Beta-galactosidase and lactose fermentation in the identification of enterobacteria including salmonellae

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SYNOPSIS One hundred and fourteen strains of non-lactose fermenters and 127 lactose fermenters on MacConkey’s agar have been compared in the 5% and 1% lactose tests and in β-galactosidase production, using ortho-nitro-phenyl-β-D-galactopyranoside (O.N.P.G.) as a test substance. The superiority of the O.N.P.G. test in the number of positive results and its rapidity is shown. In general, late or non-lactose fermenting strains of genera, usually lactose-positive, yield a rapidly positive O.N.P.G. reaction. Forty-one wild strains of Salmonella, Proteus, Providencia, and Pseudomonas aeruginosha were found negative in all three tests.

Of 1,075 stock strains of Salmonella examined in the O.N.P.G. test, all were negative except nine; four of these were lactose-positive strains. For practical purposes, Salmonella strains in Great Britain may be regarded as O.N.P.G. negative.

Among 100 stock strains of Arizona there was considerable variation of behaviour in the O.N.P.G. test and in the 5% and 1% lactose tests. Most strains of Arizona can be considered to yield a positive O.N.P.G. test but a minority give a negative result.

The test is recommended for routine use in the differentiation of Salmonella from other enterobacteria and for use in bacterial identification.

The 5% lactose fermentation test in parallel is suggested when the O.N.P.G. test is used for isolating routine pathogens, because organisms such as Shigellosis sonnei, Shigellosis dysenteriae 1, and Pasteurella pseudotuberculosis are O.N.P.G. positive.

Two major factors are concerned in the fermentation of lactose by enterobacteria, first a permease which enables the lactose to enter the cell, and secondly β-galactosidase which attacks the β-galactoside link in lactose, hydrolysing it to glucose and galactose. An organism may fail to ferment lactose if it lacks the permease or the β-galactosidase. In the absence of the permease it is possible to demonstrate the presence of β-galactosidase by using ortho-nitrophenyl-β-D-galactopyranoside (O.N.P.G.) as a substrate. This colourless substance is attacked by β-galactosidase with the release of yellow o-nitrophenol, thus providing a simple test for the presence of the enzyme. Ortho-nitrophenyl-β-D-galactopyranoside has been used in genetic and biochemical studies for some years. A short review and references were given by Le Minor and Ben Hamida (1962).

Lowe and Evans (1957), following earlier suggestions, used 5% instead of 1% lactose as a test for β-galactosidase and found that it gave both quicker and a larger number of positive results. They suggested that the increase of the percentage of lactose overcame the permeability barrier. Lowe in an essay in 1960, part of which was published in 1962, used the O.N.P.G. test in the rapid differentiation of Salmonella from late lactose-fermenting paracolons. He also compared the results of two methods for testing with O.N.P.G. by incubating benzene-treated cells with O.N.P.G., and by growing organisms in peptone water containing ortho-nitrophenyl-β-D-galactopyranoside. The second method was recommended as being simple and giving quicker results. Lowe found that both O.N.P.G. tests gave quicker results than the fermentation of 5% lactose and also that 83 late lactose-fermenting paracolons were O.N.P.G. positive. Salmonella and Proteus spp. were O.N.P.G. negative. Le Minor and Ben Hamida (1962) described the results of tests on 586 classified strains of enterobacteria which they

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The results of these papers taken together can be summarized as follows:—

Late lactose-fermenting strains of the following usually prompt lactose-fermenting species and genera, namely, E. coli, Citrobacter, Cloaca, and Klebsiella, are O.N.P.G. positive. Also O.N.P.G. positive are certain lactose-variable or non-lactose-fermenting genera and species, namely, Dispar, Hafnia, Serratia, Vibrio, Aeromonas, Past. septis, and Past. pseudotuberculosis. Non-lactose-fermenting species and genera, Salmonella, Proteus, Providencia, Alkalescens, Pseudomonas, Moraxella, Past. septica and Past. tularensis are O.N.P.G. negative. Those species and genera which gave variable results to O.N.P.G. tests are set out below.

<table>
<thead>
<tr>
<th>Arizona</th>
<th>O.N.P.G. Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late lactose-fermenting and non-lactose fermenting</td>
<td>30/40 Positive</td>
</tr>
<tr>
<td>Non-lactose fermenting Shigella</td>
<td>10/40 Negative</td>
</tr>
<tr>
<td>Shigella sonnei late lactose fermenting non-lactose fermenting</td>
<td>Positive</td>
</tr>
<tr>
<td>Shigella flexneri, non-lactose fermenting</td>
<td>Negative</td>
</tr>
<tr>
<td>Shigella dysenteriae 1, non- or late-lactose fermenting</td>
<td>Negative</td>
</tr>
<tr>
<td>Shigella dysenteriae 2, non-lactose fermenting</td>
<td>Positive</td>
</tr>
<tr>
<td>Shigella boydii 9, late lactose fermenting Non-lactose fermenting</td>
<td>Negative or weak positive</td>
</tr>
</tbody>
</table>

INVESTIGATION

We decided to investigate enterobacteria isolated in the field from human sources in order to assess the value of the O.N.P.G. tests in routine work. We compared the results obtained from 1% and 5% lactose and the two O.N.P.G. media and classified the strains. In addition a large series of Salmonella spp. was examined to see if O.N.P.G. positive strains occurred. A series of Arizona spp. was included for comparison with Salmonella.

STRAINS OF ENTEROBACTERIA EXAMINED A total of 282 strains were examined, of which 127 gave lactose-fermenting colonies and 114 gave non-lactose fermenting colonies on MacConkey's agar on primary subculture. In general, these 114 strains belonged to genera whose members usually ferment lactose.

Approximately two-thirds of the non-lactose fermenters on MacConkey's agar were isolated in Ibadan, Nigeria, from human faeces in cases of diarrhoea or from urine, pus, sputum, wounds, or similar sites. The rest of these strains giving non-lactose fermenting colonies had been sent in to the Salmonella Reference Laboratory as possible Salmonella spp. and proved not to be; the majority of these had been isolated in the United Kingdom.

A few of the lactose fermenters on primary subculture on MacConkey's agar had produced non-lactose fermenting colonies on the primary deoxycholate citrate agar.

A total of 41 strains comprising Salmonella, Proteus, Providencia and Pseudomonas aeruginosa were also examined.

METHODS

LACTOSE FERMENTATION Strains were tested by using a heavy inoculum into 5% lactose in 0-1% peptone water (Lowe and Evans, 1957) and by a needle-point inoculum into 1% lactose in 1% peptone water. The tubes were observed for 14 days. Andrade's indicator was used in each test.

O.N.P.G. tests by peptone water technique (Lowe, 1962, see appendix). Strains were plated on MacConkey's agar, which is in routine use in most laboratories in the United Kingdom. Material from a single colony was inoculated with a needle point into the O.N.P.G. peptone water, which was then incubated at 37°C and read at 18 hours.

O.N.P.G. tests by suspension method (Le Minor and Ben Hamida, 1962) Single colonies from MacConkey's agar plates were inoculated on to nutrient agar slopes which were incubated for 18 hours. The growth was washed off with 0-25 ml of 0-85% saline, a drop of toluol added and the suspension shaken in a mechanical shaker for 10 minutes; 0-25 ml of 0-4% O.N.P.G. solution in phosphate buffer was then added, and the mixture incubated at 37°C and read after 30 minutes, one hour, and four hours.

CLASSIFICATION OF STRAINS The 282 strains examined were classified by the results of the following tests:— Presence of motility; the fermentation of lactose, glucose, mannitol, sucrose, salicin, and dulcitol; the production of gas in glucose; the hydrolysis of urea; the production of indole, hydrogen sulphide, and acetyl-methyl-carbinol, and of phenylpyruvic acid from phenylalanine; the ability to grow in potassium cyanide medium and in Simmon's citrate; the utilization of sodium malonate; the methyl red test; the production of decarboxylases for lysine and ornithine and of a dihydrolase for arginine.

The distribution and classification of these 282 strains are shown in Table I. The non-lactose fermenters on MacConkey's agar were classified as follows:— Twenty-two Escherichia coli, 26 Citrobacter, two Klebsiella, 51 Aerobacter/Serratia/Hafnia, six Salmonella, 10 Providencia, 11 Proteus, 14 Pseudomonas aeruginosa, and 13 biochemically irregular strains. Of the 127 lactose fermenters on MacConkey's agar, 122 fell into the first four groups and five were biochemically irregular strains. The methods, media, and criteria for classification were, in general, those described by Edwards and Ewing (1962).
RESULTS

COMPARISON OF THE TWO O.N.P.G. METHODS OF TESTING FOR β-GALACTOSIDASE The two methods gave the same results. The suspension method was quicker because strongly positive organisms gave a positive result within 20 minutes and even with weakly positive organisms the result was definite at one hour. No additional positives were obtained after four hours. In the peptone water test using a heavy inoculum, the result was positive within six hours. All strains positive in the suspension test were positive in the peptone water test within 18 hours when a needle-point inoculum was used.

We agree with Lowe that the peptone water test is technically much simpler and recommend it for routine use.

EFFECT OF THE GROWTH MEDIUM ON THE O.N.P.G. TEST Suspensions of 30 strains from various media were tested for β-galactosidase by the suspension method. Suspensions from MacConkey's agar gave the same results as those from nutrient agar although a little more slowly. Deoxycholate-citrate agar did not prove satisfactory, because suspensions from this medium were often sticky or scanty and many Esch. coli strains failed to grow on it.

Suspensions from Gillies I slopes (Gillies, 1956) were not satisfactory, since with many strains the growth was not luxuriant enough; moreover some strains produced a marked yellow colour which made the test difficult to read. A similar difficulty was met with in Proteus strains which hydrolysed the urea in the Gillies medium and turned the indicator in the medium blue.

Centrifuging 18-hour growths in 5 ml. of peptone water and 7 ml. of nutrient broth produced a rather scanty deposit; this method was laborious and was abandoned after testing 10 strains.

Three strains of Serratia marcescens produced a red pigment in both tests which gave rise to difficulty in reading the result.

RAPIDITY OF RESULTS AND NUMBER OF POSITIVE RESULTS Table I shows the number of strains giving positive results in each test and the speed with which a positive reaction was obtained.

Lactose fermenters on MacConkey's agar One hundred and twenty-seven strains were tested; they were, as anticipated, all positive in the O.N.P.G. test. Non-lactose fermenters on MacConkey's agar One hundred and fourteen strains were examined. As Table I shows, more strains were positive in the O.N.P.G. test than in either 5% or 1% lactose. There were more positive results in 5% than in 1% lactose and, in some cases, 5% lactose gave quicker results than 1% lactose.

Six strains were O.N.P.G. negative, and negative in 5% and 1% lactose; two of these strains were Citrobacter spp. and four gave irregular biochemical reactions.

Five strains were O.N.P.G. negative but late lactose fermenters in 5% and 1% lactose or in 5% only. These strains were, however, non-lactose fermenters on MacConkey's agar. Two were Citrobacter strains and the other three were biochemically irregular.

REACTIONS OF THE STRAINS BY SPECIES AND GENERA Table II shows the results according to species and genera.

E. coli Twenty-two yielding non-lactose fermenting colonies on MacConkey's agar were all O.N.P.G. positive.

Klebsiella Two late lactose-fermenting strains were O.N.P.G. positive. Late lactose-fermenting strains of Klebsiella were rare in this material.

Aerobacter/Serratia/Hafnia This group could

### Table 1

<table>
<thead>
<tr>
<th>Fermentation of Lactose</th>
<th>Lactose-fermenting colonies</th>
<th>Non-lactose fermenting colonies</th>
<th>Non-lactose fermenting colonies</th>
<th>Non-lactose fermenting colonies</th>
<th>Non-lactose fermenting colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+ or X</td>
<td>+ or X</td>
<td>+ or X</td>
<td>+ or X</td>
</tr>
<tr>
<td>1%</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5%</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O.N.P.G.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli</td>
<td>127</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>39</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>60</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Serratia</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Hafnia</td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Biochemically</td>
<td>39</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Irregular</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

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+ = prompt positive  X = late positive  (+) = positive in 3-5 days  -- = negative
TABLE II

REACTIONS OF THE STRAINS BY SPECIES AND GENERA

<table>
<thead>
<tr>
<th>Species</th>
<th>Lactose</th>
<th>No. O.N.P.G.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>Late</td>
<td>22</td>
</tr>
<tr>
<td><strong>Citrobacter</strong></td>
<td>Late</td>
<td>24</td>
</tr>
<tr>
<td><strong>Citrobacter</strong></td>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td><strong>Klebsiella</strong></td>
<td>Late</td>
<td>2</td>
</tr>
<tr>
<td><strong>Aerobacter/Serratia</strong></td>
<td>Late</td>
<td>39</td>
</tr>
<tr>
<td><strong>Hafnia</strong></td>
<td>Negative</td>
<td>12</td>
</tr>
<tr>
<td><strong>Biochemically irregular strains</strong></td>
<td>Late</td>
<td>9</td>
</tr>
<tr>
<td><strong>Irregular strains</strong></td>
<td>Negative</td>
<td>4</td>
</tr>
<tr>
<td><strong>Salmonella</strong></td>
<td>Negative</td>
<td>6</td>
</tr>
<tr>
<td><strong>Providencia</strong></td>
<td>Negative</td>
<td>10</td>
</tr>
<tr>
<td><strong>Proteus</strong></td>
<td>Negative</td>
<td>11</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Negative</td>
<td>14</td>
</tr>
</tbody>
</table>

not be further subdivided by the tests used in classification. All strains were O.N.P.G. positive and Table II shows that 12 of 51 strains were lactose negative. Three of these were *Serratia marcescens* and the other nine gave biochemical reactions consistent with those of *Hafnia*.

Salmonella, Providencia, Proteus, and Pseudomonas aeruginosa were, as expected, negative in lactose and O.N.P.G.

THE O.N.P.G. TEST RESULTS ON-stock SALMONELLA STRAINS One thousand and seventy-five strains from the stock collection of the Salmonella Reference Laboratory, Colindale, were submitted to the O.N.P.G. test only, as the majority had been examined for lactose fermentation here or in other laboratories. All but about 30 of the known serotypes were included. In many instances several strains of each serotype were tested. A few strains were unusual either biochemically or serologically. Hence this selection was not comparable with a similar number of wild isolates, but was interesting in showing differing results within the genus.

Of these 1,075 strains, 1,066 were O.N.P.G. negative. Of the remaining nine O.N.P.G.-positive strains, four were known to be lactose fermenters, *S. ferlaci*, a lactose-positive species, isolated so far only from desiccated coconut, and wild lactose-positive strains of *S. typhi*, *S. anatum* and *S. newington*. The *S. typhi* strain was of human origin; the *S. anatum* and *S. newington* strains came from the same custard powder. Wild lactose-positive variants of these last two serotypes are known to occur very rarely.

Of the remaining five, four belonged to *Salmonella* subgenus II and one to subgenus I (Kauffmann, 1961).

Subgenus II contains about 50 of the 800 or so known serotypes. Serotypes belonging to this subgenus possess biochemical properties which place them between the typical *Salmonella* of subgenus I and *Arizona*. They are malonate and gelatin positive, but dulcitol positive and lactose negative. They are rarely isolated from human sources. The species which were O.N.P.G. positive were one strain of each of *S. lincoln*, *S. carletonis* and *S. grunty*. A diphasic strain of *S. bleedon* was O.N.P.G. positive whereas a monophasic strain was negative.

The only *Salmonella* of subgenus I which was O.N.P.G. positive, excluding the lactose-positive strains, was a wild strain of *S. kotte*.

Thus less than 0-1% of this collection of *Salmonella* strains was O.N.P.G. positive, and four of the non-lactose fermenting, O.N.P.G.-positive strains belonged to subgenus II.

As the majority of wild *Salmonella* spp. isolated in the United Kingdom are strains of either *S. typhimurium*, *S. paratyphi* B, or other common serotypes of subgenus I in serological O-groups B, C, D or E, *Salmonella* serotypes may be considered O.N.P.G. negative for all practical purposes.

THE O.N.P.G. TEST RESULTS ON STOCK ARIZONA STRAINS One hundred stock strains, almost all of different serotypes, were examined by the O.N.P.G. test and for fermentation of 5% and 1% lactose and their appearance on MacConkey plates. Of the 86 O.N.P.G.-positive strains, 44 were promptly lactose positive, 32 delayed lactose positive, and 10 lactose negative. Of the 14 O.N.P.G.-negative strains, 10 were lactose negative and four gave a delayed positive result.

Thus the genus *Arizona* shows considerable variation in behaviour in both lactose and the O.N.P.G. tests. The four O.N.P.G.-negative, lactose-positive strains are being investigated.

DISCUSSION

The O.N.P.G. test is technically simple and suitable for routine use. In genera whose members usually possess β-galactosidase, the presence of the enzyme can be rapidly demonstrated in strains giving delayed or weak lactose fermentation. The O.N.P.G. test is superior to testing in lactose for the presence of β-galactosidase both in the rapidity of the results and in the number of positive results obtained. Five per cent lactose was better than 1%. These findings are similar to those of Lowe (1962) and this study.

The O.N.P.G. test is thus useful in differentiating *Salmonella* strains from late and non-lactose fermenting strains of genera, most strains of which...
are lactose positive, and from certain usually lactose-negative genera such as *Serratia* and *Hafnia*.

Six out of 114 wild strains were found to be negative in both the O.N.P.G. and lactose tests. Of these two were *Citrobacter* spp. and four were biochemically irregular strains. Lowe (1962) also found paracolons showing similar behaviour.

The O.N.P.G. test cannot replace lactose in the routine search for pathogenic organisms because late lactose-fermenting *Shigella sonnei*, *Shigella dysenteriae* 1, *Shigella boydii* 9, and other occasional strains amongst the serotypes of *Shigella dysenteriae* and *Shigella boydii* are O.N.P.G. positive (Szturm-Rubinstein and Le Minor, 1962). Pasteurella *pestis* and *Pasteurella pseudotuberculosis* are O.N.P.G. positive (Mollaret and Le Minor, 1962). In addition five strains which were O.N.P.G. negative but gave a delayed fermentation of lactose were found in this series. The suggestion that a 5% lactose test should be included as well as an O.N.P.G. test is supported by the recognition that such strains occur and that some strains of *Arizona* behave in a similar way.

We wish to thank Dr. Joan Taylor for her help and advice and Mrs. M. M. Brookes for her help with the investigation.

REFERENCES


ADDITIONUM

Since this paper was written, Kauffmann (1963) has published his findings on testing a large number of *Salmonella* and *Arizona* cultures for β-galactosidase, using the O.N.P.G. test following the methods of Le Minor and Ben Hamida (1962). He finds that 166/169 *Salmonella* cultures of subgenus I were O.N.P.G. negative but 59/143 cultures of subgenus II were positive although many gave only a delayed or weak reaction after a few days. It appears that using this method and observing the reaction for several days a weak reaction is detected which would be missed by the one-day peptone water test of Lowe (1962) used in this investigation. However, our purpose was less in determining the taxonomic status of the genera than in establishing the validity and rapidity of the test in the differentiation of *Salmonella* cultures from other *Enterobacteriaceae*, and for this purpose the less sensitive test is perhaps of more value.

APPENDIX

The O.N.P.G. test was performed according to the method of Lowe (1962).

O.N.P.G. solution:—

**O-nitrophenyl-β-D-galactopyranoside** 0.6 g

0.01 M. sodium phosphate (Na₂HPO₄)

buffer at pH 7.5 100 ml.

Dissolve at room temperature and sterilize by Seitz filtration.

Add aseptically 1 part of O.N.P.G. solution to 3 parts of 1% peptone water² pH 7.5. The medium should be aseptically distributed in tubes in 2 ml amounts. Incubate for 24 hours at 37°C for sterility. Keeps for a month at 4°C. Inoculate and incubate overnight at 37°C.

**Positive reactions** A formation of a yellow colour (o-nitrophenol) of varying intensity. This colour change will occur rapidly (three hours) provided a heavy inoculum is used. Tubes showing no colour change in 24 hours should be discarded.

1 Obtainable from L. Light and Company, Colnbrook, Buckinghamshire.

2 Evans peptone was used in this investigation.
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