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# Identification and Characterization of the *Vibrio vulnificus malPQ* Operon

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**Abstract** It is likely that maltose could provide a good substrate for the bacteria in the intestine, when the pathogenic bacteria invade and colonize in human gut. For better understanding of this organism's maltose metabolism, a mutant that was not able to grow with maltose as a sole carbon source was screened from a library of mutants constructed by a random transposon mutagenesis. By a transposon-tagging method, *malPQ* genes encoding a maltodextrin phosphorylase and a 4-α-glucanotransferase, were identified and cloned from *Vibrio vulnificus*. The deduced amino acid sequences of *malPQ* from *V. vulnificus* were 48 to 91% similar to those of MalP and MalQ reported from other Enterobacteriaceae. Functions of *malPQ* genes were assessed by the construction of mutants whose *malPQ* genes were inactivated by allelic exchanges. When maltose was used as the sole carbon source, neither *malP* nor *malQ* mutant was able to grow to a substantial level, revealing that the MalP and MalQ are the only enzymes for metabolic utilization of maltose. The *malQ* mutant exhibited decreased adherence toward intestinal epithelial cells *in vitro*, but there was no difference in the $LD_{50}$s of the wild-type and the *malQ* mutant in mice. Therefore, it appears that MalQ is less important in the pathogenesis of *V. vulnificus* than would have been predicted by considering maltose as a most common sugar in the intestine, but not completely dispensable for virulence in mice.

**Key words:** *Vibrio vulnificus*, *malPQ*, 4-α-glucanotransferase, maltodextrin phosphorylase

*Vibrio vulnificus* is an opportunistic Gram-negative pathogen that commonly contaminates raw oysters and is the causative agent of foodborne diseases such as gastroenteritis and life-threatening septicemia. Predisposed individuals with underlying immunocompromised conditions, liver damage, or excess levels of iron, who consume raw oysters, can die within days from sepsis. Even otherwise healthy people are susceptible to serious wound infections after contact with shellfish or water contaminated with *V. vulnificus* [for reviews, see Refs. 15, 24]. Mortality from septicemia is very high (>50%), and death may occur within one to two days after the first signs of illness [15, 24].

It is likely that when the pathogenic bacteria invade human gut, many environmental changes, such as differences in types and concentrations of nutrients, would be encountered. Maltose/maltodextrin could be an interesting sugar in this respect, as it is very common in the intestine and could provide a good substrate for the colonizing bacteria [12]. The maltose system in *Escherichia coli* has been extensively characterized [for a review, see Ref. 1]. The uptake of maltose is accomplished by the maltose/maltodextrin transport system, and the components of the transport system are encoded by the two divergently oriented operons, *malEFG* and *malK-lamB-malM* [1]. In the cytoplasm, maltose-utilizing enzymes are encoded by three genes; *malP* encoding a maltodextrin phosphorylase, *malQ* encoding a 4-α-glucanotransferase, and *malZ* encoding a maltodextrin glucosidase [1]. However, very little is known about the uptake and metabolism of maltose in enteropathogenic bacteria.

From a standpoint of bacterial pathogenesis, the capability to utilize maltose as a potentially important nutrient source would be important. In the intestine, the amount of free glucose would be quite small. Therefore, to be a successful pathogen, bacteria must be able to use nutrients other than glucose. In addition to the scarcity of the specific nutrients, increased competition for the nutrients imposed by the host cells and endogenous bacterial flora would be encountered. As such, efficient utilization of maltose may play important

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roles in pathogenesis; that is, the bacteria must survive these adverse competitions to multiply in the intestine, finally resulting in local damages and systemic diseases. This suggests that *V. vulnificus* as an enteropathogenic bacterium is able to metabolize maltose in their growth environments. However, a definitive analysis of the biochemical pathway of maltose metabolism in the pathogen has so far not been made, and the genes encoding the components of the pathways have not yet been characterized at a molecular level.

Accordingly, as an effort to identify the genes involved in maltose utilization, a library of *V. vulnificus* mutants was constructed using the transposon mini-Tn5 *lacZ*1 [4, 10], and a mutant that was not able to utilize maltose as a sole carbon source was screened in the present study. By a transposon-tagging method, *malPQ* genes encoding a maltodextrin phosphorylase and a 4-α-glucanotransferase were identified and cloned from *V. vulnificus*. The nucleotide and deduced amino acid sequences of the *malPQ* genes were analyzed. *V. vulnificus* null mutants whose *malPQ* genes were separately disrupted were also constructed, and the virulence of the mutants was compared to that of the parental wild-type.

## MATERIALS AND METHODS

### Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* strains used for plasmid DNA replication

**Table 1.** Plasmids and bacterial strains used in this study.

| Strain or plasmid | Relevant characteristics[a] | Reference or source |
|---|---|---|
| **Bacterial strains** | | |
| *V. vulnificus* | | |
| ATCC29307 | Clinical isolate; virulent | Laboratory collection |
| MO6-24/O | Clinical isolate; virulent | [25] |
| HJK995 | MO6-24/O with *malQ*::mini Tn5 *lacZ*1 | This study |
| MH031 | ATCC29307 with *malP*::*nptI*; Km$^r$ | This study |
| MH032 | ATCC29307 with *malQ*::*nptI*; Km$^r$ | This study |
| MH035 | MO6-24/O with *malP*::*nptI*; Km$^r$ | This study |
| MH036 | MO6-24/O with *malQ*::*nptI*; Km$^r$ | This study |
| *E. coli* | | |
| DH5α | *supE44 Δ lacU169 (ϕ80 lacZ* ΔM15*) hsdR17 recA1 endA1 gyrA96* thi-1 relAI | Laboratory collection |
| SM10λpir | *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *λpir*; km$^r$; host for π-requiring plasmids; conjugal donor | [16] |
| SY327λpir | *Δ(lac pro) argE*(Am) *recA56 gyrA rpoB λpir*; host for π-requiring plasmids; conjugal donor | [16] |
| **Plasmids** | | |
| mini-Tn5*lacZ*1 | R6K*γori*; suicide vector; *oriT* of RP4; Ap$^r$ | [4] |
| pBR322 | CoEI *oriV*; cloning vector; Tc$^r$, Ap$^r$ | Laboratory collection |
| pLAFR3 | IncP *ori*; cosmid vector; Tc$^r$ | [23] |
| pUC4K | pUC4 with *nptI*; Ap$^r$, Km$^r$ | [16] |
| pCVD442 | R6K *γori*; *sacB*; suicide vector; *oriT* of RP4; Ap$^r$ | [5] |
| pRK415 | IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc$^r$ | [9] |
| pMH11-39 | Cosmid containing *malPQ* | This study |
| pMH0302 | 8.2-kb *Bam*HI/*Not*I fragment containing part of *malP, malQ*; cloned into pBR322; Tc$^s$, Ap$^r$ | This study |
| pMH0315 | 1.5-kb *SphI*/*Xba*I fragment containing part of *malP*; cloned into pBR322; Tc$^s$, Ap$^r$ | This study |
| pMH0316 | 1.2-kb *Pvu*II/*Eco*RV fragment containing part of *malQ*; cloned into pBR322; Tc$^s$, Ap$^r$ | This study |
| pMH0317 | pMH0315 with *malP*::*nptI*; Ap$^r$, Km$^r$ | This study |
| pMH0319 | pMH0316 with *malQ*::*nptI*; Ap$^r$, Km$^r$ | This study |
| pMH0321 | pCVD442 with *malP*::*nptI*; Ap$^r$, Km$^r$ | This study |
| pMH0322 | 5.2-kb *Hin*dIII/*Sph*I fragment containing part of *malP, malT*; cloned into pBR322; Tc$^s$, Ap$^r$ | This study |
| pMH0324 | pCVD442 with *malQ*::*nptI*; Ap$^r$, Km$^r$ | This study |
| pMH0330 | pRK415 with *malP*; Tc$^r$ | This study |
| pMH0331 | pRK415 with *malQ*; Tc$^r$ | This study |

[a]Ap$^r$, ampicillin resistant; Km$^r$, kanamycin resistant; Tc$^r$, tetracycline resistant.

**Table 2.** Oligonucleotides used in this study.

| Oligonucleotide | Oligonucleotide sequence, 5'→3'[a] | Location[b] | Use |
|---|---|---|---|
| PTn-1 | TTCTTCACGAGGCAGACCTCAGCGC | Tn5 | Amplification of Tn5 flanking region |
| PTn-2 | CCGCACTTGTGTATAAGAGTCAG | Tn5 | Amplification of Tn5 flanking region |
| BB-P1 | GGCCACGCGTCGACTAGTCANNNNNNNNNNACGCCC | Arbitrary | Amplification of Tn5 flanking region |
| BB-P2 | GGCCACGCGTCGACTAGTCA | Arbitrary | Amplification of Tn5 flanking region |
| MalP09 | GTTTGTGGGAATGTCAGGCG | 701 to 720 | Mutant construction |
| MalP10 | GATTTCAGCCATACTCACGCG | 1917 to 1897 | Mutant construction |
| MalQ01 | GAGCCGTAAGCGTCGTAAAGCA | 420 to 442 | Mutant construction |
| MalQ06 | CGCTTCTTTGATCTGGCCGTC | 1173 to 1153 | Mutant construction |
| MalPCF | AGGCTGCAGGAAATTAGAACCAAAAGTGAGA | Chromosomal DNA | Complementation of the *malP* |
| MalPCR | ATTGGATCCGTTGCTACCTTGGTTTTGTTG | Chromosomal DNA | Complementation of the *malP* |
| MalQCF | AGGCTGCAGCAACAGAAGTACCTTCGGAGAG | Chromosomal DNA | Complementation of the *malQ* |
| MalQC R | ACGGGATCCCTGTTCACTCTTATTTGCTCGC | Chromosomal DNA | Complementation of the *malQ* |

[a]Regions of oligonucleotides not complementary to corresponding genes are underlined.
[b]To where the nucleotides are hybridized, +1 is the first base in the translational start codon of each ORF.

or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (w/v) agar. Unless noted otherwise, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). When appropriate, antibiotics were added to media at the following concentrations: ampicillin 100 μg/ml, kanamycin 50 μg/ml, and tetracycline 10 μg/ml. All the media components were purchased from Difco (Detroit, MI, U.S.A.), and the chemicals from Sigma (St. Louis, MO, U.S.A.).

### General Genetic Methods

The procedures for the isolation of plasmid DNA, genomic DNA, and transformation were carried out as described by Sambrook and Russell [22]. Restriction and DNA-modifying enzymes were used as recommended by the manufacturer (New England Biolabs, Beverly, MA, U.S.A.). The DNA fragments were purified from the agarose gels using a Geneclean II kit (Bio 101, Inc., Vista, CA, U.S.A.). Primary DNA cloning and manipulation were conducted in *E. coli* DH5α, and restriction mapping was used to confirm that the transformants contained the appropriate plasmids. The PCR amplification of DNA was performed using a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, U.S.A.) and standard protocol [22]. The oligonucleotides used in this study were synthesized (Bioneer, Seoul, Korea) and listed in Table 2.

### Cloning and Sequencing of the *V. vulnificus malPQ* Operon

A mutant strain HJK995 that was not able to grow on M9 agar plate supplemented with 10 mM maltose as a sole carbon source was screened from a library of *V. vulnificus* mutants generated by a random transposon mutagenesis using a mini-Tn5 *lacZ*1 [4, 10]. A DNA segment flanking the transposon insertion was amplified by PCR, as described previously [21]. Since the deduced amino acid sequence of the resulting PCR product, a 1,034-bp DNA fragment, revealed 54% identity with that of *E. coli malQ*, the DNA was labeled with [α-$^{32}$P]dCTP and named MalQProbe.

To clone the full genes of the *malPQ* operon, a cosmid library of *V. vulnificus* ATCC29307 which was constructed with pLAFR3 [11, 23] was screened, using MalQProbe as a probe. A colony showing a positive signal was isolated, and the cosmid DNA was purified and named pMH11-39 (Fig. 1A). An 8.2-kb band and a 5.2-kb fragment from the cosmid DNA digested with *Bam*HI-*Not*I and *Hin*dIII-*Sph*I, respectively, was purified and ligated into pBR322 to result in pMH0302 and pMH0332, as shown in Fig. 1A. The nucleotide sequences of the DNA fragments in pMH0302 and pMH0332 were determined by primer walking (Macrogen, Seoul, Korea). Comparisons of the nucleotide and deduced amino acid sequences were conducted using BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information).

### Generation of the *mal*::*nptI* Mutants

The *malP* gene in pMH0315 that was constructed by ligation of a 1.5-kb *Sph*I-*Xba*I fragment of pMH0302 with pBR322 was inactivated *in vitro* by the insertion of *nptI* encoding for aminoglycoside 3'-phosphotransferase and conferring resistance to kanamycin [17]. The 1.2-kb DNA fragment carrying *nptI* was isolated from pUC4K (Pharmacia, Piscataway, NJ, U.S.A.) and inserted into a unique *Hin*dIII site present within the ORF of *malP* in pMH0315. The 2.7-kb *malP*::*nptI* cartridge from the resulting construct (pMH0317) was liberated and ligated with *Sma*I-digested pCVD442 [5], forming pMH0321 (Fig. 1B). To inactivate the *malQ*, a 2.1-kb *Pvu*II-*Eco*RV DNA fragment carrying the part of *malQ* was isolated from pMH0302 and ligated with pBR322 to yield pMH0316. The *malQ* in pMH0316 was inactivated by inserting the *nptI* fragment into the *Cla*I

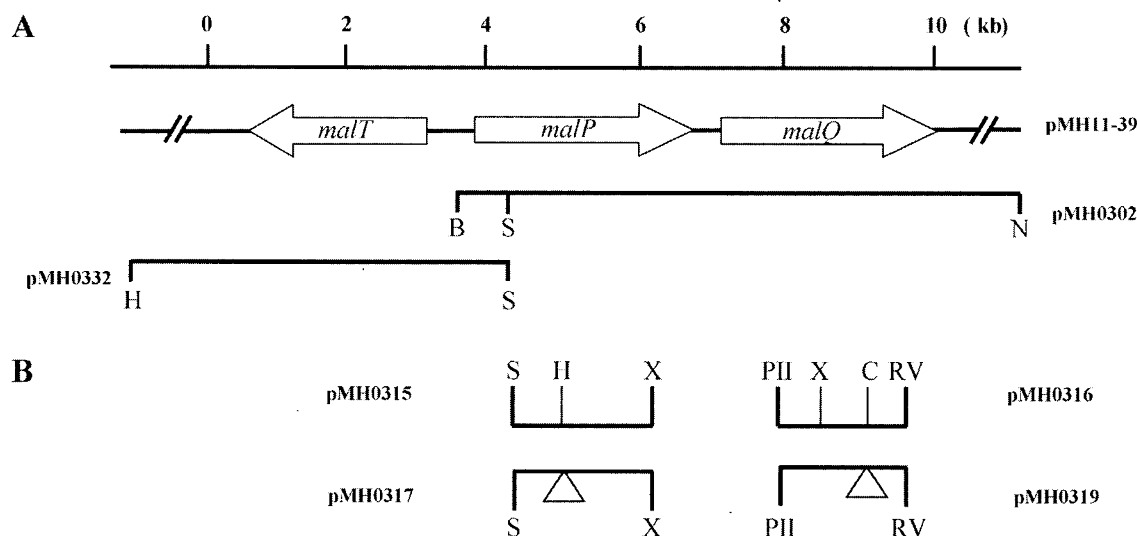


**Fig. 1.** Physical map of the *mal* genes on the *V. vulnificus* ATCC29307 chromosome and plasmids used in this study.
(A) Plasmids pMH0302 and pMH0332 were used to determine the nucleotide sequence of *malPQ*. The open boxes and thick lines represent the coding regions of *malPQ* genes and chromosomal DNA, respectively. Part of *malT* appears at the left end of *malPQ*. (B) Depicted are regions cloned in each of the plasmid used for the construction of the *mal*::*nptI* mutants. The insertion positions *nptI* cassette are indicated by open triangles. Abbreviations: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; N, *Not*I; PII, *Pvu*II; RV, *Eco*RV; S, *Sph*I; X, *Xba*I.

site of *malQ* ORF to result in pMH0319. The *malQ*::*nptI* cartridge was isolated after digestion of pMH0319 with *Pvu*II-*Eco*RV and ligated with pCVD442 to construct pMH0324 (Fig. 1B).

Since pCVD442 is a suicide vector containing the R6K $\gamma$ origin of replication, *E. coli* SM10$\lambda$*pir*, *tra* (containing pMH0321 or pMH0324) was used as a conjugal donor to generate the *mal*::*nptI* mutants of *V. vulnificus* ATCC29307 by homologous recombination (Fig. 3A). The conjugation and isolation of the transconjugants were conducted using the methods previously described [6, 7], and a double crossover, in which each wild-type *mal* gene was replaced with the *mal*::*nptI* allele, was confirmed by PCR, as shown in Fig. 3B. The *V. vulnificus* mutants chosen for further analysis were named MH031 for the *malP* mutant and MH032 for the *malQ* mutant, respectively. Similarly, an isogenic *malQ* mutant MH036 was constructed using the pMH0324 and *V. vuliníficus* MO6-24/O as a parental strain, and used for adhesion and virulence assay.

### Complementation of the *mal*::*nptI* Mutants

The ORF and upstream region of the *malP* and *malQ* were amplified by PCR using the following pairs of primers: MalPCF and MalPCR for the *malP*, and MalQCF and MalQCR for the *malQ*, as shown in Table 2. The amplified *malP* and *malQ* were digested with *Pst*I and *Bam*HI and then ligated with pRK415 [9, 20] digested with the same enzymes to result in pMH0330 and pMH0331, respectively. Since the broad-host-range vector pRK415 has an IncP1 origin and RP4 *oriT*, the resulting plasmids were mobilizable into *V. vulnificus* by conjugation.

### Measurement of Maltodextrin Phosphorylase Activity and 4-α-Glucanotransferase Activity

For measurements of enzyme activities, *V. vulnificus* strains cultured in LBS broth supplemented with 10 mM maltose at 30°C under aeration were harvested at $OD_{600}$ of 0.8 by centrifugation, washed with PBS (pH 7.4), and then kept frozen until used. The bacterial cells were resuspended in equal volume of PBS, broken by sonication (Ultrasonic processor, Sonics & Materials, Inc., CT, U.S.A.), and clarified by centrifugation. The supernatant was then used for determination of maltodextrin phosphorylase activity, 4-α-glucanotransferase activity, and cellular protein concentrations.

Enzyme reaction was initiated by adding 30 µl of the supernatant, as an enzyme source for maltodextrin phosphorylase, to 970 µl of assay solution containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM $MgCl_2$, 2 mM NADP, 5 mM maltoheptose, and auxiliary enzymes such as phosphoglucomutase (1.2U, Sigma) and glucose-6-phosphate dehydrogenase (3U, Sigma). The reaction mixture was incubated at 37°C for 20 min, and the reduction of NADP by the auxiliary enzymes was monitored to quantitate the glucose-1-phosphate [26]. For measurement of 4-α-glucanotransferase activity, the supernatant (20 µl) as an enzyme source was added to 80 µl of the reaction solution containing 100 mM MOPS (pH 7.0) and 20 mM maltose. After incubation at 37°C for 10 min, the reaction was stopped, and the glucose formed was then measured by using a glucose enzymatic kit (SIGMA, cat No. GAGO-20) [26].

Protein concentrations were determined by the method of Bradford [3], with bovine serum albumin as the standard. For statistical analysis, Student's *t* test was used to evaluate

the differences between the enzyme activities and the survival rates for the various strains (SAS software, SAS Institute Inc., Cary, NC, U.S.A.). Averages and standard errors of the mean (SEM) were calculated from at least three independent trials. One unit of maltodextrin phosphorylase and 4-α-glucanotransferase activity is defined as 1 μM of glucose-1-phosphate and glucose, respectively, released per minute per 100 μg protein.

### Adhesion Assay

Each well of 24-well culture dishes was seeded with INT-407 human intestinal epithelial cells (ATCC CCL-6) and grown overnight at 37°C in 5% $CO_2$ [13]. Cell monolayers were prepared as described previously [18], and then inoculated in triplicate with the *V. vulnificus* strains (MO6-24/O and MH036) to give different multiplicities of infection (MOI), and incubated for 15 min. The monolayer was then washed six times with prewarmed PBS to remove nonadherent bacteria. Following the last wash, the INT-407 cells were broken with 0.1% Triton X-100 treatment for 15 min. The bacteria were recovered from these cells with PBS, serially 10-fold diluted, and then plated on LBS agars. The mean number of attached bacteria per well was used to represent the adhesion index of the strains.

### Mouse Model of Infection

The 50% lethal doses ($LD_{50}$s) of wild-type and the *malQ* mutant were compared using ICR mice (Specific Pathogen-Free; Daehan Animal Co, Taejon, Korea), as described elsewhere [8, 14]. For the determination of $LD_{50}$, bacteria grown in LBS broth overnight at 30°C were harvested and suspended in PBS to appropriate concentrations, ranging from $10^2$ to $10^8$ CFU in 10-fold increments.

Group of (n=6) 7-weeks-old normal female mice were inoculated orally with 0.1 ml of serial dilutions of bacterial suspensions. The infected mice were observed for 24 h, and the $LD_{50}$s were calculated by the method of Reed and Muench [19]. Mice were intraperitoneally injected with 250 μg of iron dextran per g of body weight immediately before injection with bacterial cells.

### Nucleotide Sequence Accession Number

The nucleotide sequence of *malPQ* genes of *V. vulnificus* ATCC29307 was deposited into the GenBank under accession number AY530017.

## RESULTS

### Sequence Analysis of the *V. vulnificus malPQ* Genes

The nucleotide sequence of the DNA fragments in pMH032 and pMH0332 was determined, and the sequence data were submitted to the GenBank [Accession number AY530017]. The nucleotide sequence revealed two coding regions consisting of 2,454 nucleotides and 2,181 nucleotides, and they were 112-bp apart from each other (Fig. 1A). A database search for nucleotide sequences similar to those of the coding regions revealed *malPQ* genes cloned from *V. cholerae* with high levels of identity. The *malPQ* genes from *V. cholerae* were 83% to 91% identical in nucleotide sequences with the coding regions in pMH0302 and pMH0332 (data not shown). This information proposed that the coding regions are homologous to *malPQ* genes reported from other Enterobacteriaceae, and led us to name the coding regions *malPQ* of *V. vulnificus*.

### Comparison of Amino Acid Sequence of *V. vulnificus malP* and *malQ* with Those of Other Bacteria

The amino acid sequence deduced from the *malP* nucleotide sequence revealed a protein, maltodextrin phosphorylase, composed of 817 amino acids with a theoretical molecular mass of 92,254 Da and a pI of 6.21. The amino acid sequence of the *V. vulnificus* maltodextrin phosphorylase, MalP, was 55% to 91% identical to those of the MalP of *E. coli*, *Salmonella typhimurium*, and *V. cholerae* (Fig. 2A). The two amino acid sequences of MalP from *V. vulnificus* and *V. cholerae*, a species closely related to *V. vulnificus*, were nearly identical (91%, 743 out of 817 amino acids), and their identity appeared evenly throughout the whole proteins (Fig. 2A).

The amino acid sequence deduced from the *malQ* coding sequence revealed a protein, a 4-α-glucanotransferase, composed of 726 amino acids with a theoretical molecular mass of 81,769 Da and a PI of 5.68. The amino acid composition and molecular weight of this MalQ are quite

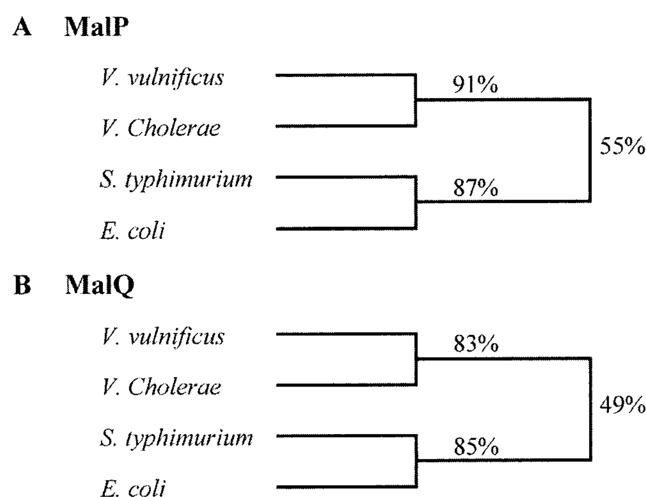


**Fig. 2.** Relatedness of amino acid sequence of MalP and MalQ of *V. vulnificus* and other bacteria.
The dendrogram showing relatedness of MalP (A) and MalQ (B) was derived using the CLUSTRALW alignment program (http://www.ebi.ac.uk/clustralw/), and based on the amino acid sequences in the GenBank databases (NCBI).

similar to those of the MalQ from other Enterobacteriaceae. The amino acid sequence of the *V. vulnificus* MalQ was 48% to 85% identical to those of the MalQ from *E. coli*, *S. typhimurium*, and *V. cholerae* (Fig. 2B). All of these information confirmed that the *malPQ* operon encodes maltodextrin phosphorylase and 4-α-glucanotransferase of *V. vulnificus* ATCC29307.

### Construction and Confirmation of the *V. vulnificus malP* Mutant and *malQ* Mutant

The insertional disruption of each *malP* gene in the mutants was confirmed by PCR. PCR analysis of genomic DNA from ATCC29307 with primers MalP09 and MalP10 (Table 2) produced a 1.3-kb fragment (Fig. 3B), whereas genomic DNA from the *malP* mutant, MH031, resulted in an amplified DNA fragment approximately 2.5-kb in length. The 2.5-kb fragment is in agreement with the projected size of the DNA fragment containing wild-type *malP* (1.3-kb) and the *nptI* gene (1.2-kb). Similarly, as shown in Fig. 3B, the construction of MH032, a *malQ* mutant, was confirmed by PCR, using primers MalQ01 and MalQ06. The 2.0-kb PCR product produced with genomic DNA from the MH032 is in good agreement with the sum of the sizes of *malQ* from the wild-type and *nptI*. To determine the stability of the insertional mutation, *V. vulnificus* MH031 and MH032 were grown overnight without kanamycin selection. The inserted *nptI* DNAs were stably maintained in both mutants, as determined by maintenance of kanamycin resistance (all of more than 500 colonies tested) and by generation of the appropriate-sized DNA fragment by PCR (data not shown).

### Effects of Mutations in *mal* Genes on Maltodextrin Phosphorylase Activity

For ATCC29307, maltodextrin phosphorylase was produced and reached a maximum 10 units (Fig. 4A). The disruption of *malP* in mutant MH031 resulted in complete loss of maltodextrin phosphorylase activity (Fig. 4A). These data demonstrated that the *malP* gene encodes the maltodextrin phosphorylase of *V. vulnificus*. The observation that the level of maltodextrin phosphorylase activity in the mutant was undetectable revealed the existence of only one maltodextrin phosphorylase produced by *V. vulnificus* ATCC29307.

Although it seemed unlikely that the decrease of maltodextrin phosphorylase activity by more than 99% resulted from polar effects of the *malP* insertional mutation on downstream genes, this possibility could not be ruled out *a priori*. Therefore, we examined if the reintroduction of recombinant *malP* into pMH0330 could complement the decrease of maltodextrin phosphorylase of MH031 cells. As shown in Fig. 4A, the maltodextrin phosphorylase activity of the MH031 (pMH0330) was restored to a level comparable to the wild-type level of ATCC29307. Therefore, the decreased maltodextrin phosphorylase activity of MH031 resulted from the inactivation of functional *malP* rather than any polar effects on genes downstream of *malP*.

### 4-α-Glucanotransferase in the *malQ* Mutant and *malP* Mutant

When the activity of 4-α-glucanotransferase was determined in the *malQ* mutant (MH032), the 4-α-glucanotransferase activity of the mutant decreased significantly and the trend of the decrease was similar to that for maltodextrin

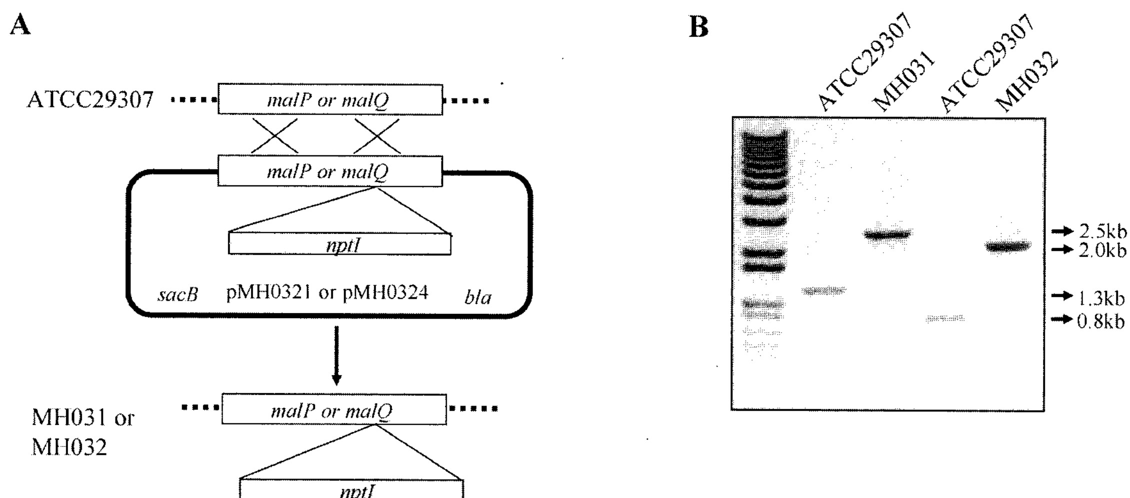


**Fig. 3.** Allelic exchange procedure and construction of *mal::nptI* isogenic mutants.
(A) Double homologous recombinations between strain ATCC29307 and plasmids pMH0321 or pMH0324 lead to an interruption of respective *mal* genes, resulting in construction of mutants MH031 or MH032, respectively. Dashed lines represent the bacterial chromosome; a full line, the plasmid DNA; open boxes, the target *mal* genes; shaded boxes, the *nptI* gene; large X's represent genetic crossing over. Abbreviations: *sacB*, levansucrase gene; *bla*, β-lactamase gene. (B) PCR analysis of ATCC29307 and isogenic mutants generated by allelic exchange. Molecular size markers (1-kb ladder, GIBCO-BRL, Gaithersburg, MD, U.S.A.) appear in the end lanes of the gel.

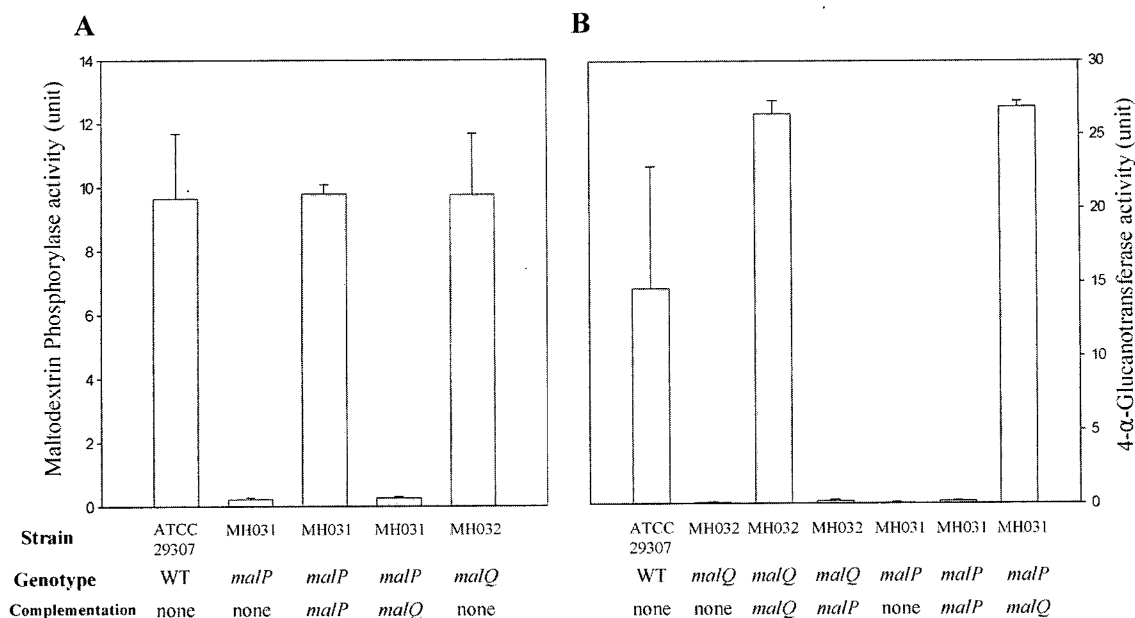


**Fig. 4.** Effects of mutations in *mal* genes on maltodextrin phosphorylase and 4-α-glucanotransferase activity.
For both panels, cultures of ATCC29307 and each isogenic mutant were grown in LBS supplemented with 10 mM maltose. Samples removed at $OD_{600}$ of 0.8 were analyzed for maltodextrin phosphorylase activity (A) and for 4-α-glucanotransferase activity (B). Complementations of the mutants with a functional *malP* (pMH0330) or *malQ* (pMH0331) are also presented as indicated. Relative activities of maltodextrin phosphorylase and 4-α-glucanotransferase were calculated as described in Materials and Methods. Error bars represent the SEM.

phosphorylase observed in the *malP* mutant (Fig. 4B). Introduction of *malQ* (pMH0331) into MH032 restored 4-α-glucanotransferase activity to the level found in the wild type. When MH031, a *malP* mutant, was compared with its parental wild-type, it appeared to produce much less 4-α-glucanotransferase, and the level of the 4-α-glucanotransferase was comparable to that of the *malQ* mutant. One possible explanation for this decrease in 4-α-glucanotransferase activity in MH031 is that the *malPQ* is one transcriptional unit, and insertional mutation of *malP* gives the effect of polarity on *malQ*. To further test this possibility, recombinant *malP* (pMH0330) was reintroduced into MH031, and the 4-α-glucanotransferase activity was determined. The level of 4-α-glucanotransferase of MH031 (pMH0330) was not restored and remained close to that of MH031. However, the reintroduction of pMH0331 carrying *malQ* into MH031 restored the decreased activity of 4-α-glucanotransferase to the level of the wild-type (Fig. 4B). These results again supported our hypothesis that *malPQ* is one transcriptional unit and the *malQ* is not functional in MH031.

## Effects of *mal* Mutations on the Growth of *V. vulnificus* Using Maltose as a Sole Carbon Source

To measure the growth of strains with maltose as a sole carbon source, M9 media [22], in which glucose was deleted and maltose (a final concentration of 10 mM) was supplemented as a sole carbon source, was used (Fig. 5). Ten mM maltose as a sole carbon source supported the growth of wild-type, and stationary-phase yield of cells corresponded to that obtainable when LBS was used. In contrast to this, either MH031 or MH032 that is deficient of functional MalPQ was not able to grow at all, revealing the conversion of maltose into glucose is essentially required

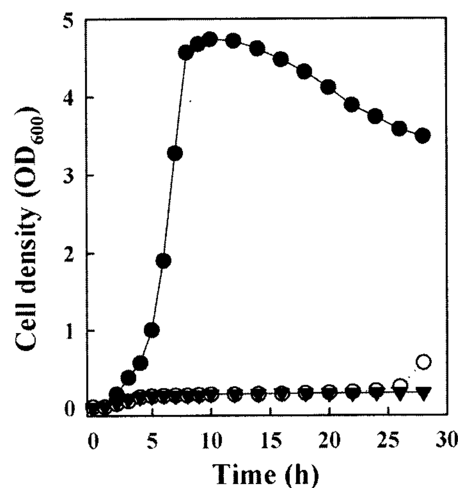


**Fig. 5.** Growth kinetics of *V. vulnificus* strains in a defined medium using maltose as a sole carbon source.
To measure the growth of strains with maltose as a sole carbon source, cultures of ATCC29307 (●), MH031 (○), and MH032 (▼) were grown in M9 media supplemented with 10 mM maltose as a sole carbon source.

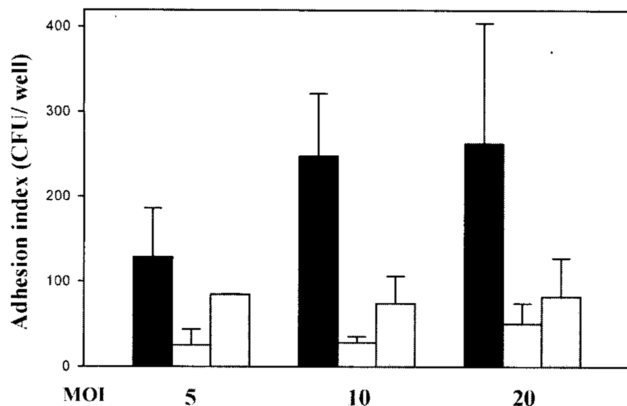


**Fig. 6.** Adhesion of *V. vulnificus* wild-type and *malQ* mutant to INT-407 cells.

INT-407 cells were cultured on 24-well culture dishes and infected with *V. vulnificus* strains at different MOIs as indicated. After incubation with the bacteria for 15 min, the INT-407 monolayers were rinsed to remove any nonadhering bacteria. The adherent bacteria were quantified and expressed as the number of bacteria per well in tissue culture. Details are described in Materials and Methods. Closed bars, wild-type (MO6-24/O); open bars, *malQ* mutant (MH036); shaded bars, *malQ* mutant complemented with pMH0331.

for growth of *V. vulnificus* with maltose as a sole carbon source. This suggests that the MalPQ proteins are responsible for maltose metabolism in *V. vulnificus*.

### MalQ is Required for Adhesion to Epithelial Cells *In Vitro*

In order to determine if MalQ plays a role in virulence of *V. vulnificus* by aiding adherence to epithelial cells, adhesion of MO6-24/O and MH036 to INT-407 cells was assayed. When INT-407 cells were infected at MOI of 5, the wild-type strain adhered to the INT-407 cells and reached an adhesion index of 115 CFU per well after 15 min of infection (Fig. 6). The adhesion index increased as the infection size (MOI) increased, and the adhesion index at a MOI of 20 represents about 250 CFU per well (Fig. 6). In contrast, MH036 was consistently and significantly less adherent than the wild-type parent strain at all MOI studied. When infected at a MOI of 5, the number of the *malQ* mutant per well of INT-407 monolayers was about 5-fold less than that of the wild-type, and the adhesion index of the *malQ* mutant was not obviously affected by increasing size of MOI (Fig. 6). It was unable to perform adhesion assays when incubation period was longer than 15 min at all MOIs, since most of the INT-407 cells were lysed (data not shown). Nonetheless, the results clearly revealed that the *malQ* mutant was significantly impaired in its ability to attach to the epithelial cells. Although the impaired adherence of MH036 to INT-407 was not completely restored by reintroduction of recombinant *malQ*, MH036 (pMH0331) substantially increased adherence to a value about half of that for the wild-type strain. These results suggested that adherence of *V. vulnificus* to INT-407 cells requires *malQ* product.

**Table 3.** Effects of the *malQ* mutation on the lethality of *V. vulnificus* to mice.

| Strain[a] | $LD_{50}$ (CFU) |
|---|---|
| MO6-24/O (n=6) | $1.3\times10^{6}$ |
| MH036 (n=6) | $2.3\times10^{6}$ |

[a]n, number of iron-treated mice for each inoculation group, ranging from $10^{2}$ to $10^{8}$ CFU in 10-fold increments.

### Effects of Mutation in *malQ* on the Virulence of *V. vulnificus*

We examined the virulence of *V. vulnificus* MO6-24/O and MH036 using iron dextran-treated and normal ICR mice (Specific Pathogen-Free; Daehan Animal Co, Taejon, Korea), as described previously [14]. As shown in Table 3, there was no difference in the $LD_{50}$s of MO6-24/O and MH036 in iron-treated mice, which were as high as $10^{6}$ CFU for both strains. In normal mice (non-iron treated), the $LD_{50}$s observed by both strains were also indistinguishable (data not shown). Therefore, in the mouse model of oral infection, in which $LD_{50}$s were compared with wild-type and *malQ* mutant strains, the MalQ appeared to be not important for disease.

## DISCUSSION

Many bacteria can utilize maltose as a sole source of carbon and energy. In *E. coli*, maltose is catabolized via the products of the maltose regulon, consisting of three operons, *malPQ*, *malK-lamB-malM*, and *malEPG* [1]. The uptake of maltose is accomplished by the combined action of gene products of *lamB*, *malE*, *malF*, *malG*, and *malK*. LamB in the outer membrane is a maltose-specific pore (maltoporin), and MalFGK, a transport complex in the cytoplasmic membrane, is a member of the superfamily of ATP-binding cassette transporters [2]. In the cytoplasm, 4-α-glucanotransferase, a gene product of *malQ*, recognizes maltotriose (or larger maltodextrins), cleaves off the reducing glucose residue, and transfers the remaining dextrinyl residues onto the incoming maltose. The resulting maltodextrins are subsequently recognized by maltodextrin phosphorylase, a gene product of *malP*, releasing glucose-1-phosphate and a maltodextrin that is smaller by one glucosyl residue [1]. The glucose and glucose-1-phosphate are both transformed into glucose-6-phosphate. Neither enzyme activities nor biochemical pathways involved in maltose metabolism of *V. vulnificus* has yet been established. However, our data revealed that the *V. vulnificus* strain with a null mutation in the *malQ* or *malP* gene is not able to exhibit growth on maltose as the sole carbon source (Fig. 5). This result

indicated that the gene products of *malPQ* probably play the primary role for maltose metabolism in *V. vulnificus* as observed in *E. coli*.

Previous studies noted that expression of the *malPQ* operon of *E. coli* is induced by maltose and controlled by a positive regulatory gene, *malT* (Fig. 1) [1]. The full expression of this regulon, like that of other genes involved in the catabolism of sugars, requires cyclic AMP (cAMP) and cyclic AMP receptor protein (CRP) [1]. In the present study, activities of maltodextrin phosphorylase and 4-α-glucanotransferase were observed only when *V. vulnificus* was grown in the presence of maltose (Fig. 4), indicating that expression of *malPQ* of *V. vulnificus* is induced by maltose as in *E. coli*. In the course of our sequencing analysis, the part of *malT* homology located upstream of *malP* was found (Fig. 1). It is not clear whether the same elements observed in the regulation of *malPQ* of *E. coli* are also involved in the regulation of the *malPQ* of *V. vulnificus*. Nevertheless, from our observation that the genetic organization and nucleotide sequences of *malPQ* are quite conserved among *E. coli* and *V. vulnificus*, and that the *malT* homology is present, it is most likely that the *malPQ* of *V. vulnificus* would be regulated in a manner analogous to the regulation of *E. coli malPQ*.

In the process that leads to colonization of the intestine, the availability of nutrients is essential to enteropathogenic bacteria. Maltose is very common in the intestine and could serve as a good carbon and energy source for the colonizing bacteria. However, compared with the substantial number of reports on the characterization of toxins of the pathogenic bacteria, there have been only a few studies on the sugar metabolism of enteropathogenic bacteria and its role in virulence. It has been proposed that maltose probably has a significant regulatory role in production of virulence factors of *V. cholerae* [12]. Mutations in the *mal* operon have a profound effect on cholera toxin production and/or secretion, and on the number of pilus structures on the cell surface [12]. The role of the *mal* operon of *V. vulnificus* in the adherence to the INT-407 human epithelial cells was also examined in the present study. As shown in Fig. 6, there was significant difference in adherence of the wild-type and *malQ* mutant to INT-407 cells, suggesting that the *malQ* and/or the product of *malQ* was necessary for the adherence of the bacteria to INT-407 cells. The major problem to be addressed is that no significant difference was observed when the *malQ* mutant was compared with parental wild-type for virulence ($LD_{50}$) in orally inoculated mice (Table 3). Although other explanations are possible, the lack of significant difference in virulence between the *malQ* mutant and the wild-type parent could be related to the presence of other sugars in the intestine. It is difficult to imagine that inability for maltose utilization is completely compensated by the presence of other sugars in the intestine; however, analysis of this hypothesis awaits additional works for identification of the amount and type of sugars in the intestine.

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