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

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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}), *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 pyorrhoea (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 & Plastridge, 1956; Ware *et al.*, 1977), 6% (Reich *et al.*, 1956) or 2·5% (Fletcher & Plastridge, 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.* (1979*a*, *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-

Abbreviations: NBT, nitroblue tetrazolium; Rm, relative mobility; SOD, superoxide dismutase.

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Table 1. *Strains of Campylobacter and other bacteria used*

Name	Received as Strain	Source*	Habitat
C. sputorum subsp. *sputorum*	C 1	NIAH	Faeces of pig
	C 2	NIAH	
	C 3	NIAH	Faeces of cattle
	C 4	NIAH	
	C 5	NIAH	
	C 6	NIAH	
	C 8	NIAH	Bull foreskin cavity wash
	C 9	NIAH	
	C 14	NIAH	
	C 15	NIAH	
C. sputorum subsp. *bubulus*	CIP 53103†	CIP	Bull sperm
C. coli	CIP 7080†	CIP	Faeces of pig
C. jejuni	CIP 702†	CIP	Faeces of cow
C. fetus subsp. *fetus*	CIP 5396†	CIP	Brain of sheep foetus
	C 16	NIAH	Bull foreskin cavity wash
	C 17	NIAH	
	C 18	NIAH	
	C 20	NIAH	
C. fetus subsp. *venerealis*	CIP 6829†	CIP	Cow
	C 21	NIAH	Bone of abortive cow embryo
	C 22	NIAH	
	C 23	NIAH	Liver of abortive cow embryo
	C 24	NIAH	Caecum of abortive cow embryo
Escherichia coli	IID 861†	IID	
Propionibacterium acnes	ATCC 11827†	ATCC	
Veillonella alcalescens subsp. *dispar*	ATCC 17748†	ATCC	Human mouth

* CIP, Collection of the Institute Pasteur, Paris, France; IID, Institute of Medical Science, University of Tokyo, Tokyo, Japan; ATCC, American Type Culture Collection, Rockville, Md., USA; NIAH, National Institute of Animal Health (Hokkaido Branch Laboratory), Sapporo, Japan.

† Type strain.

tolerance of *C. fetus* (Hoffman *et al.*, 1979*a*). 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; KH_2PO_4 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 g; peptone 10 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 M-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 M-phosphate buffer (pH 7·8) to remove Cu^{2+}, Mn^{2+}, Fe^{2+} and Fe^{3+} 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.

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 1×10^{-4} M-xanthine, $2{\cdot}5 \times 10^{-5}$ M-NBT, 1×10^{-4} M-EDTA and 0·05 M-sodium carbonate (pH 10·2) in an amount sufficient to reduce NBT to diformazan at a rate such that the A_{560} 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 Kovacs (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 negative-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). Dismutation 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*

Organism		Sp. act. of SOD* [U (mg protein)$^{-1}$]	Catalase activity	Oxidase activity
C. sputorum subsp. *sputorum*	C 1	4·8 ± 0·4	−	+
	C 2	4·8 ± 0·4	−	+
	C 3	3·2 ± 0·2	−	+
	C 4	4·5 ± 0·3	−	+
	C 5	3·8 ± 0·3	−	+
	C 6	1·8 ± 0·1	−	+
	C 8	0·9 ± 0·1	−	+
	C 9	6·5 ± 0·5	−	+
	C 14	1·7 ± 0·0	−	+
	C 15	1·9 ± 0·8	−	+
C. sputorum subsp. *bubulus*	CIP 53103	5·8 ± 1·0	−	+
C. coli	CIP 7080	3·9 ± 0·5	+	+
C. jejuni	CIP 702	9·0 ± 1·8	+	+
C. fetus subsp. *fetus*	CIP 5396	14·0 ± 2·7	+	+
	C 16	7·8 ± 3·8	+	+
	C 17	40·0 ± 2·4	+	+
	C 18	35·2 ± 3·0	+	+
	C 20	55·7 ± 2·8	+	+
C. fetus subsp. *venerealis*	CIP 6829	5·3 ± 1·6	+	+
	C 21	6·8 ± 0·1	+	+
	C 22	3·9 ± 0·2	+	+
	C 23	2·9 ± 0·3	+	+
	C 24	4·4 ± 0·1	+	+
E. coli	IID 861	1·5 ± 0·1	+	−
P. acnes	ATCC 11827	1·6 ± 0·0	−	−
V. alcalescens subsp. *dispar*	ATCC 17748	0·2 ± 0·0	+	−

* 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% O_2) (Loesch *et al.*, 1965), *C. jejuni* 6·1 kPa (6% O_2) (Hoffman *et al.*, 1979*a*), *C. fetus* subsp. *venerealis* 8·6 kPa (8·5% O_2), *C. coli* 12·7 kPa (12·5% O_2), and *C. fetus* subsp. *fetus* 18·1 kPa (17·9% O_2) (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* subspp. 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. *vener-*

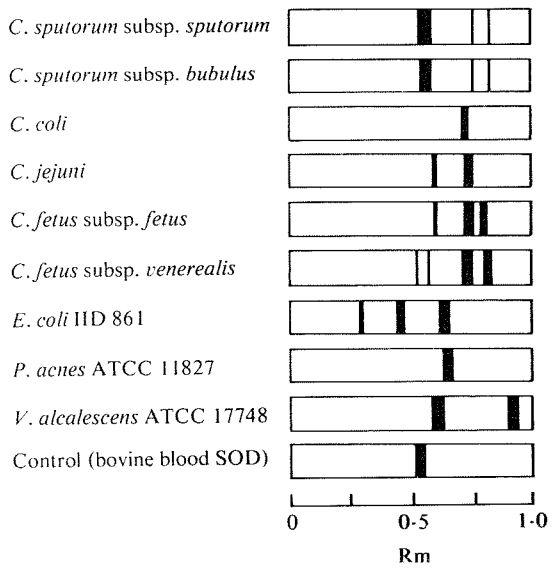

Fig. 1. Drawings of electrophoretic migration patterns of SOD of six species/subspecies of *Campylobacter*, and of *E. coli*, *P. acnes* and *V. alcalescens* (control: bovine blood SOD). Crude cell-free extracts were subjected to electrophoresis, using 0·1 to 0·3 mg protein per gel. The gels (7% acrylamide) 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.* (1979*b*) 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*.

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