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The functions of Ca^{2+} in bacteria: a role for EF-hand proteins?

Jan Michiels, Chuanwu Xi, Jan Verhaert and Jos Vanderleyden

In bacteria, Ca^{2+} is implicated in a wide variety of cellular processes, including the cell cycle and cell division. Dedicated influx and efflux systems tightly control the low cytoplasmic Ca^{2+} levels in prokaryotes. Additionally, the growing number of proteins containing various Ca^{2+}-binding motifs supports the importance of Ca^{2+}, which controls various protein functions by affecting protein stability, enzymatic activity or signal transduction. The existence of calmodulin-like proteins (containing EF-hand motifs) in bacteria is a long-standing hypothesis. Analysis of the prokaryotic protein sequences available in the databases has revealed the presence of several calmodulin-like proteins containing two or more authentic EF-hand motifs, suggesting that calmodulin-like proteins could be involved in Ca^{2+} regulation in bacteria.

EF-hand proteins are ubiquitous in eukaryotes and fulfil important regulatory or buffering roles. Calmodulin, a prototypical EF-hand protein, is a small (15–22 kDa), acidic (pI 3.9–4.3) Ca^{2+}-binding protein that can interact with >25 distinct target proteins, thereby regulating the activity of many vital enzymes, including kinases, phosphatases, nitric oxide synthases, phosphodiesterases and ion channels [1]. Calmodulin is composed of four EF-hands organized in two pairs linked by a flexible central tether (Fig. 1a). The EF-hand motif was first discovered in the crystal structure of parvalbumin [2] and comprises two nearly perpendicular α-helices (named after helices E and F in parvalbumin) separated by a 12-residue loop [3]. In general, EF-hand Ca^{2+}-binding proteins are recognized by homology in their Ca^{2+}-binding loops. Residues 1, 3, 5, 7, 9 and 12 of the loop provide the ligands for complexing Ca^{2+} ions and form the basis of a pentagonal bipyramidal coordination geometry (Fig. 2). Residues 1, 3 and 5 provide monodendate ligands, and residue 12 a bidendate ligand, via side-chain carboxylates. Residue 7 coordinates Ca^{2+} via a main-chain oxygen and residue 9 coordinates Ca^{2+} indirectly via a water molecule. The identity of the amino acids in the loop has a profound effect on the Ca^{2+}-binding characteristics of the protein [4]. Pairs rather than individual EF-hands constitute the

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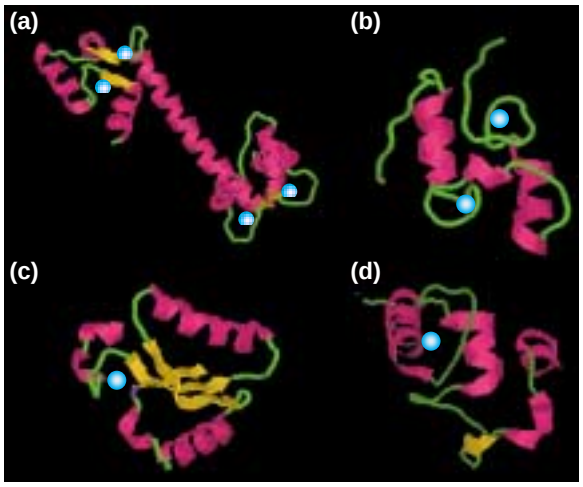

Fig. 1. Crystal structure of EF-hand Ca^{2+}-binding proteins. (a) Ca^{2+}–calmodulin complex. The carboxy-terminal (top) and amino-terminal (bottom) lobes are linked by the central helix. The central helix and the helices flanking the Ca^{2+}-binding loops are in pink, and the β-sheets in yellow. (b) Type I dockerin domain from the *Clostridium thermocellum* cellulosome. (c) Structure of the periplasmic glucose and galactose receptor from *Salmonella typhimurium* (amino acids 126–209). Glu205, occupying a position equivalent to that of the residue at coordinating position 12 of the classical EF-hand, is shown in purple. (d) Structure of the *Escherichia coli* lytic transglycosylase Slt35 (amino acids 201–264). The Ca^{2+}-ions are represented by blue spheres. The ribbon drawing was constructed using the RasMol V2.7.1.1 programme based on the coordinates in the Brookhaven Protein Data Bank (PDB) entry (a) 1CLL, (b) 1DAQ, (c) 1GCA and (d) 1QDR.

```
          ---Loop-----
-Helix E--        --Helix F--
         X Y Z-Y-X -Z
         1 3 5 7 9 12
EnXXnnXXnDXDIDDGLDXXDLXXnnXXn
           NLENPIE  EI
           SVNQ VN   V
            FSG MQ   M
            YTH CS   F
            WGR  T   Y
              K  A   W
                 G
                 C
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Fig. 2. Consensus sequence of the canonical EF-hand. The positions of the E and F helices as well as the intervening loop are given above the consensus amino acid sequence. The PROSITE EF-hand signature corresponds to the 12 amino acids of the loop and the fourth amino acid of helix F. The side chains of the coordinating residues marked X, Y, Z, –X and –Z provide oxygen atoms for the coordination of Ca^{2+}. The residue at position –Y bonds with a carbonyl oxygen; the residue at –Z provides two oxygens from its carboxylate group. Ca^{2+} is coordinated in a pentagonal bipyramid. Amino acids shown in red are not tolerated at that position. X represents any amino acid, n is a hydrophobic amino acid often found in the inside of the helices.

functional units of EF-hand proteins. Pairing of neighbouring EF-hands – yielding a globular structure – is proposed to be responsible for the cooperativity of Ca^{2+} binding and to increase the affinity of the protein for this ion [5]. The EF-hands in each lobe of calmodulin are linked by a short antiparallel β-sheet (residues 7–9) and form a compact structure. The majority of EF-hand proteins contain between two and eight helix-loop-helix structures that are organized in paired domains to facilitate contact between them.

EF-hand proteins in bacteria

Calerythrin and calsymin

The presence of calmodulin-like proteins in several prokaryotic species was suggested previously, based on functional and immunological evidence [6]. Although these results could not be corroborated by protein sequence data in most of the systems analyzed, recent sequence data tend to confirm the initial suggestion that EF-hand proteins are present in bacteria. The first prokaryotic protein shown to contain authentic EF-hand motifs was the 20-kDa protein calerythrin from the Gram-positive bacterium *Saccharopolyspora erythrea* [7,8]. Four EF-hand domains, of which three are typical and one is atypical, were reported (Table 1; Fig. 3). In agreement with the primary structure of the protein, binding experiments using Cd^{2+} ions revealed that calerythrin contains three high-affinity metal-binding sites, two of which display strong cooperativity [9]. As in calmodulin, calerythrin has a high phenylalanine:tyrosine ratio (13:1). A group of invertebrate sarcoplasmic proteins and aequorins were shown to have the highest similarity with calerythrin, indicating that this protein might function as a Ca^{2+} buffer or transporter rather than having a regulatory role [10–12].

More recently, we identified the EF-hand protein calsymin in the Gram-negative bacterium *Rhizobium etli* [13]. Calsymin has a molecular mass of 30 kDa, is highly acidic (pI 3.6) and contains six EF-hand motifs organized in pairs in three structurally similar domains, A, B and C (Fig. 3; Table 1). Calsymin has been shown to bind Ca^{2+}, and plays a key role during the complex lifestyle of *R. etli*. *R. etli* induces the formation of nodules on the roots of *Phaseolus vulgaris*; within these structures the bacteria are present inside plant cells, surrounded by a plant-derived membrane. Inactivation of the *casA* gene encoding calsymin strongly disrupts the intracellular stage of the bacterial lifecycle, thus decreasing nitrogen fixation activity. As calsymin is secreted by *R. etli*, it has been suggested that it could function as a transducer of 'information' between the bacteria and the plant [13].

Other EF-hand proteins in prokaryotes

To identify additional EF-hand proteins in prokaryotes, a motif search of several databases (Swissprot 39, PIR 68, GenPept 123 and TrEMBL 17) using the prosite EF-hand sequence PS00018 was carried out using the GCG (or 'Wisconsin') package; the analysis was further refined using the Pfam EF-hand motif PF00036. Many bacterial proteins containing the EF-hand motif were identified. Among these, 16 contain at least

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Table 1. Sequences of the Ca^{2+}-binding loops in bacterial EF-hand proteins

Protein name[a]	EF-hand[b]	Sequence motif[c]											
					1	3	5	7	9	12			
Calsymin	1	83	DVSQL	D	T	D	G	D	G	Y	V	S	KAEF VAARP
	2	126	LFDSF	D	S	E	G	T	G	S	L	S	VDAL AEAMS
	3	161	MLSDL	D	T	D	G	D	G	L	I	S	KAEF VAGRP
	4	193	LFDSF	D	S	E	G	A	G	S	L	S	VDVL TEAMS
	5	240	LLSDL	D	T	N	G	D	G	L	V	S	LEEF MAGKP
	6	273	LFALL	D	S	S	G	A	G	S	L	S	VQSA AS
Calerythrin	1	13	RFDRW	D	F	D	G	N	G	A	L	E	RADF EKEAQ
	2	64	LAKEA	G	V	G	S	D	G	S	L	T	EEQF IRVTE
	3	108	TWGMC	D	K	N	A	D	G	Q	I	N	ADEF AAWLT
	4	142	AFNQV	D	T	N	G	N	G	E	L	S	LDEL LTAVR
SC6F11.09	1	13	RFTTF	D	Q	D	G	N	G	H	I	D	RSDF SGAAK
	2	64	LAGIA	D	R	D	G	D	Q	R	I	T	REEF VTGAV
	3	107	ALGVA	D	T	D	G	D	G	A	V	T	VADT ARALT
	4	141	AAAAL	D	T	D	G	D	G	K	V	G	ETEI VPAFA
SCJ33.05c	1	13	MFNAF	D	V	N	G	D	G	C	L	E	EEDF AALAS
	2	64	LSQTV	D	T	D	N	D	G	R	I	D	MDDL LAMVD
	3	102	VFDAV	D	E	N	G	D	G	R	I	S	RNEH QRLID
	4	136	VFDRL	D	Q	D	A	D	G	H	L	S	RSEF AALWI
CC1180	1	100	WFGGA	D	A	N	H	D	G	A	L	T	RDEF VADAL
	2	124	FFATL	D	T	D	R	S	G	V	I	D	AFEV SAYEN
	3	201	PVMAS	D	G	D	F	D	R	R	I	T	RDEA TKAAK
	4	225	RFALL	D	T	D	K	D	G	Q	L	R	LAEL PRTPA
SC10F4.20	1	14	VFSLF	D	A	D	G	N	G	Y	L	E	QADF DLMSD
	2	63	LRTEL	D	A	N	G	D	G	R	I	S	PEEF TSVVL
	3	102	LAAMG	D	P	E	G	D	G	L	V	A	RSEF IALML
CabA	1	13	GFDRW	D	F	D	G	N	G	A	L	D	RADF EEERP
	2	111	KWGLC	D	K	N	T	D	G	E	I	N	ADEF ATVLT
	3	145	AFNQV	D	T	D	G	N	G	E	L	S	IDEA ALTAV
Asp24	1	67	QLKAA	D	T	N	G	D	G	V	L	S	RDEI EALAL
	2	100	MERRL	D	V	N	G	D	G	K	V	T	LDEI QNQRK
	3	124	EFAAL	D	R	N	D	D	G	K	L	D	RHEM RAAKM
MLL5457	1	73	AIMAA	D	T	D	G	D	G	K	I	S	LLEW EAFQA
	2	106	SFARI	D	S	N	H	D	G	F	I	D	RAEL DAFFA
	3	130	RFAKL	D	K	N	G	D	G	V	L	T	ADEM PSHKS
CC2752	1	40	MFLRL	D	T	N	K	D	G	K	I	T	GEEL KVRKG
	2	81	ELARM	D	A	D	K	D	R	V	I	D	RAEL DAFFA
	3	195	RFKRR	D	V	N	N	D	G	V	L	S	QEEL RAKGD
CC2226	1	37	VFKRW	D	A	N	A	D	G	A	V	D	KAEW TGAGR
	2	63	RFAMI	D	G	N	K	D	G	K	I	T	LDEL KTAFE
SC3D11.21	1	10	QFERI	D	T	D	G	D	G	L	I	T	AAEF KTALA
	2	47	IIAGR	D	L	D	G	D	K	Q	L	S	FDEF WAHLN
TAL_SYNY3	1	337	IFHAY	D	L	D	G	D	G	F	I	T	REEW AGTDV
	2	360	VFDAL	D	R	D	H	D	G	K	I	T	AAEM SAGLG
CC2193	1	299	FIKEQ	D	Q	N	G	D	G	F	V	T	KDEY AATRA
	2	323	QFAKT	D	T	D	N	S	G	A	L	S	QAEY VAEFK
MLR9645	1	133	MFAIM	D	A	N	G	D	G	A	L	S	QNEV QDLVG
	2	156	RIFNA	D	N	N	G	D	G	N	I	D	MEEI QTFFH
AE0048271	1	83	SFART	D	T	D	H	D	G	K	V	S	RAEF LAVAK
	2	110	EFDSI	D	S	D	H	D	G	I	S	E	AEAY EHLRK

[a]Proteins are from *Rhizobium etli* (calsymin); *Saccharopolyspora erythrea* (calerythrin); *Streptomyces coelicor* (SC6F11.09, SCJ33.05c, SC10F4.20 and SC3D11.21); *Streptomyces ambofaciens*, (CabA); *Brucella abortus* (Asp24); *Caulobacter crescentus* (CC1180, CC2752, CC2226 and CC2193); *Mesorhizobium loti* (MLL5457 and MLR9645); *Synechocystis* (TAL_SYN3); and *Pseudomonas aeruginosa* (AE0048271).

[b]Numbering starts from the amino-terminal end of the protein.

[c]Amino acids corresponding to the consensus and conserved in >65% or >45% of the loops are coloured red and dark blue, respectively. The D–E and S–T combinations were considered conservative substitutions. Numbers shown above the loops indicate the amino acids that provide the ligands to complex Ca^{2+}. Phenylalanine residues bordering the loop sequences are shown in green. The sequence [S/T/D/E]XX[D/E] corresponds to an N capping box sequence [52]. A second structural motif might flank the N capping box, stabilizing the α-helix through hydrophobic interactions [52]. The residues forming this 'hydrophobic staple' motif are shown in light blue.

two PF00036 motifs (Fig. 3; Table 1). In these proteins, the Ca^{2+}-binding loops are flanked by α-helices [14]. Interestingly, short β-turns encompass two to four residues (amino acids 2 to 5) at the beginning of each of the different loops, similar to eukaryotic calmodulins. Apart from calerythrin and calsymin, most of the identified proteins are hypothetical or of unknown function. One exception is the *Brucella abortus* Asp24 protein. The acid shock protein Asp24 is suggested to play a role during *B. abortus* infection of its host. *B. abortus* is the causative agent of brucellosis in cattle. Intracellular survival and growth of the bacteria inside phagocytic cells is thought to require adaptation to acidic conditions. Synthesis of Asp24 is increased either when *Brucella* is incubated under acidic conditions or following phagocytosis, suggesting a role for this protein in resistance to the acidic environment [15].

Most of the identified multi-EF-hand proteins are small (70–184 amino acids), have a high phenylalanine:tyrosine ratio and are either acidic (pI 4.2–5.0; calerythrin, SC6F11.09, SCJ33.05c, SC10F4.20, CabA, CC2226, SC3D11.21, MLR9645 and AE0048271), as described for calmodulin, or basic (pI 9.6–10.3; Asp24, MLL5457 and CC2752). The latter three proteins are similar at the amino acid level and might form a separate group. Regarding the biological function of these proteins, it is yet to be determined whether, analogous to eukaryotic EF-hand proteins [16], they have a role in buffering or transport of Ca^{2+} as suggested for *S. erythrea* calerythrin [10], or whether they act as regulators. Two proteins have an additional domain, identified as Pfam transaldolase and defensin propeptide domains (Fig. 3).

A second group of EF-hand proteins comprises several extracellular-polysaccharide-degrading enzymes from *Clostridium, Ruminococcus* and *Bacteroides* species. These proteins contain one or two EF-hand motifs, which are usually found at the carboxyl terminus (Fig. 1b; Fig. 3). In contrast to the typical helix-loop-helix structure, the first α-helix, preceding the Ca^{2+}-binding loop, is generally absent from these proteins. The two EF-hands are part of a larger ~70-residue dockerin domain that mediates the binding of the glycosyl hydrolases to a scaffoldin protein. The complex formed is a large extracellular cellulase that degrades cellulosic substrates [17]. Ca^{2+} binding induces folding of the dockerin domain, increasing its stability.

A third group of bacterial EF-hand proteins encompasses the periplasmic D-galactose-binding proteins, which are involved in active transport of galactose and glucose, and chemotaxis towards these sugars (Fig. 1c; Fig. 3). These proteins are characterized by the presence of an EF-hand structure in which the first five ligands for the coordination of Ca^{2+} are provided by a loop of nine residues (positions 157–165) and the remaining two ligands by a distantly located glutamate

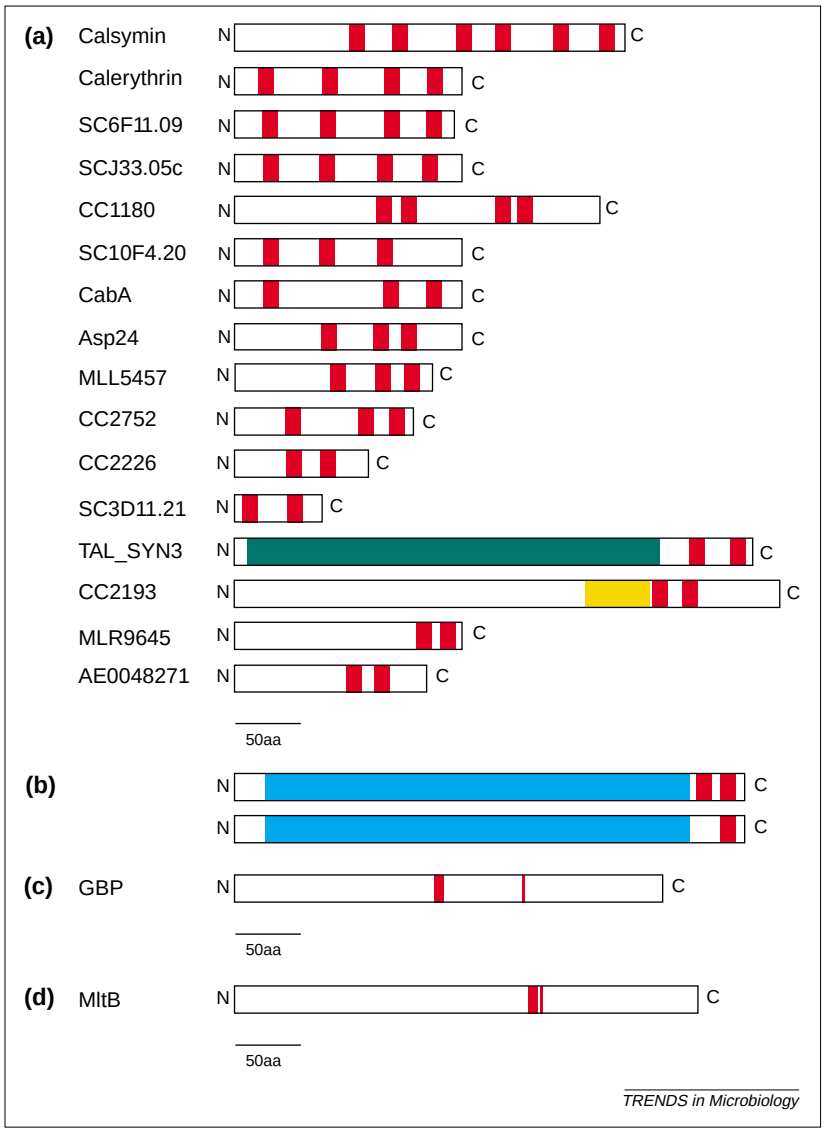

Fig. 3. Classes of EF-hand proteins in bacteria. (a) Representation of multi EF-hand proteins in Gram-negative bacteria. The identified proteins can be subdivided into four subgroups on the basis of the number of EF-hands: six (*Rhizobium etli* calsymin); four (*Saccharopolyspora erythrea* calerythrin, *Streptomyces coelicor* SC6F11.09 and SCJ33.05c, and *Caulobacter crescentus* CC1180); three (*S. coelicor* SC10F4.20, *Streptomyces ambofaciens* CabA, *Brucella abortus* Asp24, *Mesorhizobium loti* MLL5457 and *C. crescentus* CC2752); or two helix-loop-helix motifs (*C. crescentus* CC2226 and CC2193, *S. coelicor* SC3D11.21, *Synechocystis* TAL_SYN3, *M. loti* MLR9645 and *Pseudomonas aeruginosa* AE0048271). The positions of the loops in the different proteins are shown in red, the Pfam transaldolase domain in green and the Pfam defensin propeptide domain in yellow. (b) General representation (not to scale) of extracellular-polysaccharide-degrading enzymes from Gram-positive bacteria containing one or two carboxy-terminal EF-hand motifs (shown in red). The latter proteins often carry a second EF-hand-like motif, which is not recognized by the PS0018 motif. Both EF-hand motifs are part of a larger, ~70-residue dockerin domain. Proteins belonging to this group can possess different hydrolytic activities (shown in blue). (c) Schematic representation of the periplasmic glucose and galactose-binding protein (GBP) from *Salmonella typhimurium* (P23905). Amino acids 157–165 constitute the first part of the Ca^{2+}-binding region, residues 227–228 the second part (in red). (d) Representation of the membrane-bound lytic transglycosylase B (MltB) from *Escherichia coli* (P41052). Ca^{2+} is coordinated by residues 237–250 and by the carboxylate group of residue 256, occupying position 15 in the EF-hand motif (shown in red).

residue (Glu228). The position of Glu228 is equivalent to that of residue 12 in a typical EF-hand loop (Fig. 2). Although the helices are not directly linked to the loop structure, structural analysis indicates that Ca^{2+} is coordinated in an almost pentagonal bipyramid, similar to genuine EF-hands [18].

The determination of the crystal structure of Slt35, a proteolytic fragment of the membrane-bound lytic transglycosylase B (MltB) from *Escherichia coli*, revealed the presence of an EF-hand-like Ca^{2+}-binding site [19]. This fold is atypical as the loop structure is composed of 15 residues instead of 12 and it coordinates a Ca^{2+} ion with only six ligands in a distorted octahedral geometry (Fig. 1d; Fig. 3). Similar loop structures can also be found in several other MltB transglycosylases such as those from *Yersinia pestis*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Sinorhizobium meliloti*, *Mesorhizobium loti* and *Rhodobacter sphaeroides*. Similarly, members of the S100 subfamily of eukaryotic EF-hand Ca^{2+}-binding proteins are characterized by the presence of an amino-terminal binding loop comprising 14 residues instead of 12, and a chelation pattern that differs from that of prototypical EF-hands [20]. It has been shown that the binding of Ca^{2+} ions to this motif in the *E. coli* transglycosylase increases the stability of the protein [19].

Screening the 31 complete bacterial genome sequences with the prosite PS00018 consensus sequence revealed the presence of matching patterns in the majority of the species analyzed (Table 2; published as online supplementary information at http://archive.bmn.com/supp/tim/michiels.pdf). Species lacking proteins with the EF-hand signature are not clustered and are found within families and even genera that include species containing EF-hand proteins. Although the functionality of these sequences remains to be confirmed by experimental analysis, the proteins retrieved fulfil important roles in processes such as ion transport (K^+-transporting ATPase b chain and Na^+:solute symporter); DNA modification (DNA gyrase subunit A, DNA/pantothenate metabolism flavoprotein, polyA polymerase, putative polynucleotide polymerase, DNA polymerase III α subunit and prophage P4 integrase); protein metabolism (signal recognition particle and protease); the stress response (heat shock protein HtpG, acid shock proteins and DnaK); and chemotaxis (CheA and MCP). For example, the *Caulobacter crescentus* DnaK protein, which is involved in the heat shock response, was identified in this analysis. A similar observation was reported earlier for other DnaK proteins [21]. In agreement with this, it was demonstrated that autophosphorylation of *E. coli* DnaK is stimulated by Ca^{2+}. The CheA protein of *P. aeruginosa* was also retrieved. The involvement of Ca^{2+} in chemotaxis has been documented in several bacteria including *Bacillus subtilis* and *E. coli* [22]. In the latter species, the Ca^{2+} effect is mediated by the chemotaxis CheA, CheW and CheY proteins [23]. Finally, approximately 30% of the hits are hypothetical proteins. Also, many proteins that contained only one EF-hand motif were retrieved, but are not detailed further here.

Ca^{2+}-binding motifs in bacteria are diverse

Several Gram-negative species secrete proteins containing a haemolysin-type Ca^{2+}-binding domain that is organized in a parallel β-roll structure. Proteins containing this motif belong to the repeats in toxin (RTX) family, are extracellular, exported by type I secretion systems and are often determinants of pathogenesis or symbiosis. Examples include haemolysins of *E. coli*, *Proteus vulgaris*, *Morganella morganii* and *Moraxella bovis*; the nodulation protein NodO from *Rhizobium leguminosarum* and *Sinorhizobium* sp. BR816; cyclolysin from *Bordetella pertussis*; leukotoxins from *Pasteurella haemolytica* and *Actinobacillus* spp.; and zinc proteases from *Erwinia chrysanthemi* and *P. aeruginosa*. For example, in the alkaline protease of *P. aeruginosa*, the carboxyl terminus consists of a parallel β-roll structure containing multiple repeats of the GGXGXDXVX Ca^{2+}-binding motif [24] (where X is any residue but preferably small hydrophilic residues at positions 3 and 5, and U is a large hydrophobic residue). The first six residues bind Ca^{2+} and the last three residues form a β-strand. Each of the Ca^{2+} ions is bound in a six coordinate, almost octahedral site. The proteins belonging to this family have variable numbers of tandem repeats of the consensus nine residue sequence, with seven in *P. aeruginosa* alkaline protease, 12 in NodO [25], ~15 in *E. coli* haemolysin (HlyA) and ~40 in the *B. pertussis* cyclolysin precursor. Ca^{2+}-binding to *E. coli* haemolysin is an absolute requirement for cytotoxicity. The Ca^{2+}-binding regions have been suggested to function as a receptor for binding of *E. coli* haemolysin to erythrocytes or to assist the proper folding of the proteins after translocation across the membrane.

Besides these known motifs, proteins can also bind Ca^{2+} through oxygen atoms provided by several charged glutamate or aspartate residues. The *E. coli* ChaA Na^{+}/Ca^{2+} antiporter possesses such an acidic motif (EHEDDSDDDD), which is also found in the eukaryotic Ca^{+}-binding proteins calsequestrin and calreticulin [26]. However, often these motifs are less well defined and not easy to specify and, because of the absence of structural data for many proteins, are difficult to predict. For example, in the monomeric form of the *E. coli* outer membrane protein phospholipase A, Ca^{2+} is bound between loops L3 and L4 with two distantly located aspartate side chain carboxylates (Asp149 and Asp184) as ligands. In addition, only after dimerization, a second Ca^{2+}-binding site is formed at the dimer interface in the active site [27]. Also, Ca^{2+} coordination in the *P. aeruginosa* cytochrome *c* peroxidase occurs even in the absence of any negatively charged residues (an amide oxygen, main-chain carbonyls and water molecules) [28]. In this protein, the function of Ca^{2+} is to maintain the structural integrity of the protein and/or to modulate the transfer of electrons between the protein's two haem-containing domains.

Ca^{2+} homeostasis

Ca^{2+} homeostasis allows eukaryotic cells to maintain a low cytoplasmic concentration (~10–100 nM) of Ca^{2+}. Signalling is achieved by varying the cytoplasmic levels of Ca^{2+}. The concentration can increase to several micromolar and is generated either through Ca^{2+} release from intracellular stores or transport across the plasma membrane. In both cases, specific channels or pumps are required to increase Ca^{2+} concentrations locally and to reduce them again to the resting cellular level (for example, see [29]). Ca^{2+} homeostasis has also been demonstrated in several bacteria, including *E. coli* [30,31] and *B. subtilis* [32].

The analysis of fluctuations of intracellular Ca^{2+} in *E. coli* pointed to the presence of specific influx and efflux systems for Ca^{2+}, which can assure tight control of free Ca^{2+} and provide a low resting concentration of this ion (for example see [31]). This concentration is maintained at the submicromolar level (estimated at ~90 nM in *E. coli* [30]), a condition required for Ca^{2+} to be used as a cellular signal and 1000 times less than the concentrations normally found outside the cell. In addition, fluctuations of intracellular Ca^{2+} in *E. coli* can be observed [31]. Several bacteria have been shown to possess both primary ATP-dependent Ca^{2+} pumps and secondary Ca^{2+} transporters to remove Ca^{2+} from the cell [22,33]. The latter systems are antiporters using proton or sodium electrochemical gradients. The ChaA Ca^{2+}/H^{+} antiporter was described in *E. coli* [26] and homologues of this protein can be found in *Mycobacterium tuberculosis* (ChaA), *Mycobacterium leprae* (GenBank Acc. No: gi 11356650), *Yersinia pestis* (ChaA), *Streptomyces coelicolor* (gi 7480269), *Mesorhizobium loti* (gi 13475771), *Rhizobium* sp. NGR234 (Y4hA), *Agrobacterium tumefaciens* (gi 15157341), *Sinorhizobium meliloti* (gi 15073459) and *Rhizobium leguminosarum* (CpaA). Also, proteins similar to mammalian Na^{+}/Ca^{2+} exchangers are present in *E. coli* (gi 1176841), *Vibrio cholerae* (gi 11354719), *Pasteurella multocida* (gi 12720681), *Synechocystis* sp. (gi 7470257), *Bacillus halodurans* (gi 10173078), *Aquifex aeolicus* (gi 7514428), *Thermotoga maritima* (gi 7462140), *Borrelia burgdorferi* (gi 2688049), *Aquifex aeolicus* (gi 2982830) and *Treponema pallidum* (gi 3323364). P-type Ca^{2+}-ATPases were identified in *Myroides odoratus* [34], streptococci [35] and several cyanobacteria [36]. These systems transport Ca^{2+} across the membrane at the expense of ATP. In addition to these transporters, non-proteinaceous complexes between the lipidic polymer poly-3-hydroxybutyrate and inorganic polyphosphate located in the plasma membrane have been demonstrated to act as voltage-gated Ca^{2+} channels in *E. coli* [37].

Finally, it must be stressed that, although in bacteria there is no sequestration of Ca^{2+} in intracellular stores such as the eukaryotic endoplasmic reticulum, local Ca^{2+} gradients might

exist. The cell membranes and cell wall are particularly rich in Ca^{2+} and specific proteins or phospholipids might help to present this ion to the cytoplasm. For example, the poly-3-hydroxybutyrate and polyphosphate complexes in the cytoplasmic membrane can accumulate Ca^{2+} ions and could transfer them to the cytoplasm.

Roles of Ca^{2+} in bacteria

Biochemical and physiological data confirm that Ca^{2+} is involved in a variety of bacterial cellular processes, including the cell cycle and cell division [38], competence [39], pathogenesis [40], motility and chemotaxis [23,41] (reviewed in [6,22,42,43]). A well-documented example is the chemotactic behaviour of *B. subtilis* and *E. coli* cells. In *E. coli*, repellents cause a rise in intracellular Ca^{2+} and provoke tumbling of the bacteria, whereas in the presence of attractants, Ca^{2+} levels are reduced and bacteria adopt a pattern of smooth swimming [23,41,44]. Ca^{2+} also has an important structural role as it is required to stabilize and maintain the integrity of the outer lipopolysaccharide layer and the cell wall ([33] and references therein). In recent years evidence for the role of Ca^{2+} at the molecular level has also been obtained. The activity and stability of several enzymes, many of which are extracellular, is directly controlled by Ca^{2+} binding. Examples of such enzymes are subunit c of *E. coli* ATP-synthase [45], *P. aeruginosa* lipases [46], subtilisins from bacilli [47] and cellulolytic enzymes, including endoglucanases and xylanases from *Clostridium* species.

There is also substantial evidence indicating a role for Ca^{2+} in cell division [48]. During cell division in *E. coli*, the dynamic formation of the FtsZ ring is necessary before cytokinesis can occur. The assembly of this ring was shown to be stimulated *in vitro* in the presence of millimolar concentrations of Ca^{2+} [38]. It was proposed that GTP binding and hydrolysis by FtsZ, which contains several putative Ca^{2+}-binding sites [49], are altered in the presence of Ca^{2+}. It was also demonstrated recently that changes in the intracellular Ca^{2+} concentration occur when *Serratia liquefaciens* is challenged with the autoinducer molecule *N*-hexanoyl-L-homoserine lactone [50]. In this case, Ca^{2+} could act as a second messenger and the transient changes in intracellular Ca^{2+} levels might then affect quorum sensing signal transduction. In *E. coli*, expression of the outer membrane porins OmpC and OmpF is mainly regulated by the EnvZ–OmpR phosphorelay system in response to the osmolarity of the medium. Using an EnvZ hybrid protein, it was demonstrated that Ca^{2+} directly stimulates EnvZ autophosphorylation and phosphate transfer to OmpR, suggesting that Ca^{2+} is involved in signal transduction [51].

Conclusions

Several lines of evidence indicate that Ca^{2+} plays a major role in the regulation of bacterial processes as diverse as chemotaxis, cell differentiation and cell division [22,33,43,48]. Several prerequisites for Ca^{2+} signalling, including a tightly regulated intracellular level of free Ca^{2+} and several classes of Ca^{2+}-binding proteins, are present in bacteria. From several case studies, it appears that Ca^{2+} binding might stabilize bacterial proteins [17,19], modulate their enzymatic activity [38] or activate and/or repress their signal transducing functions [50,51]. By virtue of the homology to their eukaryotic counterparts, the identified EF-hand proteins could be involved in signal transduction in response to variations in Ca^{2+} concentration and thus, these proteins could be valuable targets in studying the role of Ca^{2+} in bacteria. However, from the present analysis (Table 2; published as online supplementary information at http://archive.bmn.com/supp/tim/michiels.pdf), it appears that universal Ca^{2+} regulators, such as those found in eukaryotes, might be absent from bacteria. The elucidation of the biological function of bacterial EF-hand and many other Ca^{2+}-binding proteins awaits further analysis and will require a detailed study of the Ca^{2+}-binding characteristics and interacting proteins, and evaluation of the effects of specific mutations on the function of these proteins. Such an analysis will ultimately reveal the molecular basis of Ca^{2+} functioning in bacteria. It is likely that additional Ca^{2+}-binding proteins, including EF-hand proteins, will emerge as more prokaryotic genomes are sequenced. Analysis of these proteins might reveal unexpected parallels between eukaryotic and prokaryotic cellular functions involving Ca^{2+} signal transduction.

Supplementary information

Table 2, listing EF-hand proteins found in complete bacterial genome sequences, is published online at http://archive.bmn.com/supp/tim/michiels.pdf

Acknowledgements

We thank Hans Vermeiren for his valuable assistance during the computer analyses. Our apologies to those colleagues whose work could not be cited owing to space constraints. J.M. is a postdoctoral fellow of the Fund for the Scientific Research-Flanders. C.X. is a recipient of a doctoral scholarship from the Research Council of the K.U. Leuven. J.V. is a recipient of a doctoral scholarship from the 'Instituut ter bevordering van Wetenschappelijk en Technologisch Onderzoek'.

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