Simple and Sensitive Procedure for Screening Collagenolytic Bacteria and the Isolation of Collagenase Mutants

By P. J. ROBBERTSE,* D. R. WOODS, A. H. REAY AND F. T. ROBB

Department of Microbiology, Rhodes University, Grahamstown 6140, South Africa

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INTRODUCTION

Woods *et al.* (1973) described a procedure involving liquid cultures for determining the collagenolytic activity of hide bacterial populations. Previous reports have shown that the enumeration of aerobic and anaerobic cured hide bacteria is of limited value as an indication of hide quality (Woods *et al.*, 1970*a, b*; Woods, Welton & Cooper, 1971). However, a routine agar plate assay for collagenolytic bacterial colonies would be most useful in the leather industry and would have advantages over the liquid technique. The isolation from hides of an aerobic collagenolytic *Achromobacter iophagus* strain, which produces an inducible extracellular collagenase with the highest specific activity for a collagenase, has been described (Welton & Woods, 1973, 1975; Lecroisey *et al.*, 1975). Future studies on the control of collagenase could be facilitated by the development of a simple screening procedure for the isolation of collagenase mutants.

Although techniques utilizing reconstituted collagen gels have been used for the isolation of collagenolytic bacteria (Waldvogel & Swartz, 1969; Merkel, Dreisbach & Ziegler, 1975), they have proved unsuitable for mutant screening procedures. The method of Waldvogel & Swartz (1969) involving the transfer of portions of agar covered by actively growing overnight cultures to collagen gels is not suitable for screening large numbers of colonies. Growth on collagen gels alone (Merkel *et al.*, 1975) is also unsuitable because many strains, including *A. iophagus*, do not form visible colonies on collagen gels. This paper describes an adaptation of the double layered agar plate technique used for the detection of various enzymes (e.g. lipase: Eijkman, 1901; Alford & Steinle, 1967) for the isolation of collagenase-producing strains. The characteristics of some of the mutants are also described.

METHODS

All percentage compositions are w/v and all nutrients are from Difco, unless otherwise stated.

Bacteria. The wild-type collagenolytic *A. iophagus* strain isolated from hides by Welton & Woods (1973) was used. The strain was identified as *A. iophagus* using the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957), but using the eighth edition (Buchanan & Gibbons, 1974) it was found to be of uncertain taxonomic position.

Media. Wild-type and mutant strains were maintained on the complex medium of Welton & Woods (1973). The peptone agar medium consisted of 0.25% peptone and 1.5% agar in 0.1 M-Tris/HCl buffer (pH 7.6) containing 0.4 M-NaCl and 2 mM-CaCl₂. The Casamino acids medium consisted of 2.5% vitamin-free acid-hydrolysed casein and 1.5% agar in similar Tris/HCl buffer. The minimal medium was that of Grabow & Smit (1967), supplemented with 0.4 M-NaCl and 2 mM-CaCl₂. Incubation was at 30 °C.

Mutagenesis. Collagenase mutants were produced by treating exponentially growing broth cultures with 100 µg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) ml⁻¹ for 15 min at 37 °C (Adelberg, Mandel & Chen, 1965) in 0.1 M-Tris/maleic acid buffer containing 0.4 M-NaCl, 0.4 mM-MgSO₄, 7.5 mM-(NH₄)₂SO₄ and 2 mM-sodium citrate. The treated cultures were incubated in the peptone medium at 30 °C for 4 h to allow the expression of mutations.

* Present address: Department of Genetics, University of Pretoria, Pretoria 0002, South Africa.

Preparation of collagen. Acid-soluble calfskin collagen was prepared by the method of Cooper & Davidson (1965) and kept in 0.47 M-acetic acid at 4 °C. Prior to use, the collagen was clarified by centrifugation and dialysed against 0.1 M-sodium phosphate buffer, pH 7.6. The collagen did not gel when it was kept at 4 °C and when required 4 M-NaCl was added to give a final concentration of 0.4 M-NaCl. The collagen was immediately poured over the surface of the peptone agar plates and allowed to gel at 37 °C.

Screening of collagenase mutants. Peptone agar plates were spread with NTG-treated *A. iophagus* wild-type cells and incubated overnight at 30 °C. The colonies were then overlaid with the cold, reconstituted, acid-solubilized collagen which gelled on incubation at 37 °C. After a further 1.5 or 3 h incubation at 37 °C, collagenase-positive colonies were identified by a zone of hydrolysis around the colonies. The specificity of the reconstituted collagen gel overlay was tested by placing on the surface of the gel filter paper discs containing either pronase (Miles Laboratories), trypsin (Miles Laboratories) or collagenase from *Clostridium histolyticum* (Koch-Light). The effects of glucose and Casamino acids on the production of collagenase were determined by adding 0.1, 0.5, 1.0, 1.5, 2.0 or 2.5 % glucose or Casamino acids to the peptone agar. In certain experiments either 1 % glucose or 0.1 % Casamino acids was added to the collagen overlay.

Induction of collagenase in liquid media. The induction of collagenase in broth cultures of *A. iophagus* was investigated by adding collagen, peptone, gelatin, tryptone, Casitone and Casamino acids to 0.1 M-Tris/HCl buffer (pH 7.6) containing 0.4 M-NaCl and 2 mM-CaCl₂. The cultures were shaken at 30 °C for 18 h and collagenase was assayed using the synthetic substrate phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (PZ-Pro-Leu-Gly-Pro-Arg) (Fluka, Buchs, Switzerland) by the method of Wünsch & Heidrich (1963). Collagenase activity was expressed as pkat per ml culture filtrate on the basis of 1 pkat = 0.09 units according to Wünsch & Heidrich (1963).

RESULTS

Induction of collagenase

Collagenase was induced by both collagen and peptone, giving activities of 60 pkat ml⁻¹ in the presence of 2 mg collagen ml⁻¹ and 300 and 370 pkat ml⁻¹ in the presence of 5 and 50 mg peptone ml⁻¹, respectively. Media containing gelatin, tryptone, Casitone or Casamino acids (each at 50 mg ml⁻¹) and glucose minimal salts medium all failed to induce the production of collagenase. The amount of bacterial growth was similar in all the media except the collagen and glucose minimal salts media. Further incubation of all cultures which did not contain collagenase after 18 h did not result in the induction of collagenase. Since the peptone medium induced the highest yields of collagenase, it was used in the development of a plate method for screening collagenase mutants.

Collagenase screening procedure

The double layered agar plate technique using a peptone agar layer and collagen gel overlay was investigated as a method for screening collagenase strains and mutants. The collagen gel was not liquefied by either pronase or trypsin but was hydrolysed by commercial *C. histolyticum* collagenase. The method proved to be suitable for the detection of collagenase-positive (Col⁺) wild-type colonies of *A. iophagus*. The Col⁺ colonies were surrounded by an area of hydrolysis and collagenase-negative (Col⁻) strains or mutants showed no zones of hydrolysis. A semi-quantitative estimation of the rate and amount of collagenase produced was obtained by determining the time and degree of collagen lysis. The wild-type *A. iophagus* strain produced zones of collagen hydrolysis on peptone plates after 1.5 h at 37 °C.

Isolation of collagenase mutants

The production of collagenase by the wild-type Col⁺ strain was inhibited by the addition of either 0.5 % glucose or 0.5 % Casamino acids to the peptone agar (Table 1). The addition of 0.1 % Casamino acids to the collagen overlay inhibited collagenase production by the Col⁺ strain growing on peptone agar plates containing 0.1 % Casamino acids. The inhibition of collagen hydrolysis by glucose was not reversed by the addition of 1 mM-cyclic AMP. The Col⁺ strain did not hydrolyse the collagen gel overlay after growth on the Casamino acids agar medium. The Col⁺ strain was mutagenized with NTG and screened for mutants on the

Table 1. *Collagenase production by A. iophagus wild-type Col⁺ strain and the mutant strains ColA, ColG and Col⁻*

Peptone (0.25%) or Casamino acids (CAA; 2.5%) agar plates supplemented with different concentrations of glucose, cyclic AMP or CAA were streaked with the Col⁺, ColA, ColG and Col⁻ *A. iophagus* strains. After overnight incubation at 30 °C, the plates containing visible colonies were overlaid with either collagen or collagen supplemented with 1% glucose or 0.1% CAA. Zones of hydrolysis were recorded after 3 h at 37 °C.

Medium	Strain			
	Col ⁺	ColA	ColG	Col ⁻
Peptone	++	—	++	—
Peptone+0.1% glucose	+	—	++	—
Peptone+0.5 to 2% glucose	—	—	++	—
Peptone+2.5% glucose	—	—	++	—
Peptone+0.5% glucose+1 mM-cyclic AMP	—	—	++	—
Peptone+0.1 to 2.5% glucose+1% glucose in overlay	—	—	++	—
Peptone+0.1% CAA	++	++	++	—
Peptone+0.5% CAA	—	++	++	—
Peptone+1 to 2.5% CAA	—	++	—	—
Peptone+0.1% CAA+0.1% CAA in overlay	—	++	++	—
Peptone+0.5% CAA+0.1% CAA in overlay	—	++	+	—
Peptone+1 to 2.5% CAA+0.1% CAA in overlay	—	++	—	—
CAA	—	++	—	—
CAA+0.1% CAA in overlay	—	++	—	—
Peptone+1.5% CAA+0.1 to 2.5% glucose	—	++	—	—

++, Good hydrolysis; +, hydrolysis; —, no lysis.

media indicated in Table 1. Many Col⁻ mutants were isolated and the nature of the mutations are being investigated.

Two mutants, a ColA mutant and a ColG mutant (Table 1), were isolated which differed from the Col⁺ strain in their sensitivity to repression of collagenase by Casamino acids and glucose, respectively. The ColA mutant was not only completely insensitive to the addition of 2.5% Casamino acids to the peptone agar and 0.1% Casamino acids to the collagen overlay, but actually required the addition of at least 0.1% Casamino acids to the peptone agar before collagenase was produced. Optimum levels of collagenase were obtained when 1.5% Casamino acids was added to the peptone agar. The production of collagenase by the ColA mutant in peptone agar supplemented with 1.5% Casamino acids was not repressed by the addition of 0.1 to 2.5% glucose to the agar medium. The ColG mutant was repressed by concentrations of Casamino acids greater than 0.5% but was not affected by glucose. Although many colonies have been screened, a constitutive mutant which does not require collagen or peptone for collagenase induction has not been isolated.

DISCUSSION

The peptone–collagen overlay method is a simple, sensitive and specific procedure for the isolation of collagenase strains or mutants. The method could be used in the leather and related industries as a quality control procedure for assaying collagenolytic bacteria on hides and skins.

The induction of collagenase by peptone and not gelatin is interesting. Keil-Dlouha, Misrahi & Keil (1976) showed that the synthesis of collagenase in *A. iophagus* is induced by

collagen and by its high molecular weight fragments. It is concluded that peptone contains the high molecular weight fragments but that gelatin, although it is a degradation product of collagen, does not contain the inducer.

The production of collagenase by the wild-type *A. iophagus* strain is repressed by both glucose and Casamino acids. The failure of cyclic AMP to reverse the glucose effect suggests that this effect is not due to catabolite repression (De Crombrughe *et al.*, 1969). The isolation of the ColG mutant, which is insensitive to glucose but repressed by amino acids, and the ColA mutant, which is insensitive to glucose but requires amino acids, suggests that the glucose and amino acid effects are mediated differently. The repression of extracellular protease synthesis by amino acids has been reported in a number of bacterial genera (Glenn, 1976). However, little is known about the mechanism of repression by amino acids. This study reveals a complex regulatory system for collagenase involving two independent inhibitory effects and a separate inductive effect by collagen and its high molecular weight fragments. The mechanisms of these three systems are unknown and are being investigated.

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