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The two-component cell lysis genes *holWMY* and *lysWMY* of the *Staphylococcus warneri* M phage ϕWMY: Cloning, sequencing, expression, and mutational analysis in *Escherichia coli*

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Abstract

From the genome library of *Staphylococcus warneri* M, the two successive cell-lysis genes (*holWMY* and *lytWMY*) were cloned and characterized. The *lytWMY* gene encoded a protein (LysWMY), whose calculated molecular mass and p*I* were 54 kDa and 8.95, respectively. When overproduced in *Escherichia coli*, *lysWMY* directed a protein of 45 kDa (smaller than the predicted molecular mass), having N-terminal 13 residues identical with those predicted from DNA. Comparative analysis revealed that LysWMY significantly resembles the putative *N*-acetylmuramoyl-L-alanine amidases encoded by the staphylococcal phages ϕ11, 80 alpha, and Twort. Examination of modular organization of LysWMY identified three putative domains CHAP (for D-alanyl-glycyl endopeptidase), amidase (L-muramoyl-L-alanine amidase), and SH3 (cell wall recognition). Gene knockout analysis revealed that each of the two domains of CHAP and amidase was responsible for cell-lytic activity on a zymogram gel. Site-directed mutation of Cys29Ala, His92Ala, or Asn114Ala in the CHAP domain substantially reduced cell-lytic activity, suggesting that this Cys–His–Asn triad is crucial for the enzymatic function. On the other hand, the *holWMY* gene encoded a protein (HolWMY) with molecular mass and p*I* of 16 kDa and 4.36; this protein contained two potential transmembrane helices, resembling other predicted holins (a cytoplasmic membrane-disrupting protein) encoded by the *S. aureus* phage, ϕ11, 80 alpha, and Twort. Upon mitomycin C exposure of *S. warneri* M, a prophage (ϕWMY) was induced and the virion was examined under electron microscopy. PCR amplification and sequencing revealed the presence of the *holWMY–lysWMY* genes in the phage genome.

Keywords: Amidase; Endolysin; Holin; *Staphylococcus warneri* M.

1. Introduction

Staphylococci are thought to have multiple cell wall peptidoglycan (murein) hydrolases, such as *N*-acetylglucosaminidases, *N*-acetylmuramidases, *N*-acetylmuramyl-L-alanine amidases, endo-peptidases, transglycosylases, and/or carboxypeptidase (Giesbrecht et al., 1998). Generally, these murein hydrolases have been divided into two groups of autolysin and endolysin. The autolysins are encoded by the

Abbreviations: CHAP, cysteine, histidine-dependent amidohydrolases/peptidase; SH3, src-homology 3; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; RBS, ribosome binding site; PCR, polymerase chain reaction.

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bacterial chromosome, carry a signal peptide in their N-termini, and can lead to the destruction of murein and subsequent cell lysis (Oshida et al., 1995; Hell et al., 1998). Their pivotal functions on cell growth and division have been assigned to cell wall synthesis, daughter cell separation, murein turnover and recycling, and pathogenesity (Archibald et al., 1993; Giesbrecht et al., 1998; Allignet et al., 2001).

On the other hand, the staphylococcal endolysins are encoded by bacteriophages. Contrary to autolysins, almost all of the endolysins have no signal peptides, and their translocation through the cytoplasmic membrane is thought to proceed with the help of phage-encoded holin proteins, acting as a cytoplasmic membrane-disrupting protein (Young and Blasi, 1995). Recently, these phage-encoded cell-lytic enzymes have been expected to counteract certain pathogenic and/or spoilage bacteria (Gasson, 1996; Loeffler et al., 2001). Hitherto, several putative endolysins and holins (referred to as the two component "holin–endolysin" cell lysis system) have been reported from the staphylococcal phages, including ϕ11 (Wang et al., 1991), Twort (Loessner et al., 1998), ϕPVL (Kaneko et al., 1998), and 187 (Loessner et al., 1999). In addition, recent genome sequences of *S. aureus* strains revealed various prophages and their endolysins (GenBank accession nos. AP003135; AF424782; AF424783).

These staphylococcal endolysins have a modular organization, and some functional domains such as D-alanyl-glycyl endopeptidase, L-muramoyl-L-alanine amidase, *N*-acetyl-glucosaminidase, or SH3 domains (Navarre et al., 1999; Loessner et al., 1998) have been implicated in lytic activity or recognition of the cell wall.

Recent comparative analyses have revealed the so-called CHAP domain, which has been found in N-terminal portions of the endolysins encoded by the staphylococcal phages, ϕ11, Twort, ϕPVL, and 187 (Navarre et al., 1999). Each of these CHAP domains of about 150 amino acids contains three conserved key amino acids, Cys, His, and a polar residue (Asn, His, or Asp), acting as an enzymatic active-site (Bateman and Rawlings, 2003; Rigden et al., 2003; Anantharaman and Aravind, 2003). The CHAP domain of ϕ11 has been reported to serve as a D-alanyl-glycyl endopeptidase (Navarre et al., 1999). Further details on structure and function of the staphylococcal phage endolysins remain to be elucidated at the molecular and genetic levels.

Previously, we isolated a new staphylococcal strain *Staphylococcus warneri* M from processed seafood (smoked *Watasenia scintillans*) (Yokoi et al., 2001). In the course of investigation on cell wall hydrolases of *S. warneri* M, we cloned two genes, *atlWM* and *lysWMY*, which are located at different loci in the chromosome; *atlWM* has been identified as a major autolysin gene of *S. warneri* M (GenBank accession no. AB182648).

Here, we report the molecular characteristics of the other gene, *lysWMY*, encoding an endolysin containing a typical CHAP domain in its N-terminal region: i.e., its cloning, sequencing, expression, and mutational analysis in *Escherichia coli*. In addition, we describe the holin gene *holWMY* located just upstream of *lysWMY*, and a prophage ϕWMY of *S. warneri* M, which encodes the new two-component lysis system (*holWMY–lysWMY*).

2. Materials and methods

2.1. Bacteria, plasmids, and phages

The plasmids, phages, primers, and bacteria (except for *E. coli* XL1-Blue) used in this study were summarized in Tables 1 and 2. The staphylococcal strains and *E. coli* XL1-Blue were propagated in Luria–Bertani (LB) medium at 37 °C with shaking (Yokoi et al., 2001). The growth media used for the lactic acid bacteria (Kodaira et al., 1997) were GYP (glucose–"yeast extract"–peptone) and MRS (DIFCO). The deletion mutants of *lysWMY* were constructed using the restriction- or PCR-generating fragments (Table 1; Fig. 1). The site-directed mutations were introduced using the synthetic oligonucleotide primers (Table 1) as described previously (Kakikawa et al., 2002). The prophage ϕWMY was induced by mitomycin C exposure, purified, and analyzed by electron microscopy as described previously (Kakikawa et al., 1996).

2.2. DNA analysis

Cloning, sequencing, and Southern hybridization were performed as described previously (Yokoi et al., in press). The *S. warneri* M genomic DNA was partially digested with *Sau*3AI, and the fragments were cloned into a unique *Bam*HI site of pUC119. The genome library was introduced into *E. coli* XL1-Blue. The transformants were grown at 37 °C for 24 h on a plate containing autoclaved *S. warneri* M cells (1×10^9/ml), Triton X-100 (0.1%), ampicillin (100 μg/ml), and IPTG (1 mM). The colonies grown were further incubated at 4 °C for 24 h, and screened for a Triton X-100-induced lysis phenotype. Recombinant plasmids were sequenced as described previously (Yokoi et al., 2001).

2.3. Protein analysis

Protein analysis by sodium dodecyl sulfate polyacrylamide gel elecrophoresis (SDS–PAGE) and zymography was carried out as described (Yokoi et al., in press). Proteins were stained with Coomassie brilliant blue (CBB) R-250. In zymogram analysis, SDS–polyacrylamide separating gel (pH 8.8) containing the autoclaved bacterial cells (about 2×10^9/ml) was used to detect the lytic activities. After SDS–PAGE, the gel was soaked for 30 min in distilled water at room temperature. The gel was then transferred into the renaturing buffer (50 mM Tris–HCl [pH 7.0] containing 1% Triton X-100 and 0.15 M NaCl), and gently shaken overnight at 37 °C. The renatured protein, possessing the