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 N₂ 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 N₂ 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 growth, the minimal medium was supplemented with 0.5 g of (NH₄)₂SO₄ per liter.

**L broth** (LC broth) (6 minus CaCl₂) was supplemented with 2 × 10⁻⁴ M CaCl₂, 25 μg/ml of thymidine, and 0.1% glucose to make LCTG broth and agar for phage growth and assay.

**Bacterial Strain.** *Klebsiella pneumoniae* 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 N₂ 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 N₂. The treated cells were diluted and plated on minimal medium supplemented with 15 μg/ml of (NH₄)₂SO₄ and incubated at 30°C in desiccators filled with N₂. A circular piece of filter paper was pressed into the lid of each Petri dish to prevent excess 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 10⁸ cells/ml. Thymidine, glucose, and CaCl₂ 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 × g for 5 min, and stored over chloroform.

Phage lysates were assayed as follows: exponentially growing bacteria were suspended in 0.01 M MgCl₂-0.005 M CaCl₂ at a final cell concentration of 1–2 × 10⁷ cells/ml. Phage samples, diluted in 1% Bacto tryptone-0.01 M MgCl₂ (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 × 10⁷ 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 N₂ for 4–5 days.

**Extracts.** Bacterial cultures were grown in 20-liter carboys containing minimal medium and bubbled with N₂. All strains

Abbreviation: LCTG medium, L broth supplemented with CaCl₂, thymidine, and glucose (see Methods).

In keeping with the current genetic nomenclature, the abbreviation nif is suggested for the nitrogen-fixation genes. Mutants are listed as nif⁻I, nif⁻2, . . .
Nitrogen Fixation Genes in Klebsiella 1175

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–acyetylene 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 N₂. 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-molybdenum component did not complement the nif-95 extract, which indicates that this mutant produced active iron-molybdenum 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...
and frequency comparable markers conversely, work with undertaken (Table particle activity bacteria. Transduction of the reported acid strain. P1 K. ralized transducing we were tested nitrogen fixation. Nutritional defect revertants more, ~~~~~~~~-

* Nitrate plates contained minimal medium plus 0.05% NaNO₃. † Chlorate plates contained 1% KClO₃ 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 A₅₆₅ unit/ml.

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

<table>
<thead>
<tr>
<th>Bacterial mutant</th>
<th>Reversion frequency (X10⁶) (nif⁻ → nif⁺)</th>
<th>Anaerobic utilization of nitrate* and chlorate† resistance</th>
<th>Nitrogenase activity (nmol of ethylene formed per min)</th>
<th>Cells (1 ml)</th>
<th>Extracts (1 mg)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pneumoniae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>-</td>
<td>+</td>
<td>(S)ensitive</td>
<td>5.0</td>
<td>41</td>
</tr>
<tr>
<td>nif-41</td>
<td>7.7</td>
<td>+</td>
<td>S</td>
<td>0.31</td>
<td>0.60</td>
</tr>
<tr>
<td>nif-88</td>
<td>18.4</td>
<td>+/−</td>
<td>(R)esistant</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>nif-94</td>
<td>7.4</td>
<td>+</td>
<td>S</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>nif-104</td>
<td>3.4</td>
<td>+</td>
<td>S</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>nif-105</td>
<td>1.5</td>
<td>+</td>
<td>R</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>nif-83</td>
<td>2.5</td>
<td>-</td>
<td>R</td>
<td>&lt;0.01</td>
<td>-</td>
</tr>
<tr>
<td>nif-90</td>
<td>0.57</td>
<td>+</td>
<td>S</td>
<td>&lt;0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

* Nitrate plates contained minimal medium plus 0.05% NaNO₃.
† Chlorate plates contained 1% KClO₃ 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 A₅₆₅ unit/ml.

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

<table>
<thead>
<tr>
<th>Nutritional defect of recipient strain*</th>
<th>Spontaneous revertants per 10⁹ cells</th>
<th>Transductant colonies produced per 10⁶ infectious phage (wild-type strain as donor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu⁻</td>
<td>17.1</td>
<td>61</td>
</tr>
<tr>
<td>Pro⁻</td>
<td>6.4</td>
<td>63</td>
</tr>
<tr>
<td>Trp⁻</td>
<td>2.2</td>
<td>97</td>
</tr>
<tr>
<td>Thi⁻†</td>
<td>4.8</td>
<td>38</td>
</tr>
</tbody>
</table>

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

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 X 10⁻⁴ nitrogen-fixing colonies per infectious 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 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 X nif-90) gave high frequencies of transductants, indicating that these mutations are in distant genes. All the other mutants that...
TABLE 4. Cotransduction of nitrogen-fixation genes with the genes of the histidine operon

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient*</th>
<th>Genetic analysis of his⁺ transductants</th>
<th>Number of his⁺ transductants</th>
<th>his⁺, nif⁺ transductants</th>
<th>Cotransduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type nif-95, his-1</td>
<td>242</td>
<td>137</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-95, his-2</td>
<td>185</td>
<td>108</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-95, his-3</td>
<td>100</td>
<td>48</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-4</td>
<td>144</td>
<td>47</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-5</td>
<td>114</td>
<td>41</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-6</td>
<td>88</td>
<td>35</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-7†</td>
<td>78</td>
<td>27</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-8†</td>
<td>96</td>
<td>34</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-9</td>
<td>50</td>
<td>15</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-10</td>
<td>50</td>
<td>15</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-11†</td>
<td>50</td>
<td>11</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type nif-88, his-12†</td>
<td>50</td>
<td>15</td>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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 his⁻ nif⁻ 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-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-88, 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.

We are indebted to Duane Yoch, A. J. Clark, Allan Wilson, and Gary Rhodes for helpful discussions and to Howard Nagatani for technical assistance. Several Klebsiella phages were isolated by T. Watkins. This work was supported by NSF Grant GB 7036 and by a USPHS Training Grant for S. S.