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Isolation and initial characterization of thermotolerant methylotrophic *Bacillus* strains

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1. SUMMARY

Using inocula from a variety of sources, mixed cultures of methanol-utilizing *Bacillus* strains were enriched readily at 55°C. Isolation of pure cultures was difficult; the six strains that were obtained eventually in pure culture all possessed the RuMP pathway, grew rapidly on methanol at temperatures up to 60°C, and were tolerant to very high methanol concentrations. An NAD-dependent alcohol dehydrogenase appeared to be involved in the initial oxidation of methanol.

2. INTRODUCTION

Over the years the number of methylotrophic bacteria reported to employ the ribulose monophosphate (RuMP) cycle of formaldehyde fixation has increased considerably, with new representatives steadily being isolated [1,2]. The overwhelming majority of these strains is Gram-negative and mesophilic. Among the methanol-utilizing RuMP cycle bacteria the occurrence of facultative strains is rare, the only clear example is in fact the very versatile Gram-positive actinomycete *Nocardia* sp. 239 [3]. For a number of years, we have attempted to isolate versatile methanol-utilizing bacteria, which in our opinion are most interesting organisms particularly from an applied point of view [4]. Since there is evidence that methylotrophic *Bacillus* strains exist in nature [5–8], we set out to isolate specifically pure cultures of these bacteria. A preliminary report of this work has been presented elsewhere [9].

3. MATERIALS AND METHODS

3.1. Media and cultivation conditions

Conical flasks, filled to 25% of the volume and incubated in a shaking waterbath, were used for enrichments and growth studies. A medium (pH 7.0) of the following composition (per litre) was used: Methanol (filter-sterilized), 50 mMol (or as indicated); $(NH_4)_2SO_4$, 1.5 g; K_2HPO_4, 4.65 g; $NaH_2PO_4 \cdot H_2O$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; trace elements [10], 0.2 ml. Yeast extract, casamino acids, peptone (0.5 g of each), and 1 ml of a

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Table 1

Source of inocula and procedures followed for the isolation of methylotrophic *Bacillus* strains in pure culture

Isolate	Source of inoculum	Procedures
Bacillus sp. C1	soil sample Holland	PRCSC
Bacillus sp. TS4	soil sample Malta	DC
Bacillus sp. TS1	waste water treatment system sugar beet factory	RCSC
Bacillus sp. PB1	as for strain TS1	PRTC
Bacillus sp. TS2	laboratory waterbath	RCSC
Bacillus sp. AR2	volcanic hot spring, Italy	PRDSC

P, pasteurization; R, repeated transfer in methanol mineral medium; C, selection of single colonies on methanol agar; S, serial dilutions; T, stepwise increase in temperature, D, stepwise increase in dilution rate, in methanol-limited continuous cultures.

vitamin mixture (mg/l: biotin, 100; thiamin · HCl, 100; riboflavin, 100; pyridoxalphosphate, 100; panthotenate, 100; nicotinic acid amide, 100; *p*-aminobenzoic acid, 20; folic acid, 10; vitamin B12, 10; lipoic acid, 10) were added per litre of enrichment medium, but subsequently replaced by the specific supplements required by some of the isolates. For solid medium, 15 g/l of Agar Noble was added. For cultivation in continuous cultures the medium described previously [11] was used, with 30 mM of methanol as the limiting carbon- and energy source. The pH was controlled at 7.0 by automatic adjustment with 1 M NaOH.

3.2. Enrichment and isolation

Samples were pasteurized by incubation at 80 °C for 10 min. The source of inocula and the isolation procedures followed are summarized in Table 1. Various alternative methods had to be devised in order to allow the isolation of pure cultures in a reproducible manner. The following procedure most consistently met with success. The enrichment medium (25 ml) was inoculated with 1–5 ml of liquid samples or 1–5 g of soil samples (resuspended in 5 ml of mineral medium), and incubated at 50–55 °C. Samples of dense cultures, usually obtained within 72 h, were streaked onto methanol agar plates and incubated at 50 °C. After 24 h, single colonies were transferred into fresh methanol liquid medium. At this stage failure to establish growth was frequently observed, apparently because cells lysed rapidly on plates. Repeated transfer of exponentially growing cells from one methanol liquid culture to another remained possible and resulted in almost immediate resumption of growth. After each transfer samples were plated out and the viability of the colonies obtained checked microscopically and tested by inoculating methanol mineral medium. After approximately ten transfers in liquid medium this resulted in the isolation of variants which did not lyse so readily on plating media anymore. At this stage the cultures were subjected to serial dilutions in mineral medium. The highest dilutions showing growth with methanol were plated onto methanol agar. The single colonies obtained were used to inoculate fresh methanol medium and the serial dilution procedure was repeated three times. Pure cultures of *Bacillus* sp. C1, TS1 and TS2 were isolated in this manner (Table 1).

The primary enrichment cultures were also used to inoculate methanol-limited continuous cultures, run at 55 °C and a dilution rate of 0.10 h^{-1}. After the establishment of a steady state the purity of the cultures was checked (microscopically and by plating out) and if more than one species appeared to be present either the dilution rate or the cultivation temperature was further increased. This procedure was repeated several times and at dilution rates around 0.20 h^{-1}, or temperatures around 60 °C, the number of contaminating species gradually diminished. Pure cultures of *Bacillus* sp. TS4 and sp. PB1 were obtained at D = 0.25 h^{-1}/55 °C and D = 0.10 h^{-1}/62 °C, respectively. The latter procedures were not always successful, i.e. one or two contaminants were found to remain present occasionally even at the higher temperatures employed, although at this point one *Bacillus* strain had clearly become dominant in the population. This opened up the possibility of applying the serial dilution technique described above. This procedure, combined with tests for viable colonies on methanol agar, resulted in the isolation of a pure culture of *Bacillus* sp. AR2.

Pure cultures (mid-exponential phase of growth on methanol) stored as 1 ml aliquots at −80 °C in the presence of 10% glycerol remained viable for at least 24 months.

3.3. Analytical procedures

The methods used for preparation of cell extracts, electron microscopy and measurements of growth, protein and dry weight values have been described previously [11]. Methanol was determined gaschromatographically. The Gram character of the isolates was determined electronmicroscopically and by testing for the presence of L-aminopeptidase (Merck, Bactident strips). Enzyme assays were performed at 45 °C. The following enzymes were assayed according to published procedures: Hexulose-6-phosphate synthase [11], methanol dehydrogenase (EC 1.1.99.8.; [12]), NAD-dependent methanol dehydrogenase [13]. NAD-dependent alcohol dehydrogenase (EC 1.1.1.1.) activity was determined in a reaction mixture (1 ml) of the following composition (final concentrations): glycine-KOH buffer pH 9.5, 200 μmol; NAD, 1 μmol; and extract. The reaction was started by the addition of 500 μmol methanol.

4. RESULTS AND DISCUSSION

4.1. Isolation of methylotrophic Bacillus strains in pure culture

With pasteurized or non-pasteurized inocula from various sources, batch or continuous culture enrichments at temperatures of 50–60 °C invariably resulted in the establishment of mixed cultures of spore-forming bacteria, using methanol as their sole source of carbon and energy (Fig. 1A). Similar mixed cultures have been described before [7], but the authors reported failure to obtain pure cultures. Initially we experienced similar problems, i.e. the organisms grew poorly on methanol agar (or agarose or gelrite) plates in that the colonies contained many lysed cells, and (non-methylotrophic) contaminants presumably growing on the lysis products. Not surprisingly, reinoculation of methanol liquid media with this colony material frequently resulted in a negative growth response. In further tests, with a variety of medium supplements, no indications were obtained that these mixed cultures were based on symbiotic interactions. We therefore set out to use alternative procedures, instead of the conventional plating technique for the isolation of pure cultures (Table 1). All six methanol-utilizing strains that subsequently were isolated in pure cultures (e.g. *Bacillus* sp. C1; Fig. 1B) initially produced subterminal, ellipsoidal spores in swollen sporangia (Fig. 1C), but some strains lost this ability in subsequent steps. With most of these strains there is a clear correlation between loss of ability to sporulate and good growth on methanol plates. Although it was noticed that a long, slender rod with a round terminal spore (Fig. 1A) was the dominant organism at low dilution rates in methanol-limited chemostats, all attempts to isolate this species in pure culture failed.

4.2. Characterization of methylotrophic Bacillus *strains*

The six *Bacillus* strains obtained in pure culture had many properties in common; an identical pattern was for instance observed in API 50CHB tests (data not shown). Their general properties are summarized in Table 2. All strains were found to be gram-positive, endo-sporeforming (at least initially), rod-shaped bacteria (Fig. 1) with a strictly respiratory metabolism. These properties designate them as *Bacillus* strains. Growth in pairs or chains was frequently observed. Although the strains were able to grow at temperatures upto 60 °C, their optimum temperature was 50–55 °C. They are therefore considered to be thermotolerant strains. After further work their precise taxonomic position will be determined.

The molar growth yields on methanol, measured at the optimum growth temperatures in methanol-limited chemostats, are amongst the highest reported for methylotrophic bacteria [2]. Methanol-grown cells of all isolates were found to possess high levels of hexulose-6-phosphate synthase (> 1.0 μmol/min/mg of protein), an enzyme specific for the RuMP pathway of formaldehyde fixation. Methanol-grown cells oxidized methanol at a very high rate (1.5 μmol/min/mg of protein). In cell-free extracts no activity of PQQ-dependent methanol dehydrogenase (characteristic for the Gram-negative methanol-utilizing bacteria), nor of NAD-dependent, PQQ-containing methanol dehydrogenase (as described for *Nocardia* sp. 239, [13]) could be detected. Instead, high activities (> 0.25 μmol/min/mg of protein)

Fig. 1. A. Mixed culture of spore-forming bacteria using methanol as sole carbon- and energy source. The arrows indicate the two dominant spore-forming species in these cultures (phasecontrast micrograph; ×2000). B. Phasecontrast micrograph of intact cells of *Bacillus* sp. C1 (×3000). C. Ultrathin section through a *Bacillus* sp. PB1 cell showing endospore (×45000). D. Ultrathin section through a cell of *Bacillus* sp. C1 to show the overall cell morphology (×75000). The inset (×175000) demonstrates the gram-positive character of the cell wall.

Table 2

General properties of methylotrophic *Bacillus* strains

Gram-positive, endo-sporeforming, rod-shaped bacteria	
Dimensions of cells: 0.5–1.0 μm (width) × 1–5 μm (length)	
Metabolism strictly respiratory	
Growth temperature range:	35–60 °C
t_d on methanol at 50–55 °C (optimum temperature):	40–80 min
pH range for growth:	5–9
pH optimum for growth:	7.0–7.5
Molar growth yield: 16–18 g dry weight/mole methanol	
Assimilation pathway: ribulose monophosphate pathway	
Methanol oxidation: NAD-dependent alcohol dehydrogenase	
Methanol tolerance:	1.5 M
Common growth substrates: methanol, glucose, maltose, mannitol, pyruvate, nutrient broth	
Common nitrogen sources: ammonia, glutamine	
No growth supplements required: *Bacillus* sp. TS1, AR2, C1	
Growth supplements required:	
Bacillus sp. PB1: biotin, thiamin, folic acid	
Bacillus sp. TS2: biotin, vitamin B12, lipoic acid	
Bacillus sp. TS4: vitamin B12	

of an NAD-dependent alcohol dehydrogenase were detected in all 6 isolates. This alcohol dehydrogenase is currently being purified and further characterized. The high growth yields of the *Bacillus* strains on methanol thus appear to be based on the involvement of an NAD-dependent alcohol dehydrogenase, and the RuMP cycle, which is energetically the most efficient C_1-assimilatory pathway. Compared to Gram-negative methylotrophic bacteria, all methylotrophic *Bacillus* strains were found to be very resistant to the toxic effects exerted by higher methanol concentrations. With *Bacillus* sp. C1, for instance, only a 50% reduction in growth rate was observed with methanol concentration of 1.5 M (Fig. 2). The range of growth substrates of the isolates is restricted. In addition to methanol, all strains showed good growth in mineral medium, with maltose, mannitol, glucose and pyruvate, and a variety of rich, complex, media. Fructose, glutamate, trehalose were used by 3 of the isolates, gluconate by 1 isolate only.

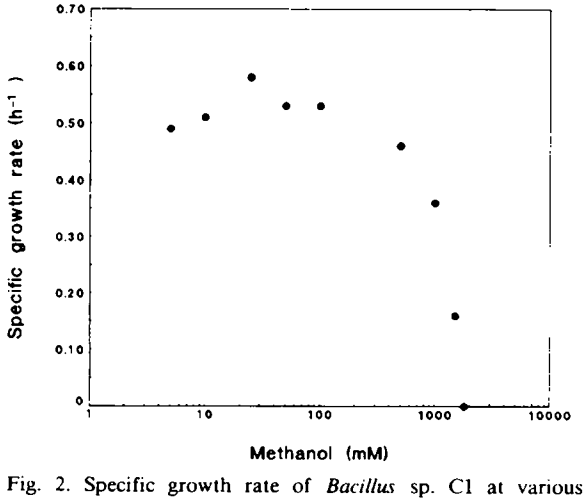

Fig. 2. Specific growth rate of *Bacillus* sp. C1 at various methanol concentrations.

From the results obtained in the present study it appears that methylotrophic *Bacillus* strains are wide-spread and ubiquitous in nature. In view of the problems experienced in obtaining pure cultures it is not surprising that they have remained undetected. Most of the pure cultures obtained, however, grow rapidly and reproducibly on solid and liquid simple salts media. In view of their characteristic properties, these organisms are of considerable interest for both fundamental and applied studies.

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