Cloning, expression, sequence analysis and biochemical characterization of an autolytic amidase of *Bacillus subtilis* 168 *trpC2*

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By use of a functional assay for activity, an autolysin structural gene was cloned on a 3 kb *EcoRI* DNA fragment from a λ gt11 expression library of *Bacillus subtilis* 168 *trpC2* genomic DNA. Sequencing of the fragment showed five open reading frames, the central one of which encoded the lytic enzyme as found by subclone activity mapping and its homology to a recently sequenced autolysin gene. The protein had a deduced sequence of 272 amino acids and a molecular mass of 29957 Da. When expressed in *Escherichia coli* DH5 α , the protein was processed to a 21 kDa form, as estimated by renaturing SDS-PAGE. The autolysin was an *N*-acetylmuramyl-L-alanine amidase and its activity was MgCl₂-dependent (20 mM optimum) and LiCl-sensitive. The enzyme could bind to and hydrolyse a wide range of peptidoglycan substrates isolated from Gram-positive bacteria; the binding was also MgCl₂-dependent. Initial mapping experiments located the autolysin gene near *aroD* on the *B. subtilis* 168 chromosome.

Introduction

Bacterial autolysins are potentially lethal enzymes capable of hydrolysing peptidoglycan, the major component of bacterial cell walls. The enzymes are characterized *in vitro* by their hydrolytic bond specificity as *N*-acetylmuramidases (muramidases), *N*-acetylglucosaminidases (glucosaminidases), *N*-acetylmuramyl-L-alanine amidases (amidases), endopeptidases and transglycosylases (Ghuysen *et al.*, 1966). Autolysins have been implicated in several important cellular functions, including cell wall growth and turnover, cell separation, flagellation, competence, differentiation and the lytic action of antibiotics (Pooley & Karamata, 1984; Ward & Williamson, 1984). The involvement of specific *B. subtilis* 168 autolysins in all of the above processes is speculative, as they have not been thoroughly investigated at the molecular level. *B. subtilis* 168 has two major vegetative cell autolysins, an amidase and a glucosaminidase of 50 and 90 kDa respectively, and of as yet

unknown function (Herbold & Glaser, 1975; Rogers *et al.*, 1984).

Direct cloning techniques, combined with a functional assay system, have resulted in the isolation of autolysin structural genes in *E. coli*. By the use of these methods, the structural genes for a 23 kDa amidase and an 80 kDa glucosaminidase from *Staphylococcus aureus* (Biavasco *et al.*, 1988; Jayaswal *et al.*, 1990) and a 27 kDa autolysin of unknown specificity from a *Bacillus* sp. have been isolated (Potvin *et al.*, 1988). This paper reports the isolation, sequence analysis and mapping of a gene encoding a low-molecular-mass autolysin of *B. subtilis* 168 *trpC2*. The mode of action and initial biochemical characterization of the cloned enzyme are also described.

Methods

Bacterial strains and growth conditions. *Bacillus subtilis* 168 *trpC2* (Rogers *et al.*, 1984) and *Bacillus megaterium* KM (Stewart *et al.*, 1981) were grown routinely in Penassay broth (Difco) at 37 °C and 30 °C respectively. *Micrococcus luteus* ATCC 4698 (Sigma), *Escherichia coli* ER1458 (New England Biolabs) and DH5 α (Hanahan, 1983; BRL) were grown in Luria-Bertani medium (LB) at 37 °C. *B. megaterium* KM endospores were prepared and stored as described by Stewart *et al.* (1981).

Preparation of purified cell wall substrates. Mid-exponential (OD₆₀₀ = 0.6) cultures or purified spores were broken, and cell walls were prepared and stored at –20 °C as described by Johnstone & Ellar

This paper is dedicated to the memory of Professor Howard Rogers, whose help and encouragement were much appreciated, and who will be sorely missed.

Abbreviations: FDNB, 2,4-dinitrofluorobenzene; ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M59232.

(1982). Purified vegetative cell walls of *Bacillus sphaericus* 9602 and *Lactobacillus arabinosus* NCIB 6376 were a gift from Dr P. J. White, University of Sheffield, UK. Procion-Red-labelled walls were produced using a method based on that of Forsberg & Rogers (1971). Purified *B. subtilis* 168 vegetative cell walls were suspended at 10 mg ml⁻¹ in 2% (w/v) NaCl. After 30 min at 60 °C with constant shaking NaOH and Procion Red MX5B (ICI) were added to a final concentration of 0.1 M and 10 mg ml⁻¹ respectively. After incubation with shaking at 60 °C for 3 d, walls were washed six times with distilled water and stored at -20 °C.

Identification of a *Bacillus subtilis* 168 autolysin structural gene. A λ gt11 library containing a partial *EcoRI* digest of *B. subtilis* 168 *trpC2* genomic DNA (gift from Dr C. Price, University of California, Davis, USA) was used to screen for autolysin activity. *E. coli* ER1458 was infected with phage and plated onto LBMg²⁺ (10 mM-MgSO₄) plates (Huynh *et al.*, 1985). The phage overlay contained 1 mM-isopropyl β -D-thiogalactopyranoside (IPTG) and 0.66 mg ml⁻¹ Procion-Red-labelled walls. After incubation at 37 °C overnight, autolysin-producing clones were identified by a zone of clearing in the red background around the plaque. The autolysin-producing clones were purified and amplified, and phage DNA was isolated. The insert was characterized by *EcoRI* digestion (Huynh *et al.*, 1985).

Subcloning and DNA sequencing of an autolysin-encoding DNA region. A 3 kb *EcoRI* λ gt11 insert was subcloned into the *EcoRI* site of plasmid pUBS1, a pUC19-based vector (from Dr G. Murphy, Institute of Plant Science Research, Cambridge, UK), to give pSFP100. This plasmid was transformed into *E. coli* DH5 α . A set of nested deletion subclones were prepared for sequencing by timed exonuclease digestion (Henikoff, 1984; Promega, Erase-a-Base). The total DNA sequence of the 3 kb insert was determined on both strands using the dideoxy-chain termination method (Sanger, 1977) with modified T7 polymerase (Sequenase, USB).

Expression of the cloned *B. subtilis* 168 autolysin in *E. coli* DH5 α . The ability of *E. coli* DH5 α with plasmid pSFP100, or subclones thereof, to express the *B. subtilis* 168 autolysin was tested using a plate assay. Clones were patched onto LBamp (50 μ g ampicillin ml⁻¹) agar with a 3 ml overlay of LBamp agar containing 0.66 mg ml⁻¹ Procion-Red-labelled *B. subtilis* cell walls. After overnight growth at 37 °C, the cells were lysed over chloroform (20 min, 20 °C) and incubated for a further 24 h. The clones expressing autolysin were identified by a zone of clearing around the colony.

Initial mapping of the autolysin gene on the *B. subtilis* chromosome. The autolysin gene was insertionally inactivated by ligation of a 1.3 kb *SmaI* fragment from pM1101, containing a chloramphenicol acetyl transferase (*cat*) gene (Youngman *et al.*, 1984; gift from Dr L. Zheng, Biological Laboratories, Harvard University, Cambridge, USA), into *NdeI*-cut, blunt-ended and phosphatased pSFP100. The unique *NdeI* site in pSFP100 occurs within the autolysin coding region. Chloramphenicol-resistant (3 μ g ml⁻¹) clones of *B. subtilis* 168 SF1 were recovered after transformation (Anagnostopoulos & Spizizen, 1961) with the *cat*-containing construct, linearized with *PstI*. The location of the autolysin gene on the *B. subtilis* chromosome was determined by PBS1 transduction (Dubnau *et al.*, 1967), using the mapping kit of Dedonder *et al.* (1977).

Preparation of autolysin-containing extract. *E. coli* DH5 α , with plasmids pUBS1 or pSFP102 (see Fig. 1) were grown overnight in 200 ml LBamp (50 μ g ml⁻¹). Cells were harvested (6000 g, 10 min, 4 °C), the pellet (0.7 g wet wt) was washed with 50 mM-Tris/HCl, pH 7.5, at 4 °C, and disrupted by sonication (6 $\times$ 30 s bursts, full power; Soniprep 150, MSE Instruments) in 10 ml 50 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂, 250 mM-LiCl and 0.5 mM-PMSF. Greater than 90% of cells were broken by this method. Unbroken cells and insoluble

cell debris was removed by centrifugation (18000 g, 5 min, 4 °C) and the supernatant used directly as a source of enzyme.

Assay for autolysin activity. Lytic activity was measured by the ability of the crude enzyme preparation to decrease the optical density of a suspension of mid-exponential vegetative cells, or purified cell walls, at 37 °C. Unless otherwise stated, the reaction contained 1 ml of walls/cells suspended to a final OD₄₅₀ of 0.2, in 25 mM-Tris/HCl, pH 7.5, with 20 mM-MgCl₂. Each assay was carried out in duplicate and 1 unit of enzyme activity (U) was defined as the amount of enzyme necessary to decrease the OD₄₅₀ by 0.001 min⁻¹.

Assay for autolysin activity in renatured SDS-PAGE gels. SDS-PAGE (11%, w/v, acrylamide) gels containing 0.1% (w/v) purified *B. subtilis* vegetative cell walls were used for detection of lytic activity (Jayaswal *et al.*, 1990). Following electrophoresis, gels were rinsed in distilled water and then renatured in 1% (v/v) Triton X-100 (Aldrich), 20 mM-MgCl₂, 25 mM-Tris/HCl, pH 7.5, (3 $\times$ 30 min, 20 °C). Gels were then incubated for 16 h at 37 °C in the same buffer but containing only 0.1% (v/v) Triton X-100. Autolysin activity appeared as a zone of clearing in the translucent background. This was enhanced by staining the gel with 0.1% (w/v) methylene blue in 0.01% (w/v) KOH prior to photography (Jayaswal *et al.*, 1990).

Characterization of the cloned autolysin. To determine the hydrolytic bond specificity of the cloned autolysin, purified *B. subtilis* 168 vegetative cell walls were suspended at 5 mg ml⁻¹ in 5 ml 20 mM-MgCl₂. Cell extract (25 μ l) from *E. coli* DH5 α with either pUBS1 or pSFP102 was added and the mixture was incubated at 37 °C. Samples (0.5 ml) were withdrawn at intervals and boiled for 10 min. Following removal of undigested walls by centrifugation (14000 g, 10 min), samples were assayed for an increase in reducing groups (Thompson & Shockman, 1968) and amino termini (Ghuysen *et al.*, 1966). To determine to which alanine isomer the new amino termini could be attributed, the method of Margot, *et al.* (1991) was used. Walls labelled exclusively at the L-alanine residue were prepared by the use of a D-alanine racemase (*dal*) mutant strain [*B. subtilis* 168 IA4 *aro1906 dal-1 purE1 trpC2* (BGSC)]. Strain IA4 showed a reversion frequency of $< 1 \times 10^{-4}$ to *dal*⁺ under the conditions used. This strain was grown to mid-exponential phase (OD₆₀₀ = 0.6) in a medium containing both unlabelled D-alanine and L-[¹⁴C]alanine (Amersham, 155 mCi mmol⁻¹, 5.74 GBq mmol⁻¹). Vegetative cell walls were prepared as described above, digested with the enzyme extract, treated with 2,4-dinitrofluorobenzene (FDNB), and acid-hydrolysed. The amino acids were separated by TLC. The radioactivity in the various TLC spots was determined by scraping the silica gel off the TLC plate, and counting its radioactivity in a Beckman LS 1801 scintillation counter.

Results

Isolation of an autolysin-encoding gene from B. subtilis 168

From approximately 120000 p.f.u. of an *EcoRI* *B. subtilis* 168 genomic DNA library in λ gt11, 27 plaques were identified as producing zones of clearing on agar plates containing Procion-Red-labelled walls of *B. subtilis* 168. The putative phage clones were purified by replating at a lower titre (200 p.f.u. per plate). The *B. subtilis* 168 DNA inserts were isolated from six purified λ gt11 clones and found in all cases to be approximately 3 kb in size. When

Fig. 1. Restriction map, sequencing strategy, subclone activity mapping and ORF positions of the 3003 bp pSFP100 insert. Each sequencing strategy arrow shows the length and direction of a sequence determination. The ability of various subclones to express activity is shown, as is the position and direction of coding of the five ORFs. The size of the predicted proteins from the complete ORFs is indicated. The restriction map uses the following abbreviations: E, *EcoRI*; Es, *EspI*; N, *NdeI*; X, *XmnI*.

this 3 kb fragment was subcloned into pUBS1 to create pSFP100 and then transformed into *E. coli* DH5 α , zones of clearing were observed around the colonies on agar containing Procion-Red-labelled walls. *E. coli* DH5 α alone, or with pUBS1, showed no zone of clearing. The hydrolysis of *B. subtilis* 168 walls was not IPTG-dependent in λ gt11 containing the 3 kb insert or in *E. coli* DH5 α (pSFP100).

DNA sequence of the 3 kb *B. subtilis* 168 DNA fragment and identification of the autolysin-encoding gene

A set of nested deletion subclones was prepared for DNA sequencing. The autolysin-encoding gene was mapped to the central region of the 3 kb insert, according to the ability of the subclones to express lytic enzyme (Fig. 1). Subclone pSFP102 showed the most activity as measured empirically by the plate assay. The DNA sequence of the 3 kb fragment was determined on both strands (Fig. 2) using the strategy shown in Fig. 1. The region has five open reading frames (ORFs) all transcribed in the same direction (Figs 1 and 2). The ORF at the beginning of the cloned DNA is N-terminally truncated. The central ORF (ORF 3) was identified as the autolysin-encoding gene by the subclone mapping (Fig. 1) and by its striking homology to a previously sequenced autolysin gene from a *Bacillus* sp. (Potvin *et al.*, 1988). The *B. subtilis* 168

autolysin is a protein of 272 amino acids and 29957 Da. The protein has 46.6% sequence identity over its N-terminal 202 amino acids with the *Bacillus* sp. autolysin described by Potvin *et al.* (1988).

The autolysin gene is preceded by a putative ribosome binding site at bases 996–1003 (Fig. 2) with an estimated free energy for the Shine–Dalgarno interaction of -11 kcal mol $^{-1}$ (-46 kJ mol $^{-1}$) (Tinoco *et al.*, 1973). Also, a putative rho-independent terminator with an estimated free energy for the stemloop structure of -17 kcal mol $^{-1}$ (-71 kJ mol $^{-1}$) (Tinoco *et al.*, 1973), is present downstream from the autolysin stop codon at bases 1839–1867. Possible σ^A -dependent promoter sequences are present upstream of the autolysin gene (-35 and -10 at bases 916–921 and 939–944, respectively). ORFs 1, 2, 4 and 5 show no significant homology to any known protein at present in the sequence database (OWL cumulative database, University of Leeds, UK, accessed by the SERC Daresbury facility).

Mapping of the autolysin gene on the *B. subtilis* chromosome

The autolysin gene, with introduced *cat* cassette, was mapped by PBS1 transduction and found to be 77% cotransduced with *aroD* (226°; Piggot, 1989) and 16% with *leuB* (247°; Piggot, 1989).

Fig. 2 (following pages). Nucleotide sequence of the cloned *B. subtilis* 168 autolysin region. Only the non-coding DNA strand is shown. The numbering of nucleotides is shown below the sequence, and the deduced amino acid sequence above. Arrows above the amino acid sequence denote the start of ORFs and asterisks denote stop codons. The putative ribosome binding site (RBS) and rho-independent terminator before and after the autolysin gene are also shown as lines above the sequence.

ORF 1

E F L L Y Q S L G A G E E D I N I N H T
GAATTCTTACTTTACAGTCATTGGGCGCAGGTGAAGAGGATATAAACATTAACCATACT
10 20 30 40 50 60

V P I T A V A P F S V Q L D K S G L T N
GTTCTTATTACAGCAGTTGCTCCGTTCTCAGTCCAGTTAGATAAAAGCGGGTTAACTAAT
70 80 90 100 110 120

D G R L K V Q T E G L N L S S L D T Q S
GATGGTCGTTTAAAGTTCAAAGTGAAGGCTTGAATCTTAGCTCATTAGACACTCAATCA
130 140 150 160 170 180

K T M D I V F H D K T E T I G E G N P F
AAAACAATGGATATTGTCTTTACAGATAAAACAGAAACCATAGGTGAGGGTAACCCATTC
190 200 210 220 230 240

T V G S F K T L L I E V Y G T A E T S E
ACCGTTGGATCATTCAAACGTTGCTCATTGAGGTTTATGGGACGGCTGAGACAAGTGAA
250 260 270 280 290 300

L K F W G K S L S G T K R A L R G Q K V
TTGAAGTTCTGGGGTAAATCCTTATCGGGAACAAAAGAGCCCTTAGAGGGCAGAAAGTG
310 320 330 340 350 360

D D G T F A T S T K G K S E A W S F S I
GATGACGGAACGTTTGGCACTAGCACAAAAGGAAAATCAGAAGCTTGGTCTTTTAGCATT
370 380 390 400 410 420

T G F K E I V M E L T A L T N G N F S V
ACTGGCTTTAAAGAAATTGTTATGGAGCTTACAGCTTTAACAATGGAACTTTTCAGTT
430 440 450 460 470 480

R G T A V S *
AGAGGGACGGCCGTCTCATAAGATCCGGCTGTCCTTTTTTATTTGCCTCGGAGGAGGTGA
490 500 510 520 530 540

→ ORF 2

M E E T S L F I N F E T L D L A R V Y
TTAGAATGGAGGAGACAAGTTTGTATTCAATTTGAAACATTAGATTTAGCAAGAGTAT
550 560 570 580 590 600

L F G G V K Y L D L L L V L S I I D V L
ATTTATTTGGAGGGGTGAAGTACCTTGATTTACTTCTAGTACTTAGCATAATTGACGTTT
610 620 630 640 650 660

T G V I K A W K F K K L R S R S A W F G
 TAACAGGAGTAATCAAGGCATGGAAATTCAAAAACTGCGAAGTCGAAGCGCATGGTTTG
 670 680 690 700 710 720

Y V R K L L N F F A V I L A N V I D T V
 GCTATGTCCGCAAGCTACTCAATTTCTTTGCGGTCATTTTAGCAAACGTGATTGATACAG
 730 740 750 760 770 780

L N L N G V L T F G T V L F Y I A N E G
 TACTCAATTTGAACGGTGTCTTAACCTTTGGTACCGTTCTTTTTTATATCGCTAATGAAG
 790 800 810 820 830 840

L S I T E N L A Q I G V K I P S S I T D
 GTTTGTCAATAACTGAAACTTAGCACAGATTGGTGTTAAAATACCATCATCAATAACAG
 850 860 870 880 890 900

R L Q T I E N E K E Q S K N N A D K A A
 ATCGATTACAAACAATTGAGAACGAAAAAGAACAGAGTAAGAATAACGCAGACAAAGCTG
 910 920 930 940 950 960

G * →
M A I
 CTGGCTAAGCCAGTGGCTTTTTTTATCTTACTGACAGAAGGAGAGAGGGTATATGGCCAT
 970 980 990 1000 1010 1020

ORF 3 (autolysin)

K V V K N L V S K S K Y G L K C P N P M
 TAAAGTTGTAAAGAATCTAGTCTCTAAATCAAAGTATGGATTGAAATGTCCTAATCCAAT
 1030 1040 1050 1060 1070 1080

K A E Y I T I H N T A N D A S A A N E I
 GAAAGCTGAATATATCACTATTCATAACACTGCGAATGATGCTTCAGCAGCCAATGAGAT
 1090 1100 1110 1120 1130 1140

S Y M K N N S S S T S F H F A V D D K Q
 TTCTTACATGAAGAATAACTCTAGCTCAACAAGTTTTCACTTTGCAGTAGACGATAAACA
 1150 1160 1170 1180 1190 1200

V I Q G I P T N R N A W H T G D G T N G
 AGTCATTCAAGGTATTCCAACGAATCGTAACGCTTGGCACACAGGAGATGGAACAAACGG
 1210 1220 1230 1240 1250 1260

T G N R K S I G V E I C Y S K S G G V R
TACAGGGAATCGCAAGTCTATTGGTGTGCGAAATTTGTTATAGCAAGTCAGGAGGGGTACG
1270 1280 1290 1300 1310 1320

Y K A A E K L A I K F V A Q L L K E R G
ATATAAGGCAGCGGAAAAGCTTGCTATTAAGTTTGTGGCTCAGCTACTTAAAGAACGTGG
1330 1340 1350 1360 1370 1380

W G I D R V R K H Q D W N G K Y C P H R
ATGGGGTATTGATCGAGTCCGCAAACATCAAGACTGGAATGGTAAGTATTGCCCGCACCG
1390 1400 1410 1420 1430 1440

I L S E G R W I Q V K T A I E A E L K K
CATTTTGTGAGAGGGAAGATGGATTCAAGTTAAGACTGCAATTGAAGCAGAATTGAAAAA
1450 1460 1470 1480 1490 1500

L G G K T N S S K A S V A K K K T T N T
GTTGGGCGGAAAAACAACTCAAGCAAAGCAAGTGTAGCTAAAAAGAAAACAACAAACAC
1510 1520 1530 1540 1550 1560

S S K K T S Y A L P S G I F K V K S P M
AAGCAGCAAAAAACGTCATATGCGCTACCATCCGGTATTTTAAAGTGAAGAGCCCAAT
1570 1580 1590 1600 1610 1620

M R G E K V T Q I Q K A L A A L Y F Y P
GATGAGAGGGGAAAAGGTAACACAAATTCAAAAAGCACTGGCTGCACTATACTTTTACCC
1630 1640 1650 1660 1670 1680

D K G A K N N G I D G V Y G P K T A D A
GGATAAAGGAGCAAAAAACAACGGCATTGACGGCGTGTATGGTCCGAAAACAGCAGATGC
1690 1700 1710 1720 1730 1740

I R R F Q S M Y G L T Q D G I Y G P K T
AATTAGACGATTCCAGTCTATGTACGGGCTTACTCAAGACGGTATTTACGGACCAAAAAC
1750 1760 1770 1780 1790 1800

K A K L E A L L K *
GAAAGCGAACTTGAAGCTCTCTTGAAGTAAATAAATAGTCTCCTTGAGTATCTTTCTCA
1810 1820 1830 1840 1850 1860

AGGAGATACTTATGTTTATTTTGTACAGATAGTTCAAAATTATTCTAATATTTGAGTAA
1870 1880 1890 1900 1910 1920

AAAGTATTATTTTCTAGTCTGAACATAATGTTATCATTTACATGTAAATAATTTAAAAG
 1930 1940 1950 1960 1970 1980

→ ORF4

M F K K L L L A T S A L T F S
 AAAGGGATTGATTTTCATGTTTAAGAAATTACTTTTAGCAACATCTGCATTAACATTCTCT
 1990 2000 2010 2020 2030 2040

L S L V L P L D G H A K A Q E V T L Q A
 TTATCATTTAGTTCTTCCGTTGGATGGACATGCCAAAGCTCAAGAGGTAACATTACAGGCA
 2050 2060 2070 2080 2090 2100

Q Q E V T Y Q T P V K L S E L P T N T T
 CAGCAGGAAGTCACTTATCAAACCTCCCGTTAAACTATCCGAATTACCAACTAATACAAC
 2110 2120 2130 2140 2150 2160

E Q S G E F H T N G I K K W I A K E A M
 GAACAATCTGGTGAATTTACACAAATGGTATTAATAAATGGATTGCTAAAGAAGCAATG
 2170 2180 2190 2200 2210 2220

K A T A S A L R H G G R I V G E V V D E
 AAGGCAACAGCCAGTGCGCTTCGACATGGTGGGAGAATTGTTGGTGAGGTAGTCGATGAG
 2230 2240 2250 2260 2270 2280

L G G S A G K T F A K H T D D V A D A L
 TTAGGTGGATCAGCAGGAAAAACATTGCGAAAACATACTGATGATGTAGCAGATGCTTTG
 2290 2300 2310 2320 2330 2340

D E L V K R G D V V E D A I I D T V S S
 GACGAGTTAGTTAAACGAGGTGACGTTGTGGAGGACGCAATTATTGACACGGTTTCCTCA
 2350 2360 2370 2380 2390 2400

Y L I D A G V K S S T A R T I A S V F T
 TATCTAATTGATGCTGGTGTAAAATCTTCTACAGCTAGAACTATTGCAAGTGTATTTACT
 2410 2420 2430 2440 2450 2460

→ ORF5

F L A F * (L) V T V E E R L D N
 TTCTTAGCTTTTTAATTGAGGTGATTTGAATTGGTAACAGTAGAAGAAAGGCTTGACAAC
 2470 2480 2490 2500 2510 2520

L E K K V E K Q A F Q L R L V Q Q L A A
 CTAGAAAAAAGTTCGAGAAGCAGGCTTTTCAACTAAGGTTAGTCCAACAATTAGCAGCT
 2530 2540 2550 2560 2570 2580

D Y D R F G L F D Q V L A Y D L S E K Q
 GATTATGATCGGTTTGGCTTATTTGATCAAGTTTTGGCCTATGATTTAAGTGAAAAACAG
 2590 2600 2610 2620 2630 2640

Y Q E L R E L T S Q Y T D K I K N G E E
 TATCAAGAATTAAGAGAGTTAACGAGCCAGTACACTGATAAGATTAAAAATGGCGAAGAG
 2650 2660 2670 2680 2690 2700

V S L H N F T E E F K R I L K D I E K E
 GTCTCTCTTCATAACTTTACTGAAGAATTTAAAAGAATTTTGAAAGATATTGAAAAAGAA
 2710 2720 2730 2740 2750 2760

V D F E K F I S L W L K G P E E G F G F
 GTGGATTTCGAAAAGTTTATTTCTCTCTGGTTAAAGGGACCAGAAGAAGGTTTTGGATT
 2770 2780 2790 2800 2810 2820

S K A L H N H F F N *
 TCTAAAGCTTTACATAACCATTTCTTTAATTAAAGATAAGGACCAGCCACATAATAAGCT
 2830 2840 2850 2860 2870 2880

GGTCTTTCTTTATTTTCCCTCAAGTTCGAACTCCTATGAACAGAATACCAGGTATCTATC
 2890 2900 2910 2920 2930 2940

CTTCCAAATATATTGTTATTAGCTTGAGGGTTTGTGTGTTTTCTTCATTAATCATAGAA
 2950 2960 2970 2980 2990 3000

TTC

Analysis of autolysin activity by renaturing SDS-PAGE

Soluble extracts of *E. coli* DH5 α containing pUBS1 or pSFP102 were prepared and subjected to SDS-PAGE. After renaturation, no lytic activity was associated with the *E. coli* DH5 α (pUBS1) extract (Fig. 3, track 4), while proteins from *E. coli* DH5 α (pSFP102) showed two bands of lytic activity at 30 and 21 kDa (Fig. 3, track 5). An exponentially growing culture (OD₆₀₀ = 0.5) of *E. coli* DH5 α (pSFP102) was harvested and resuspended in SDS-PAGE sample buffer, then boiled for 3 min. The soluble extract was separated by SDS-PAGE. After renaturation, only the 30 kDa band was present (results not shown). However, if this extract was incubated at 37 °C for 2 h, the 21 kDa band became the predominant form, with a concomitant decrease in the level of the

30 kDa band. Corresponding cloned gene products could be visualized by Coomassie Blue staining against the background of *E. coli* DH5 α proteins (Fig. 3, tracks 2 and 3).

Mode of action of the cloned autolysin

The bond specificity of the cloned lytic enzyme can be ascertained by the detection of newly exposed groups on the peptidoglycan after hydrolysis. If the enzyme is a muramidase or glucosaminidase new reducing groups should be detected; if an amidase or endopeptidase new amino termini will be detected. Fig. 4 shows the kinetics of hydrolysis of a suspension of purified *B. subtilis* 168

Fig. 3. Analysis of autolysin activity by renaturing SDS-PAGE. Samples were prepared as described in Methods, and separated by 11% (w/v) SDS-PAGE. (a) Coomassie-Blue-stained gel; (b) renaturing gel containing 0.1% purified *B. subtilis* 168 vegetative cell walls treated as described in Methods. Tracks: 1, molecular mass markers (Dalton MK VII-L) of sizes shown (kDa); 2 and 4, *E. coli* DH5 α (pUBS1) extract (1 μ l); 3 and 5, *E. coli* DH5 α (kDa) (pSFP102) extract (1 μ l). The molecular masses of the zones of lytic activity are marked.

vegetative cell walls (5 mg ml⁻¹) by the enzyme-containing extract from *E. coli* DH5 α (pSFP102). After 30 min treatment with the enzyme, the OD₄₅₀ of the wall suspension had been reduced by >90%. An equivalent amount of extract from *E. coli* DH5 α (pUBS1) had no effect on the wall suspension as measured by loss in OD₄₅₀ over 45 min. Concomitant with the decrease in OD₄₅₀ was a marked increase in the number of free amino termini (Fig. 4, $\blacktriangle$); after 30 min digestion, 637 nmol (mg walls)⁻¹ were released. In contrast over this time period only 10 nmol (mg walls)⁻¹ of new reducing groups appeared. To test whether this increase in amino termini was due to an amidase or an endopeptidase, the FDNB-labelled samples [45 min digestion with *E. coli* DH5 α (pSFP102) extract] were acid-hydrolysed and separated by TLC on silica gel plates, which allows identification of the labelled amino acids by comparison with FDNB-labelled amino acid standards (Ghuysen *et al.*, 1966). The only major spot to appear on the TLC after hydrolysis of the walls by the cloned autolysin

Fig. 4. Digestion of *B. subtilis* 168 purified vegetative cell walls by autolysin-containing extract. Walls were digested as described in Methods at 5 mg ml⁻¹ with extracts from *E. coli* DH5 α containing plasmid pUBS1 (open symbols) or pSFP102 (closed symbols). Samples were removed at various intervals for the determination of OD₄₅₀ ($\circ$, $\bullet$), of released reducing groups ($\blacksquare$) and free amino groups ($\blacktriangle$).

Fig. 5. Effect of ion concentration on enzyme activity. Enzyme activity was measured as described in Methods, using purified *B. subtilis* 168 vegetative cell walls as substrate, and the equivalent of 1 μ l of enzyme extract per 1 ml of reaction mixture. ●, Effect of MgCl_2 concentration on enzyme activity; ○, effect of LiCl concentration on enzyme activity.

corresponded to that of the FDNB-labelled amino acid derivative *N*-2,3-dinitrophenyl-DL-alanine (DNP-alanine) (results not shown). However, this does not differentiate between the possibility of the enzyme being an *N*-acetylmuramyl-L-alanine amidase (amidase) or a *meso*-diaminopimelate-D-alanine endopeptidase, both of which would cause an increase in alanine N-termini. To test which of these possibilities was correct, L-[¹⁴C]alanine-labelled walls were prepared. Digestion using the enzyme extract, FDNB-labelling, acid hydrolysis and TLC separation were as above. After counting, 80% of the radioactivity in the FDNB-labelled acid hydrolysate was found to correspond to DNP-alanine, underivatized alanine remaining at the origin. This constitutes a 14-fold increase in the number of free L-alanine N-termini on treatment with the enzyme as compared to FDNB-labelled acid-hydrolysed undigested walls. No soluble FDNB-labelled material was released over 45 min by walls treated with the *E. coli* DH5 α (pUBS1) extract. Thus, the new N-termini are likely to be L-alanine termini, and the enzyme is probably an amidase.

Effect of ion concentration on autolysin activity

The activity of the cloned autolysin was found to be highly dependent on divalent cation concentration. Activity was completely inhibited at MgCl_2 concentrations <1 mM (Fig. 5) or by the addition of 1 mM-EDTA. A sharp peak of MgCl_2 activation was found,

Table 1. Substrate specificity of the cloned *B. subtilis* 168 autolysin

The activity of the enzyme on various substrates (see Methods) was measured as a percentage of the control activity on *B. subtilis* 168 vegetative cell walls (100%, 46 U). An equivalent amount of *E. coli* DH5 α (pUBS1) enzyme preparation contained <0.1% of enzyme activity compared to the above control.

Substrate	Activity (%)
<i>B. subtilis</i> 168 vegetative cell walls	100
<i>B. subtilis</i> 168 vegetative cell walls (teichoic acid removed)	48
<i>B. sphaericus</i> 9602 vegetative cell walls	61
<i>B. megaterium</i> KM vegetative cell walls	70
<i>M. luteus</i> 4698 cell walls	65
<i>L. arabinosus</i> 6376 cell walls	46
<i>E. coli</i> ER1458 cell walls	0
<i>B. megaterium</i> KM spore cortex	295
<i>B. subtilis</i> 168 vegetative cells	17
<i>B. megaterium</i> KM vegetative cells	122
<i>M. luteus</i> 4698 cells	2

with maximal activity at 20 mM. At 50 mM- MgCl_2 , the enzyme retained <5% of its maximal activity. Mg^{2+} ions could be replaced by other divalent cations. MnCl_2 or CaCl_2 at 20 mM gave 45% and 54% of the activity, respectively, as compared with an equivalent MgCl_2 concentration (results not shown). The enzyme was inhibited by even relatively low concentrations of LiCl ; 150 mM- LiCl inhibited enzyme activity by >90% (Fig. 5).

Substrate specificity of the cloned autolysin

The enzyme extract was tested for its ability to hydrolyse a wide range of peptidoglycan substrates (Table 1). Purified cell walls from all Gram-positive bacteria tested were hydrolysed effectively. On *B. megaterium* KM spore cortex, the best substrate, the enzyme had three times more activity than on the control *B. subtilis* 168 vegetative cell walls. Intact *Bacillus* vegetative cells were also hydrolysed, although to a lesser extent than purified walls. Acid treatment to remove teichoic acids (Hughes *et al.*, 1968) led to a 52% decrease in activity.

Binding of the autolysin to purified cell walls

The autolysin bound well to vegetative cell walls of various species and to *B. megaterium* KM spore cortex (Table 2). However, this adsorption was totally Mg^{2+} -dependent; no enzyme at all became bound to *B. subtilis* 168 vegetative cell walls in the absence of Mg^{2+} .

Table 2. Binding of the *B. subtilis* 168 autolysin to purified cell walls

Enzyme extract (2 µl, 100 U) was incubated in 1 ml 25 mM-Tris/HCl (pH 7.5) with 20 mM-MgCl₂ (unless otherwise stated) containing 1 mg purified cell walls at 0 °C for 15 min. The walls were removed by centrifugation (14000 g, 5 min, 4 °C) and the supernatant was assayed for remaining enzyme activity in the standard assay. The reduction in OD₄₅₀ of the walls containing adsorbed enzyme was also measured in reaction buffer at 37 °C.

Source of wall	Adsorbed activity (U)	Soluble activity (U)	Percentage adsorbed (%)
<i>B. subtilis</i> 168 vegetative cells	55	22	71
<i>B. subtilis</i> 168 vegetative cells (no Mg ²⁺)	0	110	0
<i>B. sphaericus</i> 9602 vegetative cells	80	11	88
<i>M. luteus</i> 4698 cells	72	30	71
<i>B. megaterium</i> KM spores	70	14	83

Discussion

This work describes the cloning and analysis of a low-molecular-mass autolysin of *B. subtilis* 168. During the preparation of this manuscript Kuroda & Sekiguchi (1990) published the cloning and sequencing of this enzyme and designated the gene *cwIA*. They sequenced a 1.1 kb region containing the autolysin gene, the DNA sequence of which exactly matches that presented here and their initial mapping data gives the same approximate position on the *B. subtilis* 168 chromosome. In reference to the above work, I have retained the same nomenclature of *cwIA* for the autolysin gene. Sequencing of the *cwIA*-containing region (3 kb) reveals five ORFs, the central one of which encodes the autolysin. The expression of the autolysin gene in pSFP100 is not IPTG-inducible and thus the gene may be transcribed from its own promoter, even in *E. coli*. The transcriptional regulation of *cwIA* is at present unclear, although it is possibly controlled by the vegetative sigma factor, σ^A . Expression of autolysin genes has also been linked to the minor sigma factor, σ^D (Marquez *et al.*, 1990). Promoter mapping and expression analysis in mutant backgrounds will resolve these problems. Whether *cwIA* forms part of an operon or is transcribed singly is also uncertain.

The autolysin characterized in this work is an amidase which cleaves the peptide side chain from the glycan backbone of the peptidoglycan. In *E. coli* DH5 α , the enzyme is processed from a 30 to a 21 kDa form, although both forms show autolytic activity. A putative signal cleavage site is present after Ala₃₉ (base 1129, Fig. 2) as described by Kuroda & Sekiguchi (1990). Proteolytic processing of autolysins from higher-molecular-mass precursors has been reported in both *B. megaterium*

KM and *Streptococcus faecium* (Foster & Johnstone, 1988; Kawamura & Shockman, 1983), although whether this processing occurs *in vivo* in *B. subtilis* 168 is unknown. Both the major 50 kDa (Herbold & Glaser, 1975; Rogers *et al.*, 1984) and the 30/21 kDa (this study) amidase require Mg²⁺ for activity and have optima at 20 mM. However, the LiCl sensitivity of the enzymes differs; the 30/21 and 50 kDa amidases are inhibited by 92% and 30% by 150 mM-LiCl, respectively (Fig. 5; Herbold & Glaser, 1975).

The 30/21 kDa amidase is able to hydrolyse the isolated peptidoglycan of all Gram-positive bacteria tested, including *B. sphaericus* 9602, which is resistant to digestion by lysozyme, mutanolysin and *Charalopsis* muramidase (P. J. White, personal communication). The greater activity using *B. megaterium* KM spore cortex as substrate may be due to the low incidence of crosslinkage in the spore cortex as compared to vegetative cell peptidoglycan (Tipper & Gauthier, 1972) and thus fewer bonds need to be hydrolysed for solubilization to occur. The enzyme also binds strongly to its peptidoglycan substrate (Table 2). However, this binding is Mg²⁺-sensitive and so the dependence of activity on Mg²⁺ may be a result of the inability of the enzyme to bind to its substrate.

The physiological function of the 30/21 kDa amidase is at present unknown. The insertionally inactivated mutant was able to grow and divide ostensibly as wild-type. The roles of the major 50 kDa amidase and 90 kDa glucosaminidase are also obscure. The only autolysin to be studied in detail at the molecular level is the major amidase of *Streptococcus pneumoniae* (Garcia *et al.*, 1986). When the gene for this enzyme was insertionally inactivated the only altered properties were an inability to lyse at the end of exponential growth and an increased resistance to penicillin-induced lysis (Tomasz *et al.*, 1988). Thus there may be some redundancy in autolysin function, the enzymes acting in concert, with one autolysin being able to compensate for the loss of another. The total complement of *B. subtilis* 168 autolysins is unknown. As well as the two major vegetative autolysins, two sporulation-specific enzymes have also been identified (Guinand *et al.*, 1976). In *B. megaterium* KM, a germination-specific lytic enzyme has also been purified and characterized (Foster & Johnstone, 1987, 1988). Using renaturing SDS-PAGE, *B. subtilis* ATCC 6633 has been shown to have at least seven possible vegetative autolysins (Leclerc & Asselin, 1989), although the actual number of autolysin genes involved is unknown. An in-depth profile of the mutant inactivated in the 30/21 kDa amidase may reveal a subtle phenotype, however, autolysin function may remain obscure until multiple autolysin-deficient strains are produced, as only then will their joint role become apparent.

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