Induction of Methanethiol Production by *Brevibacterium linens* CNRZ 918

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A non-inducing medium (NID) was defined for studying the induction of methanethiol production by *Brevibacterium linens* CNRZ 918. The lowest L-methionine concentration capable of inducing maximal methanethiol production by the cells was 7 mM. The peptides L-Ala-L-Met and L-Met-L-Ala induced greater methanethiol production than free L-methionine. D-Methionine, L-cysteine, S-methyl-L-cysteine and L-ethionine were poor inducers. Culture temperature affected the duration of induction. An Na⁺ concentration of 1 M in the culture medium led to maximal methanethiol production capacity of both cells and cell extracts. L-Methionine and L-ethionine were the best substrates for the crude soluble cells extract (with release of methanethiol and ethanethiol respectively). Neither the derivatives of L-methionine that acted as inducers, nor D-methionine, were substrates for demethiolation. Demethiolation activity of the crude extract was thermolabile, not stimulated by Na⁺ and strongly inhibited by Zn²⁺, Mn²⁺ and Cu²⁺. The shortest generation time obtained for *B. linens* CNRZ 918 in NID medium + L-methionine was 4 h at 26 °C. Only coccoid forms were present when the culture temperature was 30 °C. The presence of L-methionine in the medium favoured their appearance. The strain grew best in the presence of 1% NaCl but tolerated concentrations up to 15%. The induction of methanethiol production was due to the induction of L-methionine-γ-demethylase. The level of induction was probably related to the intracellular concentration of L-methionine. The transport system of L-methionine by *B. linens* CNRZ 918 was constitutive and Na⁺ dependent.

INTRODUCTION

Some cheeses, for instance the French varieties Pont l'Évêque, Munster, Livarot, Vacherin and Camembert, exhibit characteristic flavours (see Ferchichi *et al.*, 1985) attributable to sulphur compounds which arise from the condensation of precursors such as CH₃SH and H₂S (Law, 1984). The thiols, in particular methanethiol, are produced by the predominant surface rind flora of these orange-coloured smear-ripened cheeses, which is mainly composed of bacteria related to *Brevibacterium linens* (Richard, 1984).

Methanethiol production from methionine in various organisms was reviewed by Kadota & Ishida (1972). Since that time, studies have been done with *Pseudomonas* spp. (Ito *et al.*, 1976; Laakso *et al.*, 1976; Tanaka *et al.*, 1977; Laakso & Laukkanen, 1982), *Clostridium sporogenes* (Kreiss & Hession, 1973), coryneform bacteria (Sharpe *et al.*, 1977; Ferchichi *et al.*, 1985) and rumen bacteria (Merrick & Salsbury, 1975). Several demethylases have been purified, sometimes partially, e.g. from *Pseudomonas* spp. (Tanaka *et al.*, 1976; Ito *et al.*, 1976; Tanaka *et al.*, 1977, 1985; Esaki *et al.*, 1977; Nakayama *et al.*, 1984b), *Aeromonas* sp. (Nakayama *et al.*, 1984a) and *Aspergillus* sp. (Ruiz-Herrera & Starkey, 1969). Only the enzyme of *C. sporogenes* (EC 4.4.1.11) has been fully characterized (Kreiss & Hession, 1973). The inducible character of

Abbreviations: DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; NID, non-inducing medium (for demethiolation); sMTPC, specific methanethiol-producing capacity.

methanethiol production has been reported by relatively few authors, with no attempt to characterize this induction. The present work describes the induction of methanethiol production by *B. linens* CNRZ 918 and the factors which can affect production.

METHODS

Strain and culture media. *Brevibacterium linens* CNRZ 918 was the strain used in our previous study (Ferchichi *et al.*, 1985). The basic non-inducing medium used (NID) had the following composition (g l^{-1}): sodium DL-lactate (Sigma) 5.0, yeast extract (Difco) 1.0, peptone (Bio-case low salt, Mérieux, Marcy l'Etoile, France) 1.0, L-alanine (Sigma) 5.0, K_2HPO_4 1.7, KH_2PO_4 0.24, CaCl_2 0.025, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, NaCl 10. This basic medium was supplemented with L-methionine (Sigma) at 1.0 g l^{-1} (NID + L-methionine) or with analogues. After inoculation, the NID (unsupplemented) medium contained 0.3 mM L-methionine from the carryover. The final pH of the medium varied from 7.5 to 7.3 and decreased by 0.2 unit after autoclaving for 20 min at 120°C . The final Na^+ concentration in NID + L-methionine was 5 g l^{-1} .

Cultures. One ml of the culture, prepared as previously described (Ferchichi *et al.*, 1985), was used to inoculate the preculture (100 ml of NID + L-methionine in a 500 ml Erlenmeyer flask), which was grown at 26°C with shaking for 70 h. This preculture was then used to inoculate (1–2%) the experimental culture. Experimental cultures were similarly grown in 1000 ml capacity Erlenmeyer flasks filled to 20% capacity and shaken at $100 \text{ cycles min}^{-1}$ in a water-bath reciprocal shaker.

Amino acid analysis in culture medium. Samples (3 ml) of culture medium were removed at the times indicated. After centrifugation (12500 g, 20 min) the supernatant was aseptically filtered (200 nm pore size) and then diluted in 100 mM-citrate buffer pH 2.2 containing 120 mM- Na^+ . This solution was analysed with a Biotronick LC5000 automatic amino acid analyser.

Preparation of crude extracts. After 24 h growth in NID + L-methionine, cells were harvested by centrifuging at 12500 g for 10 min at 4°C . The pellet was suspended ($\text{OD}_{650} = 50$) in 100 mM-Tris/HCl, pH 8.5, containing 0.5 mM-pyridoxal 5'-phosphate (Sigma), 0.05 mM-2-oxobutyrate (Sigma) and 1 mM- CaCl_2 (Prolabo) as stabilizing agents. The cells were broken by ultrasonic treatment (Ultrasonic NSU157 at 60% of full power, 600 mA) of 5 ml of the suspension for five 1 min periods, each followed by a resting period of 2 min in an ice-water bath. The crude extract was centrifuged at 12500 g for 10 min at 4°C and the supernatant ($1.5\text{--}2.0 \text{ g protein l}^{-1}$) constituted the crude soluble extract. To study the effect of metal ions, the extract was dialysed against 100 mM-Tris/HCl, pH 8.5, containing 0.5 mM-pyridoxal 5'-phosphate just before use. The protein concentration was determined by the method of Lowry with crystalline bovine serum albumin as reference.

Transport assays. Exponential phase cells (rod cells) and cells in the stationary growth phase (coccoid cells) were harvested by centrifugation at 12500 g for 20 min at 4°C . The cells were washed twice with 50 mM-Tris/HCl, pH 8, and resuspended in the same buffer to give a final concentration of $1.2 \text{ mg dry weight ml}^{-1}$. After preincubation for 10 min at 30°C with shaking ($120 \text{ strokes min}^{-1}$), the reaction was started by the addition of L-[3,4- ^{14}C] methionine (final concn $100 \mu\text{M}$) (purchased from the Commissariat à l'Energie Atomique, Saclay, France; final specific radioactivity in the test tube $27.4 \text{ MBq mol}^{-1}$). After 15 s, 45 s and 90 s, 200 μl of the reaction mixture was removed and rapidly filtered through a membrane filter (450 nm pore size, HA, Millipore). The residues on the filter were washed twice with 4 ml of the same buffer at 30°C . The filters were then dried under an infrared lamp and placed in scintillation vials containing 5 ml Ready-solv Hp/b (Beckman). Radioactivity was determined with a liquid scintillation spectrometer (Beckman LS90000) with a counting efficiency better than 95% for ^{14}C . The initial rate of transport (v_i , expressed in nkat per g cell dry weight) was estimated from the slope between 0 min and 1 min.

Methanethiol production capacity (MTPC) of harvested resting cells and crude extracts. The MTPC of harvested resting cells was determined as previously described (Ferchichi *et al.*, 1985) using DTNB (5,5'-dithio-bis-2-nitrobenzoic acid), which reacts with the thiols formed by the action of the L-methionine- γ -demethylase (Laakso & Nurmikko, 1976; Law & Sharpe, 1978). The substrates tested were from Sigma. The calibration curves were linear with methanethiol (up to $250 \mu\text{M}$) and ethanethiol (up to $60 \mu\text{M}$). An A_{412} value of 1.0 in a 1 cm light path cuvette corresponded to $220 \mu\text{M}$ -methanethiol or $46 \mu\text{M}$ -ethanethiol.

The specific methanethiol producing capacity (sMTPC) of the cells were defined as nmol methanethiol produced $\text{s}^{-1} (\text{g dry wt of cells})^{-1}$ [nkat ($\text{g dry wt})^{-1}$ or $\text{nmol s}^{-1} \text{g}^{-1}$] from the substrate under the conditions of the assay, and the sMTPC of the extracts as mkat ($\text{kg protein})^{-1}$.

The method of Laakso & Nurmikko (1976) was adapted for the extracts. The reaction medium (total volume 0.6 ml in a semi-micro cuvette) contained (final concn) 100 mM-Tris/HCl, pH 8.5, 0.2 mM-DTNB (dissolved in ethanol), substrate (variable concn of L-methionine or analogues), $33 \mu\text{M}$ -pyridoxal phosphate, $3.35 \mu\text{M}$ -2-oxobutyrate and $67 \mu\text{M}$ - CaCl_2 , both the latter being derived from the crude extract. After the addition of 40 μl extract (containing $\sim 5 \mu\text{g protein}$) methanethiol production at 30°C was followed at 412 nm with a Beckman 25 spectrophotometer. The rates were recorded for 2 min and the activity was calculated from the slope (70 pkat equivalent to 0.1 absorbance unit with cysteine as a reference).

Fig. 1

Fig. 1. sMTPC of harvested cells (●, ○), L-methionine concentration in the medium (▲, △) and OD₆₅₀ of *B. linens* CNRZ 918 grown at 26 °C in NID (open symbols) and NID + L-methionine (solid symbols).

Fig. 2

Fig. 2. Effect of L-methionine concentration in NID medium on the sMTPC of *B. linens* CNRZ 918 grown at 26 °C.

Thermal inactivation. Crude extract (0.5 ml) was placed in prewarmed glass test tubes (6 × 50 mm) at 25 °C, 30 °C, 35 °C and 45 °C. At different times, samples (40 µl) were removed, immediately cooled to 0 °C and tested for residual sMTPC.

Reproducibility. All the results presented are the mean of three or more replicate assays. The observed variations were consistently lower than 10%.

RESULTS

Search for a non-inducing medium. Methanethiol production by *B. linens* grown in a complex medium tripled when the medium was supplemented with 0.1% L-methionine (Ferchichi *et al.*, 1985). It was thus highly probable that an induction phenomenon occurred. After investigating the growth and methanethiol production of the strain in various maximally defined media, we chose NID (see Methods) as a non-inducing medium. Although this medium was simpler than the culture medium used by Ferchichi *et al.* (1985), it was nevertheless necessary to add a low concentration of yeast extract and peptone (each to 0.1%) to maintain an acceptable growth rate.

The sMTPC of cells grown in NID medium was very low (Fig. 1). The addition of L-methionine to this medium (NID + L-methionine) increased the sMTPC 12-fold at the maximum obtained after 24 h growth. The transport of L-methionine by the cells grown on NID + L-methionine or on NID, as rod or coccoid forms, was the same (initial rate 9 ± 2 nkat g⁻¹). When sMTPC was maximum, 88% of the L-methionine remained in the NID + L-methionine medium. After this, its concentration decreased more rapidly, as it was a growth factor, resulting in twice as much growth as that obtained with NID medium (Fig. 1). The presence of L-methionine caused an earlier appearance of coccoid cells, which constituted the major cell form when the OD₆₅₀ reached 3.5 in NID + L-methionine, as opposed to 5 in NID. Different forms, all able to divide, were observed by electron microscopy in an 18 h NID + L-methionine culture, as previously observed in the complex BM medium (Ferchichi *et al.*, 1985). Compared to this complex BM medium, the maximum sMTPC of resting cells grown on NID + L-methionine was higher and earlier (24 h as opposed to 60 h) and growth did not exhibit a latency period.

Optimal inducing concentration of L-methionine. The addition of L-methionine up to about 7 mM to NID medium increased the sMTPC of the cells; higher concentrations did not increase the sMTPC further (Fig. 2). A final L-methionine concentration of 7 mM (6.7 mM or 0.1% added to

Table 1. *Specificity of induction of methanethiol production by B. linens CNRZ 918*

Rod cells were harvested after 28 h growth at 26 °C in NID medium + 6.7 mM inducer (except for the control) and their sMTPC from L-methionine was determined as described in Methods. The results are expressed relative to the value for cells from NID + L-methionine (100% or 75 nkat g⁻¹).

Inducer added (6.7 mM) to NID medium	sMTPC (%)	Inducer added (6.7 mM) to NID medium	sMTPC (%)
Control (no supplementation)	6	L-Met-L-Ala	153
L-Methionine	100	L-Met-L-Gly	92
D-Methionine	25	L-Met-L-Phe	81
L-Ethionine	17	L-Met-L-Asp	65
L-Cysteine	11	L-Met-L-Met-L-Met	77
S-Methyl-L-cysteine	16	L-Met-L-Arg-L-Phe-L-Ala	54
S-Ethyl-L-cysteine	4	L-Ala-L-Met	150
Seleno-DL-methionine	NG	L-Ser-L-Met	99
DL-Methionine sulphoxide	8	L-Pro-L-Met	84
2-Aminobutyric acid	5	Gly-L-Met-Gly	100
2-Oxobutyric acid	5	L-Leu-L-Trp-L-Met-L-Arg	82
2-Oxo-4-methiolbutyric acid	4	N-Formyl-L-Met-L-Ala	77
L-Methioninamide	108	L-Trp-L-Met-L-Asp-L-Phenylalanyl- amide	28
N-Formyl-DL-methionine	90		
N-Benzyl-L-methionine	58		
Acetyl-L-methioninamide	8		

NG, No growth.

the medium during preparation) was chosen for subsequent studies in NID + L-methionine (see Methods). Regardless of whether L-methionine (6.7 mM) was added initially to the culture medium or after 18 h incubation, the sMTPC obtained after about 24 h of incubation, i.e. 6 h after the delayed addition, was identical.

Specificity of induction. Various compounds related to methionine (structural analogues, methionine derivatives, methionine-containing peptides or potential degradation products) were tested for their inducing ability (Table 1). The dipeptides L-Ala-L-Met and L-Met-L-Ala gave a higher sMTPC in resting cells than did L-methionine alone. Variable induction was observed with other peptides or pseudopeptides. D-Methionine, L-cysteine, S-methyl-L-cysteine and L-ethionine were poor inducers although they were used as the sole carbon and nitrogen source for growth (M. Ferchichi, D. Hemme & J. E. Baudoin, unpublished results). Neither degradation products nor S-ethyl-L-cysteine were inducers. Selenomethionine (S replaced by Se) inhibited growth.

The sMTPC of crude extracts and the transport of L-methionine by the cells obtained from cultures on NID + one peptide (L-Ala-L-Met or L-Met-L-Ala or L-methioninamide or N-formyl-L-methionine) was the same as that of cells obtained from NID or NID + L-methionine.

Specificity of demethiolation. Demethiolating activity was present only in the soluble fraction of cell extracts. Stabilization of the activity was obtained with 500 µM-pyridoxal phosphate (K_m value); maximal activity required 5 mM of this cofactor. This may explain the 40% loss of activity observed during dialysis. Only L-methionine (K_m 5 mM), DL-methionine sulphoxide, S-methyl-L-cysteine and L-ethionine (K_m 5 mM) were substrates (Table 2). In contrast to non-proliferating cells (Ferchichi *et al.*, 1985), none of the peptides or other methionine derivatives were substrates for the soluble crude extract.

Influence of growth temperature on sMTPC of cells. The maximal sMTPC for cells grown in NID + L-methionine varied from 60 nkat g⁻¹ for cells grown at 20 °C to 75 nkat g⁻¹ for cells grown at 26 °C. The sMTPC values decreased less rapidly on continued incubation at 20 °C than at 26 °C or 30 °C (50% of maximum reached 32 h after the maximum at 20 °C, compared to 21 h at 26 °C or 30 °C). The optimal growth temperature in NID + L-methionine was 26 °C. The shortest latency time, the shortest generation time (4 h ± 15 min, mean of 15 cultures) and the

Table 2. *sMTPC of crude extract of B. linens CNRZ 918 with respect to different substrates*

Activity is expressed relative to that against L-methionine (100% or 12 mkat kg⁻¹). For comparison, values obtained with non-proliferating cells (Ferchichi *et al.*, 1985) are given in parentheses.

Substrate (24 mM)	sMTPC (%)
L-Methioninamide	0 (472)
L-Ala-L-Met	0 (245)
L-Met-L-Ala	0 (245)
L-Ethionine*	123 (120)
L-Methionine (control)	100 (100)
DL-Methionine	77 (60)
S-Methyl-L-cysteine	21 (40)
2-Oxo-4-methiolbutyric acid	5 (38)
N-Formyl-DL-methionine	0 (30)
DL-Methionine-S-methylsulphonium chloride	8 (17)
DL-Methionine-2-hydroxy analogue	0 (11)
DL-Methionine sulfoxide	21 (9)
D-Methionine	0 (0)
DL-Methioninol	5 (0)

* In this case, ethanethiol was produced and the production capacity was estimated by comparison with a calibration graph obtained with ethanethiol instead of methanethiol.

Table 3. *Effect of metal ions on methanethiol production from L-methionine by non-proliferating cells and crude extract of B. linens CNRZ 918*

The results are expressed relative to demethiolating activity without added metal ions for dialysed crude extract (100% or 7.2 mkat kg⁻¹) and for non-proliferating cells (100% or 75 nkat g⁻¹). Ions (all added as chloride salts) were 1 mM for extract and 10 mM for cells. Cells were harvested after 24 h growth in NID medium + L-methionine.

Ion	sMTPC of crude extract (%)	sMTPC of cells (%)
Control	100	100
Na ⁺	92	200
K ⁺	102	118
NH ₄ ⁺	98	79
Ca ²⁺	102	63
Cu ²⁺	54	ND
Mn ²⁺	10	ND
Mg ²⁺	110	107
Zn ²⁺	0	ND
Fe ²⁺	104	ND

ND, Not determined.

highest final cell yield were observed at this temperature. At 30 °C, only cocci were present and growth reached only 5 OD₆₅₀ units.

Effect of heating on the sMTPC of crude extracts. The demethiolase activity of crude extracts was reduced by 50% after heating at 30 °C, 35 °C and 45 °C for 41 min, 9 min and 1 min, respectively. The apparent activation energy, calculated from an Arrhenius plot, was 251 kJ mol⁻¹.

Effect of metal ions. Only Na⁺ and K⁺ (to a lesser extent) stimulated methanethiol production by whole cells (Table 3). No stimulation of activity was observed with a dialysed crude extract; Zn²⁺ and Mn²⁺ strongly inhibited the activity and Cu²⁺ reduced it by 50%.

The sMTPC of both cells and crude extracts was maximal when the Na⁺ concentration in the culture medium was 1 M (Fig. 3); this maximal activity was also observed in crude extracts with 0.5 M-Na⁺ in the medium. The rate of transport of L-methionine by non-proliferating cells increased 12-fold when Na⁺ was added to the suspension buffer at a concentration of 10 mM.

Fig. 3. Effect of Na⁺ concentration in NID + L-methionine on the sMTPC of the cells (●), sMTPC of crude extract (▲) and the maximal OD₆₅₀ (○) of *B. linens* CNRZ 918 at 26 °C.

The growth increase was maximal with 0.17 M-Na⁺ in the culture medium, explaining the choice of this concentration for NID medium (Fig. 3). Above 1 M-Na⁺, growth inhibition was marked but not total, even at 2.5 M-Na⁺. Cell morphology did not change as a function of Na⁺ concentration. The same correlations were observed between OD₆₅₀, number of cells dry weight at all Na⁺ concentrations in the medium, indicating internal consistency.

DISCUSSION

The use of a non-inducing medium has enabled us to show that L-methionine is required to obtain significant methanethiol production by *B. linens* CNRZ 918. L-Methionine did not induce its own transport, which appeared to be constitutive, but it did induce the L-methionine-γ-demethylase. In a complex medium, it is undoubtedly free L-methionine, or L-methionine in the form of peptides included in the various components, which induces the L-methionine-γ-demethylase in the absence of added methionine (Ferchichi *et al.*, 1985).

The induction of one or several enzymes and permeases involved in methanethiol production has been described in *Achromobacter starkeyi* (Ruiz-Herrera & Starkey, 1970), *Pseudomonas ovalis* (Tanaka *et al.*, 1977) and *Pseudomonas fluorescens* UK1 (Laakso, 1979). In contrast, the system present in fungi is constitutive and active only on D-methionine (Ruiz-Herrera & Starkey, 1969). Among the inducers we found effective for *B. linens*, D-methionine, which acted as a weak inducer (if free of L-methionine), was not a substrate for demethiolation by resting cells or crude extracts. The absence of methanethiol production from D-methionine indicates a lack of a methionine racemase in *B. linens* CNRZ 918. Such a racemase has been found in *Pseudomonas* sp. (Kallio & Larson, 1955). Certain L-methionine-containing peptides were better inducers and substrates for whole cells than L-methionine itself. The most probable explanation for this is their better cell penetration, as has been shown for glycine and leucine in *Streptococcus lactis* (Rice *et al.*, 1978). Peptide entry into whole cells seems to be followed or accompanied by hydrolysis. Crude soluble cell extracts could not demethylate the same peptides. This might indicate that the peptidases were membrane-bound, or were destroyed during cell breakage or not active under conditions of assay. The intracellular L-methionine concentration thus probably plays an important role in the degree of induction, the more rapid entry of peptides leading to a higher internal concentration of the amino acid.

The extent of growth of *B. linens* CNRZ 918 in NID medium containing 7 mM-L-methionine was twice that in unsupplemented NID medium, showing that the amino acid is a limiting factor.

The *B. linens* demethylase is a highly specific enzyme, since thiomethyl could be replaced by only thioethyl (L-ethionine). A shorter chain (S-methyl-L-cysteine) or a chain with a modified function (2-oxo-, 2-hydroxy) resulted in lower or undetectable demethiolation.

Specific methanethiol production in cultures decreased as soon as the number of coccoid cells became significant although the transport rate by coccoid or rod cells was the same, invalidating our previous hypothesis (Ferchichi *et al.*, 1985). Our present results indicate that this decreased production was probably related to the decreased concentration of inducer in the medium.

B. linens CNRZ 918 was isolated from the surface of a cheese, where it develops in the presence of 1–2% NaCl. Our results indicate that the transport of L-methionine by this strain was sodium dependent, in contrast to its demethiolation. Such a dependence has been found in the case of threonine in *Brevibacterium flavum* (Shiio *et al.*, 1973), a strain closely related to *B. linens*, and for other amino acids in *Streptococcus lactis* (Rice *et al.*, 1978). Thus, the higher induction of L-methionine- γ -demethylase obtained in NID + L-methionine when Na⁺ was added is probably related to a higher intracellular concentration of L-methionine. Since the uptake of L-methionine was stimulated by extracellular Na⁺, the transport rate was probably the limiting step. This effect of Na⁺ for such sodium-tolerant bacteria is certainly complex and requires more study.

The various conditions which influence demethiolation activity of *B. linens* could be useful in certain aspects of cheese technology.

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