Identification and Cloning of Genes Encoding Potential Proteins Involved in Membrane Biosynthesis from *Corynebacterium ammoniagenes*

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The bacterial cell membrane, existing inside the cell wall, is composed of proteins and phospholipids and plays several critical roles in the cell functions. As a barrier it prevents the passive leakage of cytoplasmic constituents, and is the site of many proteins involved in the transport of substances into and out of the cell. The function and biosynthesis of phospholipids in the cell membrane have been reported in several organisms [Raetz and Dowhan, 1990; Zhu *et al.*, 2006]. Coryneform bacteria are rod-shaped, non-sporulating, and Gram-positive microorganisms. In the case of *Corynebacterium glutamicum*, used for the industrial production of amino acids, addition of detergents can induce overproduction of L-glutamate [Takinami *et al.*, 1965; Duperray *et al.*, 1992], by changing the permeability of the cell membrane [Demain and Birnbaum, 1968]. In addition, detergent reflected the structural complexity of the cell membrane [Schuck *et al.*, 2003]. On the other hand, other studies have found that, regardless of the membrane permeability, L-glutamate could be secreted [Hoischen and Krämer, 1990; Krämer *et al.*, 1994]. *C. ammoniagenes* has been used for the industrial production of flavor-enhancing purine nucleotides and other compounds [Koizumi *et al.*, 2000; Noguchi *et al.*, 2003; Kim *et al.*, 2006], and the fatty acid distribution in its phospholipids have been reported by Yoshihiro *et al.* However, little is known about the membrane permeability of *C. ammoniagenes*. Accordingly, we focused on proteins influenced by detergent in the cell membrane to investigate clues for developing various metabolites overproducing *C. ammoniagenes*. We cloned and identified two genes encoding potential proteins involved in the membrane biosynthesis from *C. ammoniagenes* ATCC 6872.

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *C. ammoniagenes* ATCC 6872 was used to provide the DNA template for cloning of rescuer genes for determining detergent-sensitivity. Detergent-sensitive mutant derived from *C. ammoniagenes* ATCC 6872, *C. ammoniagenes* DS-101, was made using the method of Tuttle *et al* and employed for the selection of positive clones. An *E. coli/C. glutamicum* shuttle plasmid, pECCG117 was used for molecular biological work on *C. ammoniagenes* [Kim *et al.*, 2006]. *C. ammoniagenes* was routinely grown in a YPB medium (10 g yeast extract, 10 g peptone, 10 g beef extract, 2.5 g NaCl per 1 L water; pH 7.2). Rescuer genes for detergent-sensitivity were screened on a minimal agar medium (20 g glucose, 1 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 3.6 mg $MnCl_2 \cdot 4H_2O$, 20 mg L-cysteine, 5 mg thiamine-HCl, 10 mg Ca-D-pantothenate, 30 μg biotin, 20 g agar per 1 L water; pH 7.2) [Kim *et al.*, 2006], with 50 mg L^{-1} of polyoxyethylene sorbitan monopalmitate (Tween 40, Sigma) as detergent [Kimura *et al.*, 1996]. When necessary, kanamycin was used at the final concentration of 10 mg L^{-1}. *C. ammoniagenes* was cultured at 32°C in a 500-mL baffled flask containing 50 mL medium with an agitation speed of 120 rpm [Kim *et al.*, 2006]. The wild-type strain, *C. ammoniagenes* ATCC 6872, and the detergent-sensitive mutant, DS-101, were tested for detergent-sensitivity. Growth of DS-101 decreased slightly when the concentration of Tween 40 exceeded 30 mg L^{-1}, and was inhibited at 50 mg L^{-1}, whereas no difference in the cell growth was observed in the wild-type (data not shown). To investigate the detergent-sensitivity of the rescuer genes, genomic DNA of *C. ammoniagenes* ATCC6872 was isolated using a genomic DNA purification kit (Qiagen), and partially digested with a restriction enzyme *Sau*3AI. The DNA fragments ranging from 2 to 7 kb were eluted from agarose gel and cloned into the *Bam*HI site of pECCG117. These constructs were transformed into *C. ammoniagenes* DS-101 [Dunican and Shivan, 1989], and the transformants were incubated at 32°C for 72 h on the minimal agar medium [Kim *et al.*, 2006] with kanamycin containing 50 mg L^{-1} Tween 40 as the detergent [Kimura *et al.*, 1996]. Eighteen transformants showed complementation of cell

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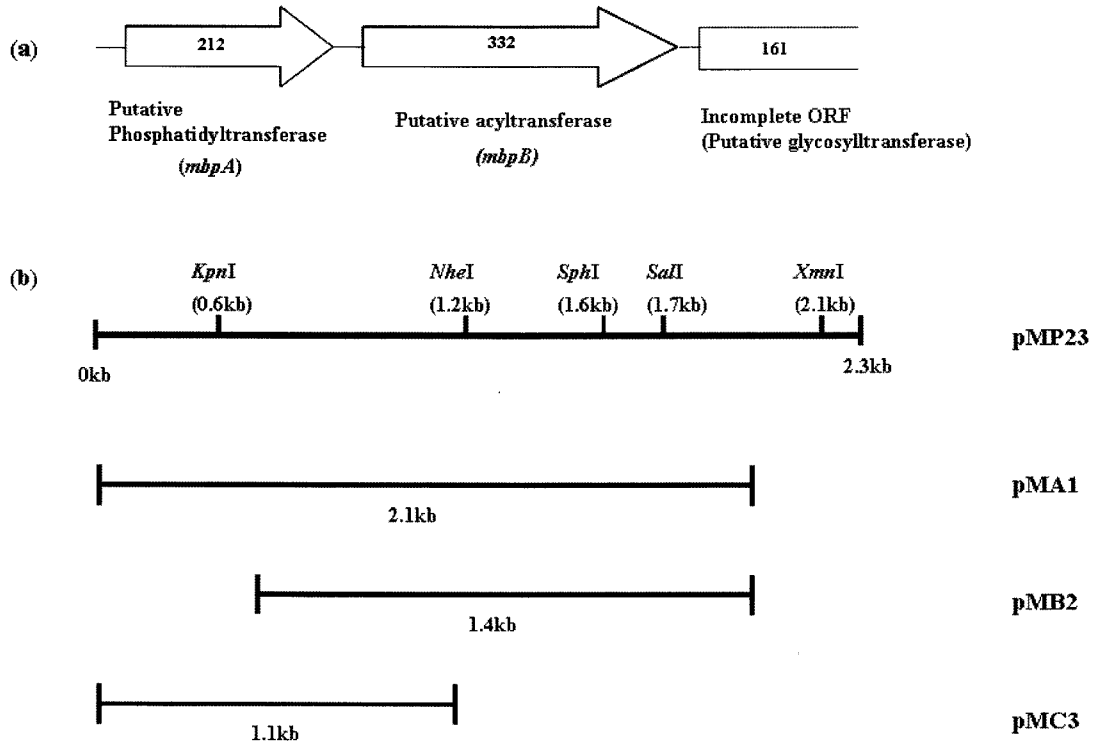

Fig. 1. Diagrammatic representation of annotated ORFs, the restriction endonuclease map of pMP23 and subclones. (a) The number of amino acids for the annotated ORFs is shown within the arrows. The arrows indicate the direction of transcription. The (putative) functions of the gene products are indicated below the arrow. (b) Restriction map of the pMP23 isolated from genomic DNA library, and subclones for complementation test on detergent-sensitivity. Fragments were amplified with *Xba*I/*BamH*I enzymes and subcloned into the vector pECCG117.

growth. In addition, all plasmid DNAs isolated from the transformants displayed approximately 1.1-kb fragments with an identical restriction enzyme pattern when digested with *Kpn*I and *Sal*I, and showed the same nucleotide sequence. Of these plasmids, one plasmid containing the longest DNA insert was selected and named pMP23 (Fig. 1). The isolated plasmid pMP23, when retransformed into *C. ammoniagenes* DS-101, gave the same result, which indicates that the products of the putative genes in plasmid pMP23 are involved in the recovery of the sensitivity of the detergent.

The insert fragment of pMP23 was sequenced. The length of the insert DNA was 2375 bp, with two complete ORFs and one incomplete ORF (Fig. 1). ORF1, encoding a product of 212 aa, with a calculated molecular mass of 22,480 Da, and ORF2, consisting of 332 aa with a predicted molecular mass of 37,670 Da, were identified. The incomplete ORF was missing a C-terminal fragment. The deduced amino acid sequences of ORF1 and ORF2 were compared to those in the NCBI database using the BLAST program server of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to search for homologous proteins. The deduced amino acid sequence encoded by ORF1 displayed a high sequence similarity to those of the putative phosphatidyltransferase of *C. diphtheriae* NCTC 13129 (NP_939738.1, 68% identities and 80% positives), *C. efficiens* YS-314 (NP_738393.1, 61% identities and 79% positives), *C. jeikeium* K411 (YP_250846.1, 57% identities and 74% positives) and phosphatidylglycerophosphate synthase of *C. glutamicum* ATCC 13032 (NP_600881.1, 62% identities and 78% positives). The protein encoded by ORF2 also showed high levels of sequence similarity to the putative acyltransferase of *C. efficiens* YS-314 (NP_738392.1, 55% identities and 68% positives), *C. glutamicum* ATCC 13032 (NP_600881.1, 50% identities and 67% positives), *C. jeikeium* K411 (YP_250845.1, 50% identities and 65% positives), and *C. diphtheriae* NCTC 13129 (NP_939737.1, 48% identities and 63% positives). All of these proteins were predicted from the whole-genome sequencing projects, but still remain uncharacterized. This comparison of the protein identity suggested that ORF1, ORF2, and incomplete ORF were related to the phospholipids biosynthesis in the membrane [Yoshihiro *et al.*, 1986; Raetz and Dowhan, 1990]. Therefore, ORF1 and ORF2, encoding proteins involved in the membrane biosynthesis, were named as MbpA and MbpB, respectively and deposited in the GenBank with the accession number DQ

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
C. ammoniagenes		
ATCC 6872	Wild-type strain	ATCC[b]
DS-101	Detergent-sensitive mutant	This work
CA-200	DS-101 carrying plasmid, pECCG117	This work
MP 412	DS-101 carrying plasmid, pMA1	This work
MP 232	DS-101 carrying plasmid, pMB2	This work
MP 511	DS-101 carrying plasmid, pMC3	This work
DR-333	DS-101 carrying plasmid, pMP23	This work
Plasmids		
pECCG117	*E. coli- C. glutamicum* shuttle vector, Km^r	[Kim *et al.*, 2006]
pMP23	pECCG117 carrying 2.4 kb of *Sau3A*I fragment from *C. ammoniagenes* ATCC 6872.	This work
pMA1	pECCG117 carrying 2.1 kb of *XbaI*/*BamH*I fragment from pMP23	This work
pMB2	pECCG117 carrying 1.4 kb of *XbaI*/*BamH*I fragment from pMP23	This work
pMC3	pECCG117 carrying 1.1 kb of *XbaI*/*BamH*I fragment from pMP23	This work

[a]Abbreviations: Km^r, kanamycin resistance.
[b]ATCC, American Type Culture Collection.

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DNA fragment was subcloned in a plasmid, pECCG117, to investigate the recovery of detergent-sensitivity. For amplification of *mbpA*, primers A (5'-TGCTCTAGACTA GAACTAGTGGATCTCGAA-3') and B (5'-CGCGGAT CCCTAGTTCACGGGTTACGTTTT-3') were used, whereas *mbpB* was constructed with primers C.D (5'-TGCTCTAGATCGAACGTCCGGAGCGCTTGAT-3' and 5'-CGCGGATCCCGTTATAAGAAATCGGGATGG AGCCGCCACC-3'). For amplification containing both *mbpA* and *mbpB*, primers A and D were chosen. The amplified fragments were double-digested with *Xba*I and *Bam*HI and ligated with *XbaI*/*Bam*HI-cleaved pECCG117. The resulting plasmids were designated pMA1, pMB2, and pMC3 (Fig. 1), and MP412, MP232, and MP511 strains were constructed with their respective plasmids (Table 1). MP511 and MP232 exhibited slight growth recovery by the introduction of *mbpA* and *mbpB*, respectively (Table 2). On the other hand, MP412 harbouring both *mbpA* and *mbpB* showed a substantial recovery of growth compared to DS-101, and a similar level of cell growth compared to DR-333. These results suggested that both *mbpA* and *mbpB* are necessary for the recovery of sensitivity to the detergent. In addition, incomplete ORF has no correlation with detergent-sensitivity, because no difference was observed between MP412 and DR-333. MP412 and DR-333 also exhibited low levels of growth in detergent compared to the wild-type strain. In *C. glutamicum* serious disadvantage of classical mutagenesis is the accumulation of uncharacterized secondary mutations [Ohnishi *et al.*, 2002]. Therefore, we supposed that the growth inhibitions of MP412 and DR-333 on detergent could be related to the unnecessary mutation of other regions in DS-101 strain. Kimura *et al* identified *dtsR*, a novel gene, which rescues detergent-sensitivity of *Brevibacterium lactofermentum*. It was similar in sequence to the β subunits of biotin-containing acyl-CoA carboxylase complexes. They also suggested that DtsR forms an enzyme complex involved in the fatty acid synthesis. In this study, when we attempted to identify all genes responsible for the recovery of detergent-sensitivity, two genes, *mbpA* and *mbpB*, related to the phospholipid biosynthesis in the membrane, were identified. In further studies, attempts will be made to

Table 2. Growth comparison for complementation test on detergent-sensitivity

Strain	With detergent	Without detergent
ATCC 6872	++	++
DS-101	-	++
CA-200[a]	-	++
DR-333	+	++
MP412	+	++
MP232	+/-	++
MP511	+/-	++

A culture grown in YPB medium overnight was washed with saline, and 1% broth was inoculated into minimal medium plate supplemented with or without 50 mg L^{-1} Tween 40 as detergent. The growth was measured after 72 h culture. The relative growth was visually quantified into four levels: ++, heavy growth; +, light growth; +/-, trace growth; -, no growth. Each experiment was performed using three independent cultures.
[a]CA-200 is negative control strain.

investigate the status of the *mbp* locus in the DS-101 strain as well as the membrane permeability of *C. ammoniagenes*.

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