Molecular characterization of an autolytic amidase of *Listeria monocytogenes* EGD

Anna M. McLaughlan and Simon J. Foster

Author for correspondence: Simon J. Foster. Tel: +44 114 222 4411. Fax: +44 114 272 8697.
e-mail: s.foster@sheffield.ac.uk

Department of Molecular
Biology and
Biotechnology, University
of Sheffield, Firth Court,
Western Bank, Sheffield
S10 2TN, UK

The gene encoding a 102 kDa autolysin has been cloned from an expression library of *Listeria monocytogenes* EGD genomic DNA, using a direct screening protocol. The encoded protein has two domains, an N-terminal enzymic domain showing a high level of homology to the amidase domain of the major autolysin (*atl*) of *Staphylococcus aureus*, and a C-terminal, putative cell-wall-binding domain containing four imperfect direct repeats. In order to examine the role of the enzyme, the autolysin-encoding gene was insertionally inactivated by site-specific integration of a temperature sensitive plasmid. The enzyme accounts for 66% of the total lytic enzyme activity when *L. monocytogenes* walls are used as substrate and several of the major autolytic bands are missing on renaturing gels when compared to the wild-type. The enzyme does not appear to be directly involved in cell separation but has a role in motility. Characterization of the recombinant enzyme expressed in *Escherichia coli* has revealed it to be an amidase and to be able to hydrolyse a range of peptidoglycan substrates.

Keywords: *Listeria monocytogenes*, peptidoglycan hydrolase, autolysin, amidase

INTRODUCTION

Bacterial peptidoglycan hydrolases are ubiquitous amongst both Gram-positive and Gram-negative bacteria. Those that are capable of hydrolysing covalent bonds in the peptidoglycan of their own cell walls are referred to as autolysins (Ghuysen *et al.*, 1966). It has been proposed that these enzymes play roles in a variety of important biological functions, including expansion of the cell wall, cell wall turnover during growth, splitting of the septum allowing cell division, motility, differentiation, and antibiotic-induced lysis (Ward & Williamson, 1984). The fact that autolysins may also play a role as virulence factors has been one of much speculation although there is now direct evidence that these enzymes do contribute to pathogenesis (Berry *et al.*, 1992; Mani *et al.*, 1994).

The development of renaturing SDS-PAGE, and the construction of mutants inactivated in specific autolysin structural genes has allowed the autolytic complement of several bacteria to be elucidated. Multiple peptido-

glycan hydrolases from *Bacillus subtilis* have been identified and several studied at the molecular level (Smith *et al.*, 1996). Renaturing gels of *Staphylococcus aureus* have revealed the presence of at least 20 lytic bands, although >97% of this profile is due to the processed forms of the major bifunctional autolysin, Atl (Foster, 1995; Oshida *et al.*, 1995). Relatively few studies have focused on the peptidoglycan hydrolases from Gram-negative bacteria apart from those in *Escherichia coli* (Höltje, 1995). However, the major autolysin from *Pseudomonas aeruginosa* has been characterized (Li *et al.*, 1996).

Listeria monocytogenes is a Gram-positive opportunistic pathogen that is environmentally widespread and causes severe food-borne infections in humans and animals (Farber & Peterkin, 1991). We have previously characterized the peptidoglycan hydrolase profile of *L. monocytogenes* EGD under a variety of different environmental and physiological conditions (McLaughlan & Foster, 1997). This revealed multiple bands of activity, which may represent many gene products or proteolytic processing of proforms, as seen in *S. aureus* (Foster, 1995). To determine the true number of autolysins that contribute to this profile, it is necessary to isolate structural genes and to create specific mutants. One extracellular protein called p60, produced by all

Abbreviations: FDNB, 2,4-dinitrofluorobenzene; PITC, phenyl isothiocyanate

The GenBank accession numbers for the sequences reported in this paper are AF042193 and AF035424.

virulent strains, has been identified and shown to have autolytic properties (Wuenschel *et al.*, 1993). Mutants with reduced p60 levels are filamentous; the cells fail to separate after cell division and as a result, show reduced ability to invade host cells. Addition of p60 to these mutants disaggregates chains and at least partially restores invasiveness of the resulting single cells, implying a role for p60 in virulence (Kuhn & Goebel, 1989).

To further understand the roles of autolysins in the physiology of *L. monocytogenes*, we have used a direct screening method for the isolation of lytic-enzyme-encoding genes. This has allowed the identification of a major autolysin encoding gene. The gene has been characterized and the function of the protein examined.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. *L. monocytogenes* strains were grown in Brain Heart Infusion broth (BHI; Oxoid) at 37 °C with constant shaking (250 r.p.m.) unless otherwise stated, or on BHI plates (1%, w/v agar). *Escherichia coli* XL0LR and XL-1 Blue MRF⁺ were grown in Luria-Bertani broth (LB) or on LB agar [1% (w/v)] at 37 °C.

Preparation of purified cell wall substrate. Purified cell wall preparations were prepared as previously described (Foster, 1992; McLaughlan & Foster, 1997). Procion-Red-labelled walls of *L. monocytogenes*, *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis* were prepared as described previously (Foster, 1991).

Identification of lytic enzyme structural genes. A λ ZAP Express library of 2–10 kb *L. monocytogenes* EGD chromosomal DNA, partially digested with *Sau*3A, was constructed according to the manufacturer's instructions (Stratagene), in *Bam*HI-cut and dephosphorylated vector. A total of 1.1×10^6

independent insert-containing clones were produced and the library amplified once. The library was screened for lytic-enzyme-producing clones by the plate assay, described previously, using Procion-Red-labelled cell walls as the substrate (Foster, 1991). Lytic-enzyme-producing clones were identified by a zone of clearing in the red wall background and were picked and purified by further rounds of plate screening. Purified clones were treated to excise the phagemid (pBKCMV) containing the *L. monocytogenes* DNA inserts, according to the manufacturer's protocol (Stratagene), and clones were checked for recombinant lytic activity by the plate test (Foster, 1991). DNA was isolated from these clones and characterized by restriction analysis. All molecular biological methods were performed as in Sambrook *et al.* (1989).

Preparation of samples and renaturing SDS-PAGE. Samples containing peptidoglycan hydrolases were prepared and analysed by renaturing gel electrophoresis as previously described (Foster, 1992); 11% (w/v) acrylamide gels containing 0.05% (w/v) purified bacterial walls were utilized. Peptidoglycan hydrolase activity was detected as zones of clearing in the blue-stained cell wall background.

Molecular analysis of the lytic enzyme clones. Nested deletions were prepared using the Erase-A-Base system (Promega) and used for sequencing. DNA sequence was determined from both strands using the dideoxy method. Synthetic oligonucleotide fragments based on DNA sequence generated during this work, or to vector sequence, were used as primers. DNA sequence was edited and analysed using the Staden package. Putative open reading frames were examined using the NIP program and the deduced amino acid sequences were compared to the NCBI Entrez protein databases by BLAST searches.

Preparation of fractions containing recombinant enzyme. For biochemical analysis, 4 l cultures of *E. coli* containing pAMB8 were grown in the presence of IPTG (1 mM), to OD₆₀₀ 1.5 after inoculation with 0.01 vol. overnight culture. The cells were harvested by centrifugation (11000 g, 4 °C, 10 min) and broken in a French press (180 MPa) in 40 ml 50 mM Tris/HCl (pH 7.6), 200 mM NaCl, 0.5 mM PMSF at

Table 1. Strains and plasmids

Strains or plasmids	Relevant genotype/phenotype	Source/reference
Strains		
<i>L. monocytogenes</i> EGD	Wild-type	Wuenschel <i>et al.</i> (1993)
<i>L. monocytogenes</i> AM101	<i>ami</i> ::pAMB15; Ery ^r	This study
<i>E. coli</i> XL-1 Blue	Tet ^r	Stratagene
<i>E. coli</i> XL0LR	Tet ^r	Stratagene
Plasmids		
pBKCMV	Kan ^r	Stratagene
pAMB8	pBKCMV with 3.2 kb insert carrying truncated <i>L. monocytogenes ami</i> gene; Kan ^r	This study
pAMB9	pBKCMV with 1.8 kb insert carrying <i>L. monocytogenes</i> phage A118 lytic enzyme gene; Kan ^r	This study
pAULA	Ery ^r	Chakraborty <i>et al.</i> (1992)
pAMB15	<i>Bam</i> HI/ <i>Eco</i> RI-cut pAULA with 768 bp <i>Bam</i> HI/ <i>Eco</i> RI internal <i>ami</i> fragment; Ery ^r	This study

4 °C. After removal of debris by centrifugation (100 000 g, 4 °C, 2 h), the supernatant was dialysed overnight against 100 vols 20 mM potassium phosphate buffer (pH 7.4) at 4 °C. The dialysate was then centrifuged (100 000 g, 4 °C, 2 h); the pellet was resuspended in 20 mM Tris/HCl (pH 7.6), 200 mM NaCl, stored at -20 °C and used directly as the enzyme source.

Assay for autolytic activity. Lytic activity of the enzyme extract was determined spectrophotometrically by the ability of the extract to decrease the optical density of a cell wall suspension. Unless stated otherwise, the reaction contained 1 ml 20 mM Tris/HCl (pH 7.6), 200 mM NaCl and purified cell walls to a final OD₄₅₀ of 0.25. One unit (U) of enzyme activity is defined as the amount of enzyme necessary to decrease the OD₄₅₀ of a wall suspension by 0.001 min⁻¹ at 37 °C, measuring the maximum initial rate (Foster, 1991).

Determination of hydrolytic bond specificity. Purified cell walls of *B. subtilis* (1 mg dry weight) in 20 mM Tris/HCl (pH 7.6), 200 mM NaCl in a total volume of 1 ml were digested for 4 h at 37 °C with 16.5 U recombinant enzyme. Digestion of the walls was followed spectrophotometrically, the OD₄₅₀ decreasing from 0.529 to 0.04. The hydrolytic bond specificity of the autolysin was determined by analysing the N-terminal amino acid ratios after digestion. The soluble digested walls were assayed for amino groups released by amidase activity by labelling with 2,4-dinitrofluorobenzene (FDNB). To 100 µl of the digested walls, 150 µl NaCO₃ (1%, w/v), 62.5 µl borax (5%, w/v) and 31.5 µl FDNB solution (130 µl in 10 ml 100% v/v ethanol) were added, and the solution incubated at 60 °C for 30 min (Margot *et al.*, 1991). The reaction was stopped by adding 50 µl 5 M HCl and the labelled samples were acid-hydrolysed for 14 h at 110 °C under vacuum. The hydrolysates were then dried and derivatized with phenyl isothiocyanate (PITC) at 110 °C (Atrih *et al.*, 1996). The derivatives were analysed by HPLC and the amount of PITC-labelled alanine determined in both digested and control samples. The constant peptidoglycan-associated glutamic acid levels were used as an internal control.

Insertional inactivation of the autolysin-encoding gene. A 768 bp internal fragment from the sequenced autolysin gene, cloned in pAMB8, was amplified by PCR and suitable restriction sites introduced at each end. The primers used were AM43 (5'-ATATCGATCCGTAGACGGTCAAGCTACTT-ATATCC-3'; *Bam*HI restriction site underlined) and AM44 (5'-GCCGGAATTCGCTGTTGCTGTTTTGACTCGGCTA-3'; *Eco*RI restriction site underlined). After *Eco*RI and *Bam*HI digestion, the PCR product was cloned into dephosphorylated *Eco*RI- and *Bam*HI-digested pAULA (Chakraborty *et al.*, 1992), to give pAMB15. This plasmid was transformed by electroporation at 30 °C (Park & Stewart, 1990) into *L. monocytogenes* EGD; transformants were selected on BHI plates containing erythromycin (1 µg ml⁻¹) at 30 °C. Transformants were grown in BHI broth containing 1 µg erythromycin ml⁻¹ at 30 °C for 12 h. The culture was diluted 1/100 into fresh BHI containing erythromycin (1 µg ml⁻¹) at 42 °C. After 18 h the culture was diluted as above and grown for a further 12 h, prior to plating of putative mutants on erythromycin-containing BHI plates at 42 °C. The correct insertional event was confirmed by Southern blotting and probing with an internal fragment of the autolysin gene.

Swarm plate assay. Swarming motility was measured using BHI 0.3% (w/v) agar. Samples from overnight liquid cultures were spotted onto the centre of each plate and incubated overnight at 25 °C, 30 °C and 37 °C. Swarming motility was

measured as a percentage of the wild-type diameter. The results are a mean of two repetitions.

Cell autolysis. Cultures of mutant and wild-type *L. monocytogenes* strains were grown to early exponential phase (OD₆₀₀ 0.25) in BHI broth. For determining the effect of penicillin-induced lysis, 1.25 µg penicillin G ml⁻¹ (10 × MIC) was added, and the lysis of the cultures followed spectrophotometrically (Unicam SP1800 ultraviolet spectrophotometer, path length of cuvette 1 cm) while continuing incubation at 37 °C with shaking. For determining the effect of Triton X-100, the cells were grown to OD₆₀₀ ~ 0.6, harvested (6000 g, room temperature, 10 min) and resuspended in 50 mM Tris/HCl (pH 7.6), 0.05% (v/v) Triton X-100. The lysis of the suspension at 37 °C was followed spectrophotometrically. The results stated are from two independent experiments.

Lithium chloride extraction of whole cells. Extraction of whole cells with 4 M lithium chloride, to release peptidoglycan hydrolases, was performed as described previously (Foster, 1992).

RESULTS

Identification of lytic enzyme structural genes

A λ ZAP library of *L. monocytogenes* genomic DNA was screened for lytic-enzyme-producing clones by a direct screening protocol. Lytic enzyme clones are characterized by a zone of clearing in the red wall overlay around the plaque. After an initial screening of approximately 2 × 10⁶ insert-containing clones, a total of 10 lytic-enzyme-producing clones were isolated using *B. subtilis*, and 19 using *M. luteus* Procion-Red-labelled walls as substrate. *L. monocytogenes* and *S. aureus* walls were also used but no expressing clones were isolated using these substrates. The putative phage clones were purified by rescreeing prior to excision as phagemids.

The ability of *E. coli* to express the recombinant lytic enzymes was tested using Procion-Red-labelled wall overlay plates (Foster, 1991). All 10 of the clones initially isolated using *B. subtilis* walls retained activity when tested once again on *B. subtilis* walls, but interestingly, all of these clones except one (containing pAMB9) were also able to hydrolyse *M. luteus* cell walls. From the total of 19 clones isolated using *M. luteus* cell walls, two retained activity on the original substrate and only one of these was also capable of hydrolysing *B. subtilis* walls. The ability of the clones to hydrolyse the cell walls was not IPTG dependent. *E. coli* expressing pBKCMV (vector without insert) showed no activity on red-wall overlays.

Restriction analysis and Southern blotting revealed that two distinct lytic-enzyme-encoding genes had been isolated. One gene was represented by nine of the clones originally isolated by plating on *B. subtilis* and *M. luteus* walls. Of these clones, pAMB8 was used for sequencing and further characterization. This plasmid contained an insert of approximately 3.2 kb. The other gene was represented by the remaining three clones isolated using *B. subtilis* walls; *E. coli* pAMB9 was chosen as a

Fig. 1. Lytic activity of recombinant clones. Samples were prepared as described in Methods. Renaturing SDS-PAGE was performed using an 11% (w/v) acrylamide gel containing 0.05% (w/v) *L. monocytogenes* cell walls. The positions of molecular mass standards separated on the same gel are indicated. Lane 1, *E. coli* XLOR(pAMB8) containing the equivalent of 100 μ l original culture (OD_{600} 1.0); lane 2, *E. coli* XLOR(pAMB9) containing the equivalent of 0.1 μ l original culture (OD_{600} 1.0).

representative clone for sequencing (insert approximately 1.8 kb).

Renaturing gel analysis

E. coli XLOR strains expressing the recombinant enzymes were grown with and without IPTG (1 mM). The cells were harvested at mid-exponential phase (OD_{600} 1.0–1.2) and SDS cell extracts prepared (Foster, 1992). Addition of IPTG did not affect the growth rate of the cells. The protein samples were analysed by renaturing SDS-PAGE (Fig. 1). A lytic band of 104 kDa was seen in extracts from *E. coli* XLOR(pAMB8) (lane 1) and a band of 33 kDa was seen from *E. coli* XLOR(pAMB9) (lane 2). The *E. coli* XLOR(pBKCMV) extract (plasmid without insert) showed no lytic enzyme activity (results not shown). The presence of IPTG in the growth medium had no effect on the bands seen on the gel.

Sequencing of the lytic enzyme clones

DNA sequence analysis of pAMB9 revealed the presence of a gene encoding a protein of 281 amino acids. This protein shows 96% identity over its entire length to the previously characterized lytic L-alanoyl-D-glutamate

peptidase of *L. monocytogenes* phage A118 (Loessner *et al.*, 1995). Upstream of the putative peptidase-encoding gene in pAMB9, most likely in the same operon, is a gene encoding a probable holin. This gene is truncated at its 5' end in pAMB9, but the truncated protein encoded shows 100% identity (over 35 amino acids) with the holin of phage A118. Thus, the recombinant peptidoglycan hydrolase cloned on pAMB9 is likely to be of phage origin and may represent that of A118 or a very closely related phage. It is at present unknown whether the pAMB9-encoded peptidoglycan hydrolase is part of a phage inserted into the chromosome of *L. monocytogenes*.

The entire DNA sequence of the pAMB8 insert (3229 bp) was determined and two putative ORFs were identified (Fig. 2). The major ORF encodes a protein that is truncated at its N-terminus in pAMB8. Despite repeated screening of the library, a clone containing the rest of the gene could not be isolated. Recently, the sequence of the gene has been reported and a similar problem was encountered (Braun *et al.*, 1997). PCR was used to obtain the final 99 bp of the sequence based on that published. Braun *et al.* (1997) have called the gene *ami* and this nomenclature has been retained. The complete gene encodes a protein of 917 amino acids with an estimated molecular mass of 102 kDa. The protein has two apparent domains (Fig. 2). The C-terminal domain shows four repeat regions, each between 145 and 160 residues long, which show 67.1% identity between all four. The C-terminal amino acid sequence also shows homology to the C-terminal repeating region of InlB from *L. monocytogenes* (54% identity over 219 aa; Fig. 2). The N-terminal domain shows significant homology (49% identity in a 183 aa overlap) to the N-terminal amidase domain of Atl, the major autolysin of *S. aureus* (Fig. 2; Foster, 1995; Oshida *et al.*, 1995).

ORF 2 is apparently transcribed convergently to *ami* and the encoded protein is N-terminally truncated by the end of the clone. ORF 2 shows homology (67.6% identity over 173 aa) to a CTP synthase from *B. subtilis*.

Characterization of the recombinant lytic enzyme

The N-terminally truncated Ami protein expressed from pAMB8 was partially purified by precipitation in a low-salt buffer (20 mM potassium phosphate). The single dialysis step increased the specific activity of the enzyme 30-fold. The precipitated enzyme was only soluble at high salt concentrations (200 mM NaCl). Partial purification by selective precipitation of recombinant lytic enzymes has been previously noted (Foster, 1991). The enzyme was able to hydrolyse a variety of cell wall types including *L. monocytogenes* (Table 2), but showed the most activity on cell walls of *B. subtilis*. The enzyme had no activity on cell walls from *S. aureus* or on spore cortex from *Bacillus megaterium*. The optimum pH for the activity of the recombinant enzyme was pH 7.6 in 20 mM potassium phosphate buffer (results not shown). The presence of K^+ (200 mM), Li^+ (200 mM), Hg^{2+} (1 mM), Zn^{2+} (2 mM) and Ca^{2+} (2 mM) all significantly

Fig. 2. Physical and genetic map of the autolysin gene region. The map shows the plasmids used for sequencing and inactivation. Restriction enzyme sites are marked (K, *KpnI*; H, *HindIII*). The positions and orientations of the two ORFs and the positions of the direct repeats are indicated. The regions of homology to other proteins are shown as shaded boxes with the name of the homologous protein and, indicated in parentheses, the percentage identity and the length of the homologous region.

Table 2. Substrate specificity of recombinant enzyme

Substrate	Activity (%)*
<i>B. subtilis</i> 168 vegetative cell walls	100
<i>L. monocytogenes</i> EGD cell walls	73
<i>M. luteus</i> 4698 ATCC cell walls	38
<i>S. aureus</i> 8325/4 cell walls	<1
<i>Bacillus megaterium</i> KM spore cortex	<1
<i>Lactobacillus arabinosus</i> NCIB 6376 cell walls	38

* Activity measured as a percentage of that using *B. subtilis* 168 vegetative walls (100%, 16.5 U) in 20 mM Tris/HCl (pH 7.6), 200 mM NaCl at 37 °C. An equivalent amount of *E. coli* XL0LR(pBKCMV) cell extract contained <0.1% of the control enzyme activity.

Fig. 3. Effect of Mg²⁺ on activity of the recombinant enzyme. Lytic activity of the enzyme extract was determined spectrophotometrically. The reaction contained 1 ml cell walls in 20 mM Tris/HCl (pH 7.6), 200 mM NaCl to a final OD₄₅₀ of 0.25 and different concentrations of MgCl₂.

inhibited activity (by 92%, 85%, 62%, 54% and 23% respectively). Activity was stimulated by low concentrations of Mg²⁺ (Fig. 3), with an apparent optimum of

1 mM, and inhibited by the presence of 5 mM EDTA (by 62% of control).

Determination of hydrolytic bond specificity

Sequence homology suggested that *ami* encoded an amidase. To determine the hydrolytic bond specificity of the recombinant enzyme, amino acids from digested *B. subtilis* cell walls were analysed. If Ami is an amidase then its activity would be expected to liberate free L-alanine amino groups, which could be labelled with FDNB. If the digested, FDNB-labelled material is subsequently acid hydrolysed, all the amino groups of the peptidoglycan-associated amino acids will be exposed, apart from those previously labelled with FDNB. The newly exposed free amino groups could then be labelled with PITC and quantified. If Ami is an amidase then it would be expected that the amount of PITC-labelled alanine would decrease in the enzymically digested sample versus an untreated control, as a proportion would be present as a dinitrophenyl derivative (after the FDNB reaction) and so not available for PITC substitution. Thus, by comparison of the amount of PITC-labelled alanine in the peptidoglycan before and after Ami digestion, it is possible to determine whether Ami is likely to be an amidase. In the digested sample labelled with FDNB, there was a decrease of about 53% in the amount of free alanine N-termini detectable by PITC labelling and HPLC analysis, compared to that from FDNB-labelled undigested walls. This decrease in the amount of free alanine shows that the Ami enzyme is likely to have amidase activity.

Analysis of the role of Ami

To determine the function of Ami, a mutant of *L. monocytogenes* EGD insertionally inactivated in the *ami* gene was constructed. An internal fragment of 768 bp from the *ami* gene was amplified by PCR and cloned into pAULA in *E. coli* to create pAMB15. The plasmid was transformed into *L. monocytogenes* EGD and maintained at a permissive temperature (30 °C). The plasmid contains a temperature sensitive replicon and when the cells were successively subcultured at the non-permissive temperature (42 °C), the plasmid

Fig. 4. Autolysin profiles of *L. monocytogenes* strains. Cultures were grown in BHI to OD₆₀₀ of 1–1.5 at 37 °C prior to harvesting, SDS extraction, separation by SDS-PAGE and renaturation as described in Methods. Each lane contains the extract from the equivalent of 600 µl of original culture (OD₆₀₀ 1.0). The positions of molecular mass standards separated on the same gels are indicated. The gels contained 0.05 % (w/v) cell walls from *L. monocytogenes* (a), *M. luteus* (b) or *B. subtilis* (c). Lanes: 1, EGD (wild-type); 2, AM101 (*ami*).

recombined at the region of homology with the host chromosome, thus inactivating *ami*. After two rounds of enrichment, a number of putative *ami* mutants were selected and analysed by Southern blotting for the correct integrational event (results not shown). One of these mutants, AM101, was used for the analysis of the role of Ami in cellular physiology.

The autolysin profiles of EGD (wild-type) and AM101 (*ami*) were compared by renaturing SDS-PAGE, using various substrates (Fig. 4). Using *L. monocytogenes* walls as substrate, the wild-type showed multiple bands ranging from 33 to 104 kDa (Fig. 4a, lane 1) as previously shown (McLaughlan & Foster, 1997). In contrast, AM101 had reduced activity of most bands (Fig. 4a, lane 2), with only bands of 77 and 33 kDa remaining prominent. Thus, most of the lytic bands present in the profile may be due to proteolytic processing of the 104 kDa proform. However, when using *M. luteus* or *B. subtilis* walls as substrate, only the 104 kDa band was markedly reduced, suggesting the presence of other enzymes with different substrate specificities. As Ami is produced by *L. monocytogenes* and can hydrolyse its own walls it can be called an autolysin.

The role of the *ami* gene was examined by a phenotypic comparison of AM101 (*ami*) and EGD (wild-type). No difference in the growth rate of the two strains was detected and the mutant did not appear to be impaired in cell division over a range of temperatures (25, 30, 37 and 42 °C). Ultrastructural analysis by electron microscopy did not reveal any significant differences in the morphology of the cell wall.

The possible role of the autolysin in motility was analysed by growth on swarm plates, where motile strains can be seen forming a diffuse growth around the

central colony. Both wild-type and mutant strains were non-motile at 37 °C. There was a slight decrease in the motility of the mutant compared to the wild-type at 30 °C (85 % of wild-type diameter), at 25 °C (67.5 % of wild-type diameter) and at room temperature (81 % of wild-type diameter).

Penicillin-induced cell lysis was determined by the addition of penicillin G to growing cultures. After 24 h the treated EGD (wild-type) and AM101 (*ami*) cultures both had a final OD₆₀₀ of 0.2–0.25. Plate counts revealed that the bacteriocidal effect of the drug was unaltered in the mutant. From these results it appears that Ami does not have a role in penicillin-induced lysis. However, neither strain lysed completely. Similarly, Ami is not involved in Triton-X-100-induced lysis, as both the mutant and the wild-type lysed at the same rate (results not shown).

Lithium chloride extraction of total lytic enzyme activity from the cells revealed that AM101 (*ami*) had 34 % and 83 % of the parental (EGD) activity using *L. monocytogenes* or *M. luteus* walls respectively as the substrate, when assayed spectrophotometrically. There was no difference in the activity of the wild-type and the mutant when assayed using *B. subtilis* walls as substrate.

DISCUSSION

This paper describes the cloning and characterization of an autolytic amidase from *L. monocytogenes* EGD. During the preparation of this paper, the cloning and sequencing of the autolysin gene was published by Braun *et al.* (1997) and the gene designated *ami*. They identified the gene by the Southern blotting of *L. monocytogenes* EGD chromosomal DNA using the 3' end of the *inlB* gene as a probe.

The *ami* gene encodes a protein of 917 amino acids with a deduced molecular mass of 102 kDa. This molecular mass is in reasonable agreement with the size (104 kDa) of the lytic activity of the truncated recombinant protein observed on renaturing gels. The Ami protein is predicted to have a two-domain structure. The presence of two domains (a catalytic domain and a non-catalytic, cell-wall-binding domain) appears to be a common feature of autolysins (Chu *et al.*, 1992; Kuroda *et al.*, 1992; Romero *et al.*, 1990). The high homology (49% identity over 183 aa) of the N-terminal domain of Ami to the amidase domain of the *S. aureus* autolysin Atl (Foster, 1995; Oshida *et al.*, 1995), suggests that Ami also has amidase activity. Using an HPLC-based method we have demonstrated the likely amidase activity of the enzyme.

The C-terminal domain of Ami contains four imperfect direct repeats, which are between 145 and 160 residues long and show 67.1% identity between the four. A characteristic series of repeat sequences in the non-catalytic domain of autolysins is thought to be necessary for specific cell wall binding. The LytA amidase of *Streptococcus pneumoniae* has a two-domain structure: an N-terminal catalytic domain and a C-terminal domain with six repeat regions. By a series of deletions, Garcia *et al.* (1994) showed that at least four of the repeat regions are necessary for the efficient recognition and attachment of the autolysin to choline residues in the pneumococcal cell wall. This suggests the necessity of enzyme attachment to the cell wall in order to maintain high catalytic efficiency. Similarly, when the catalytic domain of CwlB (the major vegetative-cell autolytic amidase) from *B. subtilis* was deleted, the enzyme retained the ability to bind to *B. subtilis* cell walls, suggesting that the repeating sequences found in the non-catalytic region may be essential for binding (Kuroda & Sekiguchi, 1991). The autolysin CwlM from *Bacillus licheniformis* has an N-terminal catalytic domain and a C-terminal domain containing repeated sequences (Kuroda *et al.*, 1992). CwlM normally showed more activity on *M. luteus* cell walls than *B. subtilis* or *B. licheniformis* as substrate, but in contrast, a mutant truncated at the C terminus had lost this preference, suggesting that the (non-catalytic) C-terminus is essential for substrate specificity (Kuroda *et al.*, 1992).

The involvement of direct amino acid repeats in wall binding has also been postulated in a wall-associated protein (Wap) without autolytic activity. Processed forms of WapA constitute several of the major Waps of *B. subtilis* (Foster, 1993). WapA has two domains, an N-terminal domain containing three repeats (similar to those found in autolysins) and a C-terminal domain containing a different kind of repeat: 31 repeats of a motif in two areas (Foster, 1993). Deletion analysis showed that the N-terminal direct repeats were necessary for cell wall binding (Foster, 1993).

The C-terminal domain of Ami has homology (54% identity over 219 aa) to the InlB surface-anchoring domain. InlB is a member of the internalin family, which

have important roles in the pathogenicity of *L. monocytogenes* (Dramsi *et al.*, 1995, 1997). The C-terminal domain of InlB may therefore be involved in cell wall binding.

The 5'-truncated *ami* gene can be stably maintained and expressed in *E. coli*. However, the whole gene could not be cloned, even in strains limiting phagemid copy number. The reason for this is unclear but it may reflect the strength of the *ami* promoter causing overexpression to such levels that *E. coli* is killed. The recombinant protein is able to hydrolyse a variety of cell walls, showing most activity on those of *B. subtilis*. No activity was seen using walls of *S. aureus*, perhaps due to O-acetylation levels or the different amino acid constituents of the peptidoglycan (Rogers *et al.*, 1980).

An *ami* mutant was created in the wild-type background to determine the role of the enzyme. Renaturing SDS-PAGE revealed that Ami accounts for most of the lytic bands when using *L. monocytogenes* walls as the substrate. Therefore, it is likely that the 104 kDa proform (as observed on renaturing gels) is exported and then proteolytically processed to give the final profile. This phenomenon has been demonstrated in *S. aureus*, where the major autolysin Atl is post-translationally processed (Foster, 1995). Ami is a major autolysin of *L. monocytogenes*, as it constitutes 66% of the total activity when measured using host walls as substrate. As found in other Gram-positive bacteria (Smith *et al.*, 1996) there are, however, likely to be multiple autolysins in *L. monocytogenes*. Renaturing SDS-PAGE shows that the remaining autolysins in the *ami* mutant have different substrate specificity than Ami, which may indicate alternative functions.

Although Ami is a major autolysin, its function is not clear. The enzyme appears ostensibly not to be involved in the cell-separation stage of cell division. However, due to the compensatory nature of autolysins, it is only by the creation of multiple autolysin mutants that their combined roles can be determined (Smith & Foster, 1995; Smith *et al.*, 1996). Both the major amidase (LytC/CwlB) and the major glucosaminidase (LytD) of *B. subtilis* are involved in cell separation, but this is only apparent when a double mutant is created (Blackman *et al.*, 1998). Ami has a subtle role in motility, observed as a reduction in swarming. This effect on swarming has also been noted for both of the major vegetative-cell autolysins of *B. subtilis* (Blackman *et al.*, 1998). Even a slight decrease in the ability of the cells to separate will result in an inhibition of swarming, as unseparated cells will be unable to chemotax effectively.

Penicillin-induced lysis occurs as a post-mortem event after the cells have been killed. In *S. aureus*, the major autolysin Atl is responsible for this process. In *B. subtilis*, LytC is solely responsible for generalized cell lysis but both LytC and LytD are involved in antibiotic-induced lysis (Blackman *et al.*, 1998). However, it appears that wild-type *L. monocytogenes* is relatively resistant to lysis and seemingly Ami does not have a role in

penicillin-induced lysis, as neither the mutant nor the control cells lysed completely. Similarly, Ami does not have a role in generalized cell lysis due to Triton X-100.

Autolysins have been implicated as having a role in virulence in several studies. The pneumolysin from *Streptococcus pneumoniae* is located in the cytoplasm and is thought to be released as the cells undergo autolysis, due to the action of the major autolysin, LytA (Berry *et al.*, 1992). Similarly, the glucosaminidase from *S. aureus* has been shown to strongly depress the immune system in mice and may also interfere with human immune responses (Valisena *et al.*, 1991). *L. monocytogenes* mutants with reduced p60 levels exhibited reduced (if any) ability to invade host cells (Kuhn & Goebel, 1989). A mutant of *S. aureus* with reduced autolysin levels exhibited attenuated virulence in experimental models of rat endocarditis (Mani *et al.*, 1994). The *ami* mutant created in the wild-type background during this study provides the opportunity to determine the role of the enzyme in the invasive properties of the organism.

Even though Ami is a major autolysin of *L. monocytogenes*, there are still a number of other activities associated with the cell. It is only by the identification of the structural genes encoding the other enzymes and the creation of individual and multiple mutants in an isogenic background that the roles of the many autolysins of *L. monocytogenes* will begin to be elucidated. This forms the focus of our continuing research.

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