Conserved Organization in the cps Gene Clusters for Expression of Escherichia coli Group 1 K Antigens: Relationship to the Colanic Acid Biosynthesis Locus and the cps Genes from Klebsiella pneumoniae

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Group 1 capsules of Escherichia coli are similar to the capsules produced by strains of Klebsiella spp. in terms of structure, genetics, and patterns of expression. The striking similarities between the capsules of these organisms prompted a more detailed investigation of the cps loci encoding group 1 capsule synthesis. Six strains of Klebsiella pneumoniae and 12 strains of E. coli were examined. PCR analysis showed that the clusters in these strains are conserved in their chromosomal locations. A highly conserved block of four genes, orfX-wza-wzb-wzc, was identified in all of the strains. The wza and wzc genes are required for translocation and surface assembly of E. coli K30 antigen. The conservation of these genes points to a common pathway for capsule translocation. A characteristic JUMPstart sequence was identified upstream of each cluster which may function in conjunction with RfaH to inhibit transcriptional termination at a stem-loop structure found immediately downstream of the "translocation-surface assembly" region of the cluster. Interestingly, the sequence upstream of the cps clusters in five E. coli strains and one Klebsiella strain indicated the presence of IS elements. We propose that these elements were responsible for the transfer of the cps locus between organisms and that they may continue to mediate recombination between strains.

Escherichia coli produces a wide variety of capsular polysaccharides termed K antigens. These polymers can vary in composition, linkage specificity, and substitution, allowing for diversity among strains. The major capsule groups, traditionally designated groups I and II, were defined by serological properties as well as by the location of the K-antigen biosynthesis gene cluster, the polymer structure, and the expression patterns (23). A new and expanded classification system has recently been proposed (51). This is based on genetic and biochemical (assembly pathway) data and proposes four capsule groups. The new group 1 accommodates a subset of K antigens formerly designated group IA (23). Of the 66 structurally defined K antigens in E. coli, 16 show characteristics typical of group 1 capsules (23). Research in this laboratory focuses on the group 1 K antigens of E. coli and the structurally related capsules found in Klebsiella spp.

In E. coli, group 1 K antigens are produced in two distinct forms: a low-molecular-weight form (K_LPS), which comprises K oligosaccharides linked to lipid A-core and resembles a lipopolysaccharide (LPS)-linked O antigen, and a high-molecular-weight unlinked form, capsular K antigen, which is associated with the cell surface. A precise mechanism of attachment for capsular K antigen has not been described, but it is known that LPS is not involved (30). In Klebsiella, only the capsular form of K antigen is produced. The absence of the K_LPS form in Klebsiella may reflect differences in the LPS core structure and/or the ligase enzyme (21) that attaches polysaccharides to the lipid A-core acceptor.

The genes involved in synthesis and transport of both K_LPS and capsular K antigen in E. coli are encoded at a locus called cps. A prototype group 1 capsule cluster from E. coli E69 (O9a:K30) (cps_E69) has been analyzed (17). The 16-kb locus contains genes required for K30 polymerization and translocation. The K30 antigen is synthesized via the Wzy-dependent polymerization pathway, which has been described for the biosynthesis of certain O antigens (48). In brief, repeat units are made on the cytoplasmic face of the plasma membrane by the action of glycosyltransferases, which transfer residues to a lipid (undecaprenol pyrophosphate)-linked biosynthetic intermediate. The lipid-linked repeat units are moved to the periplasmic face of the membrane by the Wzx protein, where they are polymerized by the Wzy enzyme. To form K_LPS, short K30-antigenic oligosaccharides are ligated to lipid A-core by the ligase, WaaL. The capsular form of the K30 antigen is polymerized by the same pathway; however, surface expression is via an LPS-independent pathway that requires the products of wza and wzc (see below). These gene products are not required for the assembly of LPS-linked O antigens, and they provide features that distinguish gene clusters for biosynthesis of O and K antigens.

Comparative analysis of Klebsiella pneumoniae K2 (cps_KP2) (2) (GenBank accession no. D21242) and E. coli K30 (cps_EK30) (17) (GenBank accession no. AF104912) indicates a shared biosynthetic pathway. The objective of this study was to investigate the precise relationships between capsule gene clusters in a number of E. coli and Klebsiella strains.

A conserved region of genes required for translocation and polymerization of group 1 K antigens. Lipid-linked intermediates are polymerized to form high-molecular-weight K30 polysaccharide via the Wzy-dependent polymerization pathway and then translocated through the outer membrane as an unlinked polymer. Surface assembly of capsular group 1 K antigen min-
chloroform-isoamyl alcohol (24:1) extraction. With 70% ethanol and resuspended in 100 volumes of absolute ethanol. The DNA pellet was washed extractions and precipitated with 5% 5 M sodium chloride and 200 ml of 5 M sodium chloride and 250 µl of 10% hexadecltrimethyl ammonium bromide in 0.7 M sodium chloride were added, followed by a 30-min incubation at 65°C. The sample was then subjected to two phenol-chloroform-isooamyalcohol (25:24:1) extractions and precipitated with 5% 5 M sodium chloride and 2 volumes of absolute ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 100 µl of sterile water. Finally, the DNA was treated with RNase and subjected to a chloroform-isooamyalcohol (24:1) extraction.

The region between wza and wzc was amplified by PCR. PCRs were performed with Pwo or Expand Long DNA polymerase (Boehringer Mannheim) in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The primers used for DNA amplification are listed in Table 2 and shown in Fig. 1. PCR products identical in size were obtained with primers JD89 and JD109 and chromosomal DNA from strains representing 6 K serotypes of *Klebsiella* and 12 K serotypes of *E. coli* (Fig. 2). The sizes of the products were consistent with those predicted from the *cps*_{ECK30} and *cps*_{KPK2} sequence data.

For detailed analysis, selected PCR fragments were purified with Qiagen nucleotide removal columns and the region was sequenced at the Guelph Molecular Supercentre (University of Guelph, Guelph, Ontario, Canada). Analyses revealed that the wza-to-wzc regions in *E. coli* A295b (GenBank accession no. AF118245), E75 (GenBank accession no. AF118246), N24c (GenBank accession no. AF118247), 2151 (GenBank accession no. AF118248), and B161-42 (GenBank accession no. AF118249) had 99.5% identity to *E. coli* E69 at the nucleotide level and that the equivalent regions in *Klebsiella* and *E. coli* were 72% identical at the nucleotide level.

The observation that group 1 capsule clusters from *E. coli* and *Klebsiella* are organized with a conserved block of translocation-surface assembly genes is reminiscent of the modular structure of gene clusters required for the manufacture of group 2 capsules in *E. coli*. Group 2 (kps) clusters comprise three regions (51). Region 1 encodes proteins necessary for translocation of polysaccharide through the periplasm and across the outer membrane, as appears to be the case with the initial genes (wza to wzc) of the group 1 *cps* cluster. The serotype-specific central region (region 2) is responsible for biosynthesis and polymerization of oligosaccharide repeat units. This is equivalent to the genes downstream of wzc in the group 1 capsule clusters. Region 3 contains genes encoding the subunits for an ATP-binding cassette transporter that is required for capsule export across the cytoplasmic membrane (51). The region 3 components are not required in Wzy-dependent polysaccharides, such as the group 1 capsules (48, 51). In the group 2 clusters, regions 1 and 3 are conserved in strains that produce structurally distinct capsules and the gene products are functionally interchangeable between these strains (51). Although functional identity has not been formally shown for the translocation-surface assembly region of group 1 capsules, the high degree of conservation seems to indicate that this would be the case. Some conserved feature of the group 1 K antigens or a component in their assembly must therefore be recognized for the translocation-surface assembly processes to be completed.

**orfX** is a unique component of group 1 K antigen *cps* gene clusters. Although homologues of wza, wzb, and wzc are found in other systems, including the *cps*_{K12} cluster for colanic acid biosynthesis, *orfX* homologues have been found only in *cps*_{ECK30} and *cps*_{KPK2} (where it is designated *orf3*) (2, 17). In both cases,

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**TABLE 1. Bacterial strains used in this study**

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<thead>
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<th>Strain</th>
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<td>F. Ørskov</td>
</tr>
<tr>
<td>B161-42</td>
<td>O9:K23:H1</td>
<td>F. Ørskov</td>
</tr>
<tr>
<td>E69</td>
<td>O9a:K30:H12</td>
<td>F. Ørskov</td>
</tr>
<tr>
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<td>O9a:K35:H1</td>
<td>F. Ørskov</td>
</tr>
<tr>
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<td>O9ab:K26:H1-</td>
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<td>N24c</td>
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<tr>
<td>2178</td>
<td>O8:K42:H1</td>
<td>B. Jann</td>
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**TABLE 2. Primers used for PCR amplification of chromosomal DNA**

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<td>JD109</td>
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</tr>
<tr>
<td>JD89</td>
<td>wzc</td>
<td>5’-ACGGGCAATAACAGGAAAATA</td>
</tr>
<tr>
<td>JD90</td>
<td>wcaO</td>
<td>5’-TATGTGGGCGGCTTGACCTC</td>
</tr>
<tr>
<td>JD35</td>
<td>wzo</td>
<td>5’-TTTCCGAAAACACGGCCTAAA</td>
</tr>
<tr>
<td>JD95</td>
<td>orfX</td>
<td>5’-TGCGCTTCCATACAGAGTTA</td>
</tr>
<tr>
<td>JD99</td>
<td>orfX</td>
<td>5’-GAAGACAGCACCACAGTCAC</td>
</tr>
<tr>
<td>GALF1</td>
<td>galF</td>
<td>5’-GGGCGATCTCTCCGGAATACCT</td>
</tr>
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</table>

* Primer sequences were based on the relevant chromosomal region from *E. coli* E69 (17) (GenBank accession no. AF104912) and, for GALF1, *K. pneumoniae* Chedid (2) (GenBank accession no. D21242).
orfX represents the first gene in the cluster. PCRs were performed with primers designed to amplify the region between orfX (JD95 for Klebsiella and JD99 for E. coli) and a gene upstream of the K-antigen cluster, galF (GALF1) (Table 2; Fig. 1). The sizes of the fragments obtained varied considerably due to polymorphism upstream of cps (see below). However, sequence data showed orfX to be conserved in position and virtually identical at the nucleotide level in all of the E. coli strains (E56b, Bi161-42, A295b, N24c, and E75) and Klebsiella strains (A5054, 889/50, 6613, and 708) investigated (data not shown). In E. coli K30, orfX mutants do not appear to be impaired in translocation-surface assembly of the capsular K antigen (17) and the precise role of orfX remains unclear. However, the high degree of conservation in orfX homologues would indicate that it is important in the production of group 1 capsules.

Identical cps gene clusters in E. coli K30 and K. pneumoniae K20. There are notable structural similarities between group 1 K antigens in E. coli and K. pneumoniae (structures are available online in the Complex Carbohydrate Structure Database [14a]). In the example of Klebsiella K20 (11) and E. coli K30 (10), the capsular antigen structures are identical. The relationship between these two gene clusters was also examined by PCR and sequencing. Similar to the E. coli strains analyzed above, the wza-to-wzc region in these strains was shown to be 99.5% identical (Klebsiella K20 GenBank accession no. AF118250).

Genes downstream of wza, wzb, and wzc are involved in...
repeat-unit biosynthesis and polymerization. Genes in this region, in particular, the glycosyltransferases, will vary with the structure of the polymer being produced by any given strain. Among group 1 capsules, this region has been described in detail for *E. coli* K30 (17) and *Klebsiella* K2 (2), and although both encode the same classes of gene products, they exhibit only low levels of similarity. PCR amplification of the region from *wcaO* to *wzx* with primers JD90 and JD53 (Table 2; Fig. 1) yields products identical in size (3.6 kb) for both *E. coli* K30 and *K. pneumoniae* K20 (data not shown). At the nucleotide level, the products are 99% identical over the 550 bp sequenced from each end. The high degree of conservation at the nucleotide level for both the *wza*-wzc and the *wcaO*-to-*wzx* regions may indicate the transfer of *cps* gene clusters between *K. pneumoniae* and *E. coli* (see below).

**Conservation of regulatory regions upstream of *cps***. Gene clusters for bacterial polysaccharides are characteristically preceded by a 39-bp JUMPstart (for “just upstream of many polysaccharide starts”) element (22). The JUMPstart element has also been identified upstream of gene clusters involved in F conjugation pilus assembly and hemolysin toxin secretion (reviewed in reference 5). A second element, *ops* (for “operon polarity suppressor”) is an 8-bp motif located within the JUMPstart sequence (35). Both elements are believed to play a role in transcriptional anttermination of the gene clusters (5, 31, 35). Their role has been described in regulation of *E. coli* group 2 K-antigen (*kps* [42, 43]) and LPS O-antigen (*orfX* [31]) biosynthesis.

The role of the JUMPstart element has been best characterized for the hemolysin operon, where it has been shown to control operon polarity but not transcript stability (5, 35), in concert with a NusG homologue, RfaH (4, 28, 29). The element can function over long distances (2 kb); however, it does so only when present on the nascent transcript (35). In one possible model, the JUMPstart sequence functions by facilitating the formation of a number of stem-loop structures on the mRNA during transcript elongation (31). This region recruits RfaH and potentially other proteins. Binding of RfaH to stem-loop III may inhibit the formation of the other stem-loops, which are thought to induce premature termination when present.

To investigate the conservation of these regulatory elements, regions immediately upstream of the group 1 K-antigen clusters were amplified and sequenced. A conserved JUMPstart element was present in both *E. coli* strains (E56b, B161-42, A295b, N24c, and E75) and *Klebsiella* strains (A5054, 899/50, 6613, and 708) (Fig. 3A). This finding suggests that group 1 capsule clusters are subject to transcriptional control via anttermination. Interestingly, the *cps* gene clusters share a feature noted upstream of the O-antigen biosynthesis region of *E. coli* O7 (wb*) (31), i.e., the presence of two *ops* elements.

In the hemolysin system, anttermination is required to avoid extreme operon polarity in a situation where the structural gene for the toxin is separated from those required for toxin maturation and export by a stem-loop structure (5). Analysis of the available *cps* (EC30) and *cps* (KP22) sequences identified a stem-loop structure in the intergenic region between *wza* and the initial gene of the repeat-unit synthesis region (Fig. 3B). This potentially provides a strong transcriptional terminator, thereby allowing differential expression of structural components for capsule translocation and the highly active enzymes involved in polymer synthesis. Equivalent stem-loop structures are also predicted from the nucleotide sequence downstream of the *wza* homologue in the gene clusters for amylovoran (9) and colanic acid (44) (data not shown). Differential expression of genes required for translocation-surface assembly and synthesis is known to occur in the *E. coli* group 2 *kps* clusters. In these systems, regions 2 and 3 are organized into one transcriptional unit under the control of the region 3 promoter. Region 2 genes rely on transcriptional anttermination by RfaH to avoid operon polarity problems (42). The *kps* region 1 is not regulated via anttermination but has been shown to be thermoregulated (12, 40). In addition, the first gene of region 1, *kpsF*, plays a poorly understood role in regulation (12). It remains to be established whether the product of *orfX* plays a similar role in expression of group 1 K antigens.

Group 1 K antigens are known to be regulated by the Rcs (for “regulator of capsule synthesis”) system in both *E. coli* (24, 25) and *Klebsiella* (1, 32, 47). This two-component regulatory system is best characterized for colanic acid production in *E. coli* K12 (19); however, essentially identical systems operate in *E. amylovora* (6, 7, 14, 27). It is believed that the RcsC protein senses an environmental signal and, along with a second protein, RcsF, modulates the activity of RcsB through phosphorylation. RcsB can interact with a Lon protease-sensitive protein, RcsA, and upregulate *cps* transcription. Both RcsA and RcsB have helix-turn-helix DNA-binding motifs and bind to the promoter region of the *ams* cluster for amylovoran production in *E. amylovora* (27). A potential binding site has also been identified upstream of the colanic acid cluster based on the titration of regulatory proteins by using promoter DNA and sequence homology to the *E. amylovora* binding site (27, 45). The environmental signal sensed by the Rcs system is uncharacterized but may involve membrane perturbations (13, 18, 37) or osmotic stress (3, 18, 41).

Although group 1 capsules, colanic acid, and amylovoran are all Rcs regulated, there are some important differences in their expression patterns. Most notable is the observation that colanic acid and amylovoran are optimally produced at 20°C and are not manufactured at 37°C. This is not surprising for colanic acid since this polymer is not a virulence determinant (39) and its function may be more important in environments outside the host (18, 41). *E. amylovora* is a plant pathogen associated with infections at environmental temperatures. However, group 1 capsules are virulence determinants and are produced at 37°C (49). The ability to express *cps* gene products at 37°C may be due to altered interactions of an RcsA-RcsB dimer with the *cps* promoter or to the involvement of additional (as yet uncharacterized) regulatory proteins. The Rcs proteins themselves are highly conserved in *E. coli* K30 and *E. coli* K12 (24, 25), suggesting that they are unlikely to determine the different patterns of expression. The regions upstream of *cps* in *E. coli* and *K. pneumoniae* strains with group 1 K antigens lack the published RcsA-RcsB-binding sequences (27). Therefore, although the Rcs system may function to regulate group 1 K antigens, details of this interaction may differ considerably from those for the colanic acid and amylovoran systems.

Analysis of the *Klebsiella* K2 upstream region resulted in the identification of a partial sequence of a putative σ54 promoter (2). This is conserved in all the *E. coli* and *K. pneumoniae* strains examined here. However, this putative promoter lies downstream of the JUMPstart element and thus could not operate in situations where anttermination by RfaH is required. The precise physiological significance of this “promoter” is therefore unclear.

**Possible lateral transfer of group 1 *cps* genes**. The data presented above demonstrates that the K-antigen gene clusters of *E. coli* and *Klebsiella* are highly conserved in organization and in *cps* nucleotide sequence. This is consistent with the possibility of lateral transfer of group 1 capsule gene clusters between these organisms. Further evidence in support of this contention was obtained by the identification of IS elements...
upstream of the cps gene clusters. In E. coli K30, a partial IS1 element (250 bp) truncates an adjacent gene, orf2. orf2 is also found immediately upstream of the cpsKPK2 cluster (2) but is absent in E. coli K-12. orf2 is not essential for K-antigen production in K. pneumoniae (2). A survey of other strains showed that many of the E. coli cps clusters are flanked by IS sequences (Fig. 4). Of the six E. coli strains examined, five contained IS elements. The type of IS element present is highly variable,
Escherichia coli

\[ \text{galF} \ \text{orf2} \ \text{IS1} \ \text{orfX} \ \text{wza} \ \text{wzb} \ \text{wzc} \]

Klebsiella spp.

Chedid

A5054

889/50

IS603

6613

708

FIG. 4. Diagrammatic representation of the region upstream of \text{cps} in the \text{E. coli} and \text{K. pneumoniae} strains investigated. The regions for \text{E. coli} E69 (17) and \text{K. pneumoniae} Chedid (2) are based on published data. The location and type of IS element identified, if any, is indicated. The putative α34 promoters and conserved JUMPstart elements are located between the IS elements (or \text{orf2} and \text{orfX}), the first gene of each cluster.

with only IS1 being identified in more than one strain (\text{E. coli} E69 and E56b). In contrast, only one of the five \text{K. pneumoniae} strains had an IS element. The location of the IS sequences seems to be quite highly conserved, with only two of six strains (\text{E. coli} A295b and \text{K. pneumoniae} 889/50) showing slight variation.

The presence of IS elements adjacent to most of the tested \text{E. coli} \text{cps} clusters and their absence in all but one of the cps regions examined in Klebsiella strains suggest that IS elements may have mediated an initial transfer of the cluster to \text{E. coli} from Klebsiella and could continue to be important for the exchange of genes encoding different capsule types between strains. Support for this hypothesis can be found in an analysis of gnd (6-phosphogluconate dehydrogenase) alleles from a variety of \text{E. coli} and Klebsiella isolates. The gnd genes map near \text{his} and \text{gnd} in \text{E. coli}, and Nelson and Selander (34) have suggested that diversity in \text{gnd} represents a surprisingly high degree of recombination. This was attributed to cotransfer with adjacent loci (primarily \text{rfb}) whose activities are subject to diversifying selection because of the host immune response. Nelson and Selander also argued for lateral transfer of \text{gnd} genes from Klebsiella to \text{E. coli}. \text{E. coli} strains with the 16 known group 1 K antigens have a limited array of LPS O antigens (23); of the >170 O serotypes, only O9, O9a, O8, O20, and O101 are represented. Klebsiella strains have 77 different K antigens (36) but fewer than 10 structurally distinct O antigens (26, 46). Notably, some O-antigen structures are shared, and lateral transfer of the O3 gene cluster to \text{E. coli} has been proposed (46). Collectively, these data suggest that transfer of a large region of DNA including the \text{rfb} and \text{cps} loci may have occurred. In such a scenario, the extended region between \text{galF} and \text{his} (\text{orf2-cps-ugd-rfb-gnd}) from Klebsiella would replace the “typical” \text{E. coli} region (\text{cps} [colanic acid]-\text{rfb}-\text{gnd-ugd-wzz}). This is consistent with our previous analysis of the regions surrounding \text{gnd} that confirmed the lack of \text{wzz} in \text{E. coli} strains with group 1 capsules (15, 16). Such organization also explains why expression of colanic acid and expression of a group 1 capsule are mutually exclusive. In previous work, we proposed that the \text{cps}_{\text{K-12}} and \text{cps}_{\text{ECK30}} systems are allelic (25, 50). From the differences in organization (the presence of \text{orfX}), altered upstream sequences, and the IS/\text{orf2} region missing in \text{E. coli} K-12, these can no longer be considered alleles. Colanic acid is therefore not simply a widespread serotype of group 1 K antigen and should not be included as such.

The simple conclusion that all \text{E. coli} group 1 capsules have arisen by lateral transfer of DNA from Klebsiella is complicated by several observations. First, not all \text{E. coli} group 1 capsules have structurally identical counterparts in Klebsiella, although this could reflect further gene transfer and recombination events within \text{cps} after the initial transfer, thereby resulting in the production of novel structures. However, of the strains examined here, one \text{E. coli} isolate (E75) lacks an IS element immediately upstream of \text{cps} and only one Klebsiella strain (889/50) has an IS element (IS903). These exceptions suggest that the events that resulted in group 1 capsule diversity in \text{E. coli} and Klebsiella are more complicated. As noted above, the \text{E. coli} K30 and \text{K. pneumoniae} K20 (889/50) \text{cps} gene clusters appear to be identical, and there are two possible explanations for this. One is that the cluster was mobilized to these strains from a common (unknown) source by a process involving different IS elements. The other is that the gene exchanges between Klebsiella and \text{E. coli} are possibly not restricted to unidirectional lateral transfer events. There is currently no data to resolve these possibilities.

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**ADDENDUM IN PROOF**

While this paper was under revision, further analysis of the RcsA-RcsB binding site in *Escherichia coli* K-12 was reported (W. Ebel and J. E. Trempy, J. Bacteriol. 181:577–584, 1999). A conserved motif (the RcsA box) was identified upstream of both *cps* and *rcsA* in *E. coli* K-12. This motif is not present in the regions upstream of the group 1 capsule gene clusters reported here. The binding site for RcsA-RcsB in *Erwinia* and related bacteria has also been further elucidated (M. Weiland, C. Kiecker, D. L. Coplin, O. Kelm, W. Saenger, and F. Bernhard, J. Biol. Chem. 274:3300–3307, 1999).

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