Molecular cloning and sequencing of the upstream region of the major \textit{Bacillus subtilis} autolysin gene: a modifier protein exhibiting sequence homology to the major autolysin and the \textit{spoIIID} product

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The upstream region of the N-acetylmuramoyl-L-alanine amidase gene (\textit{cwlB}; a major \textit{Bacillus subtilis} autolysin) was cloned into \textit{Escherichia coli} by chromosome walking. Sequencing of the region showed the presence of two open reading frames, one (designated as \textit{cwbA}) which starts at a UUG codon and encodes a polypeptide of 705 amino acids with a \textit{M}, of 76725, and the other (designated as \textit{ippx}), upstream of \textit{cwbA}, comprising 102 amino acids and having a signal sequence characteristic of a lipoprotein. Purification of the CwbA protein and determination of its N-terminal amino acid sequence revealed that it contains a presumed signal peptide which is processed after Ala at position 25 from the N-terminal, and that the \textit{M}, of the mature form is 75000. The amino acid sequences of the N-terminal and C-terminal regions of CwbA were found to be highly homologous with those of the cell wall binding domain of CwlB and the \textit{spoIIID} gene product, respectively. CwbA stimulated the major autolysin activity approximately threefold in \textit{vitro}. These data indicate that CwbA is the modifier protein of the major autolysin reported by Herbold, D. R. & Glaser, L. (1975; \textit{Journal of Biological Chemistry} 250, 1676–1682). In-frame fusion between the \textit{ippx} and \textit{lacZ} genes demonstrated that \textit{ippx} is translated \textit{in vivo} and expressed during the exponential growth phase.

**Introduction**

\textit{Bacillus subtilis} produces a number of autolysins, including N-acetyl-muramoyl-L-alanine amidase (the major autolysin) and endo-\(\beta\)-N-acetylglucosaminidase (glucosaminidase) (Herbold & Glaser, 1975; Rogers et al., 1984). During sporulation, two other cell wall hydrolases, s-amidase and \(\gamma\)-D-glutamyl-(L)mesodiaminopimelyl endopeptidase, are formed which are thought to be associated with the formation of cortex peptidoglycan (Guinand et al., 1976). Additionally, some \textit{B. subtilis} strains (YS-25 and K-77) produce extracellular endo-\(\beta\)-N-acetylmuramidases (Okada & Kitahara, 1973; Murao & Takahara, 1974). Among the peptidoglycan hydrolases in \textit{B. subtilis}, the N-acetylmuramoyl-L-alanine amidase gene was first cloned in this laboratory (Kuroda & Sekiguchi, 1990). The product of this gene is not a major autolysin but a cell wall hydrolase that is highly active on spore cortex peptidoglycan (Kuroda & Sekiguchi, 1990; Foster, 1991). Recently, we cloned and sequenced the major autolysin gene, \textit{cwlB} (Kuroda & Sekiguchi, 1991). Insertional inactivation of \textit{cwlB} led to extraordinary resistance to cell lysis, even after 6 d incubation at 37°C.

During the cloning of \textit{cwlB}, we found an open reading frame (designated as \textit{cwbA}) upstream of \textit{cwlB}, which putatively encodes a 75 kDa cell wall binding protein (Kuroda & Sekiguchi, 1991). Previously Herbold & Glaser (1975) reported a modifier protein with an \textit{M}, of 80000 which specifically binds with the major autolysin in a 1:1 molar ratio and stimulates enzyme activity. We report here the cloning and sequencing of the upstream region of \textit{cwlB}, the identification of the CwbA protein as a modifier protein and its amino acid sequence.

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The nucleotide sequence data reported in this paper have been submitted to DDBJ and have been assigned the accession number D10388.

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**Methods**

**Bacterial strains, phages and plasmids.** These are listed in Table 1. Bacillus subtilis and Escherichia coli were grown in LB medium (5 g yeast extract, 10 g polypeptone and 10 g NaCl per litre, pH 7.2) at 37°C. When required, ampicillin, chloramphenicol and tetracycline were added to final concentrations of 50, 5 and 20 μg ml⁻¹, respectively.

**Assay of cell wall lytic activity.** Lytic activity was measured as described previously (Kuroda & Sekiguchi, 1990). The reaction mixture (5 ml) consisted of B. subtilis 168S cell wall, partially purified with SDS (SDS-wall) to a final A₅₄₀ of 0.3 in TK buffer (0.1 M-Tris/HCl, pH 8.0, 0.1 M-KCl), and the enzyme extract (100 μl). The mixture was incubated with shaking at 37°C. One unit of enzyme activity was defined as the amount required to decrease the A₅₄₀ by 0.001 in 1 min.

**Bacterial transformation.** Transformation of E. coli and B. subtilis was performed by the competent cell and protoplast transformation methods, respectively (Mandel & Higa, 1970; Akamatsu & Sekiguchi, 1987).

**Cloning of the upstream gene of cwlB by means of chromosome walking.** A genomic library was constructed in λEMBL3 (Stratagene). Chromosomal DNA from B. subtilis 168S was partially digested with a diluted Sau3AI solution to give the maximum yield of 10-20 kb DNA fragments, and then ligated to the HindIII site of a vector and packaged in vitro (Gigapack II Plus packaging extract; Stratagene). The 0.6 kb HindIII fragment in the insert (containing the C-terminal of the cwbA gene and the N-terminal of the cwlB gene) was radioactively labelled with a multiprime labelling system (Amersham) and [α-³²P]ATP (Amersham), and then used to screen a genomic library (3000 clones) through plaque hybridization (Maniatis et al., 1982). A 5-5 kb BgHII fragment containing the cwbA gene from one of five positive recombinant phages was subcloned into the BamHI site of pUC19, the resultant plasmid being designated as pCL7.

**DNA sequencing.** Nucleotide sequencing was performed by the dideoxy chain-termination method with a modified T7 polymerase (Sequenase; Toyobo). Phages M13mp18 and mp19 (Takara) were used to generate templates for sequencing. Electrophoresis was performed on 8% (w/v) polyacrylamide gels containing 8 M-urea. The sequences of both strands were determined for the 3 kb EcoRV-BamHI fragment of pCL7 (Fig. 1).

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**Table 1. Bacterial strains, phages and plasmids**

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<tr>
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Fig. 1. Partial restriction map of the λEMBL3 vector containing the cwlB operon. pCL4 is a pUC119 derivative containing a 2-5 kb EcoRI insert of B. subtilis chromosomal DNA (Kuroda & Sekiguchi, 1991). pCL7 was constructed by subcloning a 5-3 kb BgHII fragment into pUC19. The open box denotes the λEMBL3 vector. The thick and thin arrows indicate the genes in the cwlB operon and the sequencing strategy, respectively. A potential transcriptional terminator is also indicated. Bg, BglII; Ba, BamHI; E, EcoRI; H, HindIII; S, Sau3AI; V, EcoRV.
Construction of pCL7 derivative plasmids. A 2.3 kb BamHI–EcoRI fragment containing the cwbB gene from pCL4 (Fig. 1) was ligated into the EcoRI and BamHI sites of pHY300PLK (B. subtilis and E. coli shuttle vector; Takara), followed by the transformation of E. coli JM109. Plasmid DNA (pHWB) isolated from transformants contained a 2.3 kb BamHI–EcoRI insert. For the construction of pHWEX, the 4.0 kb Xbal–BamHI fragment of pCL7 (containing lppX and a part of cwbA) was ligated into the Xbal and BamHI sites of pHWB. For the construction of pHWA8a and pHWA8b, pHWEX was digested with BamHI and BglII, respectively, and then blunt-ended with the Klenow fragment (Takara), followed by self-ligation. For the construction of pHYSH5, the 1.4 kb EcoRV fragment of pCL7 was ligated into the SalI site of B. subtilis DNA fragment fusion vector, pMC1871 (T460 bp; Pharmacia), and then E. coli JM109 was transformed with the ligation solution, followed by selection on LB plates containing ampicillin and X-gal. Plasmid DNAs were isolated from transformants which gave a blue colour on selection plates. The orientation of the inserts was determined by checking the distance between the asymmetric HindIII site of the insert and that of the vector. Finally, a 3.9 kb HindIII–SalI fragment carrying a translational fusion of the 63 codons of lppX and the lacZ gene cartridge from one of the resultant plasmids was inserted into the HindIII and SalI sites of pHY300PLK.

Purification of the major autolysin (CwbB) and cell wall binding protein (CwbA). B. subtilis AD1 harbouring pHWA8a or pHWA8b was cultured with shaking in 3 l LB medium containing tetracycline (20 μg ml\(^{-1}\)) for 10 h at 37 °C. After centrifugation of each culture, the cells were suspended in 15 ml 5 M-NaCl in buffer A (50 mM-Tris/HCl, pH 8.0, 10 mM-MgCl\(_2\), 1 mM-EDTA) and then incubated at 0 °C for 30 min. After centrifugation, the supernatant was diluted with buffer A and applied to a column (2.2 by 10 cm) of hydroxyapatite gel (Wako), then equilibrated with 0.2 M-KCl in 0.1 M-potassium phosphate buffer (pH 7.5). The column was eluted with a linear gradient of potassium phosphate buffer (pH 8.0, 10 mM-MgCl\(_2\), 1 mM-EDTA) and then incubated at 0 °C for 30 min. After centrifugation, the supernatant was diluted with buffer A and applied to a column (2.2 by 10 cm) of hydroxyapatite gel (Wako), then equilibrated with 0.2 M-KCl in 0.1 M-potassium phosphate buffer (pH 7.5). The column was eluted with a linear gradient of potassium phosphate buffer (pH 7.5) from 0.1 to 5 M-NaCl. CwbA and CwbB were eluted with 2 to 2.5 M-NaCl. Fractions containing CwbB or CwbB were pooled (approximately 12 ml) and diluted fivefold with 0.1 M-potassium phosphate buffer (pH 7.5), and then applied to a column (2.2 by 10 cm) of hydroxyapatite gel (Wako), then equilibrated with 0.2 M-KCl in 0.1 M-potassium phosphate buffer (pH 7.5). The column was eluted with a linear gradient of potassium phosphate buffer (pH 7.5) from 0.1 to 0.5 M, containing 0.2 M-KCl. CwbA and CwbB were eluted with 0.3 and 0.4 M-potassium phosphate buffer, respectively. The peak fractions containing CwbA or CwbB were pooled (approximately 6 ml), dialysed against 1 M-KCl in buffer A and used as the purified protein sample. The yields of the purified CwbA and CwbB were approximately 8 and 5%, respectively (as calculated as a densitometer).

β-Galactosidase assay. The β-galactosidase assay was performed as described by Shimosu & Henner (1986). B. subtilis (pHY5SH) cells harvested at regular time intervals were centrifuged for 3 min in a microfuge and then stored at -80 °C. The frozen cells were suspended in 1 ml Z buffer (Miller, 1972) containing 100 μg lysozyme ml\(^{-1}\) and 0.1% (v/v) Triton X-100, and then incubated at 37 °C for 5 min. Samples were assayed for β-galactosidase activity by the method of Miller (1972).

Results

Molecular cloning of the upstream region of the cwbB gene

A 0.6 kb HindIII fragment containing the C-terminal region of the cwbA gene was used as a probe to screen a B. subtilis 168S genomic library (Fig. 1). Five clones hybridizing with the probe were isolated. Restriction mapping and Southern hybridization were carried out for a B. subtilis DNA fragment from one of these clones inserted into the λEMBL3 vector. The results indicate that the inserted fragment contained an approximately 5 kb region upstream of cwbB (Fig. 1). A 5.5 kb BglII fragment that overlapped with the 2.5 kb EcoRI fragment of pCL4 was subcloned into pUC19 and the resulting plasmid was designated pCL7 (Fig. 1).

Nucleotide sequence of the upstream region of the cwbB gene

The 3 kb EcoRV–BamHI region of pCL7 was sequenced by means of the dyeoxy chain termination reaction. The nucleotide sequence of the 168 bp EcoRI–BamHI fragment was identical to that determined previously (Kuroda & Sekiguchi, 1991). Consequently, cwbA, starting at a rare start codon, UUG (nucleotides 1287–1289), and ending at nucleotide 3401, encodes a polypeptide of 705 amino acid residues with an Mr of 76725 (Fig. 2). The presumed start codon was preceded by a possible ribosome binding site, AGGGAGG [ΔG = -15.4 kcal mol\(^{-1}\) (−64.4 kJ mol\(^{-1}\))] the consensus sequence is underlined. An ORF (tentatively designated as lppX (lipoprotein)), which starts at nucleotide 955 and ends at nucleotide 1260, encoding a polypeptide of 102 amino acid residues with an Mr of 11228, was found upstream of cwbA (Fig. 2). A possible ribosomal binding site [GAGGTGA; ΔG = -15.3 kcal mol\(^{-1}\) (−64.0 kJ mol\(^{-1}\))] was found upstream of lppX.

Computer analysis (IDEAS sequence analysis system on the NBRF protein data base, Release 25.0, Kyushu University, Japan) revealed that CwbA exhibits significant amino acid sequence homology with the B. subtilis and B. amyloliquefaciens spoIID gene products (Lopez-Diaz et al., 1986; Turner & Mandelstam, 1986) (Fig. 3). Moreover the amino acid sequences of the N-terminal portions of CwbA and CwbB are highly homologous to each other, especially in their internal repetitious regions (Fig. 4). The N-terminal amino acid sequence of LppX is highly homologous to those of several murein lipoproteins (Fig. 5). LppX contains a potential signal sequence: there are two positively charged lysine residues at positions 2 and 3 from the N-terminus, followed by 11 hydrophobic amino acid residues (F\(_2\)-I-A-L-L-F-F-I-L-L-L\(_{14}\)). Since the sequence, L\(_{13}\)-L\(_{14}\)-S\(_{15}\)-G\(_{16}\)-C\(_{17}\), of LppX is similar to the consensus sequence of a lipoprotein, the mature protein may be derived from a precursor through cleavage with a signal peptidase after Gly\(_{16}\), followed by modification of the resultant N-terminal cysteine with lipid.
**Fig. 2.** Nucleotide sequence of the *cwZB* operon. The 2.9 kb EcoRV-BamHI fragment in Fig. 1 was sequenced, and the nucleotide sequence of the 3-7 kb between the EcoRV and HindIII sites is shown (this study; Kuroda & Sekiguchi, 1991). The deduced amino acid sequences are shown below the nucleotide sequence. The putative ribosome binding sequences (SD) and restriction endonuclease sites are shown above the nucleotide sequence. Arrowheads indicate the transcription start points of the *cwZB* operon by primer extension analysis (data will be reported elsewhere). The N-terminal amino acid sequence of CwbA is underlined. Arrows indicate putative signal sequence cleavage sites. The amino acid sequences after the closed arrows correspond to the N-terminal sequences of the mature proteins. Asterisks indicate stop codons.

**Purification and determination of the N-terminal amino acid sequence of CwbA**

The CwbA protein was extracted from cell walls with 5 M-NaCl in buffer A and then chromatographed on a cell-wall-immobilized acrylamide gel column. Fractions containing CwbA or CwlB were pooled and subjected to hydroxyapatite column chromatography. Fig. 6 shows the results of SDS-PAGE of proteins from 5 M-NaCl extracts, and from the fractions obtained on the cell-wall-immobilized column and the hydroxyapatite column. CwbA is a protein with an *M*<sub>r</sub> of 75000 (Fig. 6, lane 3) and was sequenced with an automatic peptide sequencer. Its N-terminal amino acid sequence of CwbA is underlined. Arrows indicate putative signal sequence cleavage sites. The amino acid sequences after the closed arrows correspond to the N-terminal sequences of the mature proteins. Asterisks indicate stop codons.
Fig. 3. Comparison of the deduced amino acid sequence from the \textit{cwbA} gene with that from the \textit{B. subtilis spoIID} gene (Lopez-Diaz \textit{et al.}, 1986). Identical and similar amino acids are indicated by asterisks and colons, respectively. The amino acids of the \textit{B. amyloliquefaciens} SpoIID protein (Turner \& Mandelstam, 1986) which are different from those of the \textit{B. subtilis} SpoIID are shown below the \textit{B. subtilis} sequence. The numbers above the CwbA sequence and below the SpoIID sequence are the positions with respect to the N-terminal amino acids of CwbA and \textit{B. subtilis} SpoIID, respectively.

26–40 from the N-terminus, the mature protein was probably derived from a precursor through cleavage with a signal peptidase after Ala\textsubscript{25} in the sequence, F\textsubscript{24}–A\textsubscript{25}–A\textsubscript{26}–D\textsubscript{27}. The putative leader peptide has features in common with signal peptides, i.e. positively charged lysine residues at positions 2 and 5, followed by hydrophobic amino acid residues (L\textsubscript{7}–I–V–C–S–L–A–I–L–L–I\textsubscript{9}) (Fig. 2).

\textit{Characterization of CwbA}

We purified CwbA from \textit{B. subtilis} (pHWABg) and CwlB from \textit{B. subtilis} (pHWABa). CwbA exhibited no autolytic activity, but the addition of CwbA to the major autolysin in an equimolar amount stimulated the autolytic activity about threefold (as calculated from the initial slopes for cell wall turbidity) (Fig. 7). These results indicate that the CwbA protein of \textit{B. subtilis} 168S is similar in activity to the modifier protein of \textit{B. subtilis} ATCC 6051 (Herbold \& Glaser, 1975).

\textit{Expression of lppX in vivo}

To analyse the \textit{in vivo} expression of lppX, the N-terminal region of lppX was fused in-frame with the lacZ gene. Plasmid pHY5HS carried a 0.8 kb HindIII–EcoRV fragment containing the N-terminus of lppX and its upstream region (promoter region). No significant promoter activity, contributing to the transcription of the lppX–lacZ fusion gene, was exhibited by the vector itself, because no increase in cwlB activity was detected in a strain harbouring pHY300PLK inserted with the 2.3 kb BamHI–HindIII fragment of pCL4 (containing an intact cwlB gene in the same orientation) (data not shown). The production of the lppX–lacZ fusion protein by \textit{B. subtilis} AC327(pHY5HS) started during early exponential growth phase and ended at the stationary phase (Fig. 8). Since cwlB production was initiated during early exponential growth phase (Kuroda \& Sekiguchi, 1991), and there is apparently no \(\rho\)-independent terminator between lppX and cwlB, the three genes (lppX, cwbA and cwlB) are probably in the same operon and transcribed polycistronically. The promoter sequences and regulation of the cwlB operon will be reported elsewhere.

\textit{Discussion}

A modifier protein from \textit{B. subtilis} ATCC 6051, with an \(M_r\) value of 80000, which specifically combines with the major autolysin in a 1:1 ratio, was reported by Herbold \& Glaser (1975). This protein stimulates the autolysin
Fig. 4. Comparison of the deduced amino acid sequence from the \textit{cwbA} gene (upper) with that from the \textit{cwlB} gene (lower) (Kuroda & Sekiguchi, 1991). The deduced signal sequence cleavage sites are indicated by arrowheads. The repeated sequences of CwbA and CwlB are indicated by the arrows, the homologous region of CwlB to CwlM is shown by shading and identical and similar amino acids are indicated by asterisks and colons, respectively.

The results of our sequencing of the \textit{cwbA} gene indicated that the deduced amino acid composition of its product is similar to that of the modifier protein, except for (a) the total number of amino acids (744 amino acid residues for the modifier protein versus 680 residues for mature CwbA) and (b) a few amino acids (6-9 cysteine and 4 histidine residues per mole of the modifier protein versus no cysteine residues).
LppX

**Murein lipoprotein**

*E. coli*

*E. coli* (lipoprotein-28)

*S. marcescens*

*E. amylovora*

*M. morganii*

*P. mirabilis*

**Penicillin-binding protein 3 (E. coli)**

**Penicillinase**

*B. licheniformis*

*S. aureus*

**TraT protein (plasmid R100)**

**Lysis protein**

Protein H (pCloDf13)

Protein kll (pColE1)

Protein CelB (pColE2)

Protein Hic (pColE3)

Protein Cal (pColA)

**Pullulanase**

(*K. pneumoniae*)

**Consensus sequence**

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Fig. 5. Comparison of the signal sequence of LppX with those of lipoproteins (Wu & Tokunaga, 1986). The consensus cleavage sequence of lipoproteins is shown at the bottom. The arrowhead indicates the putative signal sequence cleavage site. Amino acids identical to those of the LppX in the consensus sequence are shown by shading.

Fig. 6. SDS-PAGE of CwlB and CwbA. Lanes: 1, protein standards [0.5 μg of each protein (Bio-Rad); from top to bottom, rabbit muscle phosphorylase b (*M*, 97400), bovine serum albumin (66200), hen egg white albumin (42699), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and hen egg white lysozyme (14400)]; 2 and 3, CwlB and CwbA fractions (0.5 μg protein), respectively, eluted from the hydroxyapatite column; 4 and 5, CwlB and CwbA fractions (150 μl each), respectively, eluted from the cell wall immobilized column; 6 and 7, aliquots (25 μl) of 5 M-NaCl extracts from *B. subtilis* AD1 harbouring pHWABa and pHWABg, respectively. Proteins were stained with Coomassie brilliant blue.
Fig. 7. Cell wall lytic activities of the purified CwlB and CwbA, and a mixture of CwlB and CwbA. Purified CwlB (1.0 µg, 20 pmol) and CwbA (1.5 µg, 20 pmol) were mixed and diluted two times with buffer A. As a control, CwlB or CwbA was also diluted as described above. After 15 min incubation at room temperature, they were added to reaction mixtures (final 5 ml 0.2 M-KCl in buffer A containing 0.3 mg cell wall ml⁻¹) and then the cell wall turbidity was monitored at 37 °C. ▲, CwlB; ■, CwbA; ●, CwlB + CwbA; ○, no protein.

The spoIID298 mutation creates a morphological block after the bulging of the small compartment that accompanies the transition from sporulation stage II to stage III (Coote, 1972). Therefore, it is thought that the spoIID gene product is required for the removal of the septal peptidoglycan (Illing & Errington, 1991). The spoIID genes of B. subtilis and B. amyloliquefaciens encode polypeptides of 343 and 344 amino acid residues, respectively (Lopez-Diaz et al., 1986; Turner & Mandelstam, 1986), and the amino acid sequences of about one half of their proteins are homologous to that of the C-terminal region of CwbA (Fig. 3). The differences in the SpoIID sequences of B. subtilis and B. amyloliquefaciens are mainly located in the regions of non-homology with CwbA (Fig. 3). These results suggest that the homologous regions of CwbA and SpoIID are functionally important. The SpoIID proteins may exhibit stimulatory activity toward some sporulation-specific autolysin(s). Stragier (1989) suggested that the sequences of the SpoIID proteins include a highly hydrophobic N-terminal domain, which could act as an anchor in the membrane rather than as a signal sequence. In contrast, CwbA contains a typical signal sequence which can be processed in B. subtilis.

In a previous study it was found that the major autolysin of B. subtilis (CwlB) is composed of two functional domains: an N-terminal involved in cell wall binding and a C-terminal catalytic domain (Kuroda & Sekiguchi, 1991). The amino acid sequence of the C-terminal domain of CwlB is highly homologous to that of the N-terminal (catalytic) domain of B. licheniformis cell wall hydrolase (CwlM) (A. Kuroda, Y. Sugimoto, T. Funahashi & J. Sekiguchi, unpublished results). Since there is a high degree of homology between the N-termini of CwbA and CwlB (Fig. 3), it is very likely that the N-terminal portion of CwbA binds to the B. subtilis cell wall.

The role of LppX is obscure. We are currently investigating the subcellular location of LppX and the possibility of in vitro complex formation between LppX, CwbA and/or CwlB.

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References


