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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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 *spoIID*. 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: P_D, identified as P_{28–7} (Gilman *et al.*, 1984), and an upstream P_A. 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 P_A 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*-acetylglucosaminidase (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 insertional 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 peptidoglycan 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).

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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.

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 *sigD* (*flaB*), the gene which encodes the σ factor required for the expression of the flagellar regulon (Marquez *et al.*, 1990). That mutational inactivation of *sigD* reduces the amidase activity to about 30% of its wild-type level (Marquez *et al.*, 1990) suggests that expression of this gene can proceed from another promoter. The regulatory role of the product of the gene *sin*, a repressor of *aprE*, the alkaline protease gene (Gaur *et al.*, 1991), is more complex: disruption of *sin* (*flaD*) is accompanied by a decrease of the amidase activity comparable to that in a *sigD* strain (P. Margot, unpublished), suggesting an effector role for Sin. However, more paradoxically, certain mutations in the *sin* gene (*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 *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° between *hisA* and *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 *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).

Methods

Bacterial strains, plasmids and phage. These are listed in Table 1. Fragments from the *B. subtilis* chromosomal DNA insert in phage λ 63 (Mauël *et al.*, 1989) were subcloned in pMTL20EC (Ap^r Em^r Cm^r)

Table 1. *Bacterial strains, plasmids and bacteriophage*

Name	Genotype or description	Origin
<i>Escherichia coli</i>		
DH5	F ⁻ <i>endA1 hsdR17</i> (r ⁻ m ⁺) <i>supE44 thi-1 λ-recA1 gyrA96 relA1 Δ(argF-lacZYA)U169 ϕ80ΔlacZΔM15</i>	P. Shaw, University of Lausanne
<i>Bacillus subtilis</i>		
L5047	<i>pheA1 purA16 hisA35 trpC2 metB5</i>	Mauël <i>et al.</i> (1989)
L16006	<i>pheA1 purA16 hisA35 trpC2 metB5 sigD::pLM5</i>	Integration of pLM5 into the <i>sigD</i> locus of L5047
L16013	<i>pheA1 purA16 hisA35 trpC2 metB5 lytAΩ::cat</i>	Insertion of 1.1 kb fragment (<i>cat</i>) from pL1 into the <i>lytA</i> locus of L5047
L16015	<i>pheA1 purA16 hisA35 trpC2 metB5 lytAΩ::cat lytB::p6325Δ1</i>	Integration of p6325 Δ 1 into the <i>lytB</i> locus of L16013
L16016	<i>pheA1 purA16 hisA35 trpC2 metB5 lytB::p6325Δ1</i>	Integration of p6325 Δ 1 into the <i>lytB</i> locus of L5047
L16020	<i>pheA1 purA16 hisA35 trpC2 metB5 lytR::pA2</i>	Integration of pA2 into the <i>lytR</i> locus of L5047
Phage		
λ 63	Contains 17.3 kb fragment of <i>B. subtilis</i> 168 chromosomal DNA	Mauël <i>et al.</i> (1989)
Plasmids		
pJH101	Ap ^r Tc ^r Cm ^r	Ferrari <i>et al.</i> (1983)
pLM5	Ap ^r Cm ^r	Marquez <i>et al.</i> (1990)
pMTL20EC	Ap ^r Em ^r Cm ^r	Chambers <i>et al.</i> (1988)
pMTL21	Ap ^r	Chambers <i>et al.</i> (1988)
pWD1	Cm ^r	H. Wood, Trinity College Dublin
p6325 Δ 1	Ap ^r Em ^r	Deletion of 1.1 kb <i>EcoRI</i> - <i>StuI</i> fragment from p6325
p6327 Δ 77	Ap ^r Em ^r Cm ^r	Deletion of 0.4 kb fragment comprising intergenic region between <i>lytA</i> and <i>lytR</i> from p6327
pV3	Ap ^r	Cloning of 1.6 kb <i>HindIII</i> fragment from p6324 into the same site of pMTL21; <i>EcoRV</i> site of pMTL21 lost
pL1	Ap ^r Cm ^r	Cloning of 1.1 kb <i>EcoRI</i> fragment from pWD1 into the <i>EcoRV</i> site of pV3
pA2	Ap ^r Em ^r Cm ^r	Cloning of 0.4 kb <i>HindIII</i> fragment from p6327 Δ 77 into the same site of pMTL20EC

(Chambers *et al.*, 1988) except for the insert in plasmid 6302, which was cloned in pJH101 (Ap^r Tc^r Cm^r) (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). λ 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 cloning, 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 pMTL20EC 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). Labelling reactions were performed with a Sequenase Version 2.0 kit (USB) and [α -³²S]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 erythromycin 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 alkali blotting (Reed & Mann, 1985).

Amidase and modifier detection. Cells grown to a concentration of 10⁸ cells ml⁻¹ in SA medium were washed with cold double-distilled water. Cell wall proteins were extracted with 5 M-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 10⁸ 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 10⁸ 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 sonications at 30 W with the microtip of an MSE sonifier, separated by 10 s intervals. Three extractions with phenol/chloroform/isoamyl 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'GCCGCCAGTTCGAGTACAAGAAG3' and 5'ACTATTAACCCCGCAACCCGAAG3' 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

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.

[illegible]

Fig. 2. Nucleotide sequence of the 5280-base region. Both strands of DNA are shown only in the intergenic region between *lytA* and *lytR*, and downstream from *lytC* and *lytR*. Asterisks denote stop codons. Endonuclease cleavage sites are underlined. Deduced amino acid sequences are given in single-letter code. The N-terminal amino acid signal peptide sequences (von Heijne, 1986; Yamaguchi *et al.*, 1988) of LytA, LytB and LytC proteins are underlined. Ribosome-binding sites (only bases complementary to 16S rRNA) are doubly underlined. -35 and -10 promoter regions are marked. Converging arrowheads indicate dyad symmetry. Vertical arrows indicate start points of transcription. Nonamer repeats ANAATGAAA are boxed.

Property	<i>lytR</i> 183*	<i>lytA</i> 23*	<i>lytB</i> 38*	<i>lytC</i>
Size of the ORF (bp)	918	306	2115	1488
Start codon	AUG	AUG	UUG	UUG
Stop codon	UAA	UAA	UGA	UAA
TA in wobble position (%)	0.54	0.74	0.70	0.72
CAI†	0.49	0.44	0.46	0.44
Δ <i>G</i> for RBS (kcal mol ⁻¹)‡	-13.8	-16.0	-14.4	-19.8
TA (%)	0.58	0.64	0.62	0.61

‡ Calculated according to the rules of Tinoco *et al.* (1973)
(1 kcal = 4.184 kJ).

picomoles was radiolabelled in a 50 μ l mixture with 20 units of T4 polynucleotide kinase (Biofinex) and 100 μ Ci (3.7 MBq) of [γ - 32 P]ATP (> 5000 Ci mmol^{-1} , Amersham), and purified by ethanol precipitation (Sambrook *et al.*, 1989). Primer extensions were performed with 1.5 pmol of these probes and 30 to 50 μ g of total cellular RNA according to Jones *et al.* (1985). Primers were annealed to RNA for 60 min at 55 $^{\circ}\text{C}$ in 10 μ l of 10 mM-Tris/HCl pH 8.0, 1 mM-EDTA, 1.25 M-KCl, cooled to room temperature, centrifuged briefly, and diluted with 47 μ l of reverse transcription buffer [20 mM-Tris/HCl, 10 mM-MgCl₂, 5 mM-dithiothreitol, 0.35 mM-dNTPs, 100 μ g actinomycin D ml^{-1} , 0.1 unit RNAase inhibitor (Biofinex) μl^{-1}] to which 12 units of AMV reverse transcriptase (Biofinex) was added. Primer extensions were incubated for 60 min at 37 $^{\circ}\text{C}$, extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. Pellets were resuspended in 5 μ l 50% (v/v) formamide and loaded onto polyacrylamide sequencing gels. After autoradiography, bands were cut off the gel and their radioactivity measured in a liquid scintillation spectrometer using the ^3H window (Cerenkov radiation).

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) fulfils 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). ΔG 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,

Table 3. Features of amino acid products deduced from the nucleotide sequences of the *lytR*, *A*, *B* and *C* genes

Property	LytR	LytA	LytB	LytC
Uncleaved				
No. of amino acids	306	102	705	496
Mol. mass (kDa)	34.6	11.2	76.7	52.6
Mature*				
No. of amino acids	306	86	680	472
Mol. mass (kDa)	34.6	9.4	74.1	49.9
Isoelectric point	10.0	3.9	10.3	10.6
Net charge	7	-13	26	23

* *LytA*, *LytB* and *LytC* proteins possess a signal peptide (Fig. 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 trans-membrane 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_{A,2}.

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

(a)

LytC	1	LRSYIKVLTMCFLGLILFVP---TALADNSVK-----RVGGSNRYGTAVQISKQMYSTASTAVIVGSSSYADAI	66
LytB	1	LKSCQQLIVCSLAAILLIPSVSFAADSNISVKLLNYIGNKSSISLSPTGFYKVTGDNVAVTDRFAGASRYETATLASNSQWKNPNTVILVNRDIFDAL	100
LytC	67	SAAPLAYQKNAPLLYNSDKLSYETKTRLKEMQTKNVIIVGGTPAVSSNTANQIKSLGISIKRIAGSNRYDTAARVAKAMGATSKAVILNGFLYADAPAV	166
LytB	101	PVIPLAKKLNAPVLFQPDTLTKTTERQIAKFNPNDILIIGGARSISKDVENKLKSYG AVKRISGKNRYVLSENIAKQMGSYDKAIVVTGRVFDALAI	199
LytC	167	IPYAAKNGYPILFTNKTSINSATTSVIKDKGISSTVVGGTGSISNTVYNKLPSP---RISGSNRYELAANIVQKLNLSSTVYVSNFGSPYDPSIAGAT	263
LytB	200	APYAAAHGYPILLTEKDKLPDYDLP-----KQVIIIGSSFSVSDSVENQIKKTSTVQRIPGSTRYELTANIIKQLKLKADKVVMTNGTKYADVLIGAS	292
LytC	264	LAAKKKQSLILTNGENLSTGARKIIGSKNMSNFMIIIGNTPAVSTKVNQLKNPVV---GETIFIDPGHGDQDSGAI---GNGLLEK-----	343
LytB	293	LASKKNSQILFVKQDSVPAAKSITKDKATYAYDFIGSTSSISAEVENS LADEFYLADGGTYNLKINSGLNLENIKTYGNSLRIPENYSTSNRISLDG	392

(b)

LytB	344	DEFYLADGGTYNLKINSGLK---NLENIKTYGNSLRIPENYSTSNRISLDGKQYLGTVNFSIESTKYIRPVNENIPFEDYLKGVIPNEMPASWSLEALK	440
SpoIID	2	KQFAITLSVLCALILLVPTLLVIPFQHNKEAGAS--VESEKTAVSTKPAKGAETLKASPVSI PVYRTANQSVENIPLEEYVIGVVAEMPATFKPEALK	99
LytB	441	AQTVAARTYSI-----TKTGTTPDPTTAFQVYG-----GYSWNSNTNK---AVEQTKGKVLKYNGSLITAAAYSSSNGGYTEASNEVWSSSV	518
SpoIID	100	AQALAARTFIVRLMVSNSAVEAPKGSVLDQTMFQVYKSKAELKKQWGTSYETKLKKITDAVASTQGKILTYNNQPIEASFSTSNGYTENAEAYWTSAI	199
LytB	519	PYLIACK---DTKDPQIGWTLTSLKQQLDTSKLDLTKPSSWWSSATETDSARLSGVKNWILKNKETSADSVKIASIDDLFSGTTQGGRAKTASMKVYKF	615
SpoIID	200	PYLKSVKSPWDKSPKYKATKFTTAAEFQK-----LGVK-----LDGSSAVGKIT-----GETPGHQVATAVINGK--	261
LytB	616	VKSSTGSYNLSKITTSVPTSELRTMIGATVFKSTYVTVKKDTSKYTISGKGYGHGIGMSQYGAKARAEAGDSYSSILKFYYPGTTLS	704
SpoIID	262	-----TLKGRDIREKLG---LNSADFENKRNQDITVTTRGFGHGVMSQYGANFMAKEGKTVDDIVKYYYQGTQISE	331

Fig. 3. Comparison of the amino acid sequences: (a) LytC and the *N*-terminal moiety of LytB, (b) C-terminal moiety of LytB and SpoIID. Identical (i) and conserved (:) residues are indicated. Amino acid numbers are specified for the extremities of each segment.

LytB	64	RFAGASRYETATLASNSQWK.NPNTVILVNRDIFDALPVIPLAKKLNAPVLFQPDTLTKTTERQIAKFNPNDILIIGGARSISKDVENKLKSYGAVK
	162	RISGKNRYVLSENIA QMG.SYDKAIVVTGRVFDALATAPYAAAHGYPILLTEKDKLPDYDLP.....PKQVIIIGSSFSVSDSVENQIKKTSTVQ
	252	RIPGSTRYELTANII QQLKLKADKVVMTNGTKYADVLIGASLASKKNSQILFVKQDSVPAAKSITKDKATYAYDFIGSTSSISAEVENS LADEFYLA
LytC	30	RVGGSNRYGTAVQIS QMYSTASTAVIVGSSYADAI SAAPLAYQKNAPLLYNSDKLSYETKTRLKEMQTKNVIIVGGTPAVSSNTANQIKSLGISIK
	129	RIAGSNRYDTAARVA KAM.GATSKAVILNGFLYADAPAVIPYAAKNGYPILFTNKTSINSATTSVIKDKGISSTVVGGTGSISNTVYNKLPSP
	223	RISGSNRYELAANIV QKLNLSSTVYVSNFGSPYDPSIAGATLAAKKKQSLILTNGENLSTGARKIIGSKNMSNFMIIIGNTPAVSTKVNQLKNPVV
Consensus		Ri-GsnRYelaani- kqm----skvv-vnG--yaDala-aplAakkn-pilftn-d-l---t---ik-k---n---iiGgt-siS--veNqlks-----
		t ta

Fig. 4. Alignment of domains of LytB and LytC. The consensus line shows absolutely conserved residues (upper-case), consensus (at least three out of six residues conserved; lower-case); (-) absence of consensus according to given criteria; (.) no residue.

belonging to the predicted *lytABC* and *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 *et al.*, 1989). Indeed, for *lytR* and *lytABC* transcripts, the strength of the signals was directly proportional to the amount of RNA added, over the range from 10 to 100 μg of total wild-type RNA (not presented). Start sites could be localized by comparison with a sequencing ladder (Fig. 5). For *lytABC* transcripts, one site was found 8 nucleotides downstream of the end of the characteristic P_D promoter consensus (Fig.

2) (Gilman *et al.*, 1981). This transcript was absent in strain L16006, in which the *sigD* gene was disrupted, confirming the σ^D dependence of this site. This promoter corresponds indeed to P_{28-7} , belonging to the collection of P_D promoters identified by Gilman *et al.* (1984) and sequenced by Singer (1987). That this transcript was increased about fourfold in strain L16020, in which the divergently transcribed *lytR* gene was disrupted, provides strong evidence of an interdependence between operons *lytABC* and *lytR*. Analysis of *lytABC* transcripts (Fig. 5) revealed another start site, localized 21 nucleotides upstream of that initiated by σ^D . The former corresponds to a putative P_A promoter (Fig. 2), designated $\text{P}_{\text{A},1}$ (see below), which is localized 7 nucleotides

Fig. 5. Mapping by primer extension of the transcription start sites of the *lytR* and *lytABC* operons. Lanes 5 to 8, and 9 to 12 (GATC) show DNA sequencing ladders of the regions upstream of *lytR* and *lytABC*, respectively. Lanes 1 to 4 (probe *lytR*) and 13 to 16 (probe *lytABC*) show the primer extension analysis. The same probes were used for sequencing. RNA samples were as follows: lanes 1 and 16, no RNA; 2 and 15, RNA from strain L16020 *lytR*; 3 and 14, RNA from strain L16006 *sigD*; 4 and 13, RNA from the reference strain L5047.

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 σ^A , 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 p6325Δ1 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 p6327Δ77 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_D and a more distal $P_{A,1}$ 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_D . 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⁻B⁻C⁺* genotype not only grow normally but are endowed with LytC at a

Fig. 6. Schematic drawing of the *lytRABC* divergon with an enlargement of the regulatory region.

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

Protein, organism	Total amino acids	Position of relevant motif	Motif	Reference
LytB, <i>Bacillus subtilis</i>	705	67	G A S R Y	This work
		165	G K N R Y	
		255	G S T R Y	
LytC, <i>Bacillus subtilis</i>	496	33	G S N R Y	This work
		132	G S N R Y	
		226	G S N R Y	
		406	G S E T Y	
CwlA, Cell wall hydrolase, <i>Bacillus subtilis</i>	272	83	G TgN R K	Kuroda & Sekiguchi (1990)
		100	G G V R Y	
<i>N</i> -Acetylmuramoyl-L-alanine amidase, <i>Bacillus</i> sp.	251	81	G RgN R H	Potvin <i>et al.</i> (1988)
		98	G G A R Y	
Cell wall hydrolase, <i>Streptococcus faecalis</i>	667	352	G S N N Q	Béliveau <i>et al.</i> (1991)
		419	G S N N N	
		487	G S N N N	
		555	G S N N N	
		358	G T N T Y	
		426	G T N T Y	
		494	G T N T Y	
LytA, <i>N</i> -acetylmuramoyl-L-alanine amidase, <i>Staphylococcus aureus</i>	481	562	G T N T Y	Wang <i>et al.</i> (1991)
		86	G S N Y G	
CPL-1, <i>N</i> -acetylmuramoyl-L-alanine amidase, bacteriophage CP-1	342	197	G S N P K	Garcia <i>et al.</i> (1990)
		220	G Q E R Y	
		268	G Q E R Y	
CPL-7, <i>N</i> -acetylmuramoyl-L-alanine amidase, bacteriophage CP-7	339	316	G Q E R Y	Garcia <i>et al.</i> (1990)
		216	G S K P Y	
		268	G S E W Y	
CPL-9, <i>N</i> -acetylmuramoyl-L-alanine amidase, bacteriophage CP-9	339	216	G S K P Y	Garcia <i>et al.</i> (1990)
		268	G S E W Y	
Consensus motif			G S N R Y	
			28 15 16 13 20	

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 *lytC* is expressed in the mutant investigated, could account for the translation of *lytC*, even if the ribosomes have a small chance of staying attached in the long nontranslated intergenic

region present in *lytA*⁻*B*⁻*C*⁺ strains. The transcriptional control of *lytR* is mediated by two promoters, one of which, P_{A,2}, has a good consensus to σ^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

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 a *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 *flaD2*-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 σ^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 *prs* (Kontinen & Sarvas, 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.

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