Characterization of a calcium porter of *Streptococcus pneumoniae* involved in calcium regulation of growth and competence

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It is shown that *Streptococcus pneumoniae* possesses a Ca^{2+} transporter, sensitive to the amiloride derivative 2',4'-dimethylbenzamil (DMB), which is essential for growth at high Ca^{2+} -concentrations, and which mediates the triggering by Ca^{2+} of competence for genetic transformation in the exponential phase and autolysis in the late exponential phase. DMB inhibited both Ca^{2+} transport and the Ca^{2+} response. Kinetic analysis of $^{45}\text{Ca}^{2+}$ transport in ATP-depleted *S. pneumoniae* revealed an electrogenic influx sensitive to DMB. This transport was cooperative with respect to Ca^{2+} concentration, and exhibited a Hill coefficient (nH) of 2. In bacteria pre-loaded with $^{45}\text{Ca}^{2+}$, a DMB-sensitive efflux could be triggered by an imposed Na^+ gradient. The efflux kinetics showed the same cooperativity profile as Ca^{2+} concentration and a similar nH value to that of influx, suggesting a possible $\text{Na}^+/\text{Ca}^{2+}$ antiport. Cooperativity of transport was lowered (nH = 1) by a mutation that confers resistance to DMB and abolishes the Ca^{2+} response. These results demonstrate that DMB-sensitive Ca^{2+} transport is essential for growth and competence regulation. The role of the DMB-sensitive porter involved in Ca^{2+} circulation and in Ca^{2+} homeostasis and its possible regulation by competence factor are discussed.

Introduction

The roles of Ca^{2+} in eukaryotes, including yeast, are diverse and important, ranging from activation of enzymes to hormone release and cell cycle control. Both Ca^{2+} -porters and Ca^{2+} -binding proteins, (calmodulins) have been described (for reviews see Campbell, 1983; Carafoli, 1987, 1988). In prokaryotes, Ca^{2+} is also involved in various processes (for reviews, see Norris *et al.*, 1991; Onek & Smith, 1992), and calmodulin-like proteins have been characterized in several species (Inouye *et al.*, 1983; Fry *et al.*, 1983; Swan *et al.*, 1987, 1989; Falah *et al.*, 1988). Ca^{2+} -transporters including pumps and channels, have been identified (Kobayashi *et al.*, 1978; Vrij *et al.*, 1985; Ambudkar *et al.*, 1986). So far, the role played by Ca^{2+} in bacteria is poorly understood. It is reported that Ca^{2+} activates auto-phosphorylation of the heat-shock protein, DnaK, in *Escherichia coli* (Cegielska & Georgopoulos, 1989), and that it stimulates expression of the virulence factor haemolysin in *Actinobacillus pleuropneumoniae* (Frey & Nicolet, 1988).

In *Streptococcus pneumoniae*, Ca^{2+} is an obligatory cation for growth. However, high Ca^{2+} (1 mM) triggers, via an exported protein (competence factor, CF), the induction of competence for genetic transformation in exponentially growing cultures and lysis in early stationary phase. DNA uptake at competence prevents lysis in early stationary phase, and it has been proposed that DNA transport constitutes a homeostatic response to elevated Ca^{2+} levels (Trombe *et al.*, 1992). On the other hand, autolysis, dependent on the activity of the *N*-acetylmuramidase encoded by *lytA* (Sanchez-Puelles *et al.*, 1986), is involved in the pathogenicity of smooth derivatives of *Streptococcus pneumoniae* (Berry *et al.*, 1989). Interestingly, competence induction and culture lysis are prevented by the Ca^{2+} -channel inhibitor 2',4'-dimethylbenzamil (DMB) (Trombe *et al.*, 1992). In the present report, the kinetic parameters of the DMB-sensitive component of calcium transport have been established, both in the wild-type strain and in a DMB-resistant mutant of *S. pneumoniae*, and correlated with the Ca^{2+} response.

Methods

Strains. The RX derivative of *Streptococcus pneumoniae*, strain Cp1015, bearing the *strRI* mutation, was used as the standard wild-type strain (Morrison *et al.*, 1984). The required mutations were

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

introduced into the wild-type background by genetic transformation using DNA from the mutant strains. Two rounds of transformation with non-saturating levels of DNA were performed to reduce the chance of multiple gene transfer. 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). The relevant phenotypes of Cp1322 are its inability to produce CF (Morrison *et al.*, 1984) and its resistance to Ca^{2+} -induced lysis (Trombe *et al.*, 1992).

Screening of Cp1015 for spontaneous mutants resistant to DMB was done on plates containing $15\text{ }\mu\text{g DMB ml}^{-1}$. Their frequency in a culture was between 10^{-5} and 10^{-4} . After characterization of their natural transformability, clones altered for competence regulation were selected. Mutations conferring DMB resistance were transferred to the wild-type background by genetic transformation. Bacteria transformed to DMB resistance (DMB^R transformants) were selected on plates containing $15\text{ }\mu\text{g DMB ml}^{-1}$, as described previously (Trombe *et al.*, 1992). Strain Cp2200 was taken as a representative of pleiotropic mutants in which the DMB^R phenotype was associated with an alteration of competence regulation.

Specific materials. The source of [^3H]DNA was the thymidine-auxotrophic strain R119, which also carries the *rif*-32 mutation (Tiraby & Fox, 1973). Amilorides were from Merck Sharp & Dohme, USA, and were provided by Dr E. J. Cragoe. DMB was prepared specifically for this study. Nutrients used for growth media were from Difco. $^{45}\text{Ca}^{2+}$ was from CEA, France. Luciferase-luciferin and ATP were from Sigma.

Growth and competence induction. Growth conditions and media were identical for the wild-type and mutant strains, as described previously (Clavé *et al.*, 1987). Briefly, stock cultures were grown at pH 7.5 in a complex medium containing (g l^{-1}): NaCl, 5; yeast extract, 1; tryptone, 5; enzymic casein hydrolysate, 10; glucose, 2; K_2HPO_4 , 3. This medium contained 120 mM-Na^+ and 0.2 mM-Ca^{2+} as measured by atomic absorption spectrometry. Growth curves were measured and autolysis tests were done as described previously (Trombe *et al.*, 1992). For transport experiments, a culture frozen at an OD_{550} of 0.4 was thawed, centrifuged at 4°C and washed with uptake medium; the pellet was kept at 4°C before use (5–30 min). Such bacteria had an ATP pool of $3\text{ }\mu\text{M}$ compared with $0.25 \pm 0.05\text{ mM}$ in exponentially growing bacteria. They were taken as ATP-depleted cells. ATP measurements were made using a luciferase-luciferin assay as described by Lopez *et al.* (1989). The intracellular Na^+ content was estimated at $85 \pm 5\text{ mM}$ by atomic absorption spectrometry.

Transport measurements. In general, values for uptake at 15 s (V_{15s}) were taken to approximate to the initial rate of transport, and these values are referred to as initial rates throughout the paper. Kinetic measurements (0–60 s) were made to verify the linearity of the response during this interval. Ca^{2+} transport was assayed using the radioactive isotope $^{45}\text{Ca}^{2+}$. In streptococci, ion pumps, including a Ca^{2+} -pump, are well known (for reviews, see Heefner, 1982; Rosen, 1987). The Ca^{2+} -pump expels Ca^{2+} from the cells. Therefore, ATP-depleted bacteria were used to avoid interference between the activities of these putative pumps and of the porter being studied. ATP-depleted bacteria were suspended in the appropriate uptake medium at room temperature to give a suspension of OD_{550} between 2 and 5. A Ca^{2+} gradient (in < out) was imposed by addition of a $^{45}\text{Ca}^{2+}$ solution and 0.1 ml samples were filtered at intervals through $0.45\text{ }\mu\text{m}$ Gelman Metrical membranes. After three washes with medium containing $0.5\text{ mg CaCl}_2\text{ ml}^{-1}$, the radioactivity retained on the filters was quantified by liquid scintillation as previously described (Trombe *et al.*, 1984). It was verified that Ca^{2+} washes lowered non-specific absorption on the filters without significant displacement of intracellular $^{45}\text{Ca}^{2+}$. Transport of $^{45}\text{Ca}^{2+}$ was measured at pH 8, in bacteria incubated either in the growth medium or in a Tris-based medium at the required Na^+ concentration (Trombe *et al.*, 1984).

When the effect of amilorides was examined, these were added to the bacterial suspension before $^{45}\text{Ca}^{2+}$ addition. The initial rate of $^{45}\text{Ca}^{2+}$ transport by DMB-sensitive bacteria was taken as the difference between values obtained in the absence and in the presence of $10\text{ }\mu\text{M}$ -DMB.

For [^{14}C]isoleucine transport measurements in ATP-depleted bacteria, the same conditions as for fully energized bacteria (Trombe *et al.*, 1984) was used. The initial rates of uptake (determined in the first 15 s) were 20–30 times lower than the values obtained in fully energized cells (Trombe *et al.*, 1984).

Calculations were done on the basis that 1 ml of suspension at an OD_{550} of 1 represents an intracellular free space of $1\text{ }\mu\text{l}$ and contains 0.33 mg protein (Trombe *et al.*, 1984).

Genetic analysis. Transformation was used to carry out genetic transfers. The recipient strain was transformed with DNA extracted from the donor under conditions described previously (Trombe *et al.*, 1992). Selection of transformants was done on plates containing the selective agent. Transformed bacteria for one given marker were tested for a second marker by streaking the colonies on the appropriate selective plates containing either $2\text{ }\mu\text{g erythromycin ml}^{-1}$ or $15\text{ }\mu\text{M}$ -DMB.

Results

Evidence for a DMB-sensitive Ca^{2+} transport involved in homeostasis at high Ca^{2+}

When a $^{45}\text{Ca}^{2+}$ gradient ($[\text{Ca}^{2+}]_{\text{out}} > [\text{Ca}^{2+}]_{\text{in}}$) was imposed on ATP-depleted bacteria (see Methods) a net influx could be measured. At 1.4 mM-Ca^{2+} , uptake was linear for 30 s and was followed by efflux with a plateau corresponding to approximately 1 mM between 3 and 5 min. The Ca^{2+} -channel inhibitor DMB decreased the initial rate of influx and the steady state was not reached (Fig. 1). Initial rates of influx in media containing increasing DMB concentrations showed (Fig. 2a) 50 %

Fig. 1. DMB-sensitive $^{45}\text{Ca}^{2+}$ uptake. Energy-depleted bacteria (intracellular ATP concn $< 3\text{ }\mu\text{M}$, intracellular Na^{2+} concn 90 mM) were suspended in the complex medium, pH 7.8, containing 30 mM-NaCl and $^{45}\text{Ca}^{2+}$ (1.2 mM , sp. act. $185\text{ fmol} \equiv 1\text{ c.p.m.}$) was added. When required, DMB ($10\text{ }\mu\text{M}$ final concn) was added to the bacterial suspension before the $^{45}\text{Ca}^{2+}$. At intervals, 0.1 ml samples were filtered and washed (see Methods). The radioactivity of the filters was quantified by liquid scintillation. Calculations were done using the reference data given in Methods. Δ , $\blacktriangle$, Two independent experiments in DMB-free medium; $\circ$, uptake in the presence of DMB.

Fig. 2. Inhibition by DMB of Ca^{2+} transport (a) and growth (b, c). (a) Initial rate of Ca^{2+} transport ($\square$, $^{45}\text{Ca}^{2+}$ concn 1.2 mM, sp. act. 220 fmol $\equiv$ 1 c.p.m.; $\blacktriangle$, $^{45}\text{Ca}^{2+}$ concn 0.4 mM, 190 fmol $\equiv$ 1 c.p.m.) determined in the presence of various concentrations of DMB. The vertical bars represent SEM ($n = 3$). (b, c) Growth measurements were made in media containing 0.2 mM- Ca^{2+} (b) or supplemented with 1 mM- Ca^{2+} (c) and the following DMB concentrations (μM): $\bullet$, 0; $\circ$, 2; $\blacktriangle$, 3; $\triangle$, 4; $\blacksquare$, 5; $\square$, 6; $\blacklozenge$, 10 (c only).

inhibition at 4 μM (I_{50}) and a plateau between 8 and 12 μM . Residual uptake, at the plateau, presumably depends on a DMB-resistant system(s). When added to cultures at high Ca^{2+} , DMB slowed the growth rate, with total inhibition at 10 μM (Fig. 2b), while at low Ca^{2+} , the effect of DMB on bacterial growth related to the duration of the lag phase, rather than the growth rate (Fig. 2c). Such an effect of Ca^{2+} on growth-inhibition was not observed in the case of amiloride or its derivative

Fig. 3. Kinetics of $^{45}\text{Ca}^{2+}$ influx in response to a Ca^{2+} gradient (a) and efflux in response to a Na^{+} gradient (b). (a) The initial rate of DMB-sensitive $^{45}\text{Ca}^{2+}$ influx as a function of the external Ca^{2+} concentration was determined as explained in Methods. The inset represents the curve $\log [\text{Ca}^{2+}]$ versus $\log [v/(V-v)]$ for experiments performed in the complex medium ($\square$) or in the Tris-based medium ($\blacklozenge$) [V is the maximal rate at 1.5 mM- Ca^{2+} ; v is V_{15s} for a given Ca^{2+} concentration]. The vertical bars represent SEM ($n = 3$). (b) Bacteria from a suspension of OD₅₅₀ 5.2 were loaded with various amounts of $^{45}\text{Ca}^{2+}$ by a 4 min incubation in the Tris-based medium with a Na^{+} concentration < 1 mM, containing various concentrations of $^{45}\text{Ca}^{2+}$ at the required specific activity. The radioactivity retained was estimated by filtration of a 0.1 ml aliquot, then a Na^{+} gradient was imposed by addition of NaCl (5 mM final concn). Samples of 0.1 ml were withdrawn and filtered after 10 and 20 s. Initial rates of efflux were calculated as explained in Methods.

5-(*N,N'*-hexamethylene)amiloride (HMA), which did not affect the initial rate of $^{45}\text{Ca}^{2+}$ uptake (data not shown). Altogether, these data point to a DMB-sensitive function involved in Ca^{2+} circulation. This function is essential for homeostasis in high Ca^{2+} media.

Kinetics of calcium transport

A plot showing the initial rate for the DMB-sensitive component of $^{45}\text{Ca}^{2+}$ influx, as a function of the external Ca^{2+} concentration, exhibits sigmoidicity, with Hill coefficients (nH) of 1.9 (Fig. 3a). Similar nH values were obtained from uptake experiments performed in the

Fig. 4. Effect of Ca^{2+} on the electrogenic transport of $[^{14}\text{C}]$ isoleucine. Energy-depleted bacteria were suspended in Tris-based medium at OD_{550} 0.3 and incubated at room temperature in the presence of $[^{14}\text{C}]$ isoleucine (10^{-2} mM, $50 \text{ fmol} \equiv 1 \text{ d.p.m.}$). At intervals, 0.1 ml samples were withdrawn, filtered and rinsed as already described. Results are expressed as d.p.m. per μl intracellular free space (see Methods). $\square$, Uptake in Ca^{2+} -free medium; $\blacktriangle$, $\blacklozenge$, simultaneous addition of $[^{14}\text{C}]$ isoleucine and 1.2 mM-CaCl_2 ; ($\blacktriangle$, $10 \mu\text{M-DMB}$ was added to the suspension before $[^{14}\text{C}]$ isoleucine and CaCl_2). Inset, $[^{14}\text{C}]$ isoleucine transport in a Ca^{2+} free medium, in the absence ($\blacklozenge$) or in the presence ($\square$) of $10 \mu\text{M-DMB}$. The scale in the inset is the same as in the main figure. The initial rate of transport in Ca^{2+} -free medium (V_{15s}) was $0.18 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

Fig. 5. Effect of DMB on the growth rate of the wild-type ($\square$) and mutant Cp2200 ($\blacklozenge$) in high- Ca^{2+} medium. Doubling times of cultures growing in high- Ca^{2+} medium containing various concentrations of DMB were determined on growth curves between OD_{400} 0.1 and 0.2.

complex medium or in a Tris-buffered medium (Fig. 3a, inset). When bacteria were preloaded to obtain internal Ca^{2+} concentrations ranging from 0.3 to 1.5 mM, efflux could be triggered by shifting the NaCl concentration of the medium to 5 mM. The initial rates of $^{45}\text{Ca}^{2+}$ efflux as a function of the intracellular Ca^{2+} concentration were also sigmoidal, with an nH value of 1.7 (Fig. 3b). It is likely that this cooperativity represents an intrinsic property of the transporter functioning as a $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

Fig. 6. Kinetics of $^{45}\text{Ca}^{2+}$ influx in strain Cp2200. The experiment was performed as described in legend of Fig. 3(a). The OD_{550} of the bacterial suspension was 5.4. The values obtained by three measurements of the initial rate of influx for a given $^{45}\text{Ca}^{2+}$ concentration are plotted. For Hill plots (inset) the mean values were taken.

Electrogenicity of transport

Electrogenicity of Ca^{2+} influx was checked using the electrogenic uptake of isoleucine as a probe (Trombe *et al.*, 1984; Trombe, 1984). Inhibition of isoleucine uptake by Ca^{2+} influx should reflect membrane depolarization. Addition of Ca^{2+} to the uptake medium abolished $[^{14}\text{C}]$ isoleucine uptake after 15 s (Fig. 4.) This delay corresponds to the kinetics of Ca^{2+} influx (Fig. 1). DMB suppressed the Ca^{2+} effect. In a Ca^{2+} -free medium, DMB ($10 \mu\text{M}$) did not alter the rate of isoleucine transport (Fig. 4, inset). This suggests that Ca^{2+} inhibition of isoleucine uptake is due to electrogenic Ca^{2+} influx, via the DMB-sensitive porter.

Genetic correlation between Ca^{2+} transport, growth, competence induction and autolysis

Among spontaneous mutants resistant to $15 \mu\text{M-DMB}$ (DMB^R), clone DMB^{R1} was chosen because it showed a reduced rate of $^{45}\text{Ca}^{2+}$ transport. Mutant Cp2200 was constructed by transformation of the wild-type strain Cp1015 with DNA from mutant DMB^{R1} and its growth inhibition by DMB was verified (Fig. 5). The uptake kinetics of $^{45}\text{Ca}^{2+}$ showed a lower cooperativity than the wild-type strain, $nH = 1.3$ (Fig. 6). This mutant did not develop natural competence in high- Ca^{2+} medium (20 independent experiments) and was resistant to autolysis. This provides genetic evidence to implicate the kinetics of Ca^{2+} transport in the Ca^{2+} response. Interestingly, resistance to autolysis and the loss of natural transformability in mutant Cp2200 are reminiscent of the characteristics of the competence-defective (Com^-) mutant Cp1322 (Morrison *et al.*, 1984; Trombe *et al.*, 1992); however, strain Cp1322, selected for its defect in natural transformability, was not DMB-resistant. Genetic

analysis was performed to determine whether the gene conferring the DMB^{R1} phenotype, carried by DNA of strain CP2200, and *comA::ermAM*, carried by the recipient strain Cp1322 (Ery^R), were alleles. No Ery^S transformant was found among 100 DMB^R transformants after transformation of strain Cp1322 by DNA extracted from mutant Cp2200, suggesting that these markers were not carried by the same DNA transforming molecule. The average size of a transforming molecule being estimated around 5 kb, it is likely that the mutation responsible for the DMB^{R1} phenotype defines a new gene which regulates the Ca^{2+} response, including competence induction.

Discussion

Kinetic analysis of $^{45}\text{Ca}^{2+}$ transport in ATP-depleted *S. pneumoniae* provides evidence for Ca^{2+} transport down its electrochemical potential (Figs 1, 3a) and in response to an imposed Na^+ gradient (Fig. 3b). This transport is sensitive to the amiloride derivative DMB (Figs 1, 2a). Neither amiloride itself nor HMA inhibited the initial rate of $^{45}\text{Ca}^{2+}$ transport. Thus *S. pneumoniae* probably possesses a DMB-sensitive $\text{Na}^+/\text{Ca}^{2+}$ antiporter. Using electrogenic isoleucine transport (Trombe *et al.*, 1984) as a reporter activity, it was shown that the DMB-sensitive component of the Ca^{2+} transport was electrogenic (Fig. 4). In prokaryotes, electrogenic Ca^{2+} transport has already been described for several species (Tsujibo & Rosen 1983; Vrij *et al.*, 1985), and observed in ATP-depleted *Streptococcus faecalis* (Kobayashi *et al.*, 1978). However, no specific inhibitor was characterized. In *S. pneumoniae*, DMB defines a function which probably corresponds to a $\text{Na}^+/\text{Ca}^{2+}$ antiporter essential for growth at high Ca^{2+} concentrations. The DMB concentration (10 μM) at which the plateau of uptake-inhibition is observed, suggesting a total block of the porter (Fig. 2a), corresponds to total inhibition of growth at high Ca^{2+} (Fig. 2c). The most likely hypothesis is that, *in vivo*, the porter mediates both influx and efflux of Ca^{2+} by antiport of Na^+ and thus adjusts the intracellular Ca^{2+} concentration to a level compatible with growth.

On the other hand, the DMB concentration that lowers by 50% the rate of transport ($I_{50} = 4 \mu\text{M}$) (Fig. 2a) corresponds to the inhibitory concentration for competence induction and for autolysis (Trombe *et al.*, 1992; Fig. 2b). Finally, the reduced value for the initial rate of Ca^{2+} transport at high Ca^{2+} in mutant Cp2200 due to a loss of cooperativity (Fig. 6) can be correlated with a deficiency in natural competence and with resistance to autolysis. This shows that the initial rate of Ca^{2+} uptake via the DMB-sensitive porter is involved in the regulation of competence and of autolysis (Trombe *et al.*, 1992).

It is noteworthy that in the wild-type strain, cooperativity of transport has an nH value of 2 for influx and efflux (Fig. 3a,b). Indeed, the Ca^{2+} concentration of 1 mM, where the inflexion point is observed in kinetics studies, falls in the same order of magnitude as the critical concentration at which the Ca^{2+} response is induced (Trombe *et al.*, 1992) and corresponds to the Ca^{2+} concentration of body fluids that constitute the natural habitat of *S. pneumoniae*. Considering the transport kinetics, in this range of values tiny changes in Ca^{2+} concentration should result in large shifts of the rate of transport. This constitutes a valuable property for a putative sensor of Ca^{2+} concentration in the medium or in the cytoplasm.

The consequences of growth at high Ca^{2+} characterized to date are autolysis activation and competence induction (Trombe *et al.*, 1992). Indeed, competence has been associated with cytoplasmic alkalization, stimulation of glycolysis (Lopez *et al.*, 1989), induction of specific oligopeptides (Morrison & Baker, 1979), activation of a membrane endonuclease (Lacks *et al.*, 1975; Puyet *et al.*, 1990) and DNA transport activity (Clavé *et al.*, 1987; Clavé & Trombe, 1989). Thus competence might be considered as a 'specialized' physiological state associated with ionic signals and triggered by Ca^{2+} influx. Such a description of competence does not exist for other systems such as *Bacillus subtilis*, where the cascade of gene regulation implicated in the onset of competence is well established (Dubnau, 1991).

Amiloride-sensitive ion channels have been extensively described in eukaryotes (Cuthbert & Fanelli, 1978; Kaczorowski *et al.*, 1985; Simchowicz & Cragoe, 1986). Na^+ circulation, through an amiloride-sensitive Na^+/H^+ antiporter, appears to be a critical event in generating ionic signals. These signals are represented by an increase in cytoplasmic pH and Na^+ (Paris & Pouyssegur, 1983; Pouyssegur *et al.*, 1984). These signals, associated with an elevation of free cytoplasmic Ca^{2+} , are ubiquitous in the response of eukaryotic cells to growth factors and hormones (for reviews, see Rozengurt, 1980; Metcalfe *et al.*, 1985). On the other hand, it has been proposed that Ca^{2+} uptake is the determining step in cell differentiation and hence in the cell cycle (Miyakama *et al.*, 1985; Iida *et al.*, 1990).

The Ca^{2+} effect in *S. pneumoniae* is controlled by an exported protein (Trombe *et al.*, 1992) named competence factor (CF) (Tomasz & Hotchkiss, 1964). CF probably recognizes a specific receptor on the cytoplasmic membrane (Ziegler & Tomasz, 1970). It has been shown that CF stimulates a DMB-sensitive $^{45}\text{Ca}^{2+}$ influx but does not lower the Ca^{2+} requirement for competence induction (Trombe *et al.*, 1992). It is proposed that CF activation of the Ca^{2+} response occurs through the stimulation of Ca^{2+} influx via the DMB-sensitive porter.

The cooperativity level, as regards Ca^{2+} , that modulates the rate of cation uptake might constitute the critical parameter where regulation occurs. The behaviour of strain Cp2200, which carries a pleiotropic mutation that lowers cooperativity of Ca^{2+} transport and alters the regulation of competence and of autolysis, appears to fit this hypothesis. Other mutants showing an enhanced nH value for Ca^{2+} transport have been isolated and are now being studied.

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