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tial and temporal dynamics in the genetic diversity of algal virus populations amplified with the primers AVS-1 and AVS-2. These results demonstrate that DGGE can be used to rapidly assess the genetic diversity of algal virus communities in nature.

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Improved Determination Method for Plasmid Copy Number Using Pulsed Field Gel Electrophoresis

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The common methods of determining plasmid copy numbers calculate plasmid copies per cell (12). However, this is difficult because not all microorganisms grow as single cells (e.g., mycelial growth of actinomycetes or chain-forming streptococci). Therefore, the use of chromosomal DNA as a reference to calculate copies per chromosome is the preferred method. In most approaches, DNA is purified (1,5,7,9,13,17). As the isolation procedures involve a significant loss of DNA (6,11), all results are approximate values.

Although there are methods that use chromosomal DNA as an internal standard, precise results are obtainable only when the fragment pattern is reproducible. In the case of using strain-specific nucleases (8), a complete reproducible digestion cannot be proved. To optimize the assessability of the chromosomal fragments following the digestion by restriction endonuclease(s), the wide size range separation by pulsed field gel electrophoresis (PFGE) is most practical. One approach using Southern hybridization of the gel is described (10).

We describe a new method based on both the reproducible separation of chromosomal restriction fragments as an internal standard and the linearized plasmid DNA by means of PFGE. When

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compared with other preparation procedures, the method's main advantage is its limited loss and damage to chromosomal DNA from embedding the cells in agarose. The method is applicable for large and small plasmids with high and low copy numbers. Moreover, we improved the preparation procedure of *Streptomyces lividans* 66, overcame the problem of the nonspecific DNA cleavage (2) and are now able to determine plasmid copy numbers in this strain.

The following protocol is for *S. lividans* 66. Modifications for other microorganisms such as *Escherichia coli* are indicated. A sample from a liquid culture (*S. lividans* 0.3–1 mg dry weight, *E. coli* 0.3–0.6 mg dry weight, $1\text{--}2 \times 10^9$ cells) is immediately chilled on ice and centrifuged at 4°C. The pellet is resuspended in 1.5 mL 4 mol/L guanidine hydrochloride (Fluka Chemie, Buchs, Switzerland) in an incubation buffer of 10 mmol/L HEPES, 0.202 mol/L EDTA, pH 8.0 (both from Roche Molecular Biochemicals, Mannheim, Germany). Treatment with guanidine hydrochloride reduces DNA

degradation by nucleases and may not be needed for other bacteria, such as *E. coli*. The suspension must be incubated for 5 min at 38°C and then centrifuged for 10 min at 4160× *g* and 4°C. The pellet is resuspended in 1 mL HES-buffer, 25 mmol/L HEPES; 25 mmol/L EDTA; 0.3 mol/L sucrose, pH 8.0 (Merck Eurolab, Darmstadt, Germany) (4). Then, 200 µL of the prepared solution and 600 µL 1.5% low melting point agarose (Bio-Rad Laboratories, Hercules, CA, USA) in HES-buffer at 45°C are mixed and poured into reusable plug molds (Bio-Rad Laboratories) and left for 15 min at 4°C to solidify.

The plugs are incubated for 1.5 h at 37°C in 5 mL HES-buffer containing 5 mg/mL lysozyme (20000 U/mg); for *E. coli* 1 mg/mL (Serva Electrophoresis, Heidelberg, Germany). After removal of the lysozyme solution, 5 mL NDS-buffer, 1% N-lauroylsarcosine (Serva Electrophoresis); 0.5 mol/L EDTA, 10 mmol/L glycine, pH 9.5 (Merck Eurolab) (4) containing 1 mg/mL proteinase K, [(30 mAnsonU/mg) (Merck Eurolab)] are added and incubated overnight at 37°C with gentle shaking. This solution is removed and 3 mL HE-buffer, 10 mmol/L HEPES, 2 mmol/L EDTA, pH 8.0, and 150 µL of a stock solution of 10 mg/mL in double-dis-

tilled water of Pefabloc SC™ (a protease inhibitor; 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride; Roche Molecular Biochemicals) are added and incubated for 1 h at room temperature. The plugs are washed 4× in 3 mL HE-buffer for 30 min at room temperature with gentle shaking and can be stored in HE-buffer at 4°C. However, immediate digestion with restriction endonucleases is recommended.

Pieces (5 mm) of the plugs are washed twice with the restriction buffer (as recommended by the manufacturer) for 30 min at room temperature. The restriction enzyme is chosen to produce a suitable pattern of chromosomal fragments and to linearize the plasmid DNA. Depending on the activity of the restriction endonuclease(s), digestion is carried out between 4 h and overnight.

For PFGE, an agarose gel 1.5% Sea-Kem Gold™ (FMC BioProducts, Rockland, ME, USA) in 0.5× TBE-buffer (15) + 12 mmol/L thiourea (Merck Eurolab) was used. Thiourea is necessary to avoid the Tris-dependent, site-specific cleavage of *S. lividans* DNA during gel electrophoresis (14). The gel is stained using ethidium bromide (EtdBr), for example, and scanned using CS-1 Image Documentation System (Cybertech, Berlin, Ger-

Figure 1. Pulsed field gel electrophoresis of *Streptomyces lividans* TK23 (pGM9) following digestion with *Xba*I (linearizes plasmid)/*Ase*I (New England Biolabs, Beverly, MA, USA). Using the CHEF-DRII System (Bio-Rad Laboratories), PFGE was carried out at 160 V for 23 h with a ramp of 1–16 s. Lane 1: pGM9 ladder (ligation of linearized plasmid, prepared as *Streptomyces* DNA marker in order to determine the fragment length more exactly), 6.265 kb indicate linearized pGM9; lanes 2 and 3: *S. lividans* TK23 (pGM9); lane 4: low range PFG marker (New England Biolabs). The lines indicate the chromosomal reference bands used in estimation.

Figure 2. Pulsed field gel electrophoresis of *E. coli* K12 (F'*lac*) and a plasmid-free strain following digestion with *Xba*I. PFGE was carried out at 160 V for 20 h with a ramp of 1–20 s. Lane 1: Lambda Ladder PFG Marker (New England Biolabs), lane 2: *E. coli* K12 (F'*lac*) 128 kb indicate linearized F'*lac*; lane 3: plasmid-free *E. coli* K12. The lines indicate the chromosomal reference bands used in estimation.

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many), and the intensity of chosen bands is measured (using WinCam 2.2, 2D Densitometer, Cybertech). The copy number is calculated as follows:

plasmid copy number = (Eq. 1)

$$\frac{IP}{IC} \cdot \frac{SCB \cdot n}{SPB} = \frac{IP \cdot SCB \cdot n}{IC \cdot SPB}$$

(*IP*: intensity of the plasmid band; *IC*: intensity of the chromosomal band; *SCB*: size of chromosomal [reference] band [kb], *SPB*: size of plasmid band [kb], *n*: number of restriction fragments per chromosomal band where a multiple band is used).

To verify the method, copy numbers of different plasmids were determined and compared with published data. The most interesting result was the broad plasmid size and copy number range for which the method was applicable. Parallel determinations maximally differed by $\pm 15\%$ only.

It is necessary to determine the linear area of all experimental and technical parameters at first to be enabled to operate within its limits thereafter. The samples should be processed as soon as possible and should not be frozen.

Although the lysis procedure does not cause any loss of chromosomal DNA, there may be a loss of plasmid DNA from the plugs from diffusion. So the buffer volumes, incubation times, length of time for the wash procedures and storage should not be exceeded unnecessarily. Immediate restriction is preferable to storage.

A PFGE gel of *S. lividans* TK23 carrying the vector plasmid pGM9, 6.265 kb (10) is shown in Figure 1 as an example. We calculated 14 copies per chromosome ($\pm 15\%$, 12 parallel determinations) during the exponential growth phase in complete medium at 28°C.

The copy number of the plasmid *F' lac* (128 kb) in *E. coli* was measured to show the accuracy of the method at its limits as well (Figure 2). We found 1.1 ($\pm 15\%$) copies per chromosome in exponentially growing cultures at 28°C in LB medium (15) according to literature (3). The method should also be applicable for linear plasmids. Any differences in copy number of chromosomal bands according to their position with

respect to *oriC* should not affect the calculation. In streptomycetes, different stages of replication are statistically distributed. Plasmid copy numbers are correlated to multifork replication within other bacteria. The resulting proportions are considered in the calculation within *n* (number of restriction fragments per chromosomal band).

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