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Characterization of a Novel Integrative Element, ICE*St1*, in the Lactic Acid Bacterium *Streptococcus thermophilus*

VINCENT BURRUS, YVONNE ROUSSEL,† BERNARD DECARIS,* AND GÉRARD GUÉDON

Laboratoire de Génétique et Microbiologie, INRA UA952, Faculté des Sciences, Université Henri Poincaré (Nancy 1), 54506 Vandoeuvre-lès-Nancy, France

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The 35.5-kb ICE*St1* element of *Streptococcus thermophilus* CNRZ368 is bordered by a 27-bp repeat and integrated into the 3′ end of a gene encoding a putative fructose-1,6-biphosphate aldolase. This element encodes site-specific integrase and excisionase enzymes related to those of conjugative transposons Tn*5276* and Tn*5252*. The integrase was found to be involved in a site-specific excision of a circular form. ICE*St1* also encodes putative conjugative transfer proteins related to those of the conjugative transposon Tn*916*. Therefore, ICE*St1* could be or could be derived from an integrative conjugative element.

Cocultures of various lactic acid bacteria are used during the manufacture of dairy products. Sequence comparisons and hybridizations reveal that horizontal transfers between a large array of species of lactic acid bacteria have occurred, most likely during dairy cocultures (13, 32). The most convincing evidence indicates that insertion sequences IS*1191*, IS*981*, IS*S1*, and IS*1194* (4, 5, 14) and some open reading frames (ORFs) involved in exopolysaccharide synthesis (6) or in restriction-modification (24) were transferred between the lactic acid bacteria *Streptococcus thermophilus* and *Lactococcus lactis* in cocultures used during cheese manufacture. However, the mechanism of genetic exchange between these two species remains unknown, and no conjugative element has been previously characterized in *S. thermophilus*.

Cloning of *var1C* and localization of its limits. The Sm4 fragment of the *S. thermophilus* CNRZ368 chromosome was previously found to contain the 35-kb variable region *var1C*, which was absent from the corresponding chromosomal fragments of strains A054 and NST2280 (28). A region containing an IS*1191* copy inserted in a truncated IS*981* element (14) was cloned and found to be included in *var1C* (28). Chromosome walking using a λGEM11 genomic library of CNRZ368 (25) was performed to isolate recombinant λ bacteriophages overlapping the *var1C* region. Their inserts were subcloned in pBC KS+ and used as hybridization probes on A054 and NST2280 DNAs. S35, ES27, I132.3, ES13, and SC02 fragments hybridized to A054 and NST2280 DNAs. On the contrary, all of the probes covering the 35.5-kb region (except IS*1191* and IS*981*) and located between the *Hin*dIII sites H_L and H_R (Fig. 1) did not hybridize to A054 and NST2280 DNAs (data not shown). Furthermore, CNRZ368, A054, and NST2280 showed identical restriction maps in regions located to the left of the *Hin*dIII site H_L and to the right of the *Hin*dIII site H_R (Fig. 1). These data indicated that *var1C* limits are located near these *Hin*dIII sites. When ES27 including the left end and ES13 including the right end were hybridized to DNAs of the three strains digested by *Cla*I, *Eco*O109, *Eco*RI, *Pst*I, or *Xba*I, they revealed the same fragment from A054 and NST2280, but two different fragments from CNRZ368. Thus, the flanking regions of *var1C* in CNRZ368 are adjacent to each other in strains A054 and NST2280 (Fig. 1).

Because A054 and CNRZ368 are very closely related to each other, but distantly related to NST2280 (28), the absence of *var1C* in A054 and NST2280 probably results from an insertion in CNRZ368 rather than from two independent identical deletions in the two other strains.

***var1C* is bordered by a direct repeat and encodes an integrative system.** Sequencing of the *var1C* limits revealed that the element is bordered by a 27-bp direct repeated sequence (R1) containing a *Hin*dIII site (Fig. 2). A 362-bp fragment was obtained by PCRs performed with the DNA of *S. thermophilus* A054 by using the convergent primers O132.3 (GGACTACT AAGAGAACAT) and O131.2 (TGTTGCTGAATACGAA GC) (Fig. 3). The sequence of this fragment revealed a unique R1 copy identical to those found on either side of *var1C* in CNRZ368 (Fig. 2). Sequence comparison indicates that R1 direct repeats of CNRZ368 correspond to the boundaries of *var1C* (Fig. 2).

Two ORFs, *int* and *xis*, are located within *var1C* near the right copy of R1 (Fig. 1 and Table 1). The putative protein encoded by *int* shows significant similarities to site-specific recombinases belonging to the ϕLC3 subgroup of the integrase family (http://members.home.net/domespo/trhome.html). This subgroup includes a large array of integrases of temperate bacteriophages and conjugative transposons of lactic acid bacteria and other gram-positive low-G+C bacteria. The C terminus of Int contains the five amino acids which are perfectly conserved in this family (data not shown) (1, 3, 11). Furthermore, *xis*, located to the left of the *int* gene, encodes a small basic protein (pI 9.88) which show significant similarities to excisionases of two conjugative transposons, Tn*5252* of *Streptococcus pneumoniae* and Tn*5276* of *L. lactis* (Table 1). *int* and *xis* are located at comparable positions in many prophages and conjugative transposons.

Therefore, these ORFs probably encode an integrative system which would mediate excision of *var1C* by site-specific recombination between the two R1 copies corresponding to the cores of the left and right attachment sites *attL* and *attR*. The unique R1 sequence found in A054 would be the *attB* attachment site used for *var1C* integration. *fda*, which flanks the right of *var1C* (Fig. 1), encodes a putative fructose-1,6-biphosphate aldolase (Table 1). The 3′ end of *fda* includes 20

* Corresponding author. Mailing address: Laboratoire de Génétique et Microbiologie, INRA UA952, Université Henri Poincaré (Nancy 1), Faculté des Sciences, BP239, 54506 Vandoeuvre-lès-Nancy, France. Phone: (33) 3 83 91 21 93. Fax: (33) 3 83 91 25 00. E-mail: decaris@nancy.inra.fr.

† Present address: Department of Medical Microbiology, St. Bartholomew's Hospital, West Smithfield, London, United Kingdom.

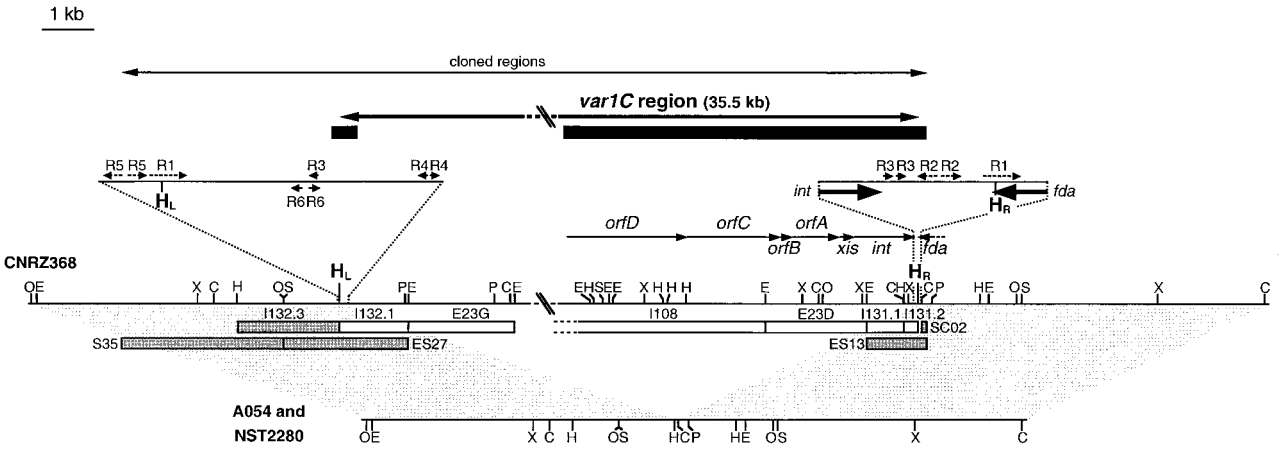

FIG. 1. Locations of *var1C* limits, ORFs, and repeated elements on a map comparison of the CNRZ368, A054, and NST2280 chromosomes. White boxes indicate probes hybridizing with CNRZ368 DNA only. Shaded boxes indicate probes hybridizing with the A054, NST2280, and CNRZ368 DNAs. Sequenced regions are shown by black boxes. Only the 3′ end of the ORF *fda* was cloned and sequenced. Arrows indicate ORF transcription direction. R1, R2, R3, R4, R5, and R6 repeated sequences are indicated by dotted arrows. H_L and H_R correspond to the *Hin*dIII sites included in the 27-bp repeated sequences delimiting *var1C*. C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; O, *Eco*O109; P, *Pst*I; S, *Sac*I; X, *Xba*I.

bp of the R1 core of *attR* (Fig. 2). Thus, *var1C* integration does not change the sequence of *fda*. Numerous integrative elements (e.g., prophages or integrative conjugative elements) integrate into the 3′ end of genes encoding tRNAs, their sequences remaining unmodified by the integration (8, 15, 17, 23, 30, 31). Other integrative elements (e.g., most of the conjugative transposons) integrate into several or numerous sites (19, 26). Only a few elements site specifically integrate into the 3′ end of protein-encoding genes. The substitution sequence is then generally only similar to the original one (10, 18).

An imperfect 14-bp inverted repeat, R2, is located 29 bp to the right of the 3′ end of the *int* gene and 21 bp to the left of the R1 core of *attR* (Fig. 1). The potential stem-loop structure ($\Delta G = -14.8$ kcal · mol^{-1}) (33), preceded by a stretch of A's and followed by a stretch of T's, could be used as a ρ-independent transcription terminator for both *int* and *fda*. A perfect 13-bp inverted repeat, R5 ($\Delta G = -18.8$ kcal · mol^{-1}), preceded by a stretch of A's, is located 2 bp to the left of the core of *attL* (Fig. 1) and could be used as a transcription termination signal for *fda* prior to the *var1C* integration. Therefore, these data suggest that the expression of *fda* would not be changed after *var1C* integration.

R3, a perfect 9-bp direct repeat, was found 2 bp downstream from the stop codon of *int* (Fig. 1). A copy of this 9-bp sequence was also found 148 bp to the right of the R1 core of *attL*. R6, an imperfect 12-bp inverted repeat, and R4, an imperfect 9-bp inverted repeat, are located 123 and 229 bp to the right of the core of *attL*, respectively. R2, R3, R4, and R6 could be binding sites for integrase or host-encoded proteins involved in the recombination.

Detection of site-specific recombination products. A nested PCR was performed to amplify the putative junction between the *var1C* termini, which could result from a site-specific recombination event between the R1 cores of *attL* and *attR*. Nested-PCR amplification was performed with the O132.5 (GATGAAATTCACATCATC)-O131.5 (CAGGAATCGAT ATTGACA) outer primer pair and the O132.4 (AGTTGAA ACTAGACTCAG)-O131.1 (TTCCGACATACGCATATC) inner primer pair (Fig. 3A) according to the method described by Manganelli et al. (21). As expected, no product was identified in strain A054 (Fig. 3B), which does not contain *var1C*. The sequence of the 536-bp PCR product obtained in CNRZ368 (*attI*, Fig. 2) is identical to the expected sequence resulting from site-specific recombination between the R1 cores of *attL* and *attR*. The PCR product was digoxigenin labelled and hybridized to *Eco*RI-digested A054 and CNRZ368 chromosomal DNA. As expected, this probe hybridizes with the two fragments containing the *var1C* termini in CNRZ368, but not with A054 DNA (data not shown). Site-specific excision of *var1C* in CNRZ368 should also lead to a junction between sequences flanking *var1C*, identical to that observed in A054. PCR amplification using the O132.3-O131.2 primer pair (Fig. 3A) was performed to detect this junction. PCR products obtained for A054 and CNRZ368 show the same size (Fig. 3B) and restriction map (data not shown).

Detection of these two junction fragments implies in

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                                        HindIII
attL   tttctggatc ttgttttta cAGTCTAATT AAGCTTTGTT TGCTGAACat aggttgattt attc
attR   cctttatttt caagttata tAGTCTAATT AAGCTTTGTT TGCTGAACca aatacatcga tacg
attB   tttctggatc ttgttttta cAGTCTAATT AAGCTTTGTT TGCTGAACca aatacatcga tacg
attI   cctttatttt caagttata tAGTCTAATT AAGCTTTGTT TGCTGAACat aggttgattt attc
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FIG. 2. Comparison of the nucleotide sequences of the four attachment sites. *attL* and *attR* include, respectively, the left and right termini of *var1C* of strain CNRZ368. *attB* corresponds to the partial sequence of a PCR product obtained from strain A054 with the primers O132.3 and O131.2. *attI* corresponds to the partial sequence of a nested-PCR product obtained from strain CNRZ368 with the primer pairs O132.5-O131.5 and O132.4-O131.1 (Fig. 3). R1 sequences are written in capital letters. The italic letters correspond to the internal sequence of *var1C*. Underlined letters indicate the bases that are complementary to the 3′ end of the *fda* gene encoding fructose-1,6-biphosphate aldolase. Sequences underlined twice correspond to the *Hin*dIII restriction sites included in R1.

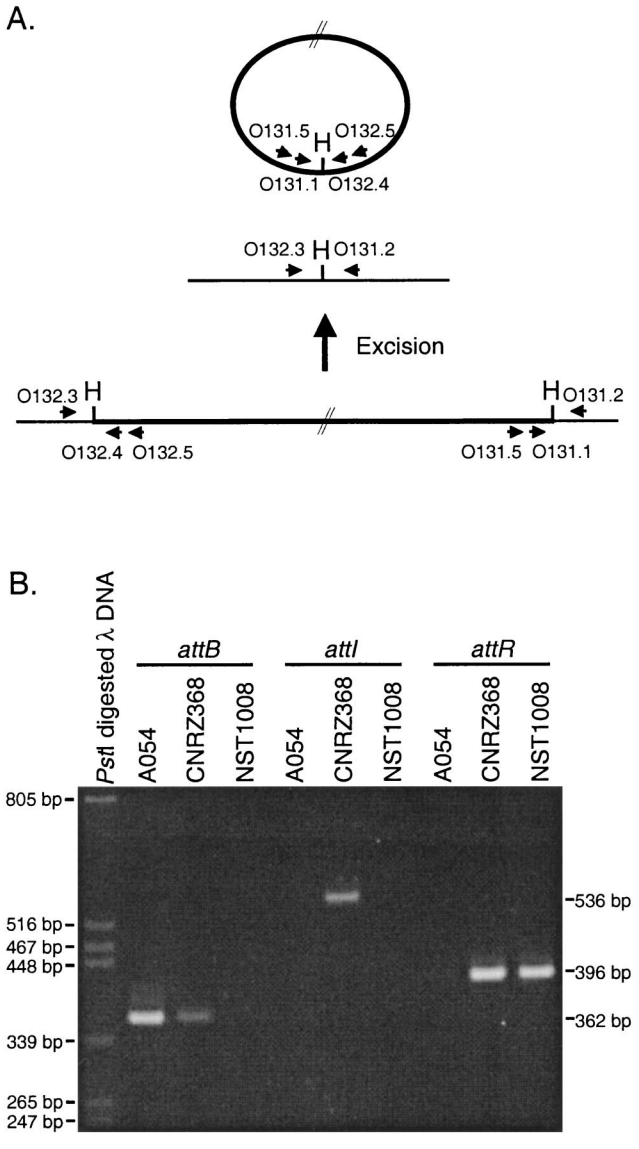

FIG. 3. PCR amplification results. (A) Model of excision of ICE*St1* and schematic localization of primer sets used to detect DNA molecules resulting from site-specific recombination events. H corresponds to the *Hin*dIII site included in the R1 cores of the *attL*, *attR*, *attB*, and *attI* attachment sites. (B) Electrophoresis of PCR products. *attB*, *attI*, and *attR* correspond to PCR with the O132.3 and O131.2 primers, nested PCR with the O131.5-O132.5 and O131.1-O132.4 primer pairs, or PCR with O131.1 and O131.2, respectively. The same volume (4 μl) of PCR product was loaded in each lane.

CNRZ368 the excision of a covalent circular molecule in some cells of the population. The R1 sequences found in the chromosome of A054, in the circular form of *var1C*, and in the ends of integrated *var1C* probably constitute the core of the *attB*, *attI*, *attL*, and *attR* attachment sites: the strand exchange reaction probably takes place by crossover events similar to those involved in λ integration and excision. The length of the core of attachment sites suggests that this element would show very strong insertional site specificity.

Disruption of the *int* gene prevents *var1C* excision. The ORF *int* was disrupted in order to prove its involvement in *var1C* excision. The thermosensitive plasmid pNST152 was constructed by subcloning the 754-bp *Hin*dIII fragment of pNST131.1 containing a fragment of *int* (region encoding residues 137 to 383 of the integrase) into pG+Host9 (20). pNST152 was used to transform *S. thermophilus* CNRZ368 by electroporation according to the method of Marciset and Mollet (22). Integration of pNST152 into the *int* gene was promoted by homologous recombination at a nonpermissive temperature (42°C). The integration site and the number of integrated copies were verified by hybridization of probe I131.1 to *Pst*I patterns of integrants (data not shown). The recombinant strain NST1008 contains two truncated copies of *int* resulting from the integration of a unique copy of pNST152 within the *int* gene of CNRZ368. Junction fragments containing *attB* or *attI* were not detected in NST1008 by PCR experiments (Fig. 3B), whereas a fragment bearing *attR* was amplified from NST1008 by using the O131.1 and O131.2 primers (Fig. 3). Therefore, *int* gene disruption causes the disappearance of the two junction fragments and, therefore, of the covalent circular molecule, showing that this gene is actually involved in *var1C* excision.

***var1C* encodes proteins related to those of some conjugative system.** The 5,881-bp region located to the left of the *xis* ORF start codon was sequenced. Four ORFs have been identified by GeneMark (http://genemark.biology.gatech.edu/GeneMark/) and/or by comparison of the putative translation products with proteins from the EMBL/GenBank databases by using BLASTX and BLASTP (2) (Fig. 1 and Table 1). All of these ORFs are preceded by a suitably located ribosome binding site (RBS) (27), have the same orientation as *xis* and *int*, and are spaced by very short sequences (Table 1). Therefore, *orfDCBA*, *xis*, and *int* could be translated from a unique transcript.

The *orfA* and *orfD* products share significant sequence similarities with proteins involved in conjugative transfer of plasmids from *Staphylococcus aureus* and Tn*916* from *Enterococcus faecalis* (Table 1). *orfC* encodes a putative protein weakly related to the translational product of *orf15* of the conjugative transposon Tn*916*. Topology predictions using the HMMTOP server (http://www.enzim.hu/hmmtop/) indicate that the proteins encoded by these two ORFs would be transmembrane proteins with similar tridimensional structures, suggesting that they are actually related. Thus, this region of *var1C* could encode conjugative functions. Various recently identified elements excise by forming a circular intermediate, promote self-transfer by conjugation into the recipient cell, and integrate by recombination between the specific site of the circular molecule and another site (17, 26, 29, 31). Therefore, the entire *var1C* sequence could be or could be derived from a site-specific integrative conjugative element. This possible conjugative element, which would be the first isolated in *S. thermophilus*, was named ICE*St1*, for integrative conjugative element of *S. thermophilus* no. 1.

The possible conjugative system of ICE*St1* is related to that of Tn*916*, but not to the system encoded by Tn*5252*. On the contrary, the ICE*St1* excisionase is related only to those of Tn*5276* and Tn*5252*. Moreover, the integrases of ICE*St1*, Tn*5276*, and Tn*5252* belong to the ϕLC3 integrase subfamily, whereas the integrase of Tn*916* belongs to another subfamily (http://members.home.net/domespo/trhome.html). Furthermore, differences in G+C content between the *xis* and *int* genes (about 34%) and *orfABCD* (about 42%) of ICE*St1* also suggest that the integration-excision system and the possible conjugative system have different origins or have undergone very different evolutions. A similar structure is observed in Tn*916* (about 36% G+C for the *xis* and *int* genes versus about 40% G+C for the conjugative system). This suggests that ICE*St1* and Tn*916* possess a modular structure which results from exchanges or acquisitions of sequences from different sources. This modular structure and evolution are similar to

TABLE 1. Characteristics of the sequenced ORFs and encoded proteins examined in this study

DNA sequence characteristic								Translation product characteristic		
ORF[a]	% G+C	Start	Stop	RBS[b]	$Dist_1$[c]	$Dist_2$[d]	Length (no. of amino acids)	Related protein[e]	Origin	% Identity (no. of amino acids)[f]
fda	38.3	ND[g]	TAA	ND	ND	ND	ND	Fructose-1,6-biphosphate aldolase Fba (AJ005697)	Chromosome of *S. pneumoniae*	80 (77)
int	33.6	ATG	TAA	TAAGGAGG	7	−1	448	Integrase Int (U93688)[h]	Pathogenicity island SaPI1 of *S. aureus*	26
								Integrase Int (M27965)[h]	Phage ϕL54a of *S. aureus*	28
								Integrase Int (M62697)[h]	Phage ϕadh of *Lactobacillus gasseri*	32 (227)
								Integrase Int (L27649)[h]	Conjugative transposon Tn*5276* of *L. lactis*	24
								Integrase Int (L29324)[h]	Conjugative transposon Tn*5252* of *S. pneumoniae*	26 (266)
xis	35.7	ATG	TAA	AAAGGAGT	5	+13	82	Excisionase Xis (L29324)	Conjugative transposon Tn*5252* of *S. pneumoniae*	41
								Excisionase Xis (L27649)	Conjugative transposon Tn*5276* of *L. lactis*	41
orfA	43.3	ATG	TAG	AAAGGAGA	4	+25	370	Putative transfer protein TraG (AF051917)	Conjugative plasmid pSK41 of *S. aureus*	30
								Putative transfer protein TrsG (L11998)	Conjugative plasmid pGO1 of *S. aureus*	30
								Immunogenic secreted protein Isp (U31811)	Chromosome of *Streptococcus pyogenes* D471	57 (325)
orfB	37.8	ATG	TAA	AGAGGAGA	5	+1	74	No similarity	ND	ND
orfC	41.5	ATG	TAG	TTAGGAGG	7	+11	626	Putative membrane protein Orf15 (U09422)	Conjugative transposon Tn*916* of *E. faecalis*	18 (267)
orfD	42.1	ATG	TAG	AAAGGAGG	4	ND	834	Putative transfer protein Orf16 (U09422)	Conjugative transposon Tn*916* of *E. faecalis*	21
								Unknown protein YddE (AB001488)	Chromosome of *B. subtilis* 168	30

[a] ORFs are listed from the right to the left of the map.
[b] The RBS consensus sequence of the gram-positive low-G+C bacterium *Bacillus subtilis* is AAAGGAGG.
[c] $Dist_1$, distance between the RBS and the start codon.
[d] $Dist_2$, distance between the start codon of an ORF and the stop codon of the previous ORF on the map (Fig. 1). A negative value indicates an overlapping of two ORFs.
[e] Functions of proteins and GenBank accession numbers (in parentheses) of nucleotide sequences encoding proteins related to the product of the ORFs sequenced in this study are indicated.
[f] Identities stretch over the entire length of each of the amino acid sequences of proteins encoded by ICE*St1*, except when indicated in parentheses.
[g] ND, not determined.
[h] Many other related integrases were found in databases, but in this table, we have only indicated a selection of the ones more related to the integrase encoded by *var1C*.

those of bacteriophages (9, 16) and enterobacterial plasmids (7).

The large size of ICE*St1* (35 kb) suggests that this element, like Tn*5276*, which encodes nisin synthesis (26), could carry industrially attractive genes. The ICE*St1* element contains a complete copy of IS*1191*, an insertion sequence probably transferred from *S. thermophilus* to *L. lactis*, and a truncated copy of IS*981*, which was probably transferred from *L. lactis* to *S. thermophilus*, most likely in cocultures of these species used during the manufacture of cheese (14). Furthermore, conjugative transposons related to ICE*St1*, like Tn*916* of *Enterococcus faecalis* and Tn*5252* of *S. pneumoniae*, are broad-host-range elements (12, 34). Therefore, ICE*St1* or elements related to ICE*St1* could be involved not only in intraspecific but also in interspecific horizontal transfers between *S. thermophilus* and other lactic acid bacteria.

Nucleotide sequence accession numbers. The GenBank accession numbers of the nucleotide sequences reported in this paper are AJ243105 (left terminus of *var1C*) and AJ243106 (right terminus of *var1C*).

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