An indigo-reducing moderate thermophile from a woad vat, *Clostridium isatidis* sp. nov.

A. Nikki Padden,¹ Vivian M. Dillon,¹ John Edmonds,² M. David Collins,^{3†} Nerea Alvarez³ and Philip John¹

Author for correspondence: A. Nikki Padden. Tel: +44 118 931 8098. Fax: +44 118 931 6577.
e-mail: a.n.padden@reading.ac.uk

¹ Department of
Agricultural Botany,
University of Reading,
Reading RG6 6AS, UK

² Chiltern Open Air
Museum, Chalfont St Giles
HP8 4AD, UK

³ Department of
Microbiology, BBSRC
Institute of Food Research,
Reading Laboratory,
Reading RG6 6AP, UK

A Gram-positive, anaerobic, moderate thermophile, strain Wv6^T, capable of reducing indigo dye, was isolated from a fermenting woad vat prepared essentially as in medieval Europe. Strain Wv6^T formed rod-shaped cells, which occurred singly, in pairs or in chains and produced terminal oval endospores. Strain Wv6^T was saccharolytic. Growth occurred at pH 5.9–9.9 (initial pH) with an optimum at 50 °C of pH 7.2±0.2 (constant pH). At pH 7.8, the temperature range for growth was 30–55 °C with the optimum at 49–52 °C. Comparative 16S rRNA gene sequence analysis demonstrated that the bacterium represents a hitherto unknown subline within rRNA cluster I *Clostridium*. Based on the results of the phylogenetic analysis and phenotypic criteria, it is proposed that the unknown moderate thermophile should be classified as *Clostridium isatidis* sp. nov., a new species of the genus *Clostridium*. The type strain of *Clostridium isatidis* is strain Wv6^T (=NCFB 3071^T).

Keywords: *Clostridium isatidis* sp. nov., phylogeny, indigo reduction, moderate thermophile

INTRODUCTION

Indigo is the only natural source of blue dye and its use dates back to the Neolithic period (Clark *et al.*, 1993). Indigo produced from the woad plant (*Isatis tinctoria* L.) was used extensively in temperate regions during the Middle Ages. The leaves were routinely harvested in summer, crushed and the resulting paste kneaded into woad balls of about 10 cm diameter, which were air-dried. The dried woad balls could be stored indefinitely but before the woad could be used for dyeing it was 'couched'. In this process, the woad balls were crushed and wetted, encouraging aerobic microbial activity which increased the temperature to 55 °C. After approximately 2 weeks, the 'couched woad' was allowed to dry (Hurry, 1930). The woad was further processed in the woad vat, in which material to be dyed was immersed. Hot water was added to the couched woad and the vat was maintained at 50 °C. During the ensuing fermentation, the pH was maintained alkaline by the periodic addition of potash

or lime. In the woad vat, the insoluble oxidized form of indigo was converted by reduction to the soluble colourless form, *leuco*-indigo. On exposure of the treated material to air it became re-oxidized and blue dyed.

The use of indigenous woad as a source of indigo declined in the early 17th century when the import of natural indigo (*Indigofera tinctoria*) to Europe from India began (Clark *et al.*, 1993). Natural indigo was capable of producing a much higher yield of the indigo pigment than woad could. In the late 19th century, production of synthetic indigo, a cheaper option that catered for increasing demand replaced the need for natural indigo. Currently wastewaters from the dyeing industry, which uses sodium dithionite as a reducing agent, are a burden to the environment (Kabdasli *et al.*, 1995) and thus a natural alternative to dithionite would be desirable.

In this study, we report on an anaerobic moderate thermophile, isolated from a woad vat, which possesses the ability to reduce the oxidized insoluble form of indigo to the soluble reduced form. On the basis of phenotypic and molecular taxonomic data, a new species, *Clostridium isatidis*, is proposed for this bacterium.

[†] Present address: Department of Food Science and Technology, University of Reading, Reading RG6 6AS, UK.

The GenBank accession number for the 16S rRNA gene sequence of strain Wv6^T (=NCFB 3071^T) is X98395.

METHODS

Sample collection and bacterial enrichment. Couched woad was used as an inoculum for the woad vat. The woad was grown at the Chiltern Open Air Museum, Chalfont St Giles and at the University of Reading; the leaves were harvested in the summer of 1994 from woad sown in spring 1994. Harvested leaves were rolled into balls and dried. The woad balls were then couched for 2 weeks and allowed to dry. Boiling tap water (500 ml) was added to the couched woad (100 g) and the vat was incubated in a water bath (50 °C) for up to 8 d. The pH was maintained at $\text{pH } 9.0 \pm 0.4$ with oak wood ash or 2 M NaOH. The reference strain, *Clostridium carnis* (NCIMB 10670^T), was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, UK. Indigo reduction was also tested for in *Clostridium aurantibutyricum* (DSM 793^T), *Clostridium celatum* (DSM 1785^T) and *Clostridium quinii* (DSM 6736^T), which were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Isolation. The bacterial populations of the woad vat were examined after 30 h and 8 d incubation. Samples were removed and diluted in peptone water (0.1%, w/v) and then spread onto selective media, as follows: Reinforced Clostridium Agar (RCA), anaerobes; Dextrose Tryptone Media, thermophiles and mesophiles; Rose Bengal Chloramphenicol, yeasts and moulds; Violet Red Bile Glucose, enterobacteriaceae; and Plate Count Agar, viable organisms (Oxoid). The plates were incubated aerobically or anaerobically as appropriate for 3 d at 47 °C. Anaerobic incubations were carried out using a 3.5 l polycarbonate anaerobic jar with an anaerobic gas generating system (Oxoid). The indigo-reducing bacteria were subsequently isolated using RCA supplemented with 0.01% (w/v) indigo, $\text{pH } 9.0 \pm 0.2$, incubated anaerobically at 47 °C for 24–48 h.

Light microscopy. Light microscopy was performed using a Reichert Polyvar 2 (Leica). Fixed slides were stained with carbol fuchsin and viewed under bright field. A sample of culture grown on RCA and Reinforced Clostridial Medium (RCM) (Oxoid) was used.

Temperature and pH ranges. The temperature range was tested in static liquid batch culture using RCM, $\text{pH } 7.8 \pm 0.2$ (test temperature). The cultures were incubated at the respective temperature and samples were taken at regular intervals. Growth rates were calculated using the increase in turbidity (OD_{600}). The pH range for growth was tested as above with an incubation temperature of 45 °C. The pH optimum was determined at 50 °C using controlled-batch culture (Brighton Systems). The pH was controlled by automatic titration of 2 M HCl and 2 M NaOH. Anaerobic conditions were maintained by flushing the culture with sterile oxygen-free nitrogen (BOC gases) between each run and the culture was stirred at 50 r.p.m. RCM without agar was used.

Substrate utilization. Growth on carbohydrates was determined using a modified PY broth ($\text{pH } 8.0$). This contained (l^{-1} distilled water): peptone, 0.5 g; tryptone, 0.5 g; yeast extract, 1 g; haemin solution (50 mg haemin dissolved in 1 ml 1 M NaOH, added to 99 ml deionized water, autoclaved separately), 10 ml; vitamin K₁ solution [0.15 ml vitamin K₁ in 30 ml 95% (v/v) ethanol], 0.2 ml; cysteine.HCl.H₂O, 0.5 g; and salts solution, 40 ml. The salts solution contained (l^{-1} distilled water): CaCl₂, 0.2 g; MgSO₄.7H₂O, 0.48 g; K₂HPO₄, 1.0 g; KH₂PO₄, 1.0 g; NaHCO₃, 10.0 g; NaCl, 2.0 g. All carbohydrate solutions were filter-sterilized, with

the exception of glycerol and starch, which were autoclaved, and added to the medium at the final concentrations given below. Broths were incubated at 45 °C for up to 48 h and the increase in turbidity at 600 nm was monitored. Cultures which exhibited an increase of at least 0.1 OD unit were considered positive for substrate utilization.

Biochemical tests. The following biochemical tests were carried out according to Harrigan & McCance (1976): gelatin hydrolysis using nutrient agar plates supplemented with gelatin (4 g l^{-1}); production of α -amylase and β -amylase using starch agar (0.2%, w/v); lecithinase activity in egg yolk agar using 10 ml egg yolk emulsion (Oxoid) added to 100 ml nutrient agar after autoclaving; the ability to clot milk in litmus milk (100 g skim milk powder l^{-1} with 10 ml 4% litmus solution (BDH Chemicals)); the digestion of meat by the production of proteolytic enzymes in cooked meat medium (Oxoid); nitrate reduction in nitrate peptone water (KNO₃, 0.2 g l^{-1} ; peptone, 10 g l^{-1} ; and agar, 0.3%); haemolysis in 100 ml blood agar base (Oxoid) supplemented with 5 ml sheep blood and 5 ml horse blood. To test for catalase, 1 ml H₂O₂ (10%, v/v) was poured onto fresh cultures and the presence of any oxygen bubbles was noted. Acid production, aesculin hydrolysis, indole production and urease were tested for using API 20A strips (bioMérieux), onto which the woad vat isolates and *C. carnis* were inoculated and incubated anaerobically at 37 °C for 24 h (according to manufacturer's instructions). Additional tests for acid production were carried out using PY broth supplemented with Bromocresol Purple (0.02 g l^{-1}) as a pH indicator.

Susceptibility to antibiotics. Resistance to antibiotics was examined using liquid broth cultures containing filter-sterilized antibiotics or Difco Dispence-O-Disc minifilters on RCA plates. An exponentially growing culture was inoculated (5%, v/v) into fresh RCM supplemented with the antibiotic and incubated at 45 °C for 48 h or spread onto RCA plates and incubated anaerobically.

Fermentation products of PYG. The fermentation products were analysed after growth on a PYG medium containing (l^{-1} distilled water): peptone, 5 g; tryptone, 5 g; yeast extract, 10 g; glucose, 10 g; salts solution, 40 ml; haemin solution, 10 ml; vitamin K₁ solution, 0.2 ml; and cysteine.HCl.H₂O, 0.5 g. The cultures were incubated statically at 45 °C at initial pH 8.0. Gases were determined by GC using a GCD chromatograph (Pye Unicam) equipped with a Porapak R column and a thermal conductivity detector. Organic acids were determined by HPLC using an Aminex HPX97H ion-exclusion column fitted with a guard column (Bio-Rad).

Determination of G + C content. The G + C content (mol%) was determined by the thermal melting point method (Garvie, 1978) using DNA extracted from strain Wv6^T grown on RCA.

16S rRNA gene sequence analysis. DNA was extracted by the method of Lawson *et al.* (1989). 16S rRNA gene fragments (corresponding to *Escherichia coli* positions 31–1521) were amplified by PCR, using primers for conserved sequences near the 5' and 3' ends of the gene (Boddinghaus *et al.*, 1990). After purification with a Prep-A-Gene kit (Bio-Rad), the PCR products were sequenced using a DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and a model 373A automatic DNA sequencer (Applied Biosystems). The FASTA program from the GCG package (Devereux *et al.*, 1984) was used to identify the nearest

relatives of the woad isolates. The 16S rRNA sequences of these strains were then obtained from the EMBL and Ribosomal Database Project data libraries to be included in further analyses. Sequences were aligned by using the program PILEUP (Devereux *et al.*, 1984) and the alignment was verified and corrected manually. Approximately the first 100 bases (which include variable region VI) were excluded from the phylogenetic analysis because of incomplete sequences for some strains and alignment uncertainties. A distance matrix was prepared using the programs PRETTY (Devereux *et al.*, 1984) and DNADIST (Felsenstein, 1989) and a phylogenetic tree was derived from the distance data using the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was assessed by bootstrap analysis using the programs SEQBOOT (500 replications), DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein, 1989).

RESULTS AND DISCUSSION

Isolation

After 30 h incubation, the bacterial population of the woad vat consisted of both aerobic and anaerobic bacteria representing 10^7 and 4×10^5 bacteria per ml slurry, respectively. After 8 d, the number of aerobic (and/or facultative anaerobic) bacteria remained constant and consisted of at least four different bacterial types. Bacterial numbers were highest on the RCA plates. The anaerobic bacterial count increased to 3×10^6 bacteria per ml slurry (RCA plates) and consisted of 10 slightly different morphological types (denoted strains Wv1–Wv10). No yeasts or moulds were present at either stage during the enrichment. As indigo reduction occurred under anaerobic conditions at an alkali pH, colonies were taken from the RCA plates and re-streaked onto RCA, pH 9, and incubated anaerobically at 47 °C. Five out of the 10 isolates grew well (strains Wv6^T–Wv10) and were re-streaked onto RCA, pH 9, supplemented with 0.01 % (w/v) indigo. Indigo reduction was indicated by the medium going colourless under anaerobic incubation conditions, and becoming blue on exposure to air. All five isolates reduced indigo. The isolates were all catalase-negative, rod-shaped, Gram-positive bacteria, which produced oval terminal endospores. Colonial morphology of the isolates was identical on horse blood agar and no isolate could grow aerobically. Strains Wv6^T–Wv10 were also shown to be genetically homogeneous (see below) and therefore strain Wv6^T was used as the representative strain which was maintained on RCA plates, pH 9.0 ± 0.2.

Colony and cell morphology of strain Wv6^T

Strain Wv6^T produced creamy white mucoid colonies on RCA (pH 9.0 ± 0.2) up to 7 mm in diameter. Colonies were circular to irregular and low convex or were raised to a central point. On sheep and horse blood agar, colonies were grey and feathery and 2 mm in diameter. Slight β-haemolysis occurred on horse blood agar but not on sheep blood agar. Cells were rod-shaped and occurred singly, in pairs or in chains

Fig. 1. Light micrographs of (a) vegetative cells grown in RCM after 6 h and (b) sporulated cells, mature spores and partially stained dead vegetative cells of strain Wv6^T grown on RCA plates after 48 h. Bar, 10 µm.

(Fig. 1a). The rods were 0.3–0.6 µm by 1.8–9.1 µm. Oval terminal endospores were produced in both liquid and solid batch culture at late exponential or early stationary phase (Fig. 1b). When grown in RCM, pH 8.6, spores could withstand heat treatment of 90 °C for 10 min but were killed after exposure to 100 °C for 4 min. Cells exhibited a tumbling motility at all stages of growth.

Biochemical and physiological characteristics

(i) Growth requirements. Strain Wv6^T was incapable of growth in air on RCA (or blood agar) plates, but was aerotolerant and remained viable after 3 months

storage at 4 °C in air. Liquid batch cultures were carried out in half-filled universals, sealed and incubated statically. Growth on RCM occurred overnight, producing turbid cultures with OD₆₀₀ of >1.5 U and an abundant amount of gas was produced. A copious amount of slime was produced during growth in RCM with pH values greater than pH 8.0. Growth occurred on PYG only in the presence of peptone, tryptone and yeast extract. The final pH of cultures grown on PYG and RCM at 45 °C with initial pH values of pH 6–9 was pH 5.2–5.4 after 5 d.

(ii) **Temperature and pH range.** Strain Wv6^T grew at 30–55 °C, but no growth was observed at 25– or 60 °C after incubation for 4 d. The optimum temperature for growth was 49–52 °C, with growth rates of 1.2–1.5 h⁻¹ (Fig. 2a). Temperature value determinations were repeated up to four times. Initial pH values of pH 5.9–9.9 supported growth, but no growth occurred at pH 5.7 or pH 11.1. The optimum pH, determined using controlled-batch culture (between pH 6.5 and 8.9), was pH 7.2 ± 0.2, with growth rates of 1.5–1.6 h⁻¹ (Fig. 2b).

(iii) **Substrate utilization and acid production.** Strain Wv6^T grew on PY broth supplemented with (% w/v): glucose, 1; sucrose, 1; fructose, 1; galactose, 1; lactose, 1; maltose, 1; mannose, 1; trehalose, 1; cellobiose, 1; starch, 1; amygdalin, 0.5; and sorbitol, 1. In all cases except lactose, growth was accompanied by acid production. Neither growth nor acid production were detected on arabinose (1%, v/v), ribose (0.5%, v/v),

Fig. 3. Phylogenetic tree based on a comparison of ca. 1320 bases constructed using the neighbour-joining method, showing the position of *C. isatidis* strain Wv6^T amongst some close relatives within rRNA cluster I *Clostridium* [see Collins *et al.* (1994) for rRNA group designation]. Bootstrap values > 90% (expressed as percentages of 500 replications) are shown at the branching points. Bar, 1.2% base substitutions.

raffinose (1%, v/v), rhamnose (1%, v/v), erythritol (0.5%, v/v), glycerol (0.8%, v/v), mannitol (1%) or inulin (1%, v/v). Strain Wv6^T produced acid from salicin but not from xylose, melezitose or glycogen (growth not tested with these substrates).

(iv) Biochemical properties. Strain Wv6^T hydrolysed aesculin but not starch or gelatin. Indole was not produced from tryptophan, and catalase, lecithinase and urease were absent. Litmus milk was not clotted and meat was not digested by the isolate. Nitrate was not reduced to nitrite. Strain Wv6^T was sensitive to tetracycline (30 µg disc⁻¹) and gentamicin (10 µg disc⁻¹), but not erythromycin (5 µg disc⁻¹). Growth in liquid broth cultures was inhibited by penicillin (2 unit ml⁻¹), ampicillin (30 µg ml⁻¹), rifampicin (30 µg ml⁻¹) or tetracycline (6 µg ml⁻¹), but occurred in the presence of chloramphenicol (30 µg ml⁻¹) and kanamycin (30 µg ml⁻¹).

(v) Fermentation products of PYG. During growth on PYG, strain Wv6^T produced an abundant amount of gas, which was identified as carbon dioxide and hydrogen. These gases were present in the ratio of 3:4. The main organic acids identified using HPLC were acetic, lactic and formic acids. Small amounts of ethanol were also produced.

16S rRNA sequence analysis

To investigate the phylogenetic interrelationships of the isolates, genes encoding 16S rRNA were amplified by PCR and subjected to sequence analysis. A short stretch of ca. 500 nucleotides (proximal to the 5' end of the rRNA, which included diagnostic variable regions V1 to V3) was determined and the isolates were found to be genealogically homogeneous, exhibiting identical

Table 1. Similarity values based on the 16S rRNA sequences of some *Clostridium* species

Species	EMBL no.	Similarity (%) with strain Wv6 ^T
<i>C. absonum</i>	X77842	94.9
<i>C. algidicarnis</i>	X77676	92.1
<i>C. aurantibutyricum</i>	X68183	96.1
<i>C. baratii</i>	X68175	95.3
<i>C. botulinum</i> (type D)	X68187	92.0
<i>C. botulinum</i> (type F)*	X68171	94.3
<i>C. butyricum</i>	X68178	94.2
<i>C. cadaveris</i>	M59068	92.5
<i>C. caliptrosporum</i>	X77843	94.6
<i>C. carnis</i>	M59091	97.8
<i>C. celatum</i>	X77844	96.9
<i>C. cellulovorans</i>	X71849	94.4
<i>C. fallax</i>	M59088	93.2
<i>C. homopropionicum</i>	X76744	92.4
<i>C. intestinalis</i>	X76740	93.2
<i>C. novyi</i>	X68188	91.5
<i>C. paraputrificum</i>	X75907	95.8
<i>C. perfringens</i>	M59103	93.7
<i>C. puniceum</i>	X73444	94.6
<i>C. quinii</i>	X76745	96.2
<i>C. thermobutyricum</i>	X72868	91.9
<i>C. thermopalmarium</i>	X72869	91.7
<i>Sarcina ventriculi</i>	X76649	93.3

* Non-proteolytic.

sequences. The almost complete 16S rRNA gene sequence of a representative strain (strain Wv6^T) of the indigo-reducing bacterium was therefore determined. Preliminary comparative sequence searches of EMBL and GenBank databases using the FASTA program (Devereux *et al.*, 1984) revealed the unknown bacterium was related to the *Clostridium* subphylum of the Gram-positive bacteria. Within this subphylum, highest sequence relatedness (generally >92% similarity) was shown with rRNA cluster I *Clostridium* [rRNA clusters as defined by Collins *et al.* (1994)]. Significantly lower levels of relatedness were shown with members of the other *Clostridium* rRNA clusters (data not shown). The new sequence and those of its closest relatives in rRNA cluster I *Clostridium* were subjected to pairwise analysis and derived evolutionary distances were used to determine their phylogenetic interrelationships. The phylogenetic analysis confirmed that the unknown bacterium was a member of rRNA cluster I *Clostridium*. A tree depicting the phylogenetic relationships of strain Wv6^T is shown in Fig. 3 and some representative sequence similarities are given in Table 1. *C. carnis* was shown to be the closest phylogenetic relative of strain Wv6^T. A pairwise sequence divergence value of ca. 2.4% (corresponding to 30 differences in 1323 bases) showed that the unknown bacterium, although highly related to *C. carnis*, is however, a phylogenetically separate species.

Distinguishing physiological and biochemical properties of the clostridial species that are closely related to

Table 2. Distinguishing physiological properties of *C. isatidis* and five closely related clostridia from rRNA cluster I

Data from Cato *et al.* (1986) and Svensson *et al.* (1992). Strains: 1, *C. isatidis*; 2, *C. carnis*; 3, *C. celatum*; 4, *C. quinii*; 5, *C. aurantibutyricum*; 6, *C. paraputrificum*.

Character	1	2	3	4	5	6
Morphology	Straight or curved rod	Straight or curved rod	Straight or curved rod	Rod then ovoid	Straight rod	Straight or curved rod
Size (µm)	0.3–0.6 × 1.8–9.1	0.5–1.1 × 1.6–9.9	0.8–3.0 × 6.3	1–6 × 2.5–10	0.5–0.8 × 2.8–6.3	0.5–1.4 × 1.9–17
Occurrence	Singly, pairs or chains	Singly or pairs	Singly, pairs or chains	Singly	Singly or pairs	Singly or pairs
Endospores	Oval terminal	Oval terminal or subterminal	Oval terminal, subterminal or central	Long central	Oval subterminal	Oval terminal
Motility	+	+ / –	–	+	+	+ / –
pH range*	5.9–9.9 (7.2)	NK; no growth at 8.5	NK	6.2–10.5 (7.4)	NK	NK
Temperature range (°C)†	30–55 (49–52)	30–37 not 25 or 45	30–37 not 25 or 45	15–50 (40)	30–37 not 25 or 45	30–45 but weak at 25
Respiration	Anaerobic but aerotolerant	Anaerobic but aerotolerant		Strictly anaerobic		
G + C (mol%)	27	28	NK	28	27	26–27

NK, Not known.

* Optimum pH for growth given in parentheses.

† Optimum temperature for growth given in parentheses.

Table 3. Distinguishing biochemical properties of *C. isatidis* and five closely related clostridia from rRNA cluster I

Data from Cato *et al.* (1986) and Svensson *et al.* (1992). Strains: 1, *C. isatidis*; 2, *C. carnis*; 3, *C. celatum*; 4, *C. quinii*; 5, *C. aurantibutyricum*; 6, *C. paraputrificum*. +, Positive for 90–100% strains; –, negative for 90–100% strains; +/–, 61–89% strains positive; –/+, 11–39% strains positive; d, 40–60% strains positive; w, weak reaction.

Character	1	2	3	4	5	6
Starch hydrolysed	–	–	–	–	+	+
Nitrate to nitrite	–	–	d	–	+	–/+
Gelatin hydrolysed	–	–	–	–	+	–
Urease	–	NK	+	–	NK	NK
Products from PYG*	ALF2	BALF (S)	AFB2 (S)	FABL2	LBA	BAL (SF)
Milk reaction†	–	–	c	NK	c	c
Indigo reduced	+	+	–	–	–	NK
Antibiotic resistance:						
Chloramphenicol	+	–	–	NK	–	1/39‡
Erythromycin	+	–	–	NK	–	13/39‡
Penicillin	–	–	–	NK	–	3/39‡
Tetracycline	–	–	–	NK	–	3/39‡
Acid from:						
Amygdalin	+	w	+	NK	–	d
Arabinose	–	–	–	NK	w	–
Fructose	+	d	+	+	+	+
Galactose	+	d	+	+	w	+
Glycogen	–	–	–	NK	w	+/-
Inulin	–	–	–	NK	–	–
Lactose	–	d	+	–	+	+
Mannitol	–	–	–	+	–	–
Mannose	+	w	+	+	w	+
Melezitose	–	–	–	NK	–	–
Raffinose	–	–	–	+	+	–
Ribose	–	–	d	–	–	w–
Sorbitol	+	–	–	–	–	–
Starch	+	w	–	–	w	+
Trehalose	+	–	+	NK	–	–/+
Xylose	–	–	–	+	–	–

NK, Not known.

* A, Acetic acid; B, butyric acid; F, formic acid; L, lactic acid; S, succinic acid; 2, ethanol. Products given in parentheses are irregular (minor and/or not present in all strains studied).

† c, Curd.

‡ Number of strains positive out of total number of strains tested.

strain Wv6^T, namely *C. carnis*, *C. celatum*, *C. quinii*, *C. aurantibutyricum* and *Clostridium paraputrificum*, are listed in Tables 2 and 3. All species are Gram-positive, do not produce indole or digest meat, are lecithinase-negative, hydrolyse aesculin and produce acid from glucose, maltose, cellobiose, sucrose and salicin, but not from rhamnose (Cato *et al.*, 1986; Svensson *et al.*, 1992). All species produce an abundant amount of hydrogen during growth and fail to grow in 6.5% NaCl (except *C. quinii* which is unknown) (Cato *et al.*, 1986). All strains occur as straight or curved rods with the exception of *C. quinii* which becomes ovoid in older cultures (Svensson *et al.*, 1992). The morphological, biochemical and physiological characteristics of strain

Wv6^T as well as the 16S rRNA sequence analysis illustrates that strain Wv6^T is a new species of the genus *Clostridium*, for which the name *Clostridium isatidis* is proposed.

Description of *Clostridium isatidis* sp. nov.

Clostridium isatidis (i.sa'ti.dis. Gr. gen. n. *isatidis* of the crucifer plant *Isatis*).

Colonies are creamy white, mucoid, circular to irregular and low convex or raised to a central point. Cells are rod-shaped (0.3–0.6 µm by 1.8–9.1 µm) and occur singly, in pairs or in chains. Oval terminal

endospores are produced. The temperature range for growth is 30–55 °C, with an optimum temperature of 49–52 °C. The pH range is initial pH 5.6–9.9 with an optimum pH 7.2 ± 0.2 (in controlled-batch culture). At 50 °C and pH 7.2 ± 0.2 , the doubling time is 26 min (μ , 1.6 h^{-1}). Cultures hydrolyse aesculin. Acid is produced from amygdalin, cellobiose, fructose, galactose, glucose, maltose, mannose, salicin, sorbitol, sucrose, starch and trehalose. Acetic, lactic and formic acids, ethanol, carbon dioxide and hydrogen are produced during growth on PYG. The G+C content of strain Wv6^T is 27 mol %. Isolated from woad vat. Type strain is strain Wv6^T (=NCFB 3071^T).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Leverhume Trust. Special thanks to Anthony Wynne (Institute of Food Research, Reading) for assistance with HPLC and Daniel Davies and Jason Osman (Food Science and Technology, Reading University) for assistance with fermentation assembly.

REFERENCES

- Boddinghaus, B., Wolters, J., Heikens, W. & Bottger, E. C. (1990). Phylogenetic analysis and identification of six different serovars of *Mycobacterium intracellulare* at the molecular level. *FEMS Microbiol Lett* **70**, 197–204.
- Cato, E. P., George, W. L. & Finegold, S. M. (1986). Genus *Clostridium*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Clark, R. J. H., Cooksey, C. J., Daniels, M. A. M. & Withnall, R. (1993). Indigo, woad, and Tyrian Purple: important vat dyes from antiquity to the present. *Endeavour* **17**, 191–199.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Garvie, E. I. (1978). *Streptococcus raffinolactis* (Orla-Jensen and Hansen) a group N *Streptococcus* found in raw milk. *Int J Syst Bacteriol* **28**, 190–193.
- Harrigan, W. F. & McCance, M. E. (1976). *Laboratory Methods in Food and Dairy Microbiology*. London: Academic Press.
- Hurry, J. G. (1930). *The Woad Plant and its Dye*. London: Oxford University Press.
- Kabdasli, I., Tünay, O. & Orhon, D. (1995). Sulfate removal from indigo dyeing textile wastewaters. *Water Sci Tech* **32**, 21–27.
- Lawson, P. A., Gharbia, S. E., Shah, H. N. & Clark, D. R. (1989). Recognition of *Fusobacterium nucleatum*, subgroups Fn-1, Fn-2 and Fn-3 by ribosomal RNA gene restricted patterns. *FEMS Microbiol Lett* **65**, 41–46.
- Svensson, H., Dubourguier, H. C., Presier, G. & Zehnder, A. J. B. (1992). *Clostridium quinii* sp. nov., a new saccharolytic anaerobic bacterium isolated from granular sludge. *Arch Microbiol* **157**, 97–103.