Cloning, sequencing and genetic mapping of a *Bacillus subtilis* cell wall hydrolase gene

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We have cloned DNA fragments from *Bacillus subtilis* 168S into *Escherichia coli*, which produced a lytic zone on an agar medium containing *B. subtilis* cell wall. Sequencing of the fragments showed the presence of an open reading frame (ORF) which encodes a polypeptide of 272 amino acids with a molecular mass of 29919 Da. The deduced amino acid sequence showed considerable homology with that of the cell wall hydrolase gene of *Bacillus* sp. (Potvin, C., Leclerc, D., Tremblay, G., Asselin, A. & Bellemare, G. (1988). *Molecular and General Genetics* 214, 241–248). Accordingly, the gene was designated *cwlA*, for cell wall lysis. The N-terminal amino acid sequence of *cwlA* gene product prepared from a *E. coli* clone was AIKVVKNLVSKSKYGLKCPN, which is consistent with that of the deduced sequence starting from Ala at second position from the initiation codon of the *cwlA* gene. A presumed σ^A promoter and a rho-independent terminator were found upstream and downstream of the ORF, respectively. A chloramphenicol-resistance determinant integrated into the ORF was mapped by PBS1 transduction, which indicated the gene sequence *dnaE-aroD-cwlA*.

Introduction

Bacillus subtilis produces cell wall lytic enzymes, some of which are known as autolysins. The major autolysins are the *N*-acetylmuramyl-L-alanine amidase (ν -amidase; Herbold & Glaser, 1975; Rogers *et al.*, 1984) and the endo- β -*N*-acetylglucosaminidase (glucosaminidase; Rogers *et al.*, 1984). These hydrolases affect cell lysis (Fein & Rogers, 1976; Rogers *et al.*, 1980), cell separation (Fein & Rogers, 1976), the formation of helices (Mendelson, 1982), flagellation (Fein, 1979) and competence (Ayusawa *et al.*, 1975; Akamatsu & Sekiguchi, 1987a). During the sporulation stage, two other cell wall hydrolases, s-amidase and γ -D-glutamyl-(L)mesodiaminopimelyl endopeptidase, are formed; they are considered to be associated with the formation of cortex peptidoglycans (Guinand *et al.*, 1976). Some *B. subtilis* strains (K-77 and YT-25) produce extracellular endo- β -*N*-acetylmuramidases (muramidases; Okada & Kitahara, 1973; Murao & Takahara, 1974), however, no muramidase activity has been detected in the culture supernatant of *B. subtilis* 168 (Rogers *et al.*, 1980). In

spite of the extensive biochemical studies, none of the cell wall hydrolase genes of *B. subtilis* have been cloned.

We report here the cloning of a gene, designated *cwlA*, that directs the synthesis of a wall hydrolytic enzyme of *B. subtilis*, together with its complete nucleotide sequence, its mapping and the N-terminal amino acid sequence of the gene product.

Methods

Bacterial strains, plasmids, phages and media. The strains, plasmids and phages used in this study are listed in Table 1. *B. subtilis* and *E. coli* were grown in NB medium (10 g meat extract, 10 g polypeptone and 3 g NaCl per litre, pH 7.0) and LB medium (5 g yeast extract, 10 g polypeptone and 10 g NaCl per litre, pH 7.2) at 37 °C, respectively. For determination of auxotrophic requirements, Spizizen minimal medium (Spizizen, 1958) was used. Schaeffer agar medium (Schaeffer *et al.*, 1965) was also used for culturing *B. subtilis*.

Preparation of *B. subtilis* cell wall. Cell walls were prepared essentially as described by Fein & Rogers (1976). *B. subtilis* cells harvested from a stationary-phase culture (15 l) were suspended in cold deionized water (50 ml) and disrupted with a bead-beater (Biospec) in a vessel containing glass beads (diam. 0.1 mm). After low-speed centrifugation (1400 g, 10 min) to remove the glass beads and unbroken cells, the crude cell wall was pelleted at 27000 g for 5 min at 4 °C, suspended in 20 ml of a 4% (w/v) sodium dodecyl sulphate solution and boiled for 10 min. After several washes with a 1 M-NaCl solution and deionized water, the cell wall was stored at –80 °C.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M37710.

Table 1. Bacterial strains, phages and plasmids

Genotype		Source or reference*
<i>Bacillus subtilis</i>		
168S	<i>trpC2 strA smo-1</i>	Sekiguchi <i>et al.</i> (1988)
AC310	<i>purB tet-1 smo-1</i>	Sekiguchi <i>et al.</i> (1988)
QB936	<i>ald-1 aroG932 leuA8 trpC2</i>	BGSC (kit 7)
AC705	<i>lys dnaE20 aroD120 asaA4 smo-1</i>	Sekiguchi <i>et al.</i> (1988)
17cmE	<i>trpC2 strA smo-1 cwlA::cat</i>	This study
<i>Escherichia coli</i>		
JM109	<i>recA1 Δ(lac-proAB) endA1 gyrA96 thi-1 hsdR17 relA1 supE44</i> [F': <i>traD36 proAB lacI^sΔM15</i>]	Takara
MV1184	<i>ara Δ(lac-pro) strA thi (φ80 lacZΔM15) Δ(srl-recA) 306::Tn10(tet^r)</i> [F': <i>traD36 proAB lacI^sΔM15</i>]	Takara
Phage		
PBS1		BGSC
M13KO7		Takara
Plasmid		
pUC19	Ap ^r Lac ⁺	Yanisch-Perron <i>et al.</i> (1985)
pUC118	Ap ^r Lac ⁺	Takara
pUC119	Ap ^r Lac ⁺	Takara
pUD1	Ap ^r Cm ^r	Sekiguchi <i>et al.</i> (1988)

* BGSC, Bacillus Genetic Stock Center, The Ohio State University; Takara, Takara Shuzo Co., Kyoto, Japan.

Preparation of plasmids, DNA manipulations and gel electrophoresis. *E. coli* plasmids were prepared by the method of Guerry *et al.* (1973). Restriction enzymes [Nippon Gene, except for *Sau3AI* (Takara)], *Bal31* (Takara) and *E. coli* DNA polymerase I (Klenow fragment, Takara) were used according to the manufacturers' recommendations. Agarose (1%, agarose S; Nippon Gene) gel electrophoresis was performed by the method of Sharp *et al.* (1973).

Gene library of *B. subtilis* chromosomal DNA in *E. coli*. *B. subtilis* 168S chromosomal DNA (10 µg), prepared as described by Saito & Miura (1963) and partially digested with *Sau3AI*, was subjected to agarose gel electrophoresis. Fragments in the size range 2–10 kb were recovered from agarose gels using Gene Clean (Bio101). The DNA solution was mixed with pUC19 DNA (10 µg), which had been digested with *Bam*HI, followed by dephosphorylation with calf intestine alkaline phosphatase (Boehringer), and ligated with a ligation kit (Takara) for 30 min. The ligated DNA solution was added to competent *E. coli* JM109 cells. The ampicillin-resistant (Ap^r) colonies were selected on LB agar plates containing 50 µg ampicillin ml⁻¹, and used as the gene library.

Selection of *E. coli* harbouring a *B. subtilis* cell wall hydrolase gene. The Ap^r transformants were transferred with toothpicks to LB agar plates containing *B. subtilis* cell wall (0.5 g l⁻¹) and ampicillin (50 µg ml⁻¹), followed by incubation for 1 to 2 d at 37 °C. Colonies (N6 and N47) exhibiting a zone of lysis (clear zone) were selected. Clones N6 and N47 harboured 6.0 kb (pBA6) and 9.1 kb (pBA47) plasmids, respectively.

Construction of pBA47 derivative plasmids. A 2.3 kb *Eco*RI fragment of pBA47, which has an overlap with the pBA6 insert, was subcloned into the *Eco*RI site of pUC118 (Fig. 1). The resulting plasmid (pBA47E) was digested at the *Pst*I site among the multicloning sites. For construction of pBA47B1 and pBA47B2, the linearized pBA47E DNA was digested with *Bal31* and the deleted fragments were made blunt with the Klenow fragment. After digestion with *Eco*RI, the *Eco*RI-blunt fragments were recovered by agarose gel electrophoresis, ligated to the *Eco*RI and *Sma*I sites of pUC118 DNA, and transformed into *E. coli* MV1184. For construction of pBA47ES, the 1.35 kb *Sau3AI* (S1)–*Eco*RI fragment of pBA47E was cloned into the *Bam*HI and *Eco*RI sites of pUC118. For pBA47ESR, the 1.35 kb *Xba*I–*Eco*RI

fragment of pBA47ES was cloned into the *Xba*I and *Eco*RI sites of pUC119. For pBA47EH, a *Hind*III site among the multicloning sites of pBA47ES was lost by partial digestion with *Hind*III followed by filling reaction with the Klenow fragment. The resultant plasmid, pBA47ES1, had only one *Hind*III site. The *Hind*III-digested pBA47ES1 fragment and a 0.9 kb *Eco*RI–*Hind*III fragment containing the *cat* gene derived from pUD1 (Sekiguchi *et al.*, 1988) were made blunt with the Klenow fragment and subjected to ligation.

DNA sequencing. Nucleotide sequencing was carried out by the dideoxy-chain termination method (Sanger *et al.*, 1977) with a modified T7 polymerase (Sequenase). Plasmids pUC118 and pUC119 (Takara) were used to generate templates for sequencing, and phage M13KO7 (Takara) was used as a helper phage. Electrophoresis was performed on 8% (w/v) polyacrylamide/8 M-urea gels. The sequence of both strands was determined for the 1.1 kb region between sites S1 and B1.

Preparation of a cell wall hydrolase. *E. coli* MV1184 cells harbouring pBA47ES, pBA47ESR or pUC119 were cultured with shaking in LB medium containing ampicillin (50 µg ml⁻¹) until early exponential phase was reached. Then isopropyl β-D-thiogalactoside (IPTG) was added to a portion of cultures at a final concentration of 1 mM. After 6 h incubation at 37 °C, the cell density was measured as OD₅₄₀, and the 1 ml cultures were exposed to ultrasonication (Tomy BH-200P; pulse time of 30 s, five times, at a power of 180 W). More than 90% of the cells were disrupted by this treatment. After centrifugation, the supernatants were used as enzyme solutions.

Assay of cell wall lytic activity. Lytic activity was measured as described by Ayusawa *et al.* (1975). The reaction mixture (5 ml) consisted of *B. subtilis* cell wall (to a final OD₅₄₀ of 0.3) in TK buffer [0.1 M-Tris/HCl (pH 8.0), 0.1 M-KCl] and the enzyme extract, and was incubated with slow shaking at 37 °C. Each assay was routinely performed twice. One unit (U) of the enzyme activity was defined as the amount of enzyme necessary to decrease the OD₅₄₀ by 0.001 in 1 min.

N-terminal amino acid sequence. The N-terminal amino acid sequence of the cloned enzyme purified from *E. coli* JM109(pBA47) was determined by an automatic peptide sequencer (Applied Biosystems model 470A).

Fig. 1. Restriction map of the inserts of pBA6, pBA47 and its derivatives, and the corresponding region of the *B. subtilis* chromosome. The cell wall lytic activities of the plasmids in *E. coli* are also given. The construction of derivative plasmids is described in Methods. Lytic activity was identified by the appearance of clear zones around colonies harbouring the plasmids. The open blocks represent pUC19 or pUC119 vector DNAs. Stippled and hatched blocks represent pUC118 and its multiple cloning site regions, respectively. Small arrows indicate the direction of the *lac* promoter. Relevant *Sau*3AI sites are indicated. Abbreviations: E, *Eco*RI sites; H, *Hind*III; P, *Pst*I; S, S1 and S2, *Sau*3AI; Sm, *Sma*I; B, *Bam*HI; B1 and B2, endpoints of deletions generated by *Bal*31 nuclease; Cm, the 1.0 kb fragment containing the chloramphenicol acetyltransferase gene (*cat*) from pUD1 (Sekiguchi *et al.*, 1988). The large arrow represents the coding region of the hydrolase gene and indicates the transcriptional direction.

Transformation and transduction of *B. subtilis*. Transformation of *B. subtilis* was performed according to the procedure of Anagnostopoulos & Spizizen (1961). Chloramphenicol-resistant (Cm^r) transformants were selected on NB agar plates containing 5 μg chloramphenicol ml^{-1} . PBS1 transduction was performed as described by Dubnau *et al.* (1967). For evaluating the competence of *B. subtilis* 17cmE and control 168S, *B. subtilis* AC310 DNA was used as donor DNA and Trp^+ transformants were selected.

Results

Cloning of a *B. subtilis* cell wall hydrolase gene

Chromosomal DNA of *B. subtilis* 168S was partially digested with *Sau*3AI and ligated with *Bam*HI-digested pUC19. *E. coli* JM109 was transformed with the ligation mixture and plated on LB agar containing ampicillin. After overnight culturing, transformants were picked onto LB agar plates containing *B. subtilis* cell wall (0.5 g l^{-1}) and ampicillin, and incubated for 1 to 2 d at 37 °C. Among 5000 Ap^r transformants, two colonies, N6 and N47, were surrounded by weak clear zones. Clone N47 showed a larger clear zone on an agar plate containing IPTG and the cell wall than did clone N6. Restriction site analysis of plasmids isolated from strains

N6 and N47 indicated the presence of 6.0 kb (pBA6) and 9.1 kb (pBA47) plasmids, which contain 3.3 kb and 6.4 kb DNA inserts, respectively. The inserts had an overlapping region of 2.2 kb.

Derivatives of pBA47 were constructed and the lytic activity of *E. coli* harbouring the plasmids was examined on LB agar plates containing ampicillin and cell wall (Fig. 1). Clear zones were observed for pBA47E, pBA47B1, pBA47B2, pBA47ES and pBA47ESR, but not for pBA47EH, suggesting that the hydrolase gene is located between *Sau*3AI site S1 and site B2. When *E. coli* MV1184 harbouring pBA47ES and pBA47ESR were incubated in LB medium containing ampicillin and IPTG (1 mM), lytic activities were 0.12 and 22.2 U ml^{-1} per OD_{540} unit, respectively. Without IPTG they were 0.39 and 1.8 U ml^{-1} per OD_{540} unit, respectively. The control, *E. coli* MV1184(pUC19), with or without IPTG, gave an activity of less than 0.03 U ml^{-1} per OD_{540} unit. The DNA fragment inserted in pBA47ES had an orientation opposite to that of the insert in pBA47ESR, downstream from the *lac* promoter. There may, therefore, be a weak promoter on the S1 site side in pBA47ES (Fig. 1), the hydrolase gene being transcribed from the S1 to the B1 site.

Fig. 2. Nucleotide sequence of the *B. subtilis* *cwlA* gene. Only the sequence of the nontranscribed DNA strand is shown, from position +1 [*Sau*3AI (S1) site]. The deduced amino acid sequence is given above the nucleotide sequence. Putative promoter sequences (–35 and –10 regions of the vegetative promoter) and terminators (inverted repeat sequences, 55–81 and 941–967), as well as a putative ribosome-binding sequence (SD) (97–107) are indicated. The N-terminal amino acid sequence of the *cwlA* protein prepared from *E. coli* JM109(pBA47) is overlined. B1 and B2 are the sites shown in Fig. 1. *** indicates a stop codon.

Fig. 3. Comparison of the deduced amino acid sequence of the *cwlA* gene to that of the *Bacillus* sp. cell wall hydrolase gene (Potvin *et al.*, 1988) – upper and lower sequences, respectively. Identical amino acids are indicated by colons. Similar and unrelated amino acids are indicated by dots and blanks, respectively. pBA47B2 and pBH703 (Potvin *et al.*, 1988) are truncated clones whose C-terminals were connected to the 22- and 3-amino acid peptides at the arrow positions, respectively. Downward pointing arrows indicate putative signal sequence cleavage sites (von Heijne, 1986). Conservative substitutions: I, L, V and M; K and R; S and T; D and E; F and Y; N and Q; G and A.

Southern hybridization of a *Hind*III and *Eco*RI double-digested chromosomal DNA from *B. subtilis* with a ³²P-labelled 2.3 kb *Eco*RI fragment of pBA47E showed three hybridized bands (0.45 kb, 0.9 kb and 1.6 kb), the former two corresponding to the 0.45 kb *Eco*RI–*Hind*III and 0.9 kb *Hind*III fragments, respectively, in the insert of pBA47E (data not shown). These data suggest that the cloned DNA was a fragment of the *B. subtilis* chromosomal DNA and did not have extensive rearrangements.

Nucleotide sequence of the *B. subtilis* cell wall hydrolase gene

The S1–B1 fragment, of approximately 1.1 kb, was sequenced (Fig. 2). The sequence showed one long ORF, starting at nucleotide 114 and ending at nucleotide 929, which encodes a polypeptide of 272 amino acid residues with a molecular mass of 29919 Da. A putative signal sequence cleavage site was found after Ala₃₉ in the sequence Ala₃₇–Ser₃₈–Ala₃₉–Ala₄₀ (Fig. 3). The first ATG was preceded by a possible ribosome-binding site, AGAAGGAGAGA ($\Delta G = -14.9$ kcal mol⁻¹; the consensus sequence is underlined) and a potential rho-independent terminator followed the ORF. Interestingly, another potential rho-independent terminator was found upstream of the ORF. The sequences at nucleotide 17 (TTGAGA, putative –35 sequence) and nucleotide 40 (AAGAAT, putative Pribnow box), and the 17 bp space between the two suggested the existence of a σ^A

promoter, which is recognized by the major *E. coli* RNA polymerase (Hawley & McClure, 1983).

The cell wall hydrolase was extracted from *E. coli* JM109(pBA47) cells and purified using hydroxyapatite column chromatography and HPLC (TSK-gel G3000SW_{XL}; results not shown). The N-terminal amino acid sequence was AIKVVKNLVSKSKYGLKCPN, which is consistent with that of the deduced sequence starting from Ala at nucleotide 117 in Fig. 2.

Comparison of the deduced amino acid sequence of the ORF with other protein sequences revealed an overall identity of 35% (on pairwise comparison) with the *Bacillus* sp. cell wall hydrolase gene, reported to be a major autolysin (Fig. 3; Potvin *et al.*, 1988). The homologous sites were located in the N-terminal and central regions of the protein. Therefore the ORF was tentatively designated *cwlA* (cell wall lysis). Plasmid pBA47B2, a truncated clone of the *cwlA* gene (57 deduced amino acid residues from the C-terminus were replaced by 22 deduced amino acid residues from pUC118) retained lytic activity (Figs 1 and 3). Therefore, it would appear that the essential site(s) for the enzyme activity is in the N-terminal to central region of the protein. Computer analysis (IDEAS sequence analysis system on the NBRF protein database, Release 16.0, Kyushu University) revealed a striking similarity between the C-terminal sequence of the *cwlA* gene product and the N-terminal sequence of muramoyl-pentapeptide carboxypeptidase of *Streptomyces albus* G

200	210	220	230	240	250	260
SGIFKVKSPMMRGEKVTQIQKALAA	LYFY	PDKGAKNNGIDGVY	GPKTADAI	RRFQSMYGLTQDGI	YGP	
:	:	:	:	:	:	:
NGCYTWSGTLSEGS	GEAVRQLQIRVAGY	PGTGAQL	AIDGQFGPATKAAV	QRFQSAYGLAADGI	AGP	
10	20	30	40	50	60	

Fig. 4. Comparison of the deduced amino acid sequence of the *cwlA* gene with that of the muramoyl-pentapeptide carboxypeptidase (Dideberg *et al.*, 1982; Joris *et al.*, 1983) – upper and lower sequences, respectively. The numbers above the *cwlA* sequence and below the carboxypeptidase sequence are the positions with respect to the N-terminal amino acids. Dots, blanks and conservative substitutions are as for Fig. 3.

Fig. 5. (a) Insertional mutation of the chromosomal *cwlA* gene by transformation with linearized pBA47EH. The hatched, stippled and open blocks, and the line represent the fragments containing the *cat* and *cwlA* genes, *B. subtilis* DNA (except *cwlA*) and pUC118 DNA, respectively. The distances are shown in kb. Abbreviations as in Fig. 1. (b) Southern transfer and hybridization of restriction-enzyme-digested DNAs from *B. subtilis* 168S and 17cmE with the *XbaI*–*EcoRI* fragment of pBA47ES as a probe. The 1.3 kb *XbaI*–*EcoRI* fragment of pBA47ES containing the *cwlA* gene was separated by gel electrophoresis and labelled with a nick-translation kit (Takara) containing [α - 32 P]dATP (Institute of Isotopes of the Hungarian Academy of Sciences). Southern transfer and hybridization were carried out as described previously (Sekiguchi *et al.*, 1988). Lanes 1 and 2, 168S and 17cmE chromosomal DNAs, respectively, digested with *HindIII*. The sizes of the λ *HindIII* fragments are shown on the right.

(EC 3.4.17.8; Dideberg *et al.*, 1982; Joris *et al.*, 1983) (Fig. 4).

Disruption of the *cwlA* gene by means of insertional inactivation

To examine the physiological role of the *cwlA* gene, *B. subtilis* *cwlA*::*cat* was constructed. A hybrid plasmid, pBA47EH, was obtained by insertion of the *cat* gene at the *HindIII* site of the *cwlA* gene. Competent *B. subtilis* 168S cells were transformed with the *EcoRI*-digested linearized pBA47EH, and Cm^r transformants were selected (Fig. 5a). To examine the resulting insertionally inactivated mutants, chromosomal DNA was extracted

from the transformants, digested with *HindIII* and subjected to agarose gel electrophoresis, followed by Southern blot hybridization analysis (Fig. 5b). The *XbaI*–*EcoRI* fragment from pBA47ES containing the *cwlA* gene as a probe hybridized to one band at 3.5 kb, whereas it hybridized to *HindIII*-digested *B. subtilis* 168S chromosomal DNA fragments at 0.9 kb and 1.6 kb. Since pBA47EH contained a 1.0 kb *cat* insert, double cross-over integration, leading to inactivation of the *cwlA* gene (Fig. 5a), is indicated. The constructed *B. subtilis* 17cmE and two similar Cm^r transformants showed normal cell morphology. Competence was retained at normal levels (1.2×10^4 and 0.82×10^4 transformants per μg DNA for 17cmE and 168S,

Table 2. Linkage relationships of an integrated antibiotic marker with standard markers, determined with PBS1

Donor (genotype)	Recipient (genotype)	Selected marker	Phenotype	Number
<i>B. subtilis</i> 17cmE (<i>trpC2</i> <i>strA smo-1 cwIA::cat</i>)	<i>B. subtilis</i> AC705 (<i>lys dnaE20</i> <i>aroD120 asaA4 smo-1</i>)	Cm ^r	Ts ⁻ * Aro ⁻	7
			Ts ⁺ Aro ⁻	0
			Ts ⁻ Aro ⁺	32
			Ts ⁺ Aro ⁺	61
	<i>B. subtilis</i> QB936 (<i>ald-1</i> <i>aroG932 leuA8 trpC2</i>)	Cm ^r	Aro ⁻ Leu ⁻	97
			Aro ⁺ Leu ⁻	0
			Aro ⁻ Leu ⁺	3
			Aro ⁺ Leu ⁺	0

* The *dnaE* mutant is temperature-sensitive. Temperature sensitivity was examined at 48 °C (non-permissive) and 30 °C (permissive): +, wild-type; -, temperature sensitive.

respectively). After 3 d incubation on Schaeffer agar medium containing *B. subtilis* cell walls, strains 17cmE and 168S showed clear zones of identical size.

Mapping of the *cwIA* gene

The *cwIA* gene was mapped by transduction of *B. subtilis* strains AC705 and QB936, transduced with a PBS1 lysate obtained on *B. subtilis* 17cmE. Cm^r was closely linked to *aroD* (227°; 93% linkage) and *dnaE* (223°; 61%) (Zeigler & Dean, 1989; Table 2), and the gene order was *dnaE-aroD-Cm(cwIA)*. Weak linkage to *leuA* (250°; 3%) was also observed (Table 2).

Discussion

The deduced amino acid sequence of the *B. subtilis* *cwIA* gene showed distinct homology with that of the *Bacillus* sp. cell wall hydrolase gene. The homology was highest for the 180 amino acids starting from the N-terminus, 93 of which were identical. Inactivation of the *cwIA* gene, by insertion of *cat* after Lys₁₀₉, was accompanied by loss of lytic activity. However, the product of pBA47B2, whose 57 C-terminal amino acid residues were replaced by another peptide (22 amino acids) (Fig. 3), and the truncated *Bacillus* sp. hydrolase, whose 55 C-terminal amino acids were replaced by three amino acids, both retained their lytic activity (Potvin *et al.*, 1988). Therefore, for both enzymes, the homologous N-terminal to central region seems to be functionally important. In spite of poor homology at the C-terminal, the average hydrophilicity profile (running average: 5 amino acids) of the *cwIA* protein was similar to that of the *Bacillus* sp. hydrolase (229–262 amino acid sequence of *cwIA* vs 216–249 of *Bacillus* sp.) (data not shown).

The carboxypeptidase of *S. albus* G, whose N-terminal amino acid sequence was homologous to the C-terminal region of the deduced amino acid sequence of *cwIA*

gene (Fig. 4), hydrolyses D-alanyl-D-alanine-terminated peptidoglycan precursors. It also effectively hydrolyses a variety of C-terminal N^α-(D-alanyl)-D- linkages that cross-link the peptidoglycan unit in certain cell walls and, consequently, causes cell wall lysis (Ghuysen *et al.*, 1970). It consists of two globular domains (Dideberg *et al.*, 1982; Joris *et al.*, 1983); the small N-terminal domain (76 residues) which contains three α-helices, and a large C-terminal domain (136 residues) which contains three histidine residues (ligands of a catalytic zinc atom) and an arginine, probably involved in substrate binding (Dideberg *et al.*, 1982; Joris *et al.*, 1983). Therefore, it is interesting to know the role of the C-terminal region of the *cwIA* product and the N-terminal domain of the *S. albus* G enzyme. The deduced molecular mass of the *cwIA* gene product is 29.9 kDa, and is likely to be smaller upon processing between the Ala₃₉–Ala₄₀ residues (Fig. 3). This hydrolase is therefore clearly distinct from the two major *B. subtilis* autolysins, the v-amidase and the glucosaminidase, which have molecular weights of 50 kDa (Herbold & Glaser, 1975), and 90 kDa (Rogers *et al.*, 1984), respectively. In addition, insertional inactivation of the *cwIA* gene (strain 17cmE) did not lead to a decrease of total cell wall lytic activity at pH 8.0, the optimal pH for the *B. subtilis* v-amidase. We have recently purified the *cwIA* protein, from *E. coli* (pBA47), and shown it to have a molecular mass of 22.5 kDa (unpublished). Since the ORF of *cwIA* encodes a polypeptide with a molecular mass of 29.9 kDa, and the deduced N-terminal amino acid, methionine, has been processed to form the mature protein, processing in the C-terminal region is also suggested. The cell wall lytic activity was uninhibited by a high concentration of ampicillin (100 mM), showed an optimal pH of 8.0, and did not cause an increase in reducing sugars after cell wall digestion (data not shown). Therefore, the cloned enzyme was neither a known v-amidase nor the glucosaminidase.

A homologous sequence followed by an inverted repeat region and a T-rich region were found in the upstream regions of the *cwlA* and *sacB* genes (Aymerich *et al.*, 1986), but not in that of the *Bacillus* sp. hydrolase gene (Potvin *et al.*, 1988). This may be the cause of the low expression of the *B. subtilis* *cwlA* gene relative to that of the *Bacillus* sp. hydrolase gene (Potvin *et al.*, 1988; unpublished result). The upstream region of *cwlA* has another curious sequence which is homologous to the upstream regions of *aprE* and *sacB* genes, which contain the targets for *sacU^h* and *sacQ^h* products (Henner *et al.*, 1988). In particular, higher homology was found between *cwlA* and *sacB* in the target sequences (18 of 22 bases homologous) as well as in the adjacent regions (9 to 13).

The *cwlA* gene does not correspond to the *flaD* gene, mutations in which cause a decrease in lytic activity, because the loci were different (*flaD*(218°)-*dnaE*-*aroD*-*cwlA* (Akamatsu & Sekiguchi, 1987*a, b*; Table 2). Moreover, there was no significant homology between the *cwlA* gene and the *Streptococcus pneumoniae* amidase gene (autolysin gene) cloned by López and his colleagues (García *et al.*, 1985, 1986).

Since the physiological role of the *cwlA* protein in *B. subtilis* is obscure, we are now researching the expression and regulation of the *cwlA* gene in *B. subtilis*.

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