ELSEVIER

Biochimica et Biophysica Acta 1448 (1998) 212–226

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Calcium signalling in *Bacillus subtilis*

Marie-Laure Herbaud [a], Annick Guiseppi [a], François Denizot [a], Jacques Haiech [b], Marie-Claude Kilhoffer [b,*]

[a] *Laboratoire de Chimie Bactérienne, UPR CNRS 9043, 31, Chemin Joseph Aiguier, BP 71, 13402 Marseille Cedex 20, France*

[b] *Laboratoire de Biophysique, URA CNRS 491, Université Louis Pasteur, Faculté de Pharmacie, BP 24, 67401 Illkirch Cedex, France*

Accepted 13 October 1998

Abstract

Few systematic studies have been devoted to investigating the role of Ca^{2+} as an intracellular messenger in prokaryotes. Here we report an investigation on the potential involvement of Ca^{2+} in signalling in *Bacillus subtilis*, a Gram-positive bacterium. Using aequorin, it is shown that *B. subtilis* cells tightly regulate intracellular Ca^{2+} levels. This homeostasis can be changed by an external stimulus such as hydrogen peroxide, pointing to a relationship between oxidative stress and Ca^{2+} signalling. Also, *B. subtilis* growth appears to be intimately linked to the presence of Ca^{2+}, as normal growth can be immediately restored by adding Ca^{2+} to an almost non-growing culture in EGTA containing Luria broth medium. Addition of Fe^{2+} or Mn^{2+} also restores growth, but with 5–6 h delay, whereas Mg^{2+} did not have any effect. In addition, the expression of alkyl hydroperoxide reductase C (AhpC), which is strongly enhanced in bacteria grown in the presence of EGTA, also appears to be regulated by Ca^{2+}. Finally, using $^{45}Ca^{2+}$ overlay on membrane electrotransferred two-dimensional gels of *B. subtilis*, four putative Ca^{2+} binding proteins were found, including AhpC. Our results provide strong evidence for a regulatory role for Ca^{2+} in bacterial cells.

Keywords: Calcium; Prokaryote; *Bacillus subtilis*; Oxidative stress; Aequorin; Alkyl hydroperoxide reductase C

1. Introduction

Calcium signalling is an intracellular communication system widely used by eukaryotic cells. During the past two decades, changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells have been found to be associated with a wide variety of cellular processes. At rest, eukaryotic cells maintain a very low $[Ca^{2+}]_i$, in the 10^{-7} M range (for review see [1,2]), despite the high Ca^{2+} concentration present in the extracellular environment or in intracellular Ca^{2+} stores. This calcium homeostasis generates a Ca^{2+} concentration gradient exploited by cells to transmit information. Indeed, $[Ca^{2+}]_i$ can be changed transiently in response to a stimulus, through a set of channels and pumps allowing Ca^{2+} fluxes across membranes. Transient changes in $[Ca^{2+}]_i$ are detected by intracellular Ca^{2+} sensors (intracellular CaBPs) able to bind Ca^{2+} under physiological conditions (i.e., in the presence of Mg^{2+} and KCl). Ca^{2+} inter-

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CaBP, calcium binding protein; EGTA, ethyleneglycolbis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; LIC, ligation independent cloning; MOPS, 3-(*N*-morpholino)propanesulfonic acid

* Corresponding author. Fax: +33 (3) 88674011; E-mail: mck@ecs.u-strasbg.fr

0167-4889/98/$ – see front matter
PII: S0167-4889(98)00145-1

action with these intracellular CaBPs then triggers the physiological event (for review see [3,4]). While Ca^{2+} signalling has been extensively studied in eukaryotes, only limited investigation has been made in bacteria. It has been suggested [1] that, for reasons similar to those prevailing in eukaryotes (insolubility of calcium phosphate), bacterial $[Ca^{2+}]_i$ has to be maintained at low levels. Using the Ca^{2+} probes fura-2 and aequorin, Ca^{2+} homeostasis has been reported in *Escherichia coli* [5–7]. $[Ca^{2+}]_i$ in unstarved cells was shown to be maintained at $\approx$90 nM [5,7], suggesting a finely tuned regulatory process. Active Ca^{2+} transport systems allowing Ca^{2+} extrusion from the cell have been described in *E. coli*, *Bacillus subtilis* and other bacteria (for review, see [8–10]) and consist of exchange systems such as Ca^{2+}/H^+ antiporters, Ca^{2+}/Na^+ antiporters or Ca^{2+}-ATPases. Also, bacterial cells may express Ca^{2+} channels, although information on this point is relatively scarce. The presence of intracellular CaBPs has been reported [8–11]. However, with the exception of *E. coli* where a clear relationship between $[Ca^{2+}]_i$ and chemotaxis has been established [7,12–14], little is still known about the physiological role of Ca^{2+} and its informational role in prokaryotes in general.

Here, we present an investigation on Ca^{2+} signalling in *B. subtilis*, a Gram-positive bacterium, the genome of which has recently been entirely sequenced [15]. Four main points were addressed and concern (1) Ca^{2+} homeostasis in *B. subtilis*, (2) the presence of putative CaBPs, (3) Ca^{2+} signalling per se, that is the ability of an external signal to modify the cell Ca^{2+} homeostasis, and finally (4) the effect of Ca^{2+} depletion on bacterial growth and protein expression.

2. Materials and methods

2.1. Materials

Oligonucleotides were purchased from Genosys (Genosys Biotechnologies, Cambridge, UK). Trypto casein soya broth (TS medium) was obtained from Diagnostics Pasteur and Goldstar DNA polymerase from Eurogentec.

2.2. Methods

2.2.1. Plasmid modification

Two plasmids were modified in order to allow ligation-independent cloning (LIC strategy [16]). pDG148 (kind gift from Dr P. Stragier, Institut Curie, France) and pMutin4 (kind gift from Drs V. Vagner, E. Dervyn and D. Ehrlich, INRA, Jouy-en-Josas, France) were modified respectively into pDG148-Stu (F. Denizot, manuscript in preparation) which was used as expression vector in BS168 and pMutin4-Fse (F. Denizot, manuscript in preparation) which was used for *ahpC* disruption and transcriptional fusion with the β-galactosidase gene. The modifications introduce a unique *Stu*I site in pDG148-Stu and a unique *Fse*I site in pMutin4-Fse. Before cloning, pDG148-Stu and pMutin4-Fse were cut with *Stu*I and *Fse*I, respectively and treated with T4 DNA polymerase in the presence of dATP, thus generating long single stranded overhangs compatible with ligation-independent cloning.

2.2.2. Apoaequorin system in Bacillus subtilis 168M (BS168M): construction and expression

Apoaequorin coding sequence was amplified by PCR using pSVAEQN plasmid (Molecular Probes) with the two oligonucleotides AQ440LICS and AQ440LICAS: 5′-AAGGAGGAAGCAGGTATG-GTCAAGCTTACATCAGACTTCGAC-3′ and 5′-GACACGCACGAGGTTTAGGGGACAGCTCCA-CCGTAG-3′, respectively, and the Goldstar DNA polymerase (Eurogentec). The PCR product was treated with T4 DNA polymerase in the presence of dTTP to generate the single-stranded overhangs compatible with the pDG148-Stu expression vector described above and then cloned into this vector by ligation-independent cloning. Annealing was performed using a molar ratio insert/vector of 2/1. The annealing product was used to transform *E. coli* DH5α competent cells (Gibco BRL). The resulting pDGAEQ vector was prepared from these *E. coli* cells using the high pure plasmid isolation kit (Boehringer). At this level, the apoaequorin primary sequence was checked by DNA sequencing. BS168 was then transformed with pDGAEQ using standard procedures [17]. pDGAEQ confers kanamycin resistance to BS168 cells. In the construct, the apoaequorin coding sequence is under the control of the *spac*

promoter and its expression can be induced with 1 mM IPTG.

2.2.3. Aequorin reconstitution and luminescence measurements

pDGAEQ transformed BS168 cells were grown in TS medium containing 20 μg/ml kanamycin, at 37°C with strong shaking. Exponentially growing cells (OD_{600} between 0.6 and 0.7) were induced by adding 1 mM IPTG. Incubation was continued for a further 1 h at 37°C. Bacteria were then harvested by low speed centrifugation, resuspended in TS medium (1/10 of the initial culture volume) containing 20 μg/ml kanamycin and 2.5 μM coelenterazine h (Molecular Probes) and incubated for 1 h at room temperature in darkness. After low speed centrifugation, loaded cells were washed once in buffer A (10 mM MOPS, 100 mM KCl, pH 7.2), containing where indicated 0.5 mM EGTA, and finally resuspended in an equal volume of buffer A (±EGTA). At this stage, cells were immediately used for luminescence measurements.

Chemoluminescence was measured using a Mediators PhL luminometer equipped with two dispenser modules and allowing reading from 96-well microplates. Typically, measurements were made on 100 μl aequorin-loaded cells resuspended in a given buffer. Reagents were injected and the kinetics of luminescence change vs. time were recorded. Total reconstituted aequorin was estimated at the end of each experiment by injecting 50 μl of 2% Nonidet, 100 mM $CaCl_2$.

2.2.4. ahpC disruption mutant

The erythromycin-resistant, chloramphenicol-sensitive, *ahpC* mutant SG82/AHPCKO2R was obtained by a double recombination event using pAHPCKO2R. This plasmid was constructed using the LIC strategy [16] by annealing four different partners: pMutin-Fse, a Cm^R-LIC cassette, and two PCR fragments (PCR1 and PCR2) comprising segments homologous to the *ahpC* region on the bacterial chromosome. The PCR fragments were amplified with two pairs of oligonucleotides: 5′-GCGTCTTCGCGGCCAGCCTAAATCCTACCTGTCACACC-3′, 5′-TCCGTGCCCTCCTGGAAGCAGAATACGCTCCATTGGC-3′ and 5′-GCTTGGCTGTGTCGGAGCGGCGAAACTCTTACACCTAGC-3′, 5′-CGCTGCGTCGGTTGACATCAACGAGAGCCAGCATGTCC-3′, respectively and the BS168 chromosome as template. PCR1 comprises a segment homologous to the region located around the *ahpC* initiation codon (150 bp upstream and 110 bp downstream) and PCR2 comprises a segment located around the stop codon of *ahpC* (45 bp upstream and 110 bp downstream from the stop codon). For annealing, pMutin4-Fse, cut with *Fse*I (see plasmid modification in this section) and the Cm^R-LIC cassette (F. Denizot, manuscript in preparation) were both treated with T4 DNA polymerase in the presence of dATP and the two purified PCR fragments (PCR1 and PCR2) with T4 DNA polymerase in the presence of dTTP. The 5′ overhangs generated at the extremities of the four partners allow them to circularize after annealing, the pMutin4-Fse being placed between PCR1 and PCR2. The annealing reaction was performed using a molar ratio PCR1/pMutin4-Fse/PCR2/cassette of 2/1/2/2. This assemblage was then used to transform *E. coli* DH5α. The resulting pAHPCKO2R was extracted using High Pure Plasmid Isolation Kit (Boehringer Mannheim). The *ahpC* disrupted mutant was obtained by integration of pAHPCKO2R into the genome of SG82, a *trpC2 lacA* mutant strain derived from *B. subtilis* 168 [18]. The correct integration was verified by PCR. As a consequence of this integration, the *lacZ* gene in SG82/AHPCKO2R is expressed under control of the *ahpCF* promoter, whereas the genes following *ahpC* in the *ahpCF* operon are expressed under the control of the *spac* promoter.

2.2.5. 2D gel electrophoresis

2.2.5.1. Sample preparation. Washed *B. subtilis* cells (equivalent to 40–50 μg of protein/sample) were resuspended in 90 μl lysis buffer (8 M urea, 4% CHAPS, 65 mM DTE, 1% Triton X-100) and sonicated on ice (4×20 s using a Branson Sonifier equipped with a cup horn). Following sonication, 0.4% (final concentration) BioLyte pH 3–10 (Bio-Rad) was added to the cell lysate. This sample was used for the 2D gel run.

2.2.5.2. 2D-PAGE. Isoelectric focussing (IEF) separation was performed at 20°C on a Multiphor II Electrophoresis unit (Pharmacia) using Immobi-

line DryStrips (IPG 4–7 linear, 180 mm long, Pharmacia). The protein sample was loaded on a rehydrated immobilized linear pH gradient (IPG) according to the method of Bjellqvist and coworkers [19,20]. After sample loading, the voltage was gradually increased from 100 V to 3900 V and then maintained at 3900 V until a total of 150 kVh was reached. Immobiline DryStrips were then equilibrated (17 min) in 50 mM Tris-HCl at pH 8.8, urea 6 M, SDS 5%, DTE 2% w/v and a trace of Bromophenol blue, followed by 5 min in 50 mM Tris-HCl at pH 8.8, urea 6 M, SDS 5%, iodoacetamide 2.5% w/v and a trace of Bromophenol blue. For the second dimension, vertical SDS-PAGE was performed on a Protean II xi Slab Cell (Bio-Rad) using a linear 12–20% polyacrylamide, 4–6% glycerol gradient, 0.1% SDS. Equilibrated strips were placed on top of the acrylamide gel and embedded in 0.5% agarose dissolved in electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Gels were run at 250 V and immediately processed for transfer onto membranes or fixed prior to silver staining with the Protein Silver Staining Kit (Pharmacia).

2.2.6. $^{45}Ca^{2+}$ overlay

For $^{45}Ca^{2+}$ overlay, *B. subtilis* proteins were first separated by 2D gel electrophoresis under the conditions described above except for a 10–15 times higher total protein load ($\approx$500 μg). The separated proteins were electrotransferred onto nitrocellulose membranes (Schleicher and Schuell, 0.2 μM) at 1.5 mA/cm^2 of membrane for 45 min at room temperature using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Transfer buffer contained 48 mM Tris, 39 mM glycine and 20% methanol. After blotting, the membrane was washed 3×20 min, at room temperature, in buffer B (10 mM imidazole (pH 6.8), 5 mM $MgCl_2$ and 60 mM KCl) and then incubated for 45 min in buffer B supplemented with 40% ethylene glycol and 10 μCi/ml Ca^{2+} (Amersham International). The calcium concentration in the solution was $\approx$15 μM (8–10 μM from the $^{45}Ca^{2+}$ solution plus endogenous calcium). The membrane was then rinsed for 5 min in MilliQ water (Millipore), dried between two sheets of Whatman 3MM Chr paper and then left at 37°C for 1 h. The dried membrane was finally exposed for 16 h to Kodak Biomax-MR film. After exposure, the membrane was stained with 0.1% Ponceau red in 10% acetic acid. The film was used for location of the putative calcium binding proteins on the membrane and on a 2D gel run under the same conditions as those used for electrotransfer and stained with 0.003% amidoblack in 45% methanol:10% acetic acid:45% H_2O.

2.2.7. Identification of putative calcium binding proteins

The most noticeable radioactive spots detected by overlay were matched to protein spots on 2D gels run under the same conditions as those used for overlay. Selected protein spots were then cut out of the gel, dried and submitted to internal peptide sequencing (Institut Pasteur, Laboratoire de Microséquençage des Protéines, Paris). The sequence of a given peptide 11–18 amino acids long was then used to identify the parent protein by running FASTA on the SubtiList database (http://www.pasteur.fr/Bio/SubtiList.html).

2.2.8. β-Galactosidase assay

β-Galactosidase activities were measured on the *ahpc* mutant using the method of Miller [21]. One unit of β-galactosidase is defined as the amount of enzyme which produces 1 nmole of *o*-nitrophenol per minute at 28°C.

2.2.9. Bacterial growth

Growth curves were obtained by inoculation of a given growth medium with a 1/100 dilution of an overnight culture. The culture was vigorously shaken and bacterial growth was monitored by the optical density at 600 nm (OD_{600}).

3. Results and discussion

3.1. Calcium homeostasis in B. subtilis

In order for *B. subtilis* to use Ca^{2+} as an intracellular messenger, the bacterium has to be able to maintain a differential Ca^{2+} gradient between, respectively, the cytosol and the environment and/or eventual intracellular Ca^{2+} pools. To investigate this point, we chose to use aequorin, a Ca^{2+}-regulated photoprotein, as a probe to monitor cytosolic free Ca^{2+}. Aequorin has been used successfully for

almost 30 years in very different types of eukaryotic cells [22–29], in prokaryotic cells [6,7], and even in subcellular organelles [30–33]. Its use has been greatly expanded by the availability of aequorin cDNA, which eliminates the need for microinjection and allows investigation of smaller cells. Recombinant apoaequorin can be reconstituted into the active photoprotein by simple addition of coelenterazine, the prosthetic group, into the medium.

B. subtilis was transformed with the expression vector pDGAEQ containing apoaequorin cDNA and aequorin was reconstituted within living cells by adding coelenterazine h to the external medium. When cells were resuspended in buffer A (10 mM MOPS, 100 mM KCl, pH 7.2), the measured luminescence was ≈100 RLU (relative light units). In the same buffer containing 0.5 mM EGTA, the recorded luminescence was ≈30 RLU. When Ca^{2+} was added to bacterial cells resuspended in this EGTA containing buffer, a transient increase in aequorin luminescence takes place within 10 s (Fig. 1). The maximum luminescence depends on the Ca^{2+} concentration added and reached 200-fold the resting level for 4.5 mM added free Ca^{2+}. Thereafter, two step decay kinetics (rapid and slow) were observed. In the rapid step, luminescence returns to a level of 100–400 RLU (depending on the added Ca^{2+} concentration) within 40 s and then within minutes slowly decreases to the resting level typical of cells in buffer A. Within the external Ca^{2+} concentrations tested (250 μM–4.5 mM) the resting levels observed are similar, pointing to a tight regulation of the cytosolic free Ca^{2+} concentration. The large spiking observed when Ca^{2+} was injected and the overall shape of the curves are similar to those observed with *E. coli* [6,7]. For *B. subtilis*, the exact value of $[Ca^{2+}]_i$ is difficult to compute as the cytosolic ion concentration and pH are not known with precision. Therefore, we have assumed that it is in the same range as in *E. coli* [5,7], probably around 90 nM. In *B. subtilis*, systems allowing Ca^{2+} translocation across the membrane have been reported. A membrane potential-dependent Ca^{2+} uptake system [34], sensitive to Ca^{2+} channel blockers such as nitrendipine, verapamil and ω-conotoxin [35,36] [37] has been described for Ca^{2+} entry. On the other hand, Ca^{2+} extrusion would be sustained by Ca^{2+}/H^+ [38] and/or Ca^{2+}/cation [34] antiporters. Thus the cell seems to be equipped to

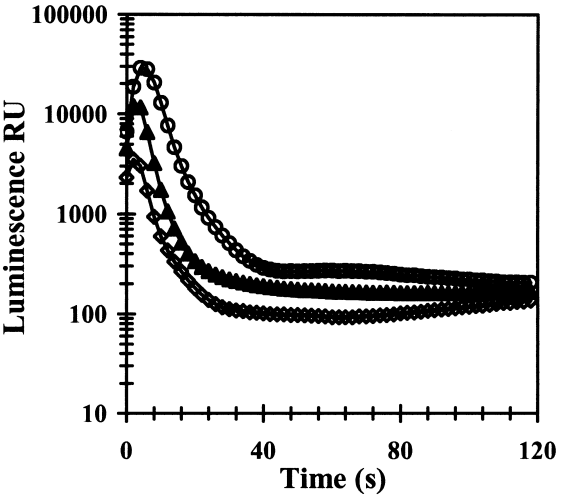

Fig. 1. Effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$ of *B. subtilis*. BS168 cells, expressing aequorin, were resuspended in buffer A containing 0.5 mM EGTA and 100 μl portions of the cell suspension were added to the wells of a 96-well microplate and placed in a luminometer. $CaCl_2$ in buffer A was injected to give 0.25 mM (◇), 0.5 mM (▲) or 4.5 mM (○) external free Ca^{2+} concentrations. Luminescence (expressed in relative units) was monitored for 120 s. At the end of the kinetics, aequorin was discharged with 2% Nonidet P40/100 mM $CaCl_2$ (data not shown). Experiments were repeated 3 times and similar results were obtained.

deal with Ca^{2+} movements. Although it has been suggested for a long time that all living cells maintain a low cytoplasmic calcium level in spite of the high concentration that prevails in the medium bathing the cells [1,39], there have been only very few experiments aimed to really investigate cytosolic Ca^{2+} in prokaryotes [5–7,12,14,40] and all the studies were performed on *E. coli*. The present study, which is the first on Ca^{2+} homeostasis in a Gram-positive bacterium, corroborates the generally accepted view that all living cells, even bacteria, maintain a low free cytosolic Ca^{2+} level.

3.2. *Ca^{2+}-binding proteins in B. subtilis*

If Ca^{2+} is to have an informational role in prokaryotic cells, what would be the molecular mechanism of Ca^{2+} regulation in these cells? Is the eukaryotic model based on the presence of 'EF-hand-type' intracellular Ca^{2+}-binding proteins valid? Several papers have reported on the presence of Ca^{2+}-binding proteins in various bacterial cells and on the identification of so-called calmodulin-like or calmodulin-

related proteins [41] (for review see [11]). In *B. subtilis*, Fry and coworkers [42,43] described the isolation, purification and characterization of a 23 000–25 000 MW calmodulin-like protein from sporulating bacteria, and Grazia-Tozzi and coworkers [44] a 38 000 MW Ca^{2+}-binding protein from exponentially growing bacteria.

In order to get further information on the presence of Ca^{2+}-binding proteins in *B. subtilis*, we used a more general Ca^{2+}-binding protein detection approach based on the ability of Ca^{2+}-binding proteins to bind $^{45}Ca^{2+}$ following SDS-PAGE and electrophoretic transfer to nitrocellulose or PVDF membranes ($^{45}Ca^{2+}$ overlay technique) [45]. *B. subtilis* proteins were therefore separated by 2D gel electrophoresis and then transferred onto nitrocellulose membranes. For maximum Ca^{2+}-binding specificity, incubation with $^{45}Ca^{2+}$ was performed in the presence of 60 mM KCl and 5 mM Mg^{2+}. Under these conditions, five major radioactive spots appeared on the autoradiogram. The spots were located on the 2D gel and the corresponding proteins identified by internal peptide microsequencing. As shown in Table 1, the putative Ca^{2+}-binding proteins appeared to be flagellin (spot 1), alkyl hydroperoxide reductase C (AhpC, spot 2), ribosomal protein B-L9 (spot 3) and the major cold shock protein (CspB, spots 4 and 5). CspB has been reported by Grauman and coworkers [46] to exist as a formylated as well as a deformylated protein and to migrate in two spots on 2D gel electrophoresis. We can assume that the same post-translational modification holds in our case. The same putative calcium-binding proteins could be identified when $^{45}Ca^{2+}$ overlay was performed on the heat stable fraction of *B. subtilis* proteins, suggesting that all these proteins are thermostable. As the sequence of all four proteins is known, we looked for the presence of EF-hand Ca^{2+}-binding motifs. However, none of these proteins appear to contain the canonical EF-hand Ca^{2+}-binding domains described previously [1,47–50] and present in calmodulin and the many others eukaryotic intracellular CaBPs involved in Ca^{2+} signalling in these cells. Furthermore, as the whole genome of *B. subtilis* is known [17], we performed a systematic search, but no such protein with such EF-hand domains could be detected in the whole genome. So far, calerythrin from *Saccharopolyspora erythraea* is the only prokaryotic protein reported to contain authentic 'EF-hand' domains [51,52]. The gene of the protein has been cloned, but there has been no further characterization.

Grazia-Tozzi and coworkers [44] isolated a 38 000 MW protein from exponentially growing *B. subtilis*, which showed heat stability and which bound Ca^{2+} with high selectivity over Mg^{2+} (as assessed by the $^{45}Ca^{2+}$ overlay technique under the same ionic conditions as we used, by electrophoretic mobility shifts and gel filtration). The protein has not been further characterized and its p*I* is not known. Nevertheless, one could speculate that their protein corresponds to flagellin as it shares some of its properties (thermostability, MW, Ca^{2+} binding in the presence of Mg^{2+}). Flagellin, the *hag* gene product in *B. subtilis*,

Table 1
Identification of the putative Ca^{2+}-binding proteins in *B. subtilis*

Spot No.	Peptide sequence	Protein	Apparent MW	Theo MW	Theo p*I*
1	EADGSIAALHSVCD	Flagellin	37 000	32 628	4.97
2	ITYAMIGDPSQTISRNFD	AhpC	23 000	20 626	4.31
3	VVREITGLGLK	B-L9	14 500	12 633	4.56
4 and 5[a]	TLEEGQAVSFEIVEGNR	CspB	11 500	7 365	4.31

B. subtilis proteins, after 2D gel electrophoresis, were transferred on a nitrocellulose membrane. The membranes were incubated with $^{45}Ca^{2+}$. Radioactive spots were revealed by autoradiography. The autoradiography was then used to locate the putative Ca^{2+}-binding proteins on a 2D gel run under the same conditions. Proteins corresponding to the major radioactive spots were cut out of the gel, dried and submitted to internal peptide sequencing. Apparent MW of the proteins were determined by comparing their electrophoretic mobility on SDS-PAGE gels vs. the mobility of standard proteins (see Fig. 2). Theo MW and Theo p*I* are the molecular weights and isoelectric points determined from the knowledge of the protein sequence (tools on: www.expasy.ch).

[a]Spots 4 and 5, with the same apparent MW but different p*I*, appeared to correspond to the same protein CspB. CspB has been reported to exist as a formylated as well as a deformylated protein [46] accounting for the two spots of different p*I*.

composes the flagellar filament, the two other parts of the flagellum being the basal body and the hook [53]. Flagellin monomers are added to the outer tip of the nascent flagellum to form the filament, suggesting that the protein has to be exported to assemble at the tip. Could flagellin be a target for Ca^{2+} regulated events? Ca^{2+} involvement in regulating chemotactic behaviour has been suggested in *B. subtilis* [36,54] and is well studied in *E. coli* [7,12–14,40]. In chemotaxis, bacteria show increased tumbling in the presence of repellents and an increased swimming in the presence of attractants. Tumbling is caused by clockwise rotation of flagella and swimming by counterclockwise rotation. The switch between tumbling and swimming has been proposed to be related to the cytosolic Ca^{2+} concentration. However, so far the mechanism of action of Ca^{2+} at the molecular level is unknown, although it has been suggested in *E. coli* [12] that the site of action of Ca^{2+} is not at the flagellar switch. In addition, considering its location and its structural role, flagellin does not seem to be a good candidate for such a regulation.

Alkyl hydroperoxide reductase C (AhpC) corresponds to the small subunit of alkyl hydroperoxide reductase (the other subunit being AhpF) and belongs to class IIIb of the sigmaB-independent general stress proteins in *B. subtilis* [55]. It is induced weakly by heat or salt stress, but strongly by oxidative stress. It is closely related to the TSA (thiol-specific antioxidant) family of eukaryotic antioxidant proteins [56,57]. No ion-binding properties have been reported so far for this protein, but its expression appears to be regulated by metal ions as will be discussed below. AhpC has been reported to regulate the expression of flagellin. Indeed, *ahpC* mutant cells overproduce flagellin [58], suggesting that under conditions where AhpC is overexpressed (such as oxidative stress conditions), flagellin synthesis would be decreased. Hartford and Dowds [59] isolated a hydrogen peroxide-resistant mutant of *B. subtilis* and showed, among other modifications, the overproduction of the two subunits of alkyl hydroperoxide reductase (AhpC and AhpF) and the repression of flagellin synthesis.

B-L9 (or L7-L12), the product of the *rplL* gene, is a ribosomal protein and has been identified by Grauman and coworkers [46] as one of the cold induced proteins (CIPs). The authors suggested that the protein may be 'essential for correct assembly of rRNA at low temperatures'.

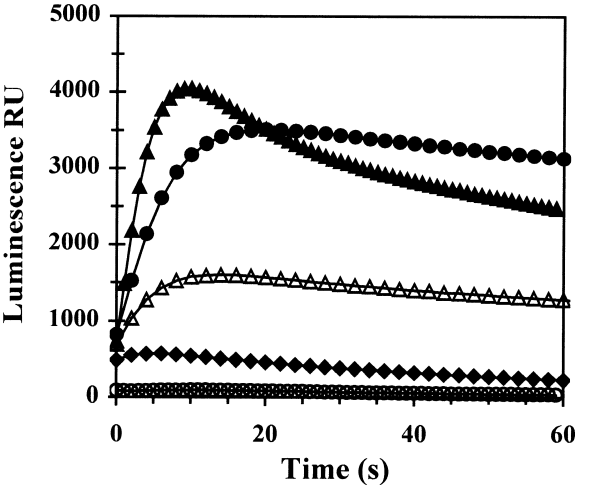

Fig. 2. Effect of oxidative stress on $[Ca^{2+}]_i$ of *B. subtilis*. BS168 cells, expressing aequorin, were resuspended in buffer A and 100 μl portions of the cell suspension were added to the wells of a 96-well microplate and placed in a luminometer. H_2O_2 in buffer A was injected at 0.17 mM (♦), 1.7 mM (△), 12.5 mM (●) or 37 mM (▲) final concentration. Aequorin luminescence (expressed in relative units) was recorded as a function of time. ○, control corresponding to the addition of buffer A. At the end of the kinetics, aequorin was discharged with 2% Nonidet P40/100 mM $CaCl_2$ (data not shown). Similar results were obtained when experiments were repeated.

CspB is the major cold-shock protein in *B. subtilis* and a member of a protein family widespread among prokaryotes and eukaryotes containing the highly conserved cold-shock domain (CSD) [60]. It has recently been suggested [61] that CspB but also CspC and CspD, which bind to RNA in a co-operative and interactive manner, may function as RNA chaperones facilitating the initiation of translation under optimal and low temperatures.

Overall, all the putative Ca^{2+}-binding proteins we identified here, appear to be related with adaptive responses of the bacterial cell. We therefore looked at the potential role of Ca^{2+} in the response to environmental changes by investigating the intracellular Ca^{2+} signalling under oxidative stress conditions.

3.3. Ca^{2+} signalling and oxidative stress

In their natural environment, all aerobic organisms must cope with reactive oxygen species (ROS) which

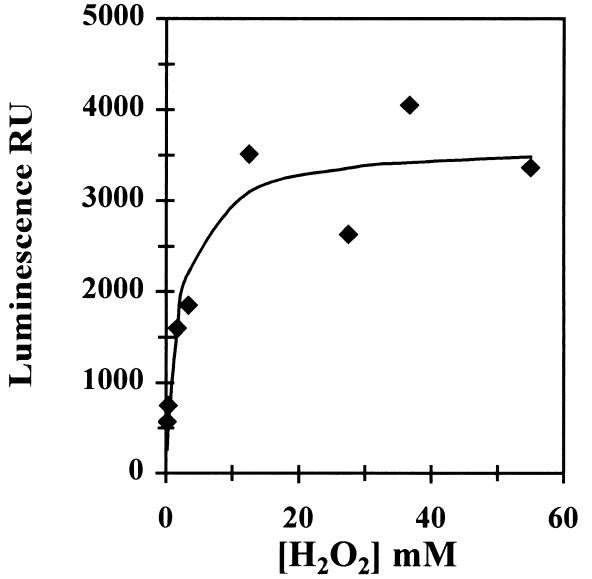

Fig. 3. H_2O_2 dose-response curve. Kinetics of luminescence changes were performed (as indicated in Fig. 2) at various $[H_2O_2]$. Luminescence taken at the maximum of the kinetic curve was plotted as a function of $[H_2O_2]$. Points correspond to the experimental data and were fitted to the theoretical curve: $L = (k[H_2O_2]/1+k[H_2O_2])L_{max}$, where L represents the theoretical luminescence at the maximum of the kinetic curve at a given $[H_2O_2]$ and L_{max} the total aequorin luminescence when Nonidet P40/$CaCl_2$ was added.

may induce various damages (lipid peroxidation, protein oxidation, DNA damage). Cells have therefore developed defense mechanisms that prevent or repair oxidative damage [62–64]. In eukaryotic cells, there have been numerous reports on Ca^{2+}, oxidative stress and cell death (for review, see [65–68]). 'Pathological' oxidative stress [69] is associated with a progressive increase in $[Ca^{2+}]_i$. At non-toxic doses, however, H_2O_2 has been shown to induce a rapid and transient rise in $[Ca^{2+}]_i$ [69–71] or even intracellular Ca^{2+} oscillations [72], similar to those observed in Ca^{2+} signalling in these cells.

In this study, we investigated the effect of H_2O_2 on cytosolic Ca^{2+}. *B. subtilis* was therefore transformed with the apoaequorin coding gene and aequorin reconstituted with coelenterazine h. In the absence of stimuli, aequorin luminescence was again at $\approx$100 RLU (Fig. 2). H_2O_2 addition caused an increase in aequorin luminescence followed by a slow and steady decrease (as observed when longer kinetics were performed). The signalling observed appeared different from the one observed in Fig. 1 where Ca^{2+} addition induced a rapid rise in $[Ca^{2+}]_i$, with $[Ca^{2+}]_i$ then falling to values close to the resting level within 40 s. However, a sustained increase in aequorin luminescence, and thus in $[Ca^{2+}]_i$, has also been observed when chemorepellents were tested on *E. coli* [7]. The effect of H_2O_2 on $[Ca^{2+}]_i$ appears to reach a plateau at $\approx$20 mM of H_2O_2 (Fig. 3). From these experiments, we cannot assess how deleterious the various $[H_2O_2]$ were to the bacterial cells. Nevertheless, during the observation times tested (kinetics were run for time scales ranging from 60 to 300 s), we never saw a steady increase in $[Ca^{2+}]_i$, as was seen for 'pathological' oxidative stresses in eukaryotic cells. Also, the H_2O_2-induced luminescence change does not arise from H_2O_2 rendering cells leaky as the total aequorin monitored by the addition of Nonidet/Ca remained similar to what was observed in the absence of H_2O_2.

In *B. subtilis*, the expression of several proteins is induced by H_2O_2 treatment, including the major vegetative catalase (KatA), alkyl hydroperoxide reductase (AhpC and AhpF), MrgA (a DNA-binding and protecting protein) and the haem biosynthesis enzymes [58,73–77]. These proteins form a peroxide regulon [78,79], repressed by PerR, a regulator en-

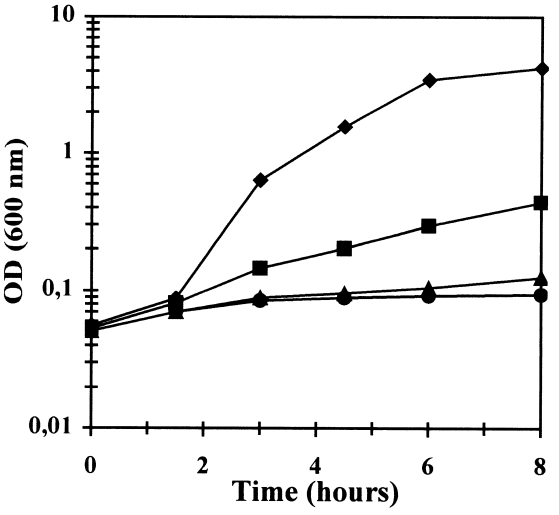

Fig. 4. Effect of EGTA on *B. subtilis* growth. LB medium containing 0 mM (♦), 1 mM (■), 5 mM (▲) or 10 mM (●) EGTA was inoculated (at a 1/100 dilution) with BS168 grown overnight in LB medium. Bacterial growth at 37°C was followed by measuring absorbance at 600 nm (OD_{600}) as a function of time. Similar curves were obtained when the experiment was repeated.

coded by *ygaG* [80]. The peroxide regulon undergoes metalloregulation [78–81]. Manganese and to a lesser extent iron, copper and cobalt appear to regulate the expression of the peroxide regulon proteins. We will show below that in addition to the metalloregulation reported previously, expression of *ahpC* is also modulated by Ca^{2+}. Would this be one more (direct or indirect) regulation for the peroxide regulon? Further studies are necessary to answer this question.

To further explore the role of Ca^{2+} in the physiology of the bacterium, we tested its potential role on bacterial growth, protein expression pattern and gene expression.

3.4. Effect of external Ca^{2+} depletion on bacterial growth

B. subtilis was grown in LB medium in the absence and in the presence of EGTA (Fig. 4). At 1 mM and 5 mM EGTA, bacterial growth is strongly slowed down and at 10 mM EGTA, OD_{600} hardly doubles after about 8 h of culture. When EGTA was added to a culture in LB medium at OD_{600} around 0.25, the growth slows down immediately and stops within 30 min (data not shown). As EGTA is not specific for Ca^{2+}, we tested the effect of Ca^{2+}, Fe^{2+}, Mn^{2+} or Mg^{2+} addition to *B. subtilis* grown in LB medium containing 5 mM EGTA. Ions were added at a total concentration of 6 mM (corresponding to $\approx$1 mM free cations). Fig. 5 shows that Ca^{2+} restores growth immediately, the growth curve being similar to the one observed in LB medium. For Fe^{2+} and Mn^{2+}, growth starts to increase slowly and takes off about 5–6 h after ion addition. The plateau corresponding to the stationary phase is the same for the three cultures (around $OD_{600} = 3$). Mg^{2+} does not show any effect at all. The following conclusions can be drawn from this study: (1) bacterial growth relies on the presence of divalent cations, (2) apparently they can replace each other (at least for the ones which have been studied here) although with different efficiencies, (3) Ca^{2+} seems to be important for bacterial growth and is the most efficient in restoring growth in EGTA-containing media, (4) bacterial growth depends on extracellular Ca^{2+} as EGTA slows down and even stops exponentially growing cultures. What factor is responsible for the difference in efficiencies between the tested ions? Manganese and iron have specific energy-dependent transport systems [82–84]. For calcium, a potential-dependent uniporter has been described [34]. Is there a difference in efficiencies between the transfer systems or does the difference lie in the switch controlling bacterial growth? Further studies will be necessary to answer these questions.

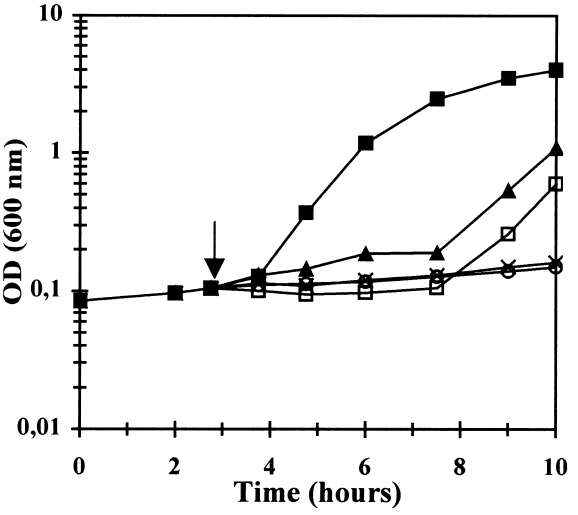

Fig. 5. Restoration of BS168 growth upon cation addition to cells in EGTA containing LB medium. An overnight culture of BS168, grown at 37°C in LB medium containing 5 mM EGTA, was divided into five subcultures. Where indicated by the arrow, Ca^{2+} (■), Fe^{2+} (▲), Mn^{2+} (□) or Mg^{2+} (○) was added at a total concentration of 6 mM (corresponding to free cation concentrations of $\approx$1 mM). × corresponds to the control where no addition was made to the subculture. Growth at 37°C was then followed by measuring the absorbance at 600 nm (OD_{600}) as a function of time. OD_{600} of the overnight culture is given at time 0. Results correspond to the mean of three experiments.

3.5. Effect of EGTA on protein expression pattern and ion regulation of ahpC expression

As EGTA had such a drastic effect on bacterial growth, which was overcome by Ca^{2+} addition, we wondered whether regulatory mechanisms exist in the bacterial cell where a (or several) Ca^{2+}-binding protein(s) could be overexpressed in response to Ca^{2+} depletion. In order to answer this question, 2D gel electrophoresis was performed on *B. subtilis* grown in LB medium in the absence and presence of 5 mM EGTA (Fig. 6). For the gels run on *B. subtilis* in the absence of EGTA, cells were taken during

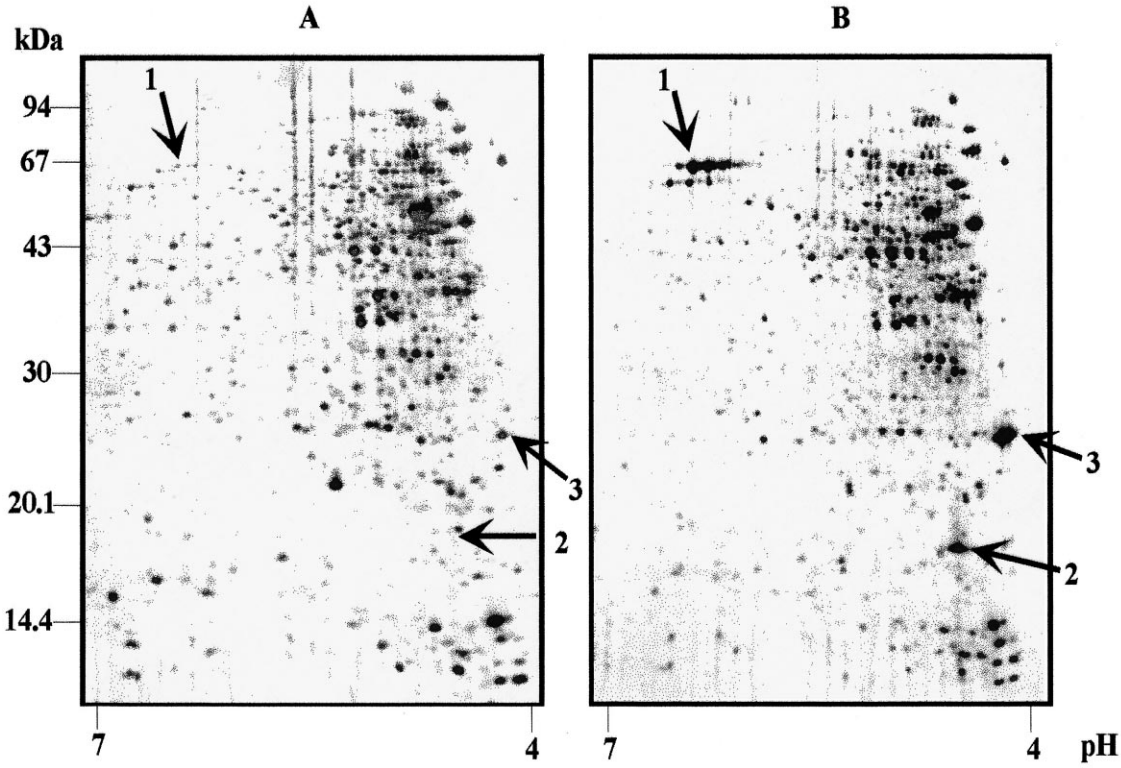

Fig. 6. Silver-stained 2D gels of *B. subtilis* cells grown in LB medium (A) or in LB medium containing 5 mM EGTA (B). 40–50 μg of total proteins were loaded onto 4–7 IPG strips. Molecular weights indicated correspond to migration on the second dimension of low molecular weight markers (Pharmacia). Arrows correspond to: (1) catalase, (2) MrgA, (3) AhpC, the three proteins which were highly overproduced in the presence of EGTA. The experiment was repeated at least 3 times and similar gels were obtained.

exponential growth at a culture density (OD_{600}) of 0.4–0.5. For cultures in the presence of EGTA, cells were taken at a culture density (OD_{600}) of 0.15, long before the cell culture reached the plateau. Comparison of the two gels reveals modifications in the expression level of many proteins, but the most noticeable are catalase (spot 1), MrgA (spot 2) and AhpC (spot 3), all three showing a strong increase in expression in EGTA-containing media. These proteins have been shown to be induced by oxidative stress (as discussed above) and by entry into the stationary phase under conditions of iron and manganese limitations [79]. Here it appears that ion depletion also induces these proteins. One could assume that EGTA addition induces in the bacteria a state similar to the stationary phase. Nevertheless, as was shown in Fig. 5, Ca^{2+} addition allows the bacteria to start growth instantaneously, without a lag phase.

Among the three proteins which were identified in this experiment, AhpC retained especially our attention. Indeed, this protein was shown to be a putative Ca^{2+} binding protein and its expression was strongly enhanced by the presence of EGTA. Previous results showed that the *ahpCF* operon (which codes for AhpC and AhpF, the two subunits of the alkyl hydroperoxide reductase) was regulated in the stationary phase by manganese and iron [79], with manganese being more potent than iron. Here, we reinvestigate AhpC expression, under experimental conditions different from those of Bsat and coworkers [79] and we looked for a potential Ca^{2+} regulation of *ahpC* expression. *ahpC* was therefore interrupted by the use of pMutin vector, which places the β-galactosidase gene under the control of the *ahpCF* promoter. As indicated in Section 2, SG82, the *B. subtilis* 168 strain interrupted for the endogenous β-galactosidase gene, was used for the construction. The SG82/AhpCKO2R mutant and *B. subtilis*

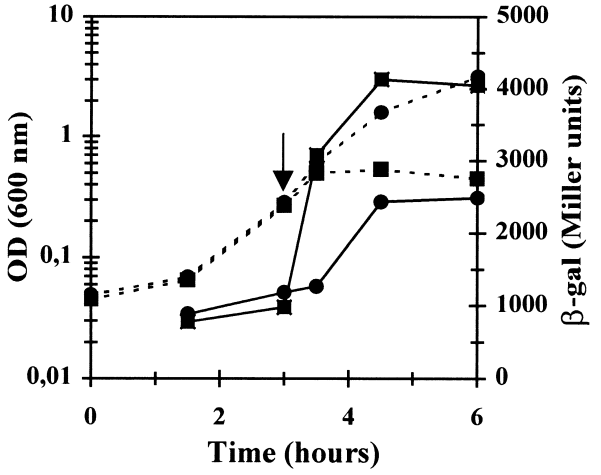

Fig. 7. *ahpC* expression as a function of growth phase and EGTA. Two cultures of SG82/AHPCKO2R were grown in LB medium. At the time given by the arrow (corresponding to exponentially growing cells), 5 mM EGTA was added to one of the cultures. OD_{600} (dotted lines) and β-galactosidase activities (full lines) were measured as a function of time. ● corresponds to the culture remaining in LB medium and ■ to the culture where EGTA was added. β-Galactosidase activities are expressed in Miller units. Values reported are the mean of three experiments.

168 had identical growth curves in LB medium. Fig. 7 shows the effect of EGTA addition on *ahpC* expression. Under normal growth conditions in LB medium, *ahpC* was slightly expressed in early-logarithmic phase growing cells, then expression was induced by a factor 3 in the stationary phase. This is comparable to the results obtained by Bsat and coworkers [79]. When EGTA was added to the culture in mid-logarithmic phase (3 h), bacterial growth immediately slows down and stops. Concomitant to EGTA addition, *ahpC* expression increases dramatically (5-fold) and stays well above the value reached in the stationary phase. To test the cation effect, SG82/AhpCKO2R was grown overnight in LB medium containing 5 mM EGTA. The culture density of this overnight culture was 0.2 and the β-galactosidase activity was 13 000 Miller units (time 0 in Fig. 8). When Ca^{2+}, Fe^{2+} or Mn^{2+} was added (time = 30 min), bacterial growth behaves as previously shown for wild type *B. subtilis* (i.e., immediate start of growth in the presence of Ca^{2+} and a delayed start for Fe^{2+} or Mn^{2+}). Concerning the β-galactosidase activity, all three ions induce a drop in activity. However, they differ in the delay before the drop occurs. For Ca^{2+}, the drop in the β-galactosidase activity is immediate and follows the starting of bacterial growth start. For Fe^{2+}, the drop also occurs soon after ion addition, but the drop is not correlated with the start of growth. For Mn^{2+}, its effect on β-galactosidase activity is delayed compared to its addition, but on the other hand, its effect on β-galac-

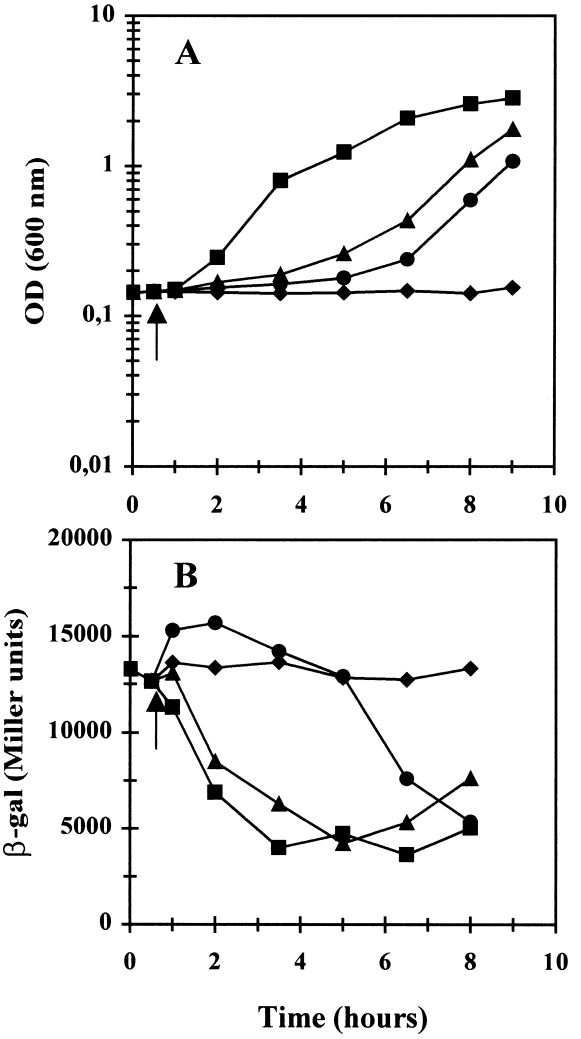

Fig. 8. Regulation of *ahpC* expression by divalent cations. An overnight culture of SG82/AHPCKO2R, grown at 37°C in LB medium containing 5 mM EGTA, was divided into four subcultures. At the time indicated by the arrow, Ca^{2+} (■), Fe^{2+} (▲), or Mn^{2+} (●) was added at a total concentration of 6 mM (corresponding to free cation concentrations of ≈1 mM). ♦ corresponds to the control with no addition to the subculture. OD_{600} (A) and β-galactosidase activities (B) were determined as a function of time. β-Galactosidase activities are expressed in Miller units. OD_{600} of the overnight culture is given at time 0. Values reported are the mean of three experiments.

tosidase activity is correlated with the start of growth. The present study clearly points to a complex ion regulation of *ahpC* expression. In addition to Mn^{2+} and Fe^{2+} regulation, Ca^{2+} regulation appears to play a major role. This raises the obvious question as to the relation between oxidative stress, cation depletion, AhpC expression and Ca^{2+} signalling. Also, is the expression of the other members of the peroxide regulon also modulated by Ca^{2+}?

3.6. Concluding remarks

The present study brings strong evidence for an involvement of Ca^{2+} in *B. subtilis* signal transduction. Taken together with results obtained on *E. coli*, a sum of evidence for an informational role of Ca^{2+} in bacterial cells starts to emerge. To extend the Ca^{2+} signalling paradigm to all living cells, one needs to get also information on Ca^{2+} regulation in Archaea, which have been reported to have a common ancestry with Eucarya [85]. So far, there is only one report on a calmodulin-like archaeal Ca^{2+}-binding protein [86].

The Ca^{2+}-binding proteins found in our study do not exhibit canonical EF-hand domains. In eukaryotes, the proteins of the EF-hand containing family play a major role in Ca^{2+} regulated processes. Nevertheless, besides this family, eukaryotes have other Ca^{2+}-binding proteins and namely those belonging to the annexin family. Bacteria may well have more ancient molecular mechanisms to handle Ca^{2+} signalling.

Finally, the results presented here clearly show a relationship between oxidative stress and Ca^{2+} signalling and thus the redox potential of the cell. This suggests coupling between Ca^{2+} and other components involved in the regulation of the redox potential of the cell, and namely other cations such as copper, iron or manganese. In eukaryotes proteins of the S100 family, which in addition to Ca^{2+} bind Zn^{2+} and Cu^{2+}, have been suggested to be involved in the protection against oxidative damage. In addition, in these proteins Ca^{2+} binding has been shown to be modulated by binding of other cations and namely Zn^{2+}. Therefore, we suggest that the bacterial Ca^{2+}-binding proteins bind also or even primarily cations such as copper, manganese, zinc or iron. Studies are underway to clarify this point.

Acknowledgements

We are indebted to J. Busuttil for skillful technical assistance in DNA sequencing and to K. Takeda for critical reading of the manuscript. We are grateful to V. Vagner and E. Dervyn for providing us with pMutin4 vector, to P. Stragier for pDG148 and J. Errington for SG82 strain. MCK wishes to acknowledge M. Chippaux for welcoming her in the Laboratoire de Chimie Bactérienne in Marseille. This research was supported by a grant from the Fondation pour la Recherche Médicale (to JH), from the Association pour la Recherche contre le Cancer (to JH), by the Programme de Recherche Fondamentale en Microbiologie et Maladies Infectieuses et Parasitaires and fundings from the Université Louis Pasteur and Centre National de la Recherche Scientifique.

References

[1] R.H. Kretsinger, The informational role of calcium in the cytosol, Adv. Cyclic Nucleotide Res. 11 (1979) 1–26.
[2] E. Carafoli, Intracellular calcium homeostasis, Annu. Rev. Biochem. 56 (1987) 395–433.
[3] L.J. Van Eldik, J.G. Zendegui, D.R. Marshak, D.M. Watterson, Calcium-binding proteins and the molecular basis of calcium action, Int. Rev. Cytol. 77 (1982) 1–61.
[4] P. Cohen, C.B. Klee, Calmodulin, Elsevier, Amsterdam, 1988.
[5] P. Gangola, B.P. Rosen, Maintenance of intracellular calcium in *Escherichia coli*, J. Biol. Chem. 262 (1987) 12570–12574.
[6] M.R. Knight, A.K. Campbell, S.M. Smith, A.J. Trewavas, Recombinant aequorin as a probe for cytosolic free Ca^{2+} in *Escherichia coli*, FEBS Lett. 282 (1991) 405–408.
[7] N.J. Watkins, M.R. Knight, A.J. Trewavas, A.K. Campbell, Free calcium transients in chemotactic and non-chemotactic strains of *Escherichia coli* determined by using recombinant aequorin, Biochem. J. 306 (1995) 865–869.
[8] R.J. Smith, Calcium and bacteria, Adv. Microb. Physiol. 37 (1995) 83–133.
[9] J. Youatt, Calcium and microorganisms, Crit. Rev. Microbiol. 19 (1993) 83–97.
[10] V. Norris, S. Grant, P. Freestone, J. Canvin, F.N. Sheikh, I. Toth, M. Trinei, K. Modha, R.I. Norman, Calcium signalling in bacteria, J. Bacteriol. 178 (1996) 3677–3682.
[11] L.A. Onek, R.J. Smith, Calmodulin and calcium mediated regulation in prokaryotes, J. Gen. Microbiol. 138 (1992) 1039–1049.
[12] L.S. Tisa, J. Adler, Calcium ions are involved in *Escherichia coli* chemotaxis [published erratum appears in Proc. Natl.

Acad. Sci. USA 1993 Mar 1;90(5):2097], Proc. Natl. Acad. Sci. USA 89 (1992) 11804–11808.
[13] L.S. Tisa, B.M. Olivera, J. Adler, Inhibition of *Escherichia coli* chemotaxis by omega-conotoxin, a calcium ion channel blocker, J. Bacteriol. 175 (1993) 1235–1238.
[14] L.S. Tisa, J. Adler, Chemotactic properties of *Escherichia coli* mutants having abnormal Ca^{2+} content, J. Bacteriol. 177 (1995) 7112–7118.
[15] F. Kunst, N. Ogasawara, I. Moszer, A.M. Albertini, G. Alloni, V. Azevedo, M.G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S.C. Brignell, S. Bron, S. Brouillet, C.V. Bruschi, B. Caldwell, V. Capuano, N.M. Carter, S.K. Choi, J.J. Codani, I.F. Connerton, A. Danchin et al., The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*, Nature 390 (1997) 249–256.
[16] C. Aslanidis, P.J. de Jong, Ligation-independent cloning of PCR products (LIC-PCR), Nucleic Acids. Res. 18 (1990) 6069–6074.
[17] F. Kunst, G. Rapoport, Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*, J. Bacteriol. 177 (1995) 2403–2407.
[18] R.A. Daniel, J. Haiech, F. Denizot, J. Errington, Isolation and characterization of the lacA gene encoding beta-galactosidase in *Bacillus subtilis* and a regulator gene, lacR, J. Bacteriol. 179 (1997) 5636–5638.
[19] B. Bjellqvist, J.C. Sanchez, C. Pasquali, F. Ravier, N. Paquet, S. Frutiger, G.J. Hughes, D. Hochstrasser, Micropreparative two-dimensional electrophoresis allowing the separation of samples containing milligram amounts of proteins, Electrophoresis 14 (1993) 1375–1378.
[20] B. Bjellqvist, C. Pasquali, F. Ravier, J.C. Sanchez, D. Hochstrasser, A nonlinear wide-range immobilized pH gradient for two-dimensional electrophoresis and its definition in a relevant pH scale, Electrophoresis 14 (1993) 1357–1365.
[21] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972, pp. 352–355.
[22] J.R. Blinks, W.G. Wier, P. Hess, F.G. Prendergast, Measurement of Ca^{2+} concentrations in living cells, Prog. Biophys. Mol. Biol. 40 (1982) 1–114.
[23] J.R. Blinks, Use of photoproteins as intracellular calcium indicators, Environ. Health Perspect. 84 (1990) 75–81.
[24] M. Brini, R. Marsault, C. Bastianutto, J. Alvarez, T. Pozzan, R. Rizzuto, Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$). A critical evaluation, J. Biol. Chem. 270 (1995) 9896–9903.
[25] A.K. Campbell, A. Patel, W.A. Houston, N.J. Scolding, S. Frith, B.P. Morgan, D.A. Compston, Photoproteins as indicators of intracellular free Ca^{2+}, J. Biolumin. Chemilumin. 4 (1989) 463–474.
[26] J. Nakajima-Shimada, H. Iida, F.I. Tsuji, Y. Anraku, Monitoring of intracellular calcium in *Saccharomyces cerevisiae* with an apoaequorin cDNA expression system, Proc. Natl. Acad. Sci. USA 88 (1991) 6878–6882.
[27] J. Nakajima-Shimada, H. Iida, F.I. Tsuji, Y. Anraku, Galactose-dependent expression of the recombinant $Ca^{2(+)}$-binding photoprotein aequorin in yeast, Biochem. Biophys. Res. Commun. 174 (1991) 115–122.
[28] Y. Yoshimoto, Y. Hiramoto, Observation of intracellular Ca^{2+} with aequorin luminescence, Int. Rev. Cytol. 129 (1991) 45–73.
[29] O. Shimomura, Preparation and handling of aequorin solutions for the measurement of cellular Ca^{2+}, Cell Calcium 12 (1991) 635–643.
[30] M. Brini, R. Marsault, C. Bastianutto, T. Pozzan, R. Rizzuto, Nuclear targeting of aequorin. A new approach for measuring nuclear Ca^{2+} concentration in intact cells, Cell Calcium 16 (1994) 259–268.
[31] M. Brini, F. De Giorgi, M. Murgia, R. Marsault, M.L. Massimino, M. Cantini, R. Rizzuto, T. Pozzan, Subcellular analysis of Ca^{2+} homeostasis in primary cultures of skeletal muscle myotubes, Mol. Biol. Cell 8 (1997) 129–143.
[32] J.M. Kendall, G. Sala-Newby, V. Ghalaut, R.L. Dormer, A.K. Campbell, Engineering aequorin to measure Ca^{2+} in defined compartments of living cells, Biochem. Soc. Trans. 20 (1992) 144S.
[33] J.M. Kendall, M.N. Badminton, R.L. Dormer, A.K. Campbell, Changes in free calcium in the endoplasmic reticulum of living cells detected using targeted aequorin, Anal. Biochem. 221 (1994) 173–181.
[34] W. de Vrij, R. Bulthuis, E. Postma, W.N. Konings, Calcium transport in membrane vesicles of *Bacillus subtilis*, J. Bacteriol. 164 (1985) 1294–1300.
[35] I. Kusaka, T. Matsushita, Characterization of a Ca^{2+} uniporter from *Bacillus subtilis* by partial purification and reconstitution into phospholipid vesicles, J. Gen. Microbiol. 133 (1987) 1337–1342.
[36] T. Matsushita, H. Hirata, I. Kusaka, Calcium channel blockers inhibit bacterial chemotaxis, FEBS Lett. 236 (1988) 437–440.
[37] H.H.T. Matsushita, I. Kusaka, Calcium channels in bacteria, Ann. NY Acad. Sci. 560 (1989) 426–429.
[38] T. Matsushita, T. Ueda, I. Kusaka, Purification and characterization of Ca^{2+}/H^{+} antiporter from *Bacillus subtilis*, Eur. J. Biochem. 156 (1986) 95–100.
[39] S. Simon, in: E.D. Weinberg (Ed.), Microorganisms and Minerals, Marcel Dekker, New York, 1977.
[40] L.S. Tisa, J. Adler, Cytoplasmic free-Ca^{2+} level rises with repellents and falls with attractants in *Escherichia coli* chemotaxis, Proc. Natl. Acad. Sci. USA 92 (1995) 10777–10781.
[41] D. Laoudj, C.L. Andersen, A. Bras, M. Goldberg, A. Jacq, I.B. Holland, EGTA induces the synthesis in *Escherichia coli* of three proteins that cross-react with calmodulin antibodies, Mol. Microbiol. 13 (1994) 445–457.
[42] I.J. Fry, L. Villa, G.D. Kuehn, J.H. Hageman, Calmodulin-like protein from *Bacillus subtilis*, Biochem. Biophys. Res. Commun. 134 (1986) 212–217.
[43] I.J. Fry, M. Becker-Hapak, J.H. Hageman, Purification and properties of an intracellular calmodulinlike protein from *Bacillus subtilis* cells, J. Bacteriol. 173 (1991) 2506–2513.
[44] M. Grazia Tozzi, U. D'Arcangelo, A. Del Corso, G.W. Or-

dal, Identification and purification of a calcium-binding protein from *Bacillus subtilis*, Biochim. Biophys. Acta 1080 (1991) 160–164.
[45] K. Maruyama, T. Mikawa, S. Ebashi, Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis, J. Biochem. 95 (1984) 511–519.
[46] P. Graumann, K. Schroder, R. Schmid, M.A. Marahiel, Cold shock stress-induced proteins in *Bacillus subtilis*, J. Bacteriol. 178 (1996) 4611–4619.
[47] R.H. Kretsinger, EF-hands embrace, Nat. Struct. Biol. 4 (1997) 514–516.
[48] H. Kawasaki, R.H. Kretsinger, Calcium-binding proteins 1: EF-hands, Protein Profile 2 (1995) 297–490.
[49] H. Kawasaki, R.H. Kretsinger, Calcium-binding proteins. 1: EF-hands, Protein Profile 1 (1994) 343–517.
[50] S. Nakayama, R.H. Kretsinger, Evolution of the EF-hand family of proteins, Annu. Rev. Biophys. Biomol. Struct. 23 (1994) 473–507.
[51] D.G. Swan, R.S. Hale, N. Dhillon, P.F. Leadlay, A bacterial calcium-binding protein homologous to calmodulin, Nature 329 (1987) 84–85.
[52] D.G. Swan, J. Cortes, R.S. Hale, P.F. Leadlay, Cloning, characterization, and heterologous expression of the *Saccharopolyspora erythraea* (*Streptomyces erythraeus*) gene encoding an EF-hand calcium-binding protein, J. Bacteriol. 171 (1989) 5614–5619.
[53] M. Silverman, M.I. Simon, Bacterial flagella, Annu. Rev. Microbiol. 31 (1977) 397–419.
[54] G.W. Ordal, Calcium ion regulates chemotactic behaviour in bacteria, Nature 270 (1977) 66–67.
[55] J. Bernhardt, U. Volker, A. Volker, H. Antelmann, R. Schmid, H. Mach, M. Hecker, Specific and general stress proteins in *Bacillus subtilis* – a two-dimensional protein electrophoresis study, Microbiology 143 (1997) 999–1017.
[56] H.Z. Chae, K. Robison, L.B. Poole, G. Church, G. Storz, S.G. Rhee, Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes, Proc. Natl. Acad. Sci. USA 91 (1994) 7017–7021.
[57] H.Z. Chae, S.G. Rhee, A thiol-specific antioxidant and sequence homology to various proteins of unknown function, Biofactors 4 (1994) 177–180.
[58] H. Antelmann, S. Engelmann, R. Schmid, M. Hecker, General and oxidative stress responses in *Bacillus subtilis*: cloning, expression, and mutation of the alkyl hydroperoxide reductase operon, J. Bacteriol. 178 (1996) 6571–6578.
[59] O.M. Hartford, B.C. Dowds, Isolation and characterization of a hydrogen peroxide resistant mutant of *Bacillus subtilis*, Microbiology 140 (1994) 297–304.
[60] K. Schroder, P. Graumann, A. Schnuchel, T.A. Holak, M.A. Marahiel, Mutational analysis of the putative nucleic acid-binding surface of the cold-shock domain, CspB, revealed an essential role of aromatic and basic residues in binding of single-stranded DNA containing the Y-box motif, Mol. Microbiol. 16 (1995) 699–708.
[61] P. Graumann, T.M. Wendrich, M.H. Weber, K. Schroder, M.A. Marahiel, A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures, Mol. Microbiol. 25 (1997) 741–756.
[62] B. Demple, Regulation of bacterial oxidative stress genes, Annu. Rev. Genet. 25 (1991) 315–337.
[63] B. Demple, C.F. Amabile-Cuevas, Redox redux: the control of oxidative stress responses, Cell 67 (1991) 837–839.
[64] S.J. Elliott, S.K. Koliwad, Oxidant stress and endothelial membrane transport, Free Radic. Biol. Med. 19 (1995) 649–658.
[65] P. Nicotera, G. Bellomo, S. Orrenius, The role of Ca^{2+} in cell killing, Chem. Res. Toxicol. 3 (1990) 484–494.
[66] D.J. Reed, Review of the current status of calcium and thiols in cellular injury, Chem. Res. Toxicol. 3 (1990) 495–502.
[67] J.L. Farber, The role of calcium in lethal cell injury, Chem. Res. Toxicol. 3 (1990) 503–508.
[68] A.W. Harman, M.J. Maxwell, An evaluation of the role of calcium in cell injury, Annu. Rev. Pharmacol. Toxicol. 35 (1995) 129–144.
[69] T. Volk, M. Hensel, W.J. Kox, Transient Ca^{2+} changes in endothelial cells induced by low doses of reactive oxygen species: role of hydrogen peroxide, Mol. Cell. Biochem. 171 (1997) 11–21.
[70] Z.W. Lee, S.M. Kweon, B.C. Kim, S.H. Leem, I. Shin, J.H. Kim, K.S. Ha, Phosphatidic acid-induced elevation of intracellular Ca^{2+} is mediated by RhoA and H_2O_2 in rat-2 fibroblasts, J. Biol. Chem. 273 (1998) 12710–12715.
[71] C.R. Hoyal, J. Giron-Calle, H.J. Forman, The alveolar macrophage as a model of calcium signaling in oxidative stress, J. Toxicol. Environ. Health B Crit. Rev. 1 (1998) 117–134.
[72] Q. Hu, S. Corda, J.L. Zweier, M.C. Capogrossi, R.C. Ziegelstein, Hydrogen peroxide induces intracellular calcium oscillations in human aortic endothelial cells, Circulation 97 (1998) 268–275.
[73] D.K. Bol, R.E. Yasbin, Characterization of an inducible oxidative stress system in *Bacillus subtilis*, J. Bacteriol. 172 (1990) 3503–3506.
[74] D.K. Bol, R.E. Yasbin, Analysis of the dual regulatory mechanisms controlling expression of the vegetative catalase gene of *Bacillus subtilis*, J. Bacteriol. 176 (1994) 6744–6748.
[75] O.M. Hartford, B.C. Dowds, Cloning and characterization of genes induced by hydrogen peroxide in *Bacillus subtilis*, J. Gen. Microbiol. 138 (1992) 2061–2068.
[76] P.C. Loewen, J. Switala, Multiple catalases in *Bacillus subtilis*, J. Bacteriol. 169 (1987) 3601–3607.
[77] L. Chen, J.D. Helmann, *Bacillus subtilis* MrgA is a Dps(PexB) homologue: evidence for metalloregulation of an oxidative-stress gene, Mol. Microbiol. 18 (1995) 295–300.
[78] L. Chen, L. Keramati, J.D. Helmann, Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions, Proc. Natl. Acad. Sci. USA 92 (1995) 8190–8194.

[79] N. Bsat, L. Chen, J.D. Helmann, Mutation of the *Bacillus subtilis* alkyl hydroperoxide reductase (ahpCF) operon reveals compensatory interactions among hydrogen peroxide stress genes, J. Bacteriol. 178 (1996) 6579–6586.
[80] N. Bsat, A. Herbig, L. Casillas-Martinez, P. Setlow, J.D. Helmann, *Bacillus subtilis* contains multiple Fur homologues: identification of iron uptake (Fur) and peroxide regulon (PerR) repressors, Mol. Microbiol. 29 (1998) 189–198.
[81] L. Chen, L.P. James, J.D. Helmann, Metalloregulation in *Bacillus subtilis*: isolation and characterization of two genes differentially repressed by metal ions, J. Bacteriol. 175 (1993) 5428–5437.
[82] E.D. Weinberg, Microorganisms and Minerals, vol. 3, Marcel Dekker, New York, 1977.
[83] M.L. Guerinot, Microbial iron transport, Annu. Rev. Microbiol. 48 (1994) 743–772.
[84] J.B. Neilands, Siderophores: structure and function of microbial iron transport compounds, J. Biol. Chem. 270 (1995) 26723–26726.
[85] C.R. Woese, O. Kandler, M.L. Wheelis, Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya, Proc. Natl. Acad. Sci. USA 87 (1990) 4576–4579.
[86] T. Rotharmel, G. Wagner, Isolation and characterization of a calmodulin-like protein from *Halobacterium salinarium*, J. Bacteriol. 177 (1995) 864–866.