Sequencing and analysis of the *Bacillus subtilis* lytRABC divergon: a regulatory unit encompassing the structural genes of the *N*-acetylmuramoyl-L-alanine amidase and its modifier

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Abbreviations: CWBP, cell-wall-bound protein; RBS, ribosome-binding site.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M87645.

(Received 16 March 1992; revised 27 May 1992; accepted 3 June 1992)

The regulatory unit of *Bacillus subtilis* strain 168 encompassing the structural genes of the *N*-acetylmuramoyl-L-alanine amidase and of its modifier has been sequenced, and found to be a divergon consisting of divergently transcribed operons lytABC and lytR. Proteins LytA, LytB and LytC are endowed with export signal peptides. Mature LytA is a 9.4 kDa, highly acidic polypeptide whose deduced amino acid sequence points to a lipoprotein. LytB and LytC, the modifier and the amidase, are highly basic. After cleavage of the signal sequence their molecular masses are 74.1 and 49.9 kDa, respectively. These two proteins share considerable homology in their N-terminal moieties and have three GSNRY consensus motifs, characteristic of nearly all amidases. The C-terminal moiety of LytB exhibits homology to the product of *spoIIID*. LytR is a 35 kDa protein which acts as an attenuator of the expression of both lytABC and lytR operons. Transcription of the lytABC operon proceeds from two promoters: P1, identified as P28-7 (Gilman et al., 1984), and an upstream P2*. The former only is subject to LytR attenuation. Translational initiation of lytB and lytC is directed by UUG start codons, suggesting that lytA, B and C undergo coupled translation. Transcription of lytR is initiated at two start sites, one of which corresponds to a highly intense P2* promoter whereas the other does not seem to share much homology with any of the known promoter consensus sequences. Both promoters are attenuated by LytR. It is confirmed that the synthesis of the amidase is controlled at least in part by SigD, i.e. that it belongs to the fla regulon and that its activity, or part of it, is co-regulated with flagellar motility. The role of the mutations conferring the Sin, Fla and Ifm phenotypes in the expression of the lytABC operon is discussed.

Introduction

During vegetative growth the cell wall of *Bacillus subtilis*, strain 168, is endowed with two major autolysins: the *N*-acetylmuramoyl-L-alanine amidase and the endo-β-*N*-acetylmuramidase (Rogers et al., 1980). Both belong to a specific class of cell-wall-bound proteins (Studer & Karamata, 1988; Margot & Karamata, 1992), and their molecular masses, derived from SDS-PAGE mobility, are about 49 and 90 kDa, respectively (Herbold & Glaser, 1975; Rogers et al., 1984). *In vitro* (Herbold & Glaser, 1975) and *in vivo* (Margot & Karamata, 1992) analyses have revealed that the amidase activity is enhanced two- to threefold by another cell-wall-bound protein (CWBP), the modifier. The modifier and the amidase were mapped near 310°, and cloned (Margot & Karamata, 1992). Their inactivation by insertion mutagenesis has revealed that they are encoded by genes *lytB* and *lytC*, respectively. That the latter genes belong to the same operon accounts for the equimolar amounts of CWBP49 (the amidase) and CWBP76 (the modifier) in native cell wall. Other enzymes hydrolysing peptido-glycan have been described: an amidase encoded by the gene cwlA (Kuroda & Sekiguchi, 1990; Foster, 1991); and two autolysins encoded by the defective bacteriophage PBSX, which were identified as an amidase and an *N*-acetylmuramidase (Ward et al., 1982).
The regulation of the expression of the operon encoding the amidase and the export of this protein are apparently complex since they are affected by mutations in a variety of genes. It was shown that the amidase activity is under the control of \textit{sigD} (\textit{flaB}), the gene which encodes the $\sigma$ factor required for the expression of the flagellar regulon (Marquez \textit{et al.}, 1990). That mutational inactivation of \textit{sigD} reduces the amidase activity to about 30\% of its wild-type level (Marquez \textit{et al.}, 1990) suggests that expression of this gene can proceed from another promoter. The regulatory role of the product of the gene \textit{sin}, a repressor of \textit{aprE}, the alkaline protease gene (Gaur \textit{et al.}, 1991), is more complex: disruption of \textit{sin} (\textit{flaD}) is accompanied by a decrease of the amidase activity comparable to that in a \textit{sigD} strain (P. Margot, unpublished), suggesting an effector role for \textit{Sin}. However, more paradoxically, certain mutations in the \textit{sin} gene (\textit{flaD2}, for example) lead to nearly total disappearance of the amidase activity; the residual activity in the latter mutants amounts to 2.5\% of that of the wild-type (Margot \textit{et al.}, 1991), and could be due to the other, minor, autolytic activities (see above). Finally, strains with the increased flagellar motility phenotype (Ifm) have a two- to sevenfold increased autolytic activity (Pooley \& Karamata, 1983; H. M. Pooley, unpublished). Overexpression of the amidase gene seems however to be due to at least two mutations, both located around 310$^\circ$ between \textit{hisA} and \textit{gtaB} genes (Pooley \& Karamata, 1984).

To further our understanding of the apparently complex control mechanism of the amidase expression we have sequenced the whole regulatory unit which contains \textit{lytC}, the structural gene of amidase. We report that this unit is under the control of at least four promoters. It consists of two divergently transcribed operons, whose expression is under the control of a protein encoded by one of them.

Part of this contribution was presented at the 6th International Conference on Bacilli, Stanford, California, USA (July, 1991). During the preparation of the manuscript part of the sequence reported here below was published (Kuroda \& Sekiguchi, 1991).

\section*{Methods}

\textit{Bacterial strains, plasmids and phage.} These are listed in Table 1. Fragments from the \textit{B. subtilis} chromosomal DNA insert in phage 163 (Mauel \textit{et al.}, 1989) were subcloned in pMTL20EC (Ap$^\prime$ Emr$^\prime$ Cm$^\prime$)

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Name} & \textbf{Genotype or description} & \textbf{Origin} \\
\hline
\textit{Escherichia coli} & & \\
DH5 & F$^-$ endA1 hsdR17 (r$^{-}$m$^{-}$) supE44 thi-l 2-recA1 gyrA96 relA1 (argF-lacZYA)U169 φ80lacZAM15 & P. Shaw, University of Lausanne \\
\hline
\textit{Bacillus subtilis} & & \\
L16006 & pheA1 purA16 hisA35 trpC2 metB5 sigD::pLM5 & Insertion of plM5 into the \textit{sigD} locus of L5047 \\
L16013 & pheA1 purA16 hisA35 trpC2 metB5 lytA::luc & Insertion of \textit{lytA} locus of L5047 \\
L16015 & pheA1 purA16 hisA35 trpC2 metB5 lytA::cat & Insertion of p6325Δ1 into the \textit{lytB} locus of L16013 \\
L16016 & pheA1 purA16 hisA35 trpC2 metB5 lytB::p6325Δ1 & Integration of \textit{lytB} locus of L5047 \\
L16020 & pheA1 purA16 hisA35 trpC2 metB5 lytR::pA2 & Integration of \textit{lytR} locus of L5047 \\
\hline
\textit{Phage} & & \\
λ63 & Contains 17.3 kb fragment of \textit{B. subtilis} 168 chromosomal DNA & \\
\hline
\textit{Plasmids} & & \\
pH101 & Ap$'$ Te$'$ Cm$'$ & Ferrari \textit{et al.} (1983) \\
plM5 & Ap$'$ Cm$'$ & Marquez \textit{et al.} (1990) \\
pWD1 & Cm$'$ & H. Wood, Trinity College Dublin \\
p6325Δ1 & Ap$'$ Emr$'$ & Deletion of 1·1 kb \textit{EcoRI}–\textit{StuI} fragment from p6325 \\
p6327Δ77 & Ap$'$ Emr$'$ Cm$'$ & Deletion of 0.4 kb fragment comprising intergenic region between \textit{lytA} and \textit{lytR} from p6327 \\
pV3 & Ap$'$ & Cloning of 1·6 kb \textit{HindIII} fragment from p6324 into the same site of pMTL21; \textit{EcoRV} site of pMTL21 lost \\
pl1 & Ap$'$ Cm$'$ & Cloning of 1·1 kb \textit{EcoRI} fragment from pWD1 into the \textit{EcoRV} site of pV3 \\
pA2 & Ap$'$ Emr$'$ Cm$'$ & Cloning of 0·4 kb \textit{HindIII} fragment from p6327Δ77 into the same site of pMTL20EC \\
\hline
\end{tabular}
\caption{\textit{Bacterial strains, plasmids and bacteriophage}}
\end{table}
(Chambers et al., 1988) except for the insert in plasmid 6302, which was cloned in pH101 (Ap spectinomycin) (Ferrari et al., 1983) and maintained in Escherichia coli strain DH5.

**Media and growth.** E. coli strains were grown in LB containing selective agents: ampicillin (50 μg ml⁻¹), erythromycin (10 μg ml⁻¹), and chloramphenicol (10 μg ml⁻¹). *B. subtilis* strains were grown in SPIZ I, SPIZ II, SA and LB media as previously described (Karamata & Gross, 1970). When required, media were supplemented with amino acids (20 μg ml⁻¹), adenine (100 μg ml⁻¹), erythromycin (0.25 μg ml⁻¹ or 25 μg ml⁻¹) and chloramphenicol (3 μg ml⁻¹).

**DNA preparation.** For sequencing, plasmid DNA was prepared by the alkaline lysis method (Birnboim & Doly, 1979). DNA was obtained as described by Grossberger (1987). After RNAase treatment, DNA was purified by phenol/chloroform extraction and precipitated by ethanol. The boiling method miniprep (Del Sal et al., 1988) was used to prepare DNA for clonation, deletion reactions and transformations. DNA fragments separated by electrophoresis on 0.8% agarose gel were extracted from gel slices and purified by centrifugation through siliconized sterile glass wool (Heery et al., 1990).

**DNA sequencing and analysis.** Unidirectional deletions of the DNA fragments cloned in pM86EC were generated by a double-stranded nested deletion kit (Pharmacia). DNA sequencing on both strands was performed using the dideoxy chain-termination method (Sanger et al., 1977). Labeling reactions were performed with a Sequenase Version 2.0 kit (USB) and [α-³²P]dATP (Amersham). To sequence regions for which no suitable clones were available, site-specific primers (17-mers) were synthesized with an SM oligonucleotide synthesizer (Beckman). The sequence was compiled and analysed by the University of Wisconsin Computer Group software (Devereux et al., 1984).

**Transformation.** Competent cells of *E. coli* DH5 were prepared and transformed by the procedure of Chung & Miller (1989). Transformation of *B. subtilis* was performed as described by Karamata & Gross (1970). Erythromycin-resistant transformants of *B. subtilis* were selected on LB medium containing 0.25 μg ml⁻¹. They were transferred to the same medium containing a 100-fold higher concentration of the antibiotic.

**Hybridization.** For Southern hybridization (Southern, 1975) endonuclease fragments of chromosomal DNA were transferred from agarose gel to Hybond-N nylon membranes (Amersham) by alkaline blotting (Reed & Mann, 1985).

**Amidase and modifier detection.** Cells grown to a concentration of 10⁶ cells ml⁻¹ in SA medium were washed with cold double-distilled water. Cell wall proteins were extracted with 5 mM-NaCl, 50 mM-Tris pH 8.0 (Margot & Karamata, 1992), and analysed by SDS-polyacrylamide (12%, w/v) electrophoresis (Laemmli, 1970).

**Autolysis.** Cells were grown with aeration in SA medium at 37 °C to a concentration of 10⁶ cells ml⁻¹. After arrest of aeration, sodium azide was added at a final concentration of 0.05 M, and the decrease of nephelometric density was followed (Margot & Karamata, 1992).

**Determination of turnover kinetics.** The procedure described by Pooley (1976) was followed.

**Isolation of total RNA.** Total cellular RNA was isolated from a culture grown in SA medium at 37 °C and harvested at 10⁶ cells ml⁻¹. The isolation procedure was essentially that of Gilman & Chamberlin (1983). A 15 ml volume of culture was transferred to a 50 ml centrifugation tube containing ice chilled to -20 °C. Cells were sedimented at 0 °C (5 min at 10000 r.p.m., Sorvall rotor SS-34), resuspended in 0.5 ml disruption buffer (30 mM-Tris/HCl pH 7.5, 100 mM-NaCl, 5 mM-EDTA, 1% w/v SDS, 100 μg proteinase K ml⁻¹), transferred onto ice, and disrupted by four 5 s sonicators at 30 W with the microtip of an MSE sonifier, separated by 10 s intervals. Three extractions with phenol/ chloroform/isooamyl alcohol (25:24:1, by vol.) at 55 °C were followed by an extraction with chloroform/isoamyl alcohol (24:1, v/v) and precipitation with ethanol. Precipitated nucleic acids were redissolved in 20 mM-Tris/HCl pH 8.0, 10 mM-MgCl₂, 2 mM-CaCl₂, and 100 μg ml⁻¹ of proteinase K-treated DNAase I (Tullis & Rubin, 1980). After incubation for 60 min at 37 °C, SDS, EDTA and NaCl were added at final concentrations of 1%, 50 mM and 200 mM, respectively. The remaining RNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), once with chloroform/isoamyl alcohol (24:1) and precipitated with ethanol.

** Primer extension.** Oligodeoxynucleotides 5’GGCGCCAGTTCCGAGTACAAGAAG3’ and 5’ACTATTACCCGGCACCACCGAGAAG3’ were prepared with the Beckman SM oligonucleotide synthesizer for the analysis of *lytR* and *lytABC* transcripts, respectively. They were purified on a 19% polyacrylamide gel. Twenty

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**Fig. 1.** Sequencing strategy. The 5280 bp sequence as a part of the 6.1 kb *SacI–EcoRI* region was determined on both DNA strands using sets of nested deleted inserts derived from plasmid subclones (——). Thin arrows correspond to the extent of DNA sequences determined from the single labelling reaction using the M13 (——) or synthesized primers (——). Position of ORFs is given under the restriction map (——). Sequences determined by using the λ63 DNA as template are indicated by dotted arrows (——–>). labelled λ63.
Table 2. Properties of the nucleotide sequence of the lytA, B, C and R genes

<table>
<thead>
<tr>
<th>Property</th>
<th>lytR</th>
<th>lytA</th>
<th>lytB</th>
<th>lytC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of the ORF (bp)</td>
<td>918</td>
<td>306</td>
<td>2115</td>
<td>1488</td>
</tr>
<tr>
<td>Start codon</td>
<td>AUG</td>
<td>AUG</td>
<td>UUG</td>
<td>UUG</td>
</tr>
<tr>
<td>Stop codon</td>
<td>UAA</td>
<td>UAA</td>
<td>UGA</td>
<td>UAA</td>
</tr>
<tr>
<td>TA in wobble position (%)</td>
<td>0-54</td>
<td>0-74</td>
<td>0-70</td>
<td>0-72</td>
</tr>
<tr>
<td>CAH</td>
<td>0-44</td>
<td>0-44</td>
<td>0-46</td>
<td>0-44</td>
</tr>
<tr>
<td>AG for RBS (kcal mol(^{-1}))</td>
<td>-138</td>
<td>-160</td>
<td>-144</td>
<td>-198</td>
</tr>
<tr>
<td>TA (%)</td>
<td>0-58</td>
<td>0-64</td>
<td>0-62</td>
<td>0-61</td>
</tr>
</tbody>
</table>

* Numbers of bp separating the adjacent ORFs. The numbers do not comprise the stop codons.
† Codon Adaptation Index (Sharp & Li, 1987).
‡ Calculated according to the rules of Tinoco et al. (1973) (1 kcal = 4.184 kJ).
Results

Nucleotide sequence of the lytRABC divergon

The DNA segment encompassing the structural genes of the amidase and of its modifier (Margot & Karamata, 1992) was sequenced on both strands between restriction sites SacI and EcoRI according to the strategy described in Fig. 1. Computer analysis of the nucleotide sequence (Fig. 2), the main features of which are summarized in Table 2, revealed a functional unit consisting of two divergently transcribed operons: the operon lytABC transcribed in the direction of DNA replication, consisting of three ORFs, and the monocistronic operon lytR, transcribed in the opposite direction. The divergon, comprising 5160 bp, is flanked on each side by a rho-independent terminator. The segment encoding the terminator of lytR ($\Delta G = -16.2$ kcal mol\(^{-1}\); Freier et al., 1986) fulfills the same function for adjacent, convergently transcribed, orfX (B. Soldo, unpublished). The terminator of lytC ($\Delta G = -16.1$ kcal mol\(^{-1}\)) is flanked by a non-coding region extending over at least 450 bp (V. Lazarevic, unpublished). Comparison of the sequenced subclones with those used for insertional inactivation of the modifier and the amidase (Margot & Karamata, 1992) revealed that the latter proteins are encoded by the lytB and lytC genes, respectively. The two operons are separated by a regulatory region of 183 bp. lytA is separated from lytB by 23 bp, and lytB from lytC by 38 bp (Table 2). It is noteworthy that both intercistronic spacers are within the limit of 66 bp which is compatible with translational coupling (Petersen, 1989). Indeed, translation of both lytB and lytC starts with the relatively unusual UUG initiation codon, which does not allow de novo translational initiation (Adhin & van Duin, 1989). $\Delta G$s for RBSs range from $-13.8$ kcal mol\(^{-1}\) for lytR to $-19.8$ for lytC. They may reflect differences at the level of the efficiency of gene expression (Sharp et al., 1990). The estimation based on the percentage of A/T base pairs in the wobble position suggests that ORFs belonging to the lytR and lytABC operons are expressed at very different levels; the expression of the former ORF would be rather moderate while that of the latter ORFs would be extremely high (Shields & Sharp, 1987). However, values (Table 2) of the codon adaptation index (CAI), considered to provide a more realistic measure of the gene expression level, suggest that all four genes are moderately expressed.

Analysis of deduced products encoded by lytA, B, C and R ORFs

The predicted products of ORFs lytA, lytB and lytC consist of 102, 705 and 496 amino acid residues, corresponding to proteins with molecular masses of 11-2, 76-7 and 52-6 kDa (Table 3). All three proteins are apparently endowed with signal peptides (Fig. 2) allowing passage through the cytoplasmic membrane. LytB and LytC were expected to possess signal peptide since they are CWBPs. The calculated molecular masses of their cleaved products – 74-1 and 49-9 kDa, respectively – correspond to those of the modifier and the amidase obtained by SDS-PAGE analysis (Margot & Karamata, 1992). Both polypeptides are highly basic, with isoelectric points of 10-3 and 10-6, respectively; this is in good agreement with their high affinity for cell wall containing teichoic acid (Herbold & Glaser, 1975; Studer, 1988). The deduced product of lytA, a 102 amino acid polypeptide, is highly acidic (Table 3). Its N-terminal peptide, a signal peptide specific to lipoprotein, is followed by a hydrophilic amino acid sequence. The deduced product of lytR, a basic protein, could be membrane-anchored by its N-terminal part – from residue 11 to 35 – which is homologous to transmembrane domains of many eukaryotic glycoproteins (Birchmeier et al., 1986; Krupinski et al., 1989). The LytB and LytC proteins show significant homology (Fig. 3). They each contain three homologous domains of about 100 amino acids in their N-terminal moieties (Fig. 4). Sequence analysis also revealed a homology between the C-terminal moiety of LytB and the spoIID gene (Lopez-Diaz et al., 1986) product (Fig. 3).

Transcription of the lytABC and lytR operons in vivo

Search for putative transcription signals has revealed that all potential promoters are grouped in the 183 bp regulatory region which separates lytR from lytA (Fig. 2). In addition, this region contains four 9 bp sequences organized as two inverse repeats, which may play a regulatory role (Bohannon & Sonenshein, 1989), as well as an operator-like structure overlapping with $P_{A2}$.

In vivo transcription starts were determined by the primer extension method. Two DNA oligonucleotides

<table>
<thead>
<tr>
<th>Property</th>
<th>LytR</th>
<th>LytA</th>
<th>LytB</th>
<th>LytC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncleaved</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of amino acids</td>
<td>306</td>
<td>102</td>
<td>705</td>
<td>496</td>
</tr>
<tr>
<td>Mol. mass (kDa)</td>
<td>34-6</td>
<td>12-2</td>
<td>76-7</td>
<td>52-6</td>
</tr>
<tr>
<td>Mature*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of amino acids</td>
<td>306</td>
<td>86</td>
<td>680</td>
<td>472</td>
</tr>
<tr>
<td>Mol. mass (kDa)</td>
<td>34-6</td>
<td>9-4</td>
<td>74-1</td>
<td>49-9</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>10-0</td>
<td>3-9</td>
<td>10-3</td>
<td>10-6</td>
</tr>
<tr>
<td>Net charge</td>
<td>7</td>
<td>-13</td>
<td>26</td>
<td>23</td>
</tr>
</tbody>
</table>

* LytA, LytB and LytC proteins possess a signal peptide (Fig. 2).
belonging to the predicted \textit{lytABC} and \textit{lytR} transcripts were $^{32}$P end-labelled at their 5'-terminus. It is expected that, in the presence of a molar excess of oligonucleotide primers, the amount of label found in a band of the extended primer will be directly proportional to the amount of RNA added to the extension reaction (Sambrook \textit{et al.}, 1989). Indeed, for \textit{lytABC} and \textit{lytR} transcripts, the strength of the signals was directly proportional to the amount of RNA added, over the range from 10 to 100 ng of total wild-type RNA (not presented). Start sites could be localized by comparison with a sequencing ladder (Fig. 5). For \textit{lytABC} transcripts, one site was found 8 nucleotides downstream of the end of the characteristic P$_D$ promoter consensus (Fig. 2) (Gilman \textit{et al.}, 1981). This transcript was absent in strain L16006, in which the \textit{sigD} gene was disrupted, confirming the $^{32}$P dependence of this site. This promoter corresponds indeed to P$_{28-7}$, belonging to the collection of P$_D$ promoters identified by Gilman \textit{et al.} (1984) and sequenced by Singer (1987). That this transcript was increased about fourfold in strain L16020, in which the \textit{lytR} gene was disrupted, provides strong evidence of an interdependence between operons \textit{lytABC} and \textit{lytR}. Analysis of \textit{lytABC} transcripts (Fig. 5) revealed another start site, localized 21 nucleotides upstream of that initiated by P$_{28-7}$. The former corresponds to a putative P$_A$, I promoter (Fig. 2), designated P$_{A,1}$ (see below), which is localized 7 nucleotides
upstream of the start site. This transcript was affected neither by the sigD nor by lytR mutations (Fig. 5). In the wild-type strain L5047, 29% of the transcripts are initiated by σA, which is in excellent agreement with the amidase activity present in strains with a deficient sigD gene (Marquez et al., 1990).

Analysis of the lytR transcript (Fig. 5) also revealed two start sites and, compared to lytABC, a high level of transcription. The first site lies 7 nucleotides downstream of the end of a putative P_A promoter (Fig. 2), designated P_A,2. Although presence of another promoter could not be inferred from the nucleotide sequence analysis, primer extension experiments have revealed an additional start site 61 nucleotides upstream of the first one. The neighbouring upstream segment bears no obvious resemblance to any of the inventoried promoter motifs. Although it could theoretically accommodate the -10 and -35 sequences of a P_A promoter, it will be designated P_X here below.

Transcription of lytR in mutant L16020 (lytR) (see below) is initiated at no less than three sites, all different from that observed in the lytR+ strain and considerably increased when compared to the latter. One of these sites, with an intensity comparable to that of the P_X site in the lytR+ strain, is localized 23 bp downstream of the latter. The two remaining sites lie between 10 and 5 bp upstream of the P_X-related site; the expression at the site closest to the latter is about seven times higher than that corresponding to P_X.

**Behaviour of lytA- and lytR-deficient mutants**

To inactivate the lytA gene, the chloramphenicol resistance (cat) gene located on the 1.1 kb EcoRI fragment of plasmid pWD1 was cloned into the unique EcoRV site of plasmid pV3, providing a new recombinant plasmid, designated pL1. Transformation of strain L5047 with the 2.7 kb HindIII fragment of plasmid pL1 yielded chloramphenicol-resistant recombinants which were shown by Southern hybridization to possess a single cat gene inserted into lytA. NaCl extracts of whole cells or native cell walls of strain L16013, a cat-bearing lytA recombinant, were subjected to SDS-PAGE analysis (not shown). It appeared that L16013 was devoid of lytB and lytC proteins, confirming that ORFs lytA, lytB and lytC belong to the same transcription unit. Search for the LytA protein, whose molecular mass is 9.4 kDa (mature protein), by inspection of 15% SDS-PAGE gels of either native cell wall or TCA-precipitated exoprotein (not shown) was unsuccessful. This supports the sequence analysis data suggesting the lipoprotein nature of LytA (see above).

To inactivate lytA while ensuring expression of lytC, we constructed strain L16015 by insertion of plasmid p6325A1 into the lytB gene of strain L16013, as revealed by Southern hybridization (not shown). In this strain, lytC was read by the constitutively expressed promoter of the erythromycin resistance gene of plasmid pMTL20EC (Brehm et al., 1987; Chambers et al., 1988). Indeed, NaCl extracts of strain L16015 revealed that CWBP49 was present. Cell autolysis, flagellar motility, as observed by the light microscope, and turnover kinetics of strains L16015 lytA-B-C+ and L16016 lytA+B-C+ did not differ significantly. Thus it appeared that LytA does not play an essential role for the amidase export and activity, at least in the absence of the modifier.

To inactivate the lytR gene, the 0-4 kb HindIII fragment from p6327A77 was cloned into plasmid pMTL20EC, and the resulting plasmid pA2 was inserted into the lytR gene. The chloramphenicol-resistant recom...
binant L16020 thus obtained was shown by Southern hybridization to have a disrupted lytR gene. Although the LytR protein seems to play an effector role in the expression of the lytABC operon (see above), our preliminary experiments did not detect any physiological alteration of the lytR strain L16020. Indeed, its turnover kinetics and cell wall autolysis were identical to those of the parent strain L5047 (not shown).

Discussion

The N-acetylmuramoyl-L-alanine amidase, one of the two major autolysins of *B. subtilis* 168, was shown not to be essential for vegetative growth (Margot & Karamata, 1992). It was suggested that the bulk of the activity of this CWBP, which has a high affinity for teichoic acid endowed peptidoglycan, is involved in flagellar morphogenesis (Pooley & Karamata, 1984). However, physical and genetic studies have revealed that the *in vivo* activity and biosynthesis of this enzyme are subject to a rather complex regulation (see Introduction). The sequencing reported here of the regulatory unit which contains lytC (Fig. 2), the structural gene of the amidase, largely confirms this view. The main features of this unit, the *lytRABC* divergon, are schematically represented in Fig. 6. The operon *lytABC*, transcribed in the direction of DNA replication, encodes an acidic, low-molecular-mass protein (LytA), as well as the modifier (LytB) and the amidase (LytC). The monocistronic operon *lytR*, transcribed in the opposite direction, encodes a 35 kDa protein (LytR) whose only function so far identified is to attenuate expression of both operons of the *lytRABC* divergon.

The analysis of the deduced amino acid sequences encoded by this divergon provides an incomplete picture of the nature and role of proteins LytA, B, C and R. Mature LytA is a 9-4 kDa, highly acidic polypeptide. Its sequence strongly suggests that it is a lipoprotein (Yamaguchi et al., 1988), anchored to the cytoplasmic membrane via the N-terminal glyceride-modified cysteinyl residue, and localized on its outer surface. By analogy to PrsA lipoprotein (Kontinen et al., 1991), it may play a role in the secretion of LytB and LytC. However, its inactivation affects neither cell growth nor export of the amidase. LytB, the so-called modifier, was shown *in vitro* to enhance the amidase activity by a factor of two to three. However, *in vivo* studies on strains with inactivated *lytB* or *lytC* genes leave open the question of its possible general amidase-enhancing activity or even of an intrinsic amidase activity (Margot & Karamata, 1992). Sequence homology studies revealed a high homology between LytB and LytC in three repetitive domains localized within the N-terminal moiety. These domains contain the consensus GSNRY motif found in nearly all amidases so far sequenced originating from different bacteria and phages (Table 4). The question of the role of this motif, i.e. recognition or catalytic activity remains open. Interestingly, the C-terminal moiety of the LytB protein exhibits a high homology with the *spoIID*-encoded protein (Lopez-Diaz et al., 1986), which supports the hypothesis that the latter is involved in cell wall metabolism during spore formation (Illing & Errington, 1991). LytC protein is responsible for the bulk of the amidase activity during vegetative growth. Strains with inactivated *lytC* are impeded neither in growth nor in cell separation. They have less than 3% of the amidase activity present in the parent strain and exhibit considerably reduced autolysis of either whole cells or native cell walls, as well as anomalies in cell wall turnover kinetics (Margot & Karamata, 1992). Finally, LytR was shown to play an attenuator role for both its own and *lytABC* operon expression as evidenced by considerably increased transcription frequency in a LytR-deficient strain (Fig. 5). It is noteworthy that an attenuator is endowed with a membrane-anchoring-like sequence. This basic protein is expressed at a rate five times higher than that of the amidase, suggesting an abundant protein which may play a role of a more general attenuator, affecting for example the expression of the other operons belonging to the flagellar regulon (see below). In addition, the motif identified in its deduced amino acid sequence is not a typical HTH one. It does not have a counterpart in inventoried DNA-binding proteins; the T region is too short to allow a full turn, and the whole motif is smaller than the 60 bp lower limit (Harrison & Aggarwal, 1990).

At the transcriptional level, the operon *lytABC* is under the control of a proximal *P*<sub>D</sub> and a more distal *P*<sub>λ,i</sub> promoter (Fig. 6) which, towards the end of the exponential growth phase, account for 70 and 30% of the amidase activity, respectively. The latter activity is in perfect agreement with amidase assays in *sigD* and *flaB* deficient strains (P. Margot, unpublished). Our observations reveal that absence of LytR is accompanied by a 4.4-fold enhancement of the *lytABC* operon transcription from *P*<sub>D</sub>. At the protein synthesis level it would appear that translation of *lytB* and *lytC* is subordinated to that of *lytA*. Indeed, the *lytB* and *lytC* parts of the polycistronic mRNA have UUG as start codon, which was reported to ensure coupled translation through translational reinitiation (Adhin & van Duin, 1989), i.e. only ribosomes fixed on a RBS of an upstream gene can translate a UUG-starting ORF. This system would account for equimolarity of LytB and LytC and suggests an absolute requirement of LytA for functioning of the operon. Nevertheless, strains with the *lytA*<sup>+</sup> *B*<sup>+</sup> *C*<sup>+</sup> genotype not only grow normally but are endowed with LytC at a
higher than normal level. This leaves open the question of the universality of the model of Adhin & van Duin (1989). However, a very strong promoter of the erythromycin resistance gene, from which \( \text{ZytC} \) is expressed in the mutant investigated, could account for the translation of \( \text{ZytC} \), even if the ribosomes have a small chance of staying attached in the long nontranslated intergenic region present in \( \text{lytA}^-\text{B}^-\text{C}^+ \) strains. The transcriptional control of \( \text{lytR} \) is mediated by two promoters, one of which, \( P_{A,2} \), has a good consensus to \( \sigma^A \)-controlled promoters while the other, \( P_x \), a relatively strong one and located upstream of \( P_{A,2} \), exhibits at best a very poor homology to a \( P_A \) consensus. LytR seems to be a self-attenuator since in a LytR-deficient strain expression

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**Table 4.** The five-amino-acid motif common to proteins endowed with an N-acetylmuramoyl-L-alanine amidase activity and the modifier of the \( B. \) subtilis amidase

<table>
<thead>
<tr>
<th>Protein, organism</th>
<th>Total amino acids</th>
<th>Position of relevant motif</th>
<th>Motif</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{LytB, Bacillus subtilis} )</td>
<td>705</td>
<td>67</td>
<td>G A S R Y</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>165</td>
<td>G K N R Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>255</td>
<td>G S T R Y</td>
<td></td>
</tr>
<tr>
<td>( \text{LytC, Bacillus subtilis} )</td>
<td>496</td>
<td>33</td>
<td>G S N R Y</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>132</td>
<td>G S N R Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>226</td>
<td>G S N R Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>406</td>
<td>G S E T Y</td>
<td></td>
</tr>
<tr>
<td>( \text{CwlA, Cell wall hydrolase, Bacillus subtilis} )</td>
<td>272</td>
<td>83</td>
<td>G T gN R K</td>
<td>Kuroda &amp; Sekiguchi (1990)</td>
</tr>
<tr>
<td>( \text{N-Acetylmuramoyl-L-alanine amidase, Bacillus sp.} )</td>
<td>251</td>
<td>81</td>
<td>G R gN R H</td>
<td>Potvin et al. (1988)</td>
</tr>
<tr>
<td>( \text{Cell wall hydrolase, Streptococcus faecalis} )</td>
<td>667</td>
<td>352</td>
<td>G S N N Q</td>
<td>Béliveau et al. (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>419</td>
<td>G S N N N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>487</td>
<td>G S N N N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>555</td>
<td>G S N N N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>358</td>
<td>G T N T Y</td>
<td></td>
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<td></td>
<td></td>
<td>426</td>
<td>G T N T Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>494</td>
<td>G T N T Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>562</td>
<td>G T N T Y</td>
<td></td>
</tr>
<tr>
<td>( \text{LytA, N-acetylmuramoyl-L-alanine amidase, Staphylococcus aureus} )</td>
<td>481</td>
<td>86</td>
<td>G S N Y G</td>
<td>Wang et al. (1991)</td>
</tr>
<tr>
<td>( \text{CPL-1, N-acetylmuramoyl-L-alanine amidase, bacteriophage CP-1} )</td>
<td>342</td>
<td>197</td>
<td>G S N P K</td>
<td>Garcia et al. (1990)</td>
</tr>
<tr>
<td>( \text{CPL-7, N-acetylmuramoyl-L-alanine amidase, bacteriophage CP-7} )</td>
<td>339</td>
<td>216</td>
<td>G S K P Y</td>
<td>Garcia et al. (1990)</td>
</tr>
<tr>
<td>( \text{CPL-9, N-acetylmuramoyl-L-alanine amidase, bacteriophage CP-9} )</td>
<td>339</td>
<td>216</td>
<td>G S K P Y</td>
<td>Garcia et al. (1990)</td>
</tr>
<tr>
<td><strong>Consensus motif</strong></td>
<td></td>
<td>( \begin{array}{c} 28 \ 15 \ 16 \ 13 \ 20 \end{array} )</td>
<td>( \begin{array}{c} 8 \ 15 \ 16 \ 13 \ 20 \end{array} )</td>
<td></td>
</tr>
</tbody>
</table>
from $P_{A,2}$ is increased twofold while that formerly associated with $P_X$ is displaced, and apparently originates from at least three sites which are close to that present in the $lytR^+$ strain. Whether the latter phenomenon is due, at least in part, to the destabilization of mRNA remains to be investigated.

The global picture of the regulatory region of the $lytRABC$ divergon, discussed above, allows a reassessment of the role of different mutants affecting the expression of the amidase. The effect of the $flaA4$ mutation (Pooley & Karamata, 1984), which was shown to cause deficiency in the synthesis of the flagellum basal body (Albertini et al., 1991; Hauser et al., 1991) reveals the possibility of a feedback inhibition at the level of SigD synthesis. Indeed, reduction of about 70% of the amidase activity in $flaA4$-bearing strain (H. M. Pooley, unpublished; S. Richard, unpublished) corresponds to absence of transcription of the $lytABC$ operon from $P_D$. Our observations shed hardly any light on the behaviour of mutants deficient at the level of the $sin$ ($flaD$) gene (Sekiguchi et al., 1990), which was reported to encode a repressor (Gaur et al., 1991). Inactivation of $sin$ by insertional mutagenesis leads again to a 70% decrease of the amidase activity, suggesting that the Sin protein is an effector of $P_D$. A possible explanation for the total absence of amidase activity in $flaD$-bearing mutants, revealed by their inability to lyse cell walls contained in solid media, could be due to mutations provoking firm binding of the protein to an operator or to an effector with the result of blocking expression from $P_D$ and thus of the $P_A$ promoter located upstream. Absence of other $\sigma^D$-controlled proteins (Mirel & Chamberlin, 1989; P. Margot, unpublished) in $flaD$-bearing strains could mean that Sin is an effector for all $P_D$ promoters which are inactivated in strains carrying $flaD1$ or $flaD2$ mutations. Finally, our observations provide a working hypothesis to explain the Ifm phenotype. Mutants with a two- to sevenfold higher amidase activity (H. M. Pooley, unpublished) owe their phenotype to at least two mutations localized in the chromosomal region encompassing the $lytRABC$ divergon (Pooley & Karamata, 1984). That the rate of the amidase expression in $lytR$-bearing strains is increased four- to fivefold may not be sufficient to ensure a comparable increase at the CWBP49 level due to difficulties with protein export. Thus, the secondary mutation(s) in Ifm strains could affect one or more $pre$ (Kontinen & Survas, 1988) or other genes whose products are involved in protein export.

The partial integration of the $lytABC$ operon within the $fla$ regulon is in agreement with a strongly impaired motility of $lytC$-deficient strains on semi-solid plates (Margot & Karamata, 1992) and provides further support for the hypothesis (Fein & Rogers, 1976; Pooley & Karamata, 1984) that the bulk of the amidase activity is required for the morphogenesis of the flagellum, in particular of its basal body. Finally, although comparison of the $P_D$ and SigD sequences with those controlling the flagellar motility regulons in $E. coli$ and $Salmonella typhimurium$ reveals a significant homology (Helmann & Chamberlin, 1987; Helmann et al., 1988; Bartlett et al., 1988; Ohnishi et al., 1990), $lyt$ genes of the latter organisms have not been shown to be integrated into the $fla$ regulon.

We are grateful to Dr M. Chamberlin, University of Berkeley, for providing plasmid pLM5, and for communicating unpublished observations.

References


Sequencing of the lytRABC divergon in B. subtilis


