Acetylene Reduction (Dinitrogen Fixation) by Clinical Isolates of *Klebsiella pneumoniae*

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Freshly isolated clinical strains of *Klebsiella* were tested for the ability to fix dinitrogen by the acetylene reduction assay. Ability to detect this trait was markedly affected by cultural conditions. When the test was run at 37°C in the presence of yeast extract (50 mg/liter), only 1.6% of the organisms were diazotrophs, whereas this temperature without yeast extract yielded 12.9% positive cultures. The optimum condition found was 28°C without yeast extract (21.9% positive); therefore, search for diazotrophy in clinical strains should not be conducted at the usual incubation temperature. There was a high incidence of indole-positive strains among diazotrophs. No such correlation was noted with any other biochemical trait or antibiotic susceptibility tested. The significance of this correlation is not apparent.

Dinitrogen fixation has been studied in *Klebsiella* sp. isolated from pulp mill effluents (11), paper mill process waters (15), soil (12), the marine environment (20), and other sources (10). Diazotrophic specimens of other genera of the *Enterobacteriaceae* have also been isolated, including *Enterobacter* (20), *Citrobacter* (6, 15), *Escherichia*, and *Erwinia* (15). These reports indicate that the process of dinitrogen fixation is common and widespread among saprophytic enteric strains. However, to the best of our knowledge, there have been no reports specifically concerned with dinitrogen fixation in clinical isolates of *Klebsiella* sp. Some surveys of the genus included clinical specimens for comparison with environmental isolates (1, 13), but the numbers of clinical isolates used in these studies were relatively small. More importantly, the subject clinical isolates were stock cultures, whereas the environmental specimens were freshly isolated. The purpose of our research was to assess the incidence of dinitrogen fixation in freshly isolated clinical strains of *Klebsiella* sp. and to determine whether this trait correlated with any characteristic of clinical interest.

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MATERIALS AND METHODS

Isolation of diazotrophs. Cultures of the *Klebsiella-Enterobacter* group were collected weekly from several local hospitals. The cultures came from four

different clinical laboratories and from different types of infectious as well as nosocomial situations and represented distinct, separate isolates. All cultures were streaked on Trypticase soy agar (Baltimore Biological Laboratories, Cockeysville, Md.) and incubated overnight at 37°C to test their purity. Mixed cultures and those that failed to grow on Trypticase soy agar were discarded. Pure cultures were tentatively identified by the results of the methyl red, Voges-Proskauer, motility, and ornithine decarboxylase tests. All media for these tests were Difco products, and testing was carried out according to the manufacturer's recommendations (4).

Acetylene reduction. For the acetylene reduction assay, all organisms were inoculated onto slants of nitrogen-free mineral salts agar of the following composition (per liter): KH_2PO_4 , 0.5 g; K_2HPO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; CaCl_2 , 0.4 g; FeCl_3 , 4.8 mg; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 mg; sucrose, 30 g; and agar, 20 g. The pH was adjusted to 7.0, and the medium was dispensed into tubes (15 by 125 mm) before autoclaving. The inoculated slants were incubated at 28°C for 3 days and then fitted with serum bottle (13 by 18 mm)-type rubber caps. Each tube was flushed twice with argon; then 0.5 ml of argon was removed and replaced with an equal amount of acetylene (C_2H_2), yielding a pC_2H_2 of approximately 0.05 atm. The gas analyses were performed on a Varian Aerograph 1400 gas chromatograph with a hydrogen flame ionizer. The column was packed with Porapak N (Waters Associates, Inc., Framingham, Mass.). The column temperature was 90°C; the injection and detection temperatures were both 140°C. The carrier gas was dry nitrogen at a flow rate of 60 ml/min. The hydrogen flow rate was 15 ml/min; that of air was 23 ml/min. Samples (0.2 ml) of the gas phase of each tube were assayed immediately after the introduction of C_2H_2 (time zero), after 2 days, and after 7 days. The reduction of acetylene to ethylene was assumed to be evidence for nitrogen fixation. The nitrogenase-positive

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controls were *Klebsiella pneumoniae* ATCC 15574 (isolated from the leaf nodules of *Psychotria bacteriophila* [3]) and K4, its nonencapsulated mutant.

Yeast extract supplementation. Nitrogen-free broth and broth containing 50 mg of yeast extract per liter were filter sterilized separately and dispensed into sterile culture tubes. The selected organisms were inoculated into each type of medium, and the absorbance in all tubes was adjusted to approximately 0.4 at 540 nm on a Bausch and Lomb Spectronic 20. The tubes were then incubated at 28°C for 3 days. The C_2H_2 reduction assay was performed as described above.

Temperature study. Two diazotrophs were selected to determine the quantitative effect of incubation temperature on acetylene reduction. Two 200-ml amounts of N-free broth were each inoculated with a loopful of one 24-h diazotroph culture, and viable plate counts were performed on N-free agar. Each suspension was then divided between two flasks and incubated for 3 days, one flask at 28°C and the other at 37°C. A viable plate count was again performed on the four total flasks. At this time (time zero), the contents of each flask were divided among six tubes, 4.0 ml per tube. All six tubes from each flask were flushed with argon, and four of them received C_2H_2 . During the remainder of the experiment, one tube without C_2H_2 and two with C_2H_2 , from each flask, were incubated at 28°C, and the remaining three of each suspension were incubated at 37°C. C_2H_2 reduction assays were performed on all tubes containing C_2H_2 at T_0 and at 12-h intervals for 48 h. Viable plate counts were performed on the tubes without C_2H_2 and on one of each two with C_2H_2 at the same intervals.

Biochemical tests. Once a sufficient number of dinitrogen-fixing *Klebsiella* was collected, further tests were undertaken to compare the biochemical characteristics of diazotrophs and nondiazotrophs. Each group contained 20 cultures; the control cultures were included with the clinical diazotrophs. Twenty-four tests were performed. A suspension of each organism in nutrient broth was used as the inoculum. Cultures were held for at least 1 week before being discarded as negative.

Susceptibility to antimicrobial agents. Susceptibility to antimicrobial agents was tested by the antibiotic-disk diffusion assay as described in the *Manual of Clinical Microbiology* (14).

RESULTS

Isolation of diazotrophs. Of 209 cultures collected, 148 (71%) were identified as *Klebsiella* sp. by initial tests. Another 32 (15%) were *Enterobacter* sp., and the remainder were unidentified. Only *Klebsiella* sp. were able to reduce C_2H_2 . A total of 18 clinical diazotrophs was found. During the course of the isolations, it appeared that conditions for the C_2H_2 reduction assay were not optimal. The procedure was then modified. The incidence of diazotrophy among *Klebsiella* cultures increased with each modification (Table 1). Both lower incubation temper-

ature and absence of yeast extract favored the detection of diazotrophy.

Yeast extract supplementation. The five organisms selected, *K. pneumoniae* ATCC 15574 and K4 (plant isolates) and CVA-50, CTG-1, and CTG-4 (clinical isolates), exhibited different responses to the incorporation of 50 mg of yeast extract per liter in N-free medium (Table 2). The plant isolates were relatively unaffected by the yeast extract, whereas in the clinical isolates C_2H_2 reduction decreased or was eliminated.

Temperature study. Figure 1 shows the effect of incubation temperature on C_2H_2 reduction for one organism (CVA-50). Cells incubated initially at 37°C were unable to reduce C_2H_2 ; shifting the cells from 28 to 37°C permitted some C_2H_2 reduction; and incubation at 28°C throughout the experiment allowed maximum C_2H_2 reduction. Viable plate counts of two diazotrophic clinical isolates showed that the temperature shift alone had no marked effect on survival either in the presence or absence of acetylene, although cultures incubated at 37°C throughout the experiment did have the lowest number of survivors.

Biochemical tests. According to the biochemical reactions, the 20 diazotrophs (18 clinical and 2 plant) proved to be *K. pneumoniae* (Table 3). There was little difference between diazotrophs and nondiazotrophs, except for the high incidence of indole production (40%) among the former.

Antimicrobial susceptibility. The results of the antimicrobial susceptibility testing can be seen in Table 4. There were no great differences between diazotrophs and nondiazotrophs. Moreover, indole producers did not reveal any unusual susceptibility patterns. All organisms

TABLE 1. Incidence of diazotrophy in *K. pneumoniae* under different cultural conditions^a

Condition	No. of cultures	No. +	% +
37°C, yeast extract	63	1	1.6
37°C, no yeast extract	62	8	12.9
28°C, no yeast extract	82	18	21.9

^a See text for details of experimental conditions.

TABLE 2. Effect of yeast extract (YE) supplementation of the growth medium on acetylene reduction by five diazotrophic *Klebsiella*

Culture	Ethylene produced within 48 h (nmol)		
	Without YE	With YE	With/without
ATCC 15574	1,953.8	2,137.0	1.09
K4	2,890.0	2,503.3	0.87
CVA-50	2,652.6	1,709.6	0.64
CTG-1	2,523.6	1,824.9	0.72
CTG-4	1,720.3	0	0

tested were completely susceptible to cephalothin, gentamicin, and nalidixic acid, and almost all were susceptible to the rest of the agents except for ampicillin and carbenicillin. No evidence of Multiple-drug resistance, as defined by Klein et al. (8), was discovered.

DISCUSSION

Published reports recommend the inclusion of yeast extract or other source of combined

nitrogen in N-free media to enhance growth and provide a source of amino acids for nitrogenase synthesis (7, 9, 13). Tubb and Postgate (19) found that yeast extract (10 μ g of N per ml) and other nitrogenous compounds, when added to a repressed population of *K. pneumoniae* M5a1, shortened the lag between repression and nitrogenase synthesis with little effect on the rate of synthesis or final activity. Neilson and Sparell (15) reported that Casamino Acids (0.5 g/liter) and individual amino acids supported growth without repressing nitrogenase synthesis. In view of these reports, 50 mg of yeast extract per liter was routinely added to the N-free medium used for the isolation of diazotrophs at the beginning of this study. However, only 1.6% of the cultures tested were able to reduce C_2H_2 , an unusually low proportion compared with other studies. Omitting the yeast extract

FIG. 1. Effect of incubation temperature upon acetylene reduction by the clinical diazotroph CVA-50. At time zero, cells were removed from the initial incubation temperature (first number) and shifted to the second temperature (second number). At each point plotted, the gas phase was assayed for ethylene.

TABLE 3. Biochemical characteristics

Test	% Positive		
	Diazo-trophs	Nondi-azo-trophs	Ex-pected ^a
Indole	40.0	4.76	6
Methyl red	0	0	13.3
Voges-Proskauer	100	100	91.1
Citrate	100	95.2	97.7
Motility	0	0	0
Ornithine decarboxylase	0	4.76	0
Arginine dihydrolase	0	0	0.9
Lysine decarboxylase	100	90.4	97.2
Urease	95.0	95.0	94.5
Malonate	100	95.2	92.5
Mucate	95.0	85.7	92.8
Gelatin	0	9.5	3.3
Nitrate reduction	100	100	99.9
Carbohydrates ^b	100	100	99.0

^a From Edwards and Ewing (5).

^b Glucose, lactose, arabinose, raffinose, rhamnose, sorbitol, salicin, cellobiose, adonitol, inositol, and glycerol.

TABLE 4. Susceptibility to antimicrobial agents

Agent	Concn (μ g)	Diazotrophs			Nondiazotrophs			Indole producers		
		% R ^a	% I	% S	% R	% I	% S	% R	% I	% S
Ampicillin	10	25	25	50	40	13	47	25	37.5	37.5
Carbenicillin	50	65	25	10	80	7	13	62.5	12.5	25
Cephalothin	30	0	0	100	0	0	100	0	0	100
Gentamicin	10	0	0	100	0	0	100	0	0	100
Kanamycin	30	5	5	90	13	0	87	12.5	12.5	75
Nalidixic acid	30	0	0	100	0	0	100	0	0	100
Nitrofurantoin	300	0	5	95	0	20	80	0	0	100
Tetracycline	30	5	0	95	7	0	93	12.5	0	87.5
Triple sulfa	250	0	0	100	7	0	93	0	0	100

^a R, Resistant; I, intermediate; S, susceptible.

increased the numbers to 12.9%. Table 2 clearly indicates that yeast extract is inhibitory for some clinical isolates at levels much lower than those reported in the literature (7, 9, 13, 15, 19). The differences between the clinical isolates and the saprophytic organisms are interesting; possibly they are due to the relative availability of combined nitrogen in the human host compared with that in soil or water.

The other initial condition that was changed was the incubation at 37°C. Postgate (16) found that *K. pneumoniae* M5a1 would grow well at 37°C with ammonium salts in the medium, but could not fix dinitrogen at this temperature; he postulated that the temperature sensitivity was due to a sensitive step in either the biosynthesis of the enzyme or in the functioning of electron transport. In view of this report, incubation of clinical cultures was changed to 28°C, with a subsequent increase in the number of diazotrophs isolated. The final percentage found was 21.9. It is quite possible that optimal conditions have not yet been determined. However, the finding that high temperature and combined nitrogen adversely affect C₂H₂ reduction is important, since it is contrary to what a clinically oriented investigator might expect. Clinical isolates would normally be incubated at the temperature of their natural habitat, and, for any other trait, 37°C is adequate. However, for C₂H₂ reduction, this temperature is completely inhibitory (Fig. 1). The inhibition cannot be due solely to cell death, since similar survival curves were seen for all suspensions regardless of temperature and presence of C₂H₂. Although the death rate was high in the first 12 h, it subsequently decreased and plateaued. (The initial rapid death rate may be the result of a lack of nitrogen in the tubes, since they were flushed with argon at time zero; the subsequent maintenance of viability may be due to the release of nitrogen from dead cells.) Cultures incubated at 28°C were able to reduce C₂H₂ in significant amounts, whereas those incubated at 37°C were inactive. It appears that temperature can be a critical factor during the induction period for nitrogenase. Perhaps the higher temperature somehow inactivates the enzyme, as Postgate (16) suggested.

As mentioned above, the only other trait investigated with obvious correlation to dinitrogen fixation was indole production. Indole production is a variable trait, according to the literature, ranging from 0% positive (in cultures from pulp mills, rivers, and Center for Disease Control, Atlanta, Ga.) to 100% positive (from trees), according to one study (18). Clinical isolates from a long-term study were 18.3% positive for

this trait (8), similar to the clinical isolates of Seidler et al., which were 17% indole positive (18). The significance of the apparent correlation between indole production and nitrogenase is not known. It is, of course, possible that the differences in indole production would disappear if larger numbers of isolates were tested. Neilson and Sparell (15), who tested 67 isolates of *K. pneumoniae* from paper mill water, divided them into two biotypes, A and B. In biotype A, 50% were nitrogenase positive and 20% were indole positive, whereas in biotype B, 80% were nitrogenase positive, but none produced indole. Thus, the findings reported in this paper for clinical isolates are different from those obtained for water organisms.

Klein et al. (8) reported that indole-producing *Klebsiella* sp. demonstrated a significant increase in drug susceptibility and decrease in multiple-drug resistance. They hypothesized that the acquisition of the genes for indole production caused the loss of the genes for drug resistance, i.e., the genes were on mutually exclusive plasmids. No evidence for this hypothesis was found in this study—indole producers, diazotrophs, and nondiazotrophs all exhibited the same patterns of drug resistance. The work of Cannon et al. (2) with *nif* plasmids that were stabilized by the genes for carbenicillin resistance suggested that a similar combination might occur in nature. Genetic exchange is common among the *Enterobacteriaceae*, and it seemed likely that, if such a plasmid could appear in vivo, it would be detectable. In this study, the diazotrophs were significantly resistant to carbenicillin and, to a lesser degree, ampicillin; however, so were the nondiazotrophs. As for patterns of resistance unrelated to dinitrogen fixation, these clinical isolates were much more susceptible to every drug used than those studied by Klein et al. (8). In one study made in Scotland, most strains were markedly antibiotic resistant; however, this did not correlate with nitrogen fixation (E. C. R. Reeve, personal communication).

In conclusion, the results of this study indicate that the ability to fix dinitrogen, as detected by C₂H₂ reduction, existed among 22% of clinical isolates of *K. pneumoniae*. This ability had no correlation with any other trait tested except indole production. The meaning of this correlation is not obvious. Thus, dinitrogen fixation appears to be a random event in *Klebsiella*. This randomness helps account for the increasing frequency of isolation of diazotrophic members of related genera (6, 15, 20). *K. pneumoniae* is a close genetic relative of both *Escherichia coli* and *Salmonella typhimurium*, especially in

the region around *nif* (17). Since conjugation and transduction are common among enteric strains, it seems likely that *nif* could easily be transferred at random in nature to related organisms. Finally, although no correlation to physiological characteristics was found, some interesting differences from soil and water isolates were revealed, for example, the response to yeast extract. These differences are fundamental enough to warrant further investigation and certainly should be considered when studying dinitrogen fixation in clinical isolates.

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