COMPARATIVE STUDIES OF OXALYL-CoA DECARBOXYLASE PRODUCED BY SOIL AND RUMINAL BACTERIA

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Abstract

Oxalic acid is an organic dicarboxylic acid which has a marked affinity for calcium and magnesium, the salts of which are insoluble in water. Its principle salts are sodium, potassium, and ammonium. The salts of oxalic acid are found in a variety of plants, some of which form the dominant pasture species. Stock losses as a result of grazing such pastures are common. Oxalate-producing fungi have also been reported to have been responsible for stock losses.

Oxalate poisoning of stock can take either of two forms. Acute poisoning usually occurs when hungry stock are placed on pasture dominated by oxalate-containing plants. Reduction of rumen microbial activity, usually due to fasting, as well as diets deficient in calcium, predispose to toxicity. Death usually occurs 4-12 hours after a lethal dose as a result of severe metabolic disturbance, although some sheep may linger on for 2 or 3 days. Chronic oxalate toxicity is common in sheep which have been adapted to grazing *Oxalis pes-caprae* (soursob). They develop a high degree of tolerance to high daily intakes of the acid oxalate contained by the plants. However, there is often low-level absorption of the oxalate with progressive damage to the kidneys as a result of deposition of masses of calcium oxalate crystals. Over a period of 2-12 months on soursob pasture, animals slowly develop renal failure and deaths occur sporadically.

Therefore, the aim of this project was to identify an enzyme responsible for the metabolism of oxalate which would be suitable for degrading oxalate in the rumen, and clone and characterise that gene.

Oxalate-degrading bacteria were isolated from soil and their ability to metabolise oxalate was compared, based on halo formation on solid growth media containing a calcium oxalate precipitate. One isolate, designated OxD, which demonstrated rapid halo formation, was characterised by the Clinical Microbiology Department of the Institute of Medical and Veterinary Science in Adelaide using the API bacterial identification system and found to be closely related to *Pseudomonas diminuta*, a species originally isolated from water and clinical

specimens. However, sequencing of the 16S subunit of rRNA showed that it was closely related to *Paracoccus* species. It was studied for growth on oxalate, substrate utilisation and volatile fatty acid production. The optimum oxalate concentration in the growth medium was found to be between 56 mM and 224 mM. Inoculation of a single colony into 100 ml of culture medium produced logarithmic growth with a lag period of 27 hours and entering stationary phase after 38 hours. It was found that the stationary phase was a result of an increase in pH rather than a lack of nutrients. It was able to grow on a range of carbohydrate sources. No volatile fatty acids were found in the growth medium after growth on oxalate.

Growth rates of all isolates in oxalate were determined and compared with that of *Pseudomonas oxalaticus*, strain Ox_{23} (ATCC 11884), a well characterised oxalate-degrading bacterium. Colony morphology, cell morphology, digestion of the DNA with a restriction endonuclease and separation of the fragments in an agarose gel, dot-blot analysis of the DNA, and analysis of restriction fragment length polymorphism in the 16S subunit of rRNA showed poor homology between *P. oxalaticus* and the other isolates.

Paracoccus sp. cells were grown in oxalate and a cell free extract was produced. The cofactor requirements and optimum conditions for oxalyl-CoA decarboxylase activity were determined. The assay required Mg^{2+}, thiamine pyrophosphate, oxalyl-CoA, oxalate, and NAD^+. The optimum pH was 6.8. The enzyme appeared to be specific for oxalyl-CoA. These conditions were the same as those for oxalyl-CoA decarboxylase activity in *P. oxalaticus*. As with *P. oxalaticus*, formate dehydrogenase was induced with the oxalyl-CoA decarboxylase. However, the specific activity of both enzymes in OxD were considerably higher than in *P. oxalaticus*.

To clone the genes for oxalyl-CoA decarboxylase, cosmid and plasmid libraries of *P. oxalaticus* were created in pHC79 and pUC19 respectively, used to transform *Escherichia coli* and tested for their ability to produce halos on calcium oxalate plates. No such colonies were found. Polyclonal antibodies were raised against a commercial preparation of an oxalate decarboxylase from *Aspergillus niger* to use as a probe for *E. coli* producing oxalyl-CoA

decarboxylase. However, they did not react with the oxalyl-CoA decarboxylase produced by any of the oxalate-degrading bacterial species.

As there were no DNA probes to select for the oxalyl-CoA decarboxylase gene cloned in *E. coli*, the plasmid pHC23 was used to create transposon mutants in *P. oxalaticus*. These were screened for the loss of halo formation on calcium oxalate plates (ox^- mutants) The DNA from any such mutants was to be cut with a suitable enzyme and the kanamycin resistance gene of the transposon and any attached *P. oxalaticus* chromosomal DNA was to be cloned. The *P. oxalaticus* DNA could then be used to probe the libraries of *P. oxalaticus* DNA in *E. coli*. However, no such colonies were detected. Southern analysis of the DNA demonstrated that the transposon was integrating into the chromosome in a random manner and some amino acid auxotrophs were recovered.

As transposon mutagenesis was unsuccessful in creating ox^- mutants, ultra-violet mutagenesis was used in an attempt to create ox^- mutants which could be used as hosts for a library of the wild-type *P. oxalaticus* DNA. Such a library would have been screened for the reversion to the ability to metabolise oxalate. However, no ox^- mutants were recovered.

As all forms of mutagenesis were unsuccessful in producing ox^- mutants, primers, approximately 1 kb apart, were created from G/C-rich regions of the published sequence of the oxalyl-CoA decarboxylase gene from *Oxalobacter formigenes*. These were used to create a PCR product approximately 1 kb in length from *P. oxalaticus* which was used as a DNA probe to probe a Southern transfer of *P. oxalaticus* DNA cut with a range of restriction endonucleases. The fragment to which the probe hybridised was cloned and sequenced. However, the sequence showed very strong homology to the elongation factor G gene of a number of organisms. No other PCR products of approximately 1 kb were produced with these primers. This would suggest that the sequence chosen for the primers was not in a conserved region of the gene and that the *P. oxalaticus* gene sequence may have poor homology with the sequence from *O. formigenes*. This was supported by results using the same primers to produce a probe from *O. formigenes* DNA. This hybridised very poorly with *P. oxalaticus* DNA and attempts to repeat the experiment were unsuccessful.

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Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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SIGNED: DATE: 5/11/99

List of Abbreviations

API	Analytical Profile Index
AR	Analytical Reagent
ATCC	American Type Culture Collection
BDH	British Drug Houses
bp	base pairs
BSA	bovine serum albumin
CoA	Coenzyme A
CPM	counts per minute
Da	Daltons
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
eg	example
ELISA	enzyme-linked immunosorbent assay
G/C	guanine/cytosine
GC	gas chromatography
HPLC	high performance liquid chromatography
ie	that is
IPTG	isopropylthiogalactoside
MCS	multiple cloning site
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RO	reverse osmosis
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecylsulphate

SSC	standard saline citrate
v/v	volume per volume
VFA	volatile fatty acid
w/v	weight per volume
Xgal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside