*nif-41*	*nif-92*	*nif-23*	*nif-105*	*nif-104*	*nif-88*	Wild type
nif-95	0	31	53	147	66	121	233	286
nif-41	26	0.6	18	27	– –*	52	43	120
nif-92	120	36	0.7	69	18	17	45	440
nif-23	120	33	14	0	10	31	31	130
nif-105	269	268	60	54	– –	23	32	388
nif-104	140	51	13	54	16	0	24	170
nif-88	160	120	50	150	11	46	0	340

* – –, not done.

TABLE 4. *Cotransduction of nitrogen-fixation genes with the genes of the histidine operon*

		Genetic analysis of *his*$^+$ transductants		
Donor	Recipient*	Number of *his*$^+$ transductant colonies analyzed	*his*$^+$,*nif*$^+$ trans-ductants	Cotrans-duction (%)
Wild type	*nif-95, his-1*	242	137	56
Wild type	*nif-95, his-2*	185	108	59
Wild type	*nif-95, his-3*	100	48	48
Wild type	*nif-23, his-4*	144	47	33
Wild type	*nif-23, his-5*	114	41	36
Wild type	*nif-23, his-6*	88	35	40
Wild type	*nif-23, his-7*†	78	27	35
Wild type	*nif-23, his-8*†	96	34	35
Wild type	*nif-88, his-9*	50	15	30
Wild type	*nif-88, his-10*	50	15	30
Wild type	*nif-88, his-11*†	50	11	22
Wild type	*nif-88, his-12*†	50	15	30

* Histidine-requiring auxotrophs were prepared from the various *nif*$^-$mutants by nitrosoguanidine treatment and penicillin enrichment, as described in *Methods*.

† These mutants are unable to convert histidinol to histidine; all other *his*$^-$ mutants utilized histidinol, but were not further classified as to their specific defect.

have been crossed with *nif-95* and *nif-88* (those shown in Table 3, and about thirty others) appear to have *nif* mutations located near *nif-95*, near *nif-88*, or between *nif-95* and *nif-88* on the chromosome. We conclude that several genes scattered across one region of the chromosome are required for nitrogen fixation.

Cotransduction of *nif* genes with genes of the histidine operon

Some of the *nif* genes were located on the chromosome near the histidine operon. This was found by use of the linked transfer of *nif*$^+$ and *his*$^+$ from wild type into *nif*$^-$ *his*$^-$ double mutants isolated from nitrosoguanidine-treated *nif-95* bacteria. Prototrophic, nitrogen-fixing colonies arising from a transductional cross between the wild-type strain and a double mutant were scored as cotransduction. When 20 different auxotrophic, double mutants were studied, only the *his* auxotrophs cotransduced with *nif-95*. Histidine-re-requiring auxotrophs of *nif-23* and *nif-88* were isolated and were used with the *nif-95 his*$^-$ mutants to establish the frequency of cotransduction. *His*$^+$ transductant colonies were picked and streaked onto minimal agar and scored for growth on nitrogen. As shown in Table 4, the cotransduction of the *nif* genes with various *his*$^-$ mutants reveals a linkage of 55% to *nif-95*, 35% to *nif-23*, and 30% to *nif-88*. It is, therefore, concluded that these *nif* genes are located on the genetic linkage map of *Klebsiella* near the *his* operon.

DISCUSSION

We now have at hand several important tools for the study of the genetic basis of biological nitrogen fixation. It is of some interest to discuss how this system might be exploited in the future. At this stage, the greatest need is for more basic knowledge about the nitrogen-fixation genes themselves. Transductional analysis of the nitrogenase system, including mapping of the essential genes and studies of the regulatory elements, is an immediate goal. Biochemical-genetic studies with various *nif*$^-$ mutants may shed light on the mechanism of nitrogen fixation.

One objective of this work was to develop a system for genetic transfer of the nitrogenase genes that might be used for studies of genetic hybridization of nitrogen-fixing organisms. We are currently performing a series of P1-mediated crosses among our *Klebsiella* strains with the aim of constructing new nitrogen-fixing hybrids from natural isolates that fix nitrogen either poorly or not at all.

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