Antonie van Leeuwenhoek **79:** 163–171, 2001.

Sequence analysis and insertional inactivation of a gene encoding *Moraxella* sp. CK-1 cell wall hydrolase

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Received 20 March 2000; accepted 25 July 2000

Key words: autolysin, cell wall hydrolase, insertional inactivation, *Moraxella* sp. CK-1

Abstract

Sequencing of the *Moraxella* sp. CK-1 autolysin (cell wall hydrolases) gene showed the presence of an open reading frame which encodes a polypeptide of 273 amino acids with a molecular mass of 33,316 Da. A presumed ribosomal binding site, a possible –10 and –35 region, and rho-dependent terminators were found. The C-terminal region of the mature protein showed considerable homology with the *Thermus* sp. serine proteinase. Enzyme assay suggests that the recombinant autolysin has amidase or endopeptidase activity. Analysis of the peptidoglycan fragments, following the treatment with the autolysin, indicates that this protein is an *N*-acetylmuramyl-L-alanine amidase. Insertional inactivation of the autolysin of *Moraxella* sp. CK-1 chromosome led to a decrease in cell wall hydrolytic activity, clumping of the cells, and color change. No lytic band present in inactivated *magA* mutant by renaturing SDS-PAGE.

Abbreviations: ATCC – American type culture collection; Bp – Base pairs; CIP – Calf intestinal alkaline phosphatase; DNFB – Dinitrofluorobenzene; IPTG – Isopropyl-β-D-thiogalactopyranoside; PAGE – Polyacrylamide gel electrophoresis; PMSF – Phenylmethylsulfonyl fluoride; RPM – Revolution per minute; SDS – Sodium dodecyl sulfate; X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Introduction

It has been known that bacteria produce several types of cell wall hydrolases, enzymes capable of hydrolysing the peptidoglycan of the cell wall. The enzymes are characterized *in vitro* by their hydrolytic bond specificity as *N*-acetylmuramidase (lysozymes), *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases (Ghuysen et al. 1966). Cell wall hydrolases have been implicated in several important cellular functions, including cell wall growth, turnover, cell separation, flagellation, differentiation, competence for genetic transformation, sporulation, and the lytic action of antibiotics (Pooley & Karamata 1984). Cell wall hydrolases are interesting materials to many bacteriologists not only because of their role in bacterial metabolism but also because of their antimicrobial potential (Banks et al. 1986).

Moraxella sp. CK-1 was isolated from an alkaline lake contaminated by 'water-bloom' (Kim et al. 1997). The organism exhibited a powerful autolytic activity through the excretion of four lytic bands (Ahn et al. 1997). Among them, one of *Moraxella* sp. CK-1 autolysin structural genes has been previously cloned into *E. coli* XL1-Blue MRF' by Ohn et al. (1997). It was proposed that this cloned autolysin is important for cell growth because of continuous expression throughout the growth phase.

Little is known about the autolysins in *Moraxella*. In this presentation, we report on the identification and nucleotide sequence of the gene (*magA*) coding for the major peptidoglycan hydrolase from *Moraxella* sp. CK-1, and compare the amino acid sequence of MagA

with those of other cell wall hydrolases. In addition, an insertionally inactivated mutant was constructed to underpin the function of the autolysin.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Moraxella* sp. CK-1 was grown in BG-11 medium, supplemented with 0.2% casitone (Difco) (Daft & Stewart 1971), at 37 °C with agitation at 150 rpm. *E. coli* and *Micrococcus luteus* ATCC4698 were grown in Luria-Bertani medium (LB) and nutrient broth, repectively. *E. coli* strains containing plasmids were grown in LB medium at 37 °C with vigorous agitation or on LB medium solidified with 1.8% (wt/vol) agar, containing 60 μg of ampicillin (Sigma) per ml, 20 μg of kanamycin (Duchefa, Netherlands) per ml, or 60 μg of erythromycin (Boehringer) per ml, when required. To confirm the existence of the F′ episome, the *E. coli* XL1-Blue MRF' strain was grown in LB medium supplemented with tetracycline at a concentration of 10 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Sigma) were used at concentration of 1 mm and 0.002% (wt/vol), respectively.

Standard DNA techniques

Standard DNA techniques were performed essentially as described by Sambrook et al. (1989). Most restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase (CIP) were purchased from Boehringer Mannheim and were used according to the instructions of the supplier. The λ/*Hin*dIII DNA size marker and the 1 kb ladder were purchased from TaKaRa. RNase A and the electro-elution system were obtained from Bio-Rad. The DNA purification system for sequencing was from Qiagen®. T7 Sequenase v2.0 from Amersham Pharmacia Biotech was used for DNA sequencing. ECL™ direct nucleic acid labeling and detection systems were purchased from Amersham Life Science, United Kingdom. Plasmid pMPT1200 was used to generate templates for sequencing. M13 forward and reverse primers were from Invitrogen®. Ampicillin, proteinase K, and lyophilized *Micrococcus luteus* ATCC 4698 cells were purchased from Sigma.

Preparation of autolysin-containing intracellular extract

The *E. coli* XL1-Blue MRF' strains which harbored pMPT1200 was cultured in 200 ml LB medium supplemented with ampicillin, (60 μg/ml) at 37 °C. The suspension was incubated at 37 °C for 15 h and the cells harvested by centrifugation (3,000 × *g*, 15 min at 4 °C) and suspended in 10 ml 20 mM Tris-HCl (pH 8.0, 4 °C) buffer containing 10 mM-$MgCl_2$ 250 mM LiCl and 0.5 mM PMSF. The cell suspension was subjected to ultrasonication (Sonics & Materials model number VC100; 30 pulses each of 6 s, at a power of 65 W). The sonicated solution was centrifuged (12,000 × *g*) for 5 min at 4 °C; the supernatant kept at 4 °C was used as the source of intracellular enzyme.

Preparation of Moraxella sp. CK-1 genomic DNA

Moraxella sp. CK-1 genomic DNA was isolated according to the method described by Murray & Thompson (1980).

DNA sequencing

Nucleotide sequencing was carried out by the dideoxy-chain termination method (Sanger et al. 1977) using a modified T7 polymerase (T7 Sequenase v2.0 from Amersham). The plasmid pMPT1200 was used to generate templates for sequencing. M13 forward and reverse primers (Invitrogen) were used for sequencing. Electrophoresis was performed on 8% (wt/vol) polyacrylamide/8 M-urea gels. The sequence of both strands was determined for the 1.0 kb region between the *Pst*I and *Bam*HI sites. Protein homology searches were carried out against the SWISSPROT (release 27) database using the FASTA program.

Assay of lytic activity and enzymatic specificity

Heat-killed cells of *Micrococcus luteus* ATCC 4698 and the *Moraxella* sp. CK-1 cell wall were used as standard substrates for the lytic enzymes. Lytic activity was assayed by following the rate of decrease in the turbidity of the cell suspension, as described previously (Sugai et al. 1989). Each assay was carried out in duplicate and 1 unit of enzyme activity was defined as the amount of enzyme necessary to decrease OD_{660} by 0.001 min^{-1}. One milliliter of *Micrococcus luteus* (5 mg/ml) in 50 mM Tris-HCl (pH 8.0) 5 mM 2-mercaptoethanol 10 ml was digested for 3 h with 20 U of recombinant enzyme at 37 °C. Samples

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Reference(s)
Strains		
Moraxella sp. CK-1	Car^r, Str^r, Wild-type strain	Kim et al. (1997)
Moraxella sp. CK-1::pKHM	Car^r, Str^r, km^r, derivative of CK-1 containing 1,282 bp kanamycin resistance gene in *magA* gene	This work
Micrococcus luteus ATCC4698		Sigma
E. coli XL1-Blue MRF'	Δ (*mcr*A)183Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac*[F'proAB $lacI^q$ZΔ M15 Tn10 (Tet^r)]	Stratagene
Plasmids		
pT7T3 19U	Ap^r, phagemid vector	Pharmacia
pUC4K	km^r, phagemid vector	Pharmacia
pMPT1200	Ap^r, pT7T3 19U carrying 1.0 Kb *Moraxella* sp. CK-1 chromosomal DNA insert with *magA* gene	Ohn et al. (1997)
PKHM	Ap^r, km^r, pMPT1200 with 1.3 Kb *Sal*I fragment of pUC4K	This work

were removed at intervals for measurements of turbidity and concentrations of reducing sugars and free amino groups. The appearances of reducing sugars and free amino groups in soluble fragments of the enzymatically hydrolyzed *Moraxella* sp. CK-1 cell wall were determined by a modified Park-Johnson procedure (Thompson & Shockman 1969) and a Ghuysen procedure using 2,4-dinitrofluorobenzene (Ghuysen et al. 1966), respectively.

Disruption of the magA gene by means of insertional inactivation

To examine the function of the *magA* gene, *magA*::Km was constructed. A hybrid plasmid, namely pKHM, was obtained by inserting the kanamycin resistant gene at the *Sal*I site of the *magA* gene. Competent cell preparation was performed by using the TSS method, as described by Miller (1992). The ligation mixture (50 ng) was added to 150 μl of XL1-Blue MRF' competent cells and 850 μl of TSS solution mix. After gentle mixing, the tube was left in ice for 30 min and induced for transformation by incubating gently in a shaker at 37 °C for 100 min. About 200 μl of induced cells were plated on an LB agar plate supplemented with X-Gal 20 mg/ml, IPTG 200 mg/ml, ampicillin 60 μg/ml, and kanamycin 25 μg/ml. The cells were incubated at 37 °C for 18 h.

Chromosomal inactivation of magA

The hybrid plasmid pKHM was constructed as described above. To obtain a *magA* mutant, plasmid pKHM was used for transformation of competent cells of *Moraxella* sp. CK-1 and kanamycin-resistant transformants were selected on CA agar plates supplemented with kanamycin (20 μg/ml). Integrants, which were subject to crossovered via portions 1 and 2, were used for further experiments (Figure 1). Since *magA*::Km contained a 1.3 kb km insert, double crossover integration led to inactivation of the *magA* gene (Figure 1). Excision of the integrated plasmid was established after nonselective growth for 24–30 generations in CA. Cells were plated on CA plates supplemented with X-Gal, kanamycin (20 μg/ml), and 0.2% (wt/vol) lyophilized *Micrococcus luteus* cell walls and screened for loss of blue staining and halo formation.

Southern blotting and hybridization

To examine the resulting insertional inactivated mutants, chromosomal DNA was extracted from transformants, digested with *Pst*I and subjected to agarose gel electrophoresis followed by Southern blot hybridization analysis. A 0.7% agarose gel was used to carry out electrophoresis with the digested DNA samples at 4 V/cm for 80 min. The DNA was transferred onto nylon membrane (Nytran 0.2 μm, Schleicher &

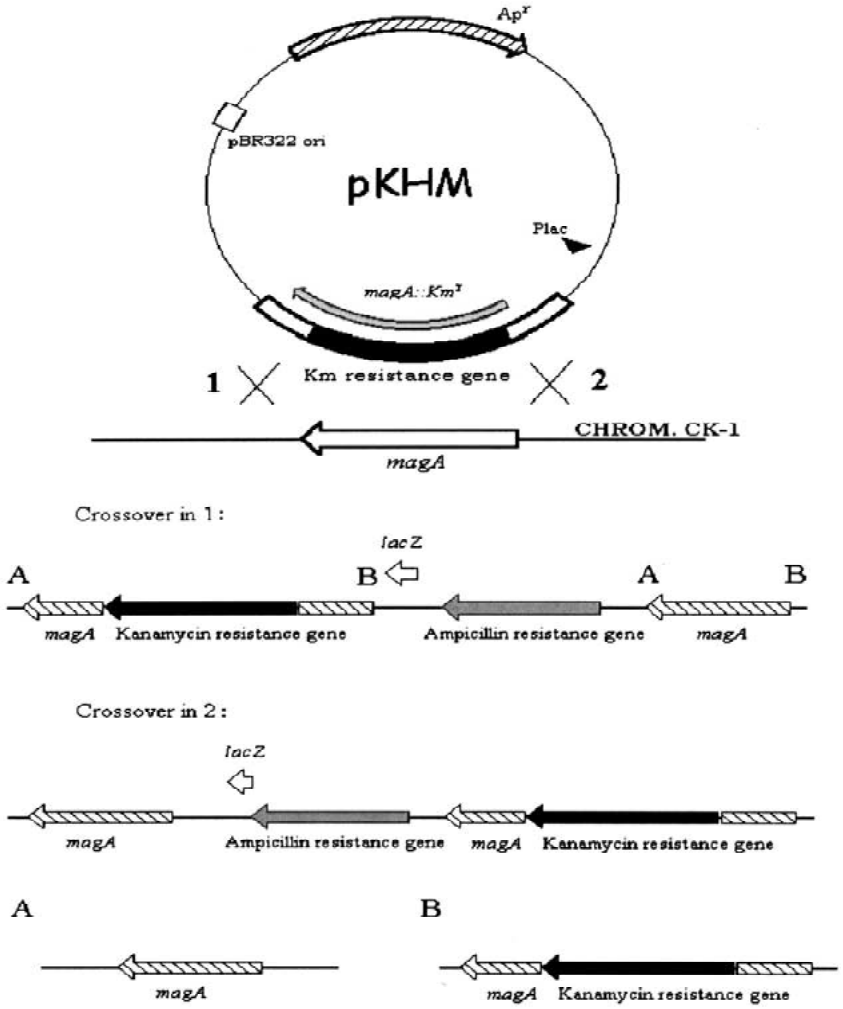

Figure 1. Schematic representation of the construction of the chromosomal *magA* mutant of *Moraxella* sp. CK-1 with pKHM. km^r, kanamycin resistance gene; *lacZ*, ß-galactosidase gene of *E. coli*; open square, origin of replication of *E. coli* plasmid pBR322; 1 and 2, possible sites for the first crossover; A and B, possible regions for the second crossover and their products.

Schuell) by the capillary transfer method. The 1.3 kb *Pst*I fragment from pUC4K, containing the kanamycin resistance gene, was used as a probe. Probe labeling and hybridization were done using the ECL labeling and detection system according to the instructions of the manufacturer (Amersham Life Science, UK).

Detection of autolysins by renaturing SDS-PAGE

Renaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide gels supplemented with 0.35% (w/v) heat killed *Micrococcus luteus* ATCC 4698, as described by Ahn et al. (1997). Electrophoresis (20 mA for the stacking gel and 40 mA for the running gel) gels were soaked in ten volumes of distilled water for 30 min at room temperature with gentle agitation. The gels were then transferred to renaturation buffer solution (20 mM sodium phosphate, pH 8.0, 1% Triton X-100, 1 mM $MgCl_2$) at 30 °C and gently agitated for 15 h at 37 °C. The enzyme activities appeared as clear bands on the opaque gels. For the determination of the molecular weight of the lytic enzymes, standard marker proteins (Bio-Rad) were electrophoresed using the same gels and stained with staining solution (0.025% coomassie blue R-250, 40% methanol, 7% acetic acid).

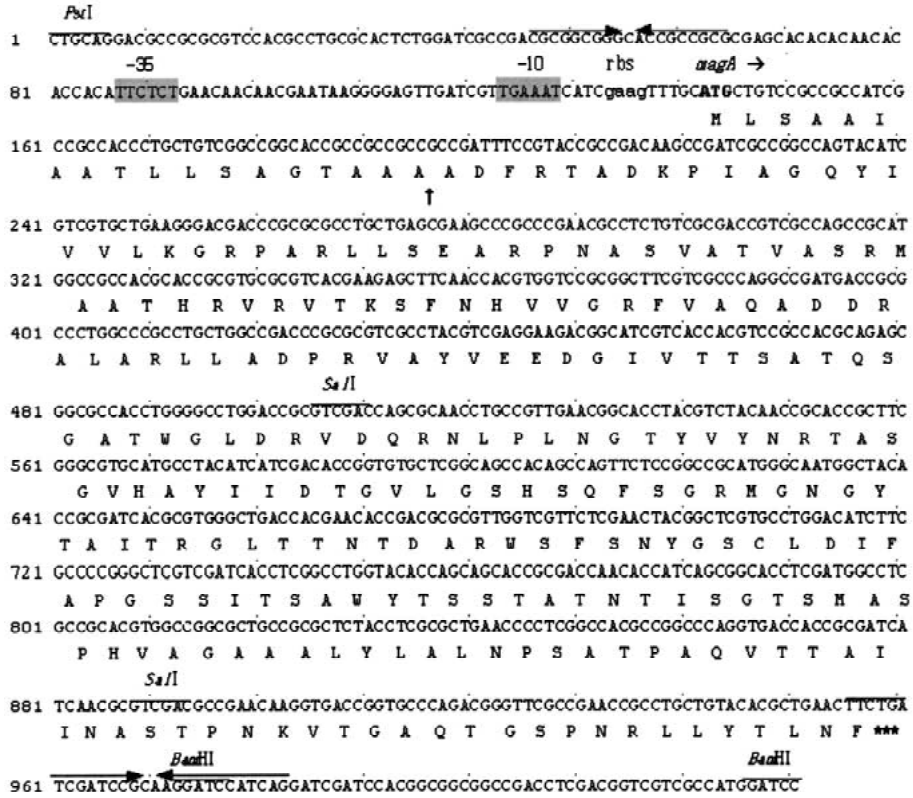

Figure 2. Nucleotide sequence and deduced amino acid sequences of *magA* of *Moraxella* sp. CK-1. Putative ribosome-binding sites (rbs and lowercase letters), -10 and -35 sequences (shaded), start codons (boldface), and stop codons (asterisks) are indicated. Two possible transcriptional terminators are indicated by horizontal arrows above the sequences. The possible signal peptide cleavage site is indicated by a vertical arrow. A number of relevant restriction sites are also indicated.

Nucleotide sequence accession number

The nucleotide sequence data reported in this study (Figure 2) have been deposited in GenBank (NCBI, National Center for Biotechnology Information) under the accession number AF064522.

Results

Nucleotide sequence of the Moraxella *sp. CK-1 cell wall hydrolase gene*

The nucleotide sequence of the 1,032-bp *Pst*I-*Bam*I fragment of pMPT1200 (Figure 2) shows that it contains one complete ORF (tentatively designated *magA* [*Moraxella* sp. CK-1 autolysin gene]) with a length of 819 bp. The autolysin gene is preceded by a putative ribosome binding site at bases 233–236 (Figure 2) with an estimated free energy for the Shine-Dalgarno interaction of -5.6 kcalmol^{-1} (-23.40 kJmol^{-1}) (Tinoco et al. 1973). Two putative rho-dependent terminators, with an estimated free energy of the stem-loop structure of -25.6 kcalmol^{-1} (-106.98 kJmol^{-1}) and -14.6 kcalmol^{-1} (-61.01 kJmol^{-1}), are present upstream from the autolysin start codon at bases 46–64 and downstream from the autolysin stop codon at bases 957–983, respectively. A possible -10 and -35 region with a spacing of 30 bp is present upstream of *magA* (Figure 2).

Deduced amino acid sequences and homology comparisons

magA could encode a protein with 273 amino acids with a deduced molecular weight of 33,316 Da. The first 18 amino acids constitute a putative signal peptide (von Heijne 1986). Cleavage of this putative signal peptide would result in a protein with a size of 31,406 Da. An identity of approximately 83.1% was found between the C-terminal region of MagA (amino acids 168 to 269) and a *Thermus* sp. extracellular ser-

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CK-1   : 168 AITRGLTTNTDARWSFSNYGSCLDIFAPGSSITSAWYTSSTATNTISGTSMA 227
Rt41A  : 305 AITVGATTSTDYRASFSNYGRCLDLFAPGQSITSAWYTSSTATNTISGTSMA 356
O-7    : 297 AITVGSTTSSDARSSFSNYGNCLDIYAPGSSITSAWYNSDTSTNTISGTSMA 348

CK-1   : 228   SPHVAGAAALYLALNPSATPAQVTTAIINASTPNKVTGAQTGSPNRLLYT 269
Rt41A  : 357   TPHVTGAAALYLQWYPTATPSQVASALLYYATPNVVKNAGRYSPNLLLYT 406
O-74   : 349   APHVAGAV  YLDENPSLSPSQIDSLLSQRSSKGKVSNPQSGSPNELLYT 398
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Figure 3. Similarity of the deduced amino acid sequence of MagA to the *Thermus* sp. Rt41A serine proteinase (Peek et al. 1992) and to the alkaline serine protease gene from *Alteromonas* sp. strain O-7 (Tsujibo et al. 1993). Amino acid identities are indicated by shading and amino acids are numbered from the N-termini of the proteins.

ine proteinase (Peek et al. P80146 1992) (Figure 3). An identity of 67% was observed with the precursor aqualysin I of *Thermus aquaticus* (Kwon et al. 1988).

Mode of autolytic action

The generation of free amino groups and reducing sugars during enzymatic hydrolysis of the *Moraxella* sp. CK-1 cell wall by the cell wall hydrolase was monitored. No increase in reducing groups was noted, while 190 nmole of free amino acids per mg was found when compared with the control (Figure 4). These results suggested that the cell wall hydrolase has amidase or endopeptidase activity (Sugai et al. 1990). For further study of this enzymatic action, the supernatant of the reaction mixture was incubated with DNFB followed by hydrolysis with 4 N HCl. 2,4-Dinitrophenol-alanine was identified by thin-layer chromatography (data not shown). We failed to detect 2,4-dinitrophenol-amino acid by dinitrophenylation of the cell mixture after hydrazinolysis. These results indicate that the cell wall hydrolase is an *N*-aceytlmuramyl-L-alanine amidase.

Southern blotting and hybridization

Chromosomal DNA of the putative mutant clone was digested with *Pst*I and subjected to Southern blot analysis. The *Pst*I fragment of the kanamycin resistance gene from pUC4K was used as a probe DNA. One strong band was seen at 1.3 kb (data not shown).

Autolytic activity of the magA mutant

For the determination of cell wall lytic activity, protein extracts from *Moraxella* sp. CK-1, the *magA* mutant, and the *E. coli* XL1-Blue MRF' strain were subjected to SDS-PAGE in the presence of 0.35% *M. luteus* ATCC 4698 for detection of cell wall hydrolases (Laemmli 1970). After renaturation in a Triton X-100

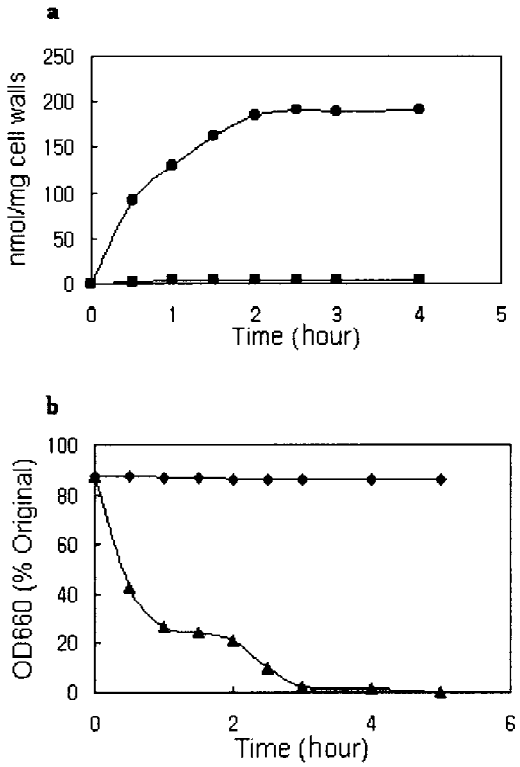

Figure 4. Digestion of *M. luteus* cell walls by autolysin-containing extract. (a) Samples were removed at various intervals for the determination of OD660 (●,■), of released reducing groups (■), and free amino groups (●); (b) Walls were digested as described in methods at 5 mg/ml with extracts from *E. coli* XL1-Blue MRF' (♦) or *E. coli* XL1-Blue MRF' containing pMPT1200 (▲).

containing buffer, cell wall hydrolases appeared as clear lytic bands against an opaque background. As we prepared intracellular proteins of *Moraxella* sp. CK-1, only two lytic bands of 32 and 40 kDa, exhibiting hydrolytic activity against *M. luteus* cells, were observed (Figure 5, lane 1). Activity was also observed when *Moraxella* sp. CK-1 cell walls were used (data not shown). No lytic band was found in extracts from the *magA* mutant (Figure 5, lane 3).

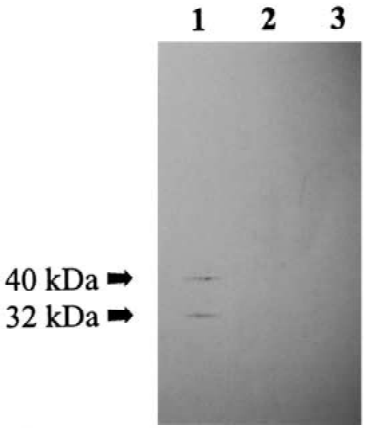

Figure 5. Zymogram of *Moraxella* sp. CK-1 and mutant extract proteins separated by renaturing SDS-PAGE (12% polyacrylamide gel containing 0.35% heat-killed *M. luteus* cells as substrate). Arrows show clearing zones due to lytic enzymes. Lane 1, Intracellular proteins of *Moraxella* sp. CK-1; lane 2, *E. coli* XL1-Blue MRF' (negative control); lane 3, *magA* mutant extract proteins.

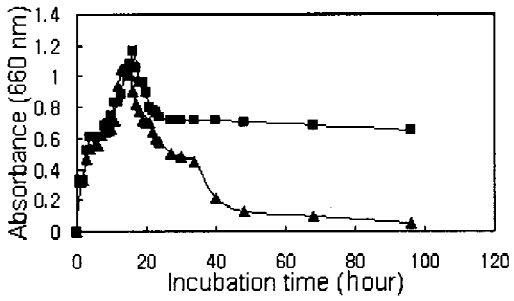

Figure 6. Digestion of *M. luteus* cell walls by *magA* mutant extract. Walls were digested as described in methods at 5 mg/ml with extracts from *Moraxella* sp. CK-1 (▲) or *magA* mutant (■). The lytic activity of *magA* mutant was measured by the ability of the crude enzyme preparation to decrease the optical density of a suspension of purified *M. luteus* cell walls at 37 °C.

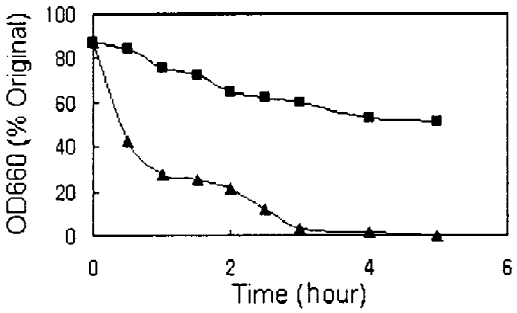

Figure 7. Growth curve of *Moraxella* sp. CK-1 (▲), and the *magA* mutant (■).

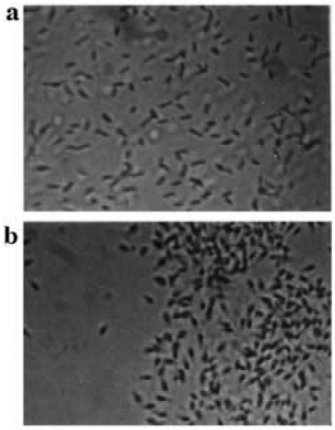

Figure 8. Light microscope features of *magA* mutant (× 1000 magnification). (a) *Moraxella* sp. CK-1; (b) *magA* mutant.

Discussion

The deduced molecular mass of the *magA* gene product is 33,316 Da (31,406 Da without signal peptide), and is likely to be smaller upon processing between Ala_{18}-Ala_{19} residues (Figure 2). Protease secretion usually involves a signal peptide and the mature protein (Wandersman 1989). Secretion of extracellular proteins has been studied in both Gram-positive and Gram-negative bacteria (Pugsley & Schwatz 1985; Wandersman 1989). The expression of the autolysin in pMPT1200 is not inducible by IPTG and hence the gene may be transcribed from its own promoter in *E. coli.* XL1-Blue MRF'. The transcriptional regulation of *magA* is still unclear.

The bond specificity test has shown that the autolysin characterized in this study has amidase or endopeptidase acivity (Sugai et al. 1990). Biochemical characterization of the products released by *magA* indicated that the cell wall hydrolase was indeed an *N*-acetylmuramyl-L-alanine amidase.

No significant homology was found between the complete *magA* sequence and other published sequences of cell wall hydrolases. The C-terminal of MagA protein was similar to that of the *Thermus* sp. serine proteinase. It is possible to find up to 81.3% identity in 102 amino acids encoded by the gene from *Thermus* sp., which codes for serine proteinase. Lazarevic et al. (1992) has reported a consensus motif (GSNRY) found in nearly all amidases, but this motif is not conserved in *magA*. The predicted consensus motifs GTAAA, GTYVY, and GTSMA have been found in the in MagA positions 14–18, 131–135, and 216–220, respectively. It may cleave *N*-linked car-

bohydrates from glycoproteins, but the role of these motifs remains to be unraveled.

Inactivation of the *magA* gene, by insertion of the kanamycin resistance gene after Val_{122}, was accompanied by loss of lytic activity. No cell wall hydrolase activity was present. No lytic band was detected by renaturing SDS-PAGE performed with intra- and extracellular proteins from the *magA* mutant. Ohn et al. (1997) have shown that the existence of a *magA* homologous region in *Moraxella* sp. CK-1. From this result, it is likely that the *magA* gene is crossed-over with the other types of homologous regions in *Moraxella* sp. CK-1. Further analysis of the deletion mutant will reveal whether *magA* is the only gene encoding a peptidoglycan hydrolase or whether *Moraxlla* sp. CK-1 contains a second muramidase, as is the case in *E. hirae* (Shockman 1992).

The *magA* mutant led to a decrease of total cell wall lytic activity at pH 10.0, the optimal pH for *Moraxella* sp. CK-1 autolysin (Figure 6). The insertionally inactivated mutant showed an increased growth yield and reduced lysis rate (Figure 7). It is interesting to note that colonies carrying *magA* mutant were white in color (Potvin et al. 1988). Though Buist et al. (1995) reported that the autolysin deletion mutant formed chains of cells, there was no indication of the formation of chains or filaments of cells in this study. However, the *magA* mutant grew in clusters when it was cultured overnight (Figure 8). This result suggests that *magA* is involved in cell separation, as previously postulated by Sugai et al. (1995).

Acknowledgements

This research was supported by Hanyang University research fund (HY-2000). The authors are grateful to Chang-Ho Shin for his assistance.

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