JOURNAL OF BACTERIOLOGY, Apr. 1994, p. 1992–1996
0021-9193/94/$04.00+0

Vol. 176, No. 7

Mutations Which Alter the Kinetics of Calcium Transport Alter the Regulation of Competence in *Streptococcus pneumoniae*

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Received 28 October 1993/Accepted 16 January 1994

In *Streptococcus pneumoniae*, Ca^{2+} induces a stress response which is regulated by a proteic activator known as competence factor (CF). This stress response is expressed as the induction of competence for DNA uptake and genetic transformation in exponentially growing cultures and by autolysis in late exponential phase. DNA transport during competence can be described as a homeostatic response that prevents autolysis of the cultures. Electrogenic and cooperative calcium transport with a Hill number (nH) of 2 appears to mediate this Ca^{2+} response. Mutant strains altered in their kinetics for Ca^{2+} transport, with nHs of 1 and 4, were isolated and characterized in order to address the role of the kinetics of Ca^{2+} transport in the Ca^{2+} response. The reduced cooperativity of Ca^{2+} uptake in mutant strain Cp2200 was associated with an absolute requirement for added CF to develop competence and with resistance to autolysis. The enhanced cooperativity of Ca^{2+} uptake in mutant strain Cp3300 was associated with facilitated competence and hypersensitivity to autolysis. Moreover, the mutation carried by strain Cp3300 increases the CF response of previously described competence-defective mutants. The pleiotropic mutants Cp2200 and Cp3300 allowed us to demonstrate that cooperativity of transport determines the Ca^{2+} response in *S. pneumoniae*.

Ca^{2+} ions have been extensively described as second messengers in eukaryotes. In prokaryotes, Ca^{2+} is also involved in a variety of cellular processes (for reviews see references 17 and 19) such as DNA transport during competence in *Streptococcus pneumoniae* (3, 7, 10, 22) and mobility in several species, e.g., chemotaxis in *Escherichia coli* (14, 20, 23, 24). Ca^{2+} activates autophosphorylation of the heat shock protein DnaK (2) and of the sensor EnvZ (21) as well as proteolysis during sporulation in *Bacillus subtilis* (18). Ca^{2+} has also been shown to modulate gene expression in *Actinobacillus pleuropneumoniae* (8). While Ca^{2+} transporters and pumps have been identified in several bacterial species (1, 5, 9), their role in Ca^{2+} metabolism is poorly understood. It is likely that a voltage-dependent Ca^{2+} channel is involved in *E. coli* chemotaxis (24).

In the case of *S. pneumoniae*, whose niche is in biological fluids containing 1 mM Ca^{2+}, 0.15 mM Ca^{2+} is required for growth. A Ca^{2+} transporter, sensitive to the amiloride derivative 2′-4′-dimethylbenzamil (DMB), is essential for Ca^{2+} homeostasis. Remarkable properties of this Ca^{2+} transporter are its cooperativity (Hill number [nH] of 2 in the wild-type strain) and an inflection point at around 1 mM. We have shown that 1 mM Ca^{2+} induces competence for genetic transformation in exponentially growing cultures and autolysis, involving an *N*-acetylmuramylalanine-amidase, at the end of exponential growth phase. Both processes are inhibited by concentrations of DMB that do not inhibit growth (25, 26). The DMB resistance mutation of strain Cp2200 confers a reduced nH value for Ca^{2+} transport, loss of natural competence irrespective of the culture medium (3, 7), and resistance to autolysis (25). This suggested that the kinetics of transport could determine the extent of the Ca^{2+} response.

In order to further check this hypothesis, we isolated a mutant with enhanced cooperativity for Ca^{2+} transport and characterized its Ca^{2+} response. In addition, we have analyzed the effect of this mutation when it is introduced into a previously described competence-defective (*com*) strain (16). Finally, we have examined the complementation of mutant Cp2200 by exogenous activator. The results presented in this article demonstrate that changes in the kinetics of Ca^{2+} transport alter the Ca^{2+} response in *S. pneumoniae*.

MATERIALS AND METHODS

Strains. Strain Cp1015, a derivative of RX bearing the *str-r1* mutation, which confers resistance to streptomycin, was used as the standard strain (16). The construction of isogenic derivatives by genetic transformation as well as the procedure for mutant isolation is described elsewhere (25, 26).

Growth and competence induction. Growth and competence induction are described elsewhere (26). Briefly, the source of DNA was the thymidine-auxotrophic strain R119, which also carries the *rif-23* mutation and is resistant to 2 μg of rifampicin per ml. Competence was measured either for precompetent cultures kept frozen before utilization (tester cells) or as a function of the growth phase in media containing 1 mM Ca^{2+}. The indications of competence were the degradation of extracellular pneumococcal [^{3}H]DNA, resulting in acid-soluble material, and genetic transformation for the marker *rif-23* (26). For $^{45}Ca^{2+}$ transport experiments a frozen culture at an optical density (OD) of 0.4 was thawed, centrifuged at 4°C, and washed with the uptake medium, and the pellet was kept at 4°C before utilization (5 to 30 min). Such bacteria had an ATP pool of 3 μM compared with 0.25 mM for exponential-phase bacteria. They were considered ATP-depleted cells and were used for $^{45}Ca^{2+}$ transport assays. ATP measurements were performed by using a luciferase-luciferin assay as previously described (12).

Transport measurements. In general, $^{45}Ca^{2+}$ uptake increased linearly during the first 60 s. Measurements at 15 s approximate the initial rate of transport ($V_{15\ s}$). The DMB-

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sensitive component of transport was defined as the difference between values obtained in the absence and in the presence of 10 or 15 μM DMB. The other conditions were described previously (25, 27). nH determinations were obtained graphically from the slope of the curve log $v/V - v = f(\log Ca^{2+})$, where V represents the maximum uptake rate and v is the $V_{15\ s}$ for a given Ca^{2+} concentration.

RESULTS

Phenotypic characterization of a pleiotropic DMB-resistant mutant altered for competence and autolysis regulation. Among 150 spontaneous mutants forming colonies on plates containing 1.5 mM Ca^{2+} and 15 μM DMB, a single clone was found to exhibit increased sensitivity to autolysis. The *DMB-2* mutation carried by this mutant was then transferred into the wild-type strain Cp1015 to produce the isogenic derivative Cp3300. The frequency of transformed bacteria for the DMB-resistant character was compatible with a single-marker transfer as shown previously for *DMB-1* (25). The DMB-resistant character of Cp3300 was determined more precisely by measuring doubling times in liquid media containing 1 mM calcium and various concentrations of DMB (Fig. 1A). Interestingly, Cp3300 inherited hypersensitivity to Ca^{2+}-induced autolysis. The decreases in ODs for strains Cp1015, Cp1322, Cp2200, and Cp3300 after 1-h incubations at 37°C in cultures which had reached their plateau cell density (OD at 400 nm of 1, which was considered the 100% value) were measured, and the ΔOD values were 44%, 2%, 12%, and 76 and 80%, respectively. For the first three strains, 10 independent cultures were monitored in parallel, and for Cp3300, two independent experiments (10 measurements each) were done. It was also interesting that Cp3300 inherited full competence development in growth medium containing 10 μM DMB. In the wild-type parent, competence was totally inhibited by 5 μM DMB (Fig. 1B). Moreover, in the case of strain Cp3300, competence developed naturally in early exponential growth phase, in cultures growing at neutral pH, without addition of the competence activator known as competence factor (CF), as shown by genetic transformation tests (Table 1). Under the same conditions the wild-type strain requires one CF unit to be activated (26). Thus, the spontaneous mutation *DMB-2*, which is transmitted as a single genetic marker by transformation, confers several phenotypes, a property which characterizes pleiotropic mutations.

DMB resistance in Cp3300 correlates with an increased cooperativity of $^{45}Ca^{2+}$ transport compared with that of the wild type. Inhibition of the initial rate of $^{45}Ca^{2+}$ uptake in strain Cp3300 clearly shows that a DMB-sensitive component was abolished at a DMB concentration between 10 and 15 μM as in the parent strain. The concentration which reduced the uptake rate by 50% was 3 μM (Fig. 2). By contrast, the kinetics of the DMB-sensitive component of $^{45}Ca^{2+}$ transport as a function of increasing $^{45}Ca^{2+}$ concentration (as previously described [25]) showed a typical cooperative-type profile with an nH of 4. This is similar to an nH of 2 for the wild-type strain. For both mutant and wild-type strains, the inflection point falls around 1 mM Ca^{2+} (Fig. 3). The major effect of the enhanced cooperativity for strain Cp3300 is an increased initial rate of Ca^{2+} transport for external concentrations around 1 mM. This property correlates with DMB resistance. Indeed, when DMB lowers Ca^{2+} uptake by 50%, residual transport is still higher in strain Cp3300 than in the parent strain, even in the absence of an inhibitor.

Involvement of the kinetics of Ca^{2+} transport in competence regulation. Interestingly, the use of DMB allowed us to isolate

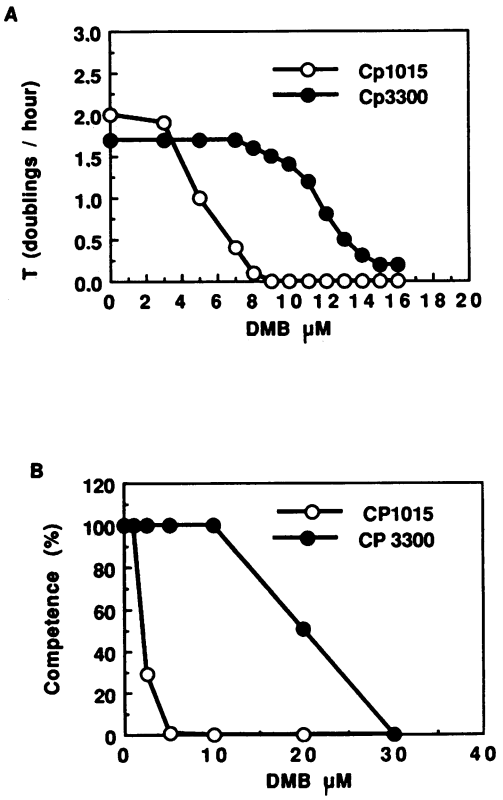

FIG. 1. DMB inhibition of growth (A) and of competence induction (B) in the wild-type strain and the DMB-resistant mutant Cp3300. (A) T values (number of doublings per hour) were deduced by measuring doubling times at 37°C in liquid cultures with ODs at 400 nm in the range from 0.1 to 0.2. Growth medium was supplemented with 1 mM Ca^{2+} and the appropriate DMB concentrations. (B) Tester cells were incubated for 30 min at 37°C in competence medium with various concentrations of DMB. ^{3}H-labelled DNA (1 μg, 60,000 dpm ml^{-1}) was added, and following a 20-min incubation period, the quantity of acid-soluble radioactivity was determined by liquid scintillation counting. At 100% competence, 20% of the initial radioactivity was converted to acid-soluble material by 10^7 CFU in 100 μl of competence medium. This result indicates that 5% of the population was transformed by chromosomal DNA for a given genetic marker.

both a mutant with reduced cooperativity, Cp2200 (25), and a mutant with enhanced cooperativity, Cp3300. These mutant strains were modified in their regulation of competence and of autolysis. They permitted us to establish the functional relationship between the kinetics of Ca^{2+} transport and the physiological response to Ca^{2+}.

Firstly, we evaluated competence in Cp2200 following the addition of exogenous CF. The data presented in Fig. 4A show that Cp2200 can indeed be fully complemented by CF addition. Thus, the Com^- phenotype associated with expression of the mutation *DMB-1* can be corrected by extra CF. However, the CF requirement of the mutant strain is 30 times higher than that of the wild-type parent.

Secondly, the CF response of strain Cp3322, carrying both the mutation *DMB-2* and a *com* mutation leading to a high CF requirement and to a defect in CF activity production (16), was compared with the CF responses of the strains carrying single mutations, *com* (Cp1322) and *DMB-2* (Cp3300). Data shown in Fig. 4B reveal a significantly higher competence response at suboptimal CF concentrations for Cp3322 than for Cp1322.

TABLE 1. Natural competence in strains Cp1015 and CP3300 as a function of growth and external pH values[a]

Strain and pH of medium	% of Rif-r transformants[b] after incubation time (min)								
	0	30	60	90	120	150	180	210	250
Cp1015									
pH 7	0	0	0	0	0	0.03	0.05	0	0
pH 8	0	0	0.2	1.2	0.75	1	0.5	0.1	0.06
Cp3300									
pH 7	0	0	0.35	2	1.5	0.75	ND[c]	0.5	0
pH 8	0	0	0.4	1.8	1.5	1	ND	0.6	0

[a] Competence medium at the indicated pH was inoculated (1/60) with cultures of each strain kept frozen at an OD at 400 nm of 0.4, and incubation at 37°C was monitored. In such cultures, the end of the exponential phase was reached between 210 and 250 min, and culture lysis could not be detected during the experiment regardless of the strain or the pH of the medium.

[b] Percentages of Rif-r transformants in aliquots of cultures withdrawn at intervals and checked at 32°C for their transformability by DNA from strain R119 (see Materials and Methods). The standard deviation was 10% for CFU counts (triplicate plating).

[c] ND, not done.

However, the threshold CF concentration required to trigger competence is still higher than that for Cp3300. This indicates that mutation *DMB-2* lowers the CF requirement caused by the *com* mutation. The functions encoded by both loci likely belong to the same signaling pathway.

DISCUSSION

Competence for genetic transformation in *S. pneumoniae* has been associated with the stimulation of primary metabolism resulting in an elevation of the ATP level, cytoplasmic alkalinization, and the channeling of pyruvate catabolism into poly-β-hydroxybutyrate synthesis (12). It has been proposed that such regulation of the metabolic network might create conditional auxotrophy, in response to which the bacterium induces expression of the competence regulon, allowing the uptake of oligodeoxyribonucleotides. Indeed, DNA transport during competence does protect the bacterium from precocious autolysis at the end of the exponential growth phase (26). Cooperative Ca^{2+} transport (nH = 2), sensitive to DMB, is clearly involved in this regulation (25, 26). The addition of CF-containing extracts to cultures causes an abrupt increase in the rate of $^{45}Ca^{2+}$ transport (26). A reduction of cooperativity (nH = 1) in strain Cp2200 can be correlated with resistance to autolysis and to growth inhibition by DMB and with a loss of natural competence (Com$^-$) (25). Interestingly, Cp2200 can be complemented by exogenous CF (Fig. 4A). This trait is similar to the phenotypes of *comA* and *comB* mutants (16). However, *DMB-1* is genetically independent (25) of the previously described *comA* and *comB* loci. Indeed, once stimulated, strain Cp2200 produces the activator in the same manner as the wild-type parent, suggesting that there is a defect in the Ca^{2+} triggering of CF production rather than in CF production per se. This might be due to the reduced cooperativity of Ca^{2+} transport as a function of its electrochemical gradient, which is a characteristic of mutant Cp2200.

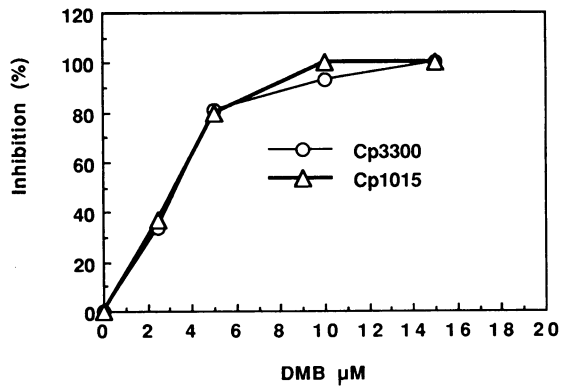

FIG. 2. DMB inhibition of the initial rate of $^{45}Ca^{2+}$ transport in Cp3300 compared with Cp1015. The transport of $^{45}Ca^{2+}$ (1.2 mM, 220 fmol/cpm) during 15-s incubations in medium with various concentrations of DMB was measured for the wild-type and mutant strains (see Materials and Methods). The 100% values were 29.7 ± 3 and 11 ± 2 nmol/min/mg of protein for strains Cp3300 and Cp1015, respectively. Measurements were performed as described in reference 26.

The results presented in Fig. 4B show that the mutation *DMB-2*, when introduced into a *comA* genetic background, increases the amplitude of the response to suboptimal CF concentrations (Fig. 4B). It is remarkable that this mutation on the one hand increases the cooperativity (nH = 4) of Ca^{2+} transport (Fig. 3) and on the other hand confers hypersensitivity to autolysis (see Results) as well as facilitated competence; natural competence levels at neutral pH in early-exponential-phase cultures (Table 1) and levels of competence development in 10 μM DMB-containing medium (Fig. 1B) were higher in the mutant than in the wild-type cells. Thus, the increased cooperativity of Ca^{2+} transport can be correlated to enhanced physiological Ca^{2+} responses. It is noteworthy that DMB at a concentration of 10 μM produces a plateau of inhibition for $^{45}Ca^{2+}$ transport, suggesting that the DMB-sensitive component is totally abolished. By contrast, competence induction as well as growth rate is hardly reduced in growth medium containing 10 μM DMB. A possibility might be that, in rapidly growing bacteria, the interplay of ionic traffic shifts the DMB sensitivity of Ca^{2+} transport, for example, by increasing the Na^+ gradient. Indeed, DMB-sensitive Na^+-Ca^{2+} exchange occurs in *S. pneumoniae* (26). Other aspects of cell physiology were not altered in the mutant strain, however. The mutant strain was dependent on the same levels of Ca^{2+} for growth (0.15 mM) and for competence induction (1 mM) as the wild-type parent (data not shown).

Taken together, these results demonstrate that the mutations which alter the cooperativity of Ca^{2+} transport also effect the Ca^{2+} response under the control of CF, competence induction, and autolysis activation.

It is well established that competence for DNA uptake in *B. subtilis* is the result of a complex network of gene regulation, mediated by phosphotransfer through sensors and regulators (6). Preliminary findings also suggest that gene regulation occurs during competence development in *S. pneumoniae* (15).

Calcium might have intracellular targets such as metabolic enzymes or kinases involved in signal transduction. In addition, a remarkable trait of Ca^{2+} transport is its electrogenic character, resulting in membrane depolarization (25). If it is true that the role of CF is to increase the rate of Ca^{2+} transport, membrane depolarization might be part of the competence induction network. For example, it could activate the F_1F_0 ATPase which hydrolyzes glycolytic ATP to generate $\Delta\mu H^+$. One consequence might be an increase in the cytoplasmic levels of phosphodonors required for the phosphorylation of gene regulators (11, 13).

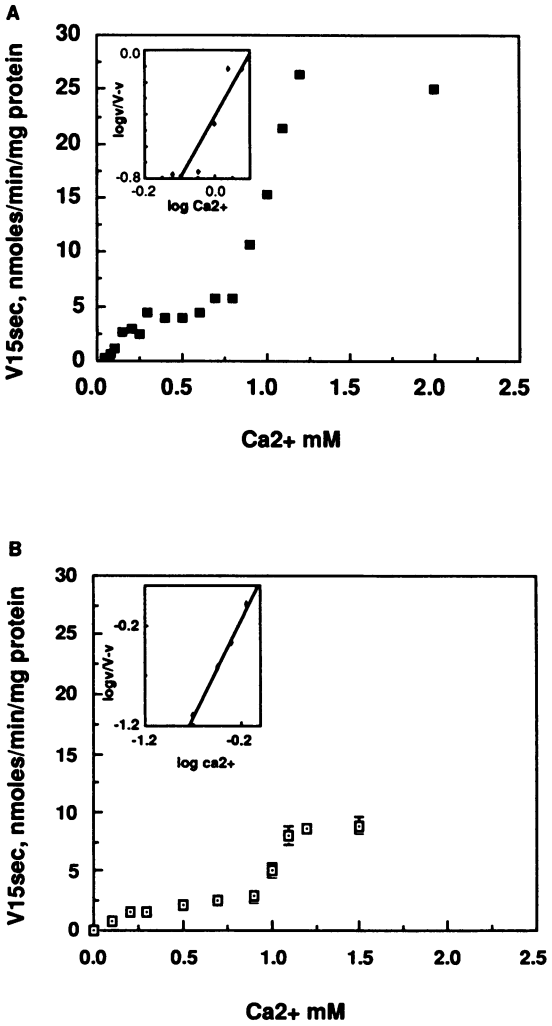

FIG. 3. Kinetics of $^{45}Ca^{2+}$ transport in Cp3300 (A) compared with that in Cp1015 (B). Initial rates (15-s transport) of DMB-sensitive $^{45}Ca^{2+}$ influx were measured as a function of increasing Ca^{2+} concentration. For each $^{45}Ca^{2+}$ concentration, the $V_{15\ s}$ was deduced graphically from kinetic measurements showing a linear response during the interval 0 to 60 s. It was measured as the difference between the values obtained in the absence and in the presence of DMB (10 μM) in the uptake medium (25). The insets represent the curve log $v/V - v = f(\log Ca^{2+})$, where V represents the maximum uptake rate and v is the uptake rate at 15 s ($V_{15\ s}$) for the given Ca^{2+} concentration. (A) Cp3300, nH = 4; (B) Cp1015, nH = 2. The vertical bars represent standard errors of the means (n = 3).

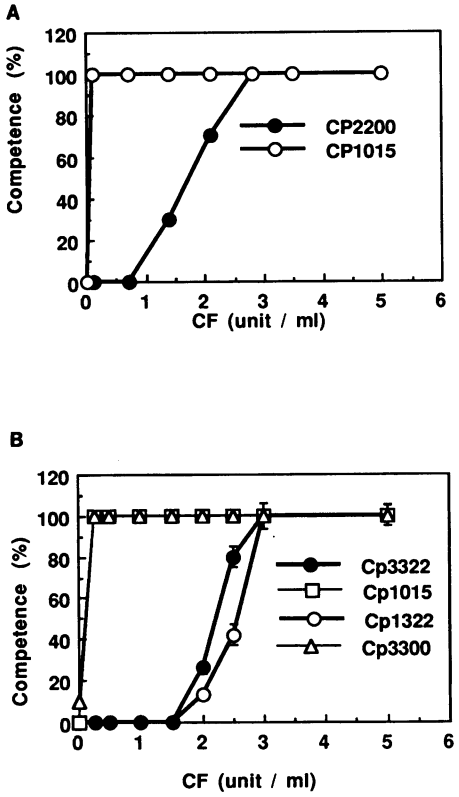

FIG. 4. *DMB-r* mutations interfere with CF regulation of competence. (A) Complementation of the *DMB-1* mutant, Cp2200, by CF extracts. Bacterial cultures from the wild-type strain Cp1015 and the mutant Cp2200 were incubated for 20 min at 37°C in media containing increasing amounts of CF-containing extracts. After addition of [^{3}H]DNA (1 μg, 60,000 dpm ml^{-1}), incubation was continued for 20 min, and then the quantity of acid-soluble radioactivity was determined by liquid scintillation counting. The 100% value corresponds to the plateau value. One CF unit induces competence of 1-ml cultures (2×10^8 CFU) of strain Cp1015 at pH 7 in 30 min (25). (B) *DMB-2* enhances the CF response of a Com$^-$ strain. The CF response of strain Cp3322, carrying both *com* and *DMB-2* mutations, was compared with the response of the *com* mutant Cp1322. Responses of the wild-type strain and of Cp3300, a mutant bearing the *DMB-2* mutation, are presented as references. Cultures from each strain, at 2×10^8 CFU/ml, were incubated for 20 min at 37°C in competence medium containing increasing amounts of CF. The level of competence induction was estimated as described in the legend to Fig. 1. The vertical bars represent standard errors of the means (n = 4).

ACKNOWLEDGMENTS

This work was supported by CNRS L.P.008201, Université Paul Sabatier, and the Beecham Foundation.

We thank David Barker for editing the manuscript.

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