



High-frequency rearrangements in the chromosome of *Mycoplasma pulmonis* correlate with phenotypic switching

B. Bhugra¹ and K. Dybvig^{1,2*}

Departments of ¹Microbiology and ²Comparative Medicine, 503 Volker Hall, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA.

Summary

Mycoplasma pulmonis is a murine pathogen that causes chronic respiratory disease in laboratory rats and mice. Several examples of high-frequency phenotypic switching have been reported for *M. pulmonis*, the molecular basis of which is unknown. We report here that during growth the *M. pulmonis* chromosome undergoes DNA rearrangements at a high frequency. Some of the rearrangements we examined correlated with changes in the susceptibility of the cells to mycoplasma virus P1, an example of phenotypic switching involving changes in surface antigen structure. Other rearrangements, unrelated to phenotypic switching, involved a DNA element present in the chromosome in multiple copies. The high level of DNA recombination that occurred in *M. pulmonis* indicates that this may be one of the most variable genomes studied to date. High levels of DNA recombination may contribute to the unusually high rate of evolution that mycoplasmas are thought to be undergoing. Understanding the molecular basis for this phenomenon may provide an insight into the chronic nature of many mycoplasmal infections.

Introduction

The genus *Mycoplasma* contains about 100 characterized species, many of which are pathogens producing significant diseases in humans, animals, insects and plants. Mycoplasmal diseases of humans and animals often involve colonization of respiratory or genital tracts and are noted for their chronic nature (Tully and Whitcomb, 1979). *Mycoplasma pulmonis* causes chronic respiratory and reproductive tract diseases in laboratory rats and mice, providing a model system for studying mechanisms of

mycoplasmal disease chronicity (Cassell *et al.*, 1978; Cassell, 1982).

The chronicity of mycoplasmal diseases may be related to high-frequency phenotypic and antigenic changes that occur during growth of some species, particularly with *M. pulmonis*. By examining subclones derived from a single strain of *M. pulmonis*, it has been established that high-frequency phenotypic switching affects the growth of the organism on agar (Dybvig *et al.*, 1989), the susceptibility of the organism to infection with mycoplasma virus P1 (Dybvig *et al.*, 1988), the adherence of the organism to plastic surfaces, and the haemadsorption of red blood cells (Watson *et al.*, 1990a). All of these phenomena correlate with changes in V-1, a major surface antigen of *M. pulmonis*. V-1 has numerous, structurally distinct forms that vary at a rate of about 2×10^{-3} variants per colony-forming unit (cfu) per generation (Watson *et al.*, 1988). Variation in V-1 occurs *in vivo* and *in vitro*, and *in vivo* variation correlates with disease severity and chronicity (Talkington *et al.*, 1989).

The present studies demonstrate that the chromosome of *M. pulmonis* undergoes rearrangements at a high frequency. Some rearrangements correlated with changes in phenotype, and therefore DNA recombination may be the principal mechanism by which mycoplasmas vary their surface structures. Other DNA rearrangements, apparently unrelated to phenotypic switching, involved an element present in the chromosome in multiple copies.

Results

Restriction-fragment polymorphisms (RFPs) in subclones of M. pulmonis strain KD735

These studies began as an attempt to obtain genetically homogeneous populations of *M. pulmonis*. To investigate the degree of genetic variation that occurs within a single strain of *M. pulmonis*, strain KD735 was subcloned as depicted in Fig. 1. DNA from each of these subclones was digested with restriction endonuclease *Hind*III and analysed by agarose gel electrophoresis.

The ethidium bromide-stained DNA banding pattern of most first-generation subclones was similar to that of the parent strain, but notable differences were sometimes

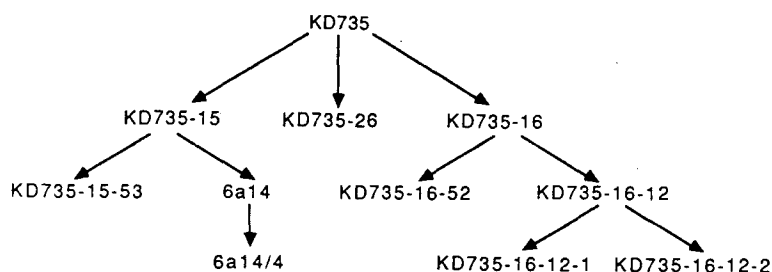


Fig. 1. Schematic diagram showing the relationship of RFP-containing strains to the parental strain KD735.

observed. Examples of some of these banding patterns are shown in Fig. 2. The banding pattern from strain KD735-15 (lane 1) is representative of the parental pattern of KD735. Strains KD735-16 (lane 3) and KD735-26 (lane 7) are subclones of KD735 that have DNA fragments of about 9.5 kb and 8.5 kb, respectively, that are absent in the parent DNA. The variant genomes present in these cells are referred to in this paper as RFPs, all of which were identified by digestion with *Hind*III.

RFPs were also present in second- and third-generation subclones. Strain KD735-15-53 (Fig. 2, lane 2) lacked a 5.9 kb fragment found in its parent strain, KD735-15 (Fig. 2, lane 1). Strain KD735-16-52 (Fig. 2, lane 4) lacked two fragments of about 6.3 and 5.9 kb that were present in its parent strain KD735-16 (Fig. 2, lane 3). Strain KD735-16-12 (Fig. 2, lane 6) is a subclone of KD735-16 that lacks the 9.5 kb fragment characteristic of its parent and instead had a new 8.2 kb fragment. Strain KD735-16-12 was still heterogeneous; for example, one of its derivatives, strain KD735-16-12-1 (Fig. 2, lane 5), lacked the 6.3 and 5.9 kb fragments.

Frequency of isolation of subclones with RFPs

Cultures of *M. pulmonis* were sufficiently heterogeneous that subclones containing RFPs were routinely isolated. All strains (subclones) were derived by filter cloning methods (see the *Experimental procedures*). Some RFP-containing subclones were isolated by immediate subcloning of filter-cloned strains. However, most of the RFP-containing subclones were isolated from cultures that had been passaged twice prior to the next round of subcloning. Cells were passaged by daily transfer (1:250 dilution) from an actively growing culture into fresh mycoplasma medium followed by overnight incubation. Five out of 12 subclones (about 40%) isolated from the passaged cultures had RFPs.

RFPs and a repetitive DNA element

One of the RFPs was associated with a repetitive DNA element. Plasmid pBK85 contains the cloned 8.5 kb *Hind*III fragment from strain KD735-26 (see Fig. 2, lane 7). When

used as a probe for analysing Southern blots of *M. pulmonis* DNA, pBK85 hybridized to several *Hind*III fragments ranging in size from 1.6 to 4.5 kb, in addition to the 8.5 kb fragment from KD735-26 (Fig. 3, lane 1). The hybridization profile of DNA from strain KD735-15 (representative of the parent strain) was similar to that of KD735-26, except that KD735-15 had a strongly hybridizing 7.2 kb fragment instead of the 8.5 kb fragment (Fig. 3, lane 3). These data suggest that the 8.5 kb variant fragment of KD735-26 arose from a 7.2 kb precursor fragment.

The RFP from strain KD735-16

M. pulmonis strain KD735-16 had a RFP unrelated to the repetitive element identified on pBK85. Plasmid pIR95 contains the cloned 9.5 kb variant fragment from this strain. On Southern blots, a pIR95 probe hybridized to the 9.5 kb fragment from KD735-16 (Fig. 4A, lane 4) and to two fragments of 5.7 kb and 4.9 kb from the parent strain, KD735 (Fig. 4A, lane 1). The probe also hybridized to a 2.7 kb fragment that is present in all of the strains examined to date, except for strain KD735-16-12-2, which has this fragment shifted to a lower molecular weight (Fig. 4A, lane 6).

One hypothesis suggests that the 9.5 kb variable fragment was generated in KD735-16 by the joining of two adjacent parental fragments of 4.9 and 5.7 kb, through the loss of about 1.1 kb of sequences containing the intervening *Hind*III site. This putative deletion was not a copy of the repetitive element discussed above; as shown in Fig. 3, DNAs from KD735-15 and KD735-16 had the same hybridization profiles when probed with pBK85. Furthermore, plasmid pIR49, which contained the cloned 4.9 kb parental fragment from KD735-15, did not hybridize to the repetitive element. pIR49 hybridized to the 9.5 and 4.9 kb fragments from KD735-16 and KD735-15, respectively, as well as to the 2.7 kb fragment that is common to these strains (Fig. 4B). In addition, pIR49 hybridized weakly to the 5.7 kb fragment from KD735-15.

Correlation of RFPs with phenotypic switching

A previous report from our laboratory described the

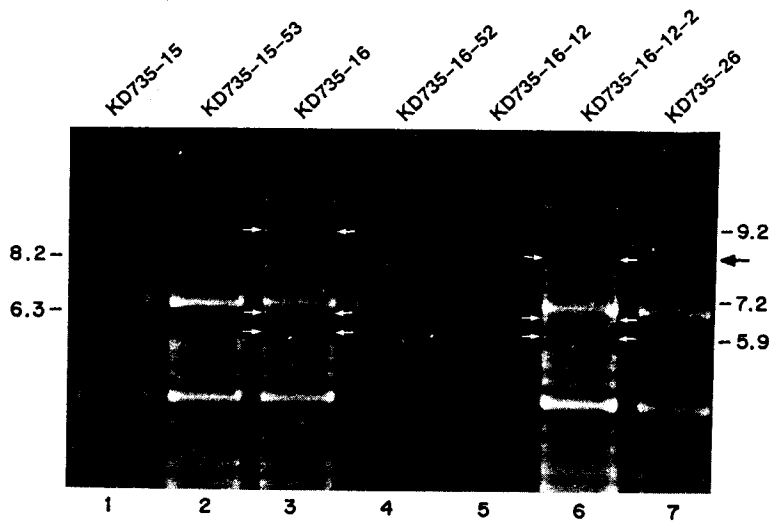


Fig. 2. RFPs in derivatives of strain KD735. *Hind*III-digested chromosomal DNAs were analysed on an agarose gel stained with ethidium bromide. Lane 1, strain KD735-15 (derived from KD735); lane 2, strain KD735-15-53 (derived from KD735-15); lane 3, strain KD735-16 (derived from KD735); lane 4, strain KD735-16-52 (derived from KD735-16); lane 5, strain KD735-16-12-1 (derived from KD735-16-12); lane 6, strain KD735-16-12 (derived from KD735-16-12); lane 7, strain KD735-26 (derived from KD735). Arrows refer to the variant 9.5, 6.3 and 5.9kb fragments from strain KD735-16 and the variant 8.2, 6.3 and 5.9kb fragments from strain KD735-16-12.

isolation of mutants of *M. pulmonis* that were resistant to infection by mycoplasma virus P1 (Dybvig *et al.*, 1988). Resistance was due to failure of the virus to adsorb to the cells, and the mutants contained an altered form of the variable surface antigen V-1. Studies were undertaken to determine whether chromosomal DNA rearrangements correlated with changes in P1 virus susceptibility. All of the strains examined to date that contained the 5.7 and 4.9kb fragments, identified by probing with pIR95, were sensitive to infection with P1 virus. However, all of the virus-resistant mutants (isolated in our previous study) to which

P1 virus did not adsorb lacked the 4.9 and 5.7kb fragments. Replacing these fragments was a higher molecular-weight fragment similar in size to the 9.5kb fragment of KD735-16 (data not shown).

Variation in DNA fragments of 6.3 and 5.9kb also correlated with changes in P1 virus susceptibility, as documented in Table 1. Strain KD735-16 was virus-resistant. In addition to the 9.5kb fragment, KD735-16 had a 6.3kb fragment that was not present in the parent strain. This fragment did not hybridize with the probes described above; its presence was directly detected as a variant band on ethidium bromide-stained gels (see Fig. 2). Other strains that contained the 6.3kb fragment were KD735-16-12 and 6a14; both of these strains were also P1 virus-resistant. We have isolated derivatives of each of these strains, designated KD735-16-52, KD735-16-12-1, and 6a14/4 (see Fig. 1), which no longer possessed the 6.3kb DNA fragment. Each of these derivatives had also reverted to a P1 virus-sensitive phenotype. In addition to the 6.3kb fragment, these strains also lacked a 5.9kb

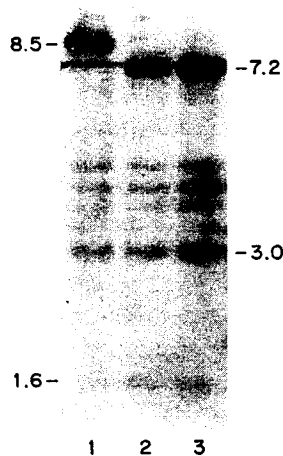


Fig. 3. Detection of a repetitive DNA element by Southern hybridization. *Hind*III-digested, chromosomal DNAs were analysed on an agarose gel, transferred to nitrocellulose, and probed with pBK85. Analysed in lanes 1-3 was DNA from KD735-26, KD735-16, and KD735-15, respectively. Numbers in the margins refer to the size (in kb) of selected fragments. The fragment at 3.0kb is actually a doublet that was poorly resolved in this particular experiment.

Table 1. Correlation between variable DNA fragments and susceptibility (susc.) of *M. pulmonis* to infection with P1 virus.^a

Strain	Parent	4.9	5.7	5.9	6.3	8.2	9.5	P1 susc.
KD735	UAB 6510	+	+	+	-	-	-	S
KD735-15	KD735	+	+	+	-	-	-	S
KD735-15-53	KD735-15	+	+	-	-	-	-	S
KD735-16	KD735	-	-	+	+	-	+	R
KD735-16-52	KD735-16	-	-	-	-	-	+	S
KD735-16-12	KD735-16	-	-	+	+	+	-	R
KD735-16-12-1	KD735-16-12	-	-	-	-	+	-	S
6a14	KD735-15	-	-	+	+	-	+	R
6a14/4	6a14	-	-	-	-	-	+	S

a. Numbers in the top row refer to the sizes of variable DNA fragments in kb. '+' and '-' signs refer to the presence and absence of DNA fragments, respectively. S and R refer to P1 virus-sensitive and -resistant phenotypes, respectively.

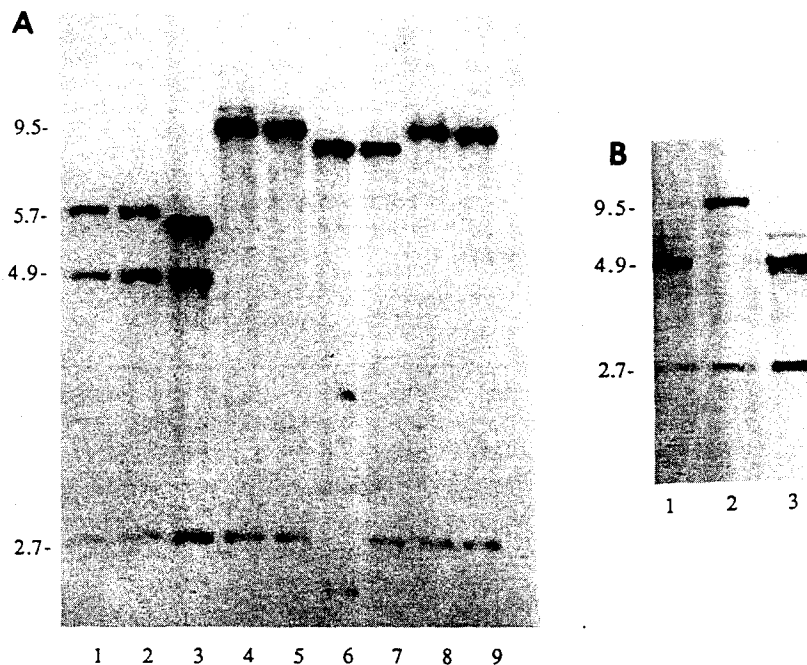


Fig. 4. Southern hybridization analysis of the variable 9.5, 5.7, and 4.9 kb fragments. Chromosomal DNA was digested with *Hind*III, analysed on an agarose gel, and transferred to nitrocellulose. Numbers in the margins refer to the sizes (in kb) of selected fragments.

A. Southern blot probed with pIR95. Lane 1, KD735; lane 2, KD735-15; lane 3, KD735-15-53; lane 4, KD735-16; lane 5, KD735-16-52; lane 6, KD735-16-12-2; lane 7, KD735-16-12-1; lane 8, 6a14; and lane 9, 6a14/4.

B. Southern blot probed with pIR49. Analysed in lanes 1-3 was DNA from KD735-15, KD735-16, and KD735-26, respectively.

fragment. These strains, however, retained their high molecular-weight fragment that hybridized with pIR95 and still lacked the 5.7 and 4.9 kb fragments (see Fig. 4A, lanes 4-9).

The correlation between specific RFPs and susceptibility to P1 virus was observed regardless of the order in which the phenotypes were identified, i.e. strain KD735-16 and its derivatives were initially identified as containing RFPs, and they were subsequently found to have altered virus susceptibilities. In contrast, strain 6a14 was initially identified as a virus-resistant mutant of strain KD735-15 that also had an altered form of V-1 antigen, and it was subsequently determined that this strain contained an RFP. Similarly, strain 6a14/4 was initially identified as a virus-sensitive revertant of strain 6a14, and it was subsequently found to contain an RFP. No correlation was observed between virus susceptibility and RFPs other than the one summarized in Table 1. For example, the locations of the repetitive element identified by probing chromosomal DNA with pBK85 did not correlate with virus susceptibility. There was also no apparent correlation between RFPs and the previously described phenomenon of colony size switching.

Discussion

In this study, RFPs were frequently detected in subclones of a single strain of *M. pulmonis*. Southern hybridization data using variant DNA fragments associated with RFPs as probes indicate that RFPs arose via insertions, deletions or other rearrangements in the chromosome.

The data do not support a model in which RFPs arose as a result of point mutations or changes in DNA methylation affecting restriction enzyme recognition sites.

About 40% of the subclones from strains that had been passaged twice were identified as containing RFPs. Because of the large number of fragments that are obtained by digestion of *M. pulmonis* DNA with *Hind*III, many RFPs would not be recognized because of comigration of variable DNA fragments with other DNA fragments of similar size. Variation within *Hind*III fragments smaller than 5 kb would almost certainly go unobserved using our methods, and we assume that the actual percentage of subclones from the passaged cultures that had RFPs is much greater than 40%.

The rate at which RFPs were generated in the passaged cultures of *M. pulmonis* can be estimated. As described in the *Experimental procedures*, subclones were obtained by picking entire colonies (in the form of agar plugs) and growing them in 1 ml cultures. These cultures reached a titre of about 10^9 cfu, suggesting that about 30 generations of growth occurred ($2^{30} = 10^9$). Serial passage of the cells should have resulted in about 16 additional generations of growth; each passage of cells diluted 1:250 required about eight generations ($2^8 = 256$) to regain titre. Therefore cells were grown for about 46 generations prior to subcloning. Because 40%, and possibly many more, of the subclones contained RFPs, we estimate that RFPs were generated at a rate of about 10^{-2} to 10^{-3} variants per cfu per generation. This high rate of DNA recombination would make the mycoplasma chromosome one of the most variable genomes known, rivalling the extreme

examples of genetic instability described for halobacteria and streptomyces (Pfeifer and Blaseio, 1989; Sapienza *et al.*, 1982; Leblond *et al.*, 1989; 1990).

The data described here indicate that in one case, strain KD735-26, a RFP contained a copy of a repetitive element. Southern hybridization analysis (data not shown) has indicated that this repetitive sequence is present in most, if not all, strains of *M. pulmonis*, including strains unrelated to KD735. The nucleotide sequence of this element has been determined recently (our unpublished data), revealing homology with the *Escherichia coli* insertion sequence IS3. Transposition of IS elements is thought to be one of the major mechanisms by which some genomes (e.g. halobacteria) undergo rearrangements (Sapineza *et al.*, 1982; DasSarma *et al.*, 1988).

Some of the DNA rearrangements that occurred in *M. pulmonis* correlated with changes in the susceptibility of the cells to mycoplasma virus P1 and, by implication, changes in V-1 antigen. The strongest correlation was with DNA fragments of 6.3 and 5.9 kb. Strains containing these fragments were P1 virus-resistant, and strains lacking them were P1 virus-sensitive. However, this correlation was complicated by the fact that DNA fragments of 4.9 and 5.7 kb were invariably missing and replaced by a single fragment of higher molecular weight (e.g. 9.5 kb) in those strains that had the 6.3 kb fragment. The connection between the 4.9 and 5.7 kb fragments and the 6.3 and 5.9 kb fragments is unclear.

DNA rearrangements have been shown to be associated with phenotypic switching in many bacterial systems. DNA inversions, gene conversions, duplications or deletions of tandem homologous blocks of DNA, and movement of transposable elements are frequently employed mechanisms of phenotypic switching in other bacterial systems (Hollingshead *et al.*, 1987; Borst and Greaves, 1987; Hoiseth *et al.*, 1986; Seifert and So, 1988). One or more of these mechanisms is/are likely to be involved in the phenomenon described here. Phenotypic switching similar to that of *M. pulmonis* has also been described in other mycoplasmal species (Olson *et al.*, 1991; Rosengarten and Wise, 1990; 1991; Watson *et al.*, 1990b), and elucidation of the genetic basis of this phenomenon in *M. pulmonis* may provide insight into how mycoplasmas in general vary their phenotypic traits.

The high frequency of genetic variation in mycoplasmas would allow for the rapid generation of diverse subpopulations which could enable these organisms to survive in a variety of niches or evade immune responses, contributing to the pathogenesis of mycoplasmal infections. With hindsight, a high rate of genetic recombination in these cells is predictable given the small size of the chromosome. The chromosomes of some mycoplasmas are as small as 600 kb (Colman *et al.*, 1990), and creating new coding regions by rearranging the chromosome would

increase the coding capacity of the cells without increasing genome size. Ribosomal RNA analysis has revealed that these organisms are evolving much faster than most eubacteria (Woese, 1987), and it will be interesting to learn how DNA recombination contributes to this evolutionary process.

Experimental procedures

Mycoplasmas

M. pulmonis was propagated in mycoplasma medium, and cfu were assayed on agar as previously described (Dybvig and Cassell, 1987). All *M. pulmonis* strains used in this study were subclones of strain KD735 (Dybvig *et al.*, 1989), a derivative of strain UAB 6510 that is susceptible to mycoplasma virus P1 (Dybvig *et al.*, 1988). Subclones were derived by filter cloning methodology, which involves removal of cell aggregates by gently passing cultures through a 0.2 µm filter immediately prior to assaying for cfu (Tully, 1983; Dybvig *et al.*, 1989). The filter cloning technique is designed to generate colonies derived from single cells. Well-separated colonies were randomly picked in agar plugs, propagated at 37°C in 1 ml cultures, and assigned a new strain designation. These cultures were stored at -70°C and used as stocks for subsequent experiments.

Electrophoresis of mycoplasma DNA

DNA was isolated from 25 ml cultures of *M. pulmonis* as described (Dybvig and Alderete, 1988). For agarose gel electrophoresis, about 5 µg of mycoplasma DNA was digested with a restriction enzyme, usually *Hind*III, and analysed on a 0.8% gel. Electrophoresis for 16 h at 2.5 V cm⁻¹ was generally sufficient to resolve individual DNA fragments of about 5.5 kb and greater, such that they could be directly observed by staining with ethidium bromide.

DNA manipulations

Restriction enzymes and T4 DNA ligase were used according to the specifications of the supplier (Gibco/BRL Life Technologies Inc.). Conditions for agarose gel electrophoresis, transfer of DNA on to nitrocellulose membranes, and labelling of DNA probes with ³²P by nick translation were as described elsewhere (Maniatis *et al.*, 1982). Stringent conditions were used for Southern hybridizations: the hybridization solution contained 50% formamide and the hybridization temperature was 42°C as described (Maniatis *et al.*, 1982). Plasmid DNA was isolated from *E. coli* using the alkaline lysis method and further purified by CsCl-ethidium bromide density gradient centrifugation (Maniatis *et al.*, 1982).

For cloning, DNA fragments were recovered from agarose gels by electrophoresis on to DEAE cellulose paper. The eluted fragments were then ligated into the *Hind*III site of plasmid pUC18, and used to transform *E. coli* strain JM103 (Messing *et al.*, 1981). The inserts within plasmids pIR95, pIR49, and pBK85 were the 9.5 kb fragment from strain KD735-16, the 4.9 kb fragment from strain KD735-15, and the 8.5 kb fragment from strain KD735-26, respectively.

Susceptibility of host cells to P1 virus

Mycoplasma virus P1 was propagated on *M. pulmonis* strain KD735. The susceptibility of the subclones of KD735 to P1 virus infection was determined by spotting virus-containing filtrates on to lawns of cells and examining the plates for zones of clearing after incubation at 37°C for 1 or 2 d, as described previously (Dybvig *et al.*, 1987).

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