An Electrophoretic Analysis of Superoxide Dismutase in *Campylobacter* spp.

By HIROKO E. KIKUCHI* and TAKESHI SUZUKI

Department of Oral Microbiology, School of Dentistry, Hokkaido University, Sapporo 060, Japan

(Received 22 May 1984; revised 29 June 1984)

Superoxide dismutase (SOD, superoxide:superoxide reductase, EC 1.15.1.1) activity was studied in 23 strains of *Campylobacter* spp. using disc polyacrylamide gel electrophoresis. Different enzyme patterns were observed with extracts of different species of *Campylobacter*; three migration bands were found in all strains of *C. sputorum* subsp. *sputorum* and *C. sputorum* subsp. *bubulus* (relative mobilities, Rm, 0.57, 0.76 and 0.85), and *C. fetus* subsp. *fetus* (Rm 0.60, 0.72 and 0.81), while four migration bands (Rm 0.52, 0.57, 0.73 and 0.82) were found in *C. fetus* subsp. *venerealis*. One band (Rm 0.73) was found in *C. coli* CIP 7080 and two bands (Rm 0.59, 0.73) in *C. jejuni* CIP 702. Superoxide dismutase activities were very high in the *Campylobacter* strains, especially in *C. fetus* subsp. *fetus* [specific activity 7.8–55.7 U (mg protein)⁻¹] compared with those in *Escherichia coli* (1.5 U mg⁻¹) and *Propionibacterium acnes* (1.6 U mg⁻¹) and *Veillonella alcalescens* (0.2 U mg⁻¹).

**INTRODUCTION**

*Campylobacter jejuni* is the causative agent of a bacterial diarrhoea in man (Newell, 1982; Skirrow, 1977; Smibert, 1978). *Campylobacter fetus* causes sterility and abortion in cattle (Smith, 1978). *Campylobacter coli* has been isolated from pig intestines and is reported to increase in number when the pig suffers from dysentery (Doyle, 1944). Catalase-negative non-pathogenic *Campylobacter* spp. found in normal reproductive organs of cattle, the human oral cavity and porcine intestinal mucosa were named *C. sputorum* subsp. *sputorum*, *C. sputorum* subsp. *bubulus* (Loesche et al., 1965) and *C. sputorum* subsp. *mucosalis* (Lawson et al., 1975), respectively, following the classification scheme of Véron & Chatelain (1973).

*Campylobacter* spp. have also been found associated with purulent pyorrhea (Dwyer & Socransky, 1968; Slots et al., 1978). Other *Campylobacter* spp. found in dental pockets of humans with periodontitis have been identified as *C. concisus* although Tanner & Badger (1981) reported that these species were serologically distinct from other *Campylobacter* species including *C. sputorum* and *C. fetus* and that some strains showed cross activities with antiserum obtained from *Wolinella recta*. Further taxonomic and biochemical studies are required on these oral *Campylobacter* spp.

Several reports have shown the optimal growth atmosphere for *Campylobacter* spp. to be 5% (v/v) oxygen (Kiggens & Plaistridge, 1956; Ware et al., 1977), 6% (Reich et al., 1956) or 2.5% (Fletcher & Plaistridge, 1964), suggesting that they may have a special superoxide dismutase (SOD). We have observed the growth of *Campylobacter* spp. in GAM semi-solid medium, a medium for the growth of anaerobic bacteria. Isolates grew 2.5 mm below the surface of this medium; different species and subspecies of *Campylobacter* grew at different rates at the same depth.

Hoffman et al. (1979a, b) reported that two strains of *C. jejuni* and one strain of *C. fetus* subsp. *fetus* showed different SOD activity staining patterns on disc polyacrylamide gel electrophoretograms. It was suggested that a particular isoenzyme of SOD might be related to the aero-
tolerance of *C. fetus* (Hoffman et al., 1979a). In order to extend our knowledge of SOD of *Campylobacter* spp. in relation to their microaerophilic characteristics and species differences, we have studied SOD of *C. sputorum* subsp. *sputorum*, *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis* and other campylobacters by disc polyacrylamide gel electrophoresis.

### METHODS

**Organisms.** The strains used are listed in Table 1, with their sources and habitats.

**Media and cultivation.** Campylobacter strains were grown in GAM broth (Nissui Co., Tokyo, Japan) placed in Petri dishes to give a large surface area. The composition of GAM broth, which is a medium for the growth of anaerobic bacteria, is as follows (l-1): peptone 10.0 g; soybean peptone 3.0 g; proteose peptone W 10.0 g; digested serum 13.5 g; yeast extract 5.0 g; beef extract 2.2 g; liver extract 1.2 g; glucose 3.0 g; KH₂PO₄ 2.5 g; NaCl 3.0 g; soluble starch 0.3 g; L-cystine. HCl 0.3 g; sodium thioglycollate 0.3 g. The pH was adjusted to 7.3 ± 0.1. The inoculum was grown in GAM semi-solid medium (GAM broth containing 1.5 g agar l⁻¹) in Erlenmeyer flasks.

*Propionibacterium acnes* and *Veillonella alcalescens* were grown in Erlenmeyer flasks filled to the top with GAM broth. *Escherichia coli* was grown in nutrient broth containing (l-1): meat extract 5.0 g; peptone 10.0 g; NaCl 5 g (Eiken Co., Tokyo, Japan).

All bacteria were cultured at 37°C. *Escherichia coli* was harvested by centrifugation after 18 h growth and other bacteria after 48 h.

**Preparation.** Harvested cells were sedimented (7500 g for 10 min), washed three times with physiological saline (0.15 w-NaCl), and suspended in saline at 0.5 g wet wt ml⁻¹. After sonication at 20 kHz at 80 W for 3 min (Tomy, Ultrasonic UR 200, Japan), the homogenate was centrifuged at 12000 g for 60 min (Sorvall, RC-5). The supernatant was dialysed for 36 h in 0.02 w-phosphate buffer (pH 7-8) to remove Cu²⁺, Mn²⁺, Fe²⁺ and Fe³⁺ ions that may affect SOD activity and salts that may affect electrophoresis.

**Determination of protein content and enzyme activities.** Protein content was determined by the Lowry method.
Superoxide dismutase of Campylobacter spp.

SOD activity of the dialysed solutions was measured by the xanthine/xanthine oxidase/nitroblue tetrazolium (NBT) system (McCord & Fridovich, 1969). Xanthine oxidase was added to 3 ml reaction mixture solution containing \(1 \times 10^{-4}\) M-xanthine, \(2.5 \times 10^{-3}\) M-NBT, \(1 \times 10^{-4}\) M-EDTA, and 0.05 M-sodium carbonate (pH 10.2) in an amount sufficient to reduce NBT to formazan at a rate such that the \(A_{460}\) increased at 0.01 unit min\(^{-1}\) at 25 °C (Hitachi spectrophotometer 200–20). The amount of SOD required to inhibit the rate of reduction of NBT by 50% under these defined conditions is defined as 1 unit of activity.

Catalase activity was detected by effervescence in 3% (v/v) hydrogen peroxide. Oxidase activity was detected using the dimethyl-p-phenylenediamine dihydrochloride reagent of Kowacs (1956).

**Electrophoresis and staining.** A dialysed solution of protein (0·1–0·3 mg) was electrophoresed on 7% (w/v) polyacrylamide disc gels using the method of Davis & Ornstein (1964). Running gels (pH 8.9) 6 cm long with 1 cm thick spacer gels were prepared in glass gel tubes of inner diameter 5 mm, and set in a cooling device. After stacking 50 μl 40% (w/v) sucrose solution containing protein and bromophenol blue, the gels were run at 5 mA per tube for 40 min at 12 °C in a buffer solution containing 0·6 g Tris and 2·88 g glycine l\(^{-1}\) (pH 8.3).

SOD was detected by soaking the gels in 2·45 mM-NBT for 20 min followed by immersion for 15 min in a solution containing 28 mM-tetramethylenediamine, 28 μM-riboflavin and 36 mM-potassium phosphate (pH 7·8). The gels were then placed in small dry test tubes and illuminated for 5 to 15 min with a commercial 20 W fluorescent lamp (360–740 nm including line spectra at 400 and 435 nm) at a distance of 10 cm. During illumination, the gels turned blue except for the bands containing SOD. Illumination was stopped when maximum contrast was achieved between the unstained and blue zones (Beauchamp & Fridovich, 1971). Migration distances of negatively-stained bands were converted to relative mobilities (Rm) by dividing each migration distance by that of the front marker (bromophenol blue).

Each sample was electrophoresed on two gels; one gel was stained without KCN and the other was stained in the presence of 2 mM-KCN (De Rosa et al., 1979) in order to detect the presence of Zn,Cu-SOD (Weisiger & Fridovich, 1973). Dissmutation of O\(_2\) by Zn,Cu-SOD is inhibited by CN\(^-\), while Fe-SOD and Mn-SOD activities are unaffected by CN\(^-\) (Fridovich, 1974).

Three to nine cultures of each strain were analysed for all enzyme activities. Bovine blood SOD (Sigma) was used as a control.

**RESULTS**

The specific activities of SOD obtained for homogenates of each strain are given in Table 2. Extracts of *E. coli* and *P. acnes* had SOD specific activities of around 1·5 U (mg protein\(^{-1}\)). Extracts of *V. alcalescens* showed negligible activity. Extracts of *Campylobacter* spp. had SOD specific activities that ranged from about 1 U (mg protein\(^{-1}\)) in *C. sputorum* subsp. *sputorum* to above 50 U (mg protein\(^{-1}\)) in *C. fetus* subsp. *fetus*. The SOD specific activities of *C. sputorum* subsp. *sputorum* and *C. fetus* subsp. *venerealis* were similar [means ± SD 3·4 ± 1·8 and 4·7 ± 1·3 U (mg protein\(^{-1}\)], respectively. While *C. fetus* subsp. *fetus* had much higher activities and also showed large activity variations between different strains [mean ± SD 30·5 ± 17·5 U (mg protein\(^{-1}\)) for the five strains studied], variations within strains were not large in comparison with those found in *C. fetus* subsp. *venerealis*. The SOD specific activities of *C. coli*, *C. jejuni* and *C. sputorum* subsp. *bubulus* were between those of *C. sputorum* subsp. *sputorum* and *C. fetus* subsp. *fetus*. The mean SOD specific activity determined for the *Campylobacter* strains other than *C. fetus* subsp. *fetus* was 5·4 ± 2·2 U (mg protein\(^{-1}\)).

All the *Campylobacter* strains had oxidase activity, and all except *C. sputorum* subsp. *sputorum* and *C. sputorum* subsp. *bubulus* had catalase activity. *Escherichia coli*, *P. acnes* and *V. alcalescens* did not have oxidase activity and *P. acnes* did not have catalase.

Fig. 1 shows the positions of bands stained for SOD activity after polyacrylamide disc electrophoresis of extracts of *Campylobacter* spp. together with those obtained for bovine blood SOD and extracts of *E. coli*, *V. alcalescens* and *P. acnes*. Different SOD profiles were obtained with extracts of different campylobacters. *Campylobacter sputorum* subsp. *sputorum* had one clear band of Rm 0·57 and two minor bands of Rm 0·76 and 0·85. *Campylobacter sputorum* subsp. *bubulus* had a very similar profile consisting of three bands of Rm 0·56, 0·76 and 0·85; *C. coli* had only one clear band of Rm 0·73; *C. jejuni* had two bands of Rm 0·59 and 0·73; *C. fetus* subsp. *fetus* had a minor band of Rm 0·60, a major band of Rm 0·72 and an intermediate band of Rm 0·81; *C. fetus* subsp. *venerealis* had two clear bands of Rm 0·73 and 0·82 and two trace bands of Rm 0·52 and 0·57. The SOD profiles of the different isolates were identical for a given species or subspecies.
Table 2. Activities of SOD and other enzymes in Campylobacter, E. coli, P. acnes and V. alcalescens

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sp. act. of SOD* [U (mg protein)⁻¹]</th>
<th>Catalase activity</th>
<th>Oxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. sputorum subsp. sputorum C 1</td>
<td>48 ± 0.4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 2</td>
<td>48 ± 0.4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 3</td>
<td>32 ± 0.2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 4</td>
<td>45 ± 0.3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 5</td>
<td>38 ± 0.3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 6</td>
<td>18 ± 0.1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 8</td>
<td>0.9 ± 0.1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 9</td>
<td>6.5 ± 0.5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 14</td>
<td>1.7 ± 0.0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C 15</td>
<td>1.9 ± 0.8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C. sputorum subsp. bubulus CIP 53103</td>
<td>5.8 ± 1.0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C. coli CIP 7080</td>
<td>3.9 ± 0.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. jejuni CIP 702</td>
<td>9.0 ± 1.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. fetus subsp. fetus CIP 5396</td>
<td>14.0 ± 2.7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 16</td>
<td>7.8 ± 3.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 17</td>
<td>40.0 ± 2.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 18</td>
<td>35.2 ± 3.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 20</td>
<td>55.7 ± 2.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. fetus subsp. venerealis CIP 6829</td>
<td>5.3 ± 1.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 21</td>
<td>6.8 ± 0.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 22</td>
<td>3.9 ± 0.2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 23</td>
<td>2.9 ± 0.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C 24</td>
<td>44.0 ± 0.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli IID 861</td>
<td>1.5 ± 0.1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P. acnes ATCC 11827</td>
<td>1.6 ± 0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V. alcalescens subsp. dispar ATCC 17748</td>
<td>0.2 ± 0.0</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Mean of three to nine determinations, ± SD.

No inhibition of SOD activity by 2 mM-KCN was observed, indicating that the enzymes in Campylobacter spp. are of the Fe-SOD and/or Mn-SOD types.

DISCUSSION

The species and subspecies of Campylobacter we studied had SOD activities higher than those of E. coli and P. acnes. Most of the campylobacters showed specific activities of around 5 U (mg protein)⁻¹. Campylobacter fetus subsp. fetus showed much higher specific activities.

It is possible to relate to some extent the SOD activity in the Campylobacter spp. to their aerotolerance. The oxygen partial pressure at which the growth of a particular isolate is inhibited was reported as follows: C. sputorum 5.3–10.7 kPa (5.2–10.6% O₂) (Loesch et al., 1965), C. jejuni 6.1 kPa (6% O₂) (Hoffman et al., 1979a), C. fetus subsp. venerealis 8.6 kPa (8.5% O₂), C. coli 12.7 kPa (12.5% O₂), and C. fetus subsp. fetus 18.1 kPa (17.9% O₂) (Ware et al., 1977). Thus aerotolerance increases with increase in the SOD activity.

Catalase is another important factor in the aerotolerance of a bacterium. Since our C. sputorum strains had no significant catalase activity, SOD may be dominant in preventing oxygen damage in these organisms. Oxygen utilization is indicated in all the Campylobacter strains studied, by the presence of a positive oxidase reaction.

The migration patterns shown in Fig. 1 suggest that Campylobacter spp. have several electrophoretically distinct variants of SOD. An isoenzyme of Rm 0.57 was the main component in C. sputorum, while an isoenzyme of Rm 0.73 is the characteristic main component of both C. fetus subsp. and of C. jejuni. However, C. jejuni is distinguishable from C. fetus by the absence of a second major isoenzyme of Rm 0.82. Campylobacter fetus subsp. fetus and C. fetus subsp. venere-
Superoxide dismutase of Campylobacter spp.

C. sputorum subsp. sputorum
C. sputorum subsp. bubulus
C. coli
C. jejuni
C. fetus subsp. fetus
C. fetus subsp. venerealis
E. coli IID 861
P. aerogenes ATCC 11827
V. alcalescens ATCC 17748
Control (bovine blood SOD)

Fig. 1. Drawings of electrophoretic migration patterns of SOD of six species/subspecies of Campylobacter, and of E. coli, P. aerogenes and V. alcalescens (control: bovine blood SOD). Crude cell-free extracts were subjected to electrophoresis, using 0·1 to 0·3 mg protein per gel. The gels (7% acrylamide) were stained for superoxide dismutase. Migration distances of negative-stained bands were converted to relative mobilities (Rm) by dividing each migration distance by that of the front marker (bromophenol blue).

ealis have similar main bands (Rm 0·72, 0·81 and Rm 0·73, 0·82) but they can be distinguished from each other since the latter has two trace bands (Rm 0·52, 0·57), and the former only one (Rm 0·60).

Contrary to the results of Ware et al. (1977), inhibition of SOD activity by CN− was not observed. Our results are thus in line with evidence that bacteria do not have Zn,Cu-SOD (Fridovich, 1974). Hoffman et al. (1979a) reported that the SOD of C. jejuni was an Fe-type enzyme. In view of the various enzyme activities and different electrophoretic profiles of SOD in Campylobacter spp., it seems probable that some species or subspecies of Campylobacter may also have Mn-SOD. The higher SOD activities and large activity variations in C. fetus subsp. fetus might be due to an active synthesis of Fe-SOD and/or Mn-SOD.

In conclusion, the present results indicate that the electrophoretic analysis of SOD may be useful in distinguishing C. sputorum, C. coli, C. jejuni, C. fetus subsp. fetus and C. fetus subsp. venerealis.

The authors thank Dr Hashimoto, The Hokkaido Branch Laboratory, National Institute of Animal Health, for kindly giving the strains of Campylobacter.

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