Modulation of Competence for Genetic Transformation in *Streptococcus pneumoniae*

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The spontaneous development of competence by cultures of *Streptococcus pneumoniae* in casein hydrolysate medium was strongly dependent on the initial pH of the culture medium. Cells growing in cultures beginning with a wide range of initial pH values (6.8 to 8.0) all developed competence, as measured by [³H]DNA uptake, [³H]DNA degradation and genetic transformation; but the initial pH of the medium affected both the timing of the occurrence of competence and the number of times the culture became competent. In cultures grown in media of lower initial pH, competence occurred only once, at high population densities, while in more alkaline media a succession of competence cycles occurred, beginning at lower cell densities. The critical population density required for the initiation of competence varied tenfold over the pH range studied. Successive competence cycles in an alkaline medium were not equivalent: while the percentage of competent cells in the first competence cycle was high (approximately 80%), that in the second competence cycle was lower (approximately 12%). Correspondingly, competence-specific proteins were less prominent in the labelled-protein pattern of the second competence cycle than in that of the first. These features of the physiology of competence control make it possible to adjust the expression of competence to suit various experimental requirements.

INTRODUCTION

The control of the expression of competence for genetic transformation among naturally transformable bacteria is not well understood. Piliated cells are competent throughout culture growth in *Neisseria gonorrhoeae* (Sparling, 1966; Biswas *et al.*, 1977); *Bacillus subtilis* develops competence slowly during the transition from exponential-phase growth to the stationary phase (Dooley *et al.*, 1971; Nester, 1964; Bott & Wilson, 1967); and *Haemophilus influenzae* is most competent in conditions where growth is blocked but protein synthesis continues (Leidy *et al.*, 1962; Spencer & Herriott, 1965; Herriott *et al.*, 1970). In contrast, in *Streptococcus* species, competence is a transitory phenomenon occurring optimally during exponential-phase growth (Tomasz & Hotchkiss, 1964; Tomasz, 1966, 1971; Pakula & Walczak, 1963). Streptococci offer a particularly favourable system for analysis, because competence is separated from other major cell changes associated with entry into the stationary phase of growth, is inducible, and typically occurs in nearly all the cells of a culture at once.

Competence in *Streptococcus pneumoniae* is associated with the induction of a small set of new proteins (Morrison & Baker, 1979; Morrison, 1981), temporary cessation of synthesis of most other proteins, and the appearance of a number of unusual cell properties, including an efficient system for taking up DNA and promoting genetic recombination between that DNA and the cell chromosome (Avery *et al.*, 1944; Fox & Allen, 1964; McCarty, 1980; Morrison *et al.*, 1982).

Abbreviations: CF, competence factor; CTM, complete transformation medium; LM, low-methionine labelling medium; PMSF, phenylmethylsulphonyl fluoride.

A similar protein shift occurs in *Streptococcus sanguis* (Raina & Ravin, 1980). The drastic protein shift seen in competent cultures suggests that nearly all cells participate in the redirection of protein synthesis accompanying competence. Genetic and physical studies investigating the composition of a competent *S. pneumoniae* culture have independently demonstrated that nearly all cells were simultaneously competent in such a culture (Javor & Tomasz, 1968; Porter & Guild, 1969).

Competence in *S. pneumoniae* is under a very specific set of controls (Tomasz & Hotchkiss, 1964; Tomasz, 1966, 1973). As a growing culture reaches a specific cell density, the cells become competent for transformation (Porter & Guild, 1969; Javor & Tomasz, 1968). This critical cell density is determined by a mechanism involving a secreted protein, competence factor (CF), acting as a feedback signal sensitive to the population level of the culture; when CF accumulates to a certain level, it induces cells in the culture to become competent (Tomasz & Hotchkiss, 1964; Tomasz & Mosser, 1966; Tomasz, 1966). Competence terminates after a short time and an inhibitor of activation is released (Tomasz, 1973). Competence can be artificially provoked at lower cell densities by adding exogenous CF. Other details of conditions required or favourable for competence are less well defined.

In the course of developing screening procedures for isolating transformation-deficient mutants (Morrison *et al.*, 1983), we found it important to control the critical cell density at which cultures of pneumococcal strains developed competence. When the final culture density was to be lowered by limiting the energy source (sugar), yet competence development was required, it was important to use culture conditions in which competence would occur at low cell densities; otherwise growth could stop before the critical density had been achieved. In contrast, induction of competence at high culture densities is important for providing large amounts of starting material for biochemical assays of events in competent cells.

Various laboratories have reported critical cell densities varying between 2×10^6 ml⁻¹ and 2×10^8 ml⁻¹ (Ottolenghi & Hotchkiss, 1962; Tomasz & Hotchkiss, 1964; Tomasz, 1966; Gurney & Fox, 1968; Porter & Guild, 1969; Morrison, 1977). Competence appears in cultures of pneumococci growing in appropriate media at a specific cell density. This critical density is reproducible from experiment to experiment but varies considerably with medium composition, bacterial strain and other unknown variables (Tomasz & Hotchkiss, 1964; McCarty, 1980). However, control of this parameter has not been described. There have been, however, several indications that culture pH is an important variable. For example, for *S. pneumoniae* strain R6 competence occurs during growth in a complex medium with a pH of 7.6, but not in the same medium adjusted to pH 6.7 (Tomasz & Mosser, 1966). The optimum pH for response to isolated CF was near 7.7, and CF activity decreased exponentially over three orders of magnitude between pH 7.7 and 6.9 (Tomasz, 1966). Also, Tiraby *et al.* (1973) reported that transformation during surface colony growth in the presence of DNA required a high initial pH for optimum expression of competence.

METHODS

Bacterial strains. *S. pneumoniae* strain CP1015 (a *str-1* derivative of strain Rx; Morrison *et al.*, 1983) served as recipient in transformation experiments. [³H]DNA was extracted from strain CP1016 (*str-1 ery-2 nov-1 vlt*) (Morrison *et al.*, 1984) after labelling with [³H]thymidine, as described by Morrison (1977). In other cases unlabelled DNA from strain 5MC (Cato & Guild, 1968) was used as donor.

Culture conditions. Complete transformation medium (CTM) was that described by Peterson & Guild (1968), supplemented with choline to avoid choline-limitation at high culture densities. A mixture of 5 g tryptone (Difco), 10 g enzymic casein hydrolysate (ICN Nutritional Biochemicals), 1 g yeast extract (Difco), 5 mg choline and 5 g NaCl l⁻¹ was sterilized for 50 min at 121 °C and then brought to 0.2% glucose, 0.2% BSA, 1 mM-CaCl₂ and 0.166 M-dipotassium phosphate before use. In addition, the initial pH was adjusted by adding 0–10 mM of HCl or NaOH l⁻¹. pH was measured at 25 °C with a model 26 Radiometer pH meter equipped with an Orion #91-103 combination electrode, after standardization with Beckman buffers at pH 7 and pH 10. Culture density was measured as optical density at 550 nm in a Coleman Jr II colorimeter using 18 mm diameter culture tubes. Competence was assayed by determining [³H]DNA solubilization or uptake, and DNAase-resistant transformants, as described previously (Morrison *et al.*, 1983). Low-methionine labelling medium (LM) was prepared as described previously (Morrison, 1978).

Protein labelling and analysis. This was done by procedures similar to those outlined previously (Morrison & Baker, 1979; Morrison, 1981). Samples to be pulse-labelled were harvested by adding glycerol to 10% (v/v), and freezing in a dry-ice-acetone bath. For labelling, the culture samples were thawed at 0 °C, washed in LM, and suspended in LM at 4×10^8 cells ml⁻¹. A 400 µl portion of this suspension, mixed with 10 µCi (370 kBq) [³⁵S]methionine, was incubated for 10 min at 37 °C. Incorporation was terminated by adding 200 µl cold CTM (+ 30%, v/v, glycerol), and the labelled cells were frozen at -80 °C. To prepare an extract for analysis, the frozen labelled cells were thawed at 0 °C, washed in 0.01 M-Tris, 1 mM-EDTA pH 8.0 in the cold, and suspended in 300 µl lysis buffer, containing 0.01 M-Tris pH 8.0, 1 mM-EDTA, 1 mM-PMSF, 0.4% Triton X-100, 4 mM-NaCl, 10 µg DNAase I ml⁻¹, 50 µg RNAase ml⁻¹, and 5 mM-MgCl₂. After incubation at 37 °C for 5 min, Sarkosyl was added to 0.05% and EDTA to 5 mM, and incubation continued for 3 min. The chilled lysate was mixed with 150 µl 1% tRNA and 150 µl 40% (w/v) TCA. The TCA precipitates, after storage for up to 12 h at 0 °C, were washed twice with 1 ml acetone, dried *in vacuo*, and redissolved in 25 µl 100 mM-Tris/HCl pH 8.0, 2% (w/v) SDS, 1% mercaptoethanol. After mixing with 25 µl sucrose-dye solution to bring the whole to 20 mM-EDTA, 0.01% bromphenol blue, 10% (w/v) sucrose, 50 mM-Tris/HCl pH 8.0, the extract was heated in a boiling water bath for 5 min, and either stored at -20 °C or analysed immediately. After TCA-insoluble ³⁵S had been determined for each sample, processing losses were accounted for by loading 10000 c.p.m. in each well of a 12-25% (w/v) PAGE gel. The gel was prepared, run and analysed as described by Laemmli (1970). Fluorography was done as described by Laskey & Mills (1975).

Congression analysis. Competent cultures were prepared under conditions like those described in Fig. 2, panel 8 (double cycle), and panel 3 (single late cycle). Samples were exposed to DNA for 10 min at 10 min intervals, diluted 10-fold in DNAase-CTM, incubated at 37 °C for 120 min to complete segregation, diluted, and plated on drug agar plates. For selection of drug-resistant transformants, the top layer of agar contained erythromycin at 0.5 µg ml⁻¹ and/or novobiocin at 10 µg ml⁻¹. Competence peaks were identified by immediate analysis of the TCA-soluble products of DNA transport and degradation measured in a duplicate set of samples exposed to [³H]DNA for the same 10 min intervals. The genetic analysis was done for samples identified as representing the first half of each competence cycle.

RESULTS AND DISCUSSION

Critical cell density for competence determined by initial pH

To examine the influence of pH on development of competence, we followed both parameters in detail during growth of strain CP1015 in CTM adjusted to various initial pH values between 7.2 and 8.2 (Figs 1 and 2). During exponential growth, the pH decreased continuously and

Fig. 1. Effect of the initial pH of the culture medium on culture acidification and on development of competence. Ten parallel cultures, identical except for addition of the indicated amounts of NaOH (or HCl, '-1'), were prepared in CTM and inoculated with 0.01 vol. of a culture frozen at OD₅₅₀ 0.2. During incubation at 37 °C, OD₅₅₀, competence (as DNA digestion activity and pH (circles)) were measured at 20 min intervals. Growth (OD₅₅₀; —) is shown for the two extreme cultures (treated with 1 mM-HCl and with 8 mM-NaOH, respectively). ●. Samples in which competence exceeded 15% of the maximum level.

Fig. 2. Kinetics of competence development in cultures initiated at pH values between 6.79 and 8.25. Twelve cultures were inoculated as in Fig. 1 after adjustment of the pH by addition of the indicated amounts of HCl or NaOH, producing initial pH values of 6.79, 6.95, 7.04, 7.16, 7.24, 7.29, 7.37, 7.45, 7.61, 7.70, 7.93 and 8.25, respectively. Competence was measured as acid-soluble label produced from [^3H]DNA (45000 c.p.m. per 0.5 ml sample) incubated with culture samples for 20 min. The vertical line in each panel indicates the time at which that culture reached OD_{550} 0.1.

Fig. 3. Relation between initial culture pH and the population density (OD_{550}) at the first peak of competence. The data shown are for the cultures described in Fig. 1.

reproducibly as acid products of metabolism titrated the culture medium. The cell density at which competence first appeared varied in a continuous fashion with the initial pH of the growth medium: competence first appeared at lower densities for higher initial pH values. [A similar phenomenon may also be noted in the competence experiments reported by Grist & Butler (1981), for some other combinations of pneumococcal strains and media.] The pattern

seen in Figs 1 and 2 is not consistent with a single 'optimum pH' for competence; competence induction actually occurred over a wide pH range (6.7 to 7.9). Rather, the critical cell density required to elicit competence was lower in more alkaline media. This appears to be consistent with earlier data (Tomasz & Mosser, 1966) showing that the activity of isolated CF increases continuously from pH 6.9 to 7.7.

By extrapolation of the summary data in Fig. 3, it appears that, while competence can occur at a stage in culture growth with pH less than 6.7 (see Fig. 1), it never appears in media with an initial pH below approximately 7.0 because the critical cell density is then set near or above the normal limit of culture growth; i.e. stationary phase is approached below the critical cell density.

It has not previously been reported that the cell density at which a culture develops competence varies continuously with the initial pH of the culture medium. Since small pH changes have strong effects on the competence cycle, this is an effect that can easily be overlooked. This phenomenon makes it possible to ensure competence early in culture growth when desired, but also allows the controlled delay of induction of competence until late-exponential phase for higher yields of competent cells.

This phenomenon also appears to account for the high optimum pH found for two kinds of competence experiments described elsewhere (Morrison *et al.*, 1983): (a) colonies grown on limiting sucrose in agar medium containing donor DNA, and (b) liquid cultures grown on limiting glucose to early-stationary phase, in otherwise complete competence medium. For cultures growing under sugar limitation, reliable competence depends on setting the critical cell density below the artificially reduced growth limit; it appears from these results that an appropriate choice of initial pH can achieve this effect.

We suggest that a simple hypothesis accounting for these results is that they represent the consequences for the model of Tomasz (1966) of the strong pH dependence of CF activity (Tomasz & Mosser, 1966). Thus, CF may be produced constitutively by non-competent cells at a fixed rate per cell, independent of pH or population level, while the critical CF level for inducing competence (and thus the population level required to achieve this concentration) depends strongly on pH.

Successive competence cycles are not equivalent

In cultures initiated at a pH which allowed competence to develop significantly earlier than the last two generations of growth, a second wave of competence appeared approximately 1–2 doubling times after the first (Figs 1 and 2). This second competence cycle is often seen when competence is measured throughout the period from early-exponential phase to stationary phase, and was described by Hotchkiss (1954) for the case of competence in thermally synchronized cultures. The nature of competence in this second cycle, however, has not been investigated in detail. Since pH regulation provided two different possible sources of high-density competent cultures (the first peak in a low-pH culture, or the second peak in a more alkaline culture), we compared the nature of the competent cells found at the first and second competence cycles in a culture initiated at high pH. The result shown in Fig. 4(a) was typical: cells from the second peak yielded fewer or only modestly more transformants than those from the first, despite a much higher total cell number. This suggested that competence in the second cycle was in some way less efficient than that in the first. Assay of the appearance of new proteins (Fig. 4b, c) confirmed this impression biochemically: the characteristic competence-specific eclipse complex protein (M_r 19000; arrowed) was induced during the second cycle of competence, but at a clearly lower level than during the first.

To distinguish between a population of less competent cells and one in which normally competent cells comprised a minority, we used the congression method of Porter & Guild (1969) to estimate genetically the fraction of competent cells. The fraction of competent cells in the first cycle of competence was very high, as expected, but was about seven-fold lower in the second (Table 1). Competence in the delayed cycle of the more acidic culture was just as high as that for the first cycle of the alkaline culture.

Fig. 4 (continued on next page)

Fig. 4. Comparison of two successive competence cycles. A culture was initiated at pH 7.8 as described for Fig. 1. Sets of samples were harvested at 10 min intervals. (a) A sample taken at each assay time was thawed and tested for competence and DNA-processing activity during a 10 min exposure to DNA at 37 °C. —, Total cell number ml⁻¹; ●, novobiocin-resistant transformants; ○, DNA degradation; △, DNA uptake. (b) Protein synthesis was examined by labelling cells from a parallel set of frozen samples resuspended in LM, by incubating for 10 min with [³⁵S]methionine. The fluorograph obtained after SDS-PAGE is shown. The time (min) during culture growth at which samples were harvested is indicated. (c) A third sample from the first peak (at 160 min), and second peak (at 240 min) was examined in more detail by pulse-labelling in successive 5 min periods, beginning at the indicated times (min) during incubation in LM.

Concluding remarks

We have extended earlier observations by showing that control of competence is susceptible to two kinds of modulation involving culture pH. First, modest alterations in the initial pH of the culture medium, in the range 6.8–8.0, strongly affect the specific cell density at which competence first appears during subsequent growth. Second, in media adjusted to a pH supporting two successive waves of competence, the fraction of cells competent is much lower in the second wave of competence than in the first. The existence of qualitatively different competence cycles succeeding one another in a single culture has not previously been reported. The results suggest that it is possible for a competent pneumococcal population to be a mixture of two cell types, competent and non-competent, much like the situation for *B. subtilis* (Dooley *et al.*, 1971). However, the data here are not precise enough to show whether the competent cells in

Table 1. *Comparison of the fraction of cells competent in competent populations*

Congression analysis was done for the first competent sample for each method, after exposure to 0.1 µg DNA ml⁻¹ for 10 min. Competence was determined by measurement of DNA-degrading activity, as described in Methods. The initial pH of CTM was adjusted to the indicated values. Culture OD₅₅₀ at each competence cycle is indicated. Diluted samples were plated after DNAase treatment, 120 min outgrowth at 37 °C, and adjustment to OD₅₅₀ 0.10. About 200–400 colonies were counted for each point in duplicate plates; the 95% confidence limit estimated for each individual frequency is 10%, and for f_c , 20%.

Selection	No. of cells ml ⁻¹		
	pH 8 culture		pH 7.2 culture (OD ₅₅₀ 0.11)
	Peak I (OD ₅₅₀ 0.05)	Peak II (OD ₅₅₀ 0.20)	
Viable cells (N_c)	2.0×10^8	2.6×10^8	2.4×10^8
Novo ^R (N_1)	6.3×10^5	1.2×10^5	8.5×10^5
Ery ^R (N_2)	4.8×10^5	9.8×10^4	7.2×10^5
Novo ^R Ery ^R ($N_{1,2}$)	1.9×10^3	3.6×10^2	3.2×10^3
f_c^*	0.80	0.12	0.83

* f_c , fraction of cells competent, calculated from $f_c = N_1 N_2 / N_{1,2} \times N_c$, to account for segregation of transformable sites during outgrowth (Porter & Guild, 1969).

such a mixed population are identical to those in a culture at full competence. This finding has an important practical implication; for biochemical studies of competent cells, the 'first cycle' of competence can be expected to provide the better starting material.

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