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# Phenotypic and genotypic differences between certain strains of *Clostridium acetobutylicum*

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**Abstract**

It has become evident that several of the strains of *Clostridium acetobutylicum* that have been employed in physiological studies of the acetone-butanol fermentation, are heterogeneous. Studies of the phenotypic and genotypic characteristics of several of these strains (involving *inter alia* both pyrolysis mass spectrometry and 16S rRNA sequence determinations) demonstrated that the type strain obtained from ATCC was not identical with that supplied by NCIMB, and that NCIMB $8052^T$ is in fact *Clostridium beijerinckii*. We therefore suggest that the name *Clostridium acetobutylicum* should be restricted to those strains that are genetically closely related to ATCC $824^T$ (which include strains DSM 792 and DSM 1731 but not strain P262).

*Keywords:* *Clostridium acetobutylicum*; Pyrolysis mass spectrometry; 16S rRNA; Phylogeny

## 1. Introduction

The large-scale commercial exploitation of the acetone-butanol fermentation that was set in train by Weizmann in 1916 at the behest of the UK government, exploited a starch-utilising clostridial isolate (BY) that was later designated as a strain of *Clostridium acetobutylicum.* Since then a succession of production strains and many original isolates that resemble the Weizmann strain in producing acetone and butanol (together with a little ethanol) from starchy substrates have all been assigned to the same species [1]. Certain isolates that were better able to ferment sucrose (molasses) were designated as strains of *Clostridium saccharoperbutylacetonicum* [2], whilst *Clostridium beijerinckii* (*Clostridium butylicum*) known to be genetically distinct from *Clostridium acetobutylicum* differed from it relatively little in phenotypic characteristics, though some strains produced *iso*propanol in place of acetone, and solventogenesis could apparently be accomplished at neutral pH [3]. *Clostridium aurantibutyricum* produced both acetone and *iso*propanol together with *n*-butanol, *Clostridium tetanomorphum* produced ethanol plus *n*-butanol but little or no acetone, whilst the pectinolytic *Clostridium puniceum* produced *n*-butanol as the major product of fermentation of glucose or starch together with only small

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quantities of acetone and ethanol. Even organisms more usually notable for their acidogenic butyric acid fermentations could, under suitable growth conditions, be observed to produce acetone and *n*-butanol, e.g. *Clostridium butyricum* and *Clostridium pasteurianum*. The genus *Clostridium* as currently delineated is extraordinarily heterogeneous [4], and attempts to determine phylogenetic relationships are being made via DNA/DNA hybridisation studies and 16S rRNA sequence similarities [5]. The solventogenic saccharolytic clostridia will undoubtedly be multirooted in any phylogenetic tree that might be constructed, and it will be particularly interesting to discover how many of the recorded strains of *Clostridium acetobutylicum* will prove to be true members of this species.

The purpose of the present study was far less ambitious. During an investigation of the physiological behaviour of *Clostridium acetobutylicum* NCIMB $8052^T$ (type strain) in continuous flow culture, it became evident that this strain differed substantially from some other strains of *C. acetobutylicum* that had been examined in a like manner [6]. In particular, though it was supposedly identical with type strain ATCC $824^T$ this was manifestly not the case [6]. It is the chief purpose of the present paper therefore to report the findings made in a limited comparative study of phenotypic and genotypic differences between the few strains of *C. acetobutylicum* that have most often been used in physiological and genetic studies of solventogenesis by this organism.

## 2. Materials and methods

### 2.1. Organisms and cultivation

The various strains of *Clostridium acetobutylicum* were obtained from a variety of sources. Strains ATCC $824^T$, DSM 1731, and NCIMB $8052^T$ were purchased from the respective culture collections, viz. American Type Culture Collection, Rockville, MD, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, National Collections of Industrial and Marine Bacteria, Aberdeen, UK. Strain P262 (i.e. NCP 262) was kindly supplied by Prof. D.R. Woods, University of Cape Town, South Africa, and strain NI-4081 [7] was donated by Dr. M. Sebald, Institut Pasteur, Paris. Strain AA219 *gutD::erm* and its origin have been described previously [8].

All of the strains were maintained as well-sporulated slant cultures on Reinforced Clostridial Medium (RCM; Lab M). For most purposes they were grown anaerobically at 37°C in clostridial basal medium, CBM [9], solidified as appropriate with 1.5% (w/v) agar.

### 2.2. Pyrolysis mass spectrometry (PyMS)

Disposable plastic loops were used to remove quantities of organisms from the upper surfaces of one or more well-isolated colonies, with great care being taken to avoid the surface of the agar-solidified medium. The samples were evenly applied onto iron-nickel foils to give a thin uniform surface coating. Prior to pyrolysis the samples were oven-dried at 50°C for 30 min. Samples were analysed five times. The pyrolysis mass spectrometer used was the Horizon Instruments PYMS-200 ×; for full operational procedures see Goodacre et al. [10]. The sample tube carrying the foil was heated, prior to pyrolysis, at 100°C for 5 s. Curie-point pyrolysis was at 530°C for 3 s, with a temperature rise time of 0.5 s. Data were collected over the $m/z$ range 51 to 200 and normalised to the total ion count to remove any influence of sample size.

The normalised data were processed with the GENSTAT package which runs under Microsoft DOS 6.2 on an IBM-compatible PC. The method has been described previously [11]. In essence, the first stage was the reduction of the data by principal components analysis keeping only those principal components whose eigenvalues accounted for more than 0.1% of the total variance. Canonical variates analysis then separated the samples into groups on the basis of the retained principal components and some *a priori* knowledge of the appropriate number of groupings. The next stage was the construction of a percentage similarity matrix by transforming the Mahalanobis' distance between *a priori* groups in canonical variates analysis with the Gower similarity coefficient $S_G$. Finally, hierarchical cluster analysis was employed to produce a dendrogram, using average linkage clustering.

*2.3. Sequence analysis*

The SEQNET facility at Daresbury was employed to analyse DNA sequences abstracted from the GenBank database using the Bestfit and Gap programs of the GCG Sequence Analysis Software Package [12].

*2.4. Determination of 16S rRNA gene sequences*

This was performed as previously described [13]. The generated sequences were aligned and similarity values were determined using the Phylip (version 3.5c) computer program run on Digital VAX-VMS (version V5.5). Nucleotide substitution rates ($K_{nuc}$) were calculated and the strains were incorporated into an unrooted phylogenetic tree constructed as in previous studies [5] by the Neighbour-Joining method [14]. The 16S rRNA gene sequences determined in the present study are available from the EMBL Data Library under the following accession numbers; X78070 *C. acetobutylicum* ATCC 824$^T$, X78071 *C. acetobutylicum* DSM 1731, X78073 *C. acetobutylicum* P262, X81021 *C. acetobutylicum* NCIMB 8052$^T$.

## 3. Results and discussion

*3.1. Phenotypic differences*

On the bases of their colony morphology, ability to grow on CBM, microscopic appearance of the organisms during the course of their sporulation, and a variety of physiological characteristics, it was concluded that whereas strains ATCC 824$^T$ and DSM 1731 were virtually indistinguishable, strain NCIMB 8052$^T$ was quite distinctive and resembled strain P262 more closely than it did either of the other two strains [6]. In particular, unlike strains ATCC 824$^T$ and DSM 1731, strain NCIMB 8052$^T$ would not grow at a pH more acid than pH 5.0 but could produce substantial amounts of acetone and butanol in batch cultures maintained at pH 7.0. Furthermore, in chemostat culture, strain NCIMB 8052$^T$ ultimately always gave rise to a population dominated by mutant(s) which were not capable of solvent production, whilst ATCC 824$^T$ and DSM 1731 under similar culture conditions sustained solventogenesis even though asporogenous or oligosporogenous mutant(s) lacking the ability to synthesise granulose were selected. However, strain NCIMB 8052$^T$ was not identical with strain P262 since it neither formed distinctive clostridial forms during sporulation nor produced capsular material: strain P262 *inter alia* uniquely fermented inositol and melibiose whilst strain NCIMB 8052$^T$ alone fermented sorbitol, D- and L-arabitol and 2-ketogluconate [6]. Other investigators have drawn attention to substantial strain differences evidenced in the composition of their cell wall teichoic acids and their cell wall lytic enzymes [15,16].

Since pyrolysis mass spectrometry (PyMS), with a typical sample time of less than 2 min, provides the means to sensitively and rapidly discriminate between phenotypically distinctive bacterial strains [11,17], the four strains in question were analysed by this technique together with strain NI-4081 which is an autolysin-deficient mutant of strain NI-4080 which in turn was a derivative of strain NI-4 (‘*Clostridium saccharoperbutylacetonicum*’) [15]. As a test of the sensitivity of the procedure, strain AA219 was also analysed; this strain is isogenic with the NCIMB 8052$^T$ strain except for a 4.7-kb insertion of an integrational plasmid (pJ1) in the *gutD* (sorbitol dehydrogenase) gene. The findings are summarised in the ordination plot (Fig. 1) derived from the PyMS

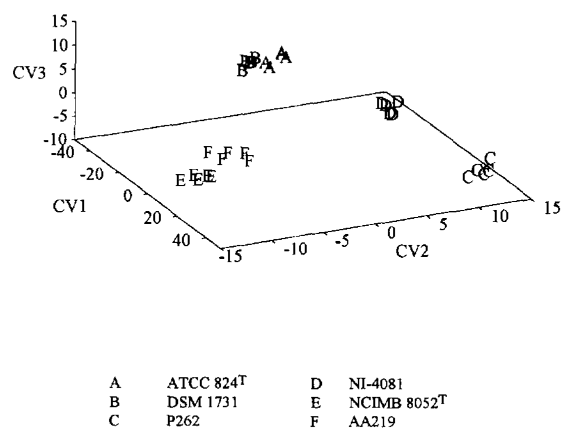


Fig. 1. Ordination plot based on PyMS data analysed by GENSTAT showing the relationship between the strains of *Clostridium acetobutylicum*. The first, second and third canonical variates accounted for 69.9%, 13.3% and 11.3% of the total variance (94.5% total).

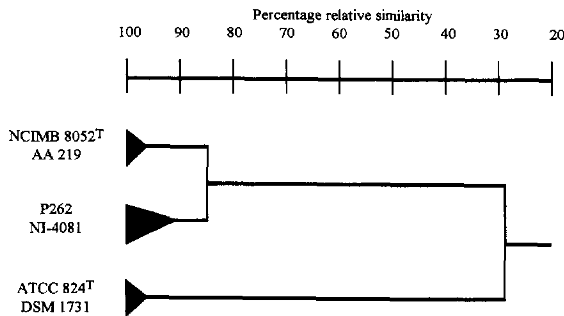


Fig. 2. Dendrogram representing the relationships between all of the examined strains of *Clostridium acetobutylicum*, based on PyMS data analysed by GENSTAT.

data. This shows that even strains NCIMB 8052$^T$ and AA219 were distinguishable by this method, thus authenticating the revealed closeness of the relationship between strains ATCC 824$^T$ and DSM 1731 and the relatively substantial difference between these and strain NCIMB 8052$^T$. Interestingly, strains P262 and NI-4081 though readily distinguishable were much more closely related to each other than to any of the alternative strains. Fig. 2 summarises these findings in the form of an abridged dendrogram of phenotypic relationships.

### 3.2. Genotypic differences

In a previous study [18] the minimal sizes of the genomes of some of these strains were estimated by summing the sizes of macro-restriction fragments produced after cleavage of their DNA with *Sma*I and *Apa*I. Three discrete groups were discernible, with NCIMB 8052$^T$ having a larger genome (6.5 Mbp) than either ATCC 824$^T$ (4.0 Mbp) or P262 (2.85 Mbp). It was again evident that strains ATCC 824$^T$ and DSM 1731 (3.5 Mbp) were very similar.

Genetic analysis in the NCIMB 8052$^T$, ATCC 824$^T$ and P262 strains has drawn attention to numerous differences between these strains. For example, there is an active restriction system in ATCC 824$^T$ [19]. Another difference was noted by Williams et al. [20], who were able to mobilise IncP-based plasmids from *E. coli* to NCIMB 8052$^T$ but not to ATCC 824$^T$ or P262. Several investigators have noted that DNA segments isolated from one strain often fail to hybridise with DNA from another strain, even under conditions of low stringency (e.g. [18]).

The sequences of more than 40 *C. acetobutylicum* genes have now been reported (GenBank release 83). Excluding the 16S rRNA gene sequences (considered separately below), there are only eight instances in which equivalent genes have been sequenced from two different strains (Table 1). This very limited comparison indicates that the ATCC 824$^T$ and DSM 792 strains are probably identical, whereas the ATCC 824$^T$ and NCIMB 8052$^T$ strains are very dissimilar (70–76% DNA sequence identity). The ATCC 4259 strain, which also appears in Table 1 is very similar to the ATCC 824$^T$ strain (D. Jones, personal communication).

The 16S rRNA gene fragments of four of the strains were amplified using PCR and their nucleotide sequences were determined directly. The sequencing strategy employed resulted in the derivation of approximately 95% of their complete 16S rRNA primary structures (approx. 1450 nucleotides

Table 1
Comparison of gene sequences in the GenBank database

| Gene | Strains | Accession numbers | DNA identity (%) | Protein similarity (%) | Protein identity (%) |
|---|---|---|---|---|---|
| *ptb* | ATCC 824$^T$ vs. NCIMB 8052$^T$ | L14744 L04468 | 73 | 84 | 69 |
| *butK* | ATCC 824$^T$ vs. NCIMB 8052$^T$ | L14744 L04468 | 70 | 79 | 64 |
| *spo0A* | ATCC 4259 vs. NCIMB 8052$^T$ | CAU09978 CAU09979 | 76 | 82 | 73 |
| *aad (adhE)* | ATCC 824$^T$ vs. DSM 792 | L14817 X72831 | 100 | 100 | 100 |
| *ctfA* | ATCC 824$^T$ vs. DSM 792 | M93363 X72831 | 100 | 100 | 100 |
| *ctfB* | ATCC 824$^T$ vs. DSM 792 | M93363 X72831 | 100 | 100 | 100 |
| *adc* | ATCC 824$^T$ vs. DSM 792 | M93363 M55392 | 100 | 100 | 100 |
| *sigE* | ATCC 824$^T$ vs. DSM 792 | U07420 Z23079 | 100 | 100 | 100 |

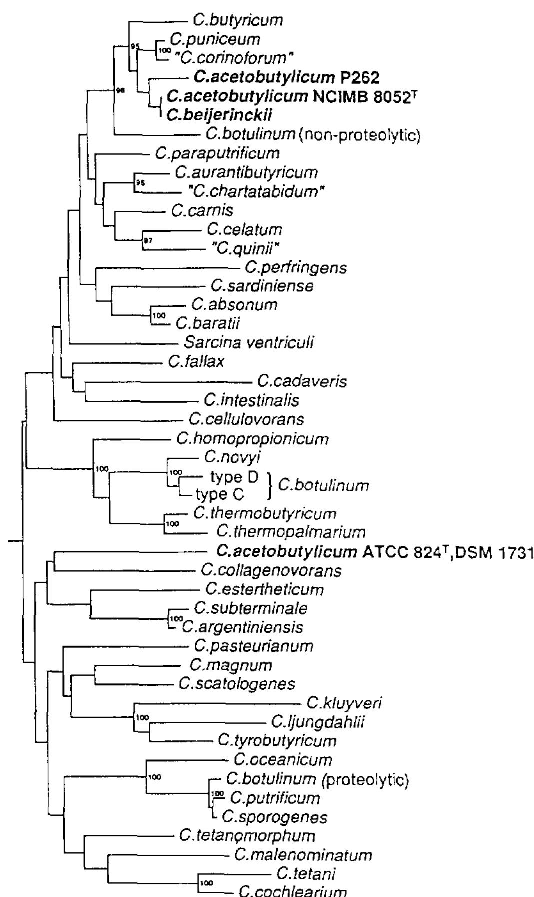


Fig. 3. Neighbour-Joining dendrogram of small-subunit rRNA sequences showing the relationships of the strains of *Clostridium acetobutylicum* to other clostridial species. The dendrogram is based on comparison of approx. 1320 nucleotides (ranging from positions 101 to 1432 of the *E. coli* numbering system). The evolutionary distance between two species is the sum of the horizontal lengths between them.

per gene). The derived sequences have been deposited in the EMBL Data Library (see above). These new sequences were aligned with those of other (chiefly) clostridial sequences. Percent pairwise similarities for an approx. 1320-nucleotide region (ranging from positions 101 to 1432 of the *Escherichia coli* numbering system) were computed and used to construct a phylogenetic tree. Some nucleotides at the 5′ end of the rRNAs were omitted from these calculations to eliminate possible alignment errors due to the extremely variable V1 region. A dendrogram depicting the genealogical interrelationships of the strains was constructed as in Fig. 3. The results confirmed the close relationship of strains ATCC 824$^T$ and DSM 1731 which displayed identical 16S rRNA sequences. Strain NCIMB 8052$^T$ showed approximately 7% sequence divergence with strains ATCC 824$^T$ and DSM 1731. Significantly, strain NCIMB 8052$^T$ also exhibited 100% 16S rRNA sequence relatedness with the type strain of *Clostridium beijerinckii*, thereby indicating that these strains represent a single genomic species. *C. acetobutylicum* strain P262 was also found to be closely related to, albeit distinct from NCIMB 8052$^T$ and *C. beijerinckii*. A 16S rRNA sequence divergence of approximately 1.5% indicates that these may represent two closely related genomic species, although chromosomal DNA–DNA pairing studies are necessary to confirm their separateness.

It is evident from both phenotypic and genotypic data that the strains currently designated *C. acetobutylicum* are heterogeneous and do not constitute a single species. Furthermore, it is clear from these investigations that the type strains obtained from ATCC and NCIMB were not identical and that NCIMB 8052$^T$ is in fact *C. beijerinckii*. We therefore suggest that the name *C. acetobutylicum* should be restricted to those strains genetically closely related to ATCC 824$^T$.

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