σ²⁹-Like Protein Is a Common Sporulation-Specific Element in Bacteria of the Genus Bacillus

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A monoclonal antibody specific for an antigenic determinant on the Bacillus subtilis sporulation-induced sigma factor σ²⁹ reacted with proteins similar in size to σ²⁹ in extracts of sporulating Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus cereus, Bacillus natto, and Bacillus pumilus but not in extracts prepared from vegetatively growing cultures of these bacteria. These results indicate that RNA polymerase modifications, initially described for B. subtilis, are likely to be common among sporulating Bacillus spp. and that at least some of the specific modifications that are observed in sporulating B. subtilis are conserved among members of this genus.

In response to nutrient deprivation, bacteria of the genus Bacillus undergo a simple form of cellular differentiation that converts the vegetative form of the organism into a dormant endospore. In B. subtilis, the program of gene expression during this process may, in part, be regulated by alterations in the template specificity and subunit composition of the RNA polymerase of the developing cell (7). Multiple sigma-like proteins have been isolated from this organism (2-4, 11, 17), and one of them (σ²⁹) appears to be directly related to the sporulation process (1, 2, 6). σ²⁹ accumulates in B. subtilis by 2 h into development (16) and is encoded by a gene (spoIIG) which is essential for endospore formation but dispensable for vegetative growth (14a). Are the sporulation-specific RNA polymerase modifications observed in B. subtilis a common feature of the sporulation process in Bacillus spp. or are they unique to B. subtilis? Studies of RNA polymerase structure and template specificities in other Bacillus spp. have suggested that RNA polymerase modifications, if they occur at all, are likely to be highly species specific. B. thuringiensis, for example, has been reported to proteolytically modify the β' and σ subunits of its vegetative cell RNA polymerase as well as synthesize several new RNA polymerase-binding proteins (5), whereas B. cereus RNA polymerase appears to undergo no change in either subunit composition or template specificity as the organism proceeds into sporulation (8). The synthesis of σ²⁹ and its association with RNA polymerase is currently the most obvious sporulation-specific change in the structure of B. subtilis RNA polymerase (1, 2, 6). If the major B. subtilis sporulation-specific RNA polymerase modifications are shared by other members of the genus Bacillus, the synthesis of a counterpart to σ²⁹ would also be likely in these bacteria.

We had previously isolated a monoclonal antibody which recognized an antigenic determinant that is present on no B. subtilis protein other than σ²⁹ (apparent molecular weight, 29,000) and its putative precursor P³¹ (apparent molecular weight, 31,000) (15, 16). In an assay in which crude extracts of B. subtilis SMY were fractionated on sodium dodecyl sulfate-polyacrylamide gels, electrophoretically transferred to nitrocellulose, and probed with this monoclonal antibody, P³¹ and σ²⁹ were readily observed in the sporulation extract but not in the vegetative cell extract (Fig. 1). Assuming that the antigenic determinant that is recognized by our antibody might be conserved on homologous proteins, we attempted to detect P³¹- and σ²⁹-like proteins in six additional Bacillus species (obtained from P. Lovett).

Extracts of B. amyloliquefaciens (BGSC 10A1), B. cereus (BGSC 6A1), B. licheniformis (BGSC 5A7), B. natto (BGSC 27A1), B. pumilus (231-3-5), and B. sphaericus (BGSC 13A1) were prepared from cells that had been vegetatively growing in DS medium (10) or from cells that were harvested at 2 and 5 h after the end of logarithmic growth in this medium. Analysis of the extracts with monoclonal antibody revealed P³¹- and σ²⁹-like proteins in four of the six Bacillus species (Fig. 2). Although the precise times at which these proteins appeared varied among the cultures, in each instance the

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proteins were absent from vegetatively growing bacteria and appeared only after logarithmic growth had ceased. The proteins detected in the *B. licheniformis*, *B. amyloliquifaciens*, and *B. natto* extracts had identical mobilities on sodium dodecyl sulfate-polyacrylamide gels to those of authentic P31 and σ29, while the two proteins detected in the *B. cereus* extracts migrated to positions between those of P31 and σ29 (Fig. 2A, lane 6).

In *B. subtilis*, the synthesis of P31 and σ29 requires the onset of sporulation and not merely movement of a culture into the stationary phase (16). In our hands, wild-type strains of *B. pumilus* (231-3-5) and *B. sphaericus* (BGSC 13A1) failed to sporulate appreciably in DS medium (Table 1) or S medium (13; data not shown). Therefore, the failure to detect P31 and σ29-like proteins in these species could merely reflect their low sporulation efficiency. We tested this possibility with mutant strains of *B. pumilus* (W20) and *B. sphaericus* (1593 and 2362) (obtained from P. Lovett) which sporulate at a higher frequency than do their wild-type parent strains. Using these mutant strains, we observed proteins with the mobilities of P31 and σ29 which reacted with the anti-σ29 monoclonal antibody in cultures of sporulating *B. pumilus* (Fig. 3, lanes 6 and 7) but not *B. sphaericus* (Fig. 3, lanes 2 to 5), even though at least one of the mutant *B. sphaericus* strains (2362) formed heat-resistant cells to approximately the same extent as did the wild-type cultures (Table 1). Thus, sporulating *B. sphaericus* apparently does not synthesize a protein which carries the epitope that is recognized by our anti-σ29 monoclonal antibody.

Remarkably, a single antigenic determinant that is present on the *B. subtilis* protein σ29 was also present on similarly sized sporulation-specific proteins in five of the six *Bacillus* species examined. Does the conservation of σ29-like proteins extend to the level of the genes encoding these proteins? Are the base sequences that code for these proteins similarly conserved? We investigated these questions by hybridizing a 32P-labeled, 1.1-kilobase pair PstI fragment of *B. subtilis* DNA (14) which carries the entire structural gene for P31, σ29 (14a) to restriction endonuclease *EcoRI*-digested DNA from seven *Bacillus* species. The DNA samples had been electrophoretically separated on an agarose gel and transferred to nitrocellulose (12). The hybridizations were accomplished.

![FIG. 2](http://jb.asm.org/) Analysis of six wild-type *Bacillus* species for synthesis of a σ29-like protein. Extracts were analyzed as described in the legend to Fig. 1. Lanes for each species are for harvest midway through vegetative growth, at 2 h after the end of logarithmic growth, and at 5 h after the end of logarithmic growth, respectively; the *B. subtilis* control was harvested at 2 h after the end of logarithmic growth. (A) Lanes: 1 to 3, *B. licheniformis*; 4 to 6, *B. cereus*; 7 to 9, *B. amyloliquifaciens*; 10, *B. subtilis* (control). (B) Lanes: 1, *B. subtilis* (control); 2 to 4, *B. sphaericus*; 5 to 7, *B. natto*; 8 to 10, *B. pumilus*.

![FIG. 3](http://jb.asm.org/) Analysis of *B. pumilus* and *B. sphaericus* mutants for synthesis of a σ29-like protein. Cultures were grown in DS medium, and extracts were analyzed as described in the legend to Fig. 1. Lanes for each species are for harvest at 2 and 5 h after the end of logarithmic growth, respectively; the *B. subtilis* control was harvested at 2 h after the end of logarithmic growth. Lanes: 1, *B. subtilis* (control); 2 and 3, *B. sphaericus* 1593; 4 and 5, *B. sphaericus* 2362; 6 and 7, *B. pumilus* W20.

### Table 1. Acquisition of heat-resistant cells

<table>
<thead>
<tr>
<th>Species</th>
<th>CFU/ml</th>
<th>% Heat resistant</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unheated</td>
<td>Heated</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>2.0 × 10⁶</td>
<td>1.9 × 10⁷</td>
</tr>
<tr>
<td><em>B. natto</em></td>
<td>3.8 × 10⁶</td>
<td>9.8 × 10⁷</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>8.8 × 10⁶</td>
<td>1.1 × 10⁷</td>
</tr>
<tr>
<td><em>B. amyloliquifaciens</em></td>
<td>1.1 × 10⁹</td>
<td>1.4 × 10⁹</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>1.2 × 10⁹</td>
<td>1.3 × 10⁹</td>
</tr>
<tr>
<td><em>B. pumilus</em> 231-3-5</td>
<td>1.1 × 10⁹</td>
<td>&lt;10⁵</td>
</tr>
<tr>
<td><em>B. pumilus</em> W20</td>
<td>3.9 × 10⁶</td>
<td>2.1 × 10⁵</td>
</tr>
<tr>
<td><em>B. sphaericus</em> BGSC 13A1</td>
<td>8.8 × 10⁶</td>
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</tr>
<tr>
<td><em>B. sphaericus</em> 1593</td>
<td>1.9 × 10⁹</td>
<td>&lt;10⁵</td>
</tr>
<tr>
<td><em>B. sphaericus</em> 2362</td>
<td>9.4 × 10⁸</td>
<td>7.3 × 10⁷</td>
</tr>
</tbody>
</table>

* Cultures of the indicated *Bacillus* species were analyzed at 8 h after the end of exponential growth in DS medium (10). Cells were diluted 1/10 in 0.1 M NaCl, placed in an 80°C water bath for 1 h, appropriately diluted, and plated on LB plates. Nontreated, appropriately diluted cells were plated on LB plates for the viable cell count.
under conditions of relatively high (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 68°C) or low (6× SSC, 55°C) stringency. Figure 4A illustrates that under the more stringent hybridization conditions the DNA sequences encoding σ29 only hybridized to EcoRI fragments (approximately 10 kilobase pairs) from *B. subtilis* (lane 2) and *B. natto* (lane 5). When less stringent conditions were used (Fig. 4B), additional hybridizations became detectable to fragments of approximately 5 and 2 kilobase pairs from *B. licheniformis* (lane 6) and *B. amyloliquifaciens* (lane 8). None of the other *Bacillus* DNAs hybridized with the probe to any greater extent than did the DNA isolated from *Escherichia coli* (Fig. 4, lanes 1). Thus, aspects of the base sequence of the σ29 gene are conserved among *Bacillus* species but to a lesser extent than the protein structure that is recognized by the anti-σ29 antibody.

We conclude from this study that proteins that are homologous to σ29 and P31 are common among members of the genus *Bacillus*. We detected sporulation-specific proteins with molecular weights of approximately 29,000 and 31,000 which reacted with an anti-σ29 antibody in six of seven *Bacillus* species that we examined. Inasmuch as these proteins appeared under the same general developmental conditions (i.e., early-intermediate sporulation), we believe that they probably perform similar functions. The failure to detect a σ32-like protein in *B. sphaericus* does not necessarily mean that this species lacks a counterpart to σ29; it may instead merely indicate that this “σ32” lacks the antigenic determinant that is recognized by our monoclonal antibody. In view of the occurrence of σ32-like proteins in other *Bacillus* species, we consider this to be the more probable explanation. We infer from these results that RNA polymerase modifications are likely to be common among sporulating *Bacillus* spp. and that some of the specific modifications characterized in *B. subtilis* will have general relevance to sporulation gene regulation within the genus.

We thank C. L. Truitt for a critical reading of the manuscript. This work was supported by National Science Foundation grant PCM-8021300 and Welch Foundation grant AQ-932.

**LITERATURE CITED**


FIG. 4. Southern blot analysis of chromosomal DNA. Purified DNA (10 μg) from the indicated bacteria was digested with restriction endonuclease EcoRI, electrophoresed through a gel of 1% agarose, and transferred to nitrocellulose paper (12). The immobilized DNA fragments were hybridized in 6× SSC to a purified 1.1-kilobase-pair PstI DNA fragment of plasmid pGS11G11 (14) labeled with 32P by the nick translation activity of DNA polymerase I (19). The temperature of hybridization was either 68 (A) or 55°C (B)