APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2006, p. 7954–7958 Vol. 72, No. 12
0099-2240/06/$08.00+0 doi:10.1128/AEM.01213-06
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Evidence for Horizontal Gene Transfer as Origin of Putrescine Production in *Oenococcus oeni* RM83[▽]

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Received 25 May 2006/Accepted 9 October 2006

The nucleotide sequence of a 17.2-kb chromosomal DNA fragment containing the *odc* gene encoding ornithine decarboxylase has been determined in the putrescine producer *Oenococcus oeni* RM83. This DNA fragment contains 13 open reading frames, including genes coding for five transposases and two phage proteins. This description might represent the first evidence of a horizontal gene transfer event as the origin of a biogenic amine biosynthetic locus.

Wines are highly selective media and support the growth of only a few species of lactic acid bacteria, mainly, *Oenococcus oeni* and several lactobacilli. *O. oeni* is often responsible for wine malolactic fermentation and is frequently utilized as a starter culture to promote malolactic conversion.

In acidic media like wine, decarboxylation of amino acids to their corresponding amines is thought to provide energy through electrogenic transport as well as assist in maintaining an optimal internal pH (11). Some of these amines are considered "biogenic" and may cause intoxication when consumed. The biogenic amine putrescine, which can potentiate the action of histamine, is the most prevalent amine in wine and is found in almost all analyzed wines (12, 15).

Biogenic amines are formed primarily by decarboxylation of the corresponding amino acids by microorganisms through substrate-specific decarboxylases. The capability of biogenic amine production appears to be strain dependent rather than species specific. Previously, we reported the identification of the *odc* gene in the putrescine producer *O. oeni* RM83 (formerly *O. oeni* BIFI-83) for the first time (14). The *odc* gene encodes a deduced 745-amino-acid putative ornithine decarboxylase (ODC) (EC 4.1.1.17) which catalyzes the conversion of ornithine to putrescine. The *odc* gene is seldom present in the *O. oeni* genome, as it has not been detected in a screen of 42 *O. oeni* strains tested to date (14). Moreover, in silico analysis of the draft *O. oeni* PSU-1 genome did not reveal the presence of any *odc* homologs (16).

Recently, Lucas et al. described that the potential for producing histamine in *Lactobacillus hilgardii* 0006 is encoded on an unstable 80-kb plasmid (13); the authors further suggested that it is very likely that the histamine producer *Tetragenococcus muriaticus* and *O. oeni* 9204 harbor the same plasmid (13). However, the localization of the *odc* gene in *O. oeni* RM83 remains unknown.

This study was undertaken to gain deeper insight into the origin of putrescine production in *O. oeni* RM83. Additionally, *O. oeni* RM83 ODC was expressed in *Escherichia coli* and biochemically characterized.

Genetic location of the *odc* locus in *O. oeni* RM83. The putrescine producer *O. oeni* RM83, formerly *O. oeni* BIFI-83, was previously isolated from lees of a Spanish red wine (14). Putrescine production by *O. oeni* RM83 was maintained without ornithine pressure, suggesting that the *odc* locus was stable. To determine if *O. oeni* RM83 harbored any plasmids, total DNA was extracted and analyzed by standard agarose gel electrophoresis (19). This assay revealed the absence of small plasmids in *O. oeni* RM83 (data not shown). Subsequently, native total DNA was embedded in agarose plugs and analyzed by pulsed-field gel electrophoresis as described previously (1). Again, plasmids were not detected. Moreover, Southern hybridization with a 1.4-kb DNA probe targeted to an internal *odc* fragment (14) yielded a positive signal only in the chromosomal DNA (data not shown). Therefore, it was concluded that *odc* in *O. oeni* RM83 is located on the chromosome.

Characterization of the *odc* region in *O. oeni* RM83. Since (i) in silico analysis of the *O. oeni* PSU-1 draft genome did not reveal the presence of an *odc* gene (16), (ii) the presence of the *odc* gene appears to be infrequent in the *O. oeni* genome (14), and (iii) the *odc* gene is chromosomally located in *O. oeni* RM83, we decided to identify the chromosomal DNA region involved in putrescine production in *O. oeni* RM83. The 17.2-kb sequence flanking the previously described 2.3-kb *odc* region was determined earlier (14). This sequence was ascertained by creating a phage library of *O. oeni* RM83 genomic DNA and by several inverse PCR experiments.

To construct the *O. oeni* RM83 DNA library, chromosomal DNA was partially digested with Sau3AI restriction enzyme and ligated to the ZAP Express vector (Stratagene, La Jolla, CA) digested with BamHI. The screening of the library using the 1.4-kb internal *odc* DNA fragment as a probe yielded five positive clones. Since the inserts of three of them were included in pAM4 and pAM8, only these plasmids were sequenced (Fig. 1). A 10,891-bp *O. oeni* RM83 DNA fragment was sequenced from the pAM4 and pAM8 plasmids. Two successive reverse PCR experiments, utilizing SnaBI and EcoRV, allowed us to sequence the 5′ end of the fragment (Fig. 1). Similarly, a reverse PCR experiment using SpeI allowed for the sequencing of the total 17,270-bp EcoRV-SpeI

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▽ Published ahead of print on 20 October 2006.

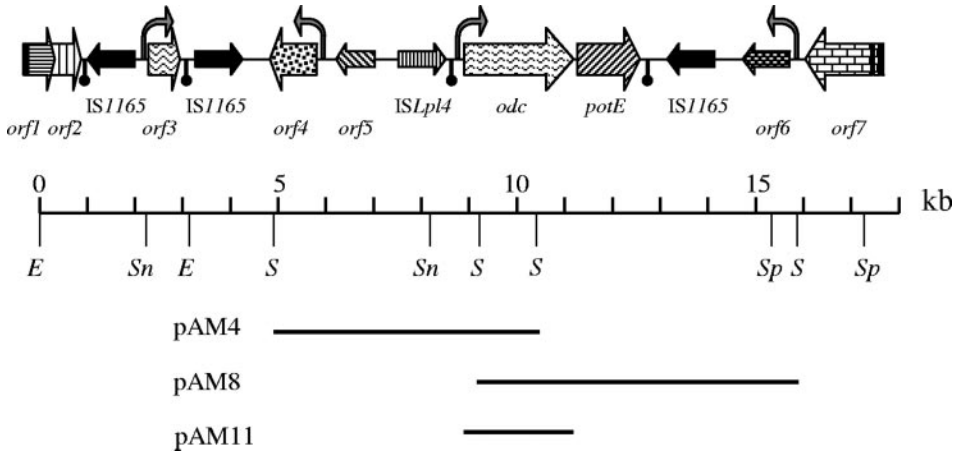

FIG. 1. Genetic organization of the *O. oeni* RM83 17.2-kb DNA region containing the *odc* gene. Thick and thin arrows represent complete and interrupted ORFs, respectively. The locations of putative promoters (vertical bent arrow) and predicted transcriptional terminator regions (ball and stick) are indicated. Some of the plasmids used in this study are indicated, as are relevant restriction sites: E, EcoRV; S, Sau3AI; Sp, SpeI; Sn, SnaBI. Only some of the corresponding restriction sites present in this fragment are represented.

chromosomal DNA fragment surrounding the *O. oeni* RM83 *odc* gene (Fig. 1).

Sequence analysis of this DNA fragment showed the presence of 11 complete (albeit some interrupted) and 2 partial open reading frames (ORFs) in the *odc* region (Fig. 1; Table 1). Two interesting features were observed: the presence of two putative phage proteins and the presence of five transposase-coding genes. The first incomplete ORF (*orf1*) is predicted to code for a protein showing the highest similarity (>30% identity) to *Streptococcus thermophilus* bacteriophage proteins. Notably, it has been reported previously that the genomes of currently characterized *S. thermophilus* phages exhibit homology to each other in a modular fashion (2). Furthermore, *orf2* is predicted to encode a protein similar to a DNA replication protein from an *Enterococcus faecalis* prophage. The *O. oeni* PSU-1 draft genome does not contain any intact temperate bacteriophage or larger tracts of obvious bacteriophage origin, although several prophage integration sites have been found previously (16).

Contiguous to and divergently transcribed from *orf2*, we found a variant copy of the insertion sequence (IS) IS*1165* (99% nucleotide identity) (10). The existence of two additional copies of IS*1165*, at positions 3121 to 4675 and 12879 to 14433/c ("/c" indicates that the sequence corresponds to the strand complementary to that included in the EMBL/GenBank database), was observed. All three of the IS*1165* copies are identical and contain the canonical terminal inverted repeats. Although IS*1165* was originally described for *Leuconostoc mesenteroides* subsp. *cremoris*, copies of this IS have been described for other lactic acid bacteria, such as *Leuconostoc lactis*, *O. oeni*, *Pediococcus* sp., *Lactobacillus helveticus*, and *Lactobacillus casei* (10).

Upstream of *orf4*, there is an 876-bp region that might correspond to an insertion sequence-like element on the basis of sequence similarity (58.5% nucleotide identity to a *Lactobacillus plantarum* transposase). Almost-perfect 17-bp inverted repeats were found at positions 6124 to 6140 and 6984 to 7000. Another IS, a copy of IS*Lpl4*, is found 722 nucleotides further upstream (4).

The *odc* gene is located downstream of IS*Lpl4*. The ODC protein is predicted to possess 745 amino acid residues, including conserved residues involved in enzymatic activity as well as the consensus sequence containing the pyridoxal-5-phosphate binding domain (14). The highest sequence identity (67%) was found between *O. oeni* and *Lactobacillus* sp. strain 30a (Table 1). Surprisingly, *O. oeni* RM83 ODC showed lower identity with similar proteins found in other members of the lactic acid bacteria (49% with *Lactobacillus johnsonii* and *Lactobacillus acidophilus*) than with enzymes from unrelated microorganisms, such as *Haemophilus influenzae* (64%), *Pasteurella multocida* (64%), and *E. coli* (58%) (14).

The next identified ORF is a putative *potE* gene. It encodes a 441-amino-acid, 47.6-kDa protein showing 67% identity to the putrescine-ornithine antiporter (PotE) from *Haemophilus influenzae* and 66% to PotE proteins from some *Enterobacteriaceae*. Unexpectedly, *O. oeni* PotE only shows a 14% identity to amino acid transporters from lactic acid bacteria (data not shown). PotE can catalyze both the uptake and the excretion of putrescine (8).

Functional expression of the *odc* gene from *O. oeni* RM83. To confirm that the *odc* gene from *O. oeni* RM83 encodes a functional ODC, we expressed this gene in *E. coli* HT414 (CGSC strain 6856), as this strain is defective for ODC activity (20). First, the gene was PCR amplified from *O. oeni* RM83 DNA by using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and oligonucleotides PIN-ODC-up (5′-GGAACTCTAG AGGGTATTAATAATGGATAGCGAAATAAATGATGA TTC) and PIN-ODC-down (5′-CGCATTGCGTTCACGTCG TTGCTCAATTATCATCTTTTTTCTTCATCTTTTGAC). The purified PCR fragment was inserted into pIN-III(lpp^{P}-5)A3 (9) by using a strategy described by Geiser et al. (7).

Cell extracts for the ODC enzymatic assay were obtained from *E. coli* HT414 cells harboring the control plasmid pIN-III(lpp^{P}-5)A3 or the recombinant plasmid pAM11 as previously described (18). The ODC assay was performed in 50 mM sodium phosphate buffer (pH 6.5) in the presence of 3.6 mM ornithine and 0.4 mM pyridoxal-5-phosphate. The reaction mixture was incubated at 37°C for 1 h. Subsequently, the pu-

TABLE 1. *odc* region-encoded proteins: properties and similarities to proteins in the databases

Gene or IS	Location in nucleotide sequence	G+C (%)	Predicted protein (aa/kDa)[a]	Similar polypeptide(s) (aa)[a]	Proposed function	Accession no.	% Identity[a]	Organism
orf1	0–237	36.3		Str0776 (286)	Replication protein, phage or plasmid associated	Q5M098	32.5 (in 81-aa overlap)	*S. thermophilus* CNRZ 1066
				ORF12 (269)	Conserved protein in *S. thermophilus* phages	O34043	30.1 (in 63-aa overlap)	*S. thermophilus* temperate bacteriophage φ O1205
				ORF35 (271)	Hypothetical protein	Q9XJC8	30.5 (in 59-aa overlap)	*S. thermophilus* lytic bacteriophage DT1
orf2	230–784	32.8	184/20.5	EF1279	DNA replication protein	Q835U3	27.6	*Enterococcus faecalis* V583 putative prophage 02
IS*1165*	1175–2236/c	48.1	353/39.5	IS*1165* transposase	Transposase	Q48788	98.8 (in 335-aa overlap)	*Leuconostoc mesenteroides* subsp. *cremoris*
orf3	2502–3095/c	35.2	197/21.6	EfaeDRAFT_2583	Hypothetical protein	Q3Y3B9	55.7	*Enterococcus faecium* DO
				Spr0580	Hypothetical protein	Q8DQN6	55.6	*Streptococcus pneumoniae* R6
IS*1165*	3258–4319	48.1	353/39.5	IS*1165* transposase	Transposase	Q48788	98.8 (in 335-aa overlap)	*L. mesenteroides* subsp. *cremoris*
orf4	4767–5837/c	35.2	356/39.5	LJ1779	Major facilitator superfamily permease	Q74HG7	50.4	*Lactobacillus johnsonii* NCC533
orf5	6541–6927/c	46.0	128/13.6	lp_3570	Transposase	Q88S70	58.5	*Lactobacillus plantarum* WCFS1
IS*Lpl4*	7828–7953	43.2	41/4.7	IS*Lpl4* transposase	Transposase	CAI93853.1	97 (in 34-aa overlap)	*L. plantarum* CECT4645
odc	8866–11103	36.3	745/81	OdcI	Ornithine decarboxylase	P43099	67.1	*Lactobacillus* sp. strain 30a
potE	11184–12509	39.2	441/47.6	PotE	Putrescine-ornithine antiporter	P44768	67.3	*Haemophilus influenzae* Rd
IS*1165*	13235–14295/c	48.1	353/39.5	IS*1165* transposase	Transposase	Q48788	98.8 (in 335-aa overlap)	*L. mesenteroides* subsp. *cremoris*
orf6	15240–15800/c	30.4	186/22.0	BCE3325150	Probable membrane protein	Q630E9	36.8 (in 148-aa overlap)	*Bacillus cereus* ZK
orf7	15966+/c	40.2		Ooen02001060	Hypothetical protein	ZP_00319317	78.2 (in 307-aa overlap)	*O. oeni* PSU-1

[a] aa, amino acids.

trescine formed in the reaction mixture was derivatized and detected by thin-layer chromatography (6) and by reverse-phase high-pressure liquid chromatography (14). Extracts from the strains harboring pAM11 were able to decarboxylate the supplied ornithine to putrescine, whereas extracts prepared from control cells containing the vector plasmid alone did not (Fig. 2). Therefore, we have provided experimental evidence that the *odc* gene encodes a functional ODC.

Recombination as origin of the *odc* region in *O. oeni* RM83. A careful comparison of nucleotide positions 15271 to 17270 (2 kb) of the sequenced *O. oeni* RM83 DNA fragment with positions 60644 to 62643 of the draft genome sequence of *O. oeni* PSU-1, GenBank accession number NZ_AABJ03000005, reveals three distinct regions based on their nucleotide sequence identity (Fig. 3). The leftmost 649 nucleotides of both sequences showed 47% nucleotide identity, the next 827 nucleotides were 61.5% identical between RM83 and PSU-1, and the rightmost 524 nucleotide residues exhibited perfect identity between both strains.

The proteins encoded by these 2-kb sequences are remarkable as well. From nucleotide position 61293, *O. oeni* PSU-1 encodes a 156-residue protein, Ooen02001059, annotated as a carbamoyl phosphate synthase, and Ooen02001060, a 631-amino-acid hypothetical protein (Fig. 3). This hypothetical protein contains an N-terminal putative signal peptide extend-

FIG. 2. Putrescine production by soluble cell extracts of IPTG (isopropyl-β-D-thiogalactopyranoside)-induced cultures of *E. coli* HT414 harboring pAM11. The putrescine produced during the enzymatic reaction was subjected to an automatic precolumn derivatization with *o*-ophthaldialdehyde prior to injection. Putrescine was determined by reverse-phase high-pressure liquid chromatography as previously described (14). (A) Results from reaction with *E. coli* HT414 bearing the control pIN-III(lpp^p-5)A3 plasmid. (B) Results from reaction with *E. coli* HT414 bearing the recombinant pAM11 plasmid.

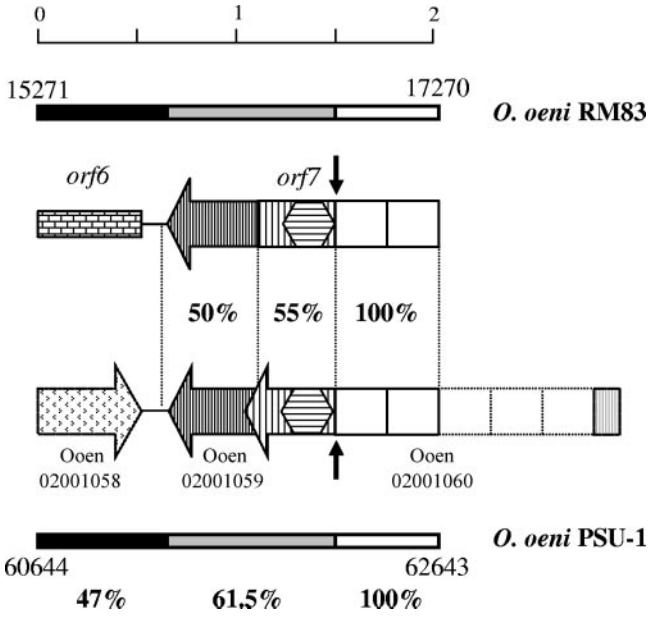

FIG. 3. Schematic overview of the sequence conservation between *O. oeni* PSU-1 and *O. oeni* RM83 chromosomal regions containing the proposed recombination site. Genes are represented by arrows. The rectangle corresponds to the interrupted gene. The genes present in these regions are indicated: *orf6* and *orf7* in *O. oeni* RM83 and Ooen02001058 (encoding a putative transcriptional terminator), Ooen02001059 (encoding the carbamoylphosphate synthase large subunit), and Ooen02001060 (coding for a hypothetical protein, ZP_00319317) in *O. oeni* PSU-1. The complete ORF coding for the hypothetical protein in *O. oeni* PSU-1 is also represented. Open squares and hexagons represent GW domains and MucBP domains, respectively. ORF regions with identical shading correspond to regions having the same degree of sequence identity. The degrees of amino acid identity between the protein fragments encoded by these ORFs are also shown. The colors of the upper and lower bars indicate the degrees of nucleotide identity between the 2-kb DNA regions: black, 47% identity; gray, 61.5%; and white, 100%. Two black arrows indicate the recombination site. The nucleotide positions corresponding to both sequences are also indicated. The *O. oeni* PSU-1 nucleotide sequence appears in the GenBank database under accession number NZ_AABJ03000005.

ing to amino acid 33, followed by five GW domains and a 130-amino-acid C-terminal end containing a MucBP (mucin-binding protein) domain (Fig. 3). GW and MucBP domains are found in bacterial cell wall bound proteins. Some putative surface proteins bind the bacterial surface by way of noncovalent interactions mediated by their C-terminal GW domains. These ~80-amino-acid-long domains contain the dipeptide Gly-Trp (GW modules). The MucBP domains consist of sequences of around 50 residues in length found in bacterial peptidoglycan bound proteins.

It is noteworthy that *orf7* in *O. oeni* RM83 appears to be a chimeric protein originating from the fusion of a gene encoding a protein 50% identical to the *O. oeni* PSU-1 putative carbamoyl phosphate synthase (Ooen02002059) and a gene encoding a protein 55% identical on its MucBP domain to the PSU-1 hypothetical protein (Ooen02002060). Taking into account that GW and MucBP domains are found in a variety of bacterial proteins, it is possible that the unknown donor protein could have domains encoded by DNA regions showing high nucleotide similarity with the corresponding *O. oeni* regions. This similarity could facilitate the crossover between this unknown donor DNA and *O. oeni* chromosomal DNA. Upon examination of regions of maximal identity, the crossover point appears to reside at nucleotide position 16747 of the *O. oeni* RM83 sequence described in this work and position 62120 of *O. oeni* PSU-1 (GenBank accession number NZ_AABJ03000005). This recombination site is located in the junction of the MucBP domain and the first GW domain. Interestingly, in *O. oeni* PSU-1 the gene coding for the hypothetical protein Ooen02001060 is found less than 5 kb downstream of the gene *recP*, coding for a transketolase. Recently, de las Rivas et al. described for the *recP* locus a possible example of a recombinatorial event from an unknown source (3). The description of two recombinatorial events in the same DNA region indicates a region of great flexibility in the *O. oeni* chromosome, as described recently for *L. plantarum* (17).

It is now understood that horizontal gene transfer provides an important mechanism for generating genotypic and phenotypic diversity in bacteria. This phenomenon has been studied extensively in relation to bacterial adaptability or fitness under certain growth conditions. Accordingly, it has been reported widely that adaptability traits can be encoded by mobile genetic elements. Genomic islands (GI) are clusters of chromosomal genes that have been described as horizontally acquired DNA regions (5). They often possess genes (or pseudogenes) coding for mobility-related elements, such as phage genes, insertion sequences, transposases, and origins of replication. A typical GI carries genes encoding traits that may increase bacterial adaptability under certain growth conditions. All of these observations taken together suggest that the 16.7-kb *O. oeni* RM83-specific DNA may be a fragment of a GI transferred by horizontal gene transfer.

Nucleotide sequence accession number. The DNA sequence of the *O. oeni* RM83 *odc* region has been deposited in the EMBL/GenBank database under accession number AJ746165.

This work was supported by grants AGL2005-00470 (CICYT), RM03-002 (INIA), and S-0505/AGR/000153 (CAM).

We thank E. García and D. Llull for their help with the pulsed-field gel electrophoresis experiments. We thank the *E. coli* Genetic Stock Center (http://cgsc.biology.yale.edu) for generously providing the *E. coli* HT414 strain. We also thank D. Sela for correcting the English version of the manuscript. The technical assistance of M. V. Santamaría and A. Gómez is greatly appreciated.

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