Anaerobic metabolism in *Bacillus licheniformis* NCIB 6346

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The products of anaerobic metabolism of glucose and its derivatives sorbitol, gluconate and glucuronate by *Bacillus licheniformis* have been determined by proton NMR. Glucose was fermented through mixed-acid fermentation pathways to acetate, 2,3-butanediol, ethanol, formate, lactate, succinate and pyruvate. However, the bacterium was incapable of fermenting the three glucose derivatives. When *B. licheniformis* cells were incubated anaerobically with glucose in the presence of nitrate, the reduced products and formate did not appear and acetate was formed as the major metabolite. Growth and formation of acetate was also observed when *B. licheniformis* cells were incubated anaerobically with each of the three glucose derivatives, in the presence of nitrate. A formate-nitrate oxido-reductase system was induced under anaerobic conditions, with increased activities when nitrate was added to the anaerobic growth medium. However no activity was detected when cells were grown in the presence of molecular oxygen. Formate-nitrate oxido-reductase activity was absent in chlorate-resistant mutants isolated spontaneously or following Tn917 insertional mutagenesis. The spontaneous mutants fermented glucose in the presence of nitrate suggesting that they were incapable of nitrate respiration, due to a deficiency in one or more components of the formate-nitrate oxido-reductase system. Two insertional mutants exhibited elevated β -galactosidase activity when grown in the presence of nitrate.

Keywords: *Bacillus licheniformis*, anaerobic metabolism, proton NMR, chlorate resistance

INTRODUCTION

The genus *Bacillus* comprises Gram-positive, rod-shaped bacteria which differentiate into endospores under aerobic conditions. Physiologically these bacteria are heterogeneous, and at least six major groups have been recognized on the basis of phenotypic characteristics and rRNA sequences (reviewed by Priest, 1993). The largest group includes *B. subtilis* and relatives; bacteria which catabolize a range of carbohydrates and related compounds through the Embden–Meyerhof and pentose phosphate pathways. Most of these bacteria use nitrate as an electron acceptor in the absence of oxygen (*B. megaterium* and *B. pumilus* are notable exceptions) and a few, like *B. cereus*, *B. licheniformis* and *B. thuringiensis*, ferment sugars in the absence of exogenous electron acceptors. *B. subtilis* is the best studied of all Gram-positive bacteria, but its failure to ferment sugars has hindered studies of energy-generating metabolism and its regulation.

It is not known whether bacilli have regulatory mechanisms equivalent to those found in Gram-negative facultative anaerobes such as *Escherichia coli*. In this bacterium, the chosen pathway of energy-generating metabolism depends on the availability of oxygen or alternative electron acceptors and on the fermentability of the carbon source. Such adaptive responses are controlled by regulatory mechanisms which ensure that the most energetically favourable metabolic mode is adopted in a specific environment (Spiro & Guest, 1991). Like *E. coli*, *B. licheniformis* strains are capable of the mixed-acid fermentation of glucose, resulting in the formation of a variety of products including acetate, 2,3-butanediol, carbon dioxide, ethanol, formate, glycerol and lactate. However no hydrogen gas is generated (Raspoet *et al.*, 1991). They are also capable of using nitrate as a terminal electron acceptor although it is not known if other exogenous electron acceptors can be used. *B. licheniformis* produces many enzymes of industrial and commercial importance and is a popular host for the industrial

production of cloned gene products (Priest, 1977; Van Leen *et al.*, 1991). An understanding of the physiology of this micro-organism, especially its growth response to variations in culture redox potential, is therefore of importance in the optimization of industrial fermentations.

This study was undertaken to examine the end-products of the anaerobic metabolism of glucose and its derivatives by *B. licheniformis* in the presence and absence of nitrate, with the aid of proton NMR spectroscopy. This method has advantages over other analytical techniques for such purposes, including the simultaneous analysis of all the metabolic products and the elimination of inaccuracies due to sample manipulation and fractionation (Alam & Clark, 1989).

METHODS

Organism and growth conditions. *Bacillus licheniformis* NCIB 6346 was maintained on nutrient agar slopes with subculturing every 4 weeks. Minimal salts medium (BSS; Anagnostopoulous & Spizizen, 1961) was used throughout, and was sterilized by autoclaving at 121 °C for 15 min. Glucose, D-gluconic acid (sodium salt), sorbitol and KNO₃ were autoclaved separately. D-Glucuronic acid (sodium salt) and Casamino acids were filter-sterilized. The appropriate carbon sources were added to the cooled media at a concentration of 30 mM, and Casamino acids were added at a concentration of 0.1% (w/v). When required in the growth media, KNO₃ was added at a final concentration of 1% (w/v).

Inocula for aerobic and anaerobic growth were prepared by growing cultures in BSS media containing the appropriate carbon sources at 37 °C in an orbital incubator with shaking at 200 r.p.m. The overnight cultures were used to inoculate fresh 100 ml vols BSS medium containing the appropriate carbon sources. Aerobic conditions were provided by growing the cultures in 250 ml conical flasks containing metal springs at 200 r.p.m. to provide complete aeration. Incubation was continued at 37 °C as before, and the cultures were used for subsequent growth curve measurement and analysis. Anaerobic growth conditions were provided by static incubation of 100 ml cultures in stoppered bottles at 37 °C. Growth measurements were recorded as the OD₆₀₀.

Procedure for *in vivo* NMR spectroscopy. Normally, when the OD₆₀₀ of the culture reached 1.0, the cells were collected by centrifugation at 6000 g for 2 min at 5 °C. The cell pellets were suspended in BSS buffer (salts medium lacking the carbon source and Casamino acids) and washed twice with the same buffer. The cells were finally suspended in BSS buffer with the appropriate carbon source (30 mM) and Casamino acids (0.1%, w/v) to a cell density of approximately 5×10^8 cells ml⁻¹. A 3.0 ml sample of the suspension was placed in a quartz cuvette, which was then sealed with a cap and incubated statically at 37 °C for 4 h. Afterwards the suspension was centrifuged at 6000 g for 5 min and the supernatant was removed and used for proton NMR analysis (Alam & Clark, 1989). For analysis of aerobically grown cells, cultures were harvested at an OD₆₀₀ of 0.2, to ensure that oxygen limitation would not influence the results. These cells were then sparged with oxygen during subsequent incubation at 37 °C.

A 0.5 ml sample of supernatant was placed in a 5-mm-diameter NMR tube, to which 0.1 ml D₂O was added. The proton NMR spectrum of the supernatant was recorded with a Bruker WP-

200 SY spectrometer operating at 200 MHz. The following parameters were employed: pulse width was 90° (10 µs: delay time between data acquisitions was 5 s, during which the strong water peak was suppressed by irradiation); the number of acquisitions (scans) collected was typically 100–500. Following Fourier transformation the residual broad wings of the water signal were removed by a polynomial baseline fitting procedure. The field was locked onto the solvent D₂O, and internal H₂O was used as a reference peak (4.78 p.p.m.). The relative concentration of metabolites was calculated by the integration of peak heights. Proton chemical shifts are reported in p.p.m. downfield from 3-(trimethylsilyl)propanesulphonic acid, sodium salt.

Authentic fermentation product samples (acetate, 2,3-butanediol, ethanol, glycerol, lactate, pyruvate and succinate) were also used to assign the NMR signals of the metabolites. These were dissolved in BSS buffer at a concentration of 50 mg ml⁻¹.

Formate-nitrate oxido-reductase assay. Cultures were harvested at mid-exponential phase (OD₆₀₀ = 0.5) by centrifugation at 6000 g for 15 min at 4 °C. The pellets were washed in potassium phosphate buffer (50 mM, pH 7.4) and suspended in a suitable volume of buffer. Lysozyme and DNase I were added to the suspension at final concentrations of 0.5 mg ml⁻¹ and 10 µg ml⁻¹, respectively. The suspension was vortexed for 30 s and then incubated in a water-bath at 37 °C for 30–45 min. A mixture of formate and KNO₃ (2.5 ml of 500 mM of each) was added to 0.5 ml lysed cells. The mixture was vortexed and incubated at 37 °C for 50 min, during which time 0.5 ml samples were removed every 10 min. The nitrite content in each sample was determined by adding 1 ml each of Griess–Ilosvay's reagents no. 1 and no. 2 (BDH) and the colour was recorded at 540 nm. Activity was calculated as µmol nitrite produced min⁻¹ (mg protein)⁻¹, by reference to a standard curve constructed using different concentrations of nitrite.

β-Galactosidase assay. An appropriate dilution of lysozyme-treated cells was added to 5 ml potassium phosphate buffer (50 mM, pH 7.4), containing 1 mM ONPG. Reaction mixtures were incubated at 37 °C. The A₄₂₀ of the sample was measured to give an estimate of the amount of o-nitrophenol released. A unit is defined as an absorbance change of 0.01 min⁻¹ at pH 7.5 and 37 °C.

Estimation of protein. Protein concentrations were estimated using a Bio-Rad dye-binding assay kit according to the manufacturer's instructions. Bovine gamma globulin was used as the protein standard.

Isolation of mutants. (i) Chlorate-resistant mutants were obtained by spreading 0.1 ml of an appropriate dilution of a nutrient broth culture onto nutrient agar plates which were supplemented with 10 mM KClO₃, 1% (w/v) KNO₃ and 30 mM glucose. The plates were then incubated at 37 °C in an anaerobic cabinet (Forma Scientific) under an atmosphere of N₂:H₂:CO₂ (80:10:10, by vol.) for 24 h. The resulting colonies were picked and grown overnight at 37 °C in 5 ml nutrient broth cultures supplemented with 0.1% (w/v) KNO₃ and tested for the presence of nitrite by using Griess–Ilosvay reagents. Those cultures which did not produce nitrite were examined further. Samples of the culture (200 µl) were transferred into test-tubes containing 5 ml BSS medium supplemented with an appropriate carbon source, either with or without KNO₃. The sealed test-tubes containing the cultures were incubated statically overnight at 37 °C. The overnight cultures were then tested for the reduction of nitrate.

(ii) *B. licheniformis* NCIB 6346 cells with chromosomes carrying the integrated erythromycin resistance transposon Tn917 were derived from *B. licheniformis* carrying the plasmid vector pTV51 (Youngman, 1990; Wati *et al.*, 1990). The cells were spread on nutrient agar plates containing 10 mM KClO₃, 1% (w/v) KNO₃, 30 mM glucose and 20 µg X-Gal ml⁻¹, and supplemented with erythromycin and lincomycin at 1 µg ml⁻¹ and 25 µg ml⁻¹, respectively. The plates were incubated at 37 °C in an anaerobic cabinet for 48 h. Chlorate-resistant blue colonies were tested for their ability to reduce nitrate.

RESULTS

Proton NMR analysis of products of metabolism

Under fully aerated conditions, *B. licheniformis* NCIB 6346 grew well on a range of carbon sources including glucose, the reduced derivative sorbitol, and the oxidized derivatives gluconate and glucuronate. Anaerobically, this strain could ferment glucose, but not the more oxidized or reduced carbon sources, while in the presence of nitrate it grew on these three derivatives with increased doubling times relative to glucose (Table 1). Other potential electron acceptors, including nitrite, DMSO, trimethylamine-*N*-oxide and tetrathionate, did not allow anaerobic growth on sorbitol, gluconate or glucuronate, or stimulate the rate of growth on glucose (data not shown). In comparison, *B. subtilis* was totally unable to ferment all four carbon sources but did grow anaerobically with glucose, gluconate and sorbitol in the presence of nitrate (Table 1).

A series of proton NMR experiments was carried out for *B. licheniformis* grown under different conditions. During the standard 4 h incubation prior to NMR analysis of supernatants, there was little or no cell growth and no lysis as judged by invariant optical densities. When incubated with glucose under fermentation conditions, the metabolites identified (Fig. 1a) were, from low to high field: succinate, pyruvate, acetate, lactate, ethanol and 2,3-butanediol. In addition to these metabolites, formate was easily identified as a single resonance at δ 8.39, while the

cluster of signals occupying a region corresponding to δ 3.0–5.5 p.p.m. of the NMR spectrum relates mostly to the many hydrogen nuclei of residual glucose. The inability to ferment the other three carbon sources, sorbitol, gluconate and glucuronate, was reflected in the absence of metabolites in their respective NMR profiles (data not shown). The positive growth responses to all four carbon sources in the presence of nitrate correlated with the presence of acetate as the major metabolite released into the incubation medium (Fig. 1b). This suggests that the oxidation of the carbon source is coupled to reduction of the electron acceptor, nitrate, rather than pyruvate.

For comparative purposes, proton NMR was used to study aerobic respiration of glucose by *B. licheniformis*. An aerobically grown culture was harvested during exponential phase and a 10 ml suspension of cells was aerated by bubbling with oxygen for 4 h at 37 °C. The OD₆₀₀ of the suspension doubled during this period. The only significant product detected under these conditions was acetate, consistent with the anticipated transfer of electrons to oxygen via the respiratory chain (data not shown).

Similar NMR experiments were carried out with *B. subtilis*. However, the cells lysed during the standard 4 h incubation with glucose as the carbon source, except when nitrate was present in the incubation medium. The NMR spectrum of the resulting supernatant showed acetate as the major product, with small amounts of lactate and succinate also formed. Trace amounts of other unknown compounds were also detected (Fig. 2).

Nitrate reduction by cell-free extracts of *B. licheniformis*

Cells were grown in the presence of glucose, with and without nitrate, under aerobic and anaerobic conditions. At mid-exponential phase, cells were harvested and used

Table 1. Growth of *B. licheniformis* and *B. subtilis* on glucose and glucose derivatives

Yield was measured as maximal growth during incubation for 10 h. NG, No growth.

Organism and growth conditions	Carbon source (30 mM)							
	Glucose		Sorbitol		Gluconate		Glucuronate	
	Doubling time (h)	Yield (OD ₆₀₀)	Doubling time (h)	Yield (OD ₆₀₀)	Doubling time (h)	Yield (OD ₆₀₀)	Doubling time (h)	Yield (OD ₆₀₀)
<i>B. licheniformis</i>								
Aerobic	0.67	1.91	0.67	2.0	0.5	2.0	2.0	1.4
Anaerobic	1.80	1.05	NG	NG	NG	NG	NG	NG
Anaerobic + nitrate	1.23	1.01	3.6	0.98	2.0	1.04	5.0	0.5
<i>B. subtilis</i>								
Aerobic	0.83	1.8	0.92	2.1	0.97	2.0	3.1	1.3
Anaerobic	NG	NG	NG	NG	NG	NG	NG	NG
Anaerobic + nitrate	3.65	0.6	4.0	0.45	4.0	0.38	NG	NG

Fig. 2. The 200 MHz ^1H NMR spectrum of a supernatant obtained following incubation of *B. subtilis* cell suspension in BSS medium with glucose as sole carbon source, in the presence of nitrate, at 37 °C. The cell suspension was obtained from an anaerobically grown BSS culture, in the presence of nitrate. Assignments as in Fig. 1.

Table 2. Production of nitrite from nitrate by cell extracts of *B. licheniformis*

Cell extracts were incubated with nitrate and an electron donor as indicated. Results are presented as the mean of duplicate determinations. ND, Not determined.

Growth conditions	Activity with electron donor [μmol nitrite produced min^{-1} (mg protein $^{-1}$)]			
	Formate	DL-Lactate	Glycerol	None
Anaerobic	0.75	0.05	0.07	0.05
Anaerobic (+ nitrate)	1.87	0.12	0.20	0.11
Aerobic	0	ND	ND	ND
Aerobic (+ nitrate)	0	ND	ND	ND

for formate-nitrate oxido-reductase assays (Table 2). Only formate was able to provide electrons for nitrate reduction; lactate and glycerol generated low levels of

nitrite which were the same as the control without an electron donor, and presumably due to the presence of endogenous electron donors. Nitrate reduction was

Fig. 1. The 200 MHz ^1H NMR spectra of supernatants obtained following incubation of *B. licheniformis* cell suspensions in BSS medium with glucose as sole carbon source, in the absence (a) and presence (b) of nitrate, at 37 °C. Cell suspensions were obtained from anaerobically grown BSS cultures, with glucose as carbon source. Assignments: F, formate; S, succinate; P, pyruvate; A, acetate; L, lactate; E, ethanol; 2,3-BD, 2,3-butanediol (see text).

Fig. 3. The 200 MHz ^1H NMR spectrum of a supernatant obtained following incubation of a chlorate-resistant mutant of *B. licheniformis* in BSS medium with glucose as sole carbon source, in the presence of nitrate, at 37 °C. Cell suspensions were obtained from anaerobically grown BSS cultures. A similar NMR scan was also obtained for cell suspensions incubated in the absence of nitrate (data not presented). Assignments as in Fig. 1.

induced about 2.5-fold when *B. licheniformis* cells were grown anaerobically in the presence of glucose and nitrate compared with glucose alone (Table 2). Absence of nitrite production by aerobically grown cells suggested a repressive effect of oxygen on the enzymes involved in the anaerobic respiration of nitrate.

Isolation of mutants deficient in nitrate respiration

Since nitrate reductase reduces chlorate to the highly toxic chlorite, mutants lacking the enzyme activity can be selected on the basis of their resistance to chlorate (Glaser & De Moss, 1972; Rajagopalan & Johnson, 1992). Of 37 spontaneous chlorate-resistant mutants, 11 were unable to reduce nitrate to nitrite. Of these, four were selected on the basis of their inability to grow on gluconate, glucuronate and sorbitol in the presence of nitrate. The other seven grew with glucose, sorbitol or gluconate (but not glucuronate) in the presence of nitrate. The total absence of formate-nitrate oxido-reductase activity in the four mutants when grown anaerobically with glucose and nitrate, together with their ability to use only glucose when nitrate was absent from the medium, indicated that

these mutants were likely to be deficient in one or more components involved in the reaction pathway which couples formate oxidation to the reduction of nitrate. The products of glucose metabolism, under anaerobic conditions, of two of these mutants were examined by proton NMR. Unlike the wild-type strain, nitrate did not prevent formation of the typical fermentation products succinate, pyruvate, lactate, ethanol, 2,3-butanediol and formate (Fig. 3).

Eighteen Tn917-generated, chlorate-resistant mutants that produced dark-blue colonies on glucose-nitrate agar plates containing X-Gal, when grown anaerobically, were examined for nitrate reduction. Nine of these mutants were deficient in nitrite production when cultured in nitrate broth for 24 h. Cell extracts from these nine mutants grown anaerobically with glucose and nitrate failed to reduce nitrate in the presence of formate. Furthermore, loss of nitrate reduction by cells permeabilized with toluene (Tangney *et al.*, 1993) in the presence of the artificial electron donor benzyl viologen indicated a lack of the nitrate reductase itself. β -Galactosidase activity was also measured in these mutants

Table 3. β -Galactosidase specific activities of Tn917-generated chlorate-resistant mutants grown anaerobically with glucose in the absence and presence of nitrate

Results are presented as the mean of triplicate determinations with the indicated standard deviations.

Mutant	β -Galactosidase activity [U (mg protein) ⁻¹]	
	- Nitrate	+ Nitrate
Chl III	5.79 $\pm$ 0.28	21.9 $\pm$ 0.54
Chl IV	26.50 $\pm$ 1.30	36.2 $\pm$ 0.75
Chl V	18.90 $\pm$ 0.47	12.9 $\pm$ 1.40

(Table 3). Mutants Chl III and Chl IV showed increased β -galactosidase activity when grown in the presence of nitrate, while the other seven mutants, represented by Chl V, showed no induction.

DISCUSSION

E. coli can ferment glucose and its derivatives, sorbitol, gluconate and glucuronate (Alam & Clark, 1989). Substrates more reduced than glucose, such as sorbitol, yield more of the highly reduced fermentation product ethanol, whereas more oxidized substrates, such as glucuronate, yield more of the less reduced fermentation product acetate. In this manner *E. coli* is capable of maintaining redox balance. *B. licheniformis*, on the other hand, was incapable of fermenting sorbitol, gluconate and glucuronate although it could grow on these substrates aerobically, suggesting that it is unable to alter radically the flow of electrons through its fermentation pathways in order to balance substrates with products.

The dominance of acetate as a metabolic end-product, especially when nitrate was present in the medium, suggests that metabolism is regulated such that energy generation is optimal, subject to the constraint that the cells do not produce more reducing equivalents than can be oxidized by the electron transport system. Such an effect has already been observed in *E. coli*, in which anaerobic nitrate respiration leads to the formation of nitrite, which is oxidized by the principal nitrite reductase. This enzyme serves both to convert the substrate to a nitrogen source (ammonia) and to remove excess reducing equivalents during anaerobic growth (Cole, 1982). The result is excretion of surplus ammonia and release of large amounts of acetate into the medium (Cole, 1978; Cole & Brown, 1980). We detected low levels of nitrite reductase in *B. licheniformis* NCIB 6346. Other strains of *B. licheniformis*, most of which produce molecular nitrogen, also have a nitrite reductase enzyme when grown in complex media containing nitrate (Pichinoty *et al.*, 1977).

The disappearance of formate during incubations with

nitrate and glucose was likely to be the result of an active respiratory formate dehydrogenase which, together with a nitrate reductase, coupled formate oxidation to the reduction of nitrate (Fig. 1b). Formate dehydrogenase has not been reported for *B. licheniformis*, although nitrate reductase from strain S244 has been characterized. The enzyme consists of two subunits, having apparent M_r values of 150 000 (α -subunit) and 57 000 (β -subunit), and contains a non-haem iron/sulphur complex together with a molybdenum cofactor (Mo). Both subunits are located on the cytoplasmic side of the bacterial membrane (Van 'Triet *et al.*, 1979). The demonstration of different phenotypes among chlorate-resistant mutants of *B. licheniformis* NCIB 6346, some of which retained the ability to reduce nitrate to nitrite, indicates that this bacterium must have several genes responsible for chlorate resistance (Schulp & Stouthamer, 1972). Thus in addition to nitrate reductase mutants, some of those isolated may be impaired in synthesis and processing of the Mo cofactor essential for activity of molybdoenzymes such as nitrate reductase or the respiratory formate dehydrogenase (Johann & Hinton, 1987; Garzon *et al.*, 1992).

The respiratory nitrate reductase of *E. coli* has been identified as a four-gene operon designated *narGHJI* (Blasco *et al.*, 1989). *lacZ* fusions to the *nar* operon promoter show a two- to sevenfold induction by anaerobiosis and a further 11–30-fold induction in the presence of nitrate (Stewart, 1982). A similar situation is envisaged in *B. licheniformis* NCIB 6346, where formate-nitrate oxido-reductase activity was derepressed by anaerobiosis and increased further when nitrate was added to the anaerobic growth medium (Table 2). The presence of such a regulatory system is also suggested by induction of β -galactosidase activity in Tn917-generated chlorate-resistant mutants when grown anaerobically in the presence of nitrate (Table 3). Similarly, the nitrate reductase of *B. stearothermophilus* has been found to be induced by nitrate and repressed and inactivated by molecular oxygen (Downey & Kiskiss, 1969; Downey *et al.*, 1969).

The absence of the usual fermentation products in the *B. licheniformis* NMR profiles in the presence of nitrate is consistent with repressive effects of nitrate on enzymes of fermentation, but could also be explained by competition between nitrate and acetyl-CoA for reducing equivalents generated by sugar metabolism. In *E. coli*, pyruvate formate-lyase (PFL), the central enzyme of anaerobic glucose metabolism (Sawers & Bock, 1988), the formate hydrogen lyase complex, which converts the formate formed as a result of the breakdown of pyruvate by the PFL to CO₂ and H₂ (Choe & Reznikoff, 1991), and the fermentative alcohol dehydrogenase involved in ethanol generation are repressed by nitrate (Clark, 1989). It would seem that *B. licheniformis*, like *E. coli*, has developed adaptive responses that are controlled by hierarchical regulatory mechanisms which ensure that the most energetically favourable metabolic mode is adopted in a specific environment (Gunsalus, 1992). Thus there is a decreasing preference for oxygen, nitrate and endogenously generated electron acceptors.

REFERENCES

- Alam, K. Y. & Clark, D. P. (1989). Anaerobic fermentation balance of *Escherichia coli* as observed by in vivo NMR spectroscopy. *J Bacteriol* **171**, 6213–6217.
- Anagnostopoulos, C. & Spizizen, J. (1961). Requirements for transformation in *Bacillus subtilis*. *J Bacteriol* **81**, 741–746.
- Blasco, F., Iobbi, C., Giordano, G., Chippaux, M. & Bonnefy, V. (1989). Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the alpha and beta subunits in iron binding and electron transfer. *Mol & Gen Genet* **218**, 249–256.
- Choe, M. & Reznikoff, W. S. (1991). Anaerobically expressed *Escherichia coli* genes identified by operon fusion techniques. *J Bacteriol* **173**, 6139–6146.
- Clark, D. P. (1989). The fermentation pathways of *Escherichia coli*. *FEMS Microbiol Rev* **63**, 223–234.
- Cole, J. A. (1978). The rapid accumulation of large quantities of ammonia during nitrite reduction by *Escherichia coli*. *FEMS Microbiol Lett* **4**, 327–329.
- Cole, J. A. (1982). Independent pathways for the anaerobic reduction of nitrite to ammonia in *Escherichia coli*. *Biochem Soc Trans* **10**, 476–478.
- Cole, J. A. & Brown, C. M. (1980). Nitrite reduction to ammonia by fermentative bacteria: a short circuit in the biological nitrogen cycle. *FEMS Microbiol Lett* **7**, 65–72.
- Downey, R. J. & Kiskiss, D. F. (1969). Oxygen and nitrate induced modification of the electron transfer system of *Bacillus stearothermophilus*. *Microbios* **2**, 145–153.
- Downey, R. J., Kiskiss, D. F. & Nuner, J. H. (1969). Influence of oxygen on development of nitrate respiration in *Bacillus stearothermophilus*. *J Bacteriol* **98**, 1056–1062.
- Garzon, A., Li, J., Flores, A., Casadesus, J. & Stewart, V. (1992). Molybdenum cofactor (chlorate resistant) mutants of *Klebsiella pneumoniae* M5a1 can use hypoxanthine as the sole nitrogen source. *J Bacteriol* **174**, 6298–6302.
- Glaser, J. H. & De Moss, J. A. (1972). Comparison of nitrate reductase mutants of *Escherichia coli* selected by alternative procedures. *Mol & Gen Genet* **116**, 1–10.
- Gunsalus, R. P. (1992). Control of electron flow in *Escherichia coli*: coordinated transcription of respiratory pathway genes. *J Bacteriol* **174**, 7069–7074.
- Johann, S. & Hinton, S. M. (1987). Cloning and nucleotide sequence of the *chdD* locus. *J Bacteriol* **169**, 1911–1916.
- Pichinoty, F., Garcia, J.-L. & Durand, C. J. (1977). La de'nitrification chez *Bacillus licheniformis*. *Can J Microbiol* **24**, 45–49.
- Priest, F. G. (1977). Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol Rev* **41**, 711–753.
- Priest, F. G. (1993). Systematics and ecology of *Bacillus*. In *Bacillus subtilis and Other Gram-positive Bacteria*, pp. 3–16. Edited by A. L. Sonenshein, J. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Rajagopalan, K. V. & Johnson, J. L. (1992). The pterin molybdenum cofactors. *J Biol Chem* **267**, 10199–10202.
- Raspoet, D., Pot, B., De Deyn, D., Devos, P., Kersters, K. & Deley, J. (1991). Differentiation between 2,3-butanediol producing *Bacillus licheniformis* and *B. polymyxa* strains by fermentation product profiles and whole-cell protein electrophoretic patterns. *Syst Appl Microbiol* **14**, 1–7.
- Sawers, G. & Bock, A. (1988). Anaerobic regulation of pyruvate formate-lyase from *Escherichia coli* K-12. *J Bacteriol* **170**, 5330–5336.
- Schulp, J. A. & Stouthamer, A. H. (1972). Isolation and characterization of mutants resistant against chlorate of *Bacillus licheniformis*. *J Gen Microbiol* **73**, 95–112.
- Spiro, S. & Guest, J. R. (1991). Adaptive responses to oxygen limitation in *Escherichia coli*. *Trends Biochem Sci* **16**, 310–314.
- Stewart, V. (1982). Requirement of *fnr* and *narL* functions for nitrate reductase expression in *Escherichia coli* K-12. *J Bacteriol* **151**, 1320–1325.
- Tangney, M., Priest, F. G. & Mitchell, W. J. (1993). Two glucose transport systems in *Bacillus licheniformis*. *J Bacteriol* **175**, 2137–2142.
- Van Leen, R. W., Bakhuis, J. G., Van Beckhoven, R. F. W. C., Burger, H., Dorssers, L. C. J., Hommes, R. W. J., Lemson, P. J., Noordam, B., Persoon, N. L. M. & Wagemaker, G. (1991). Production of human interleukin-3 using industrial microorganisms. *Bio/Technology* **9**, 47–52.
- Van 'Triet, J., Wientjes, F. B., Van Doorn, J. & Planta, R. J. (1979). Purification and characterization of the respiratory nitrate reductase of *Bacillus licheniformis*. *Biochim Biophys Acta* **576**, 347–360.
- Wati, M. R., Priest, F. G. & Mitchell, W. J. (1990). Mutagenesis using Tn917 in *Bacillus licheniformis*. *FEMS Microbiol Lett* **71**, 211–214.
- Youngman, P. (1990). Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus*. In *Molecular Biological Methods for Bacillus*, pp. 221–226. Edited by C. R. Harwood & S. M. Cutting. Chichester: Wiley.

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