S2-1

Gene Clustering for Reuterin Production in the Probiotic Bacterium *Lactobacillus reuteri* JCM1112^T and its Functional Importance for Viability in the Mammalian Gastrointestinal Tract

Hidetoshi Morita*, Toshio Masaoka*, Hiroshi Horikawa*, Kenshiro Oshima‡, Takehito Suzuki*, Masaru Murakami*, Yukio Kato*, Shin Hisamatsu¶, Tatsuya Takizawa*, Nobuyuki Kanemaki*, Yasuhide Saito*, Hitoyuki Naito*, Seigou Kishikawa¶, Toshiho Nishita*, Masafumi Fukuyama¶, Ryoichi Sakata*, Kazuyoshi Arishima*, Akio Kiuchi*, Tomonori Wada*, Hideo Fukuoka*, Masao Shino*, Shiro Chinone*, Tadayoshi Shiba‡, Tetsuhiko Yoshimura**, and Masahira Hattori§

**School of Veterinary Medicine, Azabu University, Sagamihara 229-8501; ‡School of Science, Kitasato University, Sagamihara 228-8555; ¶School of Environmental Health Science, Azabu University, Sagamihara 229-8501; **Institute for Life Support Technology, Yamagata Public Corporation for the Development of Industry, Matsuei 990-2473; and §Kitasato Institute for Life Sciences, Kitasato University, Sagamihara 228-8555 and RIKEN Genomic Sciences Center, Yokohama 230-0045 Japan)*

Lactic acid bacteria (LAB) are considered to be totally safe on the empirical basis and therefore called "generally recognized as safe (GRAS) bacteria." LAB are now ranked at the first place of the list of probiotics important to health. The current focus of interest in this group of microbes is on the genome analysis to elucidate the genome function of lactic acid bacteria at the strain level but not the genus or species level from the viewpoints of fermented food production and other applications (*e.g.*, probiotic effects).

Lactococcus lactis subsp. *lactis* is the bacterium indispensable in cheese starter cultures. The complete genome sequence of *L. lactis* IL1403 was published by the group of Institut National de la Recherche Agronomique (INRA), France, for the first time in lactic acid bacteria in 2001 (1). In LAB, the second and the third complete genome sequences were disclosed for *Lactobacillus plantarum* WCFS1 (2) (February 2003) and *Lactobacillus johnsonii* NCC533 (3) (February 2004), respectively. The analyses based on the pulse-field gel electrophoresis and genome sequence have demonstrated that the genome size of lactic acid bacteria ranges from 1.5 to >3 Mbp.

We attempted to determine the genome sequences of *Lactobacillus reuteri* JCM1112 (type strain). The G+C-content of *L. reuteri* genomes was approximately 39%. *L. reuteri* is from animal intestines and feces (Table 1) (4) and the major findings on probiotic effects have been obtained from *L. reuteri*. To explore the difference in genetic information of these bacteria with such distinct backgrounds, we determined the genome sequences. The pulsed-field gel electrophoresis reveled that the straintwo had a genome size of about 1.9 Mb. The analysis of the genome sequences showed that the both genomes contained about 1,600 of open reading frames (ORF), and most of them encoded the genes involved in transport and energy

metabolism (Fig. 1).

L. reuteri is known to produce a non-peptidic antibacterial substance, reuterin. Unlike bacteriocins, reuterin has the antimicrobial activity against not only Gram-positive bacteria but also Gram-negative bacteria, yeasts, fungi and protozoans. We analyzed a set of genes involved in the reuterin synthesis in *L. reuteri* JCM1112, as shown in Figs. 2, 3 and 4. These genes (α, β and γ subunits of glycerol and diol dehydratase) were cloned and His_6 recombinant protein (Fig. 5).

In other bacteria that possess the *dha* regulon, the expressions of glycerol dehydratase and 1,3-propanediol dehydrogenase are subjected to synchronous transcriptional regulation to prevent the excessive production of cytotoxic reuterin. *L. reuteri* was shown to have a different regulatory system. Glycerol dehydrogenase conserved in *L. reuteri* might contribute to the maintenance of the intracellular oxidation-reduction balance when 1,3-propanediol dehydrogenase is expressed. The *pdu* cluster of *L. reuteri* contained the structural genes homologous to propanol dehydrogenase (PduQ), propionaldehyde dehydrogenase (PduP) and propionate kinase (PduW) of *S. typhimurium*. This indicates that *L. reuteri* possesses an oxidation pathway to produce 3-hydroxypropionic acid from glycerol as well as a reduction pathway from glycerol to 1,3-propanediol via reuterin. Table 2 shows homology of amino acid sequence of *pdu* operon and *dha* regulon between *L. reuteri* (this study) and the other bacteria (5-15).

We also searched for genes of the known cell-adhesion factors, and the genes for putative cell-adhesion factors were found in both of the two species. Each of these genes exists in the genome in multiple copies. This strain also has ADI pathway to produce ATP from L-arginine.

The probiotic effects of lactic acid bacteria on health of humans and other mammals are attracting the greatest attention in recent years. Probiotics are defined as "living microorganisms beneficial to health of their hosts." Vitamins, antimicrobial substances such as antibiotics and bacteriocins, and dead cells of functional bacteria including lactic acid bacteria are called biogenics. *L. reuteri* possesses an operon producing adenosyl cobalamin (coenzyme B_{12}). Biogenics are defined as "bioactive substances beneficial to hosts through direct immunostimulation and inhibition of mutagenicity, oncogenesis, hyperoxidation, hypercholesterolemia and intestinal putrefaction (Mitsuoka, 1996)." Biogenic effects are confirmed in many of the lactic acid bacteria. The ongoing genome analysis for many lactic acid bacteria will give us interesting findings on the relationship between such probiotic and biogenic effects and their functionality at genome level. Further genome analysis of lactic acid bacteria in future is expected to address answers to questions such as "what are the criteria for safety of live bacteria?" and "what are harmless bacteria?"

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Table 1. Distribution of lactobacilli in intestinal guts of human and the other mammalia

Species	Human	Pig	Chicken	Cattle	Dog	Mouse	Rat	Hamster
L. acidophilus group								
L. acidophilus	?					?	?	
L. amylovorus		M	?	+				
L. crispatus	M		M					
L. gallinarum			M					
L. gasseri	M			+				
L. johnsonii	+	+	M					
L. murinus/animalis		?	?	M	M	M	?	
L. intestinalis						M	M	
L. salivarius	M	M	M					
L. agilis		+	+					
L. ruminis	+			M				
L. vitulinis	+							
L. hamsteri								M
L. aviaries		+						
L. casei	+							
L. plantarum	+							
L. brevis	+							
L. reuteri	M	M	M	M	M	M	M	M

M : FDetected as dominant *Lactobacillus* sp.

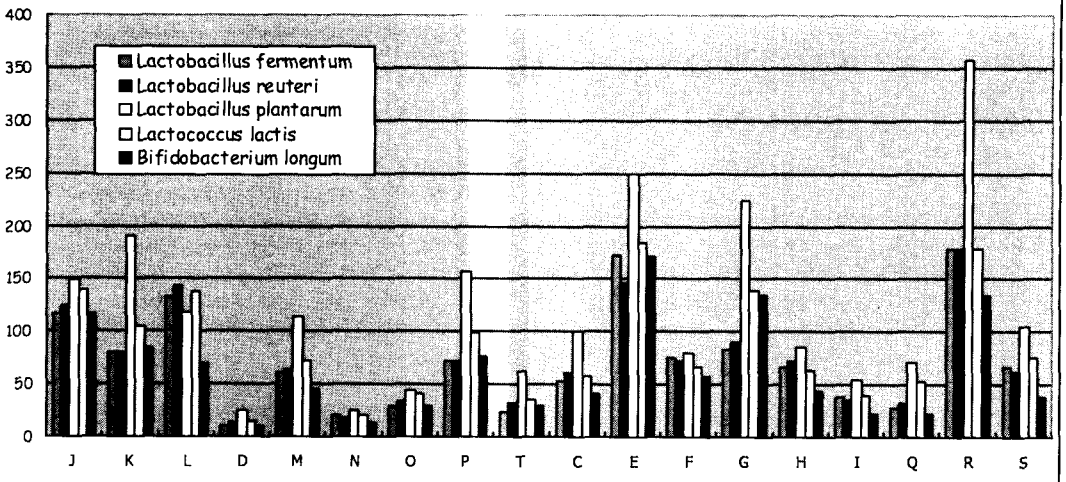
+ : FDetected rarely.

Table 2. Homology of amino acid sequence of *pdu* operon and *dha* regulon between *L. reuteri* and the other bacteria

Gene name of *L. reuteri**	*L. collinoides*[13,14,15]		*S. typhimurium*[11,12]		*C. freundii*[5,6,7,8]		*Cl. perfringens*[9,10]		Putative function
	Gene name[*1]	Identity[*2]	Gene name[*1]	Identity[*2]	Gene name[*1]	Identity[*2]	Gene name[*1]	Identity[*2]	
pduF(235)	-	-	*pduF*(264)	31 %	-	-	*glpF*(234)	44 %	Glycerol uptake facilitator and related permeases
pocR(359)	*pocR*(317)	32 %	*pocR*(303)	13 %	-	-	-	-	Transcriptional regulator
pduA(93)	*pduA*(97)	77 %	*pduA*(94)	65 %	-	-	-	-	Polyhedral organelles
pduB(238)	*pduB*(274)	64 %	*pduB*(233)	56 %	-	-	-	-	Polyhedral organelles
pduC(558)	*pduC*(558)	73 %	*pduC*(554)	64 %	*dhaB*(555)	62 %	*dhaB1*(554)	63 %	AdoCbl-dependent dehydratase large subunit
pduD(236)	*pduD*(230)	66 %	*pduD*(224)	58 %	*dhaC*(194)	50 %	*dhaB2*(190)	56 %	AdoCbl-dependent dehydratase medium subunit
pduE(172)	*pduE*(173)	67 %	*pduE*(173)	45 %	*dhaE*(142)	40 %	*dhaB3*(141)	51 %	AdoCbl-dependent dehydratase small subunit
pduG(616)	*pduG*(610)	80 %	*pduG*(610)	65 %	*dhaF*(603)	59 %	*orfZ*(616)	63 %	Dehydratase reactivation factor large subunit
pduH(119)	*pduH*(116)	52 %	*pduH*(123)	34 %	*dhaG*(117)	23 %	*orfX*(116)	42 %	Dehydratase reactivation factor small subunit
pduK(189)	*pduK*(231)	38 %	*pduK*(160)	32 %	-	-	-	-	Polyhedral organelles
pduJ(96)	*pduJ*(94)	78 %	*pduJ*(91)	74 %	-	-	-	-	Polyhedral organelles
pduL(214)	*pduL*(215)	57 %	*pduL*(210)	50 %	-	-	-	-	Unknown fuction
pduM(167)	*pduM*(167)	41 %	*pduM*(163)	15 %	-	-	-	-	Unknown fuction
pduO(202)	*pduO*(192)	65 %	*pduO*(337)	21 %	*orfW*(176)	38 %	*orfW*(170)	42 %	Adenosyltransferase
pduObis(157)	*pduObis*(164)	68 %	*pduO*(337)	17 %	*orfY*(142)	28 %	*orfY*(142)	31 %	Adenosyltransferase
pduP(477)	*pduP*(481)	69 %	*pduP*(477)	44 %	-	-	-	-	Propionaldehyde dehydrogenase
pduQ(379)	*pduQ*(373)	61 %	*pduQ*(370)	40 %	*dhaT*(387)	31 %	*dhaT*(385)	31 %	Propanol dehydrogenase
pduW(395)	*pduW*(395)	60 %	*pduW*(399)	44 %	-	-	-	-	Propionate kinase
pduU(115)	*pduU*(114)	86 %	*pduU*(116)	57 %	-	-	-	-	Polyhedral organelles
pduV(142)	-	-	*pduV*(150)	39 %	-	-	-	-	Unknown function

[*1] Figures in ( ) show the numbers of amino acid in the genes.

[*2] Homology against for the genes of *L. reuteri*.



J:Translation, ribosomal structure and biogenesis; K:Transcription; L:DNA replication, recombination and repair; D:Cell division and chromosome partitioning; M:Cell envelope biogenesis, outer membrane; N:Cell motility and secretion; O:Posttranslational modification, protein turnover, chaperones; P:Inorganic ion transport and metabolism; T:Signal transduction mechanisms; C:Energy production and conversion; E:Amino acid transport and metabolism; F:Nucleotide transport and metabolism; G:Carbohydrate transport and metabolism; H:Coenzyme metabolism; I:Lipid metabolism; Q:Secondary metabolites biosynthesis, transport and catabolism; R:General function prediction only; S:Function unknown

Fig. 1. Distribution of functional classes of predicted genes

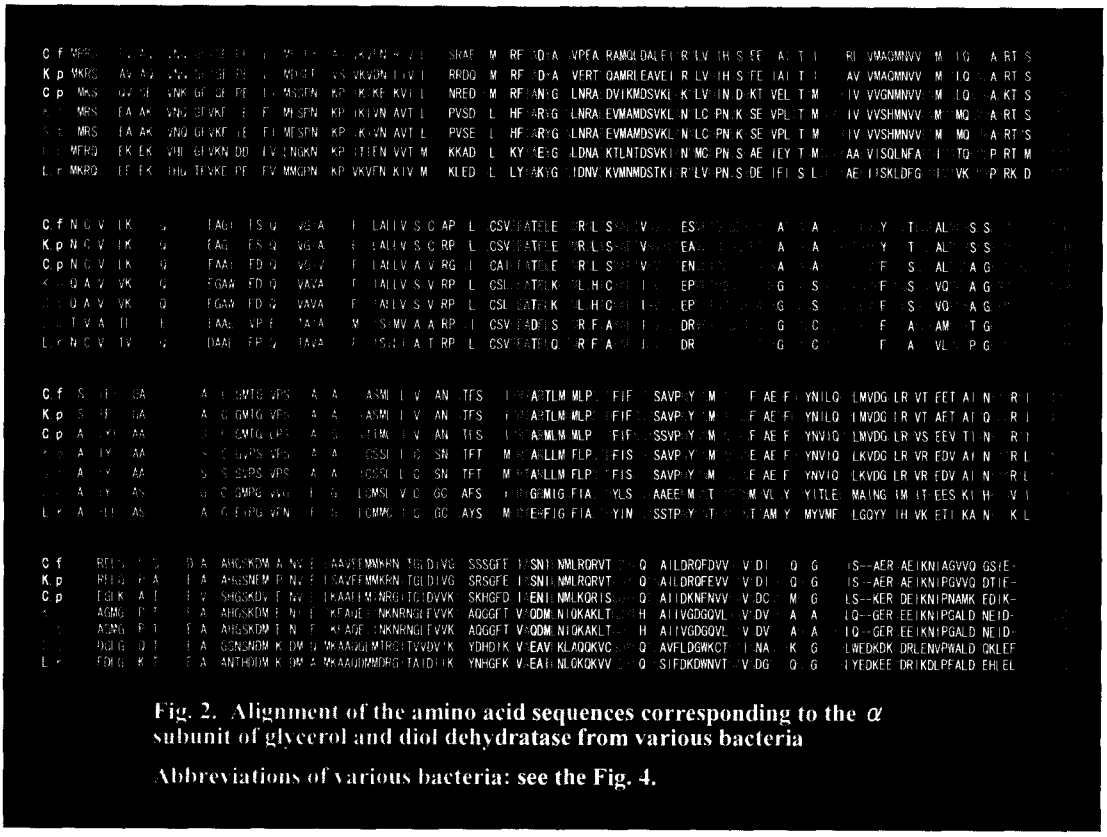


Fig. 2. Alignment of the amino acid sequences corresponding to the α subunit of glycerol and diol dehydratase from various bacteria

Abbreviations of various bacteria: see the Fig. 4.

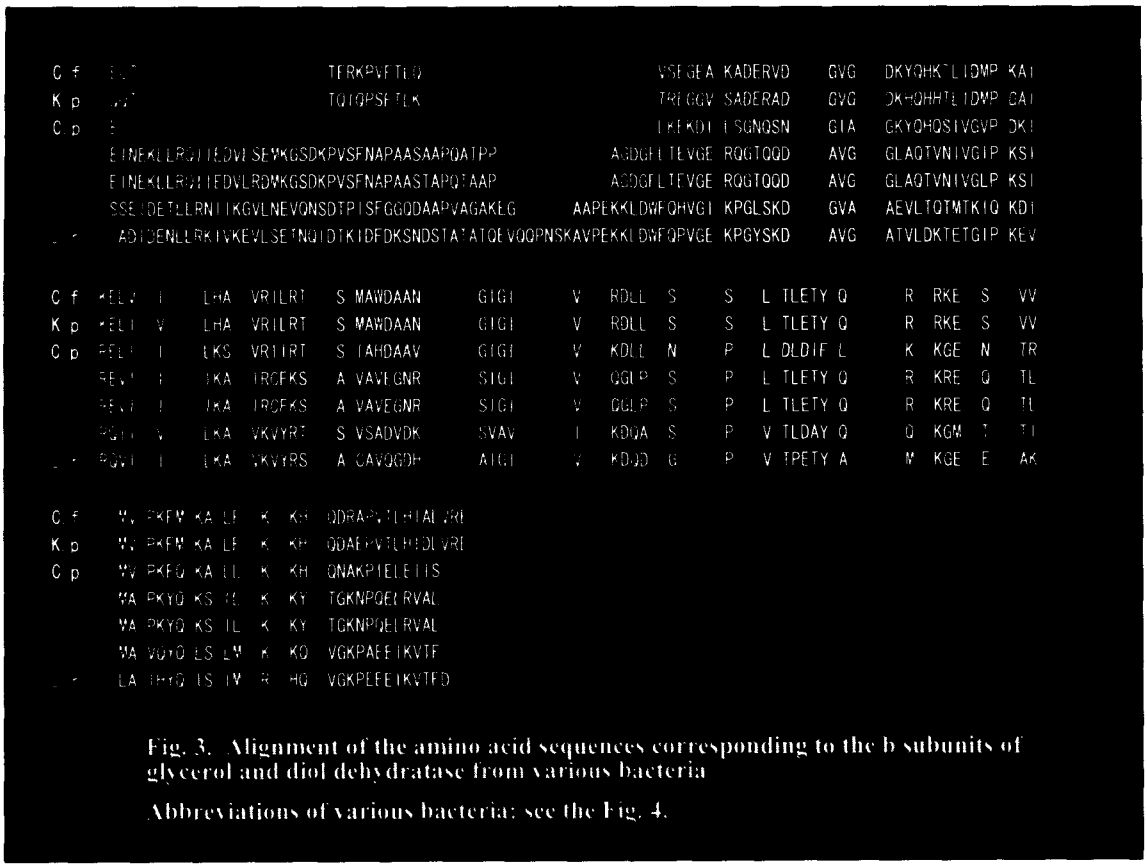


Fig. 3. Alignment of the amino acid sequences corresponding to the b subunits of glycerol and diol dehydratase from various bacteria

Abbreviations of various bacteria: see the Fig. 4.

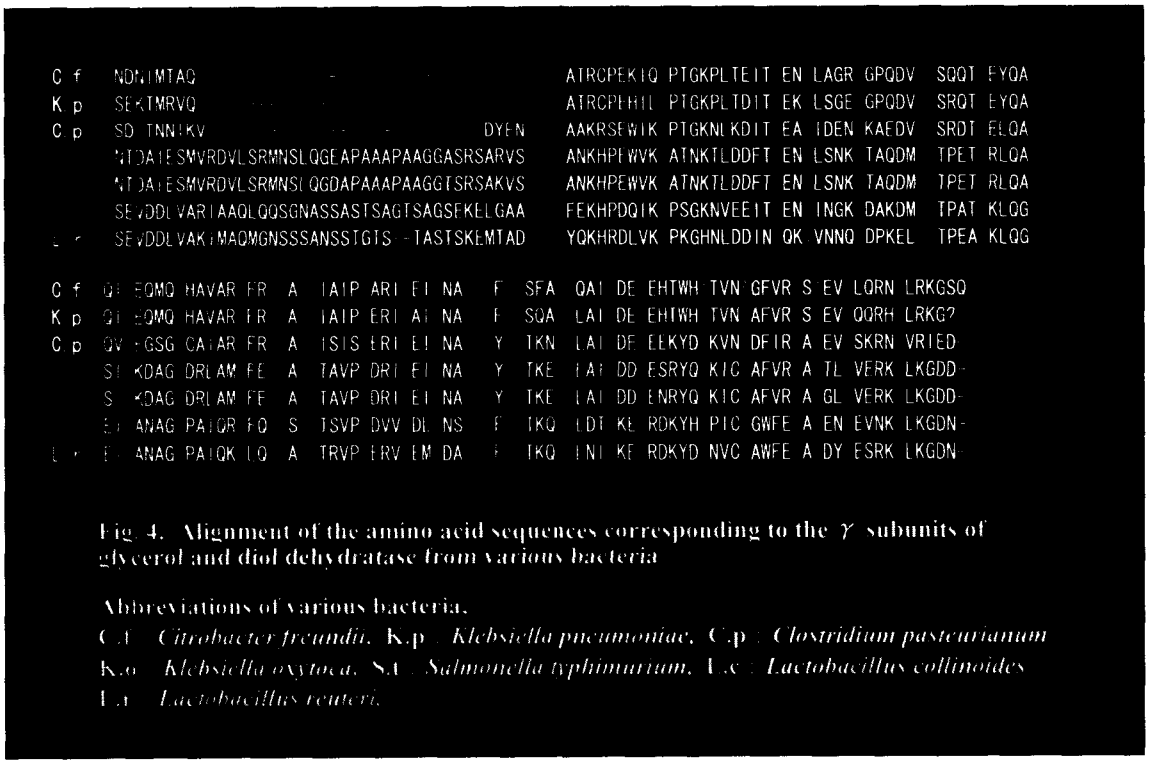


Fig. 4. Alignment of the amino acid sequences corresponding to the γ subunits of glycerol and diol dehydratase from various bacteria

Abbreviations of various bacteria.

C.f : *Citrobacter freundii*, K.p : *Klebsiella pneumoniae*, C.p : *Clostridium pasteurianum*
K.o : *Klebsiella oxytoca*, S.t : *Salmonella typhimurium*, L.c : *Lactobacillus collinoides*
L.r : *Lactobacillus reuteri*.