Biochem. J. (1975) **149**, 477–480
Printed in Great Britain

Secondary Alkylsulphatases in a Strain of *Comamonas terrigena*

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(*Received* 22 *April* 1975)

The occurrence in a strain of *Comamonas terrigena* of secondary alkylsulphatase activity towards potassium decan-5-yl sulphate is reported. A number of cell-washing and osmotic-shock procedures for releasing bacterial exocytoplasmic enzymes were ineffective in releasing this activity. Primary alkylsulphatases are not present in the organism, nor can their formation be induced under a wide variety of experimental conditions tested.

Few studies have been made on micro-organisms that are capable of degrading long-chain primary and secondary alkyl sulphate esters, although such organisms may participate in detergent biodegradation and can cause serious contamination problems in the commercial production and use of alkyl sulphate detergents (Flawn *et al.*, 1973). In the one case that has been studied in detail (*Pseudomonas* C12B) the prerequisite for biodegradation of alkyl sulphates appears to be removal of sulphate ester groups by primary and/or secondary alkylsulphatase enzymes. Subsequent utilization of the carbon chain is initiated by an alcohol dehydrogenase (Williams & Payne, 1964). *Pseudomonas* C12B is a versatile organism with regard to its ability to produce alkylsulphatases. Under appropriate growth conditions two primary alkylsulphatases (designated P1 and P2) and three secondary alkylsulphatases (S1, S2 and S3) can be detected in cell extracts (Dodgson *et al.*, 1974).

The present report is concerned with alkylsulphatase activity in another micro-organism which was co-isolated with *Pseudomonas* C12B. Surprisingly, this micro-organism, a strain of *Comamonas terrigena*, is capable of producing only secondary alkylsulphatases.

Materials and methods

Bacterium. The bacterium, a non-pigmented Gram-negative rod, was isolated from soil near a sewage outfall in Athens, Ga., U.S.A. It showed properties in common with *Comamonas terrigena* according to the classification of Weaver *et al.* (1972) and was tentatively identified as a non-flagellated strain of that organism.

Sulphate esters and other compounds. Methods of preparation or purchasing sources of sulphate esters were as described by Dodgson *et al.* (1974). Lysozyme was purchased from BDH Chemicals, Poole, Dorset, U.K.

Analytical methods. Methods for following bacterial growth and determining protein and nucleic acid in cell extracts have been described by Fitzgerald & Payne (1972*a*). Alkylsulphatase activity was measured as described by Dodgson *et al.* (1974); incubation of substrate (0.018 M final concentration) and dialysed enzyme extract was at 30°C in the presence of 0.02 M-Tris–HCl buffer, pH 7.5 (approximate optimum conditions for *C. terrigena* extracts acting on potassium decan-5-yl sulphate). The relationship between enzyme activity and concentration tended to be variable, possibly because of high concentrations of nucleic acid in cell extracts. Care was therefore taken, in any one experiment, to ensure that the same concentration of protein was present in incubation mixtures. A unit of enzyme activity is defined as 1 nmol of SO_4^{2-} liberated/min per mg of protein. Alkylsulphatases were located after electrophoresis on polyacrylamide gels by the zymographic procedure of Payne *et al.* (1974).

Culture conditions. C. terrigena was grown with shaking at 30°C in nutrient broth or in a standard buffered medium (pH 7.0) which contained: KH_2PO_4, 1.5 g; K_2HPO_4, 3.5 g; NH_4Cl, 0.5 g; $MgCl_2,6H_2O$, 0.15 g; deionized water, 1 litre. This standard medium also contained carbon and sulphur in the form and concentration specified for each experiment. L-Methionine and all sulphate esters were added to media after sterilization by Millipore filtration (GS 0.22 μm filter); other components were sterilized by autoclaving. Other details of culture conditions and preparation of cell extracts were as described by Fitzgerald & Payne (1972*a*) and Fitzgerald *et al.* (1974). Unless otherwise stated, growth of the bacterium was continued into the late stationary phase (cf. Fig. 1).

Cell-washing and osmotic-shock procedures. Bacteria were grown in nutrient broth in eight 1-litre

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Erlenmeyer flasks (500ml per flask) until 8h after reaching the stationary phase. Flask contents were pooled aseptically and cells from eight equal portions (490ml) were harvested by centrifuging. The cell pellets from one portion were resuspended in 2ml of 10mM-Tris–HCl buffer, pH7.5, and retained at −20°C as an untreated control. Pellets from five of the remaining portions were each resuspended in 25ml of the appropriate wash solution (see Table 1) and suspensions were shaken at 30°C for 2h before centrifuging at 4°C. Each supernatant was stored at −20°C until required and each pellet was resuspended in a minimum volume of buffer and stored as above. Pellets from the remaining two portions were aseptically resuspended, one in 25ml of sterile water (untreated cell suspension), the other in 25ml of 20% (w/v) sterile sucrose solution containing 10mM-Tris–HCl, pH8.0, and 0.1mM-EDTA. Both suspensions were shaken at 30°C for 20min and then centrifuged at 25°C. Supernatants were stored at −20°C. The cell pellets from the untreated cell suspension were resuspended in a minimum volume of 10mM-Tris–HCl, pH7.5, and frozen. The pellet from the sucrose-containing suspension was subjected to osmotic shock by the procedure of Neu & Heppel (1965) before centrifuging at 4°C. The supernatant (shock fluid) was stored at −20°C and the pellet was resuspended in the minimum volume of 10mM-Tris–HCl, pH7.5, and stored as usual. Subsequently, all cell suspensions were unfrozen and ruptured by passage (three times) through a chilled French pressure cell operating at 126MPa. Cell debris was removed by centrifuging for 1h at 2°C (Beckman L2-65B, 50T rotor, 165000$g_{av.}$) and each supernatant was dialysed for 36h against six changes (1 litre/supernatant) of 10mM-Tris–HCl, pH7.5. All other washings and shock fluids were unfrozen at the same time and similarly dialysed. After dialysis, the volumes of cell extracts were adjusted to 15ml and wash and shock fluids to 80ml with 10mM-Tris–HCl, pH7.5. All samples were assayed for secondary alkylsulphatase activity against potassium decan-5-yl sulphate. Protein and nucleic acid were measured in wash and shock fluids.

Results and discussion

When *C. terrigena* was grown on nutrient broth, dialysed cell extracts were able to hydrolyse decan-5-yl sulphate, maximum activity being observed in the late stationary phase (Fig. 1). Gel-electrophoresis zymograms showed the presence of at least two bands of secondary alkylsulphatase activity migrating anodically under the conditions used. The slower-moving band (designated CS1) was faint and diffuse whereas the other (designated CS2) was discrete and predominated. Neither band had mobility identical with any of the three secondary alkylsulphatases of *Pseudomonas* C12B (cf. Dodgson *et al.*, 1974). In that

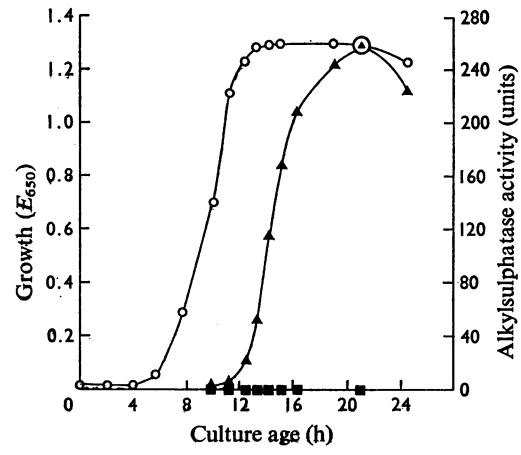

Fig. 1. *Production of alkylsulphatase activity during growth of C. terrigena*

Growth was on nutrient broth at 30°C. ○, Bacterial growth (E_{650}); ▲, activity towards potassium decan-5-yl sulphate; ■, activity towards sodium hexan-1-yl sulphate.

organism the S3 secondary alkylsulphatase is produced only under highly specific circumstances and Oronite (a commercial detergent; see Dodgson *et al.*, 1974) or a mixture of tetradecan-2-yl sulphate or hexadecan-2-yl sulphate with either tetradecan-2-ol or hexadecan-2-ol must be present in the growth medium. This S3 enzyme differs from the S1 and S2 enzymes of *Pseudomonas* C12B in being active towards potassium pentan-3-yl sulphate. Extracts of *C. terrigena*, however, were unable to hydrolyse that substrate, irrespective of whether the organism was grown in nutrient broth alone or under the specific circumstances (Dodgson *et al.*, 1974) used for the *Pseudomonas* S3 enzyme. Under those circumstances zymograms were always identical with those of extracts of broth-grown cells.

Attempts to induce formation of primary alkylsulphatases. Extracts of broth-grown cells were devoid of primary alkylsulphatase activity towards the sulphate esters of ethanol, butan-1-ol, hexan-1-ol and octan-1-ol. Nor did zymogram bands appear after gel electrophoresis when gels were incubated with decan-1-yl sulphate, dodecan-1-yl (dodecyl) sulphate or tetradecan-1-yl sulphate. Analogous extracts from *Pseudomonas* C12B possess one primary alkylsulphatase (designated P1) active towards a range of primary alkyl sulphates including those mentioned above.

When either sodium hexan-1-yl sulphate or sodium dodecan-1-yl sulphate was included in the nutrient broth (0.5%, w/v) the CS1 and CS2 secondary alkylsulphatases were present in extracts of *C. terrigena*

but no primary alkylsulphatase activity was detected. Under analogous growth conditions, *Pseudomonas* C12B produces a further primary alkylsulphatase (designated P2) in addition to the P1 enzyme.

It was possible that the presence of SO_4^{2-} ions or L-cysteine in the growth medium might repress formation of primary alkylsulphatases in *C. terrigena*. Such repression occurs with arylsulphatases (EC 3.1.6.1) from *Pseudomonas* C12B (Fitzgerald & Payne, 1972*b*) and a number of other bacteria (see, e.g., Harada & Spencer, 1964; Rammler *et al.*, 1964). In these instances arylsulphatases are formed if L-methionine serves as the only sulphur source for growth. However, growth of *C. terrigena* with citrate (or acetate or pyruvate, all at 1%, w/v) and methionine (5mM) as sole sources of carbon and sulphur respectively did not result in the formation of primary alkylsulphatases, although production of secondary alkylsulphatases was unaffected. Moreover, when either hexan-1-yl sulphate or decan-1-yl sulphate was substituted for L-methionine the organism failed to grow.

One possibility remained in relation to the induction of primary alkylsulphatases. In the case of the S3 secondary alkylsulphatase of *Pseudomonas* C12B induction of the enzyme by either tetradecan-2-yl sulphate or hexadecan-2-yl sulphate occurs only if either of the corresponding secondary alcohols is also present in culture media (Dodgson *et al.*, 1974). Attempts were therefore made to induce primary alkylsulphatase activity in *C. terrigena* by growing the organism in broth containing both primary alkyl sulphate (1mM) and parent alcohol (1mM). Four different pair combinations were selected: the C_6 sulphate ester–alcohol pair and the C_{10}, C_{14} and C_{18} pairs. Extracts of cells in all cases were still active towards secondary alkyl sulphates but were inactive towards hexan-1-yl sulphate or dodecan-1-yl sulphate.

Collectively, the results suggest that *C. terrigena* is unable to synthesize primary alkylsulphatases. However, this does not exclude the possibility of the organism utilizing primary alkyl sulphates as a carbon source for growth in the absence of a sulphatase. Denner *et al.* (1969) have shown that rats can degrade dodecan-1-yl sulphate by oxidation from the ω-carbon end of the chain. Butyric acid 4-sulphate is formed but spontaneously loses its sulphate group at pH values greater than 5 (Ottery *et al.*, 1970). Some micro-organisms are capable of ω-oxidation (Klug & Markovetz, 1971) and the possibility existed that *C. terrigena* could use primary alkyl sulphates as carbon sources in an analogous fashion. However, the organism failed to grow when hexan-1-yl sulphate (or decan-1-yl sulphate or dodecan-1-yl sulphate, all at 0.5% concn.) was used as carbon source and 5mM-Na_2SO_4 as sulphur source. Failure to grow was also observed when cells grown on broth containing decan-1-yl sulphate (0.2%, w/v) were transferred to basal salts medium containing 5mM-Na_2SO_4 and decan-1-yl sulphate (0.2%, w/v), shaken at 30°C for 4h and then inoculated into a larger volume of the

Table 1. *Effects of cell-washing and osmotic-shock procedures on release of secondary alkylsulphatase activity from C. terrigena*

Equal portions of broth-grown cells were treated as described below and in the text, and the results of the different treatments are therefore directly comparable within the limits of experimental error inherent in the procedures used. The concentrations of wash components were always as follows: sucrose, 20% (w/v); Tris–HCl buffer, 10mM, pH8.0; EDTA, 0.1mM; lysozyme, 0.5mg/ml; citrate, 85mM; $MgCl_2$, 40mM.

Treatment	Fraction	Total nucleic acid (mg)	Total enzyme activity (nmol of SO_4^{2-} released/min)	Total protein (mg)	Relative specific activity of recombined fractions (enzyme units)
Untreated control	Cell extract	—	57600	133.5	431
Washed with sucrose–Tris–HCl	Cell extract	—	42500	105.0	382
	Wash supernatant	0.37	0	6.2	
Washed with sucrose–Tris–HCl–EDTA	Cell extract	—	51000	126.0	386
	Wash supernatant	0.37	0	6.2	
Washed with sucrose–Tris–HCl–EDTA–lysozyme	Cell extract	—	57000	136.0	394
	Wash supernatant	0.42	0	8.5	
Washed with sucrose–Tris–HCl–citrate	Cell extract	—	48400	115.5	391
	Wash supernatant	0.36	0	8.1	
Washed with sucrose–Tris–HCl–citrate–$MgCl_2$	Cell extract	—	51500	124.5	390
	Wash supernatant	0.36	0	7.5	
Osmotic-shock control	Cell extract	—	49200	122.1	390
	Wash supernatant	0.15	0	3.8	
Osmotic-shock treatment	Cell extract	—	43100	100.5	394
	Wash supernatant	0.32	0	6.5	
	Shock fluid	0.15	0	2.5	

same medium. After 6 days without growth the cells were still viable. In a parallel experiment in which decan-5-yl sulphate replaced decan-1-yl sulphate as carbon source, good growth was obtained in the synthetic medium.

C. terrigena clearly has no capacity to utilize primary alkyl sulphates, a surprising finding in the light of its ability to produce secondary alkylsulphatases and to grow with decan-5-yl sulphate serving as carbon source.

Attempts to localize secondary alkylsulphatase activity. The primary (P1) and secondary alkylsulphatases (S1 and S2) of *Pseudomonas* C12B were released when cells were subjected to various cell-washing or osmotic-shock procedures, indicating an exocytoplasmic location for the enzymes (Fitzgerald & Laslie, 1975). Application of identical treatments to *C. terrigena* did not result in release of alkylsulphatases from cells grown to the late stationary phase, although some protein and nucleic acid was released (Table 1). Similar results were obtained with cells harvested at the late exponential phase of growth. Alkylsulphatase activity could subsequently be released from treated cells by passage through the French pressure cell. Collectively, these results suggest a cytoplasmic origin for the enzymes. However, the possibility is not unequivocally excluded that activity is exocytoplasmic but is associated with cell wall or plasma membrane in such a way that it is released only by severe pressure treatment. In any event, the findings reflect a further difference between alkylsulphatase activity in *C. terrigena* and *Pseudomonas* C12B. The differences as a whole may indicate variations in the significance of the enzymes to the physiology of the two organisms.

This work was supported by the Science Research Council. G. W. J. M. holds an S.R.C. Studentship. The authors thank Dr. D. Webb of the Bacteriology Unit of the U.S. Department of Health, Education and Welfare, Center for Disease Control, Atlanta, Ga., U.S.A., for the identification of the organism.

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