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Restriction Endonucleases: General Survey Procedure and Survey of Gliding Bacteria

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Among 120 strains of gliding bacteria which were screened for restriction endonucleases, 27 were found positive. Additionally, three strains carried enzymes able to release the supercoiled state of closed circular DNA. By using a new rapid method, restriction endonuclease activity was released by stirring about 0.5 g of cells (fresh weight) in a motor-driven glass homogenizer in buffer containing Triton X-101, ethylenediaminetetraacetic acid, and mercaptoethanol. A yield from 60 to 80% of the total activity present in the cells was obtained with minimal destruction of the cells. The enzyme activity in the crude extract was measured semi-quantitatively by digestion of DNA and subsequent separation of the fragments on an agarose slab gel. The method appears to be generally applicable for the extraction of restriction endonucleases from gram-negative bacteria on an analytical scale and in a modified form for large-scale preparation of restriction enzymes.

Specific endonucleases are of widespread occurrence throughout the bacterial kingdom, and the presently recognized endonucleases may be only the tip of the iceberg. Since their discovery they have become invaluable tools in research in physical mapping of DNA, DNA-sequencing, gene isolation, and genetic engineering.

Restriction and modification systems which attack foreign DNA have been described extensively. According to the model of Arber and Roulland-Dussoix and Boyer (1, 20), two ezyme activities are involved. A restriction endonuclease recognizes specific nucleotide sequences of invading foreign DNA and subsequently cleaves this DNA. Alternatively, a modification enzyme recognizing the same nucleotide sequences is able to modify these sequences by methylation. A modified DNA is protected against cleavage. Many restriction modification systems have now been identified genetically in gram-negative and gram-positive bacteria (6, 13; J. R. Awana, P. A. Meyers, and R. J. Roberts, unpublished data; R. J. Roberts, personal communication).

Restriction endonucleases can be detected by their ability to degrade unmodified DNA, as compared with modified DNA, which is resistant to their action. The biological assay normally employed to detect such restriction systems is the reduction of titers of bacteriophages coming from hosts which lack such a modification system (4, 15).

As a general approach for the discovery of new restriction enzymes, the bioassay has severe drawbacks, not the least of which is that in many bacterial species no sexual system or bacteriophage exist. Furthermore, the restriction enzymes fall into two main groups: class I, in which the endonuclease binds at a specific site but cuts randomly at an indeterminate distance from this binding site; and class II, in which the endonuclease binds at a specific sequence on the DNA and cuts within or adjacent to this sequence (type II), or at a specific distance from the recognition site (type III) (17; Roberts, personal communication). As tools for the manipulation of DNA, one of the most useful techniques in modern molecular biology, only the type II and type III enzymes are of significance. The bioassay does not distinguish between the different types of enzyme activity, and therefore more direct approaches have been developed in the search for new restriction endonucleases.

In general, screening methods for restriction endonucleases which entail the disruption of the cells by sonic oscillation are very time consuming and complicated, because nonspecific nucleases can mask the presence of the specific restriction endonuclease unless coupled to a few simple purification steps (19). Osmotic shocking has been described as a useful way to screen gram-negative bacteria for the presence of restriction endonucleases, but the efficiency of releasing for the enzyme is not very high and the procedure is time consuming (21).

We therefore developed a new rapid and efficient method for screening large numbers of strains. This method appears to be generally applicable to the screening of gram-negative bac-

teria for the presence of restriction endonucleases carried in the periplasmatic space.

Because recently in one strain of gliding bacteria endonuclease activities have been found (16), we decided to investigate gliding bacteria further for restriction endonucleases. By rescreening 120 strains, we demonstrated restriction endonuclease activities present in 27 strains belonging to 27 different species.

MATERIALS AND METHODS

Bacterial strains. All strains were taken from the collection of one of us (H. Reichenbach).

Cultures. Cells for experiments were taken from liquid cultures agitated on a rotary shaker at 30°C or, with most cytophagas, at 22°C. Media were for *Nannocystis* simplified MD1 1m, 0.3% Casitone (Difco), 0.1% $CaCl_2 \cdot 2H_2O$, and 0.2% $MgSO_4 \cdot 7H_2O$ (pH 7.2); for all other myxobacteria and the cytophagas cas 1m, 1% Casitone (Difco) and 0.1% $MgSO_4 \cdot 7H_2O$; for *Herpetosiphon* Hp74 1m, 1% sodium glutamate, 1% glucose, 0.2% yeast extract (Difco), 0.2% $MgSO_4 \cdot 7H_2O$ and 1 mM potassium phosphate buffer (pH 6.5).

DNAs. Lambda DNA was prepared from λ*t*68c*I*857*s*7 in *Escherichia coli* K-12/W8 by standard procedures (3). Plasmid DNA Rsc11 was isolated by the method of Goebel and Bonewald (9).

Endonuclease assay. Amounts of 5, 10, and 20 μl of the supernatant of the cell extract were adjusted to a final volume of 50 μl with 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8) and 10 mM $MgCl_2$ containing 0.3 μg of λDNA. Incubation was at 37°C for 1 to 3 h. Digestion was stopped with 5 μl of a mixture containing 20% (vol/vol) glycerol, 5 M urea, 0.25 M ethylenediaminetetraacetic acid, 10% (wt/vol) sodium dodecyl sulfate, and 0.025% (wt/vol) bromophenol blue (pH 7.0).

Agarose slab gel electrophoresis. Slab gels (1%) in a vertical electrophoresis apparatus were run in 30 mM NaH_2PO_4–10 mM ethylenediaminetetraacetic acid–36 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5). The gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV light.

RESULTS

Release of restriction endonucleases by stirring cells in a glass Potter tissue homogenizer. Cells from a 300-ml shaking culture were harvested by centrifugation at 24,000 × *g* for 15 min at 5°C. The pellet was resuspended in 3 to 5 ml of buffer (10 mM potassium phosphate (pH 7.5), 0.01% Triton X-101, 7 mM 2-mercaptoethanol, and 1 mM ethylenediaminetetraacetic acid) in a 50-ml glass homogenizer by stirring with a Teflon pestle at 2,000 rpm generally twice, each time for 15 s, in an ice bath. The cell suspension was centrifuged at 40,000 × *g* for 10 min at 2°C. Samples of 5, 10, and 20 μl of the supernatant were used for assaying restriction endonuclease activity. Usually, most of the cells remained undisrupted by the treatment. Proteins released by this method gave about 10 bands on disc electrophoresis (data not shown).

The amount of endonuclease activity released by homogenization was compared with the total amount of activity in the cell.

To estimate the total activity, an equal portion was sonically disrupted with a Branson Sonifier for 60 s at 60 W in an ice bath. After centrifugation at 2°C for 10 min at 40,000 × *g*, the restriction endonuclease activity was quantitatively determined in the supernatant. As shown in Fig. 1, 60 to 80% of the activity could be released by homogenization as compared with the sonically disrupted cells.

For comparison of this method with the osmotic shocking procedure, an equivalent amount of cells was osmotically shocked. The efficiency of the osmotic shocking procedure was only 40 to 60% of the total activity measured in the sonic extract. The mechanical shear method therefore allowed the most efficient detection of low quantities of endonuclease activity without extensive cell disruption.

Because this method involves only one centrifugation step, it is very rapid compared with the osmotic shocking procedure which requires two washing and centrifugation steps. This allowed us to screen 120 strains of gliding bacteria for restriction endonuclease activity in a short time.

To test the general applicability of the new method, we tested strains which were known to contain restriction endonuclease by stirring four times for 2.5 min. From *E. coli* RY13 (10) we were able to recover *Eco*RI endonuclease; from

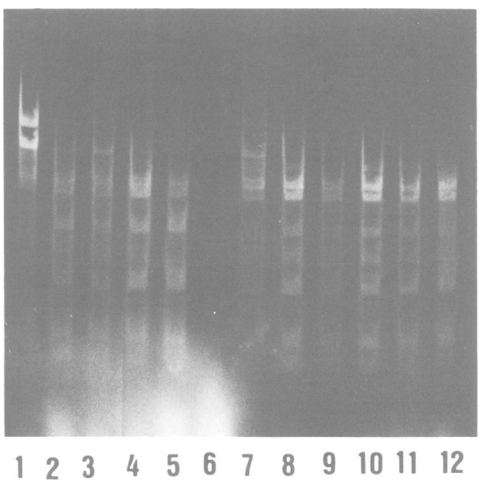

FIG. 1. *Agarose slab gel electrophoresis of Rsc11 DNA incubated for 15 min at 37°C with the extract (1, 2, 5, 10, 20, and 50 μl; positions 1 to 6) and the sonic extract (1, 2, 5, 10, 20, and 50 μl; positions 7 to 12) from C. velatus Plv9.*

Pseudomonas stuartii 164 (21) we recovered *PstI* endonuclease; and from *Enterobacter cloacae* (11) we recovered a low concentration of the *EclI* restriction endonuclease. From the gram-positive bacteria *Bacillus amyloliquefaciens* H (25) and *Bacillus subtilis* X5 (5), which contain the restriction endonucleases *Bam*HI and *Bsu*I, no restriction endonucleases were released with this method. The method seems thus to be generally applicable for releasing restriction endonucleases from gram-negative bacteria.

Specific endonuclease activity in *Myxobacterales*. We tested 29 strains of *Myxococcus fulvus* for restriction endonuclease activity. The extracts of eight strains showed definite fragments with λDNA. Two more strains showed a low restriction endonuclease activity. Mx f3 gave a restriction endonuclease activity with probably one cleavage site in λDNA (Fig. 2, panel 2). Mx f44 showed 2 fragments with λDNA (Fig. 2, panel 3). The strains Mx f2 (Fig. 2, panel 1), Mx f37 (Fig. 2, panel 5), and Mx f40 (Fig. 2, panel 6) produced very similar fragments with λDNA, and three bands, the two smaller fragments representing the products of complete digestion. By double digestion with different combinations of the extracts from the three strains, no change in the λ pattern could be obtained, which indicated that these enzymes were isoschizomers. The enzyme activity of the strain Mx f26 produced with λDNA two large fragments and one small fragment (Fig. 2, panel 4). The strain Mx f42 (Fig. 2, panel 7) showed with λDNA one large and one small fragment; Mx f45 (Fig. 2, panel 8) gave with λDNA at low enzyme concentration one large and about four small fragments; at higher enzyme concentration the fragments were lost due to degradation by nonspecific nucleases. With strains Mx f22 and Mx f25 we found a low quantity of restriction endonuclease activity with λDNA (agarose gel not shown). In the plasmid Rsc11 DNA (8.07×10^6 daltons [14]) there was no site for the endonucleases from strains Mx f2, Mx f3, and Mx f37 (not shown). In the strains Mx f26, Mx f40, and Mx f42 we found an activity which altered the supercoiled state of the closed circular form of plasmid Rsc11 (Fig. 3, panels 1, 2, and 3). Enzymes from *E. coli* 1100 and B, called omega proteins, have been purified and were able to catalyze duplex rotation in the right-handed direction only (23, 24). The known eucaryotic enzymes are able to catalyze rotation in both directions. They have been discovered in several types of animal cells (7, 12, 22). Vaccinia virus cores also contain an activity which was able to relax both left- and right-handed superhelical DNA (2). This indicates the widespread distribution of enzymes of this type. The nicking closing activity, which was found in our three strains, is the first example of such enzymes in gliding bacteria. This activity was found in addition to restriction endonuclease activity.

From nine tested strains of *M. virescens*, only strain Mx f2 showed a low endonuclease activity with probably one cut in λDNA, which would correspond to one of the activities reported by Morris and Parish (16), and one cut in Rsc11 DNA. Among six tested strains of *M. xanthus*, we found a low restriction endonuclease activity in Mx x2 which produced 10 cuts in λDNA, and in Mx x3 we found an enzyme which made probably four cuts in λDNA (gel not shown). From the two tested strains of *M. stipitatus* we obtained a high specific activity. Mx s1 (Fig. 4, panel 1) made five fragments from λDNA, and Mx s2 two major fragments (Fig. 4, panel 2). Mx s2 produced one cut in Rsc11 DNA. It was

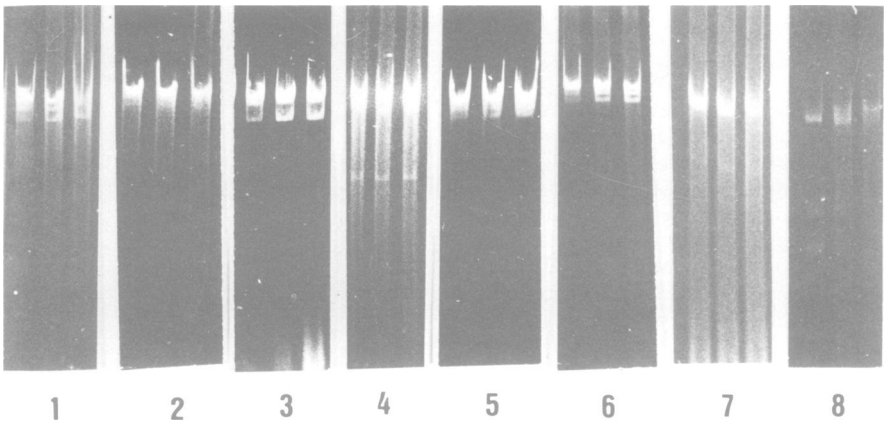

FIG. 2. *Agarose slab gel electrophoresis of λDNA fragments digested for 1 h at 37°C with 5, 10, and 20 μl of the extract from strains of Myxobacterales. Panels: 1, Mx f2; 2, Mx f3; 3, Mx f44; 4, Mx f26; 5, Mx f37; 6, Mx f40; 7, Mx f42; 8, Mx f45.*

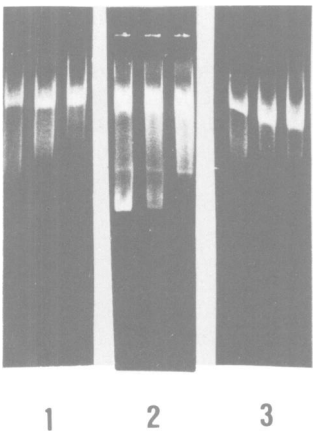

FIG. 3. *Agarose slab gel electrophoresis of Rsc11 DNA incubated for 2 h at 37°C with 5, 10, and 20 μl of the extract from strains of Myxobacterales. Panels: 1, Mx f26; 2, Mx f40; 3, Mx f42.*

thought that this activity could be identical to *Sal*GI. A double digest with *Hin*dIII, *Sal*GI and with *Hin*dIII Mx s2 using λDNA showed that the fragments are not identical. This indicated that the enzyme *Sal*GI and that from Mx s2 recognized different sites. In two strains of *Archangium serpens* activity has been detected. Strain Ar 606 showed two fragments with λDNA (Fig. 4, panel 3) and probably five fragments with plasmid Rsc11 DNA (not shown). Strain Ar 505 produced about five fragments with λDNA and five with Rsc11 DNA, but the extracted activity is very low (data not shown). From *Podangium erectum*, one tested strain, Pd e11, produced two fragments with λDNA (Fig. 4, panel 4) and one cut in Rsc11 DNA.

Specific endonuclease activity in *Cytophagales*. All of the 45 screened strains of *Cytophaga* showed very high concentrations of nonspecific nuclease activity. It was with some difficulty that we could detect a specific activity in strain PH3, which produced three fragments with Rsc11 DNA (gel not shown), whereas λDNA was completely degraded. Only strain *Cy 627* showed three sharp fragments with λDNA (Fig. 4, panel 5) and one cut with Rsc11 DNA. This activity was probably an isoschizomer to the enzyme of *M. fulvus*, Mx f26 (Fig. 2, panel 4).

With seven tested *Flexibacter* strains we found a low activity in Fx 603-3, which produced about four partial fragments with λDNA (Fig. 4, panel 6) and one cut in Rsc11 DNA.

Specific endonuclease activity in *Leucotrichales*. Five of seven tested strains of *Herpetosiphon giganteus* released specific endonuclease activities. Strain Hp a1 produced more than six fragments from λ (Fig. 5, panel 1) and also with Rsc11 DNA. The strain Hp a2 produced about three fragments from λDNA (Fig. 5, panel 2), but the extracted activity was very low. Strain Hp g5 produced two fragments with λDNA (Fig. 5, panel 3), but more than eight with that of Rsc11; this activity seemed to be similar to *Sal*GI in the pattern of λDNA, but quite different in the pattern of Rsc11 DNA, which contains only one site for *Sal*GI (14). The extract of Hp g8 showed four or more small fragments with λDNA (Fig. 5, panel 4), and only one cut with Rsc11. With strain Hp g9 we found more than eight fragments with λDNA (Fig. 5, panel 5) and more than eight fragments with Rsc11 DNA.

The number of cleavage sites in λDNA and Rsc11 DNA produced by restriction endonucleases from various gliding bacteria are summarized in Table 1.

DISCUSSION

The experiments reported here indicate that

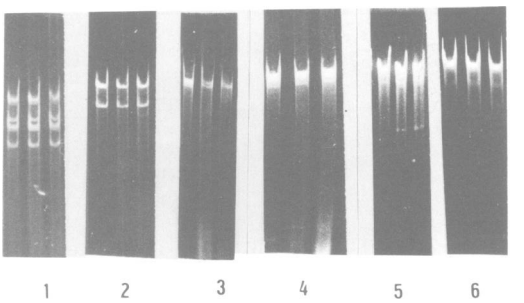

FIG. 4. *Agarose slab gel electrophoresis of λDNA fragments digested for 2 h at 37°C with 5, 10, and 20 μl of the extract from Myxobacterales. Panels: 1, Mx s1; 2, Mx s2; 3, Ar 606; 4, Pd e11; and from Cytophagales, 5, Cy 627; 6, Fx 606-3.*

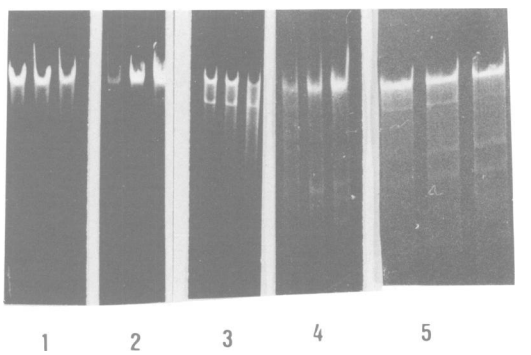

FIG. 5. *Agarose slab gel electrophoresis of λDNA incubated for 2 h at 37°C with 5, 10, and 20 μl of the extract from strains of Leucotrichales. Panels: 1, Hpa1; 2, Hpa2; 3, Hpg5; 4, Hpg8; 5, Hpg9.*

the gliding bacteria represent a rich source of restriction endonucleases. The simple releasing procedure, described here, allows a screening of 20 different strains in 1 day. The method seems to be applicable to releasing restriction endonucleases located in the periplasmatic space of gram-negative bacteria generally. The analysis by gel electrophoresis in a slab gel allows a comparison of the fragments.

The attempt to find common features among the tested bacterial strains has been disappointing. The most heterogenous source has been found to be the *Myxobacterales*. In the strains Mx f10, Mx f12, Mx f15, Mx NM, and Mx v6 we could not detect any nucleolytic activity. These strains would perhaps be suitable for developing a genetic system, like transformation or tranduction, in *Myxococcus*. All tested strains of *Cytophagales* except two contained high levels of nonspecific nucleases, which prevented the detection of specific endonuclease activity. Among the *Leucotrichales* nearly all *Herpetosiphon* strains had easily detectable restriction endonucleases.

TABLE 1. *Isolation of restriction endonucleases*

Microorganism	Code	No. of cleavage sites λDNA	RscI1 DNA
Myxobacterales			
M. fulvus	Mx f2	~2	0
	Mx f3	~1	0
	(Mx f22	~4	1)[a]
	(Mx f25	~5	1)
	Mx f26	3	Relaxed
	Mx f37	~2	0
	Mx f40	~2	Relaxed
	Mx f42	2	Relaxed
	Mx f44	1	1
	Mx f45	4	1
M. virescens	(Mx v2	1	1)
M. xantus	(Mx x2	~10	1)
	(Mx x3	~4	1)
M. stipitatus	Mx s1	4	1
	Mx s2	~1	1
A. serpens	(Ar 505	~4	~5)
	Ar 606	~1	~5
P. erectum	Pd e11	~2	1
C. velatus	Pl v9	~10	~5
Cytophagales			
Cytophaga sp.	(PH 3	?	3)
	Cy 627	3	1
Flexibacter sp.	Fx 603-3	?	1
Leucotrichales			
H. giganteus	Hp a1	~6	~6
	(Hp a2	2	4)
	Hp g5	1	~8
	Hp g8	~4	1
	Hp g9	~8	~8

[a] Parentheses indicate low restriction endonuclease activity.

The following restriction enzymes seem to be markedly different from those restriction enzymes known to us. Mx f2 and the isoschizomers Mx f37 and Mx f40 are not identical with *Sal*GI or *Kpn*I. This was proven by double digestion. Mx f26 is presumably an isoschizomer to Cy 627, because they show a very similar λ pattern. The relaxing activity in Mx f26 could be an additional enzyme. Mx s1 shows a λ pattern, which is unfamiliar. The fragments of Mx s2 are not the same as produced by *Sal*GI, as we have shown by double digestion. The λ fragments of Pd e11 are also unfamiliar. The interpretation of the endonuclease activity data from strains Mx f3, Mx f42, Mx f45, Ar 606, and Pl v9 is difficult, because the fragments obtained do not represent a limit digest. In the case of Pl v9 we know from the purified endonuclease that the fragments produced from λDNA and that of RscI1 are not comparable to any known restriction endonuclease.

In the case of the restriction endonucleases of the *Leucotrichales*, we are at the moment unable to draw conclusions as to the relationship of these activities to known endonuclease activities. The purified enzymes from Hp a2, Hp g5, and Hp g9 showed unfamiliar patterns. A more detailed analysis of the purified enzymes and the determination of the DNA recognition sites should show us whether the enzymes have new specificities. With the majority of strains, the amount of restriction endonucleases found appears to be high enough to allow a purification at a larger scale.

Myxobacteria are known to excrete an arsenal of exoenzymes (proteases, cellulases, nucleases) to attack foreign bacteria and lyse them. Perhaps the wide range of endonucleases which we encountered and which seem to be located in or under the cell surface have the function to degrade the DNA of lysed foreign bacteria. Alternatively, the restriction endonucleases could be involved in promoting site-specific genetic recombination. Site-specific genetic recombination has been shown to be promoted in vivo by the *Eco*RI endonuclease and DNA ligase (8). Such recombination could involve incoming (unmodified) DNA species, introduced by transduction, conjugation, or transformation, or rearrangement of segments of genomes of the growing cells.

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