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**A LABORATORY MODEL SYSTEM FOR ANALYSING MICROBIAL INTERACTIONS OCCURRING IN ANOXIC ESTUARINE SEDIMENTS**

R.A. HERBERT and S.M. KEITH

Department of Biological Sciences, University of Dundee, DUNDEE (UK)

**ABSTRACT** - Defined mixed populations of *Clostridium butyricum*, *Desulfovibrio desulfuricans* and *Chromatium vinosum* co-existed for long periods when grown in a single stage chemostat with glucose as sole carbon source. The nature and availability of the inorganic nitrogen source exerted a major effect on carbon flow in the experimental system. The data show that both the *Clostridium butyricum* and *Desulfovibrio desulfuricans* isolates can utilise  $\text{NO}_3^-$  as an  $e^-$  acceptor with an increase in cell yield. Under these growth conditions the free  $\text{S}^{2-}$  levels were lower resulting in more stable mixed populations. In addition the data show that the *Clostridium butyricum* produces more oxidised fermentation end-products (acetate) when  $\text{NO}_3^-$  was available and more reduced products (butyrate) when grown on  $\text{NH}_4^+$ .

**Key words** : carbon flow, *Chromatium vinosum*, strain PT121, *Clostridium butyricum*, strain SS6, *Desulfovibrio desulfuricans*, strain DT101, microbial interactions.

**RÉSUMÉ** - Des populations mixtes de *Clostridium butyricum*, *Desulfovibrio desulfuricans*, et *Chromatium vinosum* co-existent pendant de longues périodes, lorsqu'elles se développent dans un chemostat à une seule étape avec du glucose pour seule source de carbone. La nature et la disponibilité de la source d'azote inorganique exercent un effet important sur le flux de carbone du système expérimental. Les données montrent que les isolats de *Clostridium butyricum*, ceux de *Desulfovibrio desulfuricans* et ceux de *Chromatium vinosum* peuvent utiliser  $\text{NO}_3^-$  comme accepteur d'électrons, avec une augmentation de la production des cellules. Sous ces conditions de croissance les niveaux des ions  $\text{S}^{2-}$  libres sont plus faibles et il en résulte des populations mixtes plus stables. Les expériences montrent également que *Clostridium butyricum* fabrique plus de produits finaux de fermentation oxydés (acetate) lorsque  $\text{NO}_3^-$  est disponible, et plus de produits réduits (butyrate) lorsqu'elle se développe sur  $\text{NH}_4^+$ .

**Mots clés** : flux de carbone, *Chromatium vinosum*, souche PT121, *Clostridium butyricum*, souche SS6, *Desulfovibrio desulfuricans*, souche DT101, interactions microbiennes.

**INTRODUCTION**

Minéralisation of organic detritus in anoxic sediments is a highly complex process. This is primarily due to the restricted metabolic potential of the individual anaerobic micro-organisms present in such habitats. Complete mineralisation does not take place within one species of micro-organism but within associations of physiologically different types of micro-organisms. Each physiological group exploits only a small fraction of the detrital energy available and supplies the remainder in the form of excreted metabolic end-products to the next member of the detrital food chain. The microbial processes which take place in anoxic sediments are related to the quantity and quality of available organic carbon and the concentration of available electron acceptors such as  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{Mn}^{4+}$  and  $\text{CO}_2$ .

Strict anaerobes are commonly isolated from sediments in the Tay estuary (Keith, Macfarlane and Herbert, 1982, Keith, Herbert and Harfoot, 1982) and for the purposes of

this study we have used a saccharolytic clostridium identified as a strain of *Clostridium butyricum*, a sulphate reducing bacterium identified as a strain of *Desulfovibrio desulfuricans* and a purple sulphur bacterium identified as a strain of *Chromatium vinosum*. Whilst these three bacteria form only a simple experimental system they nonetheless enable the flow of carbon to be followed according to the simplified scheme presented in Figure 1. *Clostridium butyricum* SS6 ferments sugars such as glucose to yield a mixture of products including lower fatty acids, alcohols and  $H_2/CO_2$  which are in turn used as carbon and energy sources by *D. desulfuricans* DT101. The sulphate reducing bacterium uses  $SO_4^{2-}$  as the terminal  $e^-$  acceptor in anaerobic respiration with the concomitant production of  $S^{2-}$  which provides the  $e^-$  donor for the photosynthetic fixation of  $CO_2$  by *Chr. vinosum* PT 121.

The objectives of this study were to determine the effects of carbon and inorganic nitrogen source availability on the populations of these three bacteria when grown in defined mixed cultures in a single stage chemostat.

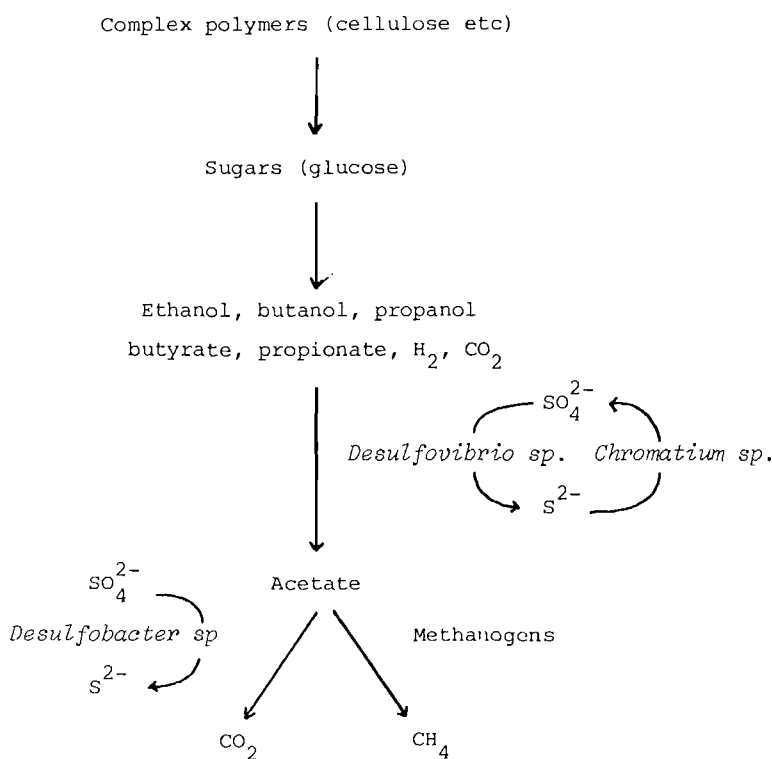


Figure 1 : Generalised scheme of carbon flow in anaerobic sediments.

## MATERIALS AND METHODS

### Isolation of anerobic bacteria

*Clostridium butyricum* strain SS6, *Desulfovibrio desulfuricans* DT101 and *Chromatium vinosum* strain PT121 were isolated and obtained in pure culture as described by Keith *et al.* (1982).

## Enumeration of saccharolytic clostridium, sulphate reducing and phototrophic bacteria populations

Population densities of *Clostridium butyricum* SS6, *Desulfovibrio desulfuricans* DT101 and *Chromatium vinosum* P1121 were determined by direct counting methods using a Neubauer counting chamber (Cruickshank, 1965).

### Growth in continuous culture

(a) Single pure cultures : -*Clostridium butyricum* SS6 and *Desulfovibrio desulfuricans* DT101 were grown in continuous culture as described by Keith, Macfarlane and Herbert (1982) and Keith and Herbert (1982).

(b) Mixed population studies : -Mixed population studies were carried out in a single stage 1 litre chemostat as described by Baker (1968). Growth medium pH was maintained at pH 7.5 using an E.I.L pH controller (E.I.L. Chertsey, Surrey) and a temperature of 25°C maintained using a thermo-circulator (Churchill Instruments, Perivale, London). Anaerobic conditions in the culture vessel and medium reservoir were maintained by sparging with high purity nitrogen (1.5 litre min<sup>-1</sup>). The growth medium had the following composition (g.l<sup>-1</sup>): MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.08; K<sub>2</sub>HPO<sub>4</sub>, 0.125; KH<sub>2</sub>PO<sub>4</sub>, 0.5; KCl, 0.33; Na<sub>2</sub>SO<sub>4</sub>, 1.0; FeSO<sub>4</sub>, 0.004; Na-citrate, 0.1; 1% w/v Fe citrate, 0.6 ml; vitamin B12 0.001% w/v, 2 ml; Yeast extract, 0.1 g. The concentration of the carbon source (glucose) and nitrogen sources (KNO<sub>3</sub> and NH<sub>4</sub>Cl) were varied as required (Fig. 2 and 3).

### Analysis of spent media

Spent media were analysed for NO<sub>2</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> according to the methods described by Brown *et al.* (1972). Free sulphide concentrations were determined using an ion-specific electrode (Orion Inc. Blackstone Street, Cambridge, MA 02139, U.S.A.).

## RESULTS -

### Growth on different nitrogen source

Initial experiments were undertaken to determine the effect of different inorganic nitrogen sources on single pure cultures of each bacterium grown in continuous culture. Data in Table 1 show that cell numbers of NO<sub>3</sub><sup>-</sup> grown cultures of *Cl. butyricum* SS6, irrespective of limitation, were significantly greater than NH<sub>4</sub><sup>+</sup> grown cultures. Spent media analyses of NO<sub>3</sub><sup>-</sup> grown cultures showed the accumulation of NH<sub>4</sub><sup>+</sup> (46% of NO<sub>2</sub><sup>-</sup> input) together with trace quantities of NO<sub>3</sub><sup>-</sup> and are consistent with the concept that NO<sub>3</sub><sup>-</sup> was functioning in a dissimilatory role and this was confirmed by the demonstra-

Growth Conditions	nitrogen source	cell density (ml culture) <sup>-1</sup>	Fermentation end-product (mM)		
			Acetate	Propionate	Butyrate
N-limitation	3.5 mM NH <sub>4</sub> <sup>+</sup>	2.4 x 10 <sup>8</sup>	3.6	1.7	14.8
	3.5 mM NO <sub>3</sub> <sup>-</sup>	3.6 x 10 <sup>8</sup>	14.8	0.8	4.6
C-limitation	7 mM NH <sub>4</sub> <sup>+</sup>	9.0 x 10 <sup>7</sup>	3.25	0.7	6.9
	7 mM NO <sub>3</sub> <sup>-</sup>	1.4 x 10 <sup>8</sup>	10.2	0.8	4.2

Table 1. Influence of nitrogen source and availability on cell density and end-products of sucrose fermentation produced by *C. butyricum* SS6 when grown in chemostat culture. D = 0.05h<sup>-1</sup>, 25°C. Sucrose concentration 10 g l<sup>-1</sup> (N-limitation) and 2 g l<sup>-1</sup> (C-limitation).

tion of functional dissimilatory  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reductases in *Cl. butyricum* SS6 (Keith, MacFarlane and Herbert, 1982). Concurrent with the increase in cell population density on  $\text{NO}_3^-$  there was a marked shift in fermentation end-products (Table 1). Ammonia grown cultures produced butyrate as the principal fermentation end-product whereas on  $\text{NO}_3^-$  acetate predominated. In addition to lower fatty acids substantial quantities of ethanol were also produced as an end-product of glucose fermentation by *Cl. butyricum* SS6 and this was the preferred carbon and energy source used by *D. desulfuricans* DT101 when grown in mixed culture.

Carbon source	N-source	15mM $\text{SO}_4^{2-}$	Cell density (ml culture) $^{-1}$	Dry Weight (mg.l) $^{-1}$
20mM lactate	7mM $\text{NH}_4^+$	+	$3.9 \times 10^8$	300
20mM lactate	7mM $\text{NO}_3^-$	+	$8.0 \times 10^8$	300
20mM lactate	7mM $\text{NH}_4^+$	-	$1.0 \times 10^8$	113
20mM lactate	7mM $\text{NO}_3^-$	-	$4.1 \times 10^8$	344

Table 2. Influence of inorganic nitrogen source on cell density of *Desulfovibrio desulfuricans* DT101 grown under C-limitation in continuous culture at a dilution rate of  $0.050 \text{ h}^{-1}$ ,  $25^\circ\text{C}$ .

In an analogous manner data in Table 2 show that *D. desulfuricans* DT101 will also utilise  $\text{NO}_3^-$  as an alternative terminal  $e^-$  acceptor to  $\text{SO}_4^{2-}$  and spent media analyses have shown that  $\text{NH}_4^+$  was the principal end-product of  $\text{NO}_3^-$  respiration. Nitrite never accumulated in more than trace quantities irrespective of  $\text{NO}_3^-$  availability. Cell yields on  $\text{NO}_3^-$  were the same as those on  $\text{SO}_4^{2-} + \text{NH}_4^+$  but when  $\text{NO}_3^-$  and  $\text{SO}_4^{2-}$  were simultaneously present cell numbers and cell yield doubled. The probable explanation for the 'nitrate enhancement' effect is that  $\text{SO}_4^{2-}$  grown cultures expend ATP in the formation of adenylylphosphosulphate (APS) whereas  $\text{NO}_3^-$  grown cultures require no energy expenditure in activating the  $e^-$  acceptor.

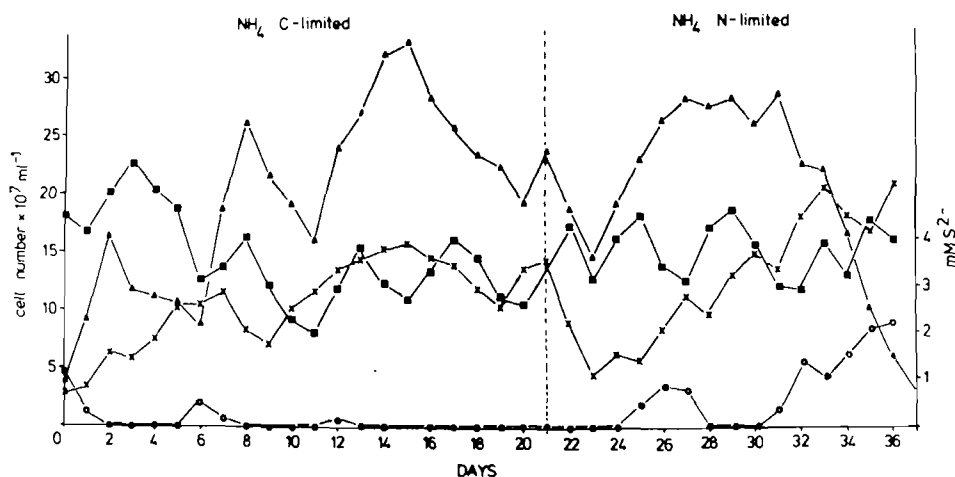


Figure 2 : Changes in population densities of *Clostridium butyricum* SS6 (■), *Desulfovibrio desulfuricans* DT101 (X) and *Chromatium vinosum* PT121 (Δ) when grown in a single stage chemostat on  $\text{NH}_4\text{Cl}$  at a dilution rate of  $0.03 \text{ h}^{-1}$  at  $25^\circ\text{C}$ . Glucose concentration 50mM, (N-limitation) and 10mM (C-limitation),  $\text{NH}_4^+$  concentration 7mM (C-limitation) and 3,5 mM (N-limitation). Open circles are concentrations of soluble  $\text{S}^{2-}$ .

### Mixed culture experiments

Having determined the physiological characteristics of the individual bacteria, Keith *et al.* 1982; Keith and Herbert, 1982, experiments were performed with defined mixed cultures of *Cl. butyricum* SS6/ *Dv. desulfuricans* DT101/ *Chr. vinosum* PT121 and *Cl. butyricum* SS6/ *Dv. desulfuricans* DT101/ *Chr. vinosum* PT121 in a single stage chemostat at a dilution rate of  $0.03 \text{ h}^{-1}$  and temperature of  $25^\circ\text{C}$ . Data presented in figure 2 show that when  $\text{NH}_4^+$  was the N-source *Chr. vinosum* PT101 predominated irrespective of limitation. Under glucose limiting conditions the free  $\text{S}^{2-}$  concentration remained low and was almost undetectable whereas under  $\text{NH}_4^+$  limitation the population of the sulphate reducing bacterium progressively increased leading to increased levels of free  $\text{S}^{2-}$  and ultimately a sharp reduction in the *Chromatium* population. In contrast  $\text{NO}_3^-$  grown cultures under C-limitation were more stable and whilst the sulphate reducer cell numbers were high there was no  $\text{S}^{2-}$  inhibition of the phototroph indicating that  $\text{NO}_3^-$  as well as  $\text{SO}_4^{2-}$  was functioning as the terminal  $e^-$  acceptor (Fig. 3a). Upon switching to  $\text{NO}_3^-$  limitation (Fig. 3b) the sulphate reducing bacteria population increased rapidly. Since the system was  $\text{NO}_3^-$  limited the *Desulfovibrio* sp. utilised an increased proportion of the available  $\text{SO}_4^{2-}$  as an  $e^-$  acceptor and as a consequence the concentration of free  $\text{S}^{2-}$  increased. The *Chromatium vinosum* strain PT121 was unable to re-oxidise the  $\text{S}^{2-}$  formed during sulphate respiration sufficiently rapidly and the levels of free  $\text{S}^{2-}$  increased (up to  $12 \text{ mM S}^{2-}$ ) such that the populations of the *Cl. butyricum* SS6 and *Chr. vinosum* PT 121 declined to a lower but stable level. Whilst the growth of the clostridium and phototroph was severely impaired by these high levels of free  $\text{S}^{2-}$  the cultures did not wash out.

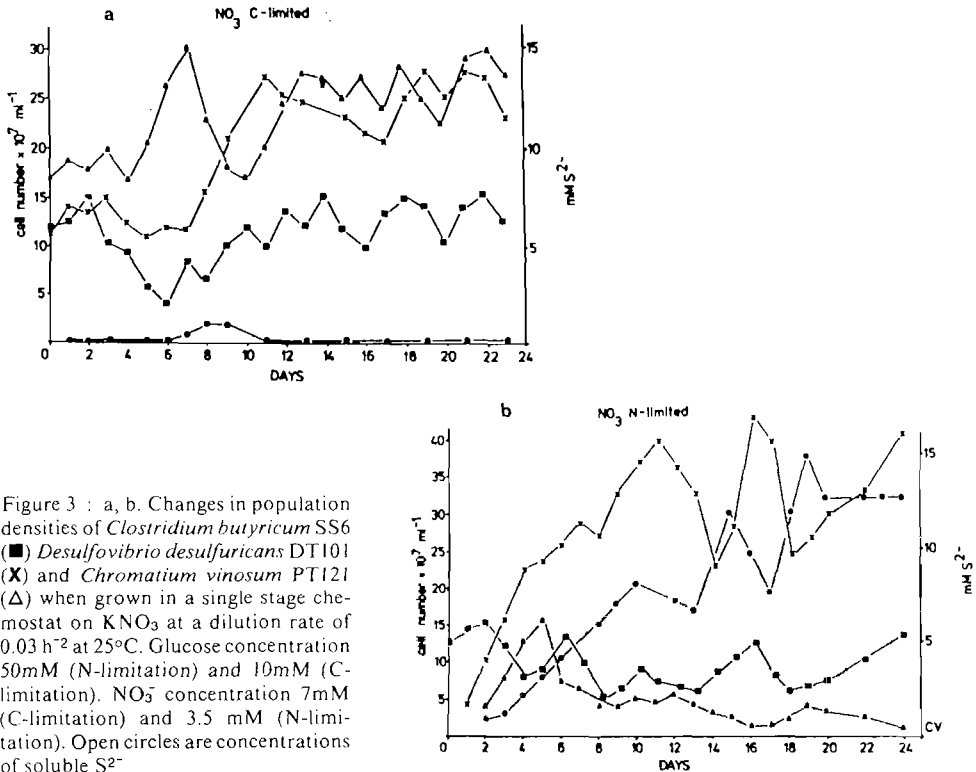


Figure 3 : a, b. Changes in population densities of *Clostridium butyricum* SS6 (■) *Desulfovibrio desulfuricans* DT101 (×) and *Chromatium vinosum* PT121 (Δ) when grown in a single stage chemostat on  $\text{KNO}_3$  at a dilution rate of  $0.03 \text{ h}^{-2}$  at  $25^\circ\text{C}$ . Glucose concentration  $50 \text{ mM}$  (N-limitation) and  $10 \text{ mM}$  (C-limitation).  $\text{NO}_3^-$  concentration  $7 \text{ mM}$  (C-limitation) and  $3.5 \text{ mM}$  (N-limitation). Open circles are concentrations of soluble  $\text{S}^{2-}$

## DISCUSSION

Data presented in this paper show that populations of *Cl. butyricum* SS6, *D. desulfuricans* DT101 and *Chr. vinosum* PT121 co-existed for extended time periods in mixed continuous culture. Experimental evidence presented in Table 1 show that the presence of  $\text{NO}_3^-$  in the growth medium resulted in increased cell populations of *Cl. butyricum* SS6 relative to  $\text{NH}_4^+$  grown cultures. These data are similar to those recorded for *Clostridium* 10H52 (Caskey and Tiejdje, 1980) and *Cl. perfringens* (Hasan and Hall, 1975) and are consistent with the concept that  $\text{NO}_3^-$  was functioning in a dissimilatory role. Analysis of fermentation products from cultures of *Cl. butyricum* SS6 showed a switch from butyrate to acetate production in the presence of  $\text{NO}_3^-$ . Similar findings have been reported by Ishimoto *et al.* (1974) for *Cl. perfringens*. Similarly data presented in Table 2 show that  $\text{NO}_3^-$  can function as an alternative  $e^-$  acceptor to  $\text{SO}_4^{2-}$  in *D. desulfuricans* DT101. Spent media analyses showed the accumulation of  $\text{NH}_4^+$  indicating a dissimilatory role for  $\text{NO}_3^-$ . In conclusion these data show that nitrate dissimilation in strict anaerobes allows these bacteria to more efficiently derive energy from carbon substrates and thus may have important ecological implications in environments such as estuaries where the supply of fermentable substrates is limited.

The mixed population study whilst only a simple experimental system does nonetheless allow the investigation under laboratory conditions some of the environmental parameters governing the growth and interactions of different physiological groups of microorganisms which can then be related back to the field environment. The results presented in Figures 2 and 3 show that an obligate commensal relationship was established between *Cl. butyricum* SS6 and *D. desulfuricans* DT101 based upon the generation of carbon substrates. *Desulfovibrio desulfuricans* DT101 in turn was involved in a mutualistic interaction with *Chr. vinosum* PT121, the relationship between the two populations being mediated by the cyclical reduction and oxidation of  $\text{SO}_4^{2-}$  and  $\text{S}^{2-}$ . Under conditions of N-limitation (Fig. 2 and Fig. 3b) free  $\text{S}^{2-}$  levels reached inhibitory levels such that growth of the phototroph was severely impaired and the relationship between *D. desulfuricans* DT101 and *Chr. vinosum* PT121 became one of ammensalism. These data show that carbon flow cannot be considered in isolation and must take into account environmental parameters such as the availability of oxygen, nitrogen and sulphur.

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