Co-existence of clpB and clpC in the Bacillaceae

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Abstract

The gene encoding ClpC in Bacillus anthracis was amplified from the chromosome by polymerase chain reaction using degenerate oligonucleotide primers. These primers also amplified a second DNA fragment identified as a clpB homolog. Both genes were suggested to be functional. Contrary to Bacillus subtilis which possesses clpC but not clpB, many Bacillus species were found to harbor both clpC and clpB. We also found that Clostridium strains could possess clpB, clpC, or both. All the Gram-negative strains tested had clpB only. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Microorganisms have developed strategies to respond rapidly to environmental stress or changes. The adaptation process involves proteolytic and chaperone activities. ClpAP is a protease in Escherichia coli. The ClpA ATPase activates the ClpP protein for proteolysis [1,2]. The Clp ATPases belong to the Clp/Hsp100 family of molecular chaperones [3]. ClpB and ClpC have regions of sequence in common with ClpA. These molecules are classified according to size, with ClpB having the largest molecular mass (reviewed in [2,4,5]). ClpB is a heat shock protein, and the ClpB of E. coli has been thoroughly characterized. It seems to be absent in Bacillus subtilis, in which ClpC, sometimes described as the alternative Gram-positive heat-inducible Clp protein, has been found [6–8]. ClpC plays a key role in the stationary phase regulatory network in B. subtilis [6]. ClpC has been identified in Listeria monocytogenes, which is required for the stress tolerance and survival in vivo of this pathogenic bacterium [9,10].

Bacillus anthracis, the etiological agent of anthrax, a mammalian disease, is usually regarded as the only pathogen belonging to the Bacillus genus [11]. B. anthracis has to respond to various stresses such as oxidative shock, after entering the host. Later in the disease the bacterium encounters growth-limiting conditions and enters a stationary phase. We therefore aimed to isolate the clpC gene so that we could analyze the function of ClpC in B. anthracis. We found that B. anthracis contained not only clpC but also clpB, both genes being functional. The pres-
ence of both these clp genes in the same species has only been described for Synechococcus sp. [8,12]. So, we investigated whether both genes occur together in other members of the Bacillaceae family, or whether, as described in B. subtilis, clpC was exclusive of clpB.

2. Materials and methods

2.1. Bacterial strains, vectors and culture media

The strains used were: B. anthracis 9131 and RBA2 [13], B. subtilis 168, Bacillus thuringiensis 4045, Bacillus sphaericus ATCC 14577, B. sphaericus 1593, Listeria monocytogenes LO28, Clostridium difficile VPI 10463, Clostridium perfringens CPN 50, Clostridium sordelli NCIB, E. coli K12, Yersinia pestis col 17, and Neisseria meningitidis clone 12. E. coli TGI [14] was used as a host for pUC19. E. coli strain HB101 harboring pRK24 T [15] was used for mating experiments. The vector pATΔS28 was constructed by digesting pATV28 by XmnI and relegating it, thus deleting the Gram-positive origin of replication ([16] and J.-C. Sirard, personal communication).

E. coli was grown in L broth or on L agar plates [17]. The Bacillaceae were grown in brain heart infusion (Difco Laboratories) medium or on brain heart infusion agar plates. Antibiotics were used in the following concentrations: ampicillin, 100 μg ml⁻¹ for E. coli; and kanamycin, 40 μg ml⁻¹, spectinomycin 60 μg ml⁻¹, for both E. coli and B. anthracis.

2.2. DNA manipulation, amplification and sequencing

Bacillaceae chromosomal DNA was extracted as described by Fouet and Sonenshein [18]. The degenerate oligonucleotides used were Clp220 (GGI GT(GAT) GGI AA(AG) AC(GAT) GC(GAT) AT(ATC) GC(GAT) GA(AG) GG) and Clp566 (CT)TC IGT (CT)TT (ATC)CC (ATC)GT IGG (ATC)CC) (and see Section 3). PCR was carried out as follows: 10 cycles of 30 s at 94°C, 45 s at 47°C, 1 min at 72°C; followed by 10 cycles of 30 s at 94°C, 45 s at 53°C, 1 min at 72°C and 10 cycles of 30 s at 94°C, 45 s at 55°C, 1 min at 72°C, with a final extension for 5 min at 72°C. When bacterial colonies were used rather than DNA, the polymerase was added after an initial step of 5 min at 99.9°C.

Sequences were determined either from PCR products, or from double-stranded DNA after cloning the fragments in pUC19 [19] by the dideoxy chain-termination procedure [20] using Sequenase kits (Amersham/USB) or the Prism AmpliTaq Dye Primer sequencing kit (Applied Biosystems) with an ABI PRISM 373A sequencer. Nucleotide and deduced amino acid sequences were analyzed using the Wisconsin Package (Genetics Computer Group Inc.).

2.3. Disruption of clpB and clpC genes, and transcriptional fusions between these genes and bgaB

Recombinant suicide plasmids were transferred from E. coli to B. anthracis by a heterogramic conjugation procedure [15]. Allelic exchange was carried out as described previously [21] using either the kanamycin- or the spectinomycin-resistance cassette ([22,23] and Trieu-Cuot, personal communication) (Fig. 1).

The clpB gene was disrupted with pB543K which was constructed as follows (Fig. 1). The bgaB gene,
encoding a thermoresistant β-galactosidase, was isolated by digesting pDL \[24\] by SmaI and Ecl136II and cloned in pATv similarly digested, giving rise to pATvbgaB. A DNA fragment harboring sequences from nucleotide +3448 (GGGAAGGA-GAATGGAAATGGGACAAAATC) to +100 (GA-GGATACCGCTAAAGATTGGGCAC), with respect to the ATG codon of the clpB gene, was PCR amplified and cloned in the SmaI site of pATvbgaB, upstream of the bgaB gene, in the same orientation of transcription giving rise to pB54. To construct pB543, the 964 (CTCATCGATGCAACAAGTAT-TAGCAGAAGAACCAAC) to 1561 (TTGGATC-TTCACCTTCTTACTTCC) nt sequence was similarly amplified and cloned downstream of bgaB in the Ecl136II site of pB54. The oligonucleotide starting at position 964 recreates an Ecl136II site when cloned in that site. The kanamycin resistance cassette was cloned in the Ecl136II site. Thus pB543K also harbors a transcriptional fusion between clpB and bgaB genes followed by a selectable marker and the 3' part of clpB.

An equivalent construction was carried out for clpC, giving rise to pC113S (Fig. 1). The SmaI-Ecl136II bgaB fragment from pDL was cloned in the SmaI site of pUC19 (pB5). pB5C5 was then constructed by ligating into the SmaI site of pB5 the PCR amplified DNA fragment −791 (AAGAATT-CAGGAACGGAATATGGAGCATGTCTCTTG) to −20 (AACATCATAGAAATCGCCTCCTTAC- TTTG), with respect to the clpC ATG codon. clpC and bgaB are in the same transcription orientation. To construct pC5S, the spectinomycin cassette was inserted downstream from bgaB in the Ecl136II site. The digestion of pC5S by StuI which is located in the spectinomycin fragment −791 (AAGAATT-CAGGAACGGAATATGGAGCATGTCTCTTG) to −20 (AACATCATAGAAATCGCCTCCTTAC-TTTG), with respect to the clpC ATG codon. clpC and bgaB are in the same transcription orientation.

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CATTACTTTACCTTTCATATCC nt clpC PCR amplified sequence gave rise to pC5SC3. The EcoRI DNA fragment of pC5SC5, harboring the transcriptional fusion followed by a selectable marker and the 3′ region of clpC, was subcloned in pAT113 [25], resulting in pC113S.

3. Results and discussion

3.1. B. anthracis possesses both clpC and clpB

To amplify B. anthracis clpC from the chromosome, by PCR, we compared the amino acid sequences of ClpC from B. subtilis [6] and L. monocytogenes [9]. The ATP-binding motifs are well conserved in all Clp ATPases and two sequences, identical in the B. subtilis and L. monocytogenes ClpCs, were identified in the A boxes of each ATP-binding motif, between residues 211–220 and 545–554 (B. subtilis numbering). Degenerate oligonucleotides (Clp220 and Clp566) were designed from these sequences.

When PCR was performed with the B. anthracis RBA2 chromosome, two fragments were obtained: one with the expected size of approximately 1 kb, and another about 150 bp larger (Fig. 2A, lane 3). The same experiment with B. subtilis 168 DNA generated a single fragment, of about 1 kb in size (Fig. 2A, lane 2). This fragment was partially sequenced and its sequence was completely identical to that of the published B. subtilis clpC sequence [6]. The spacer between the two highly conserved nucleotide-binding motifs is 60–70 residues in ClpC proteins and 120–130 residues in ClpB proteins, which suggested that the larger B. anthracis DNA fragment encoded the ClpB ATPase. We therefore determined the DNA sequence of the two fragments (accession numbers AJ224158 and AJ224159 for the 1-kb and the 1.15-kb fragments, respectively). Reverse PCR was performed to isolate the chromosomal DNA encompassing the binding sites of the initial primers, and to thus determine the hybridizing sequences. Data bases and the completed SubtiList [26,27], were searched with the deduced amino acid sequences. We found that the peptide encoded by the 1-kb fragment was highly similar to the B. subtilis ClpC (SWP37571) (90.7% identity over a 334-amino acid overlap) and that encoded by the 1.15-kb fragment was highly similar to the Synechocystis ClpB (SWP53533) [8] (63.4% identity over a 410-amino acid overlap) (Fig. 3).

We compared the B. anthracis Clp homologs and found that their level of sequence identity differed in different regions. The sequences were 62.5% identical for the first conserved nucleotide binding motif and 69% for the second. The spacer regions between

![Fig. 3. Schematic representation of Clp sequence comparison.](image-url)
these two blocks was 61 residues long for ClpC and 125 residues long for ClpB (Fig. 3). No sequence encoding ClpB was detected by PCR amplification or by sequence analysis of the complete \textit{B. subtilis} chromosome, demonstrating the specificity of its presence on the \textit{B. anthracis} chromosome.

3.2. \textit{B. anthracis} clpB and clpC are functional

The only other bacterium so far where ClpC and ClpB have been characterized is \textit{Synechococcus}. In this cyanobacterium, ClpB is a heat shock protein whereas the synthesis of ClpC increases under conditions of rapid growth. Transcriptional fusions were constructed to assay the regulation of synthesis of ClpB (pB543K) and ClpC (pC113S) in \textit{B. anthracis} (Fig. 1). CLB10 was obtained by integrating by homologous recombination the insert of pB543K in the chromosome of strain 9131, selecting for kanamycin resistance and assaying the spectinomycin sensitivity. β-Galactosidase expression was induced in given growth conditions, thus indicating that \textit{clpB} is expressed in \textit{B. anthracis}. A similar mating experiment between \textit{E. coli} harboring pC113S and \textit{B. anthracis} 9131 was carried out four times unsuccessfully, whereas these genetic exchanges are routinely done in our laboratory. This strongly suggested that \textit{clpC}, or a gene downstream, is essential for \textit{B. anthracis} growth. This implied that \textit{clpC} is transcribed in \textit{B. anthracis} and thus functional. An identical conclusion was proposed for the same reason by Clarke and Eriksson, for the \textit{Synechococcus clpC} gene [12].

3.3. The presence of \textit{clpC} and/or \textit{clpB} in other bacteria

Our data and the analysis of the complete \textit{B. subtilis} sequence [26] indicated that \textit{B. subtilis} contained only \textit{clpC}, whereas \textit{B. anthracis} possessed both \textit{clpC} and \textit{clpB}, so we carried out a larger survey of the distribution of \textit{clpC} and \textit{clpB} among bacteria. Various DNAs, or colonies, from Gram-positive and Gram-negative bacteria were used as templates for PCR amplification with the primers (Fig. 2). Two fragments of 1 kb and 1.15 kb in size were detected, for \textit{B. anthracis}, \textit{B. thuringiensis} 4045, \textit{L. monocytogenes} LO28 and \textit{B. sphaericus} ATCC 14577 (Fig. 2A, lanes 3–6). The 1-kb and 1.15-kb DNA fragments obtained by amplification from the \textit{L. monocytogenes} LO28 chromosome were partially sequenced, showing that they encoded the \textit{L. monocytogenes} LO28 ClpC (1-kb fragment) [9] and a ClpB homolog (1.15-kb fragment). Both bands were also detected in the PCR products for the \textit{B. sphaericus} 1593 strain, although the 1.15-kb band was very faint (Fig. 2A, lane 7). Our data suggest that \textit{B. subtilis} is exceptional among the \textit{Bacillus} strains tested, and that the species of the \textit{Bacillus} genus usually have both genes (Fig. 2A). However, this does not seem to be the case for the whole of the Bacillaceae family, because \textit{Clostridium} strains had all the possible gene combinations. Some had only a 1-kb fragment (\textit{C. difficile} VPI 10463), or only a 1.15-kb fragment (\textit{C. sordelli} NCIB) and some both (\textit{C. perfringens} CPN 50) (Fig. 2B). Only \textit{clpB} was detected in the Gram-negative bacteria tested (\textit{E. coli} K12, \textit{Y. pestis} col 17, \textit{N. meningitidis} clone 12) (Fig. 2C). The bacterial genomic data bases were also searched for the presence of ClpB and ClpC. The occurrence of both proteins is only described for \textit{Clostridium acetobutylicum}, reinforcing our conclusions.

Thus, our data suggest that ClpC is not an alternative to ClpB in Gram-positive bacteria, or \textit{Bacillus} spp., and that there is no obvious explanation for the distribution of \textit{clpB} and \textit{clpC} genes in the Bacillaceae.

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