地には、大腸菌群用のVRBA培地の乳糖をブドウ糖に置き換えたVRBG培地が使用されている。VRBG培地で混釈・重層し、培養後に発生した集落のうち、ブドウ糖を分解しかつオキシダーゼ陰性の菌を腸内細菌科菌群とする。MPN法では、まず選択剤を含まないBPWで前培養し、EE培地による選択増菌培養後にVRBG培地へ画線培養する。発生した集落のうち、ブドウ糖を分解しオキシダーゼ陰性の菌を腸内細菌科菌群と同定する（図6）。

EUの新しい微生物基準

2006年1月1日から施行されたCommission Regulation (EC) No 2073/2005 on Microbiological Criteria for foodstuffsでは、大腸菌群と糞便系大腸菌群による規制が微生物基準から消えた。この基準は市販食品の安全基準（Food safety criteria）と、HACCPに対応可能な製造工程での衛生管理基準（Process hygiene criteria）から成り立っている（表5）。

安全基準とは、食品のロット・バッチの微生物基準への適合性を定義づけるもので、最終製品・市場流通食品に適用され、不適合食品は回収しなければならない。衛生管理基準は製造工程が正常に機能していることを示す基準であって、市場流通食品には適用されない。

衛生指標菌には腸内細菌科菌群あるいは大腸菌が使用されている。これに不適合な場合は製造工程の衛生管理の改善や、原材料を見直す等の措置が講じられることになる。ほとんどの市販食品の安全基準は、サルモネラやリステリアのような病原菌、ブドウ球菌エンテロトキシン、ヒスタミンで規制されており、衛生指標菌が使用されているのは生食用貝類に対する大腸菌の基準のみである。合計28の食品カテゴリーの衛生管理基準うち、7カテゴリーで腸内細菌科菌群、8カテゴリーで大腸菌が採用されている。ちなみに生菌数（SPC；standard plate counts）が適用されている食品は4カテゴリーのみに留まる。米国でも糞便系大腸菌群を標的とした試験は、糞便汚染の指標性が低いとの考えから近年減少傾向にあるようである。

上述のEUの食品微生物基準は、施行されて約2年後の2007年12月5日には早くも一部改正された。Dried follow-on formula（乳児用乾燥栄養補助食品；乳児用調製粉乳）の食品安全基準にサル

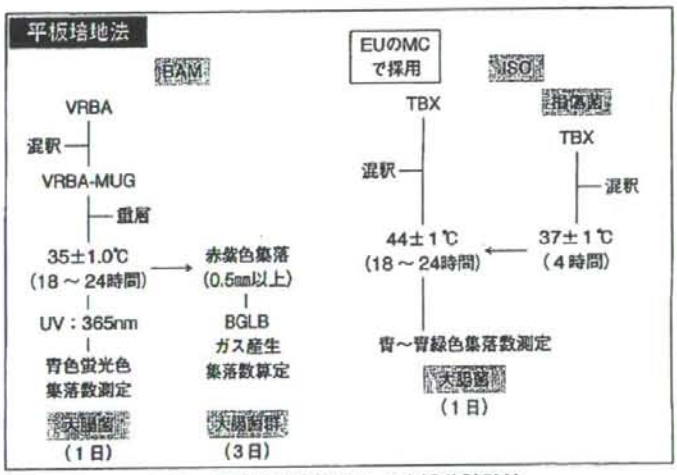

図5　酵素基質培地による大腸菌試験法

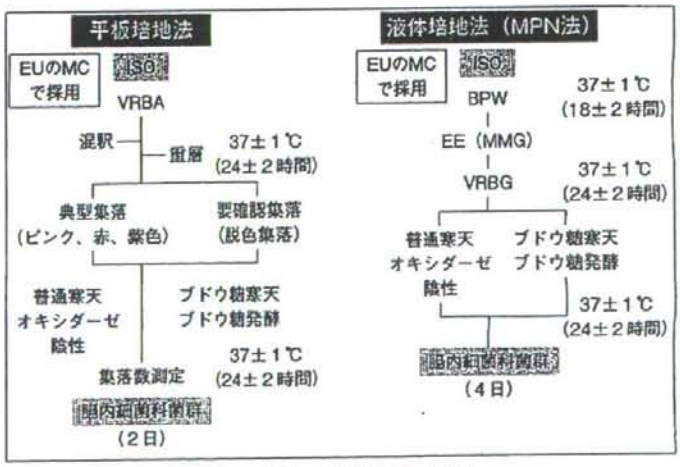

図6　腸内細菌科菌群試験法

モネラが、衛生管理基準に腸内細菌科菌群が新たに追加されたこと、乳児用および食事療法用乾燥食品に推定セレウス菌（Presumptive *B. cereus*）が衛生管理基準に採用されたことが大きな改正点である。推定セレウス菌とは、MYP（Mannitol Egg York Polymyxin Agar）培地上でマンニトール非分解かつ卵黄反応陽性の集落を、他の類似菌と鑑別試験なしに同定するものである。

この改正に際して、欧州食品安全機関（European Food Safety Authority：EFSA）は、腸内細菌科菌群をサルモネラと*Enterobacter sakazakii*の指標菌として利用できるかについて検討した。結果的には、サルモネラが分離されることは稀なため、腸内細菌科菌群とサルモネラを関連できるデータがないという理由から、乳児用および食事療法用乾燥食品に対する衛生管理基準に腸内細菌科菌群を指標とすることが見送られた。

ICMSFのサンプリングプラン

サンプリングプランとは、ハザードとリスクの要因に基づくプランであり、①食品を汚染する微生物や毒素により起こる病気の重篤度や拡散性、②食品を媒介とする感染や中毒が、ある限定された消費者（乳幼児、基礎疾患のある患者等）に感受性があるか、③微生物や毒素が製造工程、流通、調理の過程で生残、増加、破壊されるか、の3要素で規定されており、ロットの合否を判定する手段として開発された。詳細については、Microorganisms in foods 7, Microbiological testing in food safety management, ICMSF（International Commission on Microbiological Specifications for Foods；国際食品微生物規格委員会）を参照されたい。なお、日本語訳が中央法規出版から販売される予定である。

EUでは日本のような単品検査ではなく、ICMSFのサンプリングプランに基づいた試験用の検体数が規定されている。サンプリングプランは15のケースとそれに不随するプランが二次元グリッドに配置されており、ロットの合否を判定するための検査に供するサンプル数と菌数限界値を組み合わせたものである（表6）。

1ロットからランダムに採取するべき検体数n、合否判定の基準となる菌数m、n個中mを超えても許容される検体数cの組み合わせで示されているのが二階級法である。二階級法では、mは通常0である。三階級法では、mよりも多い最大菌数Mを超える検体が1

表4　日本の衛生指標菌試験法と国際標準法との比較

方法	培養温度		推定試験用培地	
	一般食品	乳および乳製品	液体	寒天
BAM	35±1.0℃	32±1.0℃	*LST	VRBA
ISO	37±1.0℃	30±1.0℃	LST	VRBA
日本	35±1.0℃	32～35℃	EC、LB BGLB	Deso.

LST-MUGやVRBA-MUGも使用される。
腸内細菌科菌群用にMMG（液体培地）がある。
*貝類ではLBまたはLSTになっている。

表5　EUにおける規制対象食品と微生物（2006年1月1日）

食品安全基準

Ready-to-eat 製品等（26種類）
L. monocytogenes, Salmonella, E. sakazakii, E. coli, Staphylococcal enterotoxins, Histamine

生の二枚貝等での MPN法のみ

衛生管理基準

肉および肉加工品（8種類）
SPC, *Enterobacteriaceae, E. coli , Salmonella*

牛乳および乳製品（9種類）
Enterobacteriaceae, E. coli, Coagulase-positive *staphylococci*

卵加工品（1種類）
Enterobacteriaceae

水産加工品（1種類）
E. coli, Coagulase-positive *staphylococci*

野菜、果物とその加工品（2種類）
E. coli

SPC：4
Enterobacteriaceae：7
E. coli：8

個でもあれば不合格とするが、菌数がm～M個の範囲内の検体数がc個以内なら条件付きの合格とされている（図7、表7－1、表7－2）。

例えば、カット野菜のEUの衛生管理基準（Process hygiene criteria）には大腸菌に対する微生物基準がある（n＝5、c＝2、m=10^2／g、M=10^3／g）。これは1ロット5検体をランダムに採取し、10^3/gを超える大腸菌を検出した検体が1個でもあれば不合格であり、10^2／gを超える不良品がない場合は合格、10^2／g～10^3／gの範囲内の検体が2個以内なら条件付きの合格ということになる。大腸菌試験法に使用する参照方法（Reference method）はISO16649－1または－2と規定されているが、ISO法との同等性がAOACインターナショナルやAFNOR（フランス規格協会）などの認証機関でバリデートされた方法も使用可能であるとされている。

わが国でICMSFのサンプリングプランが採用されている食品として「食肉製品の流通および販売の微生物指導基準」、「加熱殺菌液卵と無殺菌液卵の微生物基準」がある。これらの食品の微生物基準には“E. coli”（糞便系大腸菌群）、黄色ブドウ球菌、クロストリジアで二階級法が、生菌数で三階級法が採用されている。

菌数計測法の問題点

生菌数とは生きている菌を表現する用語であり、食品衛生小六法の成分規格の項では細菌数、試験法の項では細菌数（生菌数）が使用されている。生菌数（好気性あるいは通性嫌気性の中温菌）の測定方法は、一般的には混釈による標準平板菌数測定法（standard plate count；SPC）が広く使用されているが、これ以外にも表面塗抹平板法、乾式培地法、メンブレンフィルター法やスパイラルプレート法などがある。生菌数は一般生菌数などとも呼ばれることもあり、諸外国でもaerobic plate counts（APC）、standard plate counts（SPC）、aerobic colony counts（ACC）、total viable counts（TVC）、total colony counts（TCC）など、さまざまな呼び方がされている。

生菌数とは正確には食品中に存在する菌数ではなく、限定された一定の条件下（培地、培養温度、培養時間、酸素濃度等）で寒天平板上（中）に目に見える集落の形成が可能な菌といえる。食品に含まれる発育抑制成分、発育促進成分、食品の希釈液が生菌数の測定に影響を与える。菌の発育抑制成分として、卵白のリゾチーム、ガーリックのアリシン、タイムのチモール、マスタード・ワサビのアリルイソチアネート、バニラのバリニン、

表6　ICMSFが推奨するサンプリングプラン

ハザードの程度	検査後のリスクの変化		
	減少	無変化	増大
直接被害なし	ケース1 n=5、c=3	ケース2 n=5、c=2	ケース3 n=5、c=1
被害：低	ケース4 n=5、c=3	ケース5 n=5、c=2	ケース6 n=5、c=1
被害：中	ケース7 n=5、c=2	ケース8 n=5、c=1	ケース9 n=10、c=1
被害：高	ケース10 n=5、c=0	ケース11 n=10、c=0	ケース12 n=20、c=0
被害：甚大	ケース13 n=15、c=0	ケース14 n=30、c=0	ケース15 n=60、c=0

□ 三階級法　■ 二階級法

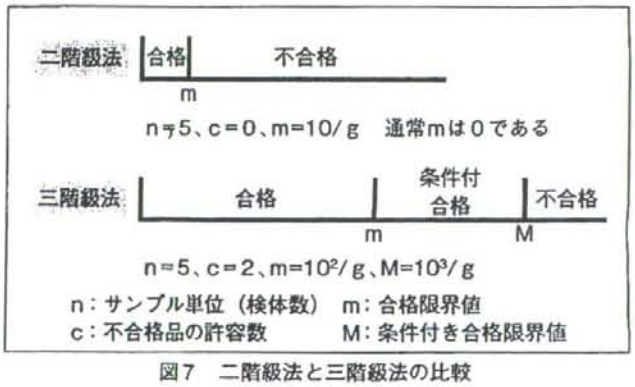

図7　二階級法と三階級法の比較

緑茶やココアに含まれるポリフェノールなどが知られている。一方、乳成分が大腸菌群（正確には乳糖非分解性のエンテロバクター）の発育を促進（支持）したことも経験した（表8）。

わが国では、生菌数の測定・計算方法は食品衛生小六法に記載されているが、定量的な成分規格がない大腸菌群の測定方法は示されていない。FDA／BAM法では、集落数の計測可能範囲を、生菌数測定法と大腸菌群ともに25～250／平板としている。生菌数を計測する場合の1平板当たりの集落数は、APHA法では25～250／平板、ISO法では15～300／平板と異なるが、大腸菌群ではいずれも15～150／平板となっている。ISO法やFDA／BAM法には同一の菌数算定用の計算式が示されており（食品衛生検査指針にも紹介されている）、この方法で計算した菌数は、食品衛生小六法と同じか少なくなる。したがって、使用する生菌数の計算方法の違いによって、成分規格への適否に影響することもある（表9～11）。

表7－1　EUにおける食品安全基準の例

第1章　食品安全基準							
食品区分	微生物/毒素代謝物	サンプリングプラン		菌数限界値		参照試験法	適用場所
		n	c	m	M		
粉ミルク ホエイパウダー	サルモネラ	5	0	陰性/25g		EN/ISO 6579	保存期限内の市販品
違反した場合							
回収またはリコールし、小売り前の食品はハザードを除く処理をする。この処理は小売りレベルではなくて、製造業者のみが実施する。 製造業者は、公衆衛生や動物衛生に危害を及ぼさないで、しかも規制当局によって認可されたHACCPとGHPの原理に基づき最初の製品以外のものに再加工するために使用する。							

表7－2　EUにおける衛生管理基準の例

第2章　衛生安全基準							
食品区分	微生物/毒素代謝物	サンプリングプラン		菌数限界値		参照試験法	適用場所
		n	c	m	M		
粉ミルク	腸内細菌科菌群	5	0	10cfu/g		ISO 21528-1	最終製品
ホエイパウダー	コアグラーゼ陽性ブドウ球菌	5	2	10cfu/g	100cfu/g	EN/ISO 6888-1 または-2	最終製品

違反結果の是正措置

腸内細菌科菌群	コアグラーゼ陽性ブドウ球菌
熱処理能力や二次汚染をチェックする	衛生管理の改善 菌数が10^5cfu/gを超えた場合は、エンテロトキシン試験を実施する

表8　乳成分が大腸菌群の発育を促進した（混釈）

希釈液	加工乳		リン酸緩衝液	
培地	×10	×100	×10	×100
VRBA	112	18	10	0
VRBG	143	18	10	0
酵素基質	210	16	62	0

リン酸緩衝液：
VRBAとVRBGでは菌数が1/10以下に減少

食品の微生物基準と試験法のあるべき姿

微生物基準と試験法のあるべき姿として、①食品に関連した新興感染症の出現や細菌試験法の進歩、あるいは食品製造技術の進歩に連動しながら、微生物基準と試験法を定期的に見直すこと、②基準となる病原菌、衛生指標菌などと食品との明確な関連性があり、消費者保護に必要であることを前提とし、しかも実現可能な規格基準を設定すること、③試験法は検査費用やマンパワーも考慮して作成することが求められる。

具体的には、妥当性が確認さ

れ、しかも国際調和が計られた標準的な試験法（Reference method）を策定することが重要である。国際的に通用する標準法が作成されると、これを基準とした代替法・迅速法の開発が促進され、同時に試験現場への導入も容易になることが期待される。標準法策定を目標とした「食品からの微生物検査標準法検討委員会」が平成17年7月に国立医薬品食品衛生研究所内で発足し、その情報は同研究所のホームページ（http://www.nihs.go.jp/index-j.html）に公開されている。

最終的には、生産者、製造業者、卸売業者、監督官庁が、決められた規格基準や試験法に対して共通の認識と理解を持つことが重要な要因となる。

食品衛生法（成分規格）がハザードでは？

わが国の食品細菌試験法の多くは食品衛生法の成分規格に組み込まれているため、法改正の手続きを取らない限り改正できない。このため、酵素基質培地を始めとして、培養法と組み合わせた簡便・迅速な細菌数測定機器が食品試験用に開発されてきたが、日本の食品衛生法上承認された方法は残念ながら未だに皆無である。日々新しい細菌試験技術が開発されている今日、試験検査に携わる人たちの多くが、わが国の成分規格（規格基準・試験法）の守旧性や無用な煩雑さに疑問を持っていることは容易に推察できる。

その最たる例が、乳製品の製造技術が飛躍的に向上したにもかかわらず、昭和26年に施行されて60年近くも基本部分は当時のままの姿を留めている、いわゆる「乳等省令」である。高度に製造管理された牛乳製品を販売する場合でも、迅速性に乏しい試験法で、しかも消費者保護のための必要性が不明

表9　菌数計算法

$$N=\frac{\Sigma C}{(n_1+0.1n_2)d} \qquad N=\left\{\frac{A+B}{2d_1}+\frac{C+D}{2d_2}\right\}/2$$

ΣC：各平板の集落数の合計
n_1：低希釈の算定対象ペトリ皿数
n_2：高希釈の算定対象ペトリ皿数
d：希釈が低いほうの希釈倍率

A、B：低希釈の集落数
C、D：高希釈の集落数
d_1：希釈が低いほうの希釈倍率
d_2：希釈が高いほうの希釈倍率

ISO、BAM法　　小六法法

⇩

$$CFU/ml=\frac{\text{各平板の集落数の合計}}{\text{各ペトリ皿に分注した試料の実量（ml）}}$$

表10　計算方法の違いによる生菌数の差異

試料番号	集落数 1:10	集落数 1:100	希釈率を考慮した集落数の比	小六法（A）	ISO、BAM（B）	A/B
1	300	30	1：1	3.0×10^3	3.0×10^3	1
2	235	31	1：1.3	2.7×10^3	2.4×10^3	1.13
3	290	52	1：1.8	4.1×10^3	3.4×10^3	1.21
4	300	60	1：2	4.5×10^3	3.3×10^3	1.36
5	150	30	1：2	2.3×10^3	1.6×10^3	1.43

有効な集落測定範囲を30～300/平板とする

小六法とISO、BAM

表11　生菌数の計算方法の違いによる成分規格への適否の影響

粉末清涼飲料、乳飲料、特別牛乳の成分規格
3万以下/ml（g）

集落数　100倍希釈（295、285/平板）
1,000倍希釈（32、34/平板）

小六法法（告示法）　　ISO、BAM法

$$\frac{(295+285)}{2\times10^{-2}}+\frac{(32+34)}{2\times10^{-3}}\Big/2 \qquad \frac{295+285+32+34}{[2+(0.1\times2)]\times10^{-2}}$$

$=3.1\times10^4$ 不適　　$=2.9\times10^4$ 適

冷凍食品（300万/g）でも同様のことが起こり得る

確な検査を実施しなければならない。制定当時はいくら優れた法律であっても、これだけ長期間経過すれば、骨董品的な価値は認められても、有用性に疑問符が付くのは当然の成り行きである。

「不祥事はなぜ繰り返されるのか―日本人のためのリスク・マネジメント―」（武井勲著、扶桑社新書、2007年11月刊行）の中の言葉を借りれば、食中毒菌とともに食品衛生法そのものがハザードではないかとすら思える。食品衛生法ハザードのリスクとして、元来は安全性や品質に問題がないかもしれない食品の廃棄とそれに伴う資源やエネルギーの無駄遣いが挙げられる。これに付随する食品価格の上昇は消費者リスクとなり、生産者リスクとしては会社の倒産という危機にもつながりかねない。経済的な損失や正当な経済活動に対するリスクを軽減するためにも、科学的根拠に基づいた合理的な規格基準とその試験法の策定が切望される（図8）。

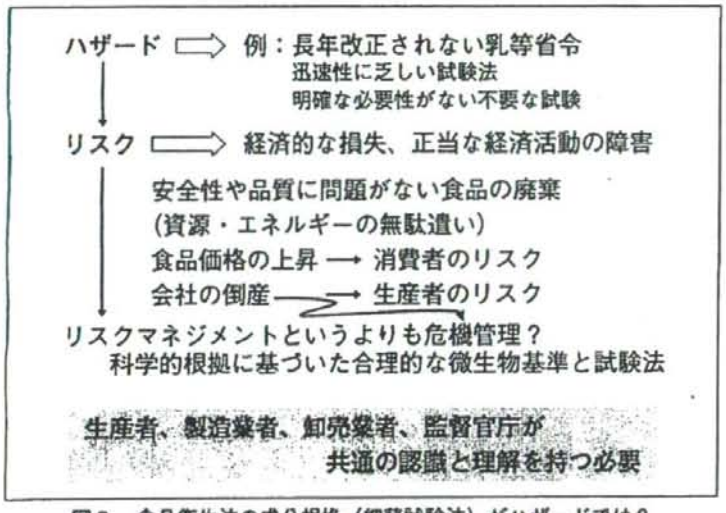

図8　食品衛生法の成分規格（細菌試験法）がハザードでは？

㈱エルメックス主催の第14回「食品衛生検査セミナー」は2月20日に神戸会場（神戸国際会議場）、3月17日に東京会場（タワーホール船堀）の2会場で開催された（写真は神戸会場の様子）。

セミナーでは本稿にて講演要旨を掲載した大阪府立公衆衛生研究所の浅尾努氏をはじめ、㈱山武の渡辺勉氏が「トレーサビリティ、HACCP、GMP、GAPだけでは食のリスクは回避できない～人を中心とした食のリスク管理の提言～」、伊藤ハム㈱の大澤頼人氏が「食品衛生技術と企業価値～品質管理を高めるための企業内のさまざまなソフトの折り合いの重要性と10年間携わってきた中国食品企業との連携生き残り競争について紹介～」、大阪大学大学院の那須正夫氏が「微生物を迅速・高精度に検出する～食の安全と安心を保証する新技術～」と題して、それぞれ講演した。

また、会場付設のエリアでは食品衛生関連資材の展示会も併催され、参加者の関心を集めた。

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ORIGINAL ARTICLE

Lysine decarboxylase of *Vibrio parahaemolyticus*: kinetics of transcription and role in acid resistance

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Keywords
acid tolerance, food, genes, lysine decarboxylase, regulation, stress response.

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Abstract

Aim: The aim of this study was to investigate the detailed mechanisms of acid resistance in *Vibrio parahaemolyticus*.

Methods and Results: All 11 strains of *V. parahaemolyticus* survived lethal acidic conditions following acid adaptation, and accumulation of cadaverine was detected. The addition of lysine improved survival, suggesting that lysine decarboxylase plays a role in the adaptive acid tolerance response. Two open reading frames (ORF) in *V. parahaemolyticus*, which are separated by a non-coding region, were found to be highly homologous to bacterial lysine decarboxylase (*cadA*) and lysine/cadaverine antiporter (*cadB*) genes. Transcriptional analyses of this operon revealed acid induction and enhanced induction by external lysine. The relative expression ratio of each transcript was found to follow the trend of *cadA* mRNA > *cadB* mRNA > *cadBA* bi-cistronic mRNA. A mutated strain, with a disrupted *cadA* gene, showed attenuated acid survival.

Conclusions: We identified the lysine decarboxylase gene operon of *V. parahaemolyticus*. Expression of this operon was induced under acidic conditions. The *cadA*-mutated strain constructed in this study showed weaker tolerance to acidic conditions than the wild-type strain.

Significance and Impact of the Study: *Vibrio parahaemolyticus* utilizes the lysine decarboxylation pathway for survival in acidic conditions.

Introduction

Vibrio parahaemolyticus, a ubiquitous marine pathogen found in seafood, causes human diarrhoea, especially through the consumption of raw fish and shellfish. *Vibrio parahaemolyticus* is a gram-negative halophilic bacterium, which is distributed worldwide in estuarine environments (Joseph *et al.* 1982). The virulent factors of this pathogen, mainly thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH), show haemolytic, cytotoxic, enterotoxic, and cardiotoxic activities against mammalian hosts (Honda and Iida 1993; Raimondi *et al.* 2000; Shimada and Arakawa 2000; Naim *et al.* 2001). While it is well established that fresh seafood generally contains *V. parahaemolyticus*, only a small fraction of bacteria carries these virulent genes (Wagatsuma 1974; DePaola *et al.* 1990; Kaysner *et al.* 1990). Over 400 cases of food poisonings caused by *V. parahaemolyticus* have been reported in Japan in 2000, illustrating the extent of this public health problem. Furthermore, the pandemic spread of *V. parahaemolyticus* serotype O3:K6, which has emerged since 1996, is a new topic in the control of this pathogen (Matsumoto *et al.* 2000). Bacteriophage f237, which is unique to the newly isolated O3:K6 clones, has been reported (Nasu *et al.* 2000), and some virulence-associated characteristics seem to be enhanced in O3:K6 clones. This newly emerging clone has also been implicated in a number of outbreaks in Japan and other countries.

Vibrio parahaemolyticus and other Vibrionaceae are generally thought to be more sensitive to low pH than other bacteria (Nishikawa *et al.* 1993; Waterman and Small 1998). Therefore, the infective dose (ID) of these pathogens is not low (Sanyal and Sen 1974; Bennish

1994). Many food- or water-borne diseases caused by *V. parahaemolyticus* (Sanyal and Sen 1974; Centers for Disease Control and Prevention 1998, 1999; Daniels *et al.* 2000) and *Vibrio cholerae* (Bennish 1994; Faruque *et al.* 1998) are important health problems worldwide. As these are gastrointestinal pathogens, it is implied that the pathogens successfully pass through the acidic stomach and colonize in the intestine. Waterman and Small (1998) reported that the surface-associated *V. cholerae* cells were protected from acidic environments. According to the report, *V. cholerae* cells cultured alone decreased over 5-log units in pH 5·0 Luria-Bertani (LB) medium, but in contrast, decreased only 3-log units even at pH 2·5 when inoculated with ground beef. As not all cases are transmitted by food, it is speculated that there is another factor participating in the acid resistance of Vibrionaceae. Merrell and Camilli (1999) reported that *V. cholerae* cells exposed to mildly acidic conditions (pH 5·7) survived lethal acid challenge (pH 4·5). Rhee *et al.* (2002) showed similar results in *Vibrio vulnificus*: their survival under acidic conditions was dependent on the lysine decarboxylase pathway. Although induced transcription of lysine decarboxylase genes under acidic pH is well described, the nature of transcription, particularly regarding the expression level of the genes, is not fully understood.

Wong *et al.* (1998) showed a similar result following mild acid exposure of *V. parahaemolyticus*. However, the detailed mechanism of acid resistance of *V. parahaemolticus* remained unknown. The phenomenon of mild acid exposure enhancing the ability of the pathogens to survive under lethal acidic conditions is now referred to as acid tolerance response (ATR). ATR was first recognized in *Salmonella typhimurium* by Foster and Hall (1990), and is now recognized by several researchers as the mechanism by which bacteria break through the gastric acid barrier (Foster 1999; Audia *et al.* 2001). ATR and its correlation with infectivity have also been reported elsewhere (Wilmes-Riesenberg *et al.* 1996; Merrell and Camilli 1999). *Vibrio parahaemolyticus*, an acid-sensitive gastric pathogen, may also utilize ATR in its mode of infection. In Japan, mildly acidic sushi (vinegar is usually added to the rice, pH 4·3–4·9) is frequently implicated in outbreaks of *V. parahaemolyticus* infection (Shimada and Arakawa 2000). This problem is suggestive of the importance of ATR in *V. parahaemolyticus* infection.

In this study, we focused on the ATR of *V. parahaemolyticus* using *in vitro* acid resistance tests. Molecular analysis for the ATR-associated gene, lysine decarboxylase, was also conducted, and the transcription patterns were precisely demonstrated by SYBR Green I real-time reverse transcriptional (RT) polymerase chain reaction (PCR) and Northern blot hybridization. Finally, a lysine decarboxylase gene-inactivated strain was constructed to clarify the role of this enzyme in ATR.

Materials and methods

Bacterial strains and growth conditions

A total of 11 *V. parahaemolyticus* strains, VpTK-(1, 3, 6, 8, 9, 10, 11, 12, 13, 17) and strain V02-64, representing the newly emerged clone of serotype O3:K6, were used. All *V. parahaemolyticus* strains (*tdh*+, Kanagawa phenomenon positive) isolated from food-poisoning patients were stored at −80°C using MicroBank microbial storage kits (Pro-Lab Diagnostics, Ontario, Canada) until use. All strains were serotyped by the slide agglutination test with O- and K-antigens using commercially available antisera (*V. parahaemolyticus* antisera Seiken set, Denka Seiken, Tokyo, Japan). Unless otherwise noted, all *V. parahaemolyticus* strains were cultured in LB broth or on LB agar (1·5% agar) with 3% sodium chloride. *Escherichia coli* strain JM109 used for genetic manipulation was grown in LB broth or on LB agar supplemented with 5 μg ml^{-1} ampicillin unless otherwise specified.

Adaptive acid tolerance assays

Overnight cultures (300 μl) of each *V. parahaemolyticus* strain were inoculated into 30 ml fresh LB broth and incubated at 30°C with shaking until the OD_{600} reached 0·16–0·20 (log-phase culture). An aliquot of this culture (1 ml) was harvested by centrifugation at 15 000 *g* for 3 min at room temperature. Cells were resuspended in 1 ml of LB-MES (100 mmol l^{-1} 4-morpholinoethanesulfonic acid (MES), pH 5·5) or LB-HEPES (100 mmol l^{-1} [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7·5) and incubated at 37°C for 1 h. Cultures were centrifuged at 15 000 *g* for 3 min at room temperature, and the pellets were resuspended in 1 ml of LB broth (pH 4·0) and incubated at 37°C for 1 h. Immediately after resuspension in LB broth (pH 4·0), portions of samples were serially diluted in phosphate-buffered saline (PBS) (pH 7·0) and plated onto trypticase soy agar (TSA; Difco Laboratories, Detroit, MI, USA) with a spiral plater (IUL Instruments, Barcelona, Spain) (initial count). After 1 h of incubation in LB broth (pH 4·0), viable cells were enumerated in the same fashion (survival count), and the per cent surviving the acid challenge was calculated by dividing the viable counts at 1 h by the initial viable counts and multiplying by 100. The amount of some polyamines (tryptamine, putrescine, cadaverine, histamine, agmatine, tyramine, and spermidine) in the LB broth was measured by HPLC as previously described (Yamanaka and Matsumoto 1989).

Lysine decarboxylase-dependent acid-tolerance assays

Overnight cultures (500 μl) of each *V. parahaemolyticus* strain were inoculated into 50 ml fresh LB broth (1 : 100) and incubated at 30°C with shaking (170 rev min^{-1}) until OD_{600} reached 0·16–0·20 (log-phase culture). Cells were precipitated and resuspended in LB-MES, pH 5·5 (acid adaptation) or LB-HEPES, pH 7·5 (no adaptation). Chloramphenicol was added at 10 μg ml^{-1} to each treatment in order to assay the ATR in the absence of *de novo* protein synthesis during acid adaptation. After incubation for 1 h, cells were washed twice with no citrate E medium (NCE; Maloy and Roth 1983) at pH 5·5 or 7·5 (pH adjusted with HCl) and resuspended in equal volumes of NCE, pH 4·0 (acid challenge) with or without 1% L-lysine hydrochloride. Viable counts were performed immediately after resuspension in NCE and at subsequent 30-min intervals.

DNA manipulation and sequencing

Chromosomal DNA of *V. parahaemolyticus* was isolated according to the standard protocol (Sambrook *et al.* 1989) and plasmid DNA was purified using Quantum prep plasmid miniprep kits (Bio-Rad Laboratories, Richmond, CA, USA). A degenerate primer pair, 5′-GTN CTW TAY TAY CAC GCN AAC TGG A-3′ and 5′-GCY TSN CRN ACC ARC ATC ATC CA-3′, for lysine/cadaverine antiporter (*cadB*) was designed from other known CadB amino acid sequences: *E. coli* (sequence accession number: NC000913), *Salmonella typhimurium* (AE008816), *V. cholerae* (NC002505), and *V. vulnificus* (AF324470). After amplifying the homologous *V. parahaemolyticus cadB* fragment using this primer pair at an annealing temperature of 58°C, the PCR products were cloned into pT7-Blue T-vector (Novagen, Madison, WI, USA) and the sequences of several clones were determined.

Unidentified regions around *cadB* were amplified and cloned using partial sequence information and LA (long and accurate) PCR *in vitro* cloning kits (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. Briefly, chromosomal DNA digested with the appropriate restriction enzymes and adapters (double-stranded oligonucleotides) were ligated. Then, the fragments containing unknown regions were amplified using an adapter primer and a primer specific to the partial *cadB* sequence. The resulting products were cloned into pT7Blue T-vectors (all restriction enzymes and DNA ligase were provided by Takara Bio). Several clones were sequenced using BigDye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) and analysed on an ABI310 genetic analyser (Applied Biosystems). Sequences were assembled and analysed by GENETYX-MAC software (Software Development Co., Ltd, Tokyo, Japan).

RNA extraction and Northern blot hybridization

Log-phase cultures (45 ml) were pelleted and resuspended in 45 ml of LB-MES (pH 5·5) or LB-HEPES (pH 7·5). After 2 h of incubation at 30°C, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by DNase I (RNase free, Wako Pure Chemical Industries, Japan) treatment for 30 min. Blotting of total RNA was performed as follows: 15 μg of total RNA was loaded onto 1% Seakem GTG (FMC BioProducts, Rockland, ME, USA) agarose gels, electrophoresed in MOPS buffer (0·4 mol l^{-1} 3-morpholinopropanesulfonic acid (MOPS), 0·1 mol l^{-1} sodium acetate, 15 mmol l^{-1} EDTA, pH 7·0) with size markers for RNA (Perfect RNA markers, 0·2–10 kb; Novagen), and blotted to Hybond-N+ membranes (Amersham Biosciences Corp., Piscataway, NJ, USA) with an electroblotter, NB-1513 (Nihon Eidoh, Tokyo, Japan). Hybridization was done with ECL (enhanced chemical luminescence) direct nucleic acid labelling kit (Amersham Biosciences) using DNA probes specific for *cadA* or *cadB*, prepared by PCR using primers 5′-TTA TCA CGC CAA CTG GAT TGG-3′ and 5′-GCG TGT AGC TTC ATG TAC TGA GC-3′ for *cadB*, and 5′-CCT CCA TTC AAC TAA AGC GCT A-3′ and 5′-CAA TGC CGT ACT GAG GTG AAG-3′ for *cadA*.

RT-PCR for mRNA quantification

In SYBR Green I real-time quantitative PCR, amplification of cDNA is measured by the increase in fluorescence resulting from the successive intercalation of SYBR Green I dye into the double-stranded DNA. The threshold cycle (*Ct*), the first PCR cycle in which the incremental increase in fluorescence can be detected, is used to calculate the initial amount of template DNA based on a standard curve plot of cDNA derived from the *Ct* values calculated from known concentrations of the standard DNA. Moreover, the specificity of all PCR reactions were verified based on the melting temperature (T_m) measured by the 'disassociation curve' analysis on an ABI 7900HT (Applied Biosystems) immediately after amplification. T_m is unique to the amplification products and is determined by the decrease in fluorescence from SYBR Green I dye during slow heating (from 60 to 95°C). Data collection and multicomponent analyses were performed with the Sequence Detection Software 2·0 supplied with ABI prism 7900HT (Applied Biosystems). An endogenous control of sample RNA, 16S rRNA, was also quantified and used for data analyses.

Primer selection

A total of four amplifications were designed to study the transcription of *cadBA* genes of *V. parahaemolyticus*. The *cadB* mRNA (519 bp) was amplified by primers 5′-ATT CGG TAG CTG GAC TGC AC-3′ and 5′-GCG TGT AGC TTC ATG TAC TGA GC-3′ and *cadA* mRNA (443 bp) was amplified by primers 5′-GTA TTC TTC CCT GTG CTT AAT GAC-3′ and 5′-GCA GTC ACA ATC GCA TGG CTA TCA-3′. The *cadBA* bi-cistronic mRNA (403 bp) was amplified by primers 5′-GCT TCG TAA TGC TGT TCT CTG G-3′ and 5′-CGT AGC CCG CTT TCT CAA GA-3′, which were designed to target the *C*-termini of *cadB* and the *N*-termini of *cadA*. The precise primer locations are shown in Fig. 2. Universal primers 510f and 920r were used to amplify 16S rRNA for endogenous reference. Reverse transcription was performed with randomly synthesized hexanucleotide [random hexamer, $d(N)_6$; Applied Biosystems].

RT of RNA

The RT reactions were carried out with the following recipe: 5·0 mmol l^{-1} of $MgCl_2$, 2·0 mmol l^{-1} of each dNTP, 2·5 μmol l^{-1} of $d(N)_6$, 0·4 U μl^{-1} of RNase Inhibitor, 1·25 U μl^{-1} of MultiScribe RTase, 200 ng of total RNA, and reacted in the supplied 10 × RT buffer at 1 × concentration. All reagents were purchased from Applied Biosystems. RNA samples extracted from each type of cells were purified by treatment with DNase I for the removal of residual DNA contamination. RT reactions were performed in a GeneAmp 9700 (Applied Biosystems) with a thermal profile of 25°C for 10 min, 37°C for 60 min followed by 95°C for 5 min to inactivate residual RTase, and a 4°C soak. Resultant cDNA were then applied to the SYBR Green I real-time quantitative PCR after purification by ethanol precipitation as described elsewhere.

Amplification

SYBR Green I real-time PCR was performed on an ABI prism 7900HT with Sequence Detector Software 2.0 (Applied Biosystems). Thermal cycling was performed with the following protocol: 50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 20 s, 58°C for 45 s, and 72°C for 45 s; and an extension phase for dissociation analysis of 95°C for 15 s followed by ramping from 60°C to 95°C in 30 min. During these cycles, fluorescent signals were measured at 521 nm every 7 s, and the data were analysed after all cycles and dissociation curve analyses had been finished. The 25-μl reaction mixture contained 1 × SYBR Green buffer, 3 mmol l^{-1} of $MgCl_2$, 1 mmol l^{-1} of d(ATP, CTP, GTP) and 2 mmol l^{-1} of dUTP, 0·2 μmol l^{-1} of each primer, 0·01 U μl^{-1} of uracil-*N*-glycosidase (AmpErase UNG), 0·025 U μl^{-1} of AmpliTaq Gold DNA polymerase, and 2 μl of template cDNA (or known concentration of genomic DNA) solution. All reagents except oligonucleotide primers were supplied from Applied Biosystems. All runs contained standard templates for calculating the standard curve, as well as controls with no reverse transcription and no templates. After checking the amplification specificity by disassociation curve analysis, results were subjected to data analysis as described next. All reaction mixtures were also electrophoresed on agarose gels followed by ethidium bromide staining for visualization.

Data analyses

The measured fluorescent signals were normalized against the reference dye (6-carboxy-X-rhodamine; ROX, included in the SYBR Green buffer) and were used to calculate the ΔRn using Rn^+ (normalized signal) – Rn^- (baseline Rn during cycles 3–15). Data were plotted as ΔRn against the PCR cycle number, with the threshold ΔRn being set at 10 times the SD of the mean baseline signal calculated for Rn^-. The obtained Ct values were plotted against the amount of DNA, and a standard curve was drawn using 10-fold serial dilutions of standard DNA. A unit of 1 × DNA standard was considered to contain 5 ng μl^{-1} (or 10^5 copies) of *V. parahaemolyticus* genomic DNA. The amount of mRNA was determined using this standard curve. In order to correct for the total cell number in the RNA extraction and extraction efficiency, the quantity of 16S rRNA in each sample was used for endogenous controls, assuming that the expression of 16S rRNA is constant for all cells used in this mRNA expression analysis.

Primer extension analysis

To determine the transcriptional start position of *cadB* and *cadA* genes, we performed primer extension analyses using 5′-Texas Red labelled primers 5′-GTA AAC AAA CGC AAG GCT CAG-3′ for *cadB* mRNA and 5′-TTA TCC CAG TCG AAC AGC AC-3′ for *cadA* mRNA. Aliquots of total RNA extracted from cells exposed to pH 5·0 were reverse transcribed with ReverTra Ace (Toyobo Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions. To map the transcriptional start site based on the fragment size, the extension products were separated on a 6% denaturing polyacrylamide gel on the DNA sequencer SQ5500E (Hitachi Ltd, Tokyo, Japan) along with a DNA sequence ladder prepared using the Thermo Sequenase primer cycle sequencing kit (Amersham Biosciences) to synthesize the target region with the same labelled primer.

Construction of the mutant strain

The *cadA* gene-inactivated strain, which was derived from *V. parahaemolyticus* V02-64 (serotype O3:K6) was con-

structed as previously described (Xu *et al.* 1994). Briefly, a partial fragment of the *cadA* gene of V02-64 was isolated by PCR using primers 5′-CAA TCT ACT TCC GTC CAA CTC G-3′ and 5′-CAA TGC CGT ACT GAG GTG AAG-3′. The amplified fragment was cloned into the pT7-Blue T-vector and then digested by *Bam*HI and *Pst*I. The digested fragment was subcloned into suicide vector, pKY719 (Xu *et al.* 1994), transformed into *E. coli* SM10*λpir*, and then re-introduced into *V. parahaemolyticus* cells through conjugation at 37°C overnight on LB agar. Cells were recovered by saline, plated onto thiosulfate citrate bile salts sucrose (TCBS; Eiken Chemicals Co. Ltd, Tokyo, Japan) supplemented with 10 $\mu g\ ml^{-1}$ of chloramphenicol, and incubated overnight at 30°C. Chloramphenicol-resistant *V. parahaemolyticus* cells with *cadA* genes inactivated by plasmid integration (single crossing-over), were screened by tests including southern blot hybridization and DNA sequencing. The lack of lysine decarboxylase activity was confirmed using an indicator broth (BBL Moeller decarboxylase broth base; Becton Dickinson, NJ, USA) and HPLC measurement as described before. Tests for biochemical characteristics were performed using ID32E API (BioMerieux, Marcy l'Etoile, France). The acid survival of the mutant and wild-type strains was measured by the same methods described before.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper is available in the DDBJ/EMBL/GenBank databases under accession number AB124819 (*V. parahaemolyticus* V02-64 *cadBA*).

Results

Vibrio parahaemolyticus possesses an adaptive ATR

The adaptive acid tolerance response in *V. parahaemolyticus* was examined in several strains (Table 1). In all strains, adapted cells (LB-MES, pH 5·5) showed better survival than nonadapted (LB-HEPES, pH 7·5) cells when exposed to acid (LB-MES, pH 4·0). As we have hypothesized that lysine decarboxylating activity plays a role in the ATR of *V. parahaemolyticus*, we analysed supernatants for the presence of polyamines. The HPLC findings show a large amount of cadaverine, which is generated from lysine through decarboxylation reactions, only in cells that survive lethal acidic conditions (adapted cells, data not shown). The recently emerged *V. parahaemolyticus* serotype O3:K6 was not more acid resistant than other *V. parahaemolyticus* strains used in this study.

Table 1 Effect of acid adaptation in the acid survival of *Vibrio parahaemolyticus* strains

Strain	Per cent survival* after 1 h at pH 4·0	
	Adapted†	Nonadapted
V02-64‡	$10{\cdot}1 \pm 6{\cdot}2_{AB}$§	$0{\cdot}23 \pm 0{\cdot}20_{A}$§
VpTK01	$32{\cdot}7 \pm 3{\cdot}4_{AB}$	$0{\cdot}21 \pm 0{\cdot}09_{A}$
VpTK03	$15{\cdot}9 \pm 3{\cdot}8_{AB}$	$0{\cdot}84 \pm 0{\cdot}14_{A}$
VpTK06	$7{\cdot}3 \pm 5{\cdot}6_{B}$	$0{\cdot}11 \pm 0{\cdot}05_{A}$
VpTK08	$36{\cdot}9 \pm 0{\cdot}6_{A}$	$0{\cdot}02 \pm 0{\cdot}01_{A}$
VpTK09	$8{\cdot}3 \pm 3{\cdot}0_{B}$	ND**
VpTK10	$18{\cdot}7 \pm 4{\cdot}5_{AB}$	$0{\cdot}03 \pm 0{\cdot}02_{A}$
VpTK11	$13{\cdot}0 \pm 0{\cdot}5_{AB}$	ND**
VpTK12	$30{\cdot}0 \pm 11{\cdot}8_{AB}$	$0{\cdot}03 \pm 0{\cdot}01_{A}$
VpTK13	$7{\cdot}0 \pm 5{\cdot}5_{B}$	$1{\cdot}00 \pm 0{\cdot}69_{A}$
VpTK17	$28{\cdot}4 \pm 6{\cdot}2_{AB}$	$0{\cdot}21 \pm 0{\cdot}07_{A}$

*Values are per cent survival after the acid challenge calculated by dividing the viable counts at 1 h by the initial viable counts and multiplying by 100. Values are the means (±SE) of three independent experiments.

†Acid adaptation was performed at pH 5·5 for 1 h at 30°C.

‡Serotype O3:K6.

§Means with the same letter within a column are not significantly different (Tukey-Kramer multiple comparison test, $P < 0{\cdot}05$).

**ND, not detected.

ATR of *Vibrio parahaemolyticus* requires external lysine

To demonstrate the dependence of ATR on lysine decarboxylase in *V. parahaemolyticus*, we determined the survival of the acid-adapted cells in NCE (pH 4·0) with or without 1% L-lysine. Tolerance of *V. parahaemolyticus* V02-64 (serotype O3:K6) to pH 4·0 NCE media was greater in the lysine supplied condition, as a threefold log reduction in survival was observed in the treatment without lysine after 60 min, while the reduction was only one order of magnitude in the treatment with lysine (Fig. 1). Nonadapted cells and adapted cells under the condition in which the protein synthesis was limited by the supplementation of the chloramphenicol in adaptation media were decreased to 10^{-3} at 30 min after resuspension in pH 4·0 NCE with lysine. This experiment was also performed for the other nine strains, and similar results were obtained. The mean per cent survival for the 10 strains at 30, 60, and 90 min at pH 4·0 was 17·8, 10·5, and 5·78, respectively, for the lysine-supplied cells, and 3·49, 0·94 and, <0·01, respectively, for the lysine-free culture conditions.

Molecular analyses of cadA and cadB genes of *Vibrio parahaemolyticus*

The high homology of bacterial lysine/cadaverine antiporter (*cadB*) genes enabled us to design degenerate

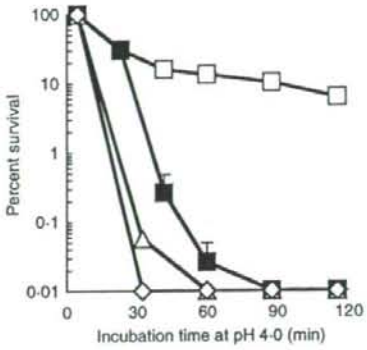

Figure 1 The fate of *Vibrio parahaemolyticus* V02-64 cells in lethally acidic environments in the presence or absence of lysine. Each cell treatment was acid adapted in Luria-Bertani (LB), pH 5·5 for 1 h and exposed to lethally acidic solution (no citrate E medium, pH 4·0) in the presence (open boxes) or absence (closed boxes) of lysine. It is notable that cells adapted with chloramphenicol (diamonds), and unadapted cells (triangles) both immediately decreased when lysine was present in the environment. All experiments were performed in triplicate, and some of the standard deviations (SD) were very small that the error bars do not extend beyond the data points.

primers based on alignments of *E. coli*, *S. typhimurium*, *V. cholerae*, and *V. vulnificus cadB* amino acid sequences. The resulting 631-bp amplification product from *V. parahaemolyticus* included six transmembrane domains as predicted by the SOSUI programme (Department of Biotechnology, Tokyo University of Agriculture and Technology; http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0E.html), and was considered to be *cadB* based on high homology of the determined DNA and the deduced amino acid sequences (82% and 92%, respectively) with *V. cholerae* sequences. The periphery sequences were completely determined using a commercial kit based on the cassette ligation-mediated PCR amplification technique. Two open reading frame (ORF) sequences coded on the same strand with lengths of 2136 and 1344 bp were matched to lysine decarboxylase and lysine/cadaverine antiporter of *V. cholerae* (91·7% and 88·3%, respectively) and other bacteria in the DNA Data Bank of Japan, (http://www.ddbj.nig.ac.jp). Therefore, considering the results from the mutational study (described next) into account, we designated the two ORF as *cadA* and *cadB* (Fig. 2a). The intergenic spacer region between *cadB* and *cadA* was 116 bp (Fig. 2a). This region has inverted repeat sequences and possibly forms a stem-loop structure in mRNA and may act as a terminator of the *cadB* sole transcript. RNA transcribed from the region has an eight-base stem and a four-base loop with no mismatches. Thus, taking into account the existence of the poly-A sequence, this region is the potential terminator (Fig. 2a). Recently, the entire genomic sequence of *V. parahaemolyticus* O3:K6 strain RIMD2210633 was reported (Makino *et al.* 2003); *cad* genes had sequence identity with those reported here of 99·7% (99·8% for the amino acid sequence) for *cadB* and 100% for *cadA*. In our study, *cadA* gene function was experimentally verified using the knockout mutant (described next).

Lysine decarboxylase genes are acid-induced operon

Northern blot hybridization revealed that *cad* genes are more highly expressed under acidic conditions than under neutral conditions (Fig. 3a, pH 5·5). However, hybridization data were not informative for the effect of lysine on transcription or for operon construction. Therefore, this RNA was subjected to the RT-PCR method using $d(N)_6$ and the primers shown in Fig. 2. Significant amounts of cDNA amplification products were produced using primers specific for *cadB*, *cadA* sole transcripts, and bi-cistronic transcripts of *cadBA* (Fig. 3b), showing that transcription of *cad* genes falls into the three patterns of *cadB*, *cadA*, and *cadBA*. Based on qualitative RT-PCR of RNA extracted from acid-stressed cells, significant amounts of amplification products (*cadB*, *cadA* and *cadBA*, Fig. 3b, lanes 1–4) were produced while only *cadA* amplification products were faintly observed in non-stressed cells (Fig. 3b, lanes 5 and 6). This shows that *cadBA* genes construct an operon, and the expression of *cadB*, *cadA*, and *cadBA* mRNA, especially *cadB* and *cadA*, are acid-inducible and low levels of transcription are also present at neutral pH.

RT-PCR gives more detailed information than the Northern method, although an accurate comparison of the expression ratio between these transcripts is not possible because of the 'plateau' effect of PCR end-point analysis and the variation in PCR efficiency between the experiments. Therefore, SYBR Green I real-time, quantitative RT-PCR was also performed to determine the relative expression of these mRNA species. Relative expression ratios showed that the expression of these genes was induced under the condition of low pH (Fig. 3c). For example, *cadA* mRNA exhibited about 40-fold greater expression at pH 5·0 (3·39 ± 1·5) than at pH 5·5 (0·082 ± 0·02). In cells exposed to pH 7·5, only the *cadA* transcript was detected, and this transcript showed the largest quantity under all conditions. In addition, the amount of *cadB* and *cadA* single transcripts was nearly 10- and 100-fold higher than the polycistronic *cadBA* transcript for each pH condition (Fig. 3c). For example, at pH 5·5 with the addition of lysine, the bi-cistronic *cadBA* transcript had a relative

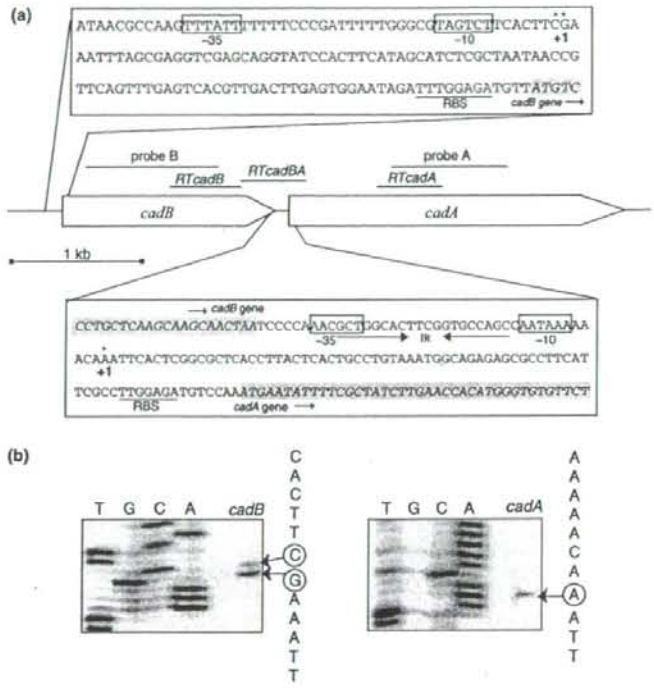

Figure 2 (a) Schematic representation of the *Vibrio parahaemolyticus* lysine decarboxylase gene cluster and intergenic region. Probes for the Northern blot hybridization of genes *cadB* and *cadA* and polymerase chain reaction (PCR) primers to measure mRNA species by quantitative reverse transcriptase (RT)-PCR are indicated by bars labelled with the probe name (probes B and A) and gene name (*RTcadB*, *RTcadBA*, and *RTcadA*), respectively. The *RTcadBA* fragment was targeted at the C-termini of *cadB* and N-termini of *cadA* to detect the bi-cistronic transcript. The periphery sequences of *cadB* and *cadA* are given in the inset. Protein coding regions are indicated by shading, the putative transcriptional terminator (inverted repeat, IR) is indicated by arrows, and the probable ribosome-binding site (RBS) is underlined. Transcriptional start sites (+1) of each gene is indicated by asterisks. Predicted promoter sites, −35 and −10, are indicated by boxes. The scale bar length of 1 kb of the sequence is indicated. (b) Primer extension analysis. The primer extension products with total RNA of *V. parahaemolyticus* were sized. Sequencing ladder obtained with the same primers were shown on the left.

expression of 0·198 ± 0·03, compared with 1·96 ± 0·89 for *cadB*, and 8·03 ± 0·08 for *cadA*. This suggests that the bi-cistronic transcript could not be visualized on Northern blot hybridization. The majority of the probe added to the total RNA might have been hybridized to the monocistronic transcripts because of their higher quantity (10- to 100-fold).

All data obtained from quantitative RT-PCR were normalized for cell numbers and extraction efficiency using the values for 16S rRNA endogenous control of 1 and 2·40 (with lysine) for pH 5·0, 0·738 and 0·643 (with lysine) for pH 5·5, and 0·487 and 0·683 (with lysine) for pH 7·5. No genomic DNA contamination was found in any of the RNA samples during 50 cycles of PCR with any of the primer pairs (data not shown).

Identification of the transcriptional start point of *cadA* and *cadB*

The transcriptional start point of *cad* genes of *V. parahaemolyticus* was determined, as shown in Fig. 2. The transcriptional start point of *cadB* mRNA was located 101 and 102 bases upstream from the start codon of *cadB*. The predicted promoter elements were TTTATT and TAGTCT located at −35 and −10, respectively. The *cadA* mRNA started from 75 bases upstream of the start codon, and the promoter elements were identified as follows: AACGCT at −35, AATAAA at −10. The promoter sequences of *cadA* and *cadB* have little resemblance to each other. Although these promoter elements have little similarity for the consensus sequences (TATAAT and TTGACA), except for the −10 box of *cadA*, they share significant homologies with the promoter elements of *V. chorelae cad* genes (Merrell and Camilli 2000).

Acid tolerance of *cadA* gene-inactivated strain

The acid tolerance of a mutant strain of the newly emergent *V. parahaemolyticus* clone, V02-64, serotype O3:K6 constructed with a disrupted *cadA* gene was also tested. The mutant strain has a plasmid integrated in its chromosome (single crossing over), with a corresponding decrease in lysine decarboxylating activity to 1/100 or less. The microscopic experiments, measurement of growth rate, and tests for biochemical properties using API ID32E (BioMerieux) revealed no changes to other characteristics by this manipulation. The 4·0-kb *Hin*cII fragment detected by southern blot hybridization using

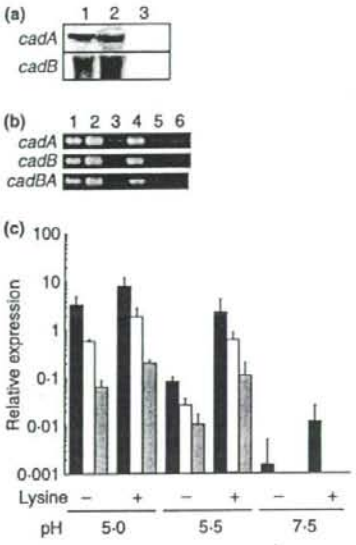

Figure 3 Transcriptional analysis of *cadBA* genes. (a) Northern blot hybridization analysis for *cadA* or *cadB* mRNA of *Vibrio parahaemolyticus* V02-64. Whole RNA (15 μg) was loaded, electrophoresed, blotted, and hybridized with probes specific for *cadA* or *cadB* under the following conditions: lane 1, pH 5·5; lane 2, pH 5·5 supplemented with 1% lysine; and lane 3, pH 7·5 with lysine. (b) Agarose gel images after 25 cycles of reverse transcriptase (RT)-polymerase chain reaction (PCR) under the following conditions: lane 1, pH 5·0; lane 2, pH 5·0 with lysine, lane 3, pH 5·5; lane 4, pH 5·5 with lysine; lane 5, pH 7·5; lane 6, pH 7·5 with lysine. (c) Relative expression analysis of *V. parahaemolyticus* lysine decarboxylase genes as detected by SYBR Green I real-time quantitative PCR under three pH conditions in the absence and presence of lysine. Total RNA extracted from the cells cultured in each condition was reverse transcribed by $d(N)_6$, and the quantity of cDNA consistent with that of the mRNA was quantified against a standard curve produced using genomic DNA. The relative amount of 1 is nearly equal to 10^4 copies of target DNA per 2 μl of the PCR template (=per reaction). Bars represent the averages of three to five experiments, and error bars indicate the SD (■, *cadA*; □, *cadB*; ▩, *cadBA*).

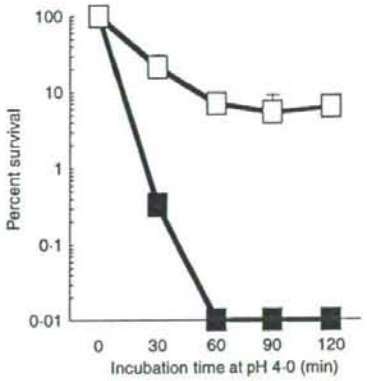

Figure 4 Acid resistance of the *Vibrio parahaemolyticus cadA* gene mutant and wild-type strains. All cell cultures were grown in Luria-Bertani (LB) media and collected at OD_{600} = 0·16–0·20 (log phase). Cells were all acid adapted in LB (pH 5·5) for 1 h to induce the acid-shock proteins. Acid-adapted cells were acid challenged in no citrate E medium (phosphate buffer, pH 4·0) with lysine at 37°C. Acid-challenged cells were then enumerated by plating onto trypticase soy agar medium at 30-min intervals. The survival of wild-type strain cells (open boxes) was reduced to one-tenths after 120 min, although survival of *cadA*-inactivated strain cells decreased immediately (closed boxes). Data represent the mean value of three measurements, and the error bars indicate the SD.

the *cadA* probe showed a band shift of 8·5 kb under the same conditions caused by plasmid integration in the mutant strain (data not shown). The acid resistance of this mutant strain at pH 4·0 in the phosphate buffer was weaker than in the parental strain; e.g. <0·01% of the mutant cells survived past 60 min of exposure, while 9·1% survival was observed for the wild-type strain (Fig. 4).

Discussion

The ATR is one of the strategies employed by the pathogens to infect and colonize mammalian hosts and has recently been reported in many enteropathogenic bacteria (Merrell and Camilli 2002). Among several proposed mechanisms of acid tolerance, amino acid decarboxylation has been confirmed in several bacteria. The molecular structure of lysine decarboxylase has been studied in some species, such as *E. coli* (Meng and Bennett 1992a,b), *S. typhimurium* (Foster and Hall 1991; Park *et al.* 1996), *V. cholerae* (Merrell and Camilli 1999, 2000), and *V. vulnificus* (Rhee *et al.* 2002). Among these species, lysine decarboxylase was confirmed to play a role in ATR in all but *E. coli*. Instead, *E. coli* utilizes glutamate decarboxylase as an alternative amino acid decarboxylase for ATR (Lin *et al.* 1995). Further, Samartzidou *et al.* (2003) have demonstrated that cadaverine, decarboxylated from lysine, plays a role in acid survival through porin inhibition in *E. coli*.

To achieve ATR via amino acid decarboxylation, two or more enzymes appear to be necessary (Bearson *et al.* 1997; Merrell and Camilli 2002); decarboxylation enzymes and their antiporters facilitate the coupled transport of amino acids and their decarboxylation products across the membrane. Each decarboxylation reaction consumes one intracellular proton to generate products including γ-aminobutyric acid (from glutamic acid) and cadaverine (from lysine), which are excreted to the extracellular space by antiporter protein and thereby decrease the

intracellular proton concentration and protect the cells from external acid stress. In our study, the molecular structure of the lysine decarboxylase gene cluster of *V. parahaemolyticus* was determined. Two ORF having sequences of 2136 and 1344 bp were identified as lysine decarboxylase (*cadA*) and lysine/cadaverine antiporter (*cadB*), respectively (Fig. 2a).

ATR of *V. parahaemolyticus* under low pH conditions is largely dependent on the presence of external lysine. Acid-adapted *V. parahaemolyticus* is resistant to low pH (4·0) only in the presence of external lysine (Fig. 1). Further, this adaptation effect is inhibited by chloramphenicol, an inhibitor of protein synthesis. Similar results have been obtained for all tested strains of *V. parahaemolyticus*, and these data suggest that *V. parahaemolyticus* gains resistance to conditions of low pH by using lysine and synthesizing proteins and that lysine-dependent pathways contribute significantly to the ATR of this pathogen. Thus, our findings corroborated previous reports that *V. parahaemolyticus* has the ability to mount a robust ATR, largely dependent on the presence of lysine.

In this study, transcriptional analyses by Northern blot hybridization and quantitative RT-PCR revealed that the *cad* genes of *V. parahaemolyticus* form an acid-inducible operon, while individual transcripts of *cadB* and *cadA* were also observed (Fig. 3a,b). The strength of induction of *cad* genes, which are related to acid adaptation, was shown to be inversely proportional to pH, suggesting that lysine decarboxylase genes are also responsible for the ATR of *V. parahaemolyticus*. The acid-inducible properties of the *cad* genes are the same in both *E. coli* and *V. cholerae*, the former has only a bi-cistronic transcript (Meng and Bennett 1992b), while the latter produces three transcripts comprising *cadBA*, *cadB*, and *cadA* (Merrell and Camilli 1999, 2000).

A small amount of *cadA* transcript is still produced under neutral pH conditions (Fig. 3c). This constitutive expression might be the result of substitution of another lysine decarboxylase (biosynthetic) found in *E. coli* (Kikuchi *et al.* 1997) as no other lysine decarboxylase was found in the entire genome of *V. parahaemolyticus* by southern hybridization (this study, data not shown) and whole genome sequencing (Makino *et al.* 2003). Cadaverine has been reported to play a role in the control of outer membrane permeability (Samartzidou *et al.* 2003) and cell division (Pruss *et al.* 1997) in *E. coli*. Therefore, decarboxylase protein CadA, the most important protein in the decarboxylation pathway, is expressed solely under neutral pH for the adjustment of the intracellular lysine or cadaverine concentration, although the expression level is low.

While the *cadB* and *cadA* genes of *V. parahaemolyticus* form an operon construct, the expression ratio between *cadB*, *cadA*, and *cadBA* was *c.* 10 : 100 : 1. The probable transcriptional terminator, the inverted repeat sequence of the *cadB* gene is indicated in Fig. 2a. Because this terminator-like sequence has sufficient length similar to a stem and a loop and includes several bases of the poly-A region, it is reasonable to assume that it acts as a transcription terminator. This likely explains the low production of *cadBA* mRNA, which is transcribed from the promoter upstream of *cadB* to the terminator of *cadA*. The *cadA* mRNA, the most abundant of the transcripts, is transcribed using its own upstream promoter. The predicted promoter regions of *cadB* and *cadA* have little resemblance. While both are acid-inducible promoters, differing transcriptional pattern were observed between *cadB* and *cadA* genes. The promoter sequence of *cadA* was more similar to the consensus sequence than that of *cadB* (Fig. 2a). Therefore, we consider that the *cadA* sole transcript is generated by its own promoter because the activity of promoter of *cadA* might be strong. When cells encounter low pH stress, *cadA* expression is increased and the *cadB* gene, which is indispensable in effecting acid tolerance, is also expressed. Some portion of the *cadB* transcripts do not terminate at the terminator located on the *cadB–cadA* intergenic region, and, thus, *cadBA* bi-cistronic transcripts are produced. In this study, the cadC-like ORF was observed in *V. parahaemolyticus* (data not shown). As *cadC* is reported to be a positive transcriptional regulator of the *cadBA* operon (Merrell and Camilli 2000; Rhee *et al.* 2005), the transcription of *cadBA* genes of *V. parahaemolyticus* might be regulated in the same way. The transcriptional regulation of the *V. parahaemolyticus cadBA* operon will be studied further.

To summarize, in the transcription of the *V. parahaemolyticus cad* operon, the *cadA* gene is constitutively expressed while *cadB* is not. Under low pH, the *cadA* gene is highly expressed, the *cadB* gene is also transcribed, and *cadB–cadA* bi-cistronic transcript is occasionally produced. This transcriptional regulation occurs because different amounts of CadA and CadB are required.

The mutant strain, with a disrupted *cadA* gene, was constructed to verify the role of *cadA* in ATR. The mutant strain was shown to have inactivated the *cadA* gene by an indicator broth, along with HPLC method, PCR, southern blot hybridization, and DNA sequencing (data not shown). All results showed the plasmid integration into the interior of the *cadA* gene, although a little cadaverine was detected from overnight cultures of the mutant strain. This remaining activity is speculated to be the result of imperfect mutation caused by the plasmid remaining in the chromosome. This mutant strain showed higher acid sensitivity than the wild-type strain, although other characteristics (growth rate, requirements for nutrition, biochemical characteristics, and colony

morphology) did not change. This mutant showed 1/1000 the acid resistance of wild-type strains under our experimental conditions. This indicates that lysine decarboxylation plays a role in the acid resistance of *V. parahaemolyticus*. This mechanism is generally considered to be related to the decreasing intracellular proton concentration through the decarboxylation reaction, and improvement of the microenvironment by excretion of the basic molecule, amine. This model has been proposed by many authors, although to the best of our knowledge, it has not been experimentally proven so far. Thus, further study is required to further develop this model. Alternatively, OmpU, an outer membrane protein, mediates organic acid resistance in *V. cholerae* (Merrell *et al.* 2001). Merrell *et al.* (2001) reported that this was accomplished by inhibition of the permeation of organic acid molecules. OMP-mediated acid resistance was also reported in *E. coli* (Samartzidou *et al.* 2003).

In conclusion, we have identified lysine decarboxylating pathway as the factor that allows survival under acidic conditions. This was confirmed by gene mutation analysis and mRNA quantification through SYBR Green I real-time RT-PCR that precisely determined the transcriptional aspects of *V. parahaemolyticus cad* genes. Acid resistance is universal in *V. parahaemolyticus* and the level does not diverge among strains. Recently, *V. parahaemolyticus* serotype O3:K6, which originated from a single clone, is spreading worldwide. Although some enhanced factor(s) might be involved in the pandemic spread of this pathogen, acid resistance is not.

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Growth and Toxin Production of Proteolytic *Clostridium botulinum* in Aseptically Steamed Rice Products at pH 4.6 to 6.8, Packed under Modified Atmosphere, Using a Deoxidant Pack

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ABSTRACT

Demand for aseptically steamed rice products has been increasing rapidly in Japan over the past 10 years. In our previous study, we showed that proteolytic *Clostridium botulinum* produce toxins in steamed rice products packaged under a modified atmosphere of ≤0.3% oxygen. In the present study, we examined the effect of pH to control botulism risk in steamed rice products packaged under modified atmosphere (5% CO_2 and 95% N_2 as the balance) with the inclusion of a deoxidant pack to produce an oxygen concentration of ≤0.3%. A mixture of 10 strains of proteolytic *C. botulinum* (5 type A strains and 5 type B strains) was inoculated into steamed rice products at pH values between 4.6 and 6.8 prior to packaging. All samples were stored at 30°C for 24 weeks. Samples at higher pH showed earlier starts of neurotoxin production. Neurotoxin was detected after 2 weeks of incubation in samples at pH 5.4 or above, whereas it took 4 weeks for the toxin to be detected in samples at pH 5.2 to 5.3 and 12 weeks in samples at pH 5.0 to 5.1. In samples at pH 4.9 or below, no toxin was detected during the experimental period. Apparent sample spoilage did not occur before *C. botulinum* produced neurotoxin in most of the samples. Based on these results, we conclude that aseptically steamed rice products must be packaged at pH 4.9 or below under modified atmosphere containing ≤0.3% oxygen, with the inclusion of a deoxidant pack.

Aseptic packaging, which is commonly used for milk, soup, pasta, and other foods, has also been used for steamed rice products in Japan. Packaged rice products can be stored for 6 months, or even longer, at room temperature and only require microwave oven heating preparation. These products are often packed under modified atmosphere: some contain 5 to 10% oxygen, and some are packed under modified atmosphere with the inclusion of a deoxidant pack so that oxygen concentration nearly reaches 0%. Modified atmosphere packaging (MAP) has become a popular means of extending shelf life of precooked foods, such as fresh pork *(12)*, fish fillets *(10, 13)*, vegetables *(6, 11, 16)*, and other food items *(3, 17)*, by preventing growth of aerobic bacteria, molds, and yeasts. However, MAP may permit growth of and toxin production by anaerobic spore-forming bacteria such as *Clostridium botulinum*. Although aseptically steamed rice products are packaged after cooking and steam sterilization, using an ultrahigh-temperature flash-heating process (generally ≥8 s at 135°C; $F_0 > 3.1$) to eliminate primary contamination, these products still carry a slight risk of secondary contamination of *C. botulinum* spores. In fact, production of neurotoxin by proteolytic *C. botulinum* was demonstrated in commercially manufactured steamed rice with MAP (pH 6.5) *(8)*. In our previous study *(8)*, therefore, we examined the effect of oxygen concentration on preventing toxin production by this pathogen in these products, and we determined 10% oxygen to be most effective. However, we still considered lower oxygen concentrations to be desirable in order to inhibit the growth of aerobic microorganisms. In fact, atmospheric conditions of nearly 0% oxygen with the inclusion of a deoxidant pack has already been adapted for the manufacture of some of these products in Japan in order to prevent the growth of aerobes, including molds.

When *C. botulinum* spores alone were inoculated into a medium made from cooked meat medium, toxin production was strictly limited below pH 4.6 *(21)*. However, since aseptically steamed rice products with pH adjusted below 4.6 taste acidic, most products currently distributed in Japan have a higher pH, mostly around 6.5.

In this study, we examined the pH threshold for controlling the risk of *C. botulinum* packaging that achieves ≤0.3% oxygen concentration through the used of modified atmosphere and the inclusion of deoxidant pack. To determine the safety level of pH that prevents *C. botulinum*–toxin production, proteolytic *C. botulinum* (types A and B) were inoculated into steamed rice adjusted to pH values between 4.6 and 6.8.

MATERIALS AND METHODS

Bacterial strains. Five type A strains (56A, 62A, 97A, Hall A, and Renkon-1 A) and five type B strains (9B, 213B, 407-1 B,

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Fukuyama B, and Okra B) of proteolytic group I were used in this study. The 56A, Hall A, Renkon-1 A, 213 B, 407-1 B, and Fukuyama B strains were kindly provided from the culture collection of H. Nakano (Laboratory of Food Microbiology and Hygiene, Hiroshima University, Hiroshima, Japan). The other *C. botulinum* strains (62A, 97A, 9B, and Okra B (proteolytic, group I) were kindly provided by the National Institute of Infectious Diseases (Tokyo, Japan).

Rice samples. Steamed, sterilized rice was prepared in a pilot plant. Briefly, after the rice bran had been sufficiently removed, the rice was washed and soaked in water for 30 min. After removing the water, the rice was steam sterilized using an ultrahigh-temperature flash-heating process (generally ≥8 s at 135°C; $F_0 >$ 3.1), followed by cooling. Packaging and the entire processing beyond the sterilization were carried out in a clean room (class 1,000). The sterilized rice was soaked in sterilized cooking water adjusted using gluconic acid to yield steamed rice with a pH of 4.6 to 6.8. The pH of water before cooking was equal to the pH of rice after cooking. And the rice was cooked at a rice:water ratio of 1:1 to produce a final cooked product with a water content of 60% (water activity of 0.98 to 0.99), which is representative of commercial products currently distributed in Japan. The soaked rice was then steam cooked at approximately 100°C, without controlling pressure for 30 min. The color of the acidically adjusted rice did not change after cooking, and it was identical to rice cooked normally. The steam-cooked rice was then aseptically packed in commercial packaging, with sample sizes of approximately 110 g, using a plastic plate and aseptically sealing with plastic film. These rice samples were stored at 30°C for 1 week and were visually inspected prior to inoculation with *C. botulinum.*

Preparation of *C. botulinum* spores. Preparation of *C. botulinum* spores and the subsequent enumeration were conducted under anaerobic conditions. *C. botulinum* strains were precultured in cooked meat medium (Eiken Chemical Co., Tokyo, Japan) at 37°C overnight. Spores of each strain were produced at 37°C over 7 days in Trypticase Peptone yeast broth (pH 7.0) with 5% (wt/vol) Trypticase Peptone (Difco, Becton Dickinson, Sparks, Md.), 0.5% (wt/vol) Bacto Peptone (Difco, Becton Dickinson), and 0.1% (wt/vol) Bacto yeast extract (Difco, Becton Dickinson). Before harvest, spores were checked with a phase contrast microscope (Olympus Co., Tokyo, Japan) for the dominance of refractile spores (>90%). Spore crops of each strain were centrifuged at 21,480 × *g* for 10 min at 4°C (SRX-201, Tomy Seiko Co., Tokyo Japan), and pellets were washed with sterile distilled water. Each crop was resuspended in sterile distilled water and frozen at −20°C until use. Spores of each strain were counted by the three-tube most-probable-number method after heat-shock treatment (80°C for 10 min, followed by rapid cooling).

Inoculation with *C. botulinum.* Equal numbers of *C. botulinum* spores from each of 10 different strains were mixed and adjusted to a concentration of approximately 4 log spores per ml. The spore mixtures were heat shocked (80°C for 10 min, followed by rapid cooling) and then 100 μl of the spore mixture was inoculated into each rice sample at each of 10 points (total of 1 ml of inoculum per rice sample) to avoid contingency differences among the samples. Cell counts of the samples and time-zero inoculum were determined by the serial dilution and pouch method *(2, 5),* using clostridia count agar (Nissui Pharmaceutical Co., Tokyo, Japan). Inoculated samples were packaged in high-gas-barrier film bags (Basela, Kureha Chemical Industry Co., Tokyo, Japan), using a Tospack V 400 gas changer (Tosei, Ohito, Shizuoka, Japan) along with a deoxidant pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) under atmospheric conditions of 0% oxygen, 5% carbon dioxide, and 95% nitrogen. All samples were incubated at 30°C. The samples were tested at weeks 0, 2, 4, 12, and 24. All the experiments were conducted in triplicate.

pH and atmospheric composition measurements. Headspace gas was analyzed for the concentration of carbon dioxide and oxygen, using a G5000A gas chromatograph (Hitachi, Tokyo, Japan). Subsamples (10 g) were placed in plastic bags (80 ml; Organo Co., Tokyo, Japan) with 10 ml of sterilized distilled water and mixed by hand for approximately 1 min to achieve a sufficiently homogeneous mixture without damaging the rice grains. Prior to the experiments, we confirmed that hand mixing was sufficient to mix the samples for microbiological counts while avoiding shredding rice grains as occurs when samples are mixed with a stomacher. pH was measured with a glass electrode pH meter (Horiba, Kyoto, Japan). The remainder of each sample (approximately 100 g) was placed in a stomacher bags (400 ml; Organo) with 100 ml of sterilized phosphate buffer (0.4% [wt/vol] Na_2HPO_4 and 0.2% [wt/vol] gelatin, pH 6.2) and mixed by hand (1 to 2 min). A portion of the mixture was used for microbiological analysis, and approximately 13 ml was transferred to sterilized centrifuge tubes (15 ml; Labcon, Petaluma, Calif) and stored at −20°C until neurotoxin assay.

Sensory evaluation. After incubation, the packages were opened, and the appearance and odor of the samples were assessed for spoilage by a panel of five judges with no special training. A sample was regarded as spoiled when all five people judged the sample as inedible, but an edible judgment by just one person was sufficient to consider the sample unspoiled.

Microbiological analysis. Anaerobic cell counts were carried out by serial dilution and pouch methods *(2, 5)* with Clostridia count agar. Portions of the same samples were used for contamination checks on PCA (Eiken) by the pour-plate method. Uninoculated samples were also checked for aerobic and anaerobic counts to confirm the absence of all bacteria.

Neurotoxin assay. Frozen rice samples were thawed, mixed, and centrifuged at 2,190 × *g* for 10 min. Two mice (approximately 20 g) were each intraperitoneally injected with 0.5 ml of supernatant. Samples were considered toxic if typical respiratory symptoms of botulism occurred and both mice died during the 48-h observation period *(19).* For samples producing toxic reactions, neurotoxin was tested for serum type by inoculating sample into mice protected with A and/or B antiserum (two mice for each treatment; six mice total) (Chiba Serum Institute, Chiba, Japan). Following the manufacturer's instructions, antiserum protection was established with a 0.5-ml intraperitoneal injection of 2 U/ml adjusted type A and/or B antiserum and the same volume of sample incubated for 1 h at room temperature. If mice protected with B antiserum died while those protected with A antiserum lived during the 48-h observation period, then the toxin was considered to be type A. Conversely, if mice protected with A antiserum died while those protected with B antiserum lived, then the toxin was considered to be type B. If mice protected with both A and B antiserum lived while the other mice died, then both toxin types A and B were considered present.

RESULTS

Measurements of pH and atmospheric composition. Atmospheric composition and pH values are shown in Table 1. The oxygen concentration at the time of packaging

TABLE 1. *Analysis of rice samples inoculated with* Clostridium botulinum *(type A, B) spores*[a]

Initial pH	Incubation time (wk)	No. of samples spoiled/no. of samples tested	Sample with toxin	Sample parameters				
				pH	O_2 (%)	CO_2 (%)	Anaerobic count (log CFU/g)	Anaerobic count (log CFU/g)[b]
4.6–4.7	0	0/1	ND[c]	4.7	0.8	1.8	2.1	ND
	2	0/3	ND	4.7 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	2.1 ± 0.1	ND
	4	0/3	ND	4.7 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.0	ND
	12	0/3	ND	4.8 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	1.8 ± 0.1	ND
	24	0/3	ND	4.8 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	1.9 ± 0.0	ND
4.8–4.9	0	0/1	ND	4.9	0.8	1.8	2.1	ND
	2	0/3	ND	4.8 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	2.3 ± 0.1	ND
	4	0/3	ND	5.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.0	ND
	12	0/3	ND	5.0 ± 0.1	0.3 ± 0.2	0.0 ± 0.0	1.9 ± 0.1	ND
	24	0/3	ND	5.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	2.5 ± 0.2	ND
5.0–5.1	0	0/1	ND	5.2	0.8	1.5	2.1	ND
	2	0/3	ND	5.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	2.3 ± 0.3	ND
	4	0/3	ND	5.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.1	ND
	12	0/2	ND	5.5 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.1	ND
		0/1	A	5.6	0.3	0	3.8	ND
	24	0/3	A (3/3)[d]	5.4 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	4.2 ± 0.2	ND
5.2–5.3	0	0/1	ND	5.4	0.8	1.6	2.1	ND
	2	0/3	ND	5.3 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	4.4 ± 0.1	ND
	4	0/3	A (1/3)[e]	5.4 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	4.5 ± 1.1	ND
	12	0/3	ND	5.5 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	2.7 ± 0.6	ND
	24	0/3	A (3/3)	5.5 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	4.4 ± 0.0	ND
5.4–5.5	0	0/1	ND	5.5	0.8	1.6	2.1	ND
	2	0/3	A (3/3)	5.5 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	4.9 ± 0.0	ND
	4	0/3	A+B (3/3)	5.6 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	4.7 ± 0.1	ND
5.6–5.7	0	0/1	ND	5.6	0.8	1.6	2.1	ND
	2	0/3	A+B (3/3)	5.7 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	5.4 ± 0.1	ND
	4	ND	ND	ND	ND	ND	ND	ND
5.8–5.9	0	0/1	ND	5.9	0.8	1.7	2.1	ND
	2	0/3	A+B (3/3)	5.9 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	5.6 ± 0.0	ND
	4	ND	ND	ND	ND	ND	ND	ND
6.0–6.1	0	0/1	ND	6.1	0.8	1.6	2.1	ND
	2	0/3	A+B (3/3)	6.0 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	5.6 ± 0.2	ND
	4	ND	ND	ND	ND	ND	ND	ND
6.2–6.3	0	0/1	ND	6.2	0.8	1.7	2.1	ND
	2	0/3	A+B (3/3)	6.2 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	6.2 ± 0.0	ND
	4	ND	ND	ND	ND	ND	ND	ND
6.8–6.9	0	0/1	ND	6.8	0.8	1.7	2.1	ND
	2	3/3	A+B (3/3)	6.8 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	6.8 ± 0.1	ND
	4	ND	ND	ND	ND	ND	ND	ND

[a] Rice sample had 60% water content.
[b] The limit of detection of viable count is 2 CFU/g.
[c] ND, not detected.
[d] Toxin was detected (0.1% oxygen, pH 5.4) in all three samples.
[e] Toxin was detected (0.3% oxygen, pH 5.4) in one of three samples.

was 0.8%, and it dropped to ≤0.3% after 2 weeks in all samples. The initial carbon dioxide concentration was 1.8%, but no CO_2 was detected after 2 weeks of incubation in all sample types. There was no significant change in the sample pH value over 24 weeks.

Growth of *C. botulinum* in steamed rice. Growth of *C. botulinum* in steamed rice at 30°C is shown in Table 1. Anaerobic counts were assumed to equal the *C. botulinum* counts because rice samples were supposed to be sterile in the absence of *C. botulinum* inoculation. Initial *C. botulinum* counts were an average 2.1 log CFU/g for all sample types. Counts after 2 weeks of incubation increased significantly in samples initially adjusted to pH 5.2 or above, ranging from 4.4 log CFU/g in samples at pH 5.2 to 6.8 log CFU/g in samples at pH 6.8. The only exception was samples at pH 5.2 to 5.3 collected at week 12, in which the anaerobic counts were lower than the counts of those collected at week 4. Sample spoilage occurred only in samples at pH 6.8. Increases in anaerobic counts were also detected in samples of lower pH later in the experimental period. One of the samples at pH 5.0 showed an increased anaerobic count of 3.8 log CFU/g after 12 weeks of incubation