Nucleotide Sequence and Characterization of the Broad-Host-Range Lactococcal Plasmid pWVO1

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The nucleotide sequence of the Lactococcus lactis broad-host-range plasmid pWVO1, replicating in both gram-positive and gram-negative bacteria, was determined. This analysis revealed four open reading frames (ORFs). ORF A appeared to encode a trans-acting 26.8-kDa protein (RepA), necessary for replication. The ORF C product was assumed to play a regulatory role in replication. Both RepA and the ORF C product showed substantial sequence similarity with the Rep proteins of the streptococcal plasmid pLS1. In addition, the plus origin of replication was identified on the basis of strong similarity with the plus origin of pLS1. Derivatives of pWVO1 produced single-stranded (ss) DNA in Bacillus subtilis and L. lactis, suggesting that this plasmid uses the rolling-circle mode of replication. In B. subtilis, but not in L. lactis, the addition of rifampicin resulted in increased levels of ssDNA, indicating that in the former organism the host-encoded RNA polymerase is involved in the conversion of the ssDNA to double-stranded plasmid DNA (dsDNA). Apparently, in L. lactis the conversion of ss to ds pWVO1 DNA occurs by a mechanism which does not require the host RNA polymerase.

The 2.2-kb cryptic plasmid pWVO1 was originally isolated from Lactococcus lactis subsp. cremoris Wg2 (Otto et al., 1982). A genetically marked derivative, pGK12 (Kok et al., 1984), was shown to replicate in a wide variety of gram-positive bacteria (such as bacilli, lactococci, streptococci, clostridia, and staphylococci) and gram-negative bacteria, including Escherichia coli.

At least two other small plasmids isolated from gram-positive bacteria, the pMV158-derived streptococcal plasmid pLS1 (Lacks et al., 1986; Van der Lelie et al., 1989) and the lactococcal plasmid pSH71 (De Vos, 1987), also replicate in gram-positive as well as gram-negative bacteria. In contrast, other plasmids, mainly isolated from Staphylococcus aureus and Bacillus species, like pT181 (Khan and Novick, 1983), pE194 (Horinouchi and Weisblum, 1982), pUB110 (McKenzie et al., 1986), pIM13 (Projan et al., 1987), pTA1060 (Bron et al., 1987), and pIJ101 (Kendall and Cohen, 1988), have a more limited host range and replicate only in a number of gram-positive bacteria. The mode of replication of most of these plasmids has been shown to involve single-stranded DNA (ssDNA) intermediates, most likely as a consequence of rolling-circle replication (RCR) (Gros et al., 1987; Gruss and Ehrlich, 1989). These plasmids will be denoted ssDNA plasmids. The rolling-circle mode of replication is analogous to that proposed for the ssDNA bacteriophages of E. coli, such as ΦX174, G4, and M13. However, these phages differ from each other in the mechanism of conversion of ssDNA to replicative form (RF) DNA (Baas and Jansz, 1988).

An essential plasmid-encoded function for RCR is the Rep protein, which introduces a single-strand nick at the plus origin. This results in the displacement of the plus strand and the concomitant synthesis of a new plus strand. The plus origins of the various plasmids from gram-positive bacteria have been classified into three groups on the basis of sequence similarities in the nick site region (Gruss and Ehrlich, 1989). Likewise, the cog-
nate replication initiator proteins have been classified in three families (Gruss and Ehrlich, 1989). All replication initiator proteins studied so far are able to act in trans (Novick et al., 1982; Projan et al., 1987).

For several ssDNA plasmids, minus origins (MOs) of replication have been described in addition to plus origins. Three families of MOs have been distinguished (Gruss and Ehrlich, 1989). These nonessential regions contain imperfect palindromic sequences which are used as initiation sites for the conversion of the displaced single strand to double-stranded (ds) plasmid forms. It is generally believed that the conversion is initiated by the host-encoded RNA polymerase (Gruss and Ehrlich, 1989), analogous to the mechanism of conversion of ssDNA to RF DNA of phage M13. The absence of a functional MO in RCR plasmids results in the accumulation of ssDNA intermediates and frequently in increased levels of plasmid instability (Boe et al., 1989; Bron et al., 1991; Del Solar et al., 1987; Gruss et al., 1987).

The ability of pWVO1 to replicate in both gram-positive and gram-negative bacteria and its wide use as a cloning vector prompted us to analyze the plasmid in more detail. The complete nucleotide sequence was determined and compared with known sequences of other plasmids. Analyses showed that pWVO1 belongs to the family of ssDNA-generating plasmids. The results also suggest that, at least in L. lactis, the priming of the lagging strand synthesis may not require the host RNA polymerase.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used are listed in Table 1. E. coli and B. subtilis were cultured in TY media (Rottlander and Trautner, 1970). M17 medium supplemented with 0.5% glucose (GM17) was used for culturing L. lactis (Terzaghi and Sandine, 1975). Erythromycin (Em) and chloramphenicol (Cm) were used at final concentrations of 5 μg/ml, both for B. subtilis and L. lactis. Ampicillin (Ap), Cm, and Em were used at final concentrations of 100, 10, and 100 μg/ml, respectively, for E. coli.

(Bio)chemicals

Chemicals were of analytical grade and were obtained from Merck (Darmstadt, FRG) or BDH (Poole, UK). Restriction enzymes, Klenow DNA polymerase, endonuclease S1, and T4 DNA ligase were used as indicated by the supplier (Boehringer-Mannheim, FRG).

DNA Techniques

DNA techniques were as described by Maniatis et al. (1982). Large-scale and mini-preparations of plasmid DNA from E. coli and B. subtilis were obtained essentially as described by Ish-Horowicz and Burke (1981) and Birnboim and Doly (1979), respectively, with minor modifications for L. lactis (Van der Lelie and Venema, 1987).

DNA Sequence Analysis

The complete nucleotide sequence of both strands of pWVO1 was determined by sequencing either single-stranded DNA of M13-derived subclones, or double-stranded plasmid DNA, using the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden) in the dideoxy chain termination method (Sanger et al., 1977). MicroGene software (Beckman, Palo Alto, CA) was used for computer-assisted sequence analysis.

Transformations

L. lactis cells were transformed by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) as described before (Leenhouts et al., 1990). Protoplasts of B. subtilis were prepared and transformed according to the method described by Chang and Cohen (1979). Competent cells of B. subtilis were prepared and transformed as described by Bron and Venema (1972). Transformation of E. coli cells
<table>
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<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant properties</th>
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<td>Bacteria</td>
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<td><em>E. coli</em></td>
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<td>JM101</td>
<td>supE, thi (lac-proAB), [F', traD36, proAB, lacZΔM15]</td>
<td>Yanisch-Perron et al. (1985)</td>
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| BL21 (DE3)                  | F'  ompT  Trp  
  R  
  lacZAM5   
  bacteriophage DE3 lysogen 
  carrying the T7 RNA polymerase gene 
  controlled by the lacUV5 promoter | Studier and Moffatt (1986) |
| *B. subtilis*               |                    |               |
| 8G5                         | Rif', trpc2, tyr1, met his, ura, nic, purA, rib | Bron and Venema (1972) |
| *L. lactis*                 |                    |               |
| MG1363                      | Rif', plasmid free derivative of L. lactis NCD0712 | Gasson (1983) |
| Plasmids                    |                    |               |
| pGKl                        | pWVO1, carrying the Cmr gene of pC194 (20) in the Mbol site | Kok et al. (1984) |
|                            | Cmr', pGK1 lacking the 431-bp Clal fragment | Kok et al. (1984) |
|                            | Ap'                 | Tabor and Richardson (1985) |
| pT713repA                   | Ap', pT713 with the 989-bp Mvnl-Mbol fragment of pWVO1 | This work |
|                            | Cmr', pGK1 lacking the 989-bp Mvnl-Mbol fragment of pWVO1 | This work |

was performed by the method of Mandel and Higa (1970).

**T7 RNA Polymerase-Directed Expression in *E. coli***

Protein samples of *E. coli* BL21 (DE3) were obtained as described by Studier and Moffatt (1986). SDS-polyacrylamide gel (12.5%) electrophoresis was as described by Laemmli (1970).

**Detection of ssDNA in Whole-Cell Lysates of *L. lactis* and *B. subtilis***

For the preparation of whole-cell lysates, cells were grown to an OD600 of 0.6–0.8. Cells from a 4.5-ml culture were harvested by centrifugation and washed once with buffer (0.15 M NaCl, 50 mM EDTA). The cells were resuspended in 193 μl lysis buffer supplemented with lysozyme (2 mg/ml) and mutanolysin (150 U/ml) and the mixtures were placed on ice for 10 min and subsequently incubated at 37°C for an additional 10 min. Lysis was completed by the addition of 7 μl 30% sarcosyl (Oramix L30; Seppic, Paris, France) and incubated for 30 min at 70°C in the presence of proteinase K (20 μg/ml). Lysates were then extracted twice with phenol and incubated at 37°C for 15 min in the presence of RNAse at a final concentration of 0.5 mg/ml. Two 30-μl portions were taken from each lysate. One of the portions was incubated with S1 nuclease (3300 U/ml) for 15 min at 37°C and the other was left untreated. After electrophoresis of the samples in 0.8% agarose/ethidium bromide gels, the DNA was transferred to GeneScreen Plus membranes (NEN Research Products, Boston, MA), as described by Chomczynski and...
Detection of the DNA was carried out using the ECL gene detection system (Amersham, Buckinghamshire, UK). Probe labeling and blot handling were carried out according to the suppliers' instructions.

Inhibition of RNA Polymerase by Rifampicin

Overnight cultures of *B. subtilis* and *L. lactis* were diluted 100-fold and grown to an OD600 of 0.6–0.8. De novo protein synthesis was then prevented by the addition of Em to a final concentration of 100 μg/ml and RNA polymerase activity was blocked by the addition of rifampicin to a final concentration of 100 μg/ml. Subsequently, the cultures were incubated for another hour at 37°C and immediately used for the preparation of whole-cell lysates.

RESULTS

Nucleotide Sequence of pWVO1

The complete nucleotide sequence of pWVO1 is shown in Fig. 1 and has a calculated GC content of 33.4%. Computer-assisted analysis revealed the presence of four open reading frames (ORF A through D), potentially capable of encoding polypeptides A, B, C, and D, with molecular sizes of 26.8, 7.6, 5.8, and 5.4 kDa, respectively. All ORFs have ATG as the putative start codon and show the same orientation. Upstream of ORF C, with a spacing of 8 bp, a potential ribosome binding site (RBS) was present. The free energy (ΔG°) of binding between this RBS (GGAG) and the 3'-end of the 16S rRNA of *L. lactis* (Ludwig et al., 1985) is −9.4 kcal/mol (Tinoco et al., 1973). Potential RBSs are also present upstream of ORFs A (AAGG) and D (GGGG) (calculated ΔG° values of −8.4 and −8.0 kcal/mol, respectively). The spacings between the putative RBSs and start codons of ORFs A and D are 10 and 7 bp, respectively. The ORF A stop codon (TGA) and ORF D start codon (ATG) partially overlap. Only one putative promoter was identified (upstream of the putative RBS of ORF C, from positions 522 to 527 [−35 region, TTGTTT] and positions 545 to 550 [−10 region, TACACT]; Van der Vossen et al., 1987). No potential transcription/translation signals could be identified upstream of ORF B which, therefore, is unlikely to encode a functional polypeptide.

Similarities of the Putative ORF A and C Products with Proteins Specified by the Streptococcal Plasmid pLS1

The putative products of ORFs A and C showed substantial similarity with two proteins encoded by the *Streptococcus agalactiae* plasmid pLS1 (Lacks et al., 1986). The ORF A protein showed 49.4% similarity to the replication initiator protein RepB; the protein encoded by ORF C showed 37.7% similarity to the pLS1 repA gene product, which was shown to be the repressor of the repB gene (Del Solar et al., 1989). An alignment of the amino acid sequences is presented in Fig. 2. The similarity of the ORFA and ORFC products of pWVO1 with known pLS1-encoded replication proteins suggests that the former proteins are involved in the replication of pWVO1.

ORF A Encodes a Trans-acting Replication Protein

To obtain further support for the idea that ORF A encodes an essential pWVO1 replication protein, we cloned the 989-bp MviI–MboI fragment, containing ORF A, between

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**Fig. 1.** Complete nucleotide sequence of pWVO1. The sequence is numbered starting at the first nucleotide at the cleavage site of the first *ClaI* restriction site upstream of ORF B. The deduced amino acid sequences of the potential ORFs are shown. Asterisks and underlined regions indicate potential RBSs and promoters, respectively. The thick arrows indicate inverted repeats (IR) and the thin arrows indicate direct repeats (DR). This nucleotide sequence has been submitted to the EMBL Data Library and assigned the accession number X56954.
Potential Secondary Structures and Iterated Sequences

Since both the plus and the minus origins in RCR plasmids from gram-positive bacteria have the potential to form secondary hairpin structures (Bron et al., 1989; Gros et al., 1987; Gruss and Ehrlich, 1989), we searched in the pWVO1 sequence for such regions of dyad symmetry. Six potential palindromic structures (Fig. 4) could be identified: from positions 50 to 181 (I, $\Delta G^o = -45.7$); 228 to 250 (II, $\Delta G^o = -14.8$); 334 to 382 (III, $\Delta G^o = -20.8$); 1656 to 1697 (IV, $\Delta G^o = -18.5$); 1719 to 1818 (V, $\Delta G^o = -12.5$); and 1929 to 2007 (VI, $\Delta G^o = -28.2$). The latter stem-loop structure can be formed within a 59-bp direct repeat, the first 18 nucleotides of which are repeated once more immediately following the 59-bp direct repeat. Due to the internal symmetry, alternative secondary structures are possible within this region. Inverted repeat (IR) III contains a region that is highly similar to the consensus sequence of the pE194-type plus origin (Gruss and Ehrlich, 1989; boxed regions in IR III of Fig. 4).
Fig. 4. Potential secondary structures and iterated sequences in pWVO1. The boxed regions in and near the stem-loop structure III represent the similarity with pE194-type plus origins. IR: inverted repeat; DR: direct repeat. The putative nick site as found for pLS1 is indicated with an arrow.
pLS1, containing a pE194-type plus origin, the nick site has been identified (De la Campa et al., 1990; see Fig. 4). This suggests that also in pWVO1 this region contains the plus origin of replication. Upstream and partially overlapping with IR III, at positions 305 to 341, a 17-bp imperfect repeat (three mismatches) is present. In addition, three direct repeats (of 11, 12, and 12 bp) are located downstream of IR III, at positions 456 to 490 (Fig. 4). Whether these regions are part of the plus origin is not clear at present.

Minus origins (MOs) are also known to be located within regions (200-300 bp) of dyad symmetry (Bron et al., 1988; Bron et al., 1989; Gruss et al., 1987; Gruss and Ehrlich, 1989; Van der Lelie et al., 1989). However, none of the potential palindromes in pWVO1 showed extensive sequence similarity with any known MO from other RCR plasmids.

Production of ssDNA Intermediates of pWVO1 Derivatives

The similarity between the plus origin and the replication protein of pWVO1 and those of the pE194-type plasmids suggested that pWVO1 is an RCR plasmid. Therefore, and, since the presence of an MO involved in the conversion of ssDNA intermediates could not be inferred from the pWVO1 sequence (see preceding section), the ability of pWVO1 to generate ssDNA was examined. In whole-cell lysates of B. subtilis containing either pGK1 or pGK11 (a deletion derivative of pGK1 which lacks the small 431-bp ClaI fragment and, consequently, the palindromic structures V and VI), significant amounts of ss plasmid DNA were present (about 30% of the total plasmid DNA mass; see Fig. 5A, lanes 1 and 2). Also in L. lactis some ss plasmid DNA was detectable; however, the amounts were low for either plasmid (<5% of the total plasmid DNA mass; Fig. 5A, lanes 3 and 4). These results confirm the idea that pWVO1 uses the rolling-circle mode of replication and that the potential stem-loop structures V and VI do not play a significant role in the conversion of ss to dsDNA.

Rifampicin Does Not Affect the Accumulation of ssDNA Intermediates in L. lactis

To examine the possible role of the host RNA polymerase in the conversion of ssDNA, rifampicin was added to cultures of rifampicin sensitive (Rif') L. lactis and B. subtilis strains carrying either pGKI or pGKII. Inhibition of RNA polymerase did not result in increased amounts of ssDNA intermediates of the pWVO1-derived plasmids pGK1 and pGK11 in L. lactis (Fig. 5B, lanes 3 and 4). In contrast, with pMV158, for which functional MOs have been described (Del Solar et al., 1987; Van der Lelie et al., 1989), an increase in the amount of ssDNA intermediates upon the addition of rifampicin was observed (results not shown). These results suggest that for plasmid pWVO1 the host-encoded RNA polymerase plays no significant role in the conversion of ss to dsDNA. In contrast, in B. subtilis the addition of rifampicin to cells harboring the pWVO1 derivatives resulted in a significant increase in the amount of ssDNA intermediates (Fig. 5B, lanes 1 and 2), indicating that in this organism RNA polymerase is involved in the conversion of ss to dsDNA.

DISCUSSION

In the studies described here the complete nucleotide sequence of the 2178-bp L. lactis subsp. cremoris Wg2 plasmid pWVO1 was determined and analyzed. Comparison of the sequence data of pWVO1 with those of pSH71, which was isolated from L. lactis subsp. lactis NCDO712, showed that these plasmids were nearly identical (W. M. de Vos, personal communication). The only significant difference was the absence of the 59-bp direct repeat in pSH71.

Four ORFs were present on the plasmid. ORF D was preceded by a putative RBS and is likely to form one transcriptional unit with ORF C and repA. Interestingly, the stopco-
don of the repA gene (TGA) partially overlaps the putative start codon (ATG) of ORF D. This suggests that the expression of ORF D might be translationally coupled to that of ORF C and repA. The putative product of ORF D was not required for replication, since ORF D could be inactivated by the insertion of an antibiotic-resistance marker in the RsaI site (position 1619) of pGK11 (unpublished results).

The similarity of RepA of pWVO1 with the RepB protein of pLS1 and the extensive homology with DNA sequences surrounding the nick sites of the pE194-family of plus origins of replication (De la Campa et al., 1990; Gruss and Ehrlich, 1989) suggests that pWVO1 is a member of the pE194-family of RCR plasmids. The putative protein C, encoded by the ORF preceding repA, showed substantial amino acid sequence similarity with RepA of pLS1. The latter protein has the characteristics of a repressor (Del Solar et al., 1989). For pSH71 it was shown that the ORF C product is a repressor at the transcriptional level for the ORF C-repA operon (W. M. de Vos, personal communication). In addition to the similarities on the amino acid and nucleotide level, there is also similarity in the structural organization of the minimal replicon of the pE194-family of RCR plasmids and that of pWVO1. In pE194, pLS1, pSH71, and pWVO1 the gene encoding the replication initiator protein is preceded by a gene encoding a regulatory protein in an operon-like structure. In all these plasmids the plus origin is located upstream of this operon (Byeon and Weisblum, 1990; Lacks et al., 1986; W. M. De Vos, personal communication).

In close vicinity to the plus origin direct repeats are present in pWVO1. Although such repeats are not present in most of the small RCR-type gram-positive plasmids, direct repeats have been found near the putative nick site of the Lactobacillus plantarum plasmids pC30i1 (Skaueng, 1989) and pLP1 (Bouia et al., 1989) and the broad-host-range plasmid pLS1 (Puyet et al., 1988). In the latter plasmid, three contiguous 11-bp direct repeats are located 73 bp downstream of the hairpin structure containing the nick site. Recently, it was shown that RepB of pLS1 binds to these direct repeats prior to the nicking of the plus origin (De la Campa et al., 1990). In pWVO1, three direct repeats of 11, 12, and 12 bp (the first has three mismatches compared to the others) are located 75 bp downstream of IR III. The repeats in these two plasmids also show mutual sequence similarity; the sequence 5'-TCGCCGCGTTT-3', present in the pLS1 repeats, resembles that in pWVO1 (5'-TCGCCAACGTTT-3'; three
mismatches). This similarity suggests an analogous role of these repeats.

In ssDNA-generating plasmids described so far, MOs are required for the efficient conversion of circular ssDNA intermediates into ds plasmid DNA (Boe et al., 1989; Bron et al., 1988; Gruss and Ehrlich, 1989; Lacks et al., 1986). Known MOs are part of large imperfect palindromic structures, generally consisting of 200–300 nucleotides. IRs V and VI could be removed from pWVO1 without affecting the production of ssDNA intermediates both in B. subtilis and L. lactis making it unlikely that these palindromic structures function as an MO. IR II and IR IV consist of only 40 nucleotides or less and, most probably, do not function as MOs. Indeed, IR IV is located just downstream of the putative transcriptional unit ORF C–repa–ORF D and has the characteristics of a transcriptional terminator.

The remaining stem–loop structure I, shown in detail in Fig. 6, shows some characteristics of an MO. The consensus sequence 5'-TAGCGT-3', present in the loop of palA-type MOs (Del Solar et al., 1987), is also located in the loop of structure I. In addition, palA-type MOs contain a so-called RSB-site at the 3'-base of the stem, containing the 18-bp consensus sequence 5'-AAGTTTTCTCGGCATAAA-3' (Gruss et al., 1987; Novick et al., 1984). A similar sequence (72.2% identical to this consensus) is present in the stem of IR I, however, at the 5'-end. At present, it is not clear whether this region constitutes a functional MO for L. lactis and B. subtilis. Although palA-type MOs are usually inefficient in B. subtilis (Gruss et al., 1987; Novick et al., 1985), it has been shown in our group that the palA MO of pLS1 has activity in this organism (W. Meijer, personal communication).

RNA polymerase has generally been considered to initiate lagging strand synthesis at MOs of RCR plasmids (Boe et al., 1989; Gruss and Ehrlich, 1989). This was, indeed, observed with pWVO1 in B. subtilis. However, the conversion of the ssDNA intermediates to ds pWVO1 DNA did not appear to require the host-encoded RNA polymerase in L. lactis, since no inhibitory effect of rifampicin was observed. This observation

![Fig. 6. Comparison of IR I of pWVO1 with complementary strand origin of ΦX174 and palA-type minus origins. The boxed regions display the similarity between IR I and the complementary strand origin of ΦX174. The asterisks indicate the similarity between IR I and a consensus sequence present in the loop of palA-type minus origins (see text). The arrow spans a sequence in hairpin I with homology to the consensus RSB sequence (see text).](image-url)
strongly suggests that initiation of lagging strand synthesis of pWVO1 in Lactococcus differs in this organism from that of other RCR plasmids in other bacteria. In this context, it is noteworthy that IR I shows some sequence similarity with the complementary strand origin of ΦX174 (Fig. 6). This bacteriophage uses the primosome complex for complementary strand synthesis, which is not inhibited by rifampicin (Baas and Jansz, 1988). Further experiments will be needed to clarify whether pWVO1 is an RCR plasmid with a new type of MO.

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REFERENCES


NUCLEOTIDE SEQUENCE OF LACTOCOCCAL PLASMID pWVO1

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