Collagenolytic Activity of Aerobic Halophiles from Hides

By JENNIFER A. THOMSON, D. R. WOODS AND
R. L. WELTON

*Department of Botany and Microbiology, Rhodes University,
Grahamstown, South Africa*

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SUMMARY

The collagenolytic activity of 110 aerobic halophilic and halotolerant bacterial strains isolated from cured hides was determined. At 2.34% (w/v) NaCl, 10% of the bacterial strains could denature purified collagen. Collagenolytic activity was not detected at 7% (w/v) NaCl. Three of the collagenolytic strains were identified as *Bacillus* spp. and eight as *Achromobacter* spp. Nine of these eleven bacteria were also gelatinolytic. Australian hides which produce decay-free leather lacked collagenolytic bacteria.

INTRODUCTION

Since leather decay is attributed to bacterial degradation of collagen, the incidence of collagenolytic bacteria in cured hides is important. Although pure collagen is not denatured by common proteolytic enzymes, it may be denatured by specific collagenases or by physical and chemical means. Denatured collagen (gelatin) is susceptible to many proteolytic enzymes. Unfortunately, frequent use of unsuitable denatured substrates has resulted in erroneous reports of collagenolytic activity (Mandl, 1961). Eight bacterial strains have been shown to have collagenolytic activity under anaerobic conditions (Waldvogel & Swartz, 1969). The only report of collagenolytic activity in aerobic bacteria, as judged by denaturation of purified collagen, implicated two strains of *Pseudomonas* (Adamcic & Clark, 1970).

The present study was undertaken to determine the incidence of collagenolytic halophilic and halotolerant bacteria in hides. The salt requirements and gelatinolytic activity of the bacteria were also investigated.

METHODS

Bacterial isolation. Plugs, 2 cm in diameter, were taken from 10 randomly selected hides from eight different batches at a tannery (Woods, Atkinson, Cooper & Galloway, 1970b). Two of the hide batches were from different Australian curers and the remainder from six different South African curers. Five plugs from each hide sample were cut into pieces and soaked in 50 ml 10% (w/v) NaCl at 4° for three days prior to plating on 10% NaCl agar to obtain isolated bacterial colonies.

Media. (Note: all percentage compositions are w/v unless otherwise stated.) Aerobic bacteria were cultured on 0.85, 10.15 and 20% NaCl agar (Woods, Atkinson, Cooper & Galloway, 1970a). Facultative anaerobes were cultured on 0.85 and 10% NaCl agar with 0.11% sodium thioglycollate. Anaerobic cultures were placed in Brewer jars under 98% N₂ and 2% H₂ (v/v). Cultures were incubated at 35°.

Gelatinolytic activity. Bacterial strains were grown on 0.85 and 10% NaCl agar containing 0.25% gelatin. Utilization of gelatin was determined by flooding with acidified 15% HgCl₂ (Cowan & Steel, 1965).

Preparation of collagen. Neutral-salt-soluble collagen extracted from calf skin and puri-

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Collagenolytic activity of *Bacillus* spp. 1, 2 and 3 at 2.34% (w/v) NaCl under aerobic conditions. ○—○, *Clostridium histolyticum* collagenase + collagen; △—△, uninoculated collagen; ●—●, bacteria + buffer; ■—■, *Bacillus* sp. 1 + collagen; ▲—▲, *Bacillus* sp. 2 + collagen; □—□, *Bacillus* sp. 3 + collagen. For clarity, zero time symbols have been omitted.

Fig. 2. Collagenolytic activity of *Achromobacter* spp. 1, 2 and 3 at 2.34% (w/v) NaCl under aerobic conditions. ○—○, *Clostridium histolyticum* collagenase + collagen; ×—×, uninoculated collagen; ●—●, bacteria + buffer; ■—■, *Achromobacter* sp. 1 + collagen; ▲—▲, *Achromobacter* sp. 2 + collagen; □—□, *Achromobacter* sp. 3 + collagen; △—△, *Achromobacter* sp. 4 + collagen. For clarity, zero time symbols have been omitted.

Fig. 3. Collagenolytic activity of *Achromobacter* spp. 5, 6, 7 and 8 at 2.34% (w/v) NaCl under aerobic conditions. ○—○, *Clostridium histolyticum* collagenase + collagen; ×—×, uninoculated collagen; ●—●, bacteria + buffer; ■—■, *Achromobacter* sp. 5 + collagen; ▲—▲, *Achromobacter* sp. 6 + collagen; □—□, *Achromobacter* sp. 7 + collagen; △—△, *Achromobacter* sp. 8 + collagen. For clarity, zero time symbols have been omitted.

fied by the method of Cooper & Davidson (1965) was used. The collagen was kept at 4° in the lyophilized form. A 0.2% solution of the collagen in 0.4 M (2.34%) NaCl, 0.01 M-tris, 0.002 M-CaCl₂, pH 7.6, was prepared by the method of Waldvogel & Swartz (1969).

Screening for collagenolytic activity. The method of Adamcic & Clark (1970) was followed, but modifications were made due to the high salt concentrations used. To 3 ml of 0.02% collagen solution in 2.34% NaCl, 0.01 M-tris, 0.002 M-CaCl₂ and 7% NaCl, 0.01 M-tris, 0.002 M-CaCl₂ at pH 7.6 was added 0.1 ml of a bacterial suspension in buffer containing 10⁸ bacteria/ml. Inoculated solutions were incubated aerobically and assayed for release of amino acids after 7 and 14 days using a ninhydrin colorimetric analysis (Rosen, 1957). Extinctions were read on a Unicam SP800 spectrophotometer at 570 nm. Controls at both 2.34 and 7% NaCl consisted of: (a) uninoculated collagen, (b) six bacterial strains, chosen at random, in buffer without collagen, (c) *Clostridium histolyticum* collagenase form II (Koch-Light Laboratories, Colnbrook, Buckinghamshire) in the proportion enzyme:collagen, 1:10. Collagenolysis was determined by comparing the amount of amino acids released with that of the uninoculated controls and expressed as μmoles glycine/ml.

Identification of strains. Collagenolytic strains were classified according to *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957).

Table 1. *Characteristics of collagenolytic Bacillus strains*

	Strain no.		
	1	2	3
Gram-variable rods	+	—	+
Gram-positive rods	—	+	—
Endospores	+	+	+
Capsules	—	—	—
Motility	+	+	+
Catalase and oxidase	+	+	+
Gelatinolytic activity	+	+	+
Starch hydrolysis	—	—	—
Production of acid, not gas from glucose, sucrose, mannose	+	+	+
Action on lactose	—	—	—
Nitrate reduction	+	+	+
Indole production	—	—	—
Obligate aerobe	—	+	+
Facultative anaerobe	+	—	—
Length (μm)	1.8	1.8	1.6
Halotolerant with optimal NaCl concentration of 2.34% (w/v)	+	+	+

RESULTS

Salt requirements of bacterial strains

Of 140 strains isolated, 51 were halotolerant (salt range of 0.85 to 20%) and 89 halophilic (salt range of 7 to 15% or 20%).

Collagenolytic activity

Eleven of 110 strains tested (76 South African, 34 Australian) at 2.34% NaCl showed collagenase activity under aerobic conditions. None of the 128 strains tested at 7% NaCl showed collagenolytic activity. Although the bacteria were isolated and subcultured for many generations on collagen-free media, only one strain, *Achromobacter* sp. 7, showed a long lag phase characteristic of the enzyme produced by one of the two *Pseudomonas* strains reported by Adamcic & Clark (1970). Significant enzymatic activity of the other strains was already detectable after seven days (Fig. 1 to 3). Collagenolytic activity was not observed when strains were tested in buffer without CaCl_2 (Gallop, Seifter & Meilman, 1957). The 11 collagenolytic strains were isolated from South African hides from five curers. The Australian hides, from two curers, did not contain collagenolytic bacteria. Thus the percentage of bacteria with collagenase from South African hides was 14.47% compared with 10% for all strains tested.

Gelatinolytic activity

Of the 11 collagenolytic strains, the nine halotolerant ones, growing from 0.85 to 20% NaCl had gelatinolytic activity while the two strains showing poor growth at 0.85% NaCl had no gelatinolytic activity. Thus degradation of gelatin by a bacterial strain is not necessarily an indication of collagenolytic activity.

Table 2. *Characteristics of collagenolytic Achromobacter strains*

	Strain no.				
	1	2, 3, 4	5, 6	7	8
Gram-negative rods	+	+	+	+	+
Endospores	—	—	—	—	—
Capsules	+	+	—	—	+
Motility	—	—	—	—	—
Catalase and oxidase	+	+	+	+	+
Gelatinolytic activity	+	+	+	—	—
Starch hydrolysis	—	—	—	—	—
Production of acid, not gas from glucose, sucrose, mannose	+	+	+	+	+
Action on lactose	—	—	—	—	—
Nitrate reduction	+	+	+	+	+
Indole production	+	—	—	—	+
Obligate aerobe	+	—	—	—	—
Facultative anaerobe	—	+	+	+	+
Length (μ m)	1.4	1.2	1.6	1.4	1.6
Halotolerant, with optimal NaCl concentra- tion of 2.34 % (w/v)	+	+	+	—	—
Halotolerant, with poor growth at 0.85 % and optimal at 7–15 % (w/v) NaCl	—	—	+	—	+

Identification of collagenolytic strains

Three of the collagenolytic strains were identified as *Bacillus* spp. and eight as *Achromobacter* spp. Their characteristics are shown in Tables 1 and 2.

DISCUSSION

Aerobic collagenolytic activity was shown in 11 bacteria which appear to belong to eight different species. Conclusive demonstrations of true bacterial collagenases have been demonstrated only in four *Clostridium* spp., three *Bacteriodes* spp., one strain of *Staphylococcus aureus* and two strains of *Pseudomonas* (Jennison, 1947; Neuman & Tytell, 1950; Gibbons & MacDonald, 1961; Waldvogel & Swartz, 1969; Adamcic & Clark, 1970).

The increased activity of the collagenase enzyme at 2.34 % NaCl indicates that although the bacteria are tolerant of a wide range of salt concentrations, there is an optimal salt concentration for enzymatic activity. The lack of activity at 7 % NaCl is likely to be due to an inability to produce the enzyme at that salt concentration or to the inactivation of the enzyme by salt. Inhibition of enzymes by salt concentrations greater than 3 % has been shown in extremely halophilic bacteria which have an optimal salt concentration for growth of 20 % (Louis & Fitt, 1971).

The observation that no Australian bacteria utilized collagen is important as it has been reported that Australian hides produce uniformly good leather with little hide decay (Hendry, Cooper & Woods, 1970).

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