The Use of M1 Medium in Transformation of *Streptococcus pneumoniae*

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Cultures of a variety of pneumococcal strains maintained competence longer and gave higher yields of transformants when incubated in M1 medium compared with NS medium. This was most probably due to the cells remaining competent for longer in M1 medium. Various parameters controlling the development of competence in M1 medium were investigated. The onset of competence was delayed in M1 medium compared with that in NS medium, probably due to the presence of Casamino acids in the former. Competence developed normally over a pH range of 7.3 to 8.3, with cultures inoculated from the same batch of frozen 'precultures' showing consistent characteristics. This was not observed when frozen 'sensitization' cultures were revived.

The average cell chain length increased with the development of competence in all the strains tested and, with the exception of cultures which had entered the stationary growth phase, declined after the culture had lost its competence. The extent of the increase in chain length was dependent upon the pH of the medium.

**INTRODUCTION**

Since the basic NS medium was described by Ephrussi-Taylor (1951) for the transformation of *Streptococcus pneumoniae*, modifications of it have been used by this laboratory and by many others (e.g. Butler, 1965; Sicard, 1964; Kohoutova, 1965; Biswas & Ravin, 1971). There are, however, drawbacks with using this medium. For instance, although competence develops in all the cells of cultures of strain C13 (Butler *et al.*, 1979), this state persists for no more than 20 to 30 min, and hence the experimental conditions have to be rigorously controlled between experiments. The pH of the medium also has to be carefully adjusted as competence will only develop over a narrow range of pH. Competence persists for longer in cultures of strains S111-1, 401 and R6x but develops in only a proportion of the culture, which can lead to spurious linkage (Butler *et al.*, 1979).

M1 medium has been mainly used for propagating pneumococcal bacteriophages (Moynet, 1976) and it was whilst screening for transducing phages that it was observed that cultures growing in M1 medium were developing high levels of competence. Subsequent work, described in this paper, has shown that this medium has a number of features which recommend it for use in pneumococcal transformation, and it is now used in preference to NS medium in this laboratory (Grist, 1979; Grist & Butler, 1979; George & Butler, 1979).

**METHODS**

*Strains and their maintenance.* All the wild-type recipient strains used in this study were ultimately derived from strain R36A of Avery *et al.* (1944) and their relationships have been summarized by Tiraby *et al.* (1975). The strain designated as strain C13T is a sample of strain C13 kindly sent more recently by Professor A. M. Sicard. The procedure for preparing 'precultures' from which the transforming cultures were prepared has been previously described (Butler, 1965).
Media. The preparation of NS medium has been described (Rotheim & Ravin, 1961; Sicard, 1964); this medium is a modification of Medium 3 of Ephrussi-Taylor (1951).

M1 medium (Moynet, 1976) has the following composition: 10 g Casamino acids (Difco), 5 g Neopeptone (Difco), 1 g yeast extract (Difco), 5 g NaCl, 50 ml Tris/HCl buffer, pH 7.8, 1 l distilled H₂O. Portions of 200 ml were autoclaved and completed with 1 ml 40% (w/v) D(+)-glucose; when used for transformation, the medium was supplemented with 0.125% (w/v) bovine serum albumin (Armour Pharmaceuticals) and 0.1% (w/v) charcoal-absorbed Difco yeast extract.

Preparation of DNA. DNA was prepared by method II of Butler & Nicholas (1973).

Transformation procedures. The competent state of a culture was measured by diluting a sample of the culture tenfold into fresh medium which contained a saturating concentration of a standard DNA carrying the str-r41 marker. The yield of transformants of a 0.1 ml sample after 20 min incubation was then used as a measure of competence.

The procedure used for measuring the linkage index between pairs of newly transformed markers has been described previously (Butler & Nicholas, 1973).

Determination of the average streptococcal chain length. The usual technique of first staining the cells with methylene blue (Butler & Smiley, 1973) could not be used on samples which contained DNA in the medium as, for reasons unknown, it resulted in the immediate lysis of the majority of the cells present. Instead, these samples were examined at the time of sampling under a phase-contrast microscope. At least 100 chains were examined in a determination.

RESULTS

Comparison between the development of competence in NS and M1 media

Table 1 summarizes the results of numerous experiments which routinely measured the development of competence in NS and M1 media. For the purpose of this study, a culture was considered competent if it yielded more than 1000 transformants on an assay plate. In the majority of cases where competence persisted for longer than 60 min, the incubation was stopped before the competence had declined, and hence the results represent minimum estimates for the duration of competence. The results show that both the average yield of transformants and the average duration of competence were higher in M1 than in NS medium in all strains tested. With strain C13, a quarter of the cultures tested maintained a high level of competence in M1 medium for more than 60 min. Duration of competence for more than 60 min was a rare event in NS medium, and it was always associated with a failure of the culture to develop to high levels of competence. This trend was apparent with the other strains tested. For instance, over half of the strain R6 cultures remained competent for longer than 100 min in M1 medium; this was never observed using NS medium. The longest duration of competence recorded occurred with strain 401 which maintained high levels of competence for longer than 4 h.

It was possible that the improved competence obtained in M1 medium could have been due either to a larger number of cells developing competence, or to the cells remaining competent for longer. With strain C13 the latter reason seemed more likely, as it is known that competence develops in all cells of C13 cultures in NS medium (Butler et al., 1979), which effectively excludes the former possibility. Two experiments gave evidence which agreed with this view. Firstly, a culture of strain C13 was incubated in both NS and M1 media in the presence of saturating concentrations of DNA which carried the str-r41 marker. At various times, samples were removed, and a portion of each was scored for str-r41 transformants whilst the rest was diluted 1:10 into medium containing DNA marked with the ery-r2 marker. After a further 20 min incubation, the ery-r2 transformants were scored. The continuing presence of the str-r41 DNA ensured that entry of this marker occurred first, to give maximum numbers of transformants, and hence a comparison of the number of str-r41 transformants obtained with each medium would indicate whether the two media contained different percentages of competent cells, whilst a comparison of the ery-r2 transformants would show any differences in the time the culture remained competent. The results given in Fig. 1 show that the yields of str-r41 transformants obtained in either medium were similar;
Table 1. *Comparison of the duration and extent of competence development of various pneumococcal strains in NS and M1 media*

The yield of transformants is expressed by $\bar{x} = 10^{-6} \times$ (average number of *str-r41* transformants per ml of the cells + DNA mixture plated, taken over the whole period of competence indicated); % frequency (% freq.) represents the percentage of the total number of competent cultures tested that gave a period of competence of that particular duration.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>No. of cultures tested</th>
<th>Duration of competence (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>C13</td>
<td>NS</td>
<td>155</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>84</td>
<td>% freq.</td>
</tr>
<tr>
<td>R6</td>
<td>NS</td>
<td>22</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>13</td>
<td>% freq.</td>
</tr>
<tr>
<td>S111-1</td>
<td>NS</td>
<td>101</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>7</td>
<td>% freq.</td>
</tr>
<tr>
<td>401</td>
<td>NS</td>
<td>31</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>9</td>
<td>% freq.</td>
</tr>
</tbody>
</table>

Transformation of pneumococci in M1 medium
However, competence appeared earlier in NS medium but lasted longer in M1 medium. Secondly, since the linkage between markers transformed at saturating concentrations into cultures incubating in NS and M1 media would be influenced by the percentage of cells that develop competence (Goodgal & Herriott, 1961), the linkage index of the ery-r2 str-r41 pair was measured, using strain S111-1 as recipient, of which only a proportion of the culture develops competence (Butler et al., 1979). The linkage index was found to be 0.20 and 0.24 in NS and M1 media respectively, and hence seen to be similar.

Strain C13 grew with a generation time of 23 min in both media and all the strains tested entered the stationary phase in M1 medium with titres between $4 \times 10^8$ and $1 \times 10^9$ cells ml$^{-1}$. However, the onset of competence was always delayed in cultures in M1 medium compared with those in NS medium. In the example shown in Fig. 1, the culture growing in NS medium developed competence at a titre of $2.4 \times 10^7$ cells ml$^{-1}$ whilst in M1 medium the onset of competence was delayed until the titre reached $4.6 \times 10^7$ cells ml$^{-1}$. The most likely cause of this delay was the presence of Casamino acids in the M1 medium, as its presence in NS medium resulted in a similar delay (Table 2). Sirotnak (1971) showed a similar effect with the addition of tryptic peptides of casein to his transformation medium. As Casamino acids is prepared from an acid hydrolysate of casein, the two effects may be due to the same cause.

Some factors concerned with the development of competence in M1 medium

The original discovery that cultures incubating in M1 medium developed competence was surprising, as the medium does not contain albumin, which has always been considered essential to the development of competence in pneumococcus (Hotchkiss & Ephrussi-Taylor, 1951; Fox & Hotchkiss, 1957). The reason for this became apparent when the effect of omitting albumin from NS medium on the development of competence was investigated. This revealed that certain strains could develop competence in the absence of albumin whilst other strains still retained a requirement (Table 2). Because of this variable response, albumin was routinely added to M1 medium used in transformations.

Competence will only develop in NS medium at pH 7.8 ± 0.10. In M1 medium, however, strains C13 and R6 develop similar levels of competence over a range of pH from 7.30 to 8.30 with only minor differences in the time of the onset and the shapes of the competence peak, as exemplified by the results given in Table 3. The growth of strain C13 was also unaffected by these changes in the pH of the medium.
Table 2. *Effect of Casamino acids and albumin on the development of competence of different strains in NS medium*

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>Medium</th>
<th>Time of sampling (min)</th>
<th>No. of <em>str-41</em> transformants per 0.1 ml plated undiluted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>C13 revived</td>
<td>NS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>from stocks</td>
<td>NS + albumin</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>frozen in 1975</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C13 revived</td>
<td>NS</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>from stocks</td>
<td>NS + Casamino acids</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>frozen in 1978</td>
<td>NS + albumin</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

* 3500–5000 transformants.
Table 3. *Effect of pH on the development of competence of strains C13 and R6 in M1 medium*

(a) *Strain C13*

<table>
<thead>
<tr>
<th>pH</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>95</th>
<th>105</th>
<th>115</th>
<th>125</th>
<th>135</th>
<th>145</th>
<th>155</th>
<th>165</th>
<th>175</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.80</td>
<td>6</td>
<td>9</td>
<td>34</td>
<td>14</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>85</td>
</tr>
<tr>
<td>7.00</td>
<td>15</td>
<td>9</td>
<td>40</td>
<td>16</td>
<td>35</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>635</td>
<td>199</td>
<td>36</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1004</td>
</tr>
<tr>
<td>7.56</td>
<td>48</td>
<td>63</td>
<td>41</td>
<td>2174</td>
<td>10000</td>
<td>1250</td>
<td>501</td>
<td>193</td>
<td>376</td>
<td>339</td>
<td>3804</td>
<td>70</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18859</td>
</tr>
<tr>
<td>8.25</td>
<td>9</td>
<td>15</td>
<td>1</td>
<td>42</td>
<td>8000</td>
<td>6316</td>
<td>142</td>
<td>71</td>
<td>249</td>
<td>595</td>
<td>330</td>
<td>163</td>
<td>207</td>
<td>190</td>
<td>70</td>
<td>16394</td>
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</table>

(b) *Strain R6*

<table>
<thead>
<tr>
<th>pH</th>
<th>70</th>
<th>90</th>
<th>110</th>
<th>130</th>
<th>150</th>
<th>170</th>
<th>190</th>
<th>210</th>
<th>230</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.00</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>26</td>
<td>1062</td>
<td>482</td>
<td>334</td>
<td>284</td>
<td>288</td>
<td>2484</td>
</tr>
<tr>
<td>7.80</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>105</td>
<td>650</td>
<td>539</td>
<td>426</td>
<td>186</td>
<td>230</td>
<td>2142</td>
</tr>
<tr>
<td>8.10</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>21</td>
<td>594</td>
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<td>344</td>
<td>423</td>
<td>2616</td>
</tr>
<tr>
<td>8.40</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>361</td>
<td>196</td>
<td>500</td>
<td>193</td>
<td>1255</td>
</tr>
</tbody>
</table>
The technique of freezing batches of precultures in 'P' medium (1% Difco Neopeptone, 0.8% charcoal-absorbed Difco yeast extract, 0.025% glucose, pH 7.6) with 10% (v/v) glycerol to −20 °C or −70 °C (Fox & Hotchkiss, 1957; Butler, 1965) has been used as a convenient method for storing cultures from which competent cultures can be prepared, and such cultures could be kept for at least 5 months at −70 °C without significantly altering their later competence development in M1 medium. Less satisfactory results were obtained by freezing the 'sensitization' culture (i.e. the culture inoculated from the preculture in which competence actually developed) in M1 medium prior to its developing competence. Although competence developed in the thawed culture, it gave very variable yields of transformants with samples from the same batch of frozen culture.

The mean chain length of cells in cultures of all the strains tested increased around the time that they developed competence to a maximum which, compared to the initial value, could be some 700% higher for strain 401, 300% for strain S111-1 and 800% for strain C13T. The extent of this increase varied in samples from the same batch of precultures, although the pattern of change was similar. The increased chain length was maintained throughout competence but, except when competence persisted into the stationary phase, chain length usually decreased as competence declined. The extent of the increase was dependent upon the pH of the medium, as exemplified in Fig. 2, in which a substantially greater increase is shown with M1 medium at pH 8.3 than with the same medium at pH 7.6, although the yields of transformants at both pH values were similar.

**DISCUSSION**

M1 medium has a composition substantially different from that used by many workers in pneumococcal transformation since the original publication of Ephrussi-Taylor and her group. It is also substantially different from the medium, known as CH medium, described by Tiraby et al. (1973). Although both contain Casamino acids, it is similar to the medium developed by Tiraby et al. (1975) for transforming pneumococcal cultures in solid medium. Although it is possible to freeze cultures in CH medium prior to their developing competence, this procedure was unsuitable in M1 medium and is not recommended.

M1 medium has a number of features which make it especially suitable for use in pneumococcal transformation. These can be summarized as follows:

1. Ease of preparation.
2. Transformation is unaffected over a range of pH from 7.3 to 8.3.
3. High levels of competence are maintained for longer in cultures in M1 medium as compared to NS medium; this allows the timing of an experiment in relation to the onset of competence to be less critical.

4. The yield of transformants is higher with strains S111-1 and 401, and hence M1 medium is particularly useful for those strains which give relatively low numbers of transformants in NS medium.

REFERENCES


