

# The API ZYM system in the identification of Gram-negative anaerobes

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**SUMMARY** The API ZYM reactions of type species of Gram-negative anaerobes representative of those encountered in human infections and of 56 clinical isolates of such organisms, identified by conventional techniques, were investigated. The API ZYM test clearly distinguished between the different genera and species examined and appears to provide a simple, reliable method for the identification of this group of organisms.

Improvements in cultural techniques have resulted in an increase in the frequency of isolation from clinical specimens of anaerobic organisms, particularly Gram-negative bacilli. Hitherto identification of these organisms has been primarily based on morphological and biochemical characteristics (McVay and Sprunt, 1952; Fisher and McKusick, 1953; Garrod, 1955) and in many cases it has been difficult to assign an organism to a particular genus or species. Subsequently, a number of new techniques have recently been applied to the taxonomic study of these bacteria.

The classification of bacteria on the basis of their chemical composition by gas chromatography was first proposed by Abel *et al.* (1963). Since then advances in gas chromatographic techniques have resulted in this method being used in the identification and differentiation of various species of obligate anaerobes (Holdeman and Moore, 1972). The disadvantage of this technique is that it is complicated and time consuming and not easily adapted to routine diagnostic bacteriology.

Finegold *et al.* (1967) and Sutter and Finegold (1971) demonstrated the use of antibiotic susceptibility patterns as aids to the characterisation of anaerobic Gram-negative bacilli. Duerden *et al.* (1976) exploited this approach by combining antibiotic susceptibility patterns with biochemical reactions and dye susceptibility tests (Suzuki *et al.*, 1966).

A simple new technique which has been used for the identification of bacteria is the API ZYM system

(API Laboratory Products). This is a semiquantitative micromethod which was originally designed to detect enzymatic activities in a variety of specimens, for example, tissues, cells, biological fluids, and bacteria. It allows the systematic and rapid study of enzymatic reactions using very small samples. The substrates are contained in a support strip, which allows contact between the enzyme and substrate; enzymatic activity is revealed by the addition of suitable indicators.

This type of system has been used successfully in the identification of staphylococci (Joubert and Buisnière, 1968), *Erwinia* (Peny, 1970), and, more recently, the API ZYM system has been applied to the identification of non-haemolytic streptococci (Waitkins *et al.*, 1977). This paper presents the results of a study of the use of the API ZYM technique in the identification of anaerobic Gram-negative organisms from human sources.

## Material and methods

### SOURCE AND ISOLATION OF STRAINS

The following type strains were obtained from the National Collection of Type Cultures: *Fusobacterium necrophorum* (NCTC 10575, 10576, 10577); *F. necrogenes* (NCTC 10723); *F. polymorphum* (NCTC 10562); *Bacteroides fragilis* ss. *fragilis* (NCTC 8560, 9343); *B. fragilis* ss. *thetaiotaomicron* (NCTC 10582); *B. fragilis* ss. *vulgatus* (NCTC 10583); *B. melaninogenicus* ss. *intermedius* (NCTC 9336 and 9338); *B. melaninogenicus* ss. *asaccharolyticus* (NCTC 9337); Hare Group V (NCTC 9816) and *Veillonella alcalescens* (NCTC 9805).

*B. oralis* (NP 333), *B. ochraceus* (VPI 2845), and *B. melaninogenicus* ss. *melaninogenicus* (ATCC 15930 and VPI 4196) were received from Professor J. G. Collee, Department of Bacteriology, Edinburgh University Medical School, Edinburgh; *B. fragilis* ss. *ovatus* (ATCC 8483) was received from Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich.

The 56 other strains studied were isolated in this laboratory from abdominal wounds, blood cultures, cerebral abscesses, lung abscess, vaginal swabs, acute dental infections (Ingham *et al.*, 1977) and an infected compound fracture of the mandible.

#### CHARACTERISATION AND IDENTIFICATION OF STRAINS

None of the isolates used in the study grew in 10% carbon dioxide and air, and all were sensitive to metronidazole when tested with a disc containing 5 µg. All Gram-negative obligate anaerobes, with the exception of Gram-negative cocci, were identified by reference to the scheme described by Duerden *et al.* (1976). The Gram-negative cocci were identified according to the criteria proposed by Rogosa (1964). Fermentation end products produced by all the strains were determined by gas liquid chromatography.

All anaerobic cultures were incubated at 37°C for at least 48 hours in an atmosphere consisting of 90% hydrogen and 10% carbon dioxide in an anaerobic jar fitted with a cold catalyst.

#### TOLERANCE TESTS

Antibiotic sensitivity and dye susceptibility tests were carried out on Oxoid sensitivity test agar containing 5% lysed horse blood.

#### ANTIBIOTIC SENSITIVITY TESTS

The antibiotic discs used were: penicillin, 1 unit, rifamide, 10 µg, and metronidazole, 5 µg (Mast). The Oxford staphylococcus was used as a control for the penicillin and rifamide discs, and *B. fragilis* (NCTC 9343) for metronidazole.

#### SENSITIVITY TO DYES

Each strain was spread on to Oxoid sensitivity test agar containing (1) no dye (control), (2) Victoria Blue 4R (1 in 80 000), and (3) ethyl violet (1 in 80 000). The plates were examined for growth after 48 hours. Each dye was initially prepared in aqueous solution and the concentrations above are those finally achieved in the test medium.

#### BILE TOLERANCE

The ability to grow in the presence of bile was tested by inoculating the strains into nutrient broth con-

taining 10% bile. *B. fragilis* (NCTC 8560) and *B. oralis* (NP 333) were used as the positive and negative controls respectively.

#### BIOCHEMICAL REACTIONS

##### *Carbohydrate fermentation tests*

Carbohydrate fermentation reactions were carried out in peptone water containing a final concentration of 1% of each of the following sugars: glucose, rhamnose, trehalose, lactose, maltose, sucrose, dulcitol, and mannitol, using an inoculum of one drop (0.03 ml) of a 48-hour culture of the strains in Robertson's cooked meat medium.

##### *Catalase test*

Each strain was grown on nutrient agar for 48 hours and, after removal from the anaerobic jar, each plate was left for at least half an hour before being tested for catalase activity. A small quantity of growth was removed from the surface of the agar using a cover slip which was then immersed in 3% hydrogen peroxide, the production of bubbles indicating a positive reaction.

##### *Nitrate reduction*

The reduction of nitrate was detected by growing each strain in nutrient broth containing 0.1% potassium nitrate. The presence of nitrite ions was indicated by a deep red colour when 1 ml nitrate solution A and 1 ml nitrate solution B (Cruikshank, 1968) were added to 48-hour cultures.

##### *Hydrogen sulphide production*

A lead acetate strip was suspended in the neck of a test tube containing tryptone broth and incubated for 48 hours. Hydrogen sulphide production was indicated by blackening of the strip.

##### *Indole production*

Indole was detected by adding 3 drops of Kovac's reagent to a 48-hour culture in Robertson's cooked meat medium, a positive result being indicated by the development of a red colour.

##### *Aesculin hydrolysis*

Each strain was spread on to a nutrient agar plate incorporating 0.1% aesculin and incubated anaerobically for five days, hydrolysis being indicated by the development of a black colour.

#### GAS LIQUID CHROMATOGRAPHY

A Pye GCD Chromatograph was used for analysis of the fermentation end products of each strain. The column consisted of 60/80 Chromosorb 101 operating at 175°C with the injector and detector at a

temperature of 250°C. The carrier gas was nitrogen and a chart recorder was used to produce a trace of the acid peaks detected.

Each strain was grown in Robertson's cooked meat medium for three days and the supernatant was removed. Six millilitres of this supernatant was acidified with 0.2 ml 18N sulphuric acid, and 1 microlitre of this was withdrawn for injection into the chromatograph. A volatile acid control solution was chromatographed to allow precise identification of the acids detected in the sample. The control solution contained approximately 0.04% of the following: n-butanol, isobutanol, n-butyric acid, and propionic acid.

#### API ZYM TESTS

The enzymes detected by the API ZYM system are listed in Table 1. Each strain tested was grown on Oxoid sensitivity test agar containing 5% lysed horse blood for 24, 48, or 72 hours according to the rate of growth of particular strains. For the purpose of the test, the growth was removed from the surface of the plate and suspended in 2 ml distilled water so as to produce a very dense suspension. Two drops (0.06 ml) of the bacterial suspensions were added to each of the 20 cupules in the API ZYM strip, which was placed in a chamber previously moistened with 5 ml distilled water. The chamber was covered with a lid to maintain a moist atmosphere and incubated at 37°C for 4 hours. After incubation, one drop of each of the API reagents A and B (*vide infra*) was added to each of the cupules, and the intensity of the colour reaction which developed within 5 minutes was graded from zero to 5 with reference to the API ZYM colour reaction chart. In order to determine the reproducibility of results all strains were examined at least twice using this technique and a number of strains from each species were tested after growing for 24, 48, or 72 hours, and, in addition, the effect of increasing the period of incubation of the strips from 4 to 6 hours was studied. The composition of the two reagents used to detect enzymatic reactions is as follows:

Reagent A:  
 Tris (hydroxymethyl) aminomethane 250 g  
 Hydrochloric acid 37% 110 ml  
 Laurylsulphate 100 g  
 Distilled water to make 1000 ml  
 pH = 7.6-7.8

Reagent B:  
 Fast Blue BB (Sigma No. F.0250) 3.5 g  
 2-methoxyethanol to make 1000 ml

#### Results

The identity of the strains of Gram-negative anaerobic bacteria isolated in this laboratory is shown in Table 2. When examined by gas liquid chromatography only those isolates allocated to the genus *Fusobacterium* produced n-butyric acid as a major fermentation end product.

The two isolates of *B. fragilis* which we were unable definitely to subspeciate were atypical in their biochemical reaction in that they fermented glucose and trehalose and gave variable results with rhamnose and were indole negative.

Ten isolates which on Gram film were seen to be Gram-negative cocci all failed to ferment glucose, lactose, maltose, sucrose, dulcitol, and mannitol, were oxidase and indole negative, nitrate and H<sub>2</sub>S positive, and grew poorly in 5% bile, and the major fermentation end product was propionic acid. Six of the strains were catalase positive, as was NCTC 9805, and were therefore identified as *V. alcalescens*. The remaining four strains and NCTC 9816 were identified as *V. parvula* on the basis of a negative catalase.

The typical patterns produced in the API ZYM test by a number of type cultures of Gram-negative obligate anaerobes are illustrated in Table 3. With the exception of *B. fragilis* the reactions of such organisms isolated and identified in this laboratory

Table 1 Enzymes detected by the API ZYM test

Test	Enzyme assayed for	Test	Enzyme assayed for
1	Control	11	Phosphatase acid
2	Phosphatase alkaline	12	Phosphoamidase
3	Esterase (C 4)	13	α Galactosidase
4	Esterase lipase (C 8)	14	β Galactosidase
5	Lipase (C 14)	15	β Glucuronidase
6	Leucine aminopeptidase	16	α Glucosidase
7	Valine aminopeptidase	17	β Glucosidase
8	Cystine aminopeptidase	18	β Glucosaminidase
9	Trypsin	19	α Mannosidase
10	Chymotrypsin	20	α Fucosidase

Table 2 Number and identity of Gram-negative anaerobes isolated

Species	Number of strains
<i>B. fragilis</i> ss <i>fragilis</i>	13
<i>B. fragilis</i> ss <i>thetaiotaomicron</i>	2
<i>B. fragilis</i> ss <i>vulgatus</i>	1
<i>B. fragilis</i> ss undetermined	2
<i>B. oralis</i>	5
<i>B. melaninogenicus</i> ss <i>asaccharolyticus</i>	7
<i>B. melaninogenicus</i> ss <i>intermedius</i>	7
<i>F. polymorphum</i>	6
<i>F. necrophorum</i>	3
<i>V. alcalescens</i>	6
<i>V. parvula</i>	4

Table 3 Reactions of type strains in the API ZYM test

Organism	API ZYM substrates																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>B. fragilis</i> NCTC 9343	0	5	2	2	0	0	0	0	0	0	5	4	3	5	4	4	0	4	0	4
<i>B. oralis</i> NP 333	0	4	2	1	0	0	0	0	0	0	4	5	3	4	0	4	3	0	0	0
<i>B. melaninogenicus</i> NCTC 9336	0	5	1	0	0	0	0	0	0	0	5	4	0	0	0	5	0	0	0	0
<i>B. melaninogenicus</i> NCTC 9337	0	5	2	4	0	0	0	0	0	0	4	4	0	0	0	0	0	0	0	1
<i>B. melaninogenicus</i> VPI 4196	0	5	1	0	0	0	0	0	0	0	3	3	1	4	0	4	0	3	0	2
<i>B. melaninogenicus</i> ATCC 15930	0	4	2	1	0	0	0	0	0	0	4	5	1	1	0	4	0	5	0	3
<i>B. ochraceus</i> VPI 2845	0	5	1	2	0	5	5	4	0	1	5	4	0	0	0	5	0	4	0	0
<i>F. polymorphum</i> NCTC 10562	0	0	3	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>F. necrophorum</i> NCTC 10575	0	5	3	4	0	0	0	0	0	0	5	1	0	0	0	0	0	0	0	0
<i>F. necrogenes</i> NCTC 10723	0	3	1	1	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
<i>Veillonella</i> NCTC 9816	0	0	1	1	0	0	0	0	0	0	2	3	0	0	0	0	0	0	0	0

Numerical values 1 to 5 indicate increasing colour intensity.

were identical with the reactions of the corresponding type cultures. All of the isolates of *Veillonella* and the type cultures NCTC 9805 and 9816 gave distinctive and identical reactions. In the case of *B. melaninogenicus* the API ZYM patterns of the three subspecies were quite distinctive. Thirteen of the clinical isolates of *B. fragilis* gave patterns identical with that produced by *B. fragilis* NCTC 9343, and the remaining five strains exhibited minor but consistent differences in reactions 15, 17, and 20. On this basis, four groups could be distinguished, which, however, do not correspond with the subspecies identified by standard techniques as shown in Table 2. The reactions of different strains of *B. fragilis* were quite consistent on repeated testing, with the exception of test number six which varied on different occasions from strongly positive to negative. This was the only reaction which varied on repeated testing with any of the strains examined in the study.

## Discussion

The results of this study indicate that the API ZYM test provides a simple, accurate, and reproducible method for the identification of Gram-negative anaerobes from human sources. Although subspeciation of *B. fragilis*, as currently defined, was not possible, the strains of *B. fragilis* were divisible into four distinct groups. The test clearly distinguished

between *B. melaninogenicus* ss. *asaccharolyticus* and *B. melaninogenicus* ss. *intermedius*, and it is of interest that all seven of the isolates from dental abscesses were ss. *intermedius* while the remaining seven from abdominal wounds, vaginal swabs, and cerebral abscesses were ss. *asaccharolyticus*, confirming a similar observation by Duerden *et al.* (1976). The type culture ATCC 15930, originally classified as *B. oralis*, but which Holbrook and Duerden (1974) have suggested should be reclassified as *B. melaninogenicus* ss. *melaninogenicus*, gave an API ZYM reaction distinct from the other two subspecies but identical with *B. melaninogenicus* ss. *melaninogenicus* VPI 4196. However, we were unable to ascertain whether or not this reaction was typical of this subspecies as no other strains were available for study. A further peculiarity of ATCC 15930 was that it was resistant to metronidazole when tested with a 5 µg disc.

The results obtained with the API ZYM test were consistent and distinct for the different species examined regardless of whether the primary incubation of the cultures was for 24, 48 or 72 hours, provided that the inoculum used for the test was a dense suspension of the organisms. No variation in results was obtained when the test was incubated for 4 or 6 hours. The only problem encountered was deterioration of reagent B when the ambient temperature was abnormally high. Deterioration was indicated by a change in colour of the reagent from

yellow to pink and a marked weakening or loss of all reactions in the test. This can be avoided by storing the reagents at 9°C.

The API ZYM patterns obtained were quite distinct for all the species which were tested, even though the genera *Fusobacterium* and *Veillonella* gave only three or four positive reactions. We are therefore of the opinion that the API ZYM system affords a reliable, simple method for the identification of Gram-negative anaerobes and as such will help to delineate more clearly their role in human infections.

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Requests for reprints should be sent to Dr H. R. Ingham, Department of Microbiology, General Hospital, Westgate Road, Newcastle upon Tyne.

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