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SWEDISH WATER AND AIR POLLUTION RESEARCH LABORATORY

INSTITUTET FÖR VATTEN- OCH LUFTVÅRDSFORSKNING

HÄLSINGEGATAN 43 · BOX 21060 · S-100 31 STOCKHOLM · SWEDEN · TEL. 08-24 96 80
STEN STUREGATAN 42 · BOX 5207 · S-402 24 GOTHENBURG · SWEDEN · TEL. 031-81 02 80

APPLICATION OF THE NITROGEN-FIXING CAPACITY OF BACTERIA FOUND IN SLUDGE SAMPLES OBTAINED FROM BIOLOGICAL TREATMENT PLANTS HANDLING WASTE FROM THE PAPER AND FOOD INDUSTRIES

by

Alasdair Neilson

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INTRODUCTION

We have previously demonstrated that substantial numbers of nitrogen-fixing Enterobacteriaceae occur in paper mill process waters. These belong to several genera of the family and are relatively resistant to inactivation of the nitrogen-fixing system by oxygen.

In order to take advantage of the nitrogen-fixing capacity of these organisms either to diminish the requirement for the addition of a nitrogen source to biological treatment plants, or to utilize sludges containing these bacteria as soil improvement products, some further questions must be answered: 1. the range of carbon substrates which can be utilized by such organisms 2. the range of nitrogen compounds which can be assimilated and their effect on nitrogen fixation.

In this study, we have isolated nitrogen-fixing bacteria from a variety of sludge samples and studies their nutritional requirements with respect both to carbon and nitrogen sources. Studies on the regulation of nitrogenase synthesis are still underway.

MATERIALS and METHODS

Isolation of bacteria. Sludge samples were obtained from five different localities. Serial dilutions in phosphate buffer (0.02 M, pH 7.0) were prepared and spread on to the surface of plates of complex galactitol medium containing (g/l distilled water) : peptone (Orthana), 5.0: yeast extract, 3.0: galactitol, 5.0: sodium dodecyl sulphate, 0.1: bromothymol blue, 0.025: agar, 15.0. The pH was adjusted to 7.2 and after autoclaving, triphenyltetrazolium chloride solution (3 ml, 1 %) sterilized by filtration was added. After incubation at 35°C for 18 h, individual colonies were picked and pure strains isolated by re-streaking twice on tryptose-soy agar.

Characterization. Fermentative capacity was assessed from the results of growth in stabs of TSI agar and confirmed in a liquid glucose-casamino acids medium containing (g/l distilled water): 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: sodium thioglycollate, 0.5: resazurin, 0.001: casamino adids, 0.75. Glucose was sterilized separately as a 40 % solution and added aseptically to the basal medium after autoclaving (25 ml). Pure strains were tested for Gramstaining, presence of oxidase (Kovacs' method), capacity for anaerobic growth with glucose, 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 %. All incubations were carried out at 37 C.

Nitrogenase assay was carried out in sterile glucose-thioglycollate N-free medium (casamino acids omitted from the medium) (5 ml) in 25 ml sterile serum bottles inoculated with 0.5 ml of a culture grown overnight in glucose-casamino acids medium. The bottles were stoppered with rubber stoppers, sealed with crimp caps and the cultures 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, it was not expected that this would significantly repress nitrogenase synthesis, particularly after the concentration was lowered by growth. After 12 h, acetylene (2 ml) was added to each bottle, the samples shaken at 30 C for 30 min., the assay terminated by adding perchloric acid (0.5 ml, 20 %) and the ethylene assayed gas chromatographically using a Poropak Q column. The suitability of the method was checked using a culture of Klebsiella pneumoniae M5al kindly supplied by Professor J.R. Postgate. Throughout, we have assumed an equivalence between acetylene reducing and nitrogen fixing activity. Specific activities are given as n mol C_2H_4/min/mg protein.

Carbon substrate utilization. The strains were screened for their ability to use a variety of substrates as sole sources of carbon. The substrates were added to the mineral basal medium of Stanier, Palleroni and Doudoroff supplemented with pantothenate (5 µg/l), thiamin (5 mg/l), biotin (50 µg/l) and vitamin B_{12} (8 µg/l). In all cases, solutions of agar (Oxoid, special) and the mineral base (with or without substrate) were prepared separately at twice the final concentration, sterilized by autoclaving and mixed aseptically on cooling. Filter-sterilized stock solutions of carbohydrates and glycerol (10 % wt/vol) were added to the sterilized medium to give a final concentration of 1 %. The polyols, amino acids and malic acid were used at concentrations of 0.5 % and putrescine, quinate 4-hydroxybenzoate and acetate at 0.2 %. These substrates were added to the mineral

base and the pH adjusted to 7.0 before autoclaving. Starch was autoclaved with the agar. Plates were inoculated by streaking and growth assessed after incubation for 48 h at 37 C.

Utilization of nitrogen was carried out in liquid medium prepared from the mineral base to which glucose was added aseptically after autoclaving to give a final concentration of 3.0 g/l. Most of the nitrogen substrates were prepared as 100 mM stock solutions, the pH adjusted to 7.0 and sterilized by filtration. These were added to the mineral base to give a final concentration of 2 mM. At this concentration, we showed that growth is limited by nitrogen. Medium was dispensed (5 ml) into tubes (20 ml), the tubes inoculated with 0.1 ml of a sulture grown with ammonium chloride, shaken vigorously at 35 C and the absorbance measured after 24, 48, 72 h at 620 nm. Most substrates were used rapidly and growth could be assessed after 24 h: results of delayed growth are given in parentheses.

RESULTS

In Table 1, we have given the nitrogen-fixing organisms present in each sample, their number and the specific activity attained under anaerobic conditions. In Tables 2A and 2B, the results of the carbon utilization experiments are summarized for these bacteria and for representatives of taxa isolated from paper mill process waters. In Tables 3A and 3B, comparable results for nitrogen utilization are given.

DISCUSSION

The organisms. It is clear that substantial numbers of nitrogen-fixing organisms were found in all of the samples. Compared with the number of genera of Enterobacteriaceae found in paper mill process waters, however, the flora of the sludge samples is rather limited.

Klebsiella pneumoniae was by far the commonest organism and Citrobacter and Enterobacter virtually absent. The klebsiella strains also differed from those found in process waters: they were less mucoid, frequently reduced triphenyltetrazolium chloride to produce dark red colonies on the isolation medium and were somewhat aberrant in their biochemical properties. Two strains (303 and 337) were Voges-Proskauer negative, methyl red positive and unable to use citrate. Four others (253, 343, 373 and 375) were indole positive and could be assigned to the oxytoca biotype which has recently been shown to differ significantly from K. pneumoniae.

Utilization of carbon

Of the carbon substrates which we have examined, it is clear that a wide range can be used effectively as sole C source. The mono- and di-saccharides (except raffinose) as well as glycerol and sorbitol are universally used. The more bizarre substrates, 4-hydroxybenzoate, quinate, putrescine and ribitol are used only by strains of K. pneumoniae. Clear-cut utilization patterns for the other substrates are not obvious. It can be seen, however, that for the types of substrates most likely to be found at least in paper-mill process waters, limitation of growth of nitrogen-fixing bacteria by C-substrate limitation is unlikely.

Utilization of nitrogen

The results of experiments on nitrogen utilization show that nitrate, amino acids, purines, putrescine and glucosamine can almost always be used as sole sources of N. In addition, some less common substrates such as ethanolamine can occasionally be used. Studies on the effect of these compounds on the derepression of nitrogenase synthesis will be carried out in the future.

On the basis of the results obtained hitherto we conclude:

1. nitrogen-fixing bacteria belonging to the family Enterobacteriaceae are present in all of the sludge samples. They are found in numbers of 10^5 to 10^6/g wet weight sludge.

2. the specific activity of nitrogenase synthesized under anaerobic conditions by pure cultures is high (30-100 n mol C_2H_4/min/mg protein.

3. all of the strains are able to use a wide range of compounds as sole source of organic carbon. Sugars and related polyols are virtually universally used.

4. nitrate, nitrite, amino acids and purines, glucosamine are used as sole source of nitrogen. It is known that nitrogenase synthesis is not usually repressed by the presence of amino acids.

Table 1. Source of N_2-fixing Enterobacteriaceae, taxa isolated, nitrogenase activity and frequency of occurrence.

Locality	Taxon	API code number	Nitrogenase specific activity (nmol/min/mg)	Number of organisms ($\log_{10}$ N_2/g
Hallsta				
250	C.intermedia	1144513	33	5
251	E.herbicola	1004773	28	5
252	E.herbicola	5004773	20	5
253	K.pneum	5245773	52	5
254	K.pneum	1215773	43	5
Hammarby				
303	K.pneum	1016773	70	6
305	E.herbicola	0004533	53	4
306	K.pneum	1215773	106	5
Bjuv A (potato production)				
335	K.pneum	1215773	115	4
336	E.herbicola	1004423	93	5
337	K.pneum	5004773	68	4
338	E.herbicola	1004423	100	4
Hylte				
341	K.pneum	5215673	130	6
342	E.herbicola	1245533	39	6
343	K.pneum	5255773		6
344	E.herbicola	1045533	65	6
345	E.herbicola	1045533	35	6
346	E.herbicola	1045533	44	6
347	K.pneum	5215773	100	6
348	K.pneum	5205773	97	6
Bjuv B (pea production)				
372	E.cloacae	3104773	57	6
373	K.pneum	5245773	81	6
374	E.herbicola	1005533	97	5
375	K.pacum	5245773	67	5
376	E.herbicola	1004753	84	5

Table 2 A. Utilization of C-substrates by Enterobacteriaceae isolated from sludge samples.

		Ribose	Adonitol	Glycerol	Galactose	Lactose	Sucrose	Maltose	Raffinose	Cellobiose	Starch	Citrate	Hydroxybenzoate	Quinate	Xylose	Acetate	Mannitol	Dulcitol	Sorbitol	Lactate	Glucosanate	Glucosamine
C. intermedius	250	+	-	+	+	-	-	+	-	+	-	+	-	-	+	+	+	-	+	+	+	+
E. herbicola	305	+	-	+	+	-	+	+	-	+	-	-	-	-	+	-	+	-	+	+	+	+
	336	+	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	-	+	+	+	+
	376	+	-	+	+	+	+	+	+-	-	+-	+	-	+	+	+	+	+	+	+	+	+
K. pneumoniae	253	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
	306	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	337	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
	341	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	347	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	373	+	+	+	+	+	+	+	+	+	+-	+	+	-	+	+	+	-	+	+	+	+
E. cloacae	372	+	-	+	+	+	+	+	+	+	-	-	-	-	+	+-	+	+	+	+	+	+

	1)	2)
250	-	-
305	-	-
336	-	-
376	-	-
253	-	+
306	+	+
337	-	-
341	+	+
347	+	+
373	-	+
372	+	-

1) L-glutamate

2) Putrescine

Table 2 B. Utilization of C substrates by Enterobacteriaceae isolated from paper mill process waters.

		Ribose	Adonitol	Glycerol	Galactose	Lactose	Sucrose	Maltose	Raffinose	Cellobiose	Starch	Citrate	Hydroxybenzoate	Quinate	Xylose	Acitate	Mannitol	Dulcitol	Sorbitol	Lactate	Glucuronate	Glucosamine	L-glutamate	Putrescine
C. freundii	29	+	-	+	+	+	+	+	±	+	±	+	-	-	+	+	+	+	+	+	+	+	+	-
	190	+	-	+	+	+	+	+	+	+	±	+	-	-	+	+	+	+	+	+	+	+	+	-
	332	+	-	+	+	+	+	+	+	+	±	+	-	-	+	+	+	+	+	+	+	+	+	-
	378	+	-	+	+	+	-	+	-	-	-	+	-	-	+	+	+	-	-	+	+	+	-	+
E. cloacae	51	+	-	+	+	+	+	+	±	+	±	+	-	-	+	+	+	+	+	+	+	+	+	-
	84	+	-	+	+	+	+	+	+	+	±	+	-	-	+	+	+	+	+	+	+	+	+	-
	296	+	-	+	+	+	+	+	+	+	±	+	-	-	+	+	+	+	+	+	+	+	+	-
E. herbicola	93	+	-	+	+	+	+	+	+	+	±	+	+	+	+	±	+	+	+	+	+	+	±	-
	252	+	+	+	+	+	+	+	+	+	±	+	+	+	+	±	+	+	+	+	+	+	+	+
K. pneumoniae	62	+	-	+	+	+	+	+	±	+	±	+	-	-	+	+	+	+	+	+	+	+	+	-
	133	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+
	303	+	-	+	+	+	+	+	+	+	±	+	-	+	+	+	+	-	+	+	+	+	+	+
	343	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+
	373	+	+	+	+	+	+	+	+	+	±	+	+	-	+	+	+	-	+	+	+	+	-	+
E. aerogenes	161	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	-	+	+	+	+	-	+
E. coli	265	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	-	+	+	+	+	-	-
A. hydrophila	262	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+
	289	+	-	+	+	+	+	+	-	+	+	+	-	-	±	±	+	-	±	+	±	±	+	±

Table 3 A. Utilization of N-substrates by Enterobacteriaceae isolated from sludge samples.

		Nitrate	Nitrite	L-glutamate	L-aspartate	L-histidine	L-methionine	Adenosine	Inosine	Uridine	Urate	Putrescine	Ethanolamine	Glycine	Glycylglycine	HMT	Glucosamine
C. intermedius	250	-	-	+	+	-	(+)	+	-	-	-	-	-	(+)	(+)	(+)	(+)
E. herbicola	305	+	+	+	+	+	-	(+)	(+)	+	+	+	-	-	-	(+)	(+)
	336	+	+	+	+	+	+	+	+	+	+	(+)	(+)	+	(+)	(+)	-
	376	+	(+)	+	(+)	(+)	-	+	(+)	+	+	-	-	-	-	-	-
K. pneumoniae	253	+	+	+	+	+	(+)	+	+	+	+	+	-	-	(+)	(+)	+
	306	+	+	+	+	+	(+)	+	(+)	+	+	+	-	+	(+)	(+)	(+)
	337	+	+	+	+	+	(+)	+	+	+	+	+	-	(+)	(+)	(+)	(+)
	341	+	+	(+)	(+)	+	(+)	+	(+)	+	+	+	-	(+)	(+)	(+)	(+)
E. cloacae	372	+	+	(+)	+	-	-	+	-	+	+	-	-	(+)	(+)	-	+

(+) delayed growth after 24 h.

Table 3 B. Utilization of N-substrates by Enterobacteriaceae isolated from paper mill process waters.

		Nitrate	Nitrite	L-glutamate	L-aspartate	L-histidine	L-methionine	Adenosine	Inosine	Uridine	Urate	Putrescine	Ethanolamine	Glycine	Glycylglycine	HMT	Glucosamine
C. freundii	190	+	+	+	+	+	-	+	(+)	(+)	(+)	-	-	(+)	(+)	(+)	(+)
E. cloacae	51	+	+	+	(+)	(+)	(+)	+	(+)	(+)	+	(+)	(+)	(+)	(+)	(+)	+
	296	+	+	+	+	(+)	(+)	+	(+)	(+)	+	(+)	-	(+)	(+)	(+)	+
E. herbicola	93	+	+	+	+	(+)	-	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	252	+	(+)	(+)	+	+	-	(+)	(+)	+	+	(+)	-	(+)	(+)	(+)	(+)
K. pneumoniae	62	+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+
	133	(+)	(+)	-	-	+	-	(+)	+	(+)	+	(+)	(+)	(+)	(+)	(+)	(+)
	303	(+)	(+)	(+)	(+)	(+)	-	(+)	(+)	(+)	+	-	(+)	(+)	(+)	(+)	(+)
E. aerogenes	161	(+)	(+)	(+)	(+)	+	-	(+)	(+)	(+)	(+)	(+)	-	(+)	(+)	(+)	-

(+) delayed growth after 24 h.