EVANS, D. G. & WARDLAW, A. C. (1953). *J. gen. Microbiol.* 8, 481-487.

Gelatinase and Collagenase Production by certain Species of *Bacillus*

BY D. G. EVANS AND A. C. WARDLAW

Department of Bacteriology, University of Manchester

SUMMARY: Fifty-three strains from six species of the genus *Bacillus* were tested for their ability to produce gelatinase and to disintegrate the collagen matrix of bone. Some strains of *B. cereus* and *B. anthracis* produced a true collagenase which brought about complete disintegration of the collagen substrate, while strains of *B. subtilis*, *B. licheniformis*, *B. brevis* and *B. megaterium* were either completely inactive or produced only partial disintegration. Collagenase production was always accompanied by gelatinase production but the reverse was not the case. Concentrated culture filtrates of *B. cereus* were shown to contain collagenase and gelatinase, whereas those of *B. subtilis* contained gelatinase only.

In a previous communication (Evans & Wardlaw, 1952) we described some of the properties of proteolytic culture filtrates from a strain of *Bacillus subtilis*. The culture filtrates were highly active in liquefying gelatin and in disintegrating hide powder which is a denatured form of skin collagen. The filtrates were, however, unable to break down undenatured tendon collagen either in the form of thin slices or thin sheets prepared from soluble collagen by the method of Delaunay, Guillaumie & Delaunay (1949). *Bacillus* strains have, however, been shown to attack the collagen-like matrix of tooth dentine (Evans & Prophet, 1950), and this has led us to extend our previous investigation. We have now tested the ability of a number of bacillus strains to produce collagenase, using as substrate a finely divided preparation of decalcified bone. This preparation was not regarded as a pure substrate but was nevertheless a rich source of native collagen. The strains were also examined for their ability to produce gelatinase, and a study was made of the relationship between this enzyme and collagenase.

MATERIALS AND METHODS

Preparation of collagen substrate. Limb-bone shafts from adult rabbits were freed from marrow and connective tissue and dried over P_2O_5 . After preliminary fragmentation the bone was ground to a fine powder in a low-temperature ball mill as used by Evans & Prophet (1950) for the preparation of powdered dentine. The powder was decalcified by suspension in a large volume of 0.2N-HCl for 1 hr. and then centrifuged. The deposit was washed free from acid by resuspending in water and centrifuging several times; the final suspension was dialysed against running water and dried from the frozen state to yield a very finely divided preparation of decalcified bone.

Test for collagenase production. The decalcified bone powder was sterilized by exposing thin layers to ultra-violet radiation. A saline suspension of the powder was mixed with melted 3% agar in saline which had been cooled

below 45° and the mixture poured into Petri dishes. For each plate 10 ml. of agar and 20 mg. of decalcified bone powder were used. Disks 6 mm. in diameter were cut from the agar plate with a sterile cork-borer and transferred to tubes containing 10 ml. sterile 1 % Evans peptone in saline at pH 7.6. Two disks were placed in each tube. The tubes of medium after the addition of the disks were incubated for 1 week to check sterility; they were then inoculated with the bacillus strains listed in Table 1 and incubated at 37°. With aseptic precautions one disk was removed from each culture after 3 days, the other disk after 5 days and each examined microscopically at $\times 15$ magnification. The degree of disintegration of the collagen particles was determined by comparison with a control disk from a tube of uninoculated medium and was estimated as none, partial or complete (Pl. 1). No attempt was made to describe grades of partial clearing.

With some of the strains the collagenase activity of culture filtrates was titrated. Serial dilutions of the filtrates were made in normal saline, and to each was added one collagen-agar disk. The tubes were incubated at 37° for 18 hr. and the end-point taken as the highest dilution which gave complete clearing of the disk. The reciprocal of the end-point dilution gave the number of minimal collagenase doses/ml. (m.c.d./ml.).

Test for gelatinase production. The same cultures which were being tested for collagenase activity and which contained collagen-agar disks, were also tested for their gelatinase activity. After 2 and 5 days of incubation, 1 ml. samples of culture were removed and the gelatinase activity titrated by the setting-time method (Evans & Wardlaw, 1952). Serial dilutions of culture were made in saline, and to each was added an equal volume of 5 % gelatin solution. This concentration of gelatin we found more sensitive than the 10 % solution used previously. The mixtures were incubated for 1 hr. at 37° and then cooled in ice water. The time taken for each mixture to set was determined and the end-point taken as that dilution which gave the same setting time as a control solution of 1.5 % gelatin alone. The activity of a culture was expressed as the number of minimal gelatinase doses/ml. (m.g.d./ml.) which was the reciprocal of the end-point dilution. Culture filtrates were titrated in a similar manner.

Strains. The fifty-three strains used are given in Table 1. They were obtained as named species from the National Collection of Type Cultures (NCTC), from the Culture Collection of the Wellcome Research Laboratories (CN) and from our own departmental collection (DBM). In addition, Dr G. P. Gladstone supplied us with three strains of *B. anthracis*—M36, Sterne and NPA. We accepted the identity of the strains as received and from the first cultures prepared fresh batches of freeze dried cultures.

RESULTS

Gelatinase and collagenase in cultures

The results of the tests on the fifty-three different strains are shown in Table 1. Species were found to differ considerably in their ability to produce gelatinase. *B. anthracis* and *B. subtilis* were the most active, some strains of which gave titres of 100–300 m.g.d./ml. after 5 days, whereas no strains of *B. brevis* and

Table 1. *The ability of fifty-three Bacillus strains from six different species to produce gelatinase and collagenase*

<i>Bacillus</i> species and strain no.		Gelatinase activity (m.g.d./ml.)		Collagenase activity Degree of disintegration of collagen	
		2 days	5 days	3 days	5 days
<i>B. subtilis</i> :	NCTC 3610	120	90	0	+
	NCTC 6276	30	60	0	0
	NCTC 6346	2	4	0	0
	NCTC 7241	50	50	0	+
	CN 788	30	25	0	+
	CN 789	12	18	0	+
	CN 808	4	10	0	0
	CN 831	140	130	+	+
	CN 1508	25	50	0	+
<i>B. licheniformis</i> :	CN 1576	25	30	0	0
	NCTC 962	2	5	0	+
	NCTC 1024	1	7	0	+
	NCTC 1025	2	6	0	0
	NCTC 1026	5	11	0	0
	NCTC 1027	5	8	0	0
	NCTC 1097	5	8	0	+
	NCTC 6349	8	6	++	++
	CN 753	<1	6	0	0
<i>B. cereus</i> :	CN 2194	10	45	+	++
	CN 2484	2	16	++	++
	CN 2501	10	16	++	++
	CN 2503	25	70	++	++
	CN 2504	10	60	++	++
	NCTC 926	.	3	0	0
	NCTC 6093	.	1	0	+
<i>B. cereus</i> var. <i>mycoides</i> :	CN 1409	10	20	0	0
	CN 1541	8	15	+	+
	CN 2195	4	10	0	0
	CN 2495	8	25	+	++
	CN 2500	<1	8	0	++
	CN 2521	2	5	0	+
	NCTC 1712	<1	3	0	0
	NCTC 2620	<1	3	0	+
<i>B. anthracis</i> :	DBM 121	<1	5	0	+
	DBM 122	20	300	0	++
	DBM 123	20	250	0	++
	M36	<1	70	0	++
	Sterne	<1	70	0	++
	NPA	<1	3	0	+
	NCTC 7577	.	<1	0	0
	CN 2904	.	<1	0	0
<i>B. brevis</i> :	CN 2918	.	<1	0	0
	CN 2922	.	<1	0	0
	CN 2934	.	<1	0	0
	CN 3005	.	<1	0	0
	CN 3006	.	<1	0	0
	NCTC 2605	.	<1	0	0
	NCTC 5636	.	<1	0	0
<i>B. megaterium</i> :	CN 737	.	<1	0	0
	CN 1759	.	<1	0	0
	CN 2496	.	<1	0	0
	CN 2529	.	<1	0	0
	CN 2623	.	<1	0	0

A titre of <1 means that the undiluted culture showed no activity. Disintegration of collagen: 0 = none; + = partial; ++ = complete.

NCTC = National Collection of Type Cultures, Colindale, London, N.W. 9.

CN = Wellcome Research Laboratories, Beckenham, Kent.

DBM = Department of Bacteriology, Manchester.

B. megaterium showed detectable activity. The strains within a single species also showed marked differences in gelatinase production. With *B. subtilis* strains, for example, the titres ranged from 4 to 130, and with *B. anthracis* there were even greater differences, from 3 to 300. With many strains the gelatinase activity at 2 and 5 days was similar, although in some cases, as with *B. anthracis* and *B. cereus*, the 5-day cultures showed a considerably higher titre. Although it was not possible with some of the strains to detect gelatinase by the titration method, this did not imply that the strains were entirely devoid of the ability to produce the enzyme. It was possible to detect gelatinase action by these strains in gelatin stab cultures incubated for long periods.

The collagenase tests showed that some strains produced complete disintegration of the collagen particles in the disks while other strains were completely inactive. The greatest activity was shown by *B. cereus*; of the seven strains tested, complete disintegration was shown by five of them in 3-day cultures and by six in 5-day cultures. The *B. anthracis* strains were also able to produce complete disintegration, although they were not so rapidly active as those of *B. cereus*. None of the eight strains of *B. anthracis* showed activity in 3-day cultures, but after 5 days of incubation four of them had produced complete disintegration and three partial disintegration. Many of the strains of other species showed partial effects, but complete disintegration was given only by two strains of *B. cereus* var. *mycoides*. No detectable collagenase activity was shown by any of the strains of *B. brevis* or *B. megaterium*.

It was evident that in the group as a whole there was some correlation between collagenase and gelatinase production. Those strains which produced collagenase always produced gelatinase, and those which showed no gelatinase activity were also inactive towards collagen. The correlation was, however, by no means complete, for gelatinase production was not always accompanied by collagenase production. Further, with those strains which produced both enzymes, there was no quantitative correlation between gelatinase activity and rapidity with which collagen was disintegrated. This lack of association between the two enzyme activities was most apparent with strains of *B. subtilis* and *B. cereus*. For example, strain NCTC 7241 of *B. subtilis* had a gelatinase titre of 50 but showed no disintegration of collagen at 3 days. On the other hand, strain NCTC 6349 of *B. cereus* produced complete disintegration of collagen at 3 days, although the gelatinase titre was only 8. This strongly suggested that the proteolytic enzymes in the two cultures differed considerably in their substrate specificity, and in order to obtain more conclusive evidence of these differences further tests were made with concentrated culture filtrates.

Gelatinase and collagenase in filtrates

Concentrated culture filtrates were prepared from two strains of each of *B. subtilis* and *B. cereus* by the method we previously used (Evans & Wardlaw, 1952). Cultures were filtered after 3–4 days growth and the filtrates precipitated by adding 500 g. ammonium sulphate/l. The precipitate was dialysed

to remove ammonium sulphate and then dried from the frozen state. To be tested for enzyme activity the dried preparations were dissolved in saline to give approximately 1% (w/v) solutions. Each preparation was titrated for gelatinase and collagenase, and the results are given in Table 2. To facilitate comparison of the two activities, the gelatinase titres of *B. cereus* have been adjusted to equal those of *B. subtilis* and the collagenase titres scaled accordingly. Neither of the *B. subtilis* filtrates, although highly active in liquefying gelatin, were able to bring about complete clearing of the collagen-agar disks. The two *B. cereus* filtrates, on the other hand, with the same gelatinase activity as those of *B. subtilis*, were able completely to clear the disks even at dilutions of 1/260 and 1/64. These results are in agreement with those obtained in the tests with cultures and clearly show that the proteolytic enzymes of these two species differ in their substrate specificity.

Table 2. Comparison of the gelatinase and collagenase activities of culture filtrates of *Bacillus subtilis* and *B. cereus*

Filtrate	Gelatinase titre (m.g.d./ml.)	Collagenase titre (m.c.d./ml.)
<i>B. subtilis</i> : NCTC 3610	300	< 1
CN 831	300	< 1
<i>B. cereus</i> : NCTC 6349	300	260
CN 2504	300	64

The results also suggest that with *B. cereus* two enzymes are involved in attacking gelatin and collagen. The two *B. cereus* filtrates attacked gelatin to the same titre but there was a four-fold difference in their collagenase titres. If the enzyme which attacked gelatin was also responsible for the breakdown of collagen, then it would have been expected that the collagenase titres of the two filtrates would have been the same. The fact that they were considerably different strongly suggests that two independent enzymes were present in the filtrates, one which attacked collagen and possibly gelatin, and one which attacked gelatin only.

The nature of the collagen substrate

It is well known that collagen can undergo changes in its susceptibility to enzyme action. We found that heating a watery suspension of decalcified powdered bone at 65° for 10 min. considerably increased its susceptibility, so that it was completely disintegrated by enzyme preparations of *B. subtilis* which were unable to attack the unheated material. On the other hand, the bone powder was made completely resistant to enzyme action by treating it with low concentrations of formaldehyde or tannic acid. Not only did this treatment make the collagen resistant to bacillus collagenases but also to the more rapidly acting collagenase of *Clostridium histolyticum*. In view of this, care was taken in preparing the bone substrate to maintain the collagen in its unaltered native form. It was not possible to use untreated powdered bone as the collagen substrate, for bone in its calcified form was found to be completely unaffected by bacillus and clostridium collagenases. A similar observation

was made by Evans & Prophet (1950) with the collagen-like matrix of dentine. It was therefore necessary to decalcify the bone and 0.2N-HCl was used for this purpose. This treatment, apart from removing the calcium salts and thereby exposing the collagen matrix to enzyme attack, probably had little or no denaturing effect on the collagen, for it was found that bone powder decalcified with phosphate buffer at pH 6.0, a mild treatment hardly likely to denature the collagen, was just as sensitive to enzyme attack as bone decalcified by 0.2N-HCl.

An experiment was also made which showed that the method we used for sterilizing decalcified bone powder by ultra-violet radiation did not alter its susceptibility to enzyme. Titrations of collagenase preparations made with agar disks containing irradiated decalcified bone powder gave the same end-points as titrations made with disks containing powder which had not been exposed to radiation.

As a result of these tests we considered that the substrate we used was native collagen which had not been denatured during its preparation. If this be accepted, then those bacillus strains which were able to disintegrate this substrate were true collagenase producers.

DISCUSSION

Although many bacteria are known to attack denatured collagen in the form of gelatin, relatively few species have been shown to break down native collagen. Our experiments indicate that some strains in the genus *Bacillus* are able to produce a true collagenase. It is possible that we have been able to detect this enzyme because of the finely divided form of substrate we have employed. The substrates commonly used in testing collagenase action are thin slices of tendon or thin sheets of precipitated collagen ('collagen paper'). These substrates are comparatively insensitive indicators and are not disintegrated by such high dilutions of enzyme as is collagen powder prepared from bone. With our most potent concentrated filtrates from *B. cereus*, we showed that agar disks containing collagen powder as indicator were at least 30 times more sensitive than collagen paper. Similar results were obtained with the collagenase of *Cl. histolyticum*. Decalcified bone we have found can be prepared as a very fine powder, and thus when treated with enzyme is intimately exposed to its action. It is possible that the collagen-agar disk method may be of value in detecting unsuspected collagenase production by other bacterial species.

It is not yet known whether the bacillus collagenases are of the same kind as those produced by *Cl. welchii* type A (Oakley, Warrack & van Heyningen, 1946) and *Cl. histolyticum* (Oakley & Warrack, 1950). The clostridium enzymes are able to break down with comparative ease the more resistant collagenase indicators such as collagen paper and even pieces of skin. This is due to the fact that they are produced in high concentration in culture and does not necessarily imply that they are of a different kind from the collagenases of the bacillus group. The collagenases of the two groups do, however, appear to differ in the rate at which they act on the substrate. We have shown, with

D. G. EVANS & A. C. WARDLAW—GELATINASE AND COLLAGENASE OF
BACILLUS SPP. PLATE 1

collagenase preparations from each group, containing the same number of m.c.d./ml., that the disintegration of bone powder is completed by the clostridium collagenases in less than half the time taken by those of the bacillus group.

The experiments have also shown that the collagenase activity of bacillus strains is not dependent on their gelatinase activity. Some strains with low gelatinase activity are highly active in disintegrating collagen, whereas others with high gelatinase activity have little or no effect on collagen. Even in the case of those culture filtrates of *B. cereus* which actively attack both gelatin and collagen, the results indicated that two independent enzymes are involved, one which attacks collagen and possibly gelatin, and one which attacks gelatin only. In this respect there is an analogy with the collagenase (κ -antigen) and gelatinase (λ -antigen) of *Cl. welchii* (Oakley, Warrack & Warren, 1948). The analogy, however, is by no means complete, for we have been unable so far to obtain conclusive evidence that either the gelatinase or the collagenase of *B. cereus* is antigenic. Further attempts are being made to obtain antibodies to these enzymes, for we consider that if it be possible to use serological tests, the proteolytic enzymes of this group will then be more clearly differentiated.

We should like to express our thanks to Mr H. Proom and Dr G. P. Gladstone for strains and to Dr G. H. Warrack for rat collagen paper. One of us (A. C. W.) wishes to acknowledge the receipt of a Scholarship from the Medical Research Council.

REFERENCES

- DELAUNAY, M., GUILLAUMIE, M. & DELAUNAY, A. (1949). Études sur le collagène. I. A propos des collagénases bactériennes. *Ann. Inst. Pasteur*, **76**, 16.
- EVANS, D. G. & PROPHET, A. S. (1950). Disintegration of human dentine by bacterial enzymes. *Lancet*, *i*, 290.
- EVANS, D. G. & WARDLAW, A. C. (1952). *In vitro* and *in vivo* properties of culture filtrates of *Bacillus subtilis* with high gelatinase activity. *J. gen. Microbiol.* **7**, 397.
- OAKLEY, C. L. & WARRACK, G. H. (1950). The alpha, beta and gamma antigens of *Clostridium histolyticum* (Weinberg & Séguin, 1916). *J. gen. Microbiol.* **4**, 365.
- OAKLEY, C. L., WARRACK, G. H. & HEYNINGEN, W. E. VAN (1946). The collagenase (κ -toxin) of *Cl. welchii* type A. *J. Path. Bact.* **58**, 229.
- OAKLEY, C. L., WARRACK, G. H. & WARREN, M. E. (1948). The kappa and lambda antigens of *Clostridium welchii*. *J. Path. Bact.* **60**, 495.

EXPLANATION OF PLATE

Collagen powder, suspended in agar disks, showing different degrees of disintegration after exposure to bacillus cultures. (1) no disintegration, (2) partial, (3) complete. $\times 9$.

(Received 17 December 1952)