Two distinct types of rRNA operons in the *Bacillus cereus* group

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The *Bacillus cereus* group includes insecticidal bacteria (*B. thuringiensis*), food-borne pathogens (*B. cereus* and *B. weihenstephanensis*) and *B. anthracis*, the causative agent of anthrax. The precise number of rRNA operons in 12 strains of the *B. cereus* group was determined. Most of the tested strains possess 13 operons and the tested psychrotolerant strains contain 14 operons, the highest number ever found in bacteria. The separate clustering of the tested psychrotolerant strains was confirmed by partial sequencing of several genes distributed over the chromosomes. Analysis of regions downstream of the 23S rRNA genes in the type strain *B. cereus* ATCC 14579 indicates that the rRNA operons can be divided into two classes, I and II, consisting respectively of eight and five operons. Class II operons exhibit multiple tRNA genes downstream of the 5S rRNA gene and a putative promoter sequence in the 23S–5S intergenic region, suggesting that 35 rRNA and the downstream tRNA genes can be transcribed independently of the 16S and 23S genes. Similar observations were made in the recently sequenced genome of *B. anthracis* strain Ames. The existence of these distinct types of rRNA operons suggests an unknown mechanism for regulation of rRNA and tRNA synthesis potentially related to the pool of amino acids available for protein synthesis.

INTRODUCTION

*Bacillus cereus* is a Gram-positive, spore-forming, soil bacterium widespread in the environment. Strains of this group can be isolated from soil (Helgason *et al.*, 1998; Vilas-Boas *et al.*, 2002), dead animal or insect bodies (Margulis *et al.*, 1998), conserved food products (Lechner *et al.*, 1998), infected humans (Helgason *et al.*, 2000; Kotiranta *et al.*, 2000) and industrial surfaces (Kotiranta *et al.*, 1998; Ronner *et al.*, 1990). The recognized species of the *B. cereus* group include *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis*. Some of these bacteria are of particular importance for human life. *B. anthracis* is the causative agent of anthrax (Read *et al.*, 2003). *B. thuringiensis* is widely used against insects threatening crops or carrying diseases (Schnepf *et al.*, 1998). Some *B. cereus* strains are used as animal probiotics (Jadamus *et al.*, 2001, 2002; Mietke *et al.*, 2000) or as plant symbionts (Stabb *et al.*, 1994). Some strains of *B. cereus* and *B. weihenstephanensis* are opportunistic food-borne pathogens. *B. weihenstephanensis* is known to be psychrotolerant (growth at 8 °C or below) (Lechner *et al.*, 1998). When ingested, pathogenic strains of *B. cereus* may cause diarrhoeic or emetic syndromes (Granum, 1994; Granum & Lund, 1997; Helgason *et al.*, 2000; Kotiranta *et al.*, 2000; Lechner *et al.*, 1998; Stenfors *et al.*, 2002).

In several previous studies, Southern hybridization and inverse PCR were used for ribotyping the *B. cereus* group strains (Johansen *et al.*, 1996; Lechner *et al.*, 1998; Patra *et al.*, 2002; Priest *et al.*, 1994). However, these works led only to approximate determination of the rRNA operon number (Patra *et al.*, 2002). Recently the entire genomic sequence of the type strain *B. cereus* ATCC 14579 was established (Ivanova *et al.*, 2003). In this study we determined the precise number of RNA operons and compared them in several strains of the *B. cereus* group distinguished by different phenotypes such as specific insect pathogenicity for *B. thuringiensis* (Schnepf *et al.*, 1998), psychrotolerance for *B. weihenstephanensis* (Lechner *et al.*, 1998), or food-related pathogenicity for *B. cereus* 391-98 (Lund *et al.*, 2000). Thirteen to fourteen rRNA operons were identified, the highest number ever detected in bacteria. To determine the relationships between these strains, we carried out parallel sequencing of several genes distributed over their chromosomes. The accurate analysis of rRNA operon sequences in the type strain revealed sequence variations, which suggest the existence of two distinct classes of rRNA operons. The eleven rRNA operons of the recently sequenced *B. anthracis* Ames (Ban Ames) (Read *et al.*, 2003) exhibit a similar polymorphism, suggesting that the presence of two distinct classes of rRNA operons is a general feature through the *B. cereus* group.

Abbreviation: LR PCR, long-range polymerase chain reaction.

The GenBank accession numbers for the sequences determined in this work are given in the text.
**METHODS**

**Strains and growth conditions.** The strains of the *B. cereus* group used in this study are listed in Table 1. Bacteria were grown in Luria Broth medium (Sambrook et al., 1989) at 30°C with agitation at 250 r.p.m. For preparation of DNA the cultures were grown overnight, diluted 1:20 and grown to an OD$_{600}$ of 3.

DNA extraction, restriction enzyme digestion and DNA blotting. Cells were hydrolysed by lysozyme (3 mg ml$^{-1}$) for 30 min at 37°C. The cells were then lysed by adding SDS to 0.5% final concentration. Proteins were degraded by proteinase K treatment (0.3 mg ml$^{-1}$ final concentration) for 2 h at 50°C, followed by two water-saturated phenol (pH 8.0) extractions at 30°C (overnight and for 2 h). DNA was precipitated by adding potassium acetate (pH 4.8) up to 0.3 M and 2 vols absolute ethanol. Restriction enzyme digestions were carried out for 2 h according to the manufacturers' instructions, at 37°C (*ClaI* and *HindIII*) or 55°C (*BclI* and *EcoRV*). Electrophoresis was performed in Tris/borate buffer in 0.8% agarose gel, overnight at 25 mA. The agarose gels were then treated in 0.5 M HCl, 0.5 M NaOH, and vacuumblotted onto Hybond-XL membrane (Amersham).

**PCR amplification.** PCR using the Expand Long Template PCR System (Roche Diagnostics) was used to obtain templates for sequencing and for preparing DNA hybridization probes. The cycling program was: 94°C for 5 min; 12 cycles of 94°C for 10 s and 68°C for 12 min; 24 cycles of 94°C for 10 s and 68°C starting from 12 min and increasing this time by 15 s each cycle. The final extension was done at 72°C for 10 min.

**Sequencing procedures.** PCR products were treated for 1 h at 37°C with exonuclease I and shrimp alkaline phosphatase (USB). Sequencing reactions were performed using an ABI PRISM sequencing kit (Applied Biosystems). Products of reactions were ethanol precipitated and analysed with an ABI 3700 sequencer (Applied Biosystems).

Separate amplification of each operon was performed using oligonucleotides listed in Table 2. Sequencing was done on each amplification product separately using a set of 32 oligonucleotides with sequences common to all operons (oligonucleotide sequences can be provided on request). PCR amplification and sequencing of the regions between closely located 58 and 16S rRNA genes of operons $rnrC$ and $rrnD$ respectively were done using the following oligonucleotides: FIAH7 (5'-GCAAGCGTTTAATCGGTAAGT-3'), FIAH8 (5'-AGGGTCCCCGGSATCCGCTTTA-3'), FIBH7 (5'-GAA'TCCCTGAAAGATGATCAGGTTG-3') and FIBH8 (5'-GCTCCCCATACCGAAGGAAGTT-3'). Assembly was performed manually using Staden's XBASE version 14.0 software (Dear & Staden, 1991) and consensus sequences of 16S, 23S and 5S rRNA genes as scaffold. The 13 RNA operon sequences were deposited at NCBI under accession numbers AY224379-AY224388.

**Preparation of 16S rDNA probe and detection of ribotypes.** A 1.4 kb region of 16S rDNA gene was amplified by PCR using total DNA from *Bce* 6A5 and oligonucleotides corresponding to the 16S rRNA genes FIBG1 (5'-GAATTGCGCAGGTAATGATTAAGAAG-3') and FIAH4 (5'-TACGCTACCTTGTTACGACTTCAC-3') as probe and the enzymes *BclI* and *ClaI* (restriction enzymes). PCR product was purified using the QIAGen PCR Purification Kit (Qiagen) and 60 ng of it was labelled using the NEBlot Kit (New England Biolabs) and 50 µCi [3-32P]dCTP (3000 Ci mmol$^{-1}$; 110 TBq mmol$^{-1}$). Hybridization of the probe to DNA blotted on membrane was done overnight at 65°C using the Rapid-hyb buffer (Amersham). Hybridized bands were detected using a STORM Scanner (Molecular Dynamics) and quantified using ImageQuant 5.2 software (Molecular Dynamics).

**Determination of the number of rRNA operons.** Total DNA of each strain was digested with *BclI*, *ClaI* or *HindIII*. An internal fragment of the 16S rRNA gene was used as the probe and the enzymes used had no recognition sites within the 16S rRNA gene but had one or more sites within the 23S rRNA gene. In these conditions, each ribosomal operon was expected to produce only one DNA fragment able to hybridize to the probe. Intensity of each hybridization band was measured using the STORM scanner. After background subtraction, an iterative process was applied to determine the number of rRNA operons corresponding to each hybridized DNA band, assuming that each operon contributed equally to the signal intensity. In iteration the signal corresponding to one rRNA operon was estimated by dividing the intensity of each hybridization band by the postulated number of rRNA operons to which the band corresponded. The relative error of the signals of one rRNA operon,

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**Table 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Strain</th>
<th>Source*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bce 6A5</td>
<td><em>B. cereus</em> ATCC 14579T (type strain) (= BGSC 6A5)</td>
<td>D. Ziegler, BGSC</td>
<td>Frankland &amp; Frankland (1887)</td>
</tr>
<tr>
<td>Bce 391-98</td>
<td><em>B. cereus</em> 391-98</td>
<td>D. Lereclus, IP</td>
<td>Lund et al. (2000)</td>
</tr>
<tr>
<td>Btc HD224</td>
<td><em>B. thuringiensis</em> subsp. <em>canadensis</em> HD224 (BGSC 4H2)</td>
<td>D. Ziegler, BGSC</td>
<td>de Barjac &amp; Bonnefoi (1972)</td>
</tr>
<tr>
<td>Btk HD1</td>
<td><em>B. thuringiensis</em> subsp. <em>kurstaki</em> HD1 (BGSC 4D1)</td>
<td>D. Ziegler, BGSC</td>
<td>Dulumage (1970)</td>
</tr>
<tr>
<td>Bti 4Q2-81</td>
<td><em>B. thuringiensis</em> subsp. <em>israelensis</em> 4Q2-81 (BGSC 4Q7)</td>
<td>D. Ziegler, BGSC</td>
<td>Clark (1987)</td>
</tr>
<tr>
<td>Bti 35646</td>
<td><em>B. thuringiensis</em> subsp. <em>israelensis</em> ATCC 35646</td>
<td>A. Lapidus, IG</td>
<td>Temeyer (1984)</td>
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<tr>
<td>Bwe 10204</td>
<td><em>B. weihenstephanensis</em> WSBC 102047</td>
<td>S. Scherer, IM</td>
<td>Lehner et al. (1998)</td>
</tr>
<tr>
<td>Bwe 10206</td>
<td><em>B. weihenstephanensis</em> WSBC 10206</td>
<td>S. Scherer, IM</td>
<td>Lehner et al. (1998)</td>
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<td><em>B. weihenstephanensis</em> WSBC 10297</td>
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<td>Pruss et al. (1999)</td>
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<td>Bwe 10315</td>
<td><em>B. weihenstephanensis</em> WSBC 10315</td>
<td>S. Scherer, IM</td>
<td>Pruss et al. (1999)</td>
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</table>

*BGSC, Bacillus Genetic Stock Center, Ohio State University, Ohio, USA; IP, Institut Pasteur, Paris, France; IG, Integrated Genomics Inc., Chicago, IL, USA; IM, Institut für Microbiologie, Freising, Germany.*
measured by using the different hybridized bands, was then calculated by the formula

\[ d = \frac{1}{k} \sum_{i=1}^{k} |x_i - \bar{x}| \]

where \( d \) is the relative error, \( x_i \) is the signal corresponding to one rRNA operon estimated for the \( i^{th} \) of total \( k \) hybridization bands and \( \bar{x} = \frac{1}{k} \sum_{i=1}^{k} x_i \) is the mean signal corresponding to one operon. The hypothesized number of rRNA operons leading to the minimal relative error was assumed to correspond to the real number of operons. Moreover, the linear regression coefficient \( R^2 \), based on the classical Pearson correlation function, was calculated to verify the linear proportionality of the hybridization signal with the number of rRNA operons for each hybridization band. The closer this coefficient is to 1 the more likely it is that the assumption about the number of operons is correct. Independent determination of the number of rRNA operons was performed for each of the digestion enzymes used and for two amounts of genomic DNA for each enzyme (see Fig. 1B).

**Phylogenetic analysis of ribotype patterns.** Hybridization profiles for each strain were divided into 10 sections and the number of rRNA operons, scored as described above, was assigned to each section. To calculate distances between strains we applied the method developed for multilocus enzyme electrophoresis data analyses (Selander et al., 1986), considering each section of the hybridization profiles as a ‘locus’ and the number of bands in this section as the ‘allele’. This enabled us to take into account the ‘allele’ frequencies and the genetic diversity. The dendrogram corresponding to the resulting distance matrix was calculated with STATISTICA 5.0 software using the UPGMA method.

**Genetic relationships.** To investigate the genetic relationships of the different strains, seven genes distributed over the 5.4 Mb chromosome of the entirely sequenced type strain Bce 6A5 (Ivanova et al., 2003) were partially sequenced in all strains. These were: *clpC* (position on the chromosome 90 kb) encoding the ClpC regulator; *purF* (300 kb), encoding glutamine phosphoribosylpyrophosphate amidotransferase; *gdpD* (574 kb), encoding glycerophosphoryl diester phosphodiesterase; *yhfL* (1068 kb), encoding long-chain fatty acid CoA ligase; *panC* (1484 kb), encoding pantoate–β-alanine ligase; *dinB* (4103 kb), encoding DNA polymerase IV; *plcR* (5255 kb), encoding transcriptional regulator PlcR. The corresponding oligonucleotides used for PCR amplification and sequencing were: 5'-GTCACATATCCCTGGTACGGC-3' (positions 101–700); 5'-GGAATACTAGATGCTGTAACCCAG-3' for *purF* (positions 101–700);

<table>
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<tr>
<th>Oligonucleotide</th>
<th>Sequence (5' to 3')</th>
<th>Position and orientation in Bce 6A5 genome (bp)*</th>
<th>Used for LR PCR of</th>
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<td>rrnA</td>
</tr>
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</tr>
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</tr>
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* Referred to NCBI accession number AE016877.
RESULTS AND DISCUSSION

Number of rRNA operons and ribotyping of different strains of the B. cereus group

As reported, the type strain B. cereus ATCC 14579 possesses 13 rRNA operons (Ivanova et al., 2003). Shotgun sequencing of the genome of B. cereus ATCC 14579 with a sixfold coverage allowed us to detect twelve 5′ ends of 16S rRNA genes and eight 5S rRNA genes. Subsequent LR PCR experiments enabled us to localize these 12 rRNA operons. Southern hybridization experiments were carried out to verify this number of rRNA operons. The results obtained with total DNA isolated from strain Bcc 6A5 (B. cereus BGSC 6A5, the same as ATCC 14579) and digested with Bcl, ClaI or HindIII are shown in Fig. 1(A). In the conditions used, each ribosomal operon was expected to produce only one DNA fragment able to hybridize to the probe. The signal intensity was measured to determine the number of DNA fragments in each band, and hence the number of rRNA operons in the chromosome (see Methods for details). For all three restriction enzymes used, the minimal statistical error was obtained if the number of rRNA operons in the chromosome was assumed to be 13, and linear regression coefficient values led to the same conclusion (Fig. 1B). This result indicates that the determination by random sequencing and LR PCR mapping missed one rRNA operon. We mapped this operon using a genomic DNA digestion by EcoRV, which does not cut 16S or 23S rRNA genes (not shown). The additional operon was located close to the operon rrmC. The unique sequence between these two operons was determined by PCR amplifications using oligonucleotides specific to 5S and 16S rRNA genes and subsequent sequencing (Fig. 1C). Finally, we succeeded in precisely locating all 13 rrm

Fig. 1. Determination of the number of rRNA operons in B. cereus BGSC 6A5. (A) Representative result of hybridization of 16S rRNA gene specific probe with total DNA digested by BclI (lane 1), ClaI (lane 2) and HindIII (lane 3). Size markers in bp are shown on the left. This experiment was carried out twice with different amounts of genomic DNA for each enzyme. (B) Mean relative statistical error (first column for each enzyme) and mean linear regression coefficients (second column for each enzyme) corresponding to the two distinct amounts of genomic DNA used, calculated for the different hypotheses of the rRNA operon number (shown in bold on the left). The smallest value of statistical error and the highest of linear regression coefficient were considered as corresponding to the correct number of operons. (C) PCR amplification of inter 5S–16S areas for closely located rRNA operons. Oligonucleotides used: FIBH7 and FIAH8 (lane 1), FIBH7 and FIAH7 (lane 2), FIBH8 and FIAH8 (lane 3), FIBH8 and FIAH7 (lane 4). The shortest DNA fragments, between 1·1 and 1·5 kb, were assumed to correspond to the newly detected inter-operon region, whereas the upper bands (between 2·3 and 2·7 kb) corresponded to the areas between rrnG and rrnH and between rml and rrnJ. Size markers in bp are shown on the left.
operons on the chromosome of \textit{Bce} 6A5 by LR PCR (the primers used are listed in Table 2).

The quantitative Southern hybridization approach used for strain \textit{Bce} 6A5 was applied to 11 other strains from the \textit{B. cereus} group. The selected strains represent the diversity of this group. According to previous studies, strains \textit{Bce} 6A5, \textit{Bce} 6A1 and \textit{B. thuringiensis} subsp. \textit{canadensis} (\textit{Btc}) HD224 are closely related (Carlson \textit{et al.}, 1994), \textit{B. thuringiensis} subsp. \textit{israelensis} (\textit{Bti}) and \textit{B. thuringiensis} subsp. \textit{kurstaki} (\textit{Btk}) strains represent the most important insecticidal groups (Hofte \& Whiteley, 1989), \textit{B. weihenstephanensis} (\textit{Bwe}) strains represent a large cluster of psychrotolerant strains (Lechner \textit{et al.}, 1998) and strain \textit{Bce} 391-98 was characterized as having a high pathogenic potential (Lund \textit{et al.}, 2000). As in the case of strain \textit{Bce} 6A5, three enzymes, \textit{Bcl}, \textit{Cla} and \textit{HindIII}, were used. Representative ribotype profiles of these strains, using digestion by \textit{HindIII}, and the deduced number of rRNA operons are shown in Fig. 2(A, B). Most of the studied strains appeared to possess 13 rRNA operons, like the type strain \textit{Bce} 6A5; 14 rRNA operons were detected for the other strains. If a correct number was used for calculation, the relative statistical error was 3–4 %. This error increased up to 9–20 % if the number used for calculation differed by 1 (Fig. 1B). In all cases, the minimal value of the statistical error correlated with the highest linear regression coefficient. This asserted that the intensity of hybridization signal varies linearly with the number of hybridized fragments, and hence with the number of rRNA operons. Thus, this approach provides an unambiguous estimation of the repeat number of a sequence in a genome even when it is as high as 14.

All the \textit{Bwe} psychrotolerant strains appeared to have a slightly higher number of rRNA operons (14 instead of 13). All other strains used in this study were unable to grow at low temperatures (8 °C) (not shown), \textit{Btc} HD224, which is close to \textit{Bce} 6A1 and \textit{Bce} 6A5 (Carlson \textit{et al.}, 1996), also exhibited 14 rRNA operons. On the other hand, \textit{Bce} 6A5 and \textit{Bce} 6A1 gave riboprofiles very similar to that of \textit{Btc} HD224, but had only 13 operons. These results indicate that the number of rRNA operons does not necessarily reflect species divergence and cannot be used as a reliable phylogenetic feature for the \textit{B. cereus} group. However, the two independently isolated \textit{Bti} strains which were characterized as very clonal in previous studies (Ankarloo \textit{et al.}, 2000; Priest \textit{et al.}, 1994) exhibited riboprofiles that were essentially indistinguishable. To obtain a better view of strain diversity, we constructed a phylogenetic tree based on the riboprofiles obtained by \textit{HindIII} digestion. This digestion was chosen because it generated the highest number of fragments and hence provided the highest resolution (Fig. 2C). The tree showed apparent divergence of the five psychrotolerant strains from the type strain \textit{Bce} 6A5 and its close relatives. It also indicated the isolation of the \textit{Bti} strains. The pathogenic strain \textit{Bce} 391-98 appeared to be isolated from all other strains.

All the \textit{Bce} 6A5 strains appeared to have 13 rRNA operons, like the \textit{B. cereus} type strain \textit{Bce} 6A5; 14 rRNA operons were detected for the other strains. If a correct number was used for calculation, the relative statistical error was 3–4 %. This error increased up to 9–20 % if the number used for calculation differed by 1 (Fig. 1B). In all cases, the minimal value of the statistical error correlated with the highest linear regression coefficient. This asserted that the intensity of hybridization signal varies linearly with the number of hybridized fragments, and hence with the number of rRNA operons. Thus, this approach provides an unambiguous estimation of the repeat number of a sequence in a genome even when it is as high as 14.

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![Fig. 2. Determination of the number of rRNA operons in different strains. (A) Representative result of hybridization of 16S rRNA specific probe with total DNA of different strains digested by \textit{HindIII}. This experiment was carried out twice with different amounts of genomic DNA for enzymes \textit{Bcl}, \textit{Cla} and \textit{HindIII}. Size markers are shown on the left. (B) Relative statistical error and linear regression coefficient calculated for the best hypotheses of the rRNA operon numbers. Strain numbers correspond to the lanes in (A). (C) Phylogenetic relationships between different strains derived from the riboprofile data (see text for the details). Distance scale is shown at the top.](http://mic.sgmjournals.org)
Genetic relationships between strains of the B. cereus group

To verify the relationships between strains presented above, we sequenced seven genes distributed over the chromosome of Bce 6A5 in the 12 studied strains. We also added the corresponding sequences of B. anthracis strain Ames (Ban Ames) (Read et al., 2003) to get a wider view of the B. cereus group. Trees were constructed using the nucleotide sequences obtained for all genes. Examples of representative dendrograms are given in Fig. 3 (gdpD, panC and plcR genes). The resulting trees were almost fully congruent, for all the genes tested, except plcR, and they confirmed the separate clustering of the five Bwe psychrotolerant strains from the type strain Bce 6A5 and its relatives. For all tested genes, no difference was found between the two Bti strains, confirming their high clonality as mentioned above. In most cases, the two pathogenic strains Bce 391-98 and Ban Ames appeared to be relatively isolated but slightly closer to the psychrotolerant strains than to others. The genes dinB (not shown), panC and plcR of strain Bce 391-98 appeared to be very close to those of strain Bwe 10204 (less than 2 bp differences in 500 bp sequenced: Fig. 3B, C), suggesting that those strains belong to the same group. However, the difference was greater for the gdpD gene (Fig. 3A) and strain Bce 391-98 lacked the ability to grow at 8 °C. In comparison, the Ban Ames strain appeared to be much more isolated. On the whole, the trees based on parallel sequencing and on the riboprofiles exhibited similar topologies.

The tree constructed for the pathogenicity-related regulator gene plcR was very particular. It is worth noting that in contrast to all other sequenced genes, the plcR alleles for the four B. thuringiensis strains clustered together, and were separated from strains Bce 6A1 and 6A5. In particular, although Btc HD224 was very close to Bce 6A5 and 6A1 for the other genes and for ribotyping (Figs 2C and 3), its plcR gene was 8% divergent from that of Bce 6A5 and very similar to those of the other insect pathogens. On the other hand, three of the psychrotolerant strains, isolated from milk powder (Pruss et al., 1999), have exactly the same plcR allele as the human pathogen Bce 391-98, also originating from food products (Lund et al., 2000) (Fig. 3C). The PlcR regulator was shown to be related to the virulence of the B. cereus group (Agaisse et al., 1999; Okstad et al., 1999; Salamitou et al., 2000). It would be interesting to investigate if there is a functional link between plcR allele and pathogenic specificity.

Comparison of the rRNA operons in Bce 6A5

Bce 6A5 appeared to possess 13 rRNA operons. As expected, all are located close to the replication origin. Physical separation of these 13 rRNA operons, amplified using LR PCR, allowed us to sequence them separately by primer walking and to study their variability in the conserved 16S, 23S and 5S regions and in the less conserved regions flanking these genes.

Sequences of the 16S, 23S and 5S genes were different in few single base positions (Fig. 4). We detected seven, seven and two alleles for the 16S, 23S and 5S rRNA genes, respectively. Seven copies of the 16S rRNA gene (A, E, G, H, I, J and M) exactly corresponded to the consensus sequence determined previously (Ticknor et al., 2001). None of the other alleles reported in this paper for different strains were detected during the present study. The 16S gene alleles corresponding to the rRNA operons B, D, F, K and L were all unique and contained one nucleotide difference compared to the consensus. The 16S gene in the rrnC operon contained two differences.

In a previous study, the existence of a ‘psychrotolerant’ signature (5′-AACATTTTGAACCGCATGGTTC3′) within the 16S rRNA genes of psychrotolerant strains was proposed for the rapid identification of such strains (Pruss et al., 1999). No 16S gene containing this sequence was detected in the Bce 6A5 strain. This result is consistent with the observation that this strain does not grow at low temperature. Mesophilic strains have three different bases in the above sequence, indicating that the ‘psychrotolerant’ signature may have been acquired by recombination. The availability of the precise map of rRNA operons would make it possible to study the exact locations of such ‘psychrotolerant’ 16S genes in the genomes of the B. cereus strains.

The intergenic 16S–23S area, differing considerably between strains, has often been used to classify strains (Bourque et al., 1995; Daffonchio et al., 1998, 2000; Harrell et al.,

![Fig. 3. Phylogenetic trees constructed from the partial nucleotide sequences of different genes (gdpD, panC and plcR). Bootstrap values calculated for 1000 trials are indicated for each node. Bars in the top corners indicate the scale of divergence for each gene.](image)
amplification of different operons by LR PCR, using the oligonucleotides reported here (Table 2), and subsequent sequencing of the intergenic 16S–23S and 23S–5S areas, can be suggested as a more precise approach for characterizing and classifying strains.

The consensus sequences of 23S and 5S genes have not been reported yet. From our data they are the same as the alleles corresponding to operons rrrnD, G, H, I and J. Concerning the 23S gene, it is remarkable that the operon rrrnB contains multiple single-nucleotide differences clustered in two regions, 310–335 and 2428–2485. This might indicate that this allele originated from two recombinational events. Alleles of operons rrrnC, E and L have several differences clustered close to position 1560, suggesting their common recombinational origin. Presence of identical 5S alleles that differ from the consensus in these three operons is consistent with this hypothesis. However, the same 5S allele was also found in operons rrrnK and M, having the 23S alleles closer to the consensus. It is therefore probable that independent recombination events took place in the 23S and 5S genes. Concerning the 5S genes, only two alleles were detected, differing at four nucleotide sites, indicating that they probably diverged due to a recombination event.

**Two distinct types of rRNA operons in Bce 6A5 and Ban Ames**

An important difference in the overall organization of rRNA operons in Bce 6A5 is the presence of multiple tRNA genes downstream of operons rrrnC, E, K, L and M (Fig. 5A). Operons K, L and M have respectively 15, 20 and 22 tRNA genes downstream, with a partially similar order of codon specificity. The closely related operons C and E both have nine tRNA genes downstream, although the codon specificities are different. All the five operons having multiple tRNA genes downstream also differ from the others by their 5S rRNA allele (Fig. 4) and 23S–5S region (Fig. 5B). These observations suggest the existence of two distinct types of tRNA operons in Bce 6A5. A phylogenetic tree constructed using the sequences of the 23S–5S region presented in Fig. 5(B) confirms the existence of two types of tRNA operons (Fig. 6). The 13 rRNA operons in Bce 6A5 could therefore be divided into two classes. The first (class I) includes operons rrrnA, B, D, F, G, H, I and J, all devoid of multiple tRNA genes downstream, and the second (class II) includes operons rrrnC, E, K, L and M, all having many (75 in total) tRNA genes downstream (Fig. 5A), and distinct 5S gene (Fig. 4) and 23S–5S intergenic region (Figs 5B and 6). It is worth noting that the 23S–5S intergenic region of all class II operons contains multiple tRNA genes downstream also differ from the class I operons (Fig. 5A). Operons K, L and M have respectively 15, 20 and 22 tRNA genes downstream, with a partially similar order of codon specificity. The closely related operons C and E both have nine tRNA genes downstream, although the codon specificities are different. All the five operons having multiple tRNA genes downstream also differ from the others by their 5S rRNA allele (Fig. 4) and 23S–5S region (Fig. 5B). These observations suggest the existence of two distinct types of tRNA operons in Bce 6A5. A phylogenetic tree constructed using the sequences of the 23S–5S region presented in Fig. 5(B) confirms the existence of two types of tRNA operons (Fig. 6). The 13 rRNA operons in Bce 6A5 could therefore be divided into two classes. The first (class I) includes operons rrrnA, B, D, F, G, H, I and J, all devoid of multiple tRNA genes downstream, and the second (class II) includes operons rrrnC, E, K, L and M, all having many (75 in total) tRNA genes downstream (Fig. 5A), and distinct 5S gene (Fig. 4) and 23S–5S intergenic region (Figs 5B and 6). It is worth noting that the 23S–5S intergenic region of all class II operons contains sequences (TTGACT as ‘−35 box’ and TA(C,A or T)AAT as ‘−10 box’) which are very similar to the Gram-positive and Gram-negative bacteria $\alpha$ promoter consensus sequence (TTGACA as ‘−35 box’ and TATAAT as ‘−10 box’). Moreover, class II rRNA operons do not have any obvious transcription terminator structure downstream of the 5S gene (Fig. 5B). Thus, the 5S rRNA gene and the

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**Fig. 4.** Polymorphic sites in the 16S, 23S and 5S rRNA genes of Bce 6A5 and Ban Ames. Only positions with differences are shown. Asterisks indicate the bases identical to the consensus. Positions of the nucleotide sites are indicated vertically above the sequences. Numbering starts from the 5' end of the corresponding rRNA gene: 5' TTTGGTGA... for 16S, 5' TTATTGGA... for 23S and 5' T(T/C)TGGT(G/A)A... for 5S. The strains are indicated on the left, and the different rRNA operons are indicated on the right.

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downstream tRNA genes might be transcribed independently from the 16S and 23S genes. In contrast, all class I operons, except *rrnF*, contain a clearly identifiable stem-loop sequence downstream of the 5S gene, which should act as a transcription terminator (Fig. 5B). The operon *rrnF* appears to be intermediate between the two classes. This operon has two tRNA genes downstream of the 5S rRNA gene and 12 tRNA genes upstream of the 16S gene. Its 5S allele is identical to those of class I rRNA operons and no promoter or terminator close to the 5S rRNA gene was detected for this operon. We assigned *rrnF* to class I, although it may have a particular significance compared to the two classes.

We observed a similar feature for the 11 rRNA operons of the recently sequenced *B. anthracis* (Ban Ames) (Read et al., 2003). As for *Bce* 6A5, rRNA-coding genes of *Ban Ames* were divergent in few positions. Similarly, eight, eight and three alleles were detected for 16S, 23S and 5S respectively (Fig. 4). The 16S consensus sequence is exactly the same as reported previously for strains *Ban Sterne* and Vollum (Ticknor et al., 2001), in accordance with the known clonality of *Ban* strains. The 16S sequence of *Ban* strains diverges at position 1015 from that of *Bce* 6A5 (shown in bold in Fig. 4). The 23S consensus sequence obtained for *Ban Ames* is also different at two positions (1562 and 2156). The 5S consensus sequence is exactly the same for the two strains. As for *Bce* 6A5, the presence of a 5S allele diverging from the consensus in operons *rrnD*, *I*, *J* and *K* of *Ban Ames* correlates with the presence of multiple tRNA genes downstream of these *rrn* operons (Fig. 5C) and a 23S–5S intergenic region distinct from the consensus (Fig. 5D). Although *Ban Ames* possesses only 11 rRNA operons, we can establish a correspondence between its operons (*Ba_rrn* operons) and those of *Bce* 6A5 (*Bc_rrn* operons) according to their sequences, the presence of multiple tRNA genes downstream and their positions in the genomes. On this basis, operons *Ba_rrnA*, *B*, *C*, *F*, *G* and *H* correspond to *Bc_rrnA*, *B*, *D*, *H*, *I* and *J* respectively (class I), operons *Ba_rrnD*, *I*, *J* and *K* correspond to *Bc_rrnE*, *K*, *L* and *M* respectively (class II) and operon *Ba_rrnE* corresponds to *Bc_rrnF* (intermediate considered as belonging to class I). Only operons *Bc_rrnC* and *G* of *Bce* 6A5 did not have counterparts in *Ban Ames*. These observations suggest that the existence of two distinct types of rRNA operons is a general feature through the *B. cereus* group.

Evidence for the diversity of rRNA sequences in a single organism has been recently accumulating. Some authors...
have already evoked the existence of distinct types of rRNA operons based on the heterogeneity of the small-subunit rRNA genes in different domains of life, indicating the wide occurrence of this phenomenon (Carranza et al., 1996; Lifting et al., 1996; Maden et al., 1987; Mylvaganam & Dennis, 1992; Reischl et al., 1998; Yap et al., 1999). Concerning the B. cereus group, a potential ‘psychrotolerant’ signature has already been found in the 16S sequence (Pruss et al., 1999). Recently the existence of different types of rRNA operons based on the whole organization and sequence of the operons was reported in Escherichia coli (Ohnishi et al., 2000) and Clostridium perfringens (Shimizu et al., 2001). However, none of these studies revealed divergence as significant as the presence of a potential alternative promoter within the 23S–5S intergenic region. The putative promoter highlighted in this study within this region in Bcc 6A5 as well as in B. Ames may enable the expression of tRNA genes independently of the global regulation of expression of the whole rRNA operon. The expression of tRNA genes could thus be regulated according to the amount of amino acids available for protein synthesis. Such a mechanism would be useful for B. cereus and B. anthracis for adaptation to the environment and the colonization of new environmental niches.

ACKNOWLEDGEMENTS

We thank Drs D. Zeigler, D. Lereclus, A. Lapidus and S. Scherer for kindly providing bacterial strains. Technical help of N. Galleron, B. Quinquis, J.-M. Batto and A. Bolotin (Génétique Microbienne, CR) INRA) in sequencing and computer programming is highly appreciated.

REFERENCES


