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Genetic Analysis of Spontaneous Lactose-Utilizing Mutants from *Vibrio vulnificus*

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Abstract Wild-type *V. vulnificus* cannot grow using lactose as the sole carbon source or take up the sugar. However, prolonged culture of this species in media containing lactose as the sole carbon source leads to the generation of a spontaneous lactose-utilizing (LU) mutant. This mutant showed strong β-galactosidase activity, whereas the wild-type strain showed a barely detectable level of the activity. A mutant with a lesion in a gene homologous to the *lacZ* of *E. coli* in the bacterium no longer showed β-galactosidase activity or generated spontaneous LU mutants, suggesting that the *lacZ* homolog is responsible for the catabolism of lactose, but the expression of the gene and genes for transport of lactose is tightly regulated. Genetic analysis of spontaneous LU mutants showed that all the mutations occur in a *lacI* homolog, which is located downstream to the *lacZ* and putative ABC-type *lac* permease genes. Consistent with this, a genomic library clone containing the *lacI* gene, when present *in trans*, made the spontaneous LU mutants no longer able to utilize lactose as the sole carbon source. Taken together with the observation that excessive amounts of exogenously supplemented possible catabolic products of lactose have negative effects on the growth and survivability of *V. vulnificus*, we suggest that *V. vulnificus* has evolved to carry a repressor that tightly regulates the expression of *lacZ* to keep the intracellular toxic catabolic intermediates at a sublethal level.

Keywords: *Vibrio vulnificus*, β-galactosidase, lactose, *lacZ*/*lacI*

The *lac* operon in *E. coli* has served as a best-studied model for gene regulation for more than 50 years [3, 17]. The classical *E. coli lac* operon constitutes *lacZ*, *lacY*, and *lacA* genes, which encode β-galactosidase, permease, and galactoside transacetylase, respectively. The first two enzymes, β-galactosidase and permease, are in charge of splitting lactose into glucose and galactose, and the import of lactose through the cell membrane, respectively. The third enzyme, galactoside transacetylas, plays no essential role in lactose metabolism. The biological role of this enzyme still remains unclear. Transcription of *lacZYA* is controlled by a negative regulator protein, LacI, which is one of the best-understood regulatory function in *E. coli*. The LacI repressor subunits form a dimer that binds the *lac* operator with a high affinity [28]. The two dimers contact each other and form the tetrameric *lac* repressor [13]. LacI is a member of the GalR/LacI family. Members of the GalR/LacI family have two distinct functional domains [15, 30, 33]. The short N-terminal domain (approximately first 60 amino acids) is the helix-turn-helix (HTH) DNA-binding domain (or headpiece). The larger C-terminal domain (approximately 280 amino acids) is the core domain for effector binding and oligomerization, and has N- and C-subdomains.

The opportunistic human pathogen *V. vulnificus* is a halophilic Gram-negative bacterium that has been isolated from seawater and shellfish in coastal waters [26, 37]. Disease caused by this organism is characterized by primary septicemia and is strongly associated with the ingestion of raw oysters and the exposure of wound to seawater [20, 34]. Most systematic infections occur in individuals with underlying predisposing conditions such as liver disease, hemochromatosis, iron overload, and immune dysfunction [4, 20]. The mortality rate from septicemic patients is greater than 50%, and death can occur within 1–2 days after infection [4]. Recent study suggested that quorum-sensing regulation responsible for a global regulation is important for the pathogenicity and survival of the bacteria [22].

V. vulnificus was originally distinguished from other *Vibrio* species by its ability to ferment lactose [16, 26]. A contradictory report, however, showed that the ability of *V. vulnificus* to utilize lactose as the sole source of carbon and energy is associated with the appearance of spontaneous

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lactose-utilizing mutants [2]. In agreement with the latter report, we found that wild-type *V. vulnificus* strains cannot utilize lactose as the sole carbon source, but these strains easily produce spontaneous LU mutants in a minimal medium containing lactose as the sole carbon source. From our genetic analyses of genes responsible for lactose metabolism, we found that this bacterium harbors a repressor regulating the expression of β-galactosidase, which is responsible for a tight regulation of the lactose utilization. The bacterium carries a strong regulation of lactose utilization, probably ensuring the viability of the pathogen from the toxic effect of accumulation of catabolic intermediates generated from lactose.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Culture Media, Growth Conditions, and Chemicals

Luria-Bertani (LB) (Difco) was used as a rich medium for *V. vulnificus* and *E. coli*, and nutrient broth (Difco) for *Agrobacterium*. TCBS agar (Difco) medium was used for selective growth of *V. vulnificus*. AB minimal medium [24] supplemented with 0.5% glucose (ABG) was used for the selective growth of *Agrobacterium* after mating. VM9 minimal medium, used to culture *V. vulnificus* strains for sugar-utilization tests, was prepared by supplementing 1% NaCl, trace elements, and vitamins for Sistrom's medium [31] to M9 minimal medium [25]. Carbon source was added to the minimal medium at a final concentration of 0.2% glucose or 0.4% lactose. *E. coli* strains were grown at 37°C, whereas *Agrobacterium* strains and *V. vulnificus* strains were grown at 28°C. For *E. coli*, ampicillin (Ap) at 100 μg/ml, kanamycin (Km) at 25 μg/ml, streptomycin (Sm) at 25 μg/ml, and tetracycline (Tc) at 10 μg/ml were used. For *V. vulnificus*, Ap at 100 μg/ml, rifampicin (Rif) at 50 μg/ml, streptomycin (Sm) at 100 μg/ml, and Tc at

Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant genotypes and characteristics[a]	Source, reference
Vibrio vulnificus strains		
ATCC29307	Clinical isolate, *lac*$^-$	[26]
29307LU	A spontaneous lactose-utilizing (LU) mutant derived from ATCC29307	This study
VvSR	A spontaneous Sm^r/Rf^r mutant derived from ATCC29307	This study
VvSR-dZ	A *lacZ*-null mutant derived from VvSR, Sm^r Rf^r	This study
VvSRLU	A spontaneous LU mutant derived from VvSR, Sm^r Rf^r	This study
MO6-24/O	Clinical isolate, *lac*$^-$	[36]
MO6LU	A spontaneous LU mutant derived from MO6-24/O	This study
MO6-dZ	A *lacZ*-null mutant derived from MO6-24/O	This study
MO6-dI1	A derivative of MO6-24/O with an in-frame deletion in *lacI1* (AY346384)	This study
MO6-dI2	A derivative of MO6-24/O with an insertion in *lacI2* (a *lacI* homolog, AY252118)	This study
MO6-dI12	A derivative of MO6-24/O with mutations in both *lacI1* and *lacI2*	This study
MO6-dZ(pRKVv12)LU	A spontaneous LU mutant derived from MO6-dZ harboring pRKVv12, Tc^r	This study
MO6-dZLU	A plasmid-cured derivative from MO6-dZ(pRKVv12)LU	This study
Plasmids		
pCHSUI-1	A suicide vector for gene replacement, R6K, Tc^r	This study
pCH-dI2	A derivative of pCHSUI-1 containing *lacI2* with an insertion mutation, Tc^r	This study
pCH-dI1	A derivative of pCHSUI-1 containing a *lacI1*-deletion construct, Tc^r	This study
pKAS-dZ	A derivative of pKAS32 containing *lacZ* with a mutation, Ap^r	This study
pKNG-dZ	A derivative of pKNG101 containing *lacZ* with a mutation, Sm^r	This study
pRKVv12	A pRK415-based genomic library clone of ATCC29307 containing *lacZ*, Tc^r	This study
pRKVv101	A pRK415-based genomic library clone of ATCC29307 containing *lacI2*, Tc^r	This study
pRKVv111	A pRK415-based genomic library clone of ATCC29307 containing *lacI1*, Tc^r	This study
p101dHP	A derivative of pRKVv101 inserted with the ΩKm-cassette from pHP45ΩKm	This study
p101dI2	pRKVv101 inserted with the ΩKm-cassette from pHP45ΩKm at the BamHI site	This study
pRK-I1	A 1,338-bp PCR product containing *lacI1* from MO6-24/O cloned into pRK415	This study
pRK-I1-LU	A 1,338-bp PCR product containing *lacI1* from MO6LU cloned into pRK415	This study
pRK-LR	A 950-bp PCR product containing the ORF in immediate downstream of *lacI1* from MO6-24/O cloned into the *Bam*HI site of pRK415, Tc^r	This study

Abbreviations: *lac*$^-$, unable to utilize lactose as sole carbon source; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance; Rf^r, reifampicin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

2 μg/ml were used. All of the antibiotics and chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

DNA Manipulation

Isolation of plasmid DNA was performed using alkaline lysis protocols as previously described [29]. Genomic DNA from *V. vulnificus* was isolated as described previously [23]. Restriction enzymes and DNA-modifying enzymes were used as recommended by the manufacturer (MBI Fermentas, New England Biolabs). The *lacI1* gene was amplified by PCR using following primer sets: 5'-AAGGATCCATCATCGTCTTCG-3' and 5'-AGGGATCCGAGTCTGACGGTGATAC G-3'.

Construction of *lacI1* Clones, pRK-I1 and pRK-I1-LU

The 2.0-kb BamHI fragment of the Ω-Km-cassette from pHP45Ω-Km [27] was inserted into the BglII and BamHI sites of pRKVv101 to construct p101-dHP and p101-dI, respectively. The 1.3-kb DNA fragments containing *lacI1* from MO6-24/O and MO6-LU were amplified by PCR using the following primers: 5'-AAGGATCCATCATCGTCTTCG-3' and 5'-AGGGATCCGAGTCTGACGGTGATACG-3'. The PCR products were digested with BamHI and ligated into the unique BamHI site of pRK415, to generate pRK-I1 and pRK-I1-LU, respectively. A 950-bp DNA fragment containing the ORF in immediate downstream of *lacI1* from MO6-24/O was amplified by PCR using the primers 5'-GAGGATCCTGAGCGCAAAGAGCAC-3' and 5'-CAGGATCCACTGGC AGTTCGGGTTTG-3'. The PCR product was digested with BamHI and inserted into the unique BamHI site of pRK415, to yield pRK-LR.

Construction of the *lacZ*-Null Mutants VvSR-dZ and MO6-dZ

A 2.6-kb EcoRI fragment containing the *lacZ* gene was cloned from pRKVv12 into the unique EcoRI site of pBluescript SK- (Stratagene). The resulting plasmid was digested with SphI and StuI. The reaction was treated with T4 DNA polymerase and T4 DNA ligase, resulting in the deletion of the 935-bp SphI/StuI fragment from the 2.6-kb EcoRI fragment to generate pBSV12-7dSS. A 1.7-kb EcoRI fragment from pBSV12-7dSS was cloned into the unique EcoRI site of a suicide vector, pKAS32 [32], to obtain pKAS-dZ. The resulting construction was introduced into *V. vulnificus* strain VvSR by biparental mating using *E. coli* strain S17-1 λ*pir* [10]. Transconjugants were selected on TCBS agar (Difco) plate containing 100 μg/ml of ampicillin (Ap), and were streaked on LB agar medium with 1 mg/ml of Sm and 40 μg/ml of X-gal. Streptomycin-resistant (Sm^R) white colonies were tested for ampicillin sensitivity (Ap^S). The *lacZ* deletions in these Sm^R-Ap^S-white clones were further confirmed by PCR and Southern hybridization (data not shown). This *lacZ*-null mutant derived from VvSR was named VvSR-dZ.

A 1.7-kb ApaI/BamHI fragment from pBSV12-7dSS was cloned into the unique ApaI/BamHI sites of pKNG101 [18]. The resulting plasmid named pKNG-dZ was introduced into the strain MO6-24/O, and the resulting transformants were spread on solid LB medium containing 10% sucrose. Among the sucrose-resistant (Suc^R) colonies, we obtained clones that are sensitive to streptomycin. The *lacZ* deletions in these Suc^R-Sm^S-white clones were further confirmed by PCR and Southern hybridization (data not shown). This *lacZ*-null mutant derived from MO6-24/O is called MO6-dZ.

Construction of the *lacI1*- and *lacI2*-null Mutants

The positive-selection suicide vector pCHSUI-1 is a derivative of pKNG101 [18]. To construct pCHSUI-1, a 4.7-kb NotI/SalI fragment from pKNG101 was blunted by treatment of Klenow, and ligated with a 2.1-kb DNA fragment containing a multiple cloning site (MCS), the *lacZ*α fragment, and the tetracycline resistance (Tc^R) gene, which was amplified from pBBR1MCS2 (21) by PCR using a primer set (5'-ATCGCAGTCGGCCTA-3' and 5'-AAGTCCAGCGCCAGAAAC-3') and blunted by Klenow.

To construct a mutation in *lacI1* (GenBank Accession No. AY346384), a 2.2-kb XbaI/SacI fragment from pRKVv111 was cloned into pCHSUI-1. A 192-bp NcoI fragment in *lacI1* on the resulting plasmid was removed by NcoI digestion and self-ligation. The resulting plasmid was named pCH-dI1. The *lacI1*-deletion derivative of MO6-24/O was obtained by an allelic exchange, and the resulting mutant was named MO6-dI1. To construct a mutation in *lacI2* (another *lacI* homolog; GenBank Accession No. AY252118), a 4.9-kb XbaI/SacI fragment from p101-dI2, which contains a mutation in AY252118 constructed by insertion of the Ω-Km cassette, was cloned into the same sites in pCHSUI-1, to generate pCH-dI2. Using this plasmid, a derivative of MO6-24/O with a mutation in *lacI2* was constructed by an allelic exchange, and named MO6-dI2. Using pCH-dI1 and pCH-dI2, a derivative of MO6-24/O with a double mutant in these two *lacI* homologs was constructed, and name MO6-dI12. All these constructs were confirmed by PCR and Southern hybridization (data not shown).

Lactose Utilization Test

β-Galactosidase activity was assayed as described previously [19]. Cells were inoculated into liquid or solid VM9L medium, which is the VM9 minimal medium containing 0.4% Bacto lactose (Difco) as the sole carbon source, and the growth was observed daily with visual inspection for a one-week period.

Paper Chromatography of Sugars in the Culture Medium

Cells in culture medium were removed by centrifugation at 12,000 ×*g* for 5 min at room temperature. The culture

supernatant was spotted on Whatman 3MM filter paper and the sugars in the supernatant were separated using 70% ethanol as a mobile phase. The separated sugars on the paper were visualized by the alkaline silver nitrate staining method as described previously [11].

Lactose Uptake Test

Uptake of lactose was estimated indirectly by measuring the disappearance of the substrates from liquid medium. Tested strains were grown in 0.5 ml of VM9 liquid medium containing 0.2% glucose for 10 h at 28°C with shaking. Cells were collected by centrifugation and washed with 0.9% NaCl solution. The collected cells were resuspended in 100 μl of VM9 minimal medium without carbon source, and the cells were starved for 2 h at 28°C with shaking. Cells were collected by centrifugation and resuspended in VM9 minimal medium containing 2 mM lactose. After 1 h, cells were removed by centrifugation and the residual lactose in the supernatants was measured quantitatively using the phenol-sulfuric acid assay as previously described [6].

RESULTS

Analysis of Spontaneous Lactose Utilization (LU) Mutants from *V. vulnificus*

Two strains of *V. vulnificus*, VvSR and MO6-24/O, formed pale-blue colonies on VM9 minimal medium containing X-gal, although these strains produced a barely detectable level of β-galactosidase activity (Table 2). These strains were tested for their ability to utilize lactose as the sole carbon source using VM9 medium containing 0.4% lactose. When examined in liquid medium culture, strain MO6-24/O could grow using lactose as the sole carbon source, whereas VvSR could not. Growth of MO6-24/O in the presence of both glucose and lactose showed a diauxic pattern that is characterized by two distinct growth phases separated by a long lag phase (Fig. 1A). In order to monitor sequential utilization of the two sugars during the diauxic growth of strain MO6-24/O, sugars remaining in the culture supernatant were measured by paper chromatography at various time intervals. As shown in Fig. 1B, the diauxic growth pattern corresponded with the disappearance of the sugars. Preferential utilization of glucose was observed in the initial growth phase of the diauxie, whereas the final growth phase after 24 h of long lag phase occurred at the expense of lactose. In contrast to this result, these cells did not grow on solid media containing lactose as the sole carbon source. We assumed that growth in liquid media and the fast consumption of lactose after a long period of a lag phase may be due to occurrence of spontaneous LU mutants. To verify this hypothesis, 20 independent clones were derived from each of the cultures at the 40-h time point (as marked with an arrow in Fig. 1A) and tested for the ability of each clone to utilize lactose as the sole carbon source. As shown in Fig. 2A, none of the clones precultured in the VM9 medium containing only glucose as the sole carbon source could utilize lactose as the sole carbon source. However, 12 out of 20 clones precultured in the

Table 2. Lactose utilization and β-galactosidase activities of *V. vulnificus* strains.

Strain	Lactose utilization[a]	β-Galactosidase activity[b]
VvSR	$-^{m}$	6±0.59
MO6-24/O	$-^{m}$	7±3.1
MO6-dZ	-	3±1.8
MO6LU	++	583±33.3
MO6-dI2	$-^{m}$	2±1.7
MO6-dI	+	526±25.2

[a]Lactose utilization was visually assessed on solid VM9 minimal media containing lactose at 0.4% (w/v) as the sole carbon source for a one-week period, and growth was recorded as -, no growth; $-^{m}$, no growth but spontaneous *lac*$^{+}$ mutants occurred; +, good growth; ++, very good growth.
[b]Cells were grown to an early stationary phase in VM9 minimal media containing 0.2% glucose at 28°C with shaking. β-Galactosidase activity was measured as previously described [24]. Mean±standard deviation was derived from values of three independent experiments.

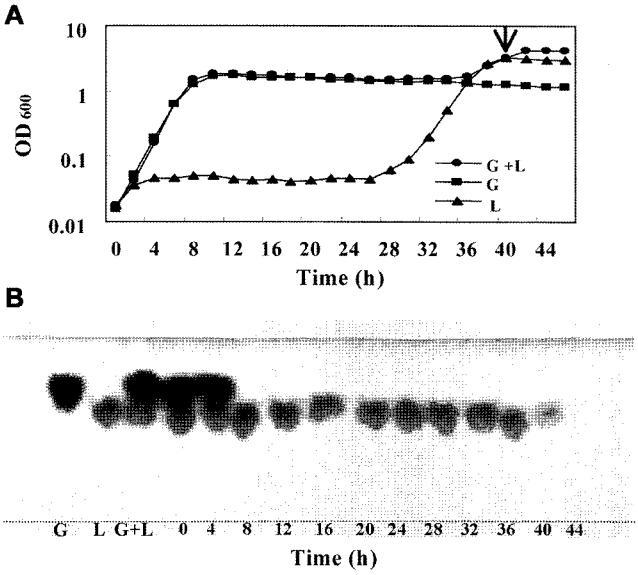

Fig. 1. Lactose and glucose utilization of *V. vulnificus* strain MO6-24/O.
A. Growth curves of MO6-24/O in VM9 minimal medium containing 0.2% glucose (G) (■), or 0.4 % lactose (L) (▲), or both (G+L) (●) as the carbon source. The cell density of each culture was measured at every 4-h intervals. The plotted data are averages of mean values from two independent experiments. Arrow (↓) represents the time point at which cells were harvested for the analysis of lactose-utilizing mutant generation (See Fig. 2). **B.** Paper chromatography for monitoring sugars remaining in culture supernatants. One ml volume of the culture was removed from each of the above cultures at the same time points, and the culture supernatant was used for the assay.

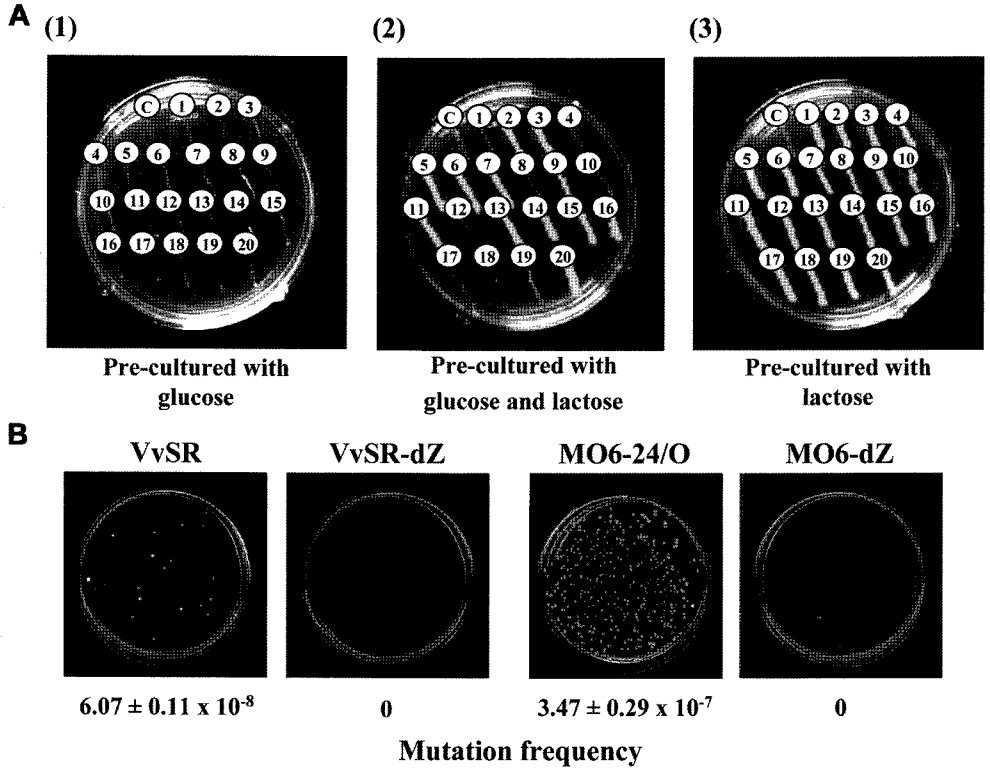

Fig. 2. Generation of spontaneous lactose-utilizing mutants.
A. Cells were obtained from each of the cultures at the 40-h time point [marked as an arrow (↓) in Fig. 1], diluted, and spread on LB agar medium. Twenty individual clones derived from each of the three cultures were patched on solid VM9 minimal medium containing 0.4% lactose and assessed for their ability to utilize lactose as the sole carbon source by visual inspection of colony formation for 48 h. **B.** Occurrence of spontaneous LU mutants from *V. vulnificus* strains. Cells were grown to late log phase in VM9 minimal medium containing 0.2% glucose, and the cells were washed with 0.9% NaCl. These cells were diluted and spread on either VM9 minimal medium containing 0.4% lactose as the sole carbon source or LB agar medium. Occurrence of spontaneous LU mutants was observed daily for a one-week period. Value±standard deviation of mutation frequency was derived from two independent experiments.

VM9 medium containing glucose and lactose, and all 20 clones precultured in the VM9 medium containing only lactose could utilize lactose as the sole carbon source. These results indicate that the outgrowth after the long lag phase with consumption of lactose is due to accumulation of the spontaneous LU mutants. The spontaneous LU mutant derived from MO6-24/O was designated MO6LU. Spontaneous LU mutants also arose from VvSR (Fig. 2B), and the mutant clone was named VvSRLU. However, the mutation frequency of VvSR was lower than that of MO6-24/O (Fig. 2B). The low frequency of spontaneous LU mutants from VvSR explained why this strain failed to utilize lactose as the sole carbon source in liquid media. In support of this idea, when the cell mass of inoculum of VvSR was increased by 10-folds, we could observe growth of the two strains in liquid medium supplemented with lactose as the sole carbon source (data not shown). The *lacZ*-null mutants VvSR-dZ and MO6-dZ derived from VvSR and MO6-24/O, respectively, could not form spontaneous LU mutants (Fig. 2B), indicating that *lacZ* is essential and functional for lactose catabolism in these *V. vulnificus* strains.

Localization of Mutations in the Spontaneous LU Derivatives

The pRK415-based genomic library clones of the wild-type *V. vulnificus* ATCC29307 were mobilized into MO6LU, a spontaneous LU mutant, and screened for ex-conjugants, which lost the ability to utilize lactose as the sole carbon source. In this way, we isolated two genomic library clones, pRKVv101 and pRKVv111. When these clones were introduced into MO6LU, the resulting strains MO6LU (pRKVv101) and MO6LU (pRKVv111) not only lost the ability to utilize lactose as the sole carbon source, but also failed to express β-galactosidase activity. However, these strains gave rise to spontaneous LU mutants (Figs. 3A and 3B). The DNA fragment in plasmid pRKVv111 contains two complete ORFs on the chromosome II (Fig. 3A). One of the two ORFs, named *lacI1* (on chromosome II), has extensive homology at the deduced amino acid sequence level with the transcriptional repressors of the GalR-LacI family in various bacteria including *V. cholerae* (identity/homology, 58/75), *V. parahemolyticus* (57/78), and *E. coli* (56/73) (The DNA sequence of the region containing *lacI1* was deposited into the GenBank database under the

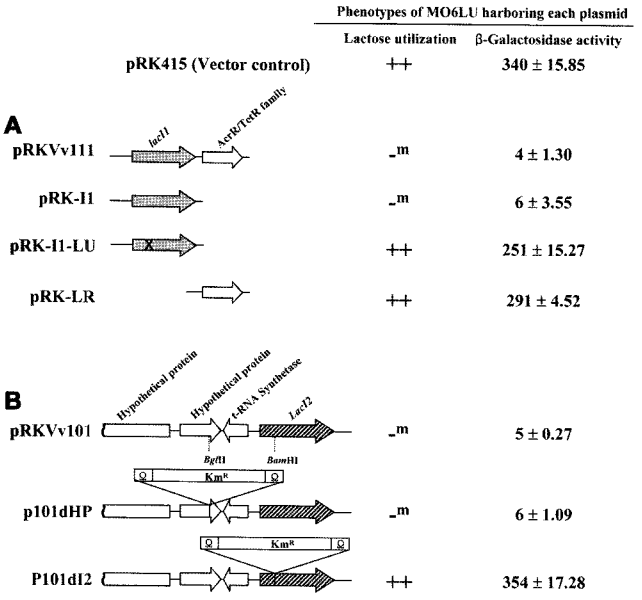

Fig. 3. Repression of lactose utilization and the *lacZ* expression by *lacI1* and *lacI2*.
Genetic maps of various subclones of regions containing *lacI1* (**A**) and *lacI2* (**B**), and their capabilities to confer the lactose utilization and β-galactosidase activities on MO6LU, which is a spontaneous LU mutant derived from *V. vulnificus* MO6-24/O.

accession number AY346384.). In the region upstream to the gene, there exists a palindromic structure containing the consensus sequences for a putative LacI repressor binding site (5'-AANC-3') [35] and the consensus element (5'-TGTGANNNNNNTCACA-3') for binding of cAMP receptor protein (CRP) [9].

The plasmid pRKVv101 carries a DNA fragment containing three ORFs on the chromosome I (Fig. 3B). Among them, one ORF, named *lacI2*, showed significant homology throughout the entire deduced amino acid sequence with the transcriptional repressors of the GalR-LacI family in various bacteria including *V. parahemolyticus* (77/90), *V. cholerae* (75/86), and *Samonella enterica* (56/72) (The DNA sequence of *lacI2* was deposited into the GenBank database under the accession number AY252118.). LacI1 and LacI2 share a significant homology (61/79) throughout the entire amino acid sequence.

We cloned the *lacI1* gene into pRK415 to construct pRK-I1. Each of pRKVv111 and pRK-I1, which contains the *lacI1* gene, was introduced into MO6LU, which is a spontaneous LU mutant derived from MO6-24/O; the resulting cells could not grow on lactose but gave rise to spontaneous LU mutants. However, when the plasmid pRK-I1-LU containing the *lacI1* gene originated from MO6LU was introduced into MO6LU, the resulting transformants grew very well on lactose and showed a high β-galactosidase activity (Fig. 3A). Similarly, when pRKVv101 or p101dHP, a derivative of pRKVv101 containing an intact *lacI2* and a mutation in a gene upstream to *lacI2*, was introduced into MO6LU, the resulting transformants could not utilize lactose as the sole carbon source, but generated spontaneous LU mutants. When, p101dI2, a derivative of pRKVv101 containing an insertion in *lacI2*, was introduced into MO6LU, the resulting cells grew on lactose and showed β-galactosidase activity as much as its parental MO6LU cell (Fig. 3B). These results indicate that *lacI1* and *lacI2* encode functional repressors responsible for a negative-control of lactose utilization.

Mapping of Mutations in LU Mutants

To verify whether the spontaneous LU mutants actually carry mutations on two *lacI* homologs, the nucleotide

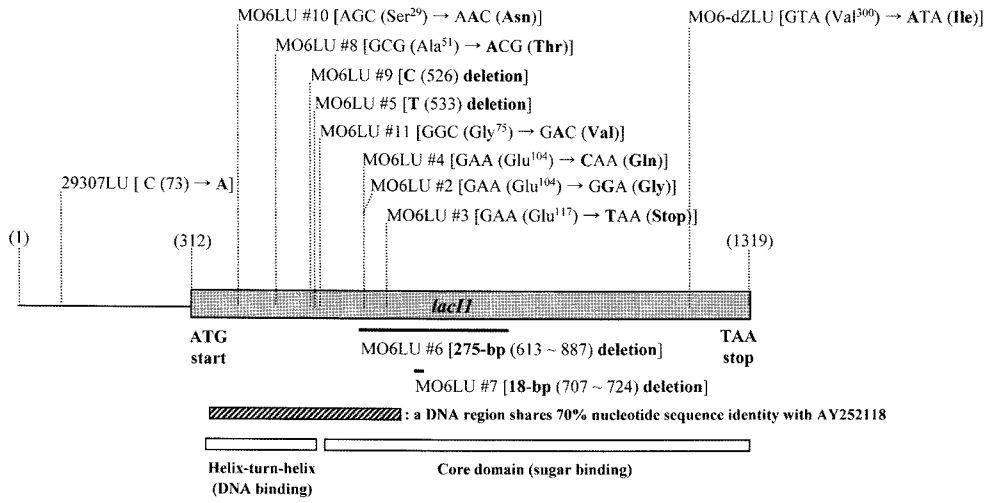

Fig. 4. Mapping of mutations in spontaneous LU mutants derived from strains MO6-24/O and 29307.
The numbers in parentheses represent coordinate numbers of the GenBank database (Accession No. AY346384).

sequences of *lacI1* and *lacI2* genes in spontaneous LU mutants were determined. We examined a total of 12 clones, and all of the spontaneous LU mutants we examined had a mutation on *lacI1* (Fig. 4). However, none of the mutations occurred on *lacI2* (data not shown). These results suggest that LacI1 plays a crucial role in repression of lactose utilization. To verify this, we constructed mutants with a deletion in each of *lacI1* and *lacI2*, and named them MO6-dI1 and MO6-dI2, respectively. These mutants were tested for abilities to utilize lactose and to express β-galactosidase activity. As summarized in Table 2, the *lacI1*-null mutant, MO6-dI1, expressed a much higher level of β-galactosidase activity than the wild-type cells, and could utilize lactose, whereas the *lacI2*-null mutant MO6-dI2 expressed a very low level of β-galactosidase activity and could not utilize lactose. These results indicate that *lacI1*, but not *lacI2*, is the actual genetic determinant responsible for the repression of utilization of lactose in *V. vulnificus*. In consistence with this result, expressed LacI1 bound to the region upstream to *lacZ*, whereas LacI2 failed to, as examined by gel-shift assay (data not shown).

Spontaneous LU Mutant Could Take up Lactose Whereas the Parental Strain Could Not

The *lacZ* gene of *V. vulnificus* on the plasmid pRKVv12 is located immediately downstream to the *E. coli lac* promoter in the vector portion of pRK415. Thus, when the plasmid pRKVv12 was introduced into a *lacZ*-null mutant MO6-dZ, the resulting strain MO6-dZ (pRKVv12) expressed a very high level of β-galactosidase (data not shown), but this strain still remains unable to utilize lactose (data not shown). From these results, we inferred that a lactose transport system is also repressed by the LacI repressor. To verify this, strains MO6-24/O, MO6LU, MO6-dZ, MO6-dZLU, MO6-dI1, MO6-dI2, MO6-dI12 (double mutant in *lacI1* and *lacI2*), MO6-dI1(pRK415), and MO6-dI1 (pRK-I1) were tested for their ability to transport lactose (Fig. 5). The spontaneous LU mutants MO6LU and MO6-dZLU, even though poorly, could transport lactose, but their parental strains MO6-24/O and MO6-dZ could not at all. Furthermore, the defined *lacI1*-null mutant MO6-dI1 could transport lactose as well as MO6LU did, but this strain lost the ability by introducing the plasmid pRK-I1, which is a *lacI1* clone (Fig. 5B). In this test, mutation in *lacI2* did not affect the phenotype (Fig. 5B). These results indicate that LacI1 is involved in repression of genes responsible for transport of lactose. However, we could not observe any phenotypic changes caused by the mutation in *lacI2* in our experimental conditions.

Catabolic Products of Lactose, Glucose and Galactose, Exert Apparent Negative Effect on Growth and Survivability of *V. vulnificus*

These results led us to ask a reason why wild-type *V. vulnificus* carries such a tight regulation of the *lacZ* expression, which keeps cells from utilizing lactose. Although *V. vulnificus* strains can utilize glucose and galactose without any toxic effects in VM9 minimal medium, we postulated that these catabolic products of lactose could exert a negative effect on growth of *V. vulnificus* in a certain complex culture condition, and therefore, this organism allows utilization of lactose under very restricted condition. To test this hypothesis, each of galactose, glucose, and lactose was added to LB medium, and their effects on growth of *V. vulnificus* strains MO6-24/O, MO6-dI1, MO6-dI2, and MO6-dI12 were tested (Fig. 6). The growth of MO6-dI2 on the solid agar media did not show a distinct difference from that of its parental strain MO6-24/O (Fig. 6A).

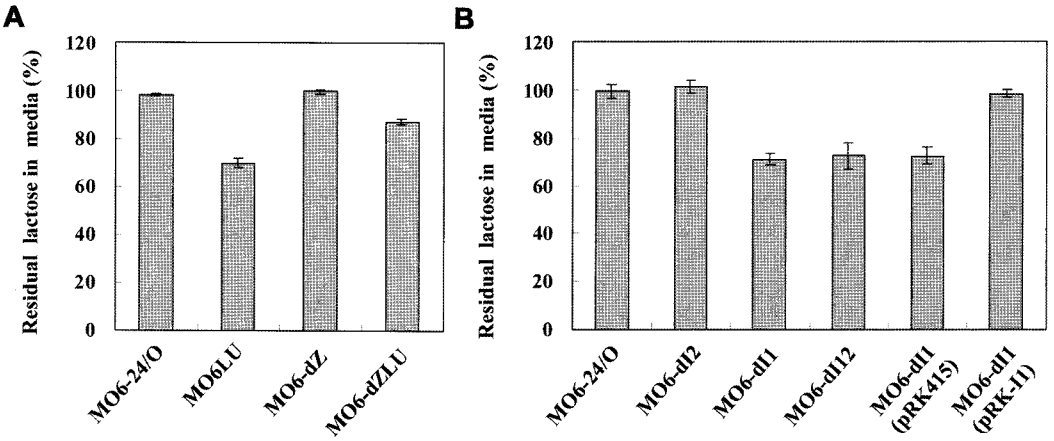

Fig. 5. Consumption of lactose is de-repressed in LU derivatives of *V. vulnificus*.
Disappearance of lactose in the culture supernatant was monitored as described in Materials and Methods. **A.** Consumption of lactose by spontaneous LU mutants and their parental strains. **B.** Consumption of lactose by the defined-deletion mutants MO6-dI1, MO6-dI2, MO6-dI12, and their derivatives. The phenol-sulfuric acid assay for quantification of lactose in the culture supernatant was performed. The means and error bars were obtained from three independent experiments.

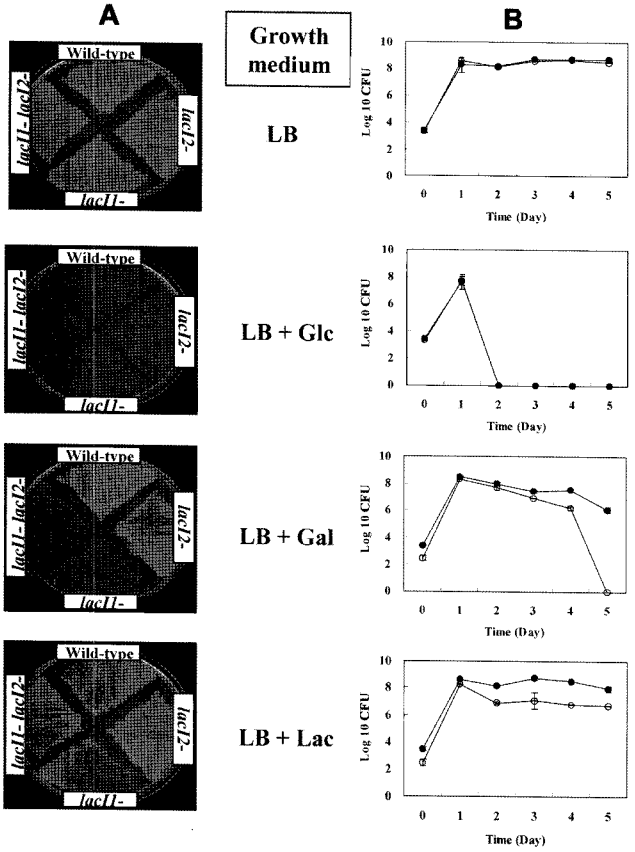

Fig. 6. Effects of lactose and its catabolic products (galactose and glucose) on viability of *V. vulnificus*.
The CFU values of wild-type MO6-24/O (-●-) and MO6-dI1, a *lacI1*-mutant derivative of MO6-24/O (-○-), in medium LB, LB containing 0.2% glucose, LB containing 0.2% galactose, and LB containing 0.4% lactose were measured. **A.** Growth of cells on the solid agar media incubated at 28°C for 24 h. **B.** Cells were inoculated into each of the broth media and incubated at 28°C and CFU (number of colonies per one ml culture) was measured daily for a five-day period.

However, a mutation in *lacI1* clearly exerted a negative effect on growth in the media containing LB supplemented with galactose. Glucose exerted a strong adverse effect on growth and survivability of all tested *V. vulnificus* strains in the culture condition. These suggest that, in spite of their nutritional values, when exposed for a long period of time, lactose itself and its catabolic products could have a negative effect on the viability of *V. vulnificus*. Based on these results, we suggest that *V. vulnificus* evolved a strong repression system for lactose utilization to protect itself from an adverse effect of those sugars on growth in a certain environmental condition.

DISCUSSIONS

In this study, we showed that MO6-24/O, a *V. vulnificus* strain known to be lactose-positive (*lac*$^+$) [8], could not utilize lactose as the sole carbon source. Another wild-type *V. vulnificus* strain, ATCC29307, is also unable to utilize lactose as the sole carbon source (data not shown). However, these strains easily produce spontaneous LU mutants. These results agreed with the results from a previous report [2]. However, early studies reported that *V. vulnificus* is distinguished from other *Vibrio* species by its ability to ferment lactose [16, 26]. We suggest that a possible reason of the discrepancy between the early study and this study is due to the large cell mass of inoculum and prolonged incubation, which may allow the accumulation of spontaneous LU mutants in early studies on *Vibrio* species [1, 16, 26]. We now know that spontaneous LU mutants are apparently accumulated in these conditions.

Our genetic and physiological studies showed that *V. vulnificus* has a functional *lacZ* responsible for catabolism of lactose, but the expression of *lacZ* and transport of lactose are strongly repressed by the transcriptional repressor LacI1. In screening of cosmid library clones of *V. vulnificus* that repress the expression of *lacZ*, we identified another *lacI* homolog, *lacI2*. This clone clearly repressed the expression of *lacZ* (Table 2). However, when we constructed a mutation in *lacI2*, the resulting mutant did not show any distinct phenotypic differences from that of wild-type cells. One possible explanation for this result is *lacI2* encodes a repressor that has a very low activity to repress *lacZ*, and when expressed at a high level by a multicopy vector, it somehow could exert a repression activity on *lacZ*. We still do not understand the biological role of this gene.

The most frequently observed evolutionary changes uncovered in which microorganisms obtain new metabolic functions fall into two broad categories: first, mutations in structural genes that lead to produce enzymes with improved activity; second, mutations in regulatory genes that lead to increased amount of enzyme synthesis [14]. In this report, we found that the latter is the case for lactose utilization in *V. vulnificus*. Considering the natural habitat, it is unlikely that this pathogen frequently encounters lactose for a nutritional source outside of the host body. However, inside of a host, it may have the chance to utilize the sugar. Therefore, a lactose-utilizing system, even if necessary, does not have to be employed frequently. Moreover, as demonstrated in this report, lactose itself and its catabolic products glucose and galactose have an adverse effect on growth and even on viability. It is possible that this pathogen evolved a strong repression system to keep the intracellular level of these sugars from being accumulated over a lethal concentration.

The mechanism by which these sugars exert a negative effect on cells is not understood. Osmotic pressure can be excluded to explain the mechanism, because only certain sugars have inhibition effects. Glucose has been implicated as an agent leading to decreased survival of *Vibrio cholerae* in growth media when heat sterilized in the presence of glucose and phosphate [12]. It has been reported that

other six-carbon (fructose and galactose) and five-carbon (arabinose, ribose, and xylose) reducing sugars also produce a toxic by-product under the same condition [5]. Although, in this study, we have separately autoclaved media and sugars, we have seen the toxic effects of sugars in LB media. It is therefore unlikely that a heat- and phosphate-catalyzed sugar by-product exerts the toxic effect. The *V. vulnificus* cells may produce a toxic metabolic by-product with unknown compounds in the LB medium. A toxic effect of sugars was not observed when tested in VM9 minimal media (data not shown). One possible explanation is this minimal medium does not contain such unknown compounds leading to production of toxic products from sugars.

It has been reported that the isolated N-terminal HTH DNA-binding domain (headpiece) of LacI retains its three-dimensional structure and its ability to bind the *lac* operator of the headpiece within the intact LacI [7]. From DNA sequence analyses of *lacI1* from the spontaneous LU mutants, we found that most mutations are located on the headpiece and the N-subdomain portion of the C-terminal core domain (Fig. 4). This suggests that the mutations disrupt the binding of the proteins to the *lac* operator.

In DNA sequence alignment between the *lacI1* and *lacI2*, we found that they share 70% nucleotide identity in a DNA region, which corresponds with the headpiece and the N-subdomain, whereas the other DNA region shares a lower sequence homology. Keeping these in mind, we suggest a spontaneous mutation on the *lacI1* gene in a *V. vulnificus* strain could have a certain disadvantage becouse of the toxic effect of sugars, and that this could be overcome by suppression mutations: a recombination between *lacI* and *lacI2* genes that leads to recovery of the regulatory function, or a mutation that leads to an increased level of expression of LacI2 product that is enough to replace the repressor function of LacI. It thus seems likely that the dual *lac* repressors (*lacI* and *lacI2*) system in *V. vulnificus* allows this organism to easily adapt and survive in certain nutritional environments. In a future study, the finding of such suppressor mutants would verify this hypothesis.

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