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Microbial Methanol Formation: A Major End Product of Pectin Metabolism

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Abstract. Various pectinolytic strains of *Clostridium, Erwinia,* and *Pseudomonas* species produced methanol as a major end product during growth on pectin but not on glucose or polygalacturonic acid. Pectin metabolism of *Clostridium butyricum* strain 4P1 correlated with a final product concentration of 16 mM at the end of growth, and a 1:1 stoichiometry for methanol production and percent initial substrate methoxylation. Growth on pectin was associated with high activity of pectin methylesterase and the absence of methanol consumption. The ecological significance of pectin metabolism and the establishment of microbial methylotrophic metabolism in nature is discussed.

Methanol is metabolized as a carbon-, hydrogen-, and oxygen-source for growth of a wide variety of eukaryotic and prokaryotic microorganisms. The known diversity of methylotrophic organisms is rapidly increasing and is perhaps best exemplified by noting the physiological diversity of methanol-consuming bacteria. Obligately respirative bacteria that include *Methylococcus, Paracoccus,* and many other genera have been emphasized most [1]. However, facultatively anaerobic *Klebsiella* species [6] and obligately anaerobic *Methanosarcina* [15] and *Butyribacterium* [18] species have been described that grow on methanol as energy source.

A major question arises when one considers microbial methanol metabolism; namely, the basis for methanol formation in nature. Only recently has microbial methanol production been reported as a metabolic end product of *Pseudomonas putida* grown on methoxylated aromatic acids [4]. The quantitative significance of this reaction in microbial habitats remains to be established. Microbial pectin metabolism may be a quantitatively significant source for methanol generation in nature because of the known activity of pectin methylesterase on methoxyl esters of galacturonic acid [11]. The purpose of this report was to determine whether pectinolytic bacteria form methanol as a significant end product and further metabolize it as a growth substrate.

Materials and Methods

All chemicals were of reagent grade quality; biochemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri). Pectin was a gift from Sunkist Growers (Corona, California); hydrolysis of Sunkist Growers pectin revealed a 10.3% methanol content by mass which correspond to a 65% degree of methoxylation.

Clostridium butyricum strain 4P1 was isolated from wetwood of a cottonwood tree [12] and strains MMP3 was isolated from mud from Lake Mendota, Wisconsin. *Clostridium thermohydrosulfuricum* strain 4B was isolated from the decomposing bacterial algal mat in Octopus Spring, Yellowstone National Park [16]; all other *Clostridium* strains and *Erwinia* and *Pseudomonas* species were isolated from rotting vegetables and obtained from A. Kelman.

Aerobic growth was carried out in 125-ml Erlenmeyer flasks, which contained 20 ml of medium and were sealed with cotton plugs and shaken during incubation. Anaerobic growth employed cultural techniques used for fastidious anaerobes [16,17], and was performed in 23-ml anaerobic culture tubes (Bellco, Vineland, New Jersey) that contained 10 ml of medium and were sealed with rubber bungs. All cultures were incubated at 30°C except for *C. thermohydrosulfuricum*, which was grown at 60°C. Growth was measured by direct insertion of anaerobic culture tubes or shake flask side arms into a Spectronic 20 colorimeter (Bausch & Lomb, Rochester, New York) and recording the optical density.

Pectin was washed twice with 95% ethanol and sterilized by ultraviolet radiation for 12 h before it was dissolved as a 10% solution in sterile water. This procedure was important because it prevented the hydrolysis of methoxyl groups that occurred when pectin was autoclaved. This sterile pectin solution was added at 0.5% (final concentration) to either a low-phosphate-buffered basal (LPBB) medium [17] that contained 0.1% yeast extract or a peptone yeast extract (PYE) medium [5]. Controls were cultured on the above media with either 0.5% glucose, methanol or polygalacturonic acid in lieu of pectin. *C. butyricum, C. thermohydrosulfuricum,* and aerobic cultures or *Erwinia* and *Pseudomonas* species were grown on LPBB-based medium, whereas the other *Clostridium* strains and anaerobic cultures of *Erwinia* species were grown on PYE-based medium.

Methanol and other fermentation products were measured by

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Table 1. Correlation of methanol formation to bacterial growth on pectin and pectin methylesterase activity (PMA).

Organism (strain)	Experimental conditions[a] Aerobic Methanol (μmol/ml)	Aerobic Growth (OD_{660})	Aerobic PMA (U/ml)	Anaerobic Methanol (μmol/ml)	Anaerobic Growth (OD_{660})	Anaerobic PMA (U/ml)
Clostridium butyricum (4P1)	—	—	—	16.0	1.1	0.62
C. butyricum (MMP3)	—	—	—	15.2	1.2	0.43
C. thermohydrosulfuricum (4b)	—	—	—	13.8	0.56	0.12
Clostridium strain (C-1)	—	—	—	9.5	0.81	0.34
Clostridium strain (C-16)	—	—	—	12.5	0.62	0.58
Clostridium strain (C-35)	—	—	—	11.5	0.67	0.48
Clostridium strain (L-3)	—	—	—	13.3	0.88	1.14
Erwinia chrysanthemi (SR 120A)	13.1	2.3	0.41	11.0	0.61	0.62
E. chrysanthemi (SR 226)	9.2	2.2	0.57	10.0	0.54	1.54
E. carotovora subsp. *atroseptica* (SR 8)	14.4	2.8	0.31	13.4	0.41	1.04
E. carotovora subsp. *carotovora* (SR 167)	9.2	3.4	0.27	12.2	0.58	0.88
Pseudomonas marginalis (G6A-3a)	12.7	3.1	0.23	—	—	—
P. marginalis (G6B)	11.4	3.5	0.46	—	—	—

[a] Assays were performed after 3 (aerobic) or 8 (anaerobic) days' incubation. Methanol and pectin methylesterase activity were not detected in uninoculated controls.

the flame ionization-gas chromatographic procedures described by Zeikus, Hegge, and Anderson [17]. Pectin methylesterase activity was quantified directly in culture samples (cells and spent medium) by measuring pH change caused by the formation of free carboxylic groups in a weakly buffered pectin solution [13].

Results and Discussion

Table 1 summarizes the results of experiments that related methanol production to pectin metabolism. The final methanol concentration generated by aerobic, facultatively anaerobic, and obligately anaerobic species ranged from 9.2 mM for *Erwinia chrysanthemi* strain SR226 to 16.0 mM for *Clostridium butyricum* strain 4P1. The methanol concentration was nearly the same for facultatively anaerobic species grown aerobically and anaerobically. Methanol formation was stoichiometrically coupled to pectin metabolism. For example, the 16 mM methanol produced by *C. butyricum* strain 4P1 was the same amount formed by the complete chemical hydrolysis of the added pectin. Growth on pectin for all strains was associated with significant activity of pectin methylesterase, which undoubtedly accounts for the production of methanol. In control experiments with glucose or polygalacturonic acid as fermentable carbohydrate, none of the strains produced methanol.

Methanol was the major nongaseous end product produced by some of the species examined. For example, the following concentration (mM) of end products was present after growth of *C. butyricum* strain 4P1 on pectin: 16, methanol; 11.4, butyrate; 7.5, acetate; 2.3, ethanol; 0.8, lactate; and 0.7, isopropanol. The methanol produced by the species examined here was not further consumed. In control experiments, these species did not grow on methanol as energy source.

Microbial methanologenesis from pectin appears to be an important mechanism that enables growth of aerobic or anaerobic methylotrophic organisms in nature. Pectin is nearly ubiquitous because it is a component of plant tissue [7], *Chlorophyta* cell walls [10], and the mucilaginous wall layers of *Cyanophyta* [3]. It is of interest to note that the pectin-metabolizing species examined here did not consume methanol. This phenomenon may help explain the need for microbial associations during pectin metabolism in nature; namely, the removal of toxic methanol by methylotrophs. If one speculates that methanol-producing, pectin-degrading species can oxidize a variety of organic components other than methanol in nature, then their metabolism helps establish the need for methylotrophs.

In certain environments, the metabolism of pectin can account for the majority of methanol produced. For example, anaerobic digestion of fruit and vegetable wastes, which are high in pectin, can yield methanol in amounts stoichiometric with the degree of fermentable substrate methoxylation. On a global scale, the primary microbial metabolic reaction(s) that form methanol are not documented. Lignin, quantitatively the most significant methoxylated biopolymer on earth, appears inert anaerobically [14], and methanol is not an end product of aerobic lignin decomposition by *Phanerochaete chrysosporium* [8]

(J. G. Zeikus and T. K. Kirk, unpublished observations). Recent studies have suggested formaldehyde as the C_1 intermediate formed during microbial degradative demethoxylation of lignin model compounds [9]. In lieu of microbial pectin or lignin metabolism, perhaps methanol excretion by methane-oxidizing bacteria is of prime importance in nature.

Conclusions

1. Methanol is a major product of microbial growth on pectin.
2. Species of aerobic, facultatively anaerobic, and obligately anaerobic pectinolytic bacteria produce but do not consume methanol.
3. Pectin metabolism, in part, establishes a niche for methylotrophs in nature.

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