Calcium regulation of growth and differentiation in *Streptococcus pneumoniae*

MARIE-CLAUDE TROMBE,* CORINNE CLAVÉ† and JEAN-MICHEL MANIAS‡

Centre de Biochimie et de Génétique Cellulaires du CNRS et Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cédex, France

(Received 28 May 1991; revised 2 September 1991; accepted 17 September 1991)

Streptococcus pneumoniae requires 0.15 mM-Ca²⁺ in the medium for optimal growth. Increasing the Ca²⁺ concentration to 1 mM triggers either a differentiative state, competence for genetic transformation during exponential growth, or partial lysis as soon as the cultures enter stationary phase. Genetic and physiological data both suggest that these responses are under the control of activator(s), excreted in the presence of high Ca²⁺ concentrations. ⁴⁵Ca²⁺ transport is also stimulated by the activator(s). The amiloride derivative 2',4'-dimethylbenzamil (DMB) inhibits ⁴⁵Ca²⁺ transport and prevents lysis and competence development. This provides evidence in favour of the involvement of Ca²⁺ transport in competence and culture lysis. On the other hand, addition of DNA to a competent culture prevents lysis of wild-type bacteria while a mutant, defective for DNA uptake, is not protected from lysis by exogenous DNA. An hypothesis is proposed for competence induction as a global metabolic response to Ca²⁺, under the control of competence factor.

Introduction

Streptococcus pneumoniae is one of the bacterial species that are able to go through a transient differentiative physiological state, competence for genetic transformation. Competent bacteria exhibit new properties with regard to surface antigens (Nava *et al.*, 1963), membrane nuclease activity (Lacks & Greenberg, 1973; Lacks & Neuberger, 1975), DNA transport (Lerman & Tolmach, 1957), recombination and repair (Love & Yasbin, 1986), expression of a set of specific proteins (Morrison & Baker, 1979; Raina & Ravin, 1980) and synthesis of the lipid polymer poly(β -hydroxybutyrate) (Reusch & Sadoff, 1983; Clavé *et al.*, 1989). Membrane hyperpolarization (Trombe, 1983), as well as a stimulation of glycolysis associated with an increased ATP pool and a cytoplasmic alkalization (Lopez *et al.*, 1989), are observed in competent *S. pneumoniae*. A remarkable feature in streptococci is that all these responses are controlled by an excreted protein, competence factor (CF) (Leonard &

Cole, 1972; Pakula & Walczak, 1963; Tomasz & Hotchkiss, 1964), which probably reacts with a receptor in the cell membrane to activate competence (Ziegler & Tomasz, 1970).

Competence in *S. pneumoniae* is routinely obtained when cells are grown in Ca²⁺-rich (1 to 1.5 mM) media. The Ca²⁺ requirement for growth in prokaryotes is not very well documented. Some evidence now exists for bacterial calmodulin-like proteins (Falah *et al.*, 1988; Fry *et al.*, 1986; Inouye *et al.*, 1983; Swan *et al.*, 1987, 1989), suggesting a regulatory function for Ca²⁺. On the other hand, Ca²⁺ circulation is described as an electrochemical process (for a review, see Rosen, 1987) associated with its export involving a Ca²⁺-ATPase in streptococci (Kobayashi *et al.*, 1978; Ambudkar *et al.*, 1986). In the work presented here, we have addressed the relationship between Ca²⁺, bacterial growth and induction of the differentiative state, permissive for DNA uptake and genetic transformation, i.e. competence.

Methods

Strains. The RX derivative *Streptococcus pneumoniae* Cp1000 was used as the standard wild-type strain for competence induction and for genetic transformation (Morrison *et al.*, 1984). The required mutations were introduced into the wild-type background by genetic transforma-

* Author for correspondence. Tel. 61 335971; fax 61 335886.

† Present address: Laboratoire de Génétique, Université Bordeaux II, 33405 Talence Cédex, France.

‡ Present address: Laboratoire de traitement des eaux, Institut National des Sciences Appliquées, Toulouse, France.

Abbreviations: CF, competence factor; DMB, 2',4'-dimethylbenzamil; HMA, 5-(*N,N'*-hexamethylene)amiloride.

Fig. 1. Ca^{2+} regulation of growth: effect of amilorides. (a) Ca^{2+} requirement. The endogenous Ca^{2+} of the growth media at pH 7 (open symbols) or pH 8 (filled symbols) was chelated with 0.2 mM-EGTA and CaCl_2 was then added to obtain final concentrations of 0.05 mM ($\diamond$, $\blacklozenge$), 0.1 mM ($\square$, $\blacksquare$) or 0.15 mM ($\circ$, $\bullet$). Growth at 37 °C was then monitored (see Methods). (b) Ca^{2+} -induced lysis: pH effect and inhibition by amilorides. Cells were grown in media containing 1.5 mM- Ca^{2+} at pH 7 (open symbols) or pH 8 (filled symbols). Amiloride or amiloride derivatives were added at time zero. Solid line, wild-type strain: $\circ$, $\bullet$, no addition; $\blacksquare$, plus 5 μM -HMA; $\blacklozenge$, plus 5 μM -amiloride; $\blacktriangledown$, plus 5 μM -DMB. Dashed line, strain Cp1095 grown at pH 8 ($\triangle$).

tion using DNA from the mutant strains. Two rounds of transformation with non-saturating levels of DNA were performed to avoid multiple gene transfer. The mutation in the *lytA* gene of strain Cp1095 comes from strain GP95, a derivative of RUP1, which carries an *ery* insertion in *lytA*, *lytA::ermC* (Tomasz *et al.*, 1988). The mutation in the *com* locus of strain Cp1322 was introduced with DNA from strain omega 22, a competence-defective mutant selected after insertional mutagenesis (Morrison *et al.*, 1984). Strain Cp1209, which carries the *ent-9* mutation, is defective in DNA transport (Morrison *et al.*, 1983; Clavé *et al.*, 1987).

Specific materials. The source of [^3H]DNA was the thymidine auxotrophic strain 119 which also carries the *rif-23* mutation (Tiraby & Fox, 1973). Amilorides were provided by Dr E. J. Cragoe, Jr, Nacogdoches, Texas, USA; 2',4'-dimethylbenzamil (DMB) was prepared specifically for this study. The calcium ionophore A23187, EGTA and tripolyphosphate (pentasodium salt) were analytical grade. Salmon sperm DNA and tRNA were from Sigma. Nutrients used for growth media were from Difco. $^{45}\text{CaCl}_2$ was from CEA, France.

Growth and competence induction. Growth conditions and media were identical for the wild-type and the mutant strains as described previously (Clavé *et al.*, 1987). Growth was routinely followed by optical density measurements at 400 nm using a Bausch and Lomb spectrophotometer. The decrease in OD₄₀₀ was taken as an indicator of culture lysis. For each experiment, a given growth curve was performed in triplicate. The curve presented is thus representative of three independent assays. Briefly, stock cultures were grown at pH 7.5 in a standard medium containing (g l⁻¹): NaCl, 5; yeast extract, 1; tryptone, 5; enzymic casein hydrolysate, 10; glucose, 2; K₂HPO₄, 3. When the culture reached an OD₄₀₀ of about 0.4, glycerol (final concn 15%, v/v) was added and samples were kept frozen at -80 °C. To obtain precompetent bacteria, samples were thawed, diluted 1 to 100 in transformation medium [standard growth medium enriched with CaCl_2 (1 mM) and bovine serum albumin (1 mg ml⁻¹)] at neutral pH, and grown for 2 h at 37 °C. Such cultures constituted the 'tester cells'. When shifted to pH 7.8 and incubated at 37 °C, they could develop 'natural' competence in 60 to 120 min or could be activated by CF-containing extract and develop competence in 20 min. Competence induction could not be observed at 32 °C but competence expression of pre-induced bacteria was maximal at this temperature.

Activator preparation. CF-containing extracts were supernatants of competent wild-type cultures fractionated with 55% saturation $(\text{NH}_4)_2\text{SO}_4$ at pH 11. The pellet dissolved in 10 mM-HCl was the source of CF; it was kept frozen at -80 °C. One CF unit induces competence of 1 ml of tester cells (2×10^8 c.f.u.) of strain Cp1000 at pH 7 in 30 min.

Extracts from competent cultures of strain Cp1322 contain no detectable CF activity (Morrison *et al.*, 1984) and were used as controls.

Assay for competence. Competence reporters (Clavé & Trombe, 1989) were the degradation of extracellular pneumococcal [^3H]DNA resulting in acid-soluble material, the uptake of [^3H]DNA in a DNAase-I-resistant form and genetic transformation for the chromosomal marker *rif-23*, which confers resistance to 2 μg rifampicin ml⁻¹.

Ion transport measurements. Ion transport was assayed using the radioactive isotope $^{45}\text{Ca}^{2+}$. Tester cells were incubated in transformation medium containing the radioactive isotope. CF-containing extract was then added and 1 ml samples were filtered at intervals through 0.45 μm Gelman Metrical membranes. After three washes with media containing 0.5 mg CaCl_2 ml⁻¹, the radioactivity retained on the filters was quantified by liquid scintillation as previously described (Trombe *et al.*, 1984). It was verified that washing with CaCl_2 lowered non-specific adsorption on the filters without significant displacement of intracellular $^{45}\text{Ca}^{2+}$.

Results

Ca^{2+} requirement for growth

The standard growth medium used for *S. pneumoniae* was found to contain 0.2 mM-calcium as measured by flame spectrophotometry. When Ca^{2+} was chelated with 0.2 mM-EGTA, growth was inhibited. Full growth could be restored by addition of a further 0.15 mM- CaCl_2 to medium containing 0.2 mM-EGTA (Fig. 1a). Therefore, Ca^{2+} is an obligatory cation for growth of *S. pneumoniae*. Similar results were obtained whether the pH of the medium was 7 or 8 (Fig. 1a). However, increasing the Ca^{2+} concentration to 1.5 mM caused precocious lysis of wild type *S. pneumoniae* when the culture reached stationary phase. Culture lysis in high- Ca^{2+} medium was strongly dependent on the initial pH of the cultures and did not occur at neutral pH (Fig. 1b). Strain Cp1095, which carries an insertion in the *lytA* gene that encodes the major autolysin (Tomasz *et al.*, 1988), was fully resistant to lysis in alkaline Ca^{2+} -containing medium (Fig. 1b). *LytA* must therefore be involved in the Ca^{2+} -triggered lysis.

Fig. 2. Specific Ca^{2+} requirement for competence induction or for competence expression. (a) Induction. Tester cells were incubated in media at pH 8 containing various Ca^{2+} concentrations with CF-free extracts of strain Cp1322 (○) or with 0.1 CF units ml^{-1} (●). Competence development was monitored at intervals by assaying the nuclease activity of the cultures using [^3H]DNA ($1 \mu\text{g ml}^{-1}$; 10^5 d.p.m. ml^{-1}) (see Methods). The highest competence level achieved for each culture is expressed as a percentage of the maximum competence level achieved at a saturating Ca^{2+} concentration (1 mM). (b) Expression. Bacteria induced to competence in a medium containing 1.5 mM- Ca^{2+} were shifted at 32°C into media containing various Ca^{2+} concentrations and [^3H]DNA ($1 \mu\text{g ml}^{-1}$; 10^5 d.p.m. ml^{-1}). CF (0.1 units ml^{-1}) was added in one series of samples (●) while the other received CF-free extracts from strain Cp1322 (○). After 15 min incubation at 32°C , the acid-soluble radioactivity was quantified by liquid scintillation counting.

Ca²⁺ requirement for differentiation: competence development

In the experimental system used for this study, competence was induced in early exponential phase when the culture was alkaline, as described by Chen & Morrison (1987). A competence cycle could be divided into two steps: induction, which required 20 min incubation at 37°C in CF-containing medium or 60 to 120 min in the absence of CF, and expression, where all the properties of the competent bacteria were expressed. The Ca^{2+} requirement for both steps was determined using Ca^{2+} -titrated media, as described for the experiments shown in Fig. 1. A difference in the Ca^{2+} requirement for optimal induction (1 mM) (Fig. 2a) and for optimal expression (0.4 mM) (Fig. 2b) suggests a specific requirement at induction. DNA transport is probably the Ca^{2+} -requiring step at the expression stage (Lacks & Greenberg, 1973; Seto & Tomasz, 1974, 1976; Lacks, 1977; Clavé *et al.*, 1989; Clavé & Trombe, 1989). Addition of CF to the medium did not change the Ca^{2+} dependence (Fig. 2), nor did the *lytA* mutation carried by strain CP1095 (data not shown). Interestingly, from the data presented, the conditions required for competence induction correspond to conditions which cause precocious lysis in the wild-type strain.

CF activation of competence and culture lysis

In cultures growing at neutral pH, competence develops naturally in the late exponential phase and at high cell density (Chen & Morrison, 1987). It was possible to induce competence in the early exponential phase, at neutral pH, by the addition of extra CF, above the concentration which induced competence in alkaline cultures (Fig. 3a). Interestingly, precocious lysis in high- Ca^{2+} medium was also obtained at neutral pH in the presence of CF (Fig. 3b). The *com* mutant Cp1322, which required high levels of CF for its activation whatever the pH used (Fig. 3a), was fully resistant to culture lysis in alkaline medium containing high Ca^{2+} , but lysed if the culture was complemented with CF (Fig. 3d). The same threshold concentration of CF was required to induce both competence and lysis (Fig. 3a, d). The single mutational event carried by the *com* strain prevents both natural lysis and competence induction. These defects are complemented under the same conditions, i.e. CF addition, suggesting a correlation between competence induction and culture lysis as proposed by Seto & Tomasz (1975). However, the *LytA*-null mutant Cp1095, which was refractory to lysis even in high-CF medium (Fig. 3c), exhibited the same CF requirement for competence induction as the wild-type strain (Fig. 3a), and was fully transformable (data not shown) as already shown for other *lyt* strains (Sanchez-Puelles *et al.*, 1986; Tomasz *et al.*, 1988), indicating that *LytA* is not involved in competence-regulation. The effect of CF in lowering the growth rate and growth yield of Cp1095 is noteworthy (Fig. 3c). This effect was not observed in media with a low (0.2 mM) Ca^{2+} concentration (Fig. 3c). Thus, competence induction might be considered as a response to Ca^{2+} stress under the control of CF. Another possibility would be that both a competence activator and a lysis activator are released at the same time in competent cultures. In any case, competence and lysis represent two manifestations of the Ca^{2+} response, regulated by the pH of the medium and under the control of released factor(s).

Prevention of lysis by exogenous DNA

In *Bacillus subtilis* it is proposed that competence is induced in response to a variety of signals including growth stage and nutritional information (for a review, see Dubnau, 1991). Culture-medium titrations showed no variation in glucose and phosphate concentrations during the development of a competence cycle in *S. pneumoniae* (Clavé, 1988). Taking into account the composition of the transformation medium (see Methods), N-starvation seems unlikely. Thus unbalanced growth with regard to C, P or N does not appear to

Fig. 3. CF-induced competence and culture lysis. (a) CF requirement for competence induction: effect of external pH. Tester cells of strains Cp1000 (○) and Cp1095 (●) were shifted to media of different pH values and stimulated by increasing amounts of CF. After 30 min incubation at 37 °C, [³H]DNA (1 µg ml⁻¹; 10⁵ d.p.m. ml⁻¹) was added and incubation was continued for 15 min at 32 °C. Acid-soluble radioactivity, used as a measure of competence, was quantified by liquid scintillation. The data plotted represent the CF concentration required, at each pH, to obtain optimal competence, i.e. 5×10^3 d.p.m. per 0.1 ml sample in the acid-soluble fraction and a transformation (Rif^r) frequency between 1 and 5%. A culture of strain Cp1322 (□) was similarly treated. (b) CF activation of culture lysis. Cultures of strain Cp1000 were started in 1.5 mM-Ca²⁺ media at pH 7 (open symbols) and pH 8 (filled symbols). Various amounts of CF were added at time zero of incubation and the optical density was monitored at intervals. △, 0.05 units CF ml⁻¹; □, 0.25 units CF ml⁻¹; ◇, 1 unit CF ml⁻¹; ○ ●, no addition. (c) Cultures of strain Cp1095 were started in media at pH 8 containing 1.5 mM-Ca²⁺ (filled symbols) or 0.2 mM-Ca²⁺ (open symbols). Various amounts of CF were added at time zero and growth was monitored. ▲, 0.05 units CF ml⁻¹; ○ ●, 0.5 units CF ml⁻¹; ■, growth at pH 8 with CF-free extract from strain Cp1322. (d) Cultures of strain Cp1322 were started at pH 8. Various amounts of CF were added at time zero and growth was monitored. ●, 0.5 units CF ml⁻¹; ◆, 1 unit CF ml⁻¹. ○, cultures with CF-free extract from strain Cp1322.

explain the Ca²⁺ response. Results in Fig. 4(a) show that addition of homologous or heterologous DNA (1 µg ml⁻¹) prevented the cultures from lysing. In these cultures, competence development was not altered by DNA (data not shown). In contrast, neither polyphosphate nor tRNA prevented lysis (Fig. 4a), suggesting that the protective effect of DNA is not caused merely by its global negative charge. Strain Cp1209, deficient in DNA transport, which develops a normal competence pattern with regard to its nuclease activation or its gene regulation (Morrison *et al.*, 1983), lysed even in DNA-supplemented medium (Fig. 4b). It is possible that the shift in the lysis profile of Cp1209, when grown in DNA-containing medium (Fig. 4b), corresponds to a partial protection resulting from its residual transport activity, estimated at 0.5 to 1% of the wild-type activity (data not

Fig. 4. Inhibition of lysis by DNA uptake. Tester cells from strains Cp1000 (a) and Cp1209 (b) were incubated at 37 °C in media containing CF (0.7 units ml⁻¹) and DNA (1 µg ml⁻¹) (solid line) from either *S. pneumoniae* (◇) or salmon sperm (■), or (dashed line) 1 µg tRNA ml⁻¹ (▽), or 1 µM polytriphosphate ml⁻¹ (□). ●, Controls with no addition. Growth was monitored as described in Fig. 3.

Fig. 5. Modulation of $^{45}\text{Ca}^{2+}$ transport by CF extracts: effect of amilorides. Tester cells from strain Cp1000 were transferred into competence medium [1.5 mM-CaCl_2 ; $1 \mu\text{Ci}$ (37 kBq) $^{45}\text{Ca}^{2+} \text{ ml}^{-1}$]. The suspension was divided into four aliquots. Three were supplemented with CF ($0.05 \text{ units ml}^{-1}$) and $5 \mu\text{M-HMA}$ (◆) or $5 \mu\text{M-DMB}$ (■) was added at the times indicated, followed by incubation at 37°C . ●, No additions. The filters were rinsed with medium containing CaCl_2 (0.5 mg ml^{-1}) and the radioactivity retained by the bacteria was determined at intervals by filtration assays. ▲, Cultures treated with CF-free extracts from strain Cp1322.

shown). In any case, these results indicate that DNA uptake is necessary to prevent lysis of cultures which have passed through a competence cycle.

Ion fluxes and competence induction

During competence induction fluctuation of the Na^+ content of the bacteria was observed and it was noted that their K^+ levels did not change (Lopez *et al.*, 1989). Measurement of the $^{45}\text{Ca}^{2+}$ flux in a CF-stimulated culture showed a transient increase in $^{45}\text{Ca}^{2+}$ retained by the cells compared to the control (Fig. 5). Treatment of cells with amiloride derivatives resulted in total inhibition of Ca^{2+} transport by DMB (Fig. 5) while HMA lowered Ca^{2+} uptake by 20% of the control. Measurements of the influx velocity, V , after 15 s corroborate these observations (data not shown). DMB has been described as an inhibitor of Ca^{2+} porters in eukaryotes (Kaczorowski *et al.*, 1985; Simchowicz & Cragoe, 1986). Interestingly, DMB prevented culture lysis (Fig. 1*b*), and was a strong inhibitor of competence induction, but under the same conditions it allowed full competence expression of pre-induced cultures (Table 1). Such an inhibition of competence induction and of culture lysis

Table 1. Effect of amilorides on competence induction and competence expression in strain Cp1000

The [^3H]DNA ($1 \mu\text{g ml}^{-1}$, $15000 \text{ d.p.m. ml}^{-1}$) degrading activity of the cultures was taken as the competence indicator. Each assay was performed in triplicate and the experiment was repeated twice. The 100% value corresponds to $10000 \text{ d.p.m. ml}^{-1} \pm 10\%$. Amiloride derivatives were added at time zero of induction (a) or to pre-induced cultures during expression (b).

Compound added	(a) Induction (% control)	(b) Expression (% control)
None (control)	100	100
Amiloride 1–10 μM	100	100
HMA 1 μM	100	100
5 μM	80	100
DMB 1 μM	70	100
5 μM	20	100

Table 2. Stimulation of competence induction in strain Cp1000, at low Ca^{2+} concentrations, by the Ca^{2+} ionophore A23187

Induction was obtained in alkaline media with Ca^{2+} concentrations adjusted as indicated, with or without the Ca^{2+} ionophore A23187. [^3H]DNA ($1 \mu\text{g ml}^{-1}$, $15000 \text{ d.p.m. ml}^{-1}$) bearing the *ery-32* genetic marker was routinely used. The standard deviation of DNA degradation and DNA transport measurements, performed in triplicate, never exceeded 10%; it was 5% for the estimation of transformants.

Ca^{2+} added	DNA degradation (d.p.m. ml^{-1})	DNA transport (d.p.m. ml^{-1})	Rif transformants (percentage of culture)
1.2 mM- CaCl_2	9120	3720	17%
0.4 mM- CaCl_2	4700	1830	8%
0.4 mM- CaCl_2 + 1 $\mu\text{M-A23187}$	11030	4470	20%

was not observed in the presence of the other derivatives (Table 1 and Fig. 1*b*). It is likely that the DMB-sensitive function is involved in competence induction as well as in triggering culture lysis. As DMB interferes with Ca^{2+} transport, Ca^{2+} porter(s) might constitute(s) the drug target. Therefore, Ca^{2+} circulation is probably a key event in competence induction and in triggering lysis of the bacteria. Indeed, using the Ca^{2+} ionophore A23187 it was possible to obtain, in the wild-type strain, full induction at a suboptimal Ca^{2+} concentration (Table 2). This suggests that Ca^{2+} influx is a determinant in the regulation of competence induction. A similar effect was not observed in strain Cp1322 (data not shown); therefore, the Ca^{2+} ionophore cannot complement the

defect in CF production. In addition, CF did not lower the Ca^{2+} requirement for induction in the wild-type strain (Fig. 2a) as does A23187 (Table 2). CF probably does not act simply as a Ca^{2+} ionophore.

Discussion

In *S. pneumoniae*, Ca^{2+} is an obligatory cation that is required at a threshold concentration of 0.15 mM (Fig. 1a). It is noteworthy that high Ca^{2+} triggers both a permissive state for DNA uptake at competence and for DNA liberation during lysis. Inhibition of Ca^{2+} uptake by DMB (Fig. 5) can be correlated with inhibition of both lysis (Fig. 1b) and competence induction (Table 1), suggesting that Ca^{2+} transport is the determinant process. The involvement of Ca^{2+} at the onset of sporulation in *B. subtilis* (Murrell, 1967) and in competence induction in *S. pneumoniae* (Fox & Hotchkiss, 1957) have already been described. However, some media allowing competence development contain less than 1 mM- Ca^{2+} , but are supplemented with a mixture of divalent cations (Tomasz & Hotchkiss, 1964). In these media, competence develops when the cultures reach an OD_{400} of 0.3 to 0.4, while competence was attained at an OD_{400} about 0.05 to 0.1 in the medium used for this study. This suggests that another set of growth parameters might trigger competence. In any case, in our system conditions that do not allow competence development, such as absence of CF production in the *com* mutant Cp1322 (Fig. 3d) and neutral pH (Fig. 1a) do not allow Ca^{2+} -induced lysis.

Complementation of mutant Cp1322 and cultures of the wild-type strain grown at neutral pH with exogenous CF restores both competence induction (Fig. 3a) and lysis (Fig. 3b, d). CF extract could not complement the lysis defect of the *lyt* mutant. It is thus unlikely that the extract was contaminated by a lytic activity. So far neither Ca^{2+} nor alkaline pH have been described as activators for the autolysin (Höltje & Tomasz, 1976; Trombe, 1981). However, pH-dependent lysis activation involving the bacterial autolysins has been described in β -lactam-treated cultures of *B. subtilis* and *S. pneumoniae* (Lopez *et al.*, 1976, 1989, 1990). Interestingly, the C medium used for bacterial growth in those experiments was one of the classical competence media (Lacks & Hotchkiss 1960). Although competence development has not been checked in those works, it is interesting to point out that culture lysis was avoided by protease addition, which is well known to block competence induction also (Lacks & Greenberg, 1973). It is possible that several growth conditions might trigger culture lysis in a pH-dependent manner. This might be the consequence of perturbation of peptidoglycan biosynthesis. On the other

hand, the leader region lying within the *lyt* promoter (Diaz & Garcia, 1990) might somehow contribute to regulation of *lyt* expression in response to deleterious growth conditions. Nevertheless, our findings support strongly the hypothesis that competence and lysis constitute responses to Ca^{2+} stress, regulated by CF or by different activators, produced in and co-purified from the same cultures.

In *lyt*⁺ cultures, lysis is overcome in the presence of exogenous DNA if the bacteria are genetically capable of DNA uptake (Fig. 4a, b). Homologous or heterologous DNA are equivalent for lysis protection (Fig. 4a). After uptake, the fate of these two substrates is quite different but in the end, both processes lead to a replenishment of the nucleotide pool. Pneumococcal DNA is integrated by homologous recombination resulting in the local replacement of one strand of the recipient molecule by the transforming strand. The parental strand is then degraded and the nucleotides are used for *de novo* DNA synthesis (Lacks *et al.*, 1967). Heterologous DNA is degraded by endonucleases and enters the nucleotide pool. Thus, DNA uptake by competent bacteria might be looked on as a process that allows nucleotide feeding. A dramatic metabolic upshift triggered by Ca^{2+} and regulated by CF occurs at competence. Stimulation of glycolysis associated with an elevation of the ATP pool, a cytoplasmic alkalinization (Lopez *et al.*, 1989) and synthesis of the lipid polymer poly(β -hydroxybutyrate) (Reusch & Sadoff, 1983; Clavé *et al.*, 1989) have been described. If one branch of the metabolic network is not activated in a concerted manner and becomes limiting, growth might, then, become unbalanced. DNA uptake prevents lysis of cultures which have passed through a competence cycle. Nucleotides might represent the limiting factor in cultures growing in high Ca^{2+} media. As an hypothesis, we propose that activation of DNA uptake at competence might be an homeostatic regulation for the restoration of balanced growth conditions.

The natural environment of *S. pneumoniae* is body fluids in which the Ca^{2+} concentration is around 1.5 mM, the concentration at which competence and lysis are obtained under laboratory conditions. Interestingly, *comA* (Morrison *et al.*, 1984; Chandler & Morrison, 1988), a gene belonging to the *com* locus, whose disruption prevents CF production, contains consensus sequences characteristic of the superfamily of ATP-dependent bacterial transporters (Hui & Morrison, 1991). The ComA primary structure, derived from the nucleic acid sequence of the *comA* gene, shows a strong homology with HlyB, which is involved in the export of the bacterial toxin haemolysin in *E. coli* (Koronakis *et al.*, 1988). By analogy, it has been proposed that the *com* locus contains an operon encoding the synthesis and excretion of CF. CF might be a molecule allowing a cycle

of consecutive lysis and transformations, resulting in cross-feeding within natural co-cultivated subpopulations. Indeed, under laboratory conditions, successive competence waves can be observed in the same culture (Chen & Morrison, 1987). Genetic exchanges at competence might be part of a more general cross-feeding event. In this highly regulated process, Ca^{2+} transport via a DMB-sensitive function plays a central role (Fig. 5, Table 1).

This work was supported by Université P. Sabatier and Centre National de la Recherche Scientifique LP008201. Corinne Clavé was supported by a grant from Ministère de la recherche et de la Technologie and Jean-Michel Manias was a graduate student.

We thank Dr Diane Stassi for helpful discussions during preparation and editing of the manuscript. Strain GP95 was kindly provided by Dr Gianni Pozzi, Università di Verona, Verona, Italy.

References

- AMBUDKAR, S. V., LYNN, A. R., MALONEY, P. C. & ROSEN, B. P. (1986). Reconstitution of ATP-dependent calcium transport from streptococci. *Journal of Biological Chemistry* **261**, 15596–15600.
- CHANDLER, M. S. & MORRISON, D. A. (1988). Identification of two proteins encoded by *com*, a competence control locus of *Streptococcus pneumoniae*. *Journal of Bacteriology* **170**, 3136–3141.
- CHEN, J. D. & MORRISON, D. A. (1987). Modulation of competence for genetic transformation in *Streptococcus pneumoniae*. *Journal of General Microbiology* **133**, 1959–1967.
- CLAVÉ, C. (1988). *Flux ioniques et énergétique cellulaire au cours de l'induction de la compétence chez Streptococcus pneumoniae, leur implication dans le transport de l'ADN*. Doctorat d'Université, Université Paul Sabatier, Toulouse, France.
- CLAVÉ, C. & TROMBE, M. C. (1989). Intracellular pH and ATP involvement in DNA uptake by *Streptococcus pneumoniae* competent for genetic transformation. *FEMS Microbiology Letters* **65**, 113–118.
- CLAVÉ, C., MORRISON, D. A. & TROMBE, M. C. (1987). Is DNA transport driven by the proton electrochemical potential difference in the naturally transformable bacteria *Streptococcus pneumoniae*? *Bioelectrochemistry and Bioenergetics* **17**, 269–276.
- CLAVÉ, C., MARTIN, F. & TROMBE, M. C. (1989). DNA uptake in *Streptococcus pneumoniae*: an insight into energetics and mechanism. In *Genetic Transformation and Expression*, pp. 27–40. Edited by L. O. Butler, C. Harwood & B. E. B. Moseley. Andover: Intercept.
- DIAZ, E. & GARCIA, J. L. (1990). Characterization of the transcription unit encoding the major pneumococcal autolysin. *Gene* **90**, 157–162.
- DUBNAU, D. (1991). The regulation of genetic competence in *Bacillus subtilis*. *Molecular Microbiology* **5**, 11–18.
- FALAH, A. M. S., BHATNAGAR, R., BHATNAGAR, N., SINGH, Y., SIDHU, G. S., MURTHY, P. S. & VENKITASUBRAMANIAN, T. A. (1988). On the presence of calmodulin-like protein in mycobacteria. *FEMS Microbiology Letters* **56**, 89–94.
- FOX, M. S. & HOTCHKISS, R. D. (1957). Initiation of bacterial transformation. *Nature, London* **179**, 1322–1325.
- FRY, I. J., VILLA, L., KUEHN, G. D. & HAGEMAN, J. H. (1986). Calmodulin-like protein from *Bacillus subtilis*. *Biochemical and Biophysical Research Communications* **134**, 212–217.
- HÖLTJE, J. V. & TOMASZ, A. (1976). Purification of the pneumococcal N-acetylmuramyl-L-alanine amidase to biochemical homogeneity. *Journal of Biological Chemistry* **251**, 4199–4207.
- HUI, F. M. & MORRISON, D. A. (1991). Genetic transformation in *Streptococcus pneumoniae*: nucleotide sequence analysis shows *comA*, a gene required for competence induction, to be a member of the bacterial ATP-dependent transport protein family. *Journal of Bacteriology* **173**, 372–382.
- INOUE, S., FRANCESCHINI, T. & INOUE, M. (1983). Structural similarities between the development-specific protein S from a Gram-negative bacterium *Myxococcus xanthus* and calmodulin. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 6829–6833.
- KACZOROWSKI, G. J., BARROS, F., DETHMERS, J. K. & TRUMBLE, M. J. (1985). Inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange in pituitary membrane vesicles by analogues of amiloride. *Biochemistry* **24**, 1394–1403.
- KOBAYASHI, H., VAN BRUNT, J., & HAROLD, F. M. (1978). ATP-linked calcium transport in cells and membrane vesicles of *Streptococcus faecalis*. *Journal of Biological Chemistry* **253**, 2085–2092.
- KORONAKIS, V. M., CROSS, M., SENIOR, B., KORONAKIS, E. & HUGHES, C. (1988). Comparison of the haemolysin secretion protein HlyB from *Proteus vulgaris* and *E. coli*; site-directed mutagenesis causing impairment of export function. *Molecular and General Genetics* **213**, 551–555.
- LACKS, S. A. (1977). Binding and entry of DNA in pneumococcal transformation. In *Modern Trends in Bacterial Transformation and Transfection*, pp. 35–44. Edited by A. Portoles, R. Lopez & M. Espinoza. Amsterdam: Elsevier/North Holland.
- LACKS, S. A. & GREENBERG, B. (1973). Competence for DNA uptake and deoxyribonuclease action external to cells in the genetic transformation of *Diplococcus pneumoniae*. *Journal of Bacteriology* **114**, 152–163.
- LACKS, S. & HOTCHKISS, R. D. (1960). A study of the genetic material determining an enzyme activity in pneumococcus. *Biochimica and Biophysica Acta* **39**, 508–517.
- LACKS, S. A. & NEUBERGER, M. (1975). Membrane location of a deoxyribonuclease implicated in the genetic transformation of *Diplococcus pneumoniae*. *Journal of Bacteriology* **124**, 1321–1329.
- LACKS, S., GREENBERG, B. & CARLSON, K. (1967). Fate of donor DNA in pneumococcal transformation. *Journal of Molecular Biology* **29**, 327–347.
- LEONARD, C. G. & COLE, R. M. (1972). Purification and properties of streptococcal competence factor isolated from chemically defined medium. *Journal of Bacteriology* **110**, 273–280.
- LERMAN, L. S. & TOLMACH, L. J. (1957). Genetic transformation. I. Cellular incorporation of DNA accompanying transformation in pneumococcus. *Biochimica and Biophysica Acta* **26**, 68–82.
- LOPEZ, R., RONDA-LAIN, C., TAPIA, A., WAKS, S. B. & TOMASZ, A. (1976). Suppression of the lytic and bactericidal effects of cell wall-inhibitory antibiotics. *Antimicrobial Agents and Chemotherapy* **10**, 697–706.
- LOPEZ, A., CLAVÉ, C., CAPEYROU, R., LAFONTAN, V. & TROMBE, M. C. (1989). Ionic and energetic changes at competence in the naturally transformable bacterium *Streptococcus pneumoniae*. *Journal of General Microbiology* **135**, 2189–2197.
- LOPEZ, R., RONDA, C. & GARCIA, E. (1990). Autolysins are directly involved in the bactericidal effect caused by penicillin in wild type and in tolerant pneumococci. *FEMS Microbiology Letters* **66**, 317–322.
- LOVE, P. E. & YASBIN, R. E. (1986). Induction of the *Bacillus subtilis* RecA protein. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 5204–5208.
- MORRISON, D. A. & BAKER, F. M. (1979). Competence for genetic transformation in pneumococcus depends on synthesis of a small set of proteins. *Nature, London* **282**, 215–217.
- MORRISON, D. A., LACKS, S. A., GUILD, W. R. & HAGEMAN, J. M. (1983). Isolation and characterization of three new classes of transformation-deficient mutants of *Streptococcus pneumoniae* that are defective in DNA transport and genetic recombination. *Journal of Bacteriology* **156**, 281–290.
- MORRISON, D. A., TROMBE, M. C., HAYDEN, M. K., WASZACK, G. A. & CHEN, J. D. (1984). Isolation of transformation-deficient *Streptococcus pneumoniae* mutants defective in control of competence, using insertion-duplication mutagenesis with the erythromycin resistance determinant pAMB1. *Journal of Bacteriology* **159**, 870–876.
- MURRELL, W. G. (1967). The biochemistry of the bacterial endospore. *Advances in Microbial Physiology* **1**, 133–251.
- NAVA, G., GALIS, A. & BEISER, S. M. (1963). Bacterial transformation: an antigen specific for competent pneumococci. *Nature, London* **197**, 903–904.

- PAKULA, R. & WALCZAK, W. (1963). On the nature of competence activator of transformable streptococci. *Journal of General Microbiology* **31**, 125–133.
- RAINA, J. L. & RAVIN, A. W. (1980). Switches in macromolecular synthesis during induction of competence for transformation of *Streptococcus sanguis*. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 6062–6066.
- REUSCH, R. N. & SADOFF, H. L. (1983). D-(–)-Poly- β -hydroxybutyrate in membranes of genetically competent bacteria. *Journal of Bacteriology* **156**, 778–788.
- ROSEN, B. P. (1987). Bacterial calcium transport. *Biochimica et Biophysica Acta* **906**, 101–110.
- SANCHEZ-PUELLES, J. M., RONDA, J. M., GARCIA, C., MENDEZ, E., GARCIA, E., GARCIA, J. L. & LOPEZ, R. (1986). A new peptidoglycan hydrolase in *Streptococcus pneumoniae*. *FEMS Microbiology Letters* **35**, 163–166.
- SETO, H. & TOMASZ, A. (1974). Early stages in DNA binding and uptake during genetic transformation of streptococci. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 1493–1498.
- SETO, H. & TOMASZ, A. (1976). Calcium-requiring step in the uptake of deoxyribonucleic acid molecules through the surface of competent pneumococci. *Journal of Bacteriology* **126**, 1113–1118.
- SIMCHOWITZ, L. & CRAGOE, E. J., JR (1986). Inhibition of chemotactic factor-activated Na^+/H^+ exchange in human neutrophils by analogues of amilorides: structure–activity relationships in the amiloride series. *Molecular Pharmacology* **30**, 112–120.
- SWAN, D. G., CORTES, J., HALE, R. S. & LEADLEY, P. F. (1989). Cloning, characterization and heterologous expression of the *Saccharopolyspora erythraea* gene encoding an EF-hand calcium-binding protein. *Journal of Bacteriology* **171**, 5614–5619.
- SWAN, D. G., HALE, R. S., DHILLON, N. & LEADLEY, P. F. (1987). A bacterial calcium-binding protein homologous to calmodulin. *Nature, London* **329**, 84–85.
- TIRABY, G. & FOX, M. S. (1973). Marker discrimination in transformation and mutation of *Streptococcus pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 3541–3545.
- TOMASZ, A. & HOTCHKISS, R. D. (1964). Regulation of the transformability of pneumococcal cultures by macromolecular cell products. *Proceedings of the National Academy of Sciences of the United States of America* **51**, 480–487.
- TOMASZ, A., MOREILLON, P. & POZZI, G. (1988). Insertional inactivation of the major autolysin of *Streptococcus pneumoniae*. *Journal of Bacteriology* **170**, 5931–5934.
- TROMBE, M. C. (1981). *Caractérisation de mutants de résistance à l'améthoptérine chez Streptococcus pneumoniae ; altération du potentiel transmembranaire. Mise en évidence d'une cible membranaire pour l'améthoptérine*. Doctorat D'état, Université Paul Sabatier, Toulouse, France.
- TROMBE, M. C. (1983). Alteration du transport actif des acides aminés au moment de la compétence chez *Streptococcus pneumoniae*. *Comptes Rendus de l'Académie des Sciences. Paris. Sciences de la Vie* **294**, 42–44.
- TROMBE, M. C., LANÉELLE, G. & SICARD, A. M. (1984). Characterization of a *Streptococcus pneumoniae* mutant with altered electric transmembrane potential. *Journal of Bacteriology* **158**, 1109–1114.
- ZIEGLER, R. & TOMASZ, A. (1970). Binding of the competence factor to receptors in the spheroplast membrane of streptococci. *Biochemical and Biophysical Research Communications* **41**, 1342–1349.