The Taxonomic Position of *Obesumbacterium proteus*, a Common Brewery Contaminant

By F. G. PRIEST, H. J. SOMERVILLE*, J. A. COLE AND
J. S. HOUGH

Department of Biochemistry, University of Birmingham, Birmingham, B15 2TT

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SUMMARY

The mole fraction of guanine plus cytosine in the DNA from *Obesumbacterium proteus* was 48 %, similar to that of *Hafnia alvei*, *Salmonella gallinarum* and the RM bacterium. DNA:DNA competition studies indicated that *O. proteus* strains fall into two groups, both of which could reasonably be included in the family Enterobacteriaceae. The same two groups were apparent from a numerical taxonomic investigation of 19 *O. proteus* strains which included 16 brewery isolates. The simple matching coefficients (%S) were calculated from results of 50 biochemical and taxonomic tests, and a dendrogram was constructed. Every *O. proteus* isolate had the characteristics of the family Enterobacteriaceae. The two subgroups united at 86 % S, and formed a cluster with *Hafnia* and *Salmonella* strains at 72.5 % S. The difference between *Hafnia alvei* and *O. proteus* was almost entirely due to their different vigour rating. Furthermore, in a simulated brewery fermentation, two strains of *H. alvei* grew in a mixed culture with yeast. This was originally the defining characteristic of the genus *Obesumbacterium*.

We propose that reference to the genus *Obesumbacterium* should be discontinued, and that *O. proteus* should be placed in the genus *Hafnia* as *Hafnia protea* comb. nov.

INTRODUCTION

Obesumbacterium proteus has for many years been a taxonomic enigma (Shimwell, 1948; Shimwell, 1964). When first isolated it was named *Flavobacterium proteus* (Shimwell, 1936; Shimwell & Grimes, 1936). Its dissimilarity with other members of this genus led to the proposal of a new genus *Obesumbacterium* with a single species whose distinctive feature was an ability to grow alongside yeast during a brewery fermentation (Shimwell, 1964).

However, cultures of yeast and bacteria are not uncommon. A bacterium C119 grows with the yeast *Toropsula glabrata* in the stomachs of new-born lambs (R. W. White, personal communication), and synergism occurs between *Proteus mirabilis* and *Saccharomyces carlsbergensis* in a steady state continuous culture (Chao & Reilly, 1972). Thus the ability to grow alongside yeast in a brewery is a poor criterion for defining a genus and has the disadvantage of excluding closely related organisms because they have not been encountered in a brewery environment. A more rational classification of *Obesumbacterium proteus* has therefore been attempted.

* Present address: Borden Microbiological Laboratory, Shell Research Limited, Sittingbourne, Kent.

Table 1. *Strains used and their source*

Organism	Source*
<i>Obesumbacterium proteus</i> 500	NCIB 8769
<i>O. proteus</i> 501	NCIB 8770
<i>O. proteus</i> 502	NCIB 8771
<i>O. proteus</i> (16 strains†)	Brewery isolates
<i>Hafnia alvei</i> NCTC 8108	NCTC
<i>H. alvei</i> NCTC 8112	NCTC
<i>H. alvei</i> NCTC 9540	NCTC
<i>Escherichia coli</i> W1485	NCIB 9481
<i>E. coli</i> B	M. Jones-Mortimer
<i>Salmonella typhimurium</i> LT2	M. Jones-Mortimer
<i>S. gallinarum</i> NCTC 5453	NCTC
<i>S. pullorum</i> NCTC 5776	NCTC
<i>Klebsiella aerogenes</i> S203	J. A. Cole
<i>K. aerogenes</i> NCTC 8172	NCTC
<i>K. edwardsii</i> var. <i>edwardsii</i> NCTC 5054	NCTC
<i>K. edwardsii</i> var. <i>atlantae</i> NCTC 9496	NCTC
<i>Proteus mirabilis</i> P9	T. W. Young
<i>P. vulgaris</i>	Medical isolate
RM bacterium RM3	NCTC 10476
<i>Enterobacter aerogenes</i> NCTC 10006	NCTC
<i>E. aerogenes</i> HG4	Brewery isolate
<i>Chromobacterium typhiflavum</i> NCTC 9381	NCTC
<i>Citrobacter freundii</i> NCTC 79	NCTC
Organism C119	R. W. White

* NCTC, National Collection of Type Cultures; NCIB, National Collection of Industrial Bacteria.

† The individual *O. proteus* strain numbers are given in Fig. 4 and 5.

METHODS

Organisms and growth conditions

Obesumbacterium proteus strains were isolated from brewery pitching yeasts. The yeast was diluted in physiological saline, plated on to MacConkey agar containing 200 µg/ml actidione (Upjohn Co., Kalamazoo, Michigan, U.S.A.) and incubated at 30 °C for 48 h. Gram-negative, lactose-negative colonies were purified by single colony isolation on the above media and retained as presumptive *O. proteus* strains. These bacteria and 18 strains representing eight genera of Enterobacteriaceae were maintained at 4 °C by monthly sub-culture on nutrient agar slopes. Their names and origins are shown in Table 1.

Obesumbacterium proteus strains were grown in nutrient broth containing 0.5 % (w/v) glucose for 48 h at 30 °C on a reciprocal shaker. Other organisms were grown in minimal media (Clowes & Hayes, 1968) at their optimum growth temperatures. To prepare ¹⁴C-labelled DNA, bacteria were grown in minimal medium containing 0.5 % (w/v) Casamino acids (Difco) until the extinction at 625 nm was 0.6. Adenine (8-¹⁴C; 50 µCi/200 ml medium) was added and the cells grown until the extinction had doubled. The bacteria were harvested by centrifuging and washed twice with 0.01 M-potassium phosphate buffer (pH 6.0).

Simulation of a brewery environment

Hopped wort was prepared in the model brewery of the British School of Malting and Brewing and was collected aseptically in fermentation tubes (1 m × 5 cm diam.) in 1 l quantities. The specific gravity was 1.045. *Saccharomyces cerevisiae* NCYC 1062 was grown in media containing (g/l): yeast extract, 3; malt extract, 3; peptone, 10; and glucose, 10.

Sterile medium (i.e. in 2 l conical flasks) was seeded with 10^6 yeast/ml and incubated for 72 h on a reciprocal shaker at 25 °C. The yeast was centrifuged, washed in sterile water, and resuspended in sterile water. Bacterial cultures were grown in the same medium for 48 h at 25 °C, centrifuged, washed, and resuspended in sterile isotonic saline (0.85 %, w/v).

Fermentation tubes were inoculated with 10^5 bacteria/ml and 10^7 yeast/ml and incubated at 18 °C. Samples taken through a suba seal stopper fitted halfway up the tubes were plated on to nutrient agar containing 100 µg actidione/ml after dilution in physiological saline. The plates were incubated at 30 °C for 48 h.

Conventional taxonomy and data analysis

All cultures were subjected to 50 physiological and biochemical tests as described by Bascomb, Lapage, Willcox & Curtis (1971) with two exceptions: motility was sought by phase contrast microscopy, and the carbohydrate concentration in fermentation tests was 0.5 % (w/v). All properties were recorded as positive or negative with weakly positive results scored positive. Tests that gave either all positive or all negative results for the 37 strains were not included in the calculation of the similarities.

Coded data were analysed on an Elliot 4130 computer at the Microbial Systematics Unit, Leicester University, to obtain simple matching coefficients (Sokal & Michener, 1958). A second programme used the average linkage cluster technique to sort the organisms into groups of high phenetic similarity (Sokal & Sneath, 1963), and a dendrogram was constructed.

The proportion of positive results displayed by each organism was calculated as a measure of its 'vigour' (Sneath, 1968). A second similarity matrix was constructed eliminating the influence of vigour differences between the organisms and from this the average linkage cluster technique was used to develop a second dendrogram.

DNA preparation, buoyant density and hybridization

DNA was prepared as described by Somerville & Jones (1972) and was stored at 4 °C over chloroform. Sheared DNA of molecular weight approximately 5×10^5 was prepared by passage through a French Press at 10000 to 15000 lb/in² (McCarthy & Bolton, 1963) and stored at -16 °C.

The buoyant density of DNA in caesium chloride was measured in a Spinco Model E analytical ultracentrifuge (Schildkraut, Marmur & Doty, 1962). *Proteus mirabilis* DNA was used as a reference either alone or with *Klebsiella aerogenes* s 203 DNA. The equation developed by De Ley (1970) was used to calculate the GC content from the buoyant density data.

DNA was immobilized on filters as described by Johnson & Ordal (1968). DNA (50 µg/ml, 87 ml) was denatured by boiling for 10 min in $0.1 \times$ SSC (SSC is 0.015 M-trisodium citrate and 0.15 M-NaCl) and was quickly cooled by diluting to 5 µg/ml with cooled $6 \times$ SSC. The solution was passed (60 ml/min) through a 15 cm Millipore HAWP filter. When the filter was almost dry, 30 ml of 0.02 % (w/v) bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex), 0.01 % (w/v) polyvinylpyrrolidone (Calbiochem, La Jolla, California, U.S.A.), 0.02 % (w/v) Ficoll (Pharmacia, Uppsala, Sweden) in $2 \times$ SSC was added, and after standing for 10 min, the filter was sucked dry. After drying in air at 20 °C for 16 h small filters (10.5 mm diam.) were punched from the large filter.

For hybridization experiments, 1 µg sheared, radioactive DNA in 0.05 ml $0.1 \times$ SSC and 150 µg sheared competitor DNA in 0.15 ml $0.1 \times$ SSC were added to 12 $\times$ 50 mm glass vials. Competitor DNA was omitted from some control tubes; others contained homologous

Table 2. *The GC content of DNA from Obesumbacterium proteus and some enterobacteria*

Organism	Mol % GC $\pm$ 1 %
<i>Obesumbacterium proteus</i> 501	48.0
<i>O. proteus</i> 502	48.5
<i>O. proteus</i> 510	48.0
<i>O. proteus</i> 511	48.0
<i>Hafnia alvei</i> NCTC 8105	48.0
<i>H. alvei</i> NCTC 9540	48.5
RM bacterium NCTC 10476	47.5
Organism CI 19 (three strains)	49 to 50
<i>Salmonella gallinarum</i>	50.0

* The figure for *S. gallinarum* has been taken from Hill (1966).

competitor DNA. The tubes were stoppered and heated in a boiling water bath for 10 min and then cooled quickly on ice. To each tube 0.05 ml 9.6 $\times$ SSC was added followed by a 10.5 mm filter carrying unsheared DNA from the same organism as the radioactive DNA. Incubations were for 16 h at 63 to 67 °C. Filters were washed three times with 2 $\times$ SSC at the temperature of incubation, blotted and dried at 60 °C for 1 h. The radioactivity was measured using 0.7 % (w/v) butyl PBD [2-(4'-*t*-butylphenyl)-5-(4¹¹-biphenyl)-1,3,4-oxadiazole] (Intertechnique Ltd, Portslade, Sussex) in toluene as scintillation fluid.

Results are expressed as % competition defined as:

$$\% \text{ competition} = \frac{x-y}{x-z} \times 100,$$

where x = radioactivity bound to filter in absence of competitor, y = radioactivity bound in presence of heterologous competitor, z = radioactivity bound with homologous competitor (Ballard *et al.* 1970). Each determination was the average of two duplicates.

Following a suggestion by Professor N. J. Palleroni, the following procedure was developed to determine the thermal dissociation profile. After counting, the filters were washed five times in toluene, blotted dry, washed three times in 2 $\times$ SSC at room temperature and once in 2 $\times$ SSC at the temperature of hybridization. Each filter was added to 0.5 ml 2 $\times$ SSC at a temperature approximately 5 °C higher than that of the last wash. After 10 min the filter was removed, blotted and added to fresh 2 $\times$ SSC at the same temperature. The temperature was raised by steps of about 5 °C and the process repeated to a maximum of 100 °C. Samples from the tubes containing dissociated DNA were then counted in 0.8 % (w/v) butyl PBD, 8.5 % (w/v) naphthalene (scintillation grade, Koch-Light Laboratories, Colnbrook, Buckinghamshire) in dioxane. The radioactivity is expressed as % of total disintegrations/min dissociating up to, and including, 100 °C. Less than 25 % of the radioactivity from homologous hybridizations remained bound at 100 °C and less than 5 % from heterologous hybridizations.

RESULTS

The results of 50 biochemical and physiological tests for *Obesumbacterium proteus* strains 502 and 510 were compared with those of all organisms recorded at the Computer Identification Centre. Three members of the family Enterobacteriaceae were named as nearest relatives, *Hafnia alvei*, the RM bacterium and *Salmonella gallinarum*.

Closely related organisms should contain essentially the same DNA GC content. DNA

Table 3. % Competition of DNA from various organisms with *Obesumbacterium proteus* DNA

Competitor DNA	Radioactive DNA and DNA on filter isolated from	
	<i>Obesumbacterium proteus</i> 502*	<i>Obesumbacterium proteus</i> 511†
<i>Obesumbacterium proteus</i> 511	100	100‡
<i>O. proteus</i> 502	100	100‡
<i>O. proteus</i> 501	77	85‡
<i>O. proteus</i> 510	100	95‡
<i>O. proteus</i> 530	65	20‡
RM bacterium	80	29‡
<i>Hafnia alvei</i> NCTC 8112	70	82‡
<i>Salmonella gallinarum</i>	17‡	0
<i>S. typhimurium</i> LT2	7‡	—
<i>Escherichia coli</i> W1485	53	21‡
<i>E. coli</i> B	81‡	59‡
<i>Klebsiella aerogenes</i> S203	17‡	—
<i>Proteus mirabilis</i>	0‡	0‡
<i>P. vulgaris</i>	21	5‡

* Tubes were incubated for 14 h at 65 °C.

† Tubes were incubated for 12 h at 65 °C.

‡ Average from two separate experiments.

from *Obesumbacterium proteus*, *Hafnia alvei* and the RM bacterium contained 48 % GC whereas that from *Salmonella gallinarum* and three strains of organism C119 contained slightly more GC (Table 2). These organisms were therefore included as reference strains for further studies

DNA hybridization

Table 3 shows the effect of excess DNA from various organisms on the binding of radioactive *Obesumbacterium proteus* DNA to filters containing homologous, unlabelled DNA. *Obesumbacterium proteus* 502, 510 and 511 gave 100 % competition with *O. proteus* 502 and 95 to 100 % competition with *O. proteus* 511. Strains 501 and 530 competed less effectively, but strain 501 showed greater competition than 530 with 511 DNA. *Hafnia alvei*, the RM bacterium and two strains of *Escherichia coli* gave 53 to 81 % competition with *O. proteus* 502, and 21 to 82 % competition with *O. proteus* 511 (Table 3).

When the radioactive DNA and the DNA bound to the filter were isolated from *Escherichia coli* W1485, *Obesumbacterium proteus* 502, 510 and 511 DNA gave 27 to 43 % competition, but DNA from strains 501 and 530 gave only 8 % and 11 % competition respectively (Table 4). The competition shown by strains 502, 510 and 511 was within the range found with the enterobacteria listed in Table 4.

An alternative approach to competition is provided by comparing the direct binding of heterologous and homologous DNA (Brenner *et al.* 1969). Preparations of DNA from *Escherichia coli* W1485 and *Obesumbacterium proteus* 502 were compared by this method under the same conditions used for competition, but in the absence of competitor DNA (Table 5). *Obesumbacterium proteus* DNA showed greater ability to associate with *E. coli* DNA, relative to homologous reassociation, than did *E. coli* DNA in the reciprocal experiment. A possible interpretation of these results is that *O. proteus* may have a smaller complement of DNA than *E. coli*.

Table 4. % Competition of DNA from various organisms with DNA from *Escherichia coli* W1485

Competitor DNA	Experiment 1*	Experiment 2*
<i>Obesumbacterium proteus</i> 511	30	—
<i>O. proteus</i> 502	43	22
<i>O. proteus</i> 501	11	—
<i>O. proteus</i> 510	27	—
<i>O. proteus</i> 530	8	—
RM bacterium	28	—
<i>Hafnia alvei</i> NCTC 8112	22	35
<i>Salmonella typhimurium</i> LT2	63	—
<i>Escherichia coli</i> W1485	100	100
<i>E. coli</i> B	93	79
<i>Klebsiella aerogenes</i> S203	—	27
<i>Proteus mirabilis</i>	50	—
<i>P. vulgaris</i>	0	—

* Tubes were incubated for 16 h at 63 °C in experiment 1 and for 16 h at 67 °C in experiment 2.

Table 5. Direct binding of radioactive DNA to homologous and heterologous DNA on a filter

Hybridization was carried out at 67 °C under the conditions used in Tables 3 and 4 but without competitor DNA.

DNA on filter	Radioactive DNA in solution	Radioactivity bound to filter (c.p.m.)	Relative reassociation (%)
<i>Escherichia coli</i> W1485	<i>E. coli</i> W1485	405	100
<i>Obesumbacterium proteus</i> 502	<i>E. coli</i> W1485	74	18
<i>O. proteus</i> 502	<i>O. proteus</i> 502	519	100
<i>O. proteus</i> 502	<i>E. coli</i> W1485	265	51

The thermal dissociation profiles were determined for DNA duplexes formed under conditions similar to those used in the preceding experiments (Fig. 1*a, b, 2*). The temperature for 50 % dissociation (apparent T_m) was 90 to 92 °C for homologous duplexes, 78 to 85 °C for heterologous duplexes between radioactive DNA from *Escherichia coli* W1485 and *Obesumbacterium proteus* 502 DNA on the filter, and 85 °C for duplexes between radioactive *O. proteus* 502 DNA and *E. coli* W1485 DNA. The dissociation profiles may be slightly displaced, particularly for the heterologous duplexes, as some radioactivity (< 20 %) was found in the buffer after the first incubation at 68 °C: this has not been represented in the diagrams. The apparent T_m may therefore be 2 to 3 °C too high for the heterologous duplexes.

Numerical taxonomy

A dendrogram was constructed from the simple matching coefficients (% S) (Fig. 3). It is evident that all the *Obesumbacterium proteus* strains form a cluster at the 73 % S level which includes *Salmonella gallinarum* and *S. pullorum*. The three *Hafnia alvei* strains unite at the 82 % S level and join the *O. proteus* strains at 72.5 % S. The RM bacterium shows greatest affinity to *Escherichia coli*; and *Chromobacterium typhiflavum*, *S. typhimurium* and *Citrobacter freundii* form solitary phenons at 75 % S. Similarly the two *Proteus* species form a single group whilst organism C119 joins the *Klebsiella* phenon at 80 % S. Finally the two *Enterobacter* species, comprising a typical strain and a brewery isolate, form their own cluster. Above the 86 % S level the *O. proteus* strains fall into two major groups from which the avian salmonellae are excluded. Although they were isolated from widely different

Fig. 1. Thermal dissociation of DNA duplexes formed with radioactive DNA from *Escherichia coli* w1485, and unlabelled DNA from *Obesumbacterium proteus* 502 or *E. coli* w1485 on the filter. (a) Hybridization carried out at 63 °C; (b) hybridization carried out at 67 °C. The bars indicate results from duplicate experiments. Experimental details are given in the Methods.

Fig. 2. Thermal dissociation of DNA duplexes formed with radioactive DNA from *Obesumbacterium proteus* 502, and unlabelled DNA from *Escherichia coli* w1485 or *O. proteus* 502 on the filter. Hybridization was carried out at 67 °C. The bars indicate results from duplicate experiments.

geographical locations, *O. proteus* strain 501 was identical to strain 510 and 520 identical to strain 531 over the range of tests chosen.

Vigour estimates of all the strains examined were expressed as the proportions of positive results (Table 6). *Obesumbacterium proteus* strains grew slowly, even in rich media, and this is reflected in their low vigour ratings. Only *Salmonella gallinarum*, *S. pullorum* and the RM

Fig. 3. Dendrogram derived using the simple matching coefficient, and the unweighted average linkage sorting method of cluster analysis. NCTC, National Collection of Type Cultures.

Fig. 4. Dendrogram derived only from the difference in pattern between organisms (Sneath, 1968), clustered using the unweighted average linkage sorting method.

Table 6. *Vigour estimates of Obesumbacterium proteus and reference organisms*

Organism	Vigour*	Organism	Vigour
<i>O. proteus</i> 570	0.385	<i>Enterobacter aerogenes</i> HG4	0.641
502	0.308	<i>E. aerogenes</i> NCTC 10006	0.615
511	0.282	<i>Citrobacter freundii</i> NCTC 9750	0.615
513	0.282	<i>Klebsiella edwardsii</i> NCTC 5054	0.615
580	0.282	<i>K. edwardsii</i> NCTC 9496	0.590
501	0.256	<i>K. aerogenes</i> NCTC 8172	0.564
510	0.256	<i>Proteus mirabilis</i> P9	0.573
540	0.256	<i>P. vulgaris</i>	0.487
532	0.256	<i>C. typhiflavum</i> NCTC 9381	0.487
521	0.256	Organism C119	0.487
522	0.256	<i>Hafnia alvei</i> NCTC 9540	0.487
530	0.231	<i>Escherichia coli</i> B	0.462
512	0.231	<i>H. alvei</i> NCTC 8112	0.436
500	0.231	<i>Salmonella typhimurium</i> LT2	0.436
520	0.205	<i>H. alvei</i> NCTC 8105	0.410
560	0.205	RM bacterium NCTC 10476	0.282
531	0.205	<i>S. pullorum</i> NCTC 5776	0.282
550	0.179	<i>S. gallinarum</i> NCTC 5453	0.231
561	0.179		

* The vigour of organisms was estimated according to Sneath (1968).

bacterium showed approximately equivalent levels of metabolic activity. The *Enterobacter* and *Klebsiella* species had the highest vigour ratings and fastest growth rates. The dendrogram derived from differences in metabolic pattern only is similar to that based on the total differences between the strains but the % S levels at which clusters are formed is higher (Fig. 4). Many of the *O. proteus* strains have identical patterns of metabolic activity. The two major groups are again evident and coalesce at 90 % S. The characteristics of the two *O. proteus* subgroups differ principally in the fermentation of mannitol, salicin and xylose (Table 7). The reactions for *Hafnia alvei* (Bascomb *et al.* 1971) are included for comparison.

With the exception that the characteristics of the *edwardsii* and *atlantae* varieties of *Klebsiella edwardsii* were identical in Fig. 4 but not in Fig. 3, the relationships between the marker strains were similar in both dendrograms. Organism C119 appeared to be identical to four *Obesumbacterium proteus* strains when the vigour component of the similarity was excluded, but *Salmonella gallinarum* and *S. pullorum* showed only 78 % S and 75 % S respectively.

Growth of bacteria in simulated brewery fermentations

Sterilized wort was inoculated with yeast and one of several species of enterobacteria. Numbers of surviving bacteria were determined after incubation at 30 °C for various times (Fig. 5). The population of *Obesumbacterium proteus* rose during the first 40 h of the fermentation, but subsequently fell. This decline in viable count was probably caused by the low pH and high alcohol concentration of the wort (see also Strandkov, Baker & Bocklemann, 1953; Case, 1965). *Hafnia alvei* grew during the first 30 h of the fermentation, and therefore appeared to be tolerant to the presence of the yeast. *Enterobacter aerogenes* failed to grow to any significant extent, and the viable count of the RM bacterium and of *Citrobacter freundii* declined throughout the incubation period.

Table 7. The defining characteristics of the *Obesumbacterium proteus* groups and *Hafnia alvei*

	Group 1	Group 2	<i>Hafnia alvei</i>
Acid from PWS adenitol	—	—	—
arabinose	—	d	+
cellobiose	—	—	d
dulcitol	—	—	+
glycerol	+	+	d
inositol	—	—	—
lactose	—	—	—
(ONPG)	—	—	+
maltose	+	+	+
mannitol	+	—	+
raffinose	—	—	—
rhamnose	+	+	+
salicin	+	—	—
sorbitol	—	—	d
starch	—	—	—
sucrose	—	—	—
trehalose	+	+	+
xylose	—	+	d
Gas from glucose PW	—	—	+
Motility 37 °C	—	—	d
Motility 30 °C	—	—	d
Growth on MacConkey agar	+	+	+
Liquefaction in gelatin 1 to 5 days	—	—	—
Liquefaction in gelatin 5 to 30 days	—	—	—
Citrate utilization	—	—	+
KCN tolerance	d	d	+
Gluconate	—	d	+
Malonate	—	—	d
Urease	—	—	—
Indole	—	—	—
H ₂ S (TSI Agar)	—	—	—
H ₂ S paper (nutrient broth)	d	d	d
Arginine decarboxylase	—	—	d
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
MR 37 °C	d	—	d
MR 30 °C	d	—	d
VP 37	—	—	d
VP 30	—	—	+

+, 90 to 100 % of strains yield positive result; d, 11 to 89 % of strains yield positive result; —, 0 to 10 % of strains yield positive result. PW, peptone water; PWS, peptone water and appropriate sugar; MR, methyl red test; VP, Voges-Proskauer test; TSI, triple sugar iron medium; ONPG, σ -nitrophenyl- β -D-galactoside.

DISCUSSION

The taxonomic position of *Obesumbacterium proteus* has been problematic since it was first isolated: Shimwell (1948) even suggested its exclusion from the Eubacteriales. A reassessment is now possible on the basis of its DNA GC content, DNA/DNA competition experiments and Adansonian analysis of results of 50 tests with 19 *O. proteus* isolates and 18 reference strains.

Conventional biochemical and physiological tests established that *Obesumbacterium proteus* fulfills the criteria defining the family Enterobacteriaceae and shows a high overall level of similarity with other members. The 19 strains formed two clusters which coalesce

Fig. 5. Growth of *Obesumbacterium proteus* (▲—▲), *Hafnia alvei* (□—□), the RM bacterium (●—●), *Enterobacter aerogenes* (△—△), and *Citrobacter freundii* (○—○) with yeast in a simulated brewery environment.

at 86 % S. Their DNA GC content was not inconsistent with their assignment to the Enterobacteriaceae. DNA/DNA competition studies again indicated a possible subdivision of *O. proteus* into two groups, one consisting of strains 502, 510 and 511 (Group 1) and the other of strains 501 and 530 (Group 2) (Tables 3 and 4). Comparison with other members of Enterobacteriaceae indicate that the *O. proteus* strains can rightly be included in this family. Group 1 of *O. proteus* would appear to be more closely related to *Escherichia coli* than Group 2. The studies are not extensive enough to indicate the exact position of *O. proteus* relative to members of the Enterobacteriaceae.

The relationships between polynucleotides from members of Enterobacteriaceae have been studied in detail by Brenner and co-workers (Brenner, Martin & Hoyer, 1967; Brenner & Cowie, 1968; Brenner *et al.* 1969). In the present work, heterologous duplexes formed at 63 °C and 67.5 °C between DNA from *Obesumbacterium proteus* and *Escherichia coli* showed T_m values 8 to 13 °C below that of homologous duplexes (Fig. 1 a, b, 2). Homologous duplexes had T_m values of 90 to 92 °C, close to that expected for DNA with the base composition of 48 % GC (Marmur & Doty, 1961). The thermal stability of duplexes between *E. coli* DNA and *O. proteus* DNA was similar whichever DNA preparation was radioactive.

The value of vigour and pattern analysis has again been substantiated. When the high vigour displayed by *Hafnia alvei* is excluded from the calculation of the similarities, the pattern difference with *Obesumbacterium proteus* almost disappears. Thus *H. alvei* NCTC 8112 is identical to *O. proteus* strains 512 and 580 and *H. alvei* NCTC 9540 joins at the 97 % S level. Conversely *Salmonella gallinarum* and *S. pullorum*, organisms of low vigour, showed a high overall similarity with *O. proteus*, but on pattern difference alone were more distantly related. Organism C119 has a high vigour rating and, using the simple matching coefficient, it shows high affinity with the Klebsiellae. When the vigour component is excluded from the calculation of the similarity, it identifies with four *O. proteus* strains. Organism C119 has a GC content of 49 %, very similar to the *O. proteus* value of 48 % and markedly different from the value of 55 % for the Klebsiellae (Hill, 1966).

Hafnia alvei strains NCTC 8105 and 8112 both grew with yeast in a simulated brewery fermentation, and therefore fulfilled the original criterion for the genus *Obesumbacterium* (Shimwell, 1964). We suggest that reference to the genus *Obesumbacterium* be discontinued and that *O. proteus* be included in the genus *Hafnia* and renamed *Hafnia protea* comb. nov. Although both dendrograms (Fig. 3, 4) and results of DNA/DNA competition experiments suggest that the *O. proteus* strains fall into two groups, no differences in their activity in the brewery can be discerned. They should therefore be regarded as belonging to a single species.

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