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Role of Nucleases in the Isolation of Plasmid Deoxyribonucleic Acid from *Pseudomonas cepacia* 4G9

JOHN A. WILLIAMS,* JAMES P. YEGGY, AND A. J. MARKOVETZ

Department of Microbiology, University of Iowa, Iowa City, Iowa 42242

Brij 58-cleared lysates of *Pseudomonas cepacia* 4G9 contain both exonucleolytic and endonucleolytic activities. Endonuclease activity was unaffected by 125 mM ethylenediaminetetraacetic acid, whereas the exonuclease activity was inhibited. In contrast, Sarkosyl NL97 inhibited only the endonuclease. Sodium dodecyl sulfate inhibited all nuclease activity in in vitro assays, but plasmid deoxyribonucleic acid added to *P. cepacia* 4G9 spheroplasts during sodium dodecyl sulfate lysis was degraded. Irreproducible plasmid isolation from *P. cepacia* 4G9 may be due to this nucleolytic activity.

Isolation of plasmid DNA from *Pseudomonas aeruginosa* and *Pseudomonas putida* has proved difficult, and a variety of procedures adapted to particular plasmids have been described (3, 5, 10). Factors pertinent to the isolation of large plasmids were discussed by Hansen and Olsen (4). On the basis of these considerations, they devised a novel protocol incorporating aspects of three existent procedures to achieve reproducible isolation of large plasmids from several genera (4).

Application of these procedures (3-5, 10) to *Pseudomonas cepacia* 4G9 resulted in extremely low yields of plasmid DNA. The most successful method was a combination of sodium dodecyl sulfate (SDS)-sodium chloride lysis followed by precipitation of DNA with polyethylene glycol before cesium chloride-ethidium bromide equilibrium centrifugation (12). Agarose gel electrophoresis of plasmid DNA resulted in variable and irreproducible patterns. A similar phenomenon has been described for *Serratia marcescens*, a known producer of an extracellular nuclease (8). The low and irreproducible plasmid yield from the latter strain was caused by an endonuclease, and plasmid DNA was successfully isolated from nuclease-negative mutants (9, 11).

The strains of *P. cepacia* used were as previously described (12). The plasmid pBR322 was used in all in vitro nuclease assays and was isolated by the method of Clewell and Helinski (1) after chloramphenicol amplification. Plasmid DNA was electrophoresed and photographed as previously described (12).

The lack of plasmids in all Brij 58 lysates of *P. cepacia* 4G9 suggested that if nucleases were responsible for plasmid loss they would be most active in such a lysate. Nucleases were prepared from *P. cepacia* strains grown overnight in L-broth and lysed with Brij 58 (1). This lysate was either assayed directly or chromatographed on a Bio-Gel A-15 column in 50 mM Tris, pH 8.0, to remove metal ions and contaminating nucleic acids. The nuclease activity peak was concentrated to a volume equal to that of the sample applied to the column with a Micro-ProDiCon dialysis membrane (Bio-Molecular Dynamics, Beaverton, Oreg.).

A preparation of pBR322 plasmid DNA containing open circular (OC) monomeric and dimeric DNAs, linear monomeric and dimeric DNAs, and covalently closed circular (CCC) monomeric DNA was used to assay nuclease activity. The reaction mixture contained 0.5 μg of plasmid DNA in 50 mM Tris, pH 8.0, and 5 μl of a Brij 58-cleared lysate of *P. cepacia* and was incubated at 37°C for 15 min. The reaction was stopped by the addition of 5 μl of an SDS solution to give a final concentration of 0.1%. Before agarose gel electrophoresis, 5 μl of 60% sucrose-0.5% bromophenol blue was added to the reaction mixture. *P. cepacia* 4G9 was spot inoculated on DNase test agar with methyl green (Difco Laboratories, Detroit, Mich.) and grown for 24 h at 37°C to assay for extracellular DNase. The differential solubility of mononucleotides and oligonucleotides in trichloroacetic acid and uranyl acetate-trichloroacetic acid was used to assay for the presence of both exonuclease and endonuclease (7).

Addition of the Brij 58-cleared lysate to pBR322 DNA resulted in total degradation of OC, linear, and CCC forms (Fig. 1, lane 7). The culture supernatant fluid, concentrated to a volume equal to that of the Brij 58 lysate, did not exhibit any nuclease activity, indicating that the nucleolytic activity was not extracellular (data not shown). The absence of halos around spot inocula of *P. cepacia* 4G9 grown on DNase test

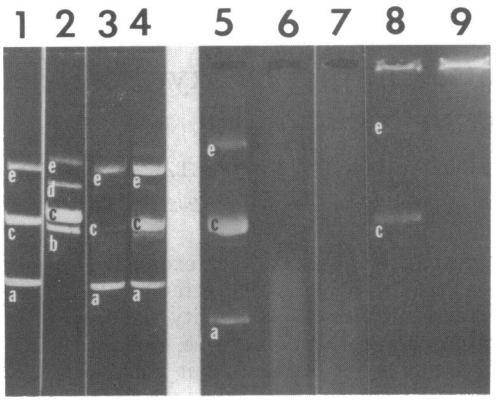

FIG. 1. *Agarose gel electrophoresis of pBR322 DNA after reaction with P. cepacia 4G9 nucleases. (1) pBR322 DNA control for lanes 2 through 4; (2) pBR322 DNA, Brij 58 lysate, and 125 mM EDTA; (3) pBR322 DNA, Brij 58 lysate, and 0.5% Sarkosyl NL97; (4) pBR322 DNA, Brij 58 lysate, and 0.5% SDS; (5) pBR322 DNA control for lanes 6 through 9; (6) pBR322 DNA and Brij 58 lysate for 40 min at 0 to 4°C; (7) pBR322 DNA and Brij 58 lysate for 15 min at 37°C; (8) pBR322 DNA (350 ng) added to spheroplasts of a 1-ml exponential-phase culture immediately before addition of SDS and potassium acetate; (9) as (8), except with a stationary-phase culture. The 1-ml lysis procedure is described elsewhere (12). Plasmid forms are marked as follows: (a) CCC monomer; (b) linear monomer; (c) OC monomer; (d) linear dimer; (e) OC dimer. Endonucleolytic activity converts forms (a) to (c) and (b) and (e) to (d), whereas exonucleolytic activity results in degradation of (b) and (d).*

agar with methyl green confirmed this result.

Standard lysis procedures incorporate EDTA to decrease metal ion concentration and thereby inhibit nuclease activity. The effect on the nuclease activity of doubling the normal EDTA concentration is shown (Fig. 1, lane 2). The conversion of CCC DNA to OC DNA and OC DNA to linear DNA was unaffected, but further degradation was prevented.

Other readily varied components during lysis are the type and percentage of detergent used. The nuclease activity from *P. cepacia* 4G9 was unaffected by the nonionic detergents Brij 58 and Triton X-100 at final concentrations of 1.0% (data not shown). Since plasmids were recovered by an SDS lysis procedure, the effect of SDS and Sarkosyl NL97, both ionic detergents, at a final concentration of 0.5% was assayed (Fig. 1, lanes 3 and 4). Sarkosyl NL97 prevented degradation of CCC DNA but permitted degradation of other forms, whereas SDS prevented all nuclease activity. The nuclease activity in the presence of SDS under normal lysis conditions is shown in Fig. 1, lanes 8 and 9. Lysis of an exponential-phase culture showed degradation of the CCC DNA only, whereas lysis of a stationary-phase culture resulted in degradation of all plasmid DNA.

The enzyme activities have been demonstrated in a Brij 58 lysate at 37°C, but for them to be of importance during plasmid isolation they must function within the normal time and temperature of lysis. The effects of a Brij 58 lysate on pBR322 DNA at 0 to 4°C for 40 min and at 37°C for 15 min are shown (Fig. 1, lanes 6 and 7). Marmur (6) suggested that heating of nuclease-producing cells before lysis may prevent DNA degradation, but heating *P. cepacia* 4G9 for 10, 20, or 30 min at 60, 65, 70, or 75°C before lysis did not improve the plasmid yield.

The possible presence of both an exonuclease and an endonuclease in the lysates was indicated by the differential effects of EDTA and detergents on plasmid degradation. Thermostability of the nucleolytic activity in the lysate was measured, and the exonuclease activity appeared to be more thermolabile than the endonuclease activity (data not shown). The presence of both exonucleolytic and endonucleolytic activities in the lysate was confirmed by the differential solubility of the reaction products in trichloroacetic acid and uranyl acetate-trichloroacetic acid (data not shown).

The inhibition of the exonuclease by EDTA (Fig. 1, lane 2) indicated a metal ion requirement, and the absence of an effect on the endonuclease implied either an independence of metal ions or a reduced requirement. A nuclease preparation fractionated on a Bio-Gel A-15 column and reconcentrated to the original sample volume had negligible nuclease activity. Addition of Mg^{2+}, Ca^{2+}, and Mn^{2+} to a final concentration of 5 mM restored both exonuclease and endonuclease activities. Addition of 20 mM EDTA or ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid to the above assays resulted in full endonuclease activity but no exonuclease activity (data not shown).

To assess whether these nucleases were specific for the strain *P. cepacia* 4G9 or characteristic of the species, several strains, *P. cepacia* ATCC 25416, *Pseudomonas multivorans* ATCC 17759, and *Pseudomonas kingii* ATCC 25609, of different origins were assayed, and all showed endonuclease activity.

From the assays described, one can calculate that the nuclease present in a Brij 58 lysate of a 100-ml culture is sufficient to degrade 300 μg of DNA. Such nuclease activity would be significant in any plasmid isolation. The successful and reproducible isolation of plasmids from *P. cepacia* 4G9 may depend on the isolation of an endonuclease-negative mutant. The selection of

such a mutant is complicated by the fact that the endonuclease is not extracellular, as is the endonuclease of *S. marcescens* (11).

Although SDS at 0.1% inhibited all nuclease activity in in vitro assays, it did not prevent the conversion of added plasmid DNA from CCC DNA to OC DNA when it was used to lyse exponential-phase cultures, nor did it prevent total DNA degradation in stationary-phase culture lysates. This stationary-phase nucleolytic activity is in contrast to endonuclease I of *Escherichia coli*, which shows little or no activity in the stationary phase (2). As the endonuclease of *P. cepacia* 4G9 is active in both growth phases, its effects cannot be avoided merely by choosing an appropriate growth stage for plasmid isolation. The difference in activity level exhibited by the growth phases may merely reflect the number of cells present in the respective 1-ml cultures. A possible explanation for the difference between in vitro and in vivo activities of SDS on the nucleases may be that, although the nucleases may convert some of the CCC DNA to OC DNA, a significant proportion could still be coprecipitating with cell debris and chromosomal DNA during low-speed centrifugation. This interpretation is supported by the observation that increased concentrations of EDTA or SDS did not improve plasmid yields or the reproducibility of agarose gel patterns. The use of diethyl pyrocarbonate as a nuclease inhibitor also failed to improve yields.

The demonstration that *P. cepacia* 4G9 has nucleases active in the presence of EDTA and SDS at concentrations normally used in standard isolation procedures provides an explanation for the irreproducible isolation of plasmids from this strain. One plasmid (pJW3) coding for ampicillin resistance has not been isolated directly from *P. cepacia* 4G9 yet has been demonstrated by transformation (12). These observations suggest that failure to isolate plasmids from a specific species by standard plasmid isolation procedures should not necessarily be taken as proof that the strain is plasmid-free.

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