APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1976, p. 197-205.

Vol. 32, No. 2
Printed in U.S.A.

Acetylene Reduction (Nitrogen Fixation) by *Enterobacteriaceae* Isolated from Paper Mill Process Waters

A. H. NEILSON* AND L. SPARELL

Institutet för Vatten-och Luftvårdsforskning, S-100 31 Stockholm, and Stockholms Universitet, Arrheniuslaboratoriet, Avdelning för Biokemi, Fack S-104 05 Stockholm 50, Sweden*

Received for publication 24 March 1976

Using selective media containing galactitol, over 130 *Enterobacteriaceae* have been isolated from paper mill process waters collected from different localities. These bacteria were extensively characterized and tested for acetylene-reducing (nitrogen-fixing) activity under anaerobic conditions. High activity was found in representatives of *Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae, Erwinia herbicola, Citrobacter freundii, Citrobacter intermedius,* and *Escherichia coli.* Under argon, nitrogenase synthesis was generally not repressed by 5 mM L-glutamate, L-aspartate, L-leucine or Casamino Acids (0.5 g/liter). In many strains, both the specific activities (nanomoles of C_2H_4 per minute per milligram of protein) and the activities (nanomoles of C_2H_4 per minute) had considerably declined after 24 h. In three selected strains, activity in intact cells grown under nitrogen was unaffected by the presence during assay of 10 mM L-amino acids or ammonium acetate. All of the strains examined were tolerant towards inactivation of nitrogen-fixing activity by 1.8% (vol/vol) oxygen during assay, and inactivation by up to 10% oxygen was partly reversible. Representatives of the six taxa synthesized nitrogenase in stirred aerobic cultures, though the protein concentrations attained were lower than under anaerobic conditions. It seems reasonable to suggest that under natural conditions, nitrogen fixation is able to contribute significantly to the nitrogen economy of the cells.

With increasing frequency, bacteria belonging to the genera *Klebsiella* and *Enterobacter* have been isolated from nonclinical sources (1, 5, 7, 12, 15, 22, 23, 26, 27). In the absence of combined nitrogen and under anaerobic conditions, a substantial proportion of all of these isolates was shown to synthesize nitrogenase.

This laboratory has been concerned with the occurrence of *Enterobacteriaceae* in paper mill process waters. In this paper we describe methods for the isolation, characterization, and demonstration of nitrogen-fixing capacity in representatives of the genera *Klebsiella, Enterobacter, Erwinia, Citrobacter,* and *Escherichia.* Previously, this has been shown only for *Klebsiella pneumoniae* isolated from such samples (22).

Several selective methods for the enumeration and isolation of such bacteria have been described. Successful results have been obtained, for example, by using plates of N-deficient medium incubated either aerobically (14) or anaerobically (23) and by using m-Endo agar (7). Encouraging results have previously been obtained in this laboratory by the use of Tergitol-7 agar, which contains lactose as carbon source and sodium heptadecyl sulfate to inhibit the growth of gram-positive organisms. Here we describe media developed from Tergitol-7 and brilliant green agars in which lactose was replaced by galactitol. Isolation was also carried out with defined media containing galactitol, glycerol, and ribitol as carbon sources and ammonium and urea as nitrogen sources.

MATERIALS AND METHODS

Isolation. Two types of media were used for primary isolation. Both contained sodium dodecyl sulfate, and in both the pH was adjusted to 7.2 before autoclaving. Throughout, concentrations are given as grams per liter of distilled water.

Media with triphenyltetrazolium chloride. (i) Complex media contained: peptone (Orthana), 5.0; yeast extract, 3.0; agar, 15.0; galactitol, glycerol, or ribitol, 5.0. (ii) Defined media were prepared by adding galactitol or glycerol or ribitol (5.0) to a basal medium containing: K_2HPO_4, 6.3; NaH_2PO_4, 1.7; $MgSO_4 \cdot 7H_2O$, 0.1; Na_2MoO_4, 0.008; ferric citrate, 0.008; yeast extract, 0.2; agar, 15.0. Nitrogen was provided as $(NH_4)_2SO_4$ (1.0) or as a urea solution (10 ml, 10% [wt/vol]) sterilized by filtration and added aseptically to the rest of the medium after autoclaving.

To both of these media were added: sodium dodecyl sulfate (0.1), bromothymol blue (0.025), and, after autoclaving, triphenyltetrazolium chloride (3 ml, 1%) sterilized by filtration.

Glucose-Casamino Acids medium was prepared by adding Casamino Acids (1.0) and glucose (50 ml, 20% [wt/vol] solution sterilized separately) to the basal medium (ii) described above. For a liquid medium the agar was omitted, and sodium thioglycolate (0.5) and resazurin (0.001) were added.

Medium with brilliant green. This medium contained peptone (Orthana), 5.0; yeast extract, 3.0; galactitol, 5.0; brilliant green (Merck), 0.0125; sodium dodecyl sulfate, 0.1; phenol red, 0.08; agar, 20.0.

The samples examined were various types of process waters obtained from eight widely separated mills. Serial dilutions in 0.05 M phosphate buffer (pH 7) were prepared and spread onto the surface of plates. After incubation at 37°C for 18 h, individual colonies were picked, and pure strains were isolated by successive streaking on glucose-Casamino Acids plates.

Characterization of the isolates. Fermentative capacity was assessed from the results of growth in stabs of triple sugar iron agar and confirmed in a liquid glucose-Casamino Acids medium. The pure strains were tested for Gram staining, presence of oxidase (Kovacs' method), capacity for anaerobic growth with glucose, and utilization of malonate, production of acid from galactitol, glycerol, and ribitol in peptone water with Andrade's indicator, subjected to the methyl red test, screened using the API 20E (Analytab Products Inc.) test kit, and assayed for acetylene-reducing (nitrogen-fixing) activity after anaerobic growth. The accuracy of identification with the API system has been shown to exceed 95%, and the average agreement with conventional tests was better than 95% (32). All incubations were carried out at 37°C.

Nitrogenase assay. (i) Under nitrogen. Sterile glucose-thioglycolate N-free medium (5 ml) in 25-ml sterile serum bottles was inoculated with 0.5 ml of a culture grown overnight at 30°C in the same medium containing Casamino Acids (1.0). The bottles were stoppered with rubber stoppers and sealed with crimp caps, and the cultures were immediately gassed with sterile nitrogen introduced through a sterile hypodermic needle dipping into the liquid; a short needle provided the gas outlet. The bottles were gassed for 12 or 24 h. During this time, visible growth was observed in all bottles due to carry-over of combined nitrogen from the original medium. The concentration of Casamino Acids could not, however, have exceeded 200 μg/ml, and from the data of Tubb and Postgate (33) it was not expected that this would significantly repress nitrogenase synthesis, particularly after the concentration was lowered by growth.

(ii) Under argon. Nitrogenase assay under argon was carried out similarly in N-free medium supplemented with 5 mM L-aspartate, L-glutamate, L-leucine, or Casamino Acids (0.75 g/liter).

In either case, after 12 or 24 h, acetylene (2 ml) was added to each bottle, the samples were shaken at 30°C for 30 min, the assay was terminated by adding perchloric acid (0.5 ml, 20%), and the ethylene was assayed by gas chromatography using a Poropak Q column (9). The suitability of the method was checked by using a culture of *K. pneumoniae* M5al kindly supplied by J. R. Postgate. Throughout, we have assumed an equivalence between acetylene-reducing and nitrogen-fixing activity. Activities are given as nanomoles of C_2H_4 per minute, and specific activities are given as nanomoles of C_2H_4 per minute per milligram of protein.

Experiments on the effects of chloramphenicol, amino acids, and oxygen were carried out as described previously (25). Growth under microaerophilic conditions was examined in N-free glucose medium lacking thioglycolate; experiments were conducted at 37°C in 250-ml bottles with 180 ml of medium stirred magnetically at 150 rpm. A bent glass tube passing through the rubber stoppers allowed free access of air.

The effects of oxygen were examined on portions removed from cultures growing anaerobically with N_2. The samples were collected anaerobically in N_2-flushed syringes and transferred to flushed (Ar or N_2) and capped bottles, and the appropriate volume of oxygen was added. For short-term experiments on inactivation, 1-ml samples in 5-ml Fernbach flasks were used with 0.5 ml of acetylene for assay. For experiments on reversibility, 10-ml samples in 50-ml serum bottles were used; acetylene (5 ml) was added, the bottles were shaken at 30°C, and samples were removed periodically for C_2H_4 analysis. After 100 min, the gas phase was replaced by pure argon, cultures were gassed for 10 min, acetylene (5 ml) was added, and incubation was continued. After a further 70 min the reaction was terminated by adding perchloric acid (1 ml, 20%).

Protein was determined by the Folin method (24) on samples prepared by digesting the intact cells from the nitrogenase assay in 5 M NaOH for 20 min at 80°C. Bovine serum albumin (fraction V, Sigma) was used as standard.

RESULTS

Isolation. Most of the strains described here were isolated on galactitol media; the complex medium seemed adequately selective, was more readily prepared, and was therefore used routinely. In a comparative study of lactose and galactitol media, it was found that, of a total of 50 isolates, all of the nitrogen-fixing ones were obtained from the galactitol medium; this effectiveness is shown also by the frequency of isolation of N_2-fixing strains of all taxa (Table 1). Media using glycerol were satisfactory but showed low selectivity, and on account of their expense ribitol media were examined only to a limited extent. Testing of our isolates showed, however, that only *K. pneumoniae* and *Enterobacter aerogenes* were able to grow at the expense of ribitol, and this medium may therefore be useful for screening suspected isolates of these taxa.

TABLE 1. *Frequency of occurrence (to nearest 10%) of characters of isolates*

Determinant	Taxon								
	Klebsiella pneumoniae		*Enterobacter cloacae*	*Enterobacter aerogenes*	*Erwinia herbicola*	*Citrobacter freundii*		*Citrobacter intermedius*	*Escherichia coli*
	A	B							
No. of isolates	48	19	27	1	14	8	3	1	4
Decarboxylase for:									
arginine	0	0	100	−	0	80	0	−	0
lysine	90	0	0	+	0	0	0	−	25
ornithine	0	0	40	+	0	20	0	+	50
Utilization of:									
citrate	90	95	100	+	10	80	100	+	0
malonate	90	80	60	−	10	20	0	−	0
Urease	80	50	0	−	0	0	0	−	0
Indole formation	20	0	0	−	10	0	0	+	100
V-P reaction 37°C	100	100	100	+	40	0	0	−	0
MR reaction 37°C	20	20	30	+	60	100	100	+	100
H_2S formation	0	0	0	−	0	0	100	−	0
Formation of acid from:									
glucose	100	100	100	+	100	100	100	+	100
mannitol	100	100	100	+	100	100	100	+	100
glucitol	100	100	100	+	100	100	100	+	100
rhamnose	100	100	100	+	100	100	100	+	100
sucrose	100	100	100	+	90	90	100	−	50
melibiose	100	100	70	+	50	90	100	−	75
amygdalin	100	100	100	+	100	100	0	+	75
arabinose	100	100	100	+	100	100	100	+	100
inositol	100	80	50	+	10	10	0	−	0
galactitol	50	95	80	−	40	60	0	−	0
ribitol	100	5	0	+	0	10	0	−	0
glycerol	100	70	10	+	10	20	30	−	0
Synthesis of nitrogenase	50	80	60	+	40	70	0	+	25

Characterization. After 18 h of incubation, two kinds of colonies were observed on plates containing triphenyltetrazolium chloride. (i) Bacteria giving rise to small, dark-red colonies less than 1.5 mm in diameter were strictly aerobic gram-negative organisms. Those which were oxidase negative and were able to grow with acetate but not glucose, mannose, maltose, or sucrose were assigned to *Acinetobacter calcoaceticus*. They were readily divided into two groups (4, 30, 34): one (var. *anitratus*) able to grow with xylose, quinate, L-leucine, adipate, and putrescine; the other (var. *lwoffii*) with more restricted metabolic capacity using only quinate. None of our strains grew with anthranilate, and we made no attempt to demonstrate carbohydrate utilization by prolonging incubation times beyond 48 h (cf. reference 20). Oxidase-positive strains belonged to the genus *Pseudomonas*. Neither of these groups of organisms will be discussed further here; we should point out, however, that *Acinetobacter calcoaceticus* is commonly found in our samples.

(ii) Bacteria that formed colonies larger than 3 mm in diameter, were mucoid, rugose, or spreading and light colored (pink, yellow, or orange) and were often with cream-colored margins were selected for further study. The vast majority were able to grow anaerobically with glucose, were oxidase and gram negative, and could be assigned to the several tribes within the family *Enterobacteriaceae*. They were uniformly negative for tryptophan deaminase, gelatin liquefaction, and generally for formation of indole and H_2S. They were uniformly positive for anaerobic growth with glucose, *o*-nitrophenyl-β-D-galactopyranoside hydrolysis, and formation of acid from glucose, mannitol, rhamnose, glucitol, amygdalin, and arabinose.

On the basis of their amino acid decarboxylase reactions and the Voges-Proskauer reaction, the isolates could clearly be divided into three groups embracing the genera *Klebsiella* and *Enterobacter*, *Erwinia*, and *Citrobacter* and *Escherichia*. In Table 1 we have assembled

the frequency of occurrence of characters for the six taxa, and in Table 2 we have shown their distribution at the eight localities.

On Tergitol-7 plates we occasionally isolated strains of *Proteus mirabilis* and *Aeromonas hydrophila* which were retained for testing in the acetylene reduction assay.

Nitrogen fixation occurrence. Our results have shown that a substantial fraction of new isolates of all of the taxa were able to synthesize nitrogenase under anaerobic conditions (Table 1). This capacity was, however, absent in three strains of H_2S-positive *Citrobacter freundii*, in the single strain of *P. mirabilis*, and in 10 strains of *Aeromonas hydrophila*. For the last three taxa, peptone (0.5 g/liter) was added to the culture media.

Values of the specific activities attained under nitrogen (Table 3) were comparable to those found for *K. pneumoniae* M5al, though comparison of our results with data for klebsiellas isolated from vegetables (7) or from paper mill effluent (22) is not possible since specific activities were not given by the authors. With plausible assumptions, however, our values seem to be comparable, though we have not systematically attempted to measure maximum values.

Lack of repression by amino acids. Experiments were carried out under argon in N-free medium supplemented with L-glutamate, L-aspartate, L-leucine, and Casamino Acids. All of these compounds supported good growth of representatives of each of the genera, though at the concentrations used (5 mM), the cell yield

TABLE 2. *The distribution and number of independent isolates of seven taxa of Enterobacteriaceae from the eight localities*

Taxon	Distribution and no. of isolates								
	Locality								
	1	2	3	4	5	6	7	8	Miscellaneous
Klebsiella pneumoniae	11	5	9		4	2	8	27	11
Enterobacter cloacae	6	4		1	5	1	7	2	2
Enterobacter aerogenes								1	
Erwinia herbicola		3	1		8		2		2
Citrobacter freundii	1	3		1	1		1		4
Citrobacter intermedius									1
Escherichia coli		1		1				1	1
Proteus mirabilis									1

TABLE 3. *Specific activities (nanomoles of C_2H_4 per minute per milligram of protein) of nitrogenase attained after 12 h in N-free medium supplemented with L-amino acids or Casamino Acids under argon, or in N-free medium under nitrogen*

Taxon	Strain no.	Compounds				
		Glutamate	Leucine	Casamino Acids	Aspartate	N_2
Klebsiella pneumoniae	62	95 (1.1)[a]	97 (0.7)	41 (0.4)	97 (0.6)	89 (0.8)
	133	124 (1.0)	89 (0.5)	13 (0.8)	98 (0.3)	73 (0.6)
Enterobacter aerogenes	161	88 (0.7)	104 (0.8)	34 (0.4)	6.6 (0.55)	64 (0.3)
Enterobacter cloacae	51	76 (0.75)	68 (0.7)	43 (0.75)	53 (1.1)	89 (0.9)
	115	89 (0.75)	74 (0.5)	52 (0.2)	56 (0.55)	129 (0.5)
Erwinia herbicola	49	5 (2.8)	12 (0.6)	0.2 (0.9)	0.1 (6.5)	10 (5.0)
	55	101 (1.5)	86 (1.6)	40 (0.8)	78 (1.25)	58 (ND)[b]
	93	90 (1.1)	127 (0.75)	53 (0.6)	49 (0.35)	167 (0.8)
	169	26 (0.4)	33 (0.5)	3.3 (0.1)	1.3 (0.4)	16 (2.8)
Citrobacter freundii	29	85 (0.7)	65 (0.7)	57 (0.2)	95 (0.1)	90 (0.15)
	97	75 (0.7)	70 (0.6)	37 (0.01)	61 (0.01)	88 (0.2)
	190	78 (1.1)	127 (0.9)	64 (0.4)	35 (0.3)	98 (1.3)
Citrobacter intermedius	250	62 (0.9)	57 (1.4)	33 (0.09)	1.6 (~0)	29 (0.3)
Escherichia coli	73[c]	58	59	6.5	30	36

[a] Numbers in parentheses give the ratios of activities measured after 24 h to those measured after 12 h.
[b] ND, Not determined.
[c] Measured only after 24 h due to low rate of enzyme derepression.

in media with L-aspartate or Casamino Acids was higher than that attained with L-glutamate or L-leucine. None of the compounds repressed nitrogenase synthesis by *K. pneumoniae, Enterobacter cloacae,* or *C. freundii.* L-Aspartate, however, appeared to repress synthesis in *Enterobacter aerogenes,* in two out of four strains of *Erwinia herbicola,* and in *Citrobacter intermedius* (Table 3). The higher specific activities observed in media supplemented with L-glutamate and L-leucine may plausibly be attributed to more severe nitrogen limitation; this is consistent with the results of the growth experiments.

Loss in acetylene-reducing activity. It was consistently noted that both activities and specific activities were frequently lower after 24 h of growth than after 12 h (Table 3, Fig. 1). For both strains of *Erwinia* in which repression by L-aspartate was observed and for one of the strains of *Escherichia coli,* activities measured after 24 h of growth under N_2 were higher than those attained after 12 h and were maximal. Further experiments were carried out with three strains: *C. freundii* 97, *K. pneumoniae* 133, and *C. intermedius* 250. It was found that inclusion in the assay medium of 10 mM L-glutamate, L-aspartate, L-alanine, L-valine, L-asparagine, L-glutamine, or ammonium acetate had essentially no effect on the acetylene-reducing activity of intact cells. This was observed not only during the period when the activity was increasing, but also in cultures in which the activity was only 25% of the maximum attained after about 12 h. Since all of these amino acids, with the possible exception of L-alanine, can serve as sole sources of nitrogen, it seems reasonable to conclude that effective systems exist for their transport into the cells. Thus, it appears that loss of N_2-fixing activity in intact cells cannot be attributed to inactivation by excessively high intracellular concentrations of any of these compounds.

Inclusion of chloramphenicol (10 μg/ml) in the assay medium had no effect on the rate of acetylene reduction, and addition to an already partly derepressed culture increased rather than decreased the rate of loss of activity.

Inactivation of acetylene-reducing activity by oxygen. All of the strains examined were somewhat tolerant towards inactivation of the N_2-fixing system by oxygen, though the degree of inactivation was highly variable (Table 4). Inactivation was partly reversible. The results of a typical experiment are given in Fig. 2, and data for representatives of the six taxa are assembled in Table 5.

At the higher concentrations of oxygen, inactivation was rapid and virtually complete (Fig. 3). After returning cultures to anaerobic conditions, some activity could always be recovered even at the highest concentrations of oxygen used; values of the specific activity indicate, however, that such cultures would be severely N limited. Inactivation by 4% oxygen appeared to be substantially reversible, although some irreversible destruction always occurred.

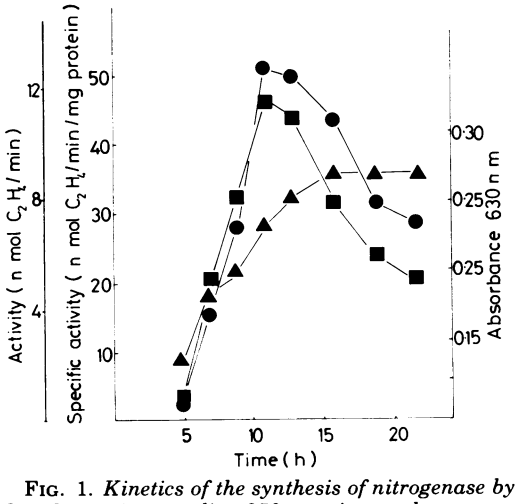

FIG. 1. *Kinetics of the synthesis of nitrogenase by Citrobacter intermedius 250 growing at the expense of nitrogen. Symbols: ●, Activity (nanomoles of C_2H_4 per minute); ■, specific activity (nanomoles of C_2H_4/ minute per milligram of protein); ▲, absorbance at 630 nm.*

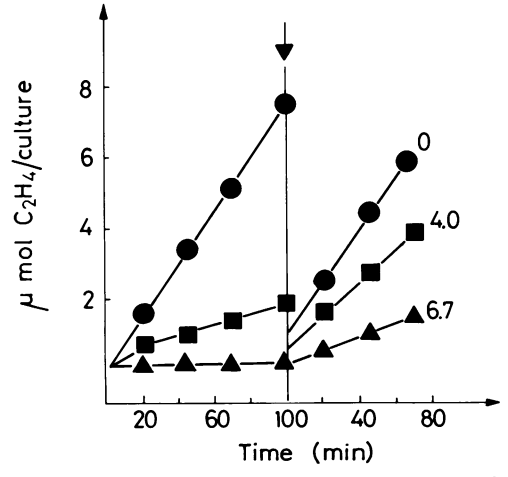

FIG. 2. *Reversibility of oxygen inactivation of the N_2-fixing system in Citrobacter intermedius 250. Acetylene reduction was measured at several oxygen tensions. Symbols: ●, Control; ■, 4% (vol/vol); ▲, 6.7% (vol/vol). At the time indicated by the arrow, the gas phase was replaced by argon, acetylene was added, and incubation was continued.*

Growth in air. For assessment of the capacity to grow aerobically at the expense of N_2, inocula of representative strains were grown anaerobically in Casamino Acids/glucose medium and transferred twice to N-free medium without thioglycolate. Acetylene-reducing activity could readily be detected after 7 h and a maximum was reached after 12 h. Generally, after 15 h the resazurin indicator became colorless.

DISCUSSION

Isolation and characterization. In the present study media based on galactitol were extensively used and proved to be highly effective. Although among our isolates only *K. pneumoniae* and *Enterobacter aerogenes* were able to grow with ribitol, it should be noted that *Escherichia coli* C, though not K-12 or B strains, has been shown to utilize ribitol (28).

TABLE 4. *Inactivation of N_2-fixing activity by oxygen in intact cells*

Organism	Strain no.	% Activity relative to anaerobic control			
		% Oxygen (vol/vol)			
		1.8	4.0	6.7	10.0
Klebsiella pneumoniae	62	55	13	5	4
	133	95	103	75	10
	159	93	13	3	1
	253	80	2	0.5	0
Enterobacter aerogenes	161	104	67	1.5	0
Enterobacter cloacae	51	56	19	2.5	1
	84	37	28	25	10
	115	95	69	2	0.5
Erwinia herbicola	49	66	3	0	ND[a]
	93	81	16	10	2
	169	12	0	0	ND
Citrobacter freundii	29	77	4	0	ND
	97	28	29	17	4
	190	0	0	0	ND
Escherichia coli	73	73	11	1	0
Citrobacter intermedius	250	43	3.5	1	0

[a] ND, Not determined.

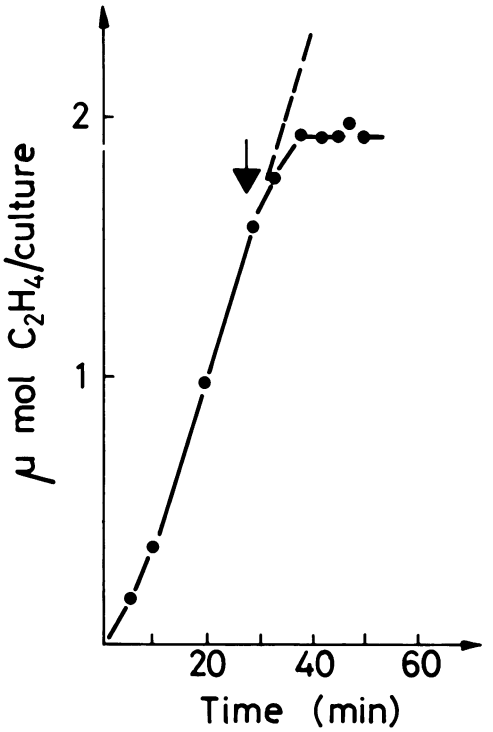

FIG. 3. *Kinetics of the inactivation of the N_2-fixing system of Citrobacter intermedius 250 by oxygen. At the time indicated by the arrow, oxygen (6.7% [vol/vol]) was introduced into a culture reducing acetylene anaerobically.*

TABLE 5. *Reversibility of oxygen inactivation of N_2-fixing activity in cultures grown anaerobically with N_2 and specific activities attained under microaerophilic conditions*

Taxon	Strain no.	% Anaerobic control activity recovered after a 70-min anaerobic incubation following 100 min of inactivation by oxygen[a]			Nitrogenase sp act (nmol of C_2H_4/min per mg of protein)
		% Oxygen (vol/vol)			
		4.0	6.7	10.0	
Klebsiella pneumoniae	253	56 (1.1)[a]	24 (114)	ND[b]	44
Enterobacter aerogenes	161	60 (1.2)	49 (70)	8 (∞)	12
Enterobacter cloacae	51	36 (52)	7 (102)	2 (∞)	62
Erwinia herbicola	93	72 (0.9)	74 (4.5)	19 (∞)	18
Citrobacter freundii	29	22 (28)	8 (63)	4 (98)	85
Citrobacter intermedius	250	67 (3)	25 (144)	3 (∞)	14
Escherichia coli	73	58 (2)	53 (2.5)	2 (∞)	0

[a] Numbers in parentheses are the ratios of activities measured after 70 min of incubation following the return to anaerobic conditions to those attained during 70 min of aerobic incubation.

[b] ND, Not determined.

Most of our isolates were phenotypically similar to established taxa given in *Bergey's Manual* (8), by Edwards and Ewing (13), by Bascomb et al. (2, 3), and by Johnson et al. (19). Few of the aberrant features seem to lie significantly outside the range allowed by the circumscriptions. Only some general comments seem necessary.

We have arbitrarily divided *K. pneumoniae* into two biotypes without making any suggestions as to their relation to those previously described. Biotype B differs from typical strains (biotype A) only in the absence of amino acid decarboxylase activities and in being unable to produce acid from ribitol. The organisms named *Enterobacter agglomerans* by Ewing and Fife (16) and named *Erwinia herbicola* by us were almost uniformly nonpigmented. It has been shown, however, (10) that incubation at 37°C induced formation of nonpigmented variants which were auxotrophic for thiamin. Since all of our incubations were carried out at this temperature and in the presence of yeast extract, we might expect a preponderance of such variants. Two distinct biotypes of *C. freundii* were isolated, one differing from typical types in being negative for production of both indole and H_2S but closely resembling those described by Crosa et al. (11). Only one isolate was assigned to *C. intermedius* (*Levinia amalonatica*). Typical strains of *Escherichia coli* were only occasionally found, and we have excluded those with atypical fermentation patterns. These are being studied further. The origin of these organisms remains unresolved. With the exception of *Escherichia coli*, all of these bacteria seem to be widely distributed in the environment. The total number of bacteria in the water coming into the mills is low but increases markedly during use from the availability of soluble organic compounds. *Escherichia coli* is rarely found and may be assumed to be an occasional fecal contaminant in the incoming water.

Finally, it should be pointed out that considerable genetic divergence can occur even within one taxon. For example, it has been shown that strains of diverse origin both of *Escherichia coli* (6) and of *K. pneumoniae* (31) showed a wide range of deoxyribonucleic acid homologies even though they were essentially indistinguishable in their biochemical reactions.

Nitrogen fixation. Hitherto, nitrogen fixation in bacteria isolated from paper mill process waters has been demonstrated only in *K. pneumoniae* (22). In the present study, high levels of activity have been demonstrated in *K. pneumoniae, Enterobacter cloacae, Enterobacter aerogenes, Erwinia herbicola, C. freundii, C. intermedius*, and *Escherichia coli* isolated from widely different localities. This appears to be the first report of nitrogen fixation by naturally occurring strains of *Escherichia coli*. Such a capacity would confer a selective advantage on these bacteria and may plausibly explain their dominance in our samples.

High levels of nitrogenase activity were found in cultures maintained under argon in media supplemented with L-amino acids; the highest activities were found with L-glutamate and L-leucine which are the poorest sources of nitrogen. These results are consistent with the view that amino acids do not generally repress nitrogenase synthesis in *K. pneumoniae* (35), and that formation of nitrogenase is regulated by derepression rather than by induction.

Incubation for periods longer than 12 h either in N-free medium under nitrogen or in amino acid-supplemented media under argon generally resulted in considerable loss of activity. At the concentrations of glucose used there can hardly be a limitation either of adenosine 5'-triphosphate or reductant. A similar loss of activity in *Rhodospirillum rubrum* could be induced with ammonium acetate, L-glutamine, or L-asparagine (25), but ammonium acetate and all of the amino acids tested were without effect on the nitrogen-fixing activity of *K. pneumoniae, C. freundii*, or *C. intermedius*. The experiments with chloramphenicol suggest that if loss of activity is due to proteolytic destruction of nitrogenase, the enzyme responsible must be present during the early stages of derepression; this seems rather unlikely. Unfortunately, none of the strains in which rapid loss of activity could be demonstrated were able to grow anaerobically with glycerol, citrate, malate, or L-aspartate. It has not, therefore, been possible to determine how loss of activity is related to the metabolism of other substrates. Because of the oxygen sensitivity of the isolated enzyme we have not attempted to measure enzyme activity in cell-free extracts.

Although we have no explanation for this loss in activity, the results suggest that determination of activities on samples removed from cultures grown longer than 12 h may lead to a substantial underestimation of nitrogen-fixing potential. Therefore, it is recommended that acetylene-reducing activity be measured routinely after 12 and 24 h of incubation.

All of the strains were somewhat tolerant to inactivation of nitrogen-fixing activity by oxygen: part of the activity could be recovered when anaerobic conditions were reestablished, though some irreversible destruction always occurred. Although oxygen represses synthesis of nitrogenase in *K. pneumoniae* (29), nitrogen

fixation has been demonstrated at oxygen tensions below 5 mm of Hg in liquid medium supplemented with yeast extract (21) and in air on a solid medium containing Casamino Acids (19). These findings are consistent with experiments which showed that at low oxygen tensions increased rates of respiration by *K. aerogenes* were able to make the culture transiently anaerobic (17). In a N-limited culture, nitrogenase synthesis would then be derepressed.

It has been shown that nitrogenase synthesis can take place under microaerophilic conditions in all of the strains of *Enterobacteriaceae* that were examined. Values of the specific activities were lower than those attained anaerobically and depend on a number of factors including the degree of nitrogen limitation, the oxygen sensitivity of the nitrogen-fixing system, and the rate of respiration. The protein concentration was about 25% of that found in cultures growing anaerobically with N_2 and was generally about 80 μg/ml. The importance of nitrogen fixation to the cell under natural conditions is hard to assess and depends on what mechanisms exist to produce and maintain lowered oxygen tensions. Probably the two most important are respiration and synthesis of polysaccharide under conditions of carbon excess and nitrogen limitation. Polysaccharide slime formation certainly occurs in paper mills and indeed can be a major problem. We feel that a combination of the potential for synthesis of nitrogenase of high specific activity, together with a degree of tolerance towards inactivation of the N_2-fixing system by oxygen, enables nitrogen fixation to make a significant contribution to the nitrogen economy of the cell under natural conditions. It may also provide the nitrogen required for the growth of non-N_2-fixing bacteria, e.g., *Acinetobacter*, which can also be found in substantial numbers in process waters.

ACKNOWLEDGMENTS

We thank Herrick Baltscheffsky for his continued interest and support and Agneta Assarsson and Margareta Wikström for expert assistance.

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