Theta replication of the lactococcal plasmid pWVO2

Rense Kiewiet, Sierd Bron,* Karel de Jonge, Gerard Venema and Jos F. M. L. Seegers
Department of Genetics, Centre of Biological Sciences, Kerklaan 30, 9715 NN, Haren, The Netherlands.

Summary
pWVO2 is a 3.8 kb narrow-host-range plasmid from Lactococcus lactis ssp. cremoris Wg2, which does not replicate in Bacillus subtilis or Escherichia coli. Single-stranded pWVO2 DNA was not observed in lactococcal cells, indicating that this plasmid does not replicate via a rolling-circle mechanism. The sequence of pWVO2 neither showed the structural organization typical for rolling-circle plasmids, nor were sequence similarities with known rolling-circle plasmids present. By 2-D agarose gel electrophoresis of replication intermediates, it was shown that pWVO2 replicates via a theta mechanism. This is the first proof for the existence of theta-replicating plasmids in lactococci. The pWVO2 minimal replicon is strongly related to that of several other lactococcal plasmid replicons. It contains one open reading frame encoding the replication protein, which is preceded by a 22 bp sequence tandemly repeated three and a half times. Further upstream is another 10 bp direct repeat present in an A/T-rich sequence. This structural organization resembles that of several iteron-containing theta-type plasmids from E. coli. Derivatives of pWVO2 were stably maintained in L. lactis and are good candidates for the development of stable food-grade cloning vectors for this organism.

Introduction
The best-characterized lactococcal plasmids pWVO1 and pSH71, from which most of today’s cloning vectors for lactococci are derived (Leenhouts et al., 1991; De Vos, 1987; Gruss and Ehrlich, 1989), belong to the rolling-circle-replicating (RCR) family of plasmids. Although based on DNA sequence information, Hayes et al. (1991) and Horng et al. (1991) suggested that other lactococcal plasmids may use theta replication, proof for this mode of replication has not yet been described.

A characteristic difference between RCR and theta plasmids is that the former generate single-stranded DNA (ssDNA) replication intermediates (te Riele et al., 1986a,b; Gruss and Ehrlich, 1989), whereas the latter do not. The absence of single-stranded plasmid DNA is, however, no proof that RCR does not occur. With some RCR plasmids the ssDNA is so rapidly converted to double-stranded DNA that it can hardly be detected. Proof for theta replication, therefore, requires additional evidence. Further indirect support can be obtained from the nucleotide sequence of the plasmid. All RCR plasmids characterized so far are highly interrelated (Gruss and Ehrlich, 1989). New RCR plasmids are therefore expected to show sequence similarity with known RCR plasmids and to show a comparable structural organization.

Direct proof for theta replication can be obtained from the analysis of replication intermediates, either by 2-D agarose gel electrophoresis (Brewer et al., 1988; Brewer and Fangman, 1987; Bruand et al., 1991; Le Chatelier et al., 1993), or by electron microscopy. Theta replication proceeds through branched intermediates which, because of their distinct topological properties, migrate slower in agarose gels than unbranched, linear DNA molecules of comparable mass. The 2-D pattern of replication intermediates of theta plasmids differs markedly from that of RCR plasmids. The 2-D electrophoresis pattern can also give indications about both the location of the origin and the direction of replication.

The aim of the present studies was to analyse the mode of replication of the lactococcal plasmid pWVO2. Since this plasmid is relatively small and previous hybridization experiments suggested a relationship between pWVO2 and the RCR plasmid pWVO1 (Van der Lelie et al., 1988), we suspected that it might replicate via the RC mechanism.

The entire nucleotide sequence of pWVO2 was determined and we showed by 2-D agarose gel electrophoresis of replication intermediates that this plasmid uses the theta mode of replication. This is the first proof for the existence of theta-type plasmids in lactic acid bacteria. pWVO2 derivatives were found to be stably maintained in lactococci.

Results
General properties of pWVO2
Lactococcus lactis ssp. cremoris Wg2 contains five different plasmids, including pWVO2 (3.8 kb), which range in
Absence of single-stranded DNA in cells harbouring pWVO2

RCR plasmids produce ssDNA replication intermediates (te Riele et al., 1986a,b; Gruss and Ehrlich, 1989). To study whether pWVO2 generates ssDNA, total cellular DNA extracts of L. lactis (pWVO2) were separated on agarose gels. After blotting onto nitrocellulose membranes, plasmid DNA was detected with a pWVO2-derived probe. No single-stranded pWVO2 DNA could be detected, even after prolonged exposure of the film (Fig. 2). In parallel experiments with pGS003, a pWVO1-derived RCR plasmid lacking its SSO (single-strand origin of replication) for the conversion to double-stranded plasmid DNA, ssDNA could clearly be detected. Also with pGK1, a pWVO1 derivative that carries the efficient SSO of this plasmid (J. Seegers, unpublished results), some ssDNA was still detectable after prolonged exposure of the film. The absence of single-stranded pWVO2 DNA is a first indication that this plasmid does not replicate via the RCR mechanism.

Nucleotide sequence of pWVO2

pWVO2 was linearized at its unique Clal site (Fig. 1) and cloned into the Clal site of pBluescript-IISK+ (Stratagene). The resulting plasmid was denoted pJR02. This plasmid did not replicate in L. lactis. A set of deletion derivatives of pJR02 was generated using the Exo III/mung bean method. Deletions in one direction were created using the SacI site as the 3' protected end and the EcoRI site as the 5' starting point for exonuclease III. Deletions in the other direction were created with the KpnI site as the 3' protected end and the SalI site as the 5'.

Fig. 1. Map of pLR300. The region representing pWVO2 is indicated with a heavy line. The insert (indicated with a thin line), containing the erythromycin-resistance gene of pE194, was inserted into the HpaI site of pWVO2, located at position 1 of the sequence (see Fig. 3). Relevant restriction sites are indicated. Drs: 22 bp sequence repeated three and a half times. The detailed structure of the region with large direct repeats ('repeats') is given in Fig. 3B.

pLR300 (Fig. 1) is a genetically marked derivative of pWVO2 which carries the EmR gene from plasmid pE194 (the functions indicated in the inner circle of Fig. 1 are discussed in the paragraph on the sequence analysis). pLR300 replicates in lactic acid bacteria, like L. lactis and Pediococcus acidilactici, but attempts to establish this plasmid and other derivatives of pWVO2 in Bacillus subtilis or Escherichia coli failed. This indicates that, in contrast to the broad-host-range RCR plasmids based on pWVO1 (Kok et al., 1984), pWVO2 has a narrow host range. The copy number of pWVO2 and pLR300 in L. lactis was about 5–10 per chromosome equivalent.

Fig. 2. Detection of ssDNA in total cellular DNA extracts of L. lactis strains carrying either A, the pWVO1-derived RCR plasmid pGS003 lacking its SSO; B, the pWVO1-derived RCR plasmid pGK1 containing the SSO; or C, pWVO2. The ssDNA in lane B could only be detected after prolonged exposure of the film. The position of ss DNA is indicated. Probes for detection of DNA were prepared from either pGK1 (lanes A and B) or pLR300 (lane C).
starting point. The resulting deletion derivatives were sequenced using the M13 universal and reverse primers (Short et al., 1988). The sequence (Fig. 3A) revealed an open reading frame (ORF) with extensive homology (75% similarity at the deduced amino acid level) to the gene encoding the replication protein of pCI305 (Hayes et al., 1991) and several other lactococcal plasmids (Horng et al., 1991; Jahns et al., 1991). In the case of pCI305 the non-coding region upstream of this ORF was denoted repA and the ORF itself repB (Hayes et al., 1991). Analogous to this plasmid, we denoted the related ORF of pWVO2 as repB. repB is followed by an inverted repeat with features typical for transcriptional terminators. The ClaI site used for cloning was located within repB. This explains why pJR02 did not replicate in L. lactis. In pWVO2 the repB gene is preceded by a 22 bp sequence tandemly repeated three and a half times (Fig. 1; Drs).

Two additional inverted repeats (IRs) are present within the promoter region. Within the 22 bp repeats, the DnaA box consensus sequence for B. subtilis can be identified three times (co-ordinates 455 to 463, 477 to 485 and 499 to 507 of the sequence, complementary to the sequence shown in Fig. 3A). An additional putative DnaA box is located between co-ordinates 176 to 184 of the sequence.

Upstream of the 22 bp repeats, an AT-rich sequence (co-ordinates 250 to 400) is present containing an additional 10 bp imperfect direct repeat (co-ordinates 356 to 380). Outside this region, from nucleotides 1985 to 3774, a 1.8 kb fragment was identified that consists entirely of an AT-rich sequence.

The results are summarized in Fig. 4. The 1.8 kb region containing the a-lacZ gene was denoted pLR351 (Table 1). A set of deletion derivatives was created in the manner described for the sequencing of pWVO2. The resulting plasmids were tested for their ability to replicate and to be maintained in L. lactis. Deletion endpoints of relevant plasmid derivatives were determined by DNA sequence analysis. The results are summarized in Fig. 4. The 1.8 kb region containing the long direct repeats A, B, and C (Figs 3A and 3B) could be deleted without interfering with the replication or the stable maintenance of the plasmid (plasmid loss was not observed after 100 generations of growth in the absence of erythromycin). Unstable derivatives (40% of the cells were plasmid-free after 60 generations of growth in the absence of erythromycin) were obtained, however, when the deletions extended into the inverted repeat (nucleotide 1900), which we consider to be the transcriptional terminator of repB. The smallest functional replicon was delimited to a 1.7 kb fragment (relevant deletion endpoints: nucleotides 203 and 1900) containing the repB gene and its upstream region. A functional repB

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Properties</th>
<th>Source/Reference</th>
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<tr>
<td>JM101</td>
<td>pIL252</td>
<td>EmR&lt;sup&gt;+&lt;/sup&gt;, 4.7 kb; unstable pAMB1 deletion derivative</td>
<td>Simon and Chopin (1988)</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pWVO2</td>
<td>3.8 kb; cryptic plasmid from L. lactis</td>
<td>Otto et al. (1982)</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pLR300</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt;, 5.6 kb; pWVO2 derivative containing in the HpaI site the Em&lt;sup&gt;+&lt;/sup&gt; gene from pMTL23E as NruI-EcoRV fragment</td>
<td>This work</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pJR02</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, 6.7 kb; pBSK+ with pWVO2 in its ClaI site</td>
<td>This work</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pLR351</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, 7.8 kb; fusion between pWVO2 and pBSK+</td>
<td>This work</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pGK1</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt;, 2.0 kb; pGK1 without the SSO</td>
<td>Kok et al. (1984)</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pGJS003</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt;, 4.0 kb; pMTL23 derivative (Chambers et al. 1998) containing Em&lt;sup&gt;+&lt;/sup&gt; in the ClaI site (pE194cop6 co-ordinates 3140 to 1938) and the T1T2 transcriptional terminator (550 bp, (Brogiolo et al., 1981)) in the EcoRI site</td>
<td>This work</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pBSK+</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, 2.9 kb; pUC-like vector containing the a-lacZ gene</td>
<td>This work</td>
</tr>
<tr>
<td>L. lactis</td>
<td>pEBSK+</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, 4.0 kb; pBSK+ containing the Em&lt;sup&gt;+&lt;/sup&gt; gene (see pMTL23E) between its SspI sites</td>
<td>This work</td>
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**Minimal replicon of pWVO2**

To determine the minimal region of pWVO2 required for replication, pWVO2 was linearized with HpaI and inserted into pBluescript-IISK+. The plasmid was marked with the erythromycin-resistance marker of pE194 in the SspI site of pBluescript-IISK+ for selection in L. lactis. This plasmid was denoted pLR351 (Table 1). A set of deletion derivatives was created in the manner described for the sequencing of pWVO2. The resulting plasmids were tested for their ability to replicate and to be maintained in L. lactis. Deletion endpoints of relevant plasmid derivatives were determined by DNA sequence analysis. The results are summarized in Fig. 4. The 1.8 kb region containing the long direct repeats A, B, and C (Figs 3A and 3B) could be deleted without interfering with the replication or the stable maintenance of the plasmid (plasmid loss was not observed after 100 generations of growth in the absence of erythromycin). Unstable derivatives (40% of the cells were plasmid-free after 60 generations of growth in the absence of erythromycin) were obtained, however, when the deletions extended into the inverted repeat (nucleotide 1900), which we consider to be the transcriptional terminator of repB. The smallest functional replicon was delimited to a 1.7 kb fragment (relevant deletion endpoints: nucleotides 203 and 1900) containing the repB gene and its upstream region. A functional repB
gene was essential: interruption of this gene by cloning the Em\(^R\) gene from pE194 into its CiaI site abolished autonomous replication in *L. lactis*. A deletion extending downstream from co-ordinate 1643, removing the last 43 amino acids from the putative replication protein, also abolished replication. In the upstream region of repB the three and a half times repeated 22 bp sequence, as well as the twice repeated sequence in the AT-rich region, were essential for replication. So far, we do not know whether the region upstream of the latter repeats (from position 356 to 203) is essential for replication, since no deletions ending in this region were studied.

**Fig. 3.** A. Nucleotide sequence of pWVO2. Possible promoter and ribosome-binding-site regions upstream of repB are indicated by lines under the sequence. The deduced amino acid sequence of the RepB protein is indicated by the single letter code. The direct repeats upstream of the repB gene are indicated by single line arrows; the inverted repeats by double line arrows. Putative DnaA boxes are indicated in bold letters. The inverted repeat (indicated as double line arrows) downstream of repB represents the putative transcriptional terminator. The locations of other regions of interest are indicated in the text. Start and endpoint of the large repeats A, B and C are indicated. This sequence is available in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number Z22704.

B. Structure of the 1.8 kb direct repeated region, located between nucleotide positions 1990 and 3773. It consists of three different repeats: A, 50 bp; B, 106 bp; and C, 204 bp, in a head-to-tail arrangement without spacing sequences.

two branches, depending on the position of the restriction site relative to the origin of replication. In the first dimension of the 2-D gel electrophoresis, the linearized DNA molecules are separated according to mass and in the second dimension the migration rates are mainly determined by the number, length and topology of the branches.

**Analysis of replication intermediates by 2-D agarose gel electrophoresis**

Upon linearization with restriction enzymes, theta-replicating molecules will contain either a bubble, or one or more than one bubble. The bubble in the middle of the molecule is between the two points of replication, and the size of the bubble varies depending on whether the enzyme cuts the linear molecule directly or whether a number of restriction sites are present.
position of linear monomers and extending towards the position of circular dimers, is strong evidence for theta replication. The arc represents bubble-shaped replication intermediates which can only be formed by theta replication.

Discussion

In this paper we describe the characterization of the lactococcal plasmid pWVO2 and show that this small, naturally occurring, plasmid (3.8 kb) replicates via a theta mechanism. In contrast to pWVO2, most plasmids from Gram-positive bacteria smaller than about 10 kb are of the RCR type (Gruss and Ehrlich, 1989). The evidence for theta replication of pWVO2 was based on three observations: (i) unlike RCR plasmids, pWVO2 did not generate detectable amounts of ssDNA replication intermediates; (ii) the minimal replicon of pWVO2 showed similarities with known theta plasmids from E. coli, but not with the highly conserved replication regions of RCR plasmids (Gruss and Ehrlich, 1989); (iii) 2-D agarose gel electrophoresis revealed replication intermediates which can only be explained on the basis of theta replication.

The nucleotide sequence of pWVO2 (3828 bp) revealed a sequence that was homologous to the replication regions of a number of recently described lactococcal plasmids, such as pCI305 (Hayes et al., 1991), pSK11L (Horng et al., 1991), and pSL2 (Jahns et al., 1991). All of the latter sequences contain an ORF that was shown to specify the replication protein. Evidence that this ORF (denoted as repB) also encodes the replication protein of pWVO2 was obtained from two experiments. First, the insertional interruption of repB at the internal Clal site destroyed the plasmid’s ability to replicate in L. lactis. Second, repB was shown to form part of the minimal replicon. Upstream of this ORF, all plasmids of this family contain a 22 bp sequence repeated three and a half times, preceded by an AT-rich sequence in which a 10 bp direct repeat was present. Structures of dyad symmetry could be identified within the promoter regions of all these plasmids. Replication of a pMTL23E-derived vector, containing this upstream region, could be obtained when the repB gene was supplied in trans (R. Kiewiet et al., in preparation). This experiment shows that this region forms the minimal origin required for replication. In the 22 bp iterons three potential DnaA box sequences, resembling those of B. subtilis (Moriya et al., 1988), are present. These boxes are also present in the 22 bp
repeats of the previously described lactococcal plasmids of this family. An additional potential DnaA box, located at position 175 to 183 of the sequence, could not be detected within the sequence of the other plasmids. Although it is tempting to speculate that the DnaA boxes play a role in the initiation of replication of these plasmids, this possibility has not yet been analysed.

The 2-D agarose gel electrophoresis patterns of replication intermediates gave the most direct proof for the theta mode of replication of pWVO2. In particular, the presence of bands representing bubble-shaped molecules was convincing evidence for this conclusion. Although we had anticipated being able to identify the start site of replication (origin), and the direction of replication of pWVO2, the 2-D gel patterns obtained did not allow us to draw conclusions in this respect. The great variety of replication intermediates obtained, combined with the similarity in the patterns obtained with the BamHI and BglII digests, leaves severa possible configurations open. Both bidirectional and unidirectional replication, starting from one unique origin, should reveal ‘switchpoints’ in at least one of the 2-D patterns. These switchpoints result from the passage of the replication fork through the restriction site that is used for linearization of the molecules. In the case of unidirectional replication the pattern should switch from bubbles to simple Y structures, while in the case of bidirectional replication a switch from bubbles to complex Y structures should occur. The patterns obtained however, did not reveal any switchpoints. At least two possible explanations can be envisaged for this observation. The first is that replication starts at various sites on the plasmid molecule. As far as we are aware, this type of replication has only been described for eukaryotic systems (Mahbubani et al., 1992; Hyrien and M’chali, 1992; Heck and Spradling, 1990; Vaughn et al., 1992; Burnett et al., 1989). The second explanation is that replication starts from a fixed origin, but proceeds in an asymmetric, bidirectional manner. In that case each single replicating molecule would show a switchpoint, which would be located at different positions in different molecules. The overall 2-D pattern would then have a smooth appearance. Asymmetric replication from a fixed origin has only been described for the E. coli plasmid R6K (Lovett et al., 1975). In that case, however, replication initially proceeds in one direction until it is stopped at a termination site, after which replication in the opposite direction starts. Depending on the restriction site used, such a mode of replication would result in patterns similar to that of unidirectional replication. The present results do not allow us to discriminate between these two possibilities.

Based on the results obtained so far, we favour the following model for pWVO2 replication. We speculate that, like the iterated sequences of several theta plasmids from Gram-negative bacteria, the repeated sequences of pWVO2 are involved in the initiation of replication through the binding of the RepB protein (Abeles, 1986; Vocke and Bastia, 1983). Possibly, RepB binding is stimulated by DnaA binding to its recognition boxes in the repeated region (Mukhopadhyay and Chattoraj, 1992). The RepB protein is believed to bind to the 22 bp iterons and to mediate, directly or indirectly, the opening of the DNA helix at several possible sites on the plasmid, including sites more distantly located from the RepB binding site. Once replication has started from one of these sites, no other start sites will be used on the same molecule. The latter idea is supported by the observation that structures predicted to result from multiple initiations (like bubble/Y shapes or double-bubble shapes) were not observed.

The present results showed that the region upstream of the 22 bp Drs forms part of the minimal replicon of pWVO2. This region consists of an AT-rich sequence and a 10 bp repeat. Analogous to the E. coli plasmid pSC101, in which a 13 bp direct repeat, present within an AT-rich sequence, is assumed to function as the entry site for DnaB and DnaC, it is tentative to speculate that analogous host functions recognize these repeats in pWVO2 and are involved in replication of this plasmid.

The observation that the sequence and structural organization of the replication regions of several lactococcal plasmids are similar to those in pWVO2 makes it likely that all plasmids belonging to this family replicate via the same (theta) mechanism.

pWVO2 derivatives, including its minimal replicon, were segregationally totally stable for at least 100 generations of growth of host cells in the absence of selective pressure, which can be expected with a copy number of 10 per chromosome. No additional functions that might contribute to its stable inheritance could be identified. Deletion of the large repeated region did not affect its copy number or segregational stability. In the past it has frequently been shown that derivatives of RC plasmids show a high level of instability, segregationally or structurally (Bron et al., 1987; 1991; Gruss and Ehrlich, 1989; Kiewiet et al., 1993), whereas derivatives of the theta replicating plasmid are considerably more stable (Jan-nière et al., 1990; Kiewiet et al., 1993). Based on these observations, we anticipate that pWVO2 has a good potential for the development of stable food-grade, cloning vectors for lactic acid bacteria.

Experimental procedures

Bacterial strains, biochemicals and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. E. coli strain JM101 (supE, thi(lac-proAB) [F’traD36 proAB lacI’ZM15]) (Yanisch-Perron et al., 1985) was used for cloning and sequencing. E. coli cells were
transformed as described previously (Mandel and Higa, 1970) and plated on LB agar or grown in LB medium (Sambrook et al., 1989). When required, the media were supplemented with 100 μg ml⁻¹ erythromycin. L. lactis ss. lactis MG1363 (Gasson, 1983) was used as a host for plasmid DNA preparations and as recipient in transformations. L. lactis cells were transformed by electroporation (Holo and Nes, 1989) and plated on M17 agar, containing 0.5 M sucrose. Transformants were grown in M17 medium (Terzaghi and Sandine, 1975). When required, the media were supplemented with 5 μg ml⁻¹ erythromycin. Chemicals used were obtained from Merck or BDH. Enzymes for DNA manipulations were from Boehringer or Promega Biotec.

**Cloning procedures and the isolation of DNA**

Cloning procedures and the isolation of DNA were essentially as described by Sambrook et al. (1989). Double-stranded DNA sequencing was performed by the dideoxyribonucleotide chain termination method of Sanger et al. (1977). Deletions for sequencing and determination of the minimal replicon were made with Exo III and mung bean nuclease as described by the supplier (Stratagene).

**Copy number determination, detection of single-stranded DNA and plasmid stability assays**

Plasmid copy numbers per chromosome equivalent were determined by the densitometric procedure as described by Jannière et al. (1985). The method used for the detection of single-stranded plasmid DNA was described before by Leenhouts et al., 1991. Assays for segregational plasmid stability were performed as described by Kiewiet et al. (1993).

**Isolation of plasmid replication intermediates**

Replication intermediates of the pWWVO2 derivative pLR300 were isolated as described for the isolation of plasmid DNA from cleared lysates of B. subtilis (Bron, 1990) with minor modifications. In this method, alkaline treatments, which would cause the loss of the desired replication intermediates, were avoided. The following modifications were used. Lactococcal cells were grown at 30°C, without shaking. To enhance lysis, mutanolysin was added to the lysis solution. Replication intermediates were recovered from CsCl/ethidium bromide gradients as the DNA fraction present between the covalently closed circular plasmid and chromosomal DNA, as described by Bruand et al. (1991).

**Detection of replication intermediates**

Total cellular DNA was treated with restriction enzymes that linearized the plasmid. Two-dimensional agarose gels were run as described by Brewer et al. (1988). Transfer of DNA from gels onto nitrocellulose filters was performed as described by Sambrook et al. (1989). The preparation of DNA probes, hybridization and detection of DNA were conducted with the ECL gene detection system (Amersham) as recommended by the manufacturer.

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**References**


