A Novel Probiotic Approach for Reducing Bovine Methane Emissions

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Introduction: We propose a plan to address the significant contributions of cattle to rising levels of methane, a greenhouse gas that is produced in large amounts as a metabolic waste product by microbes residing in the rumen of cattle. We describe a probiotic strategy in which a methane-consuming bacterium (a methanotroph) will be added to cattle feed. Following the principles of probiotic therapy, the ingested methanotroph will colonize the rumen of cattle, where it will grow and metabolize the methane produced there by rumenal bacteria. The methanotroph will convert methane to biomass that will benefit the cattle nutritionally and significantly reduce the amount of methane emitted by the treated cattle.

Background: *Methylococcus capsulatus* is a gram-negative obligate aerobe can metabolize methane (i.e. a "methanotroph"; Ward et al., 2004; Chistoserdova, 2011). One well-characterized strain of *M. capsulatus* was isolated from the Roman baths of Bath, England (so it is referred to as M. capsulatus [Bath]). The complete genome of this microbe has been sequenced, revealing 3,304,697 base pairs containing genes encoding an estimated 3,000 proteins (Ward et al., 2004). Some of these proteins are enzymes that allow *M. capsulatus* to metabolize methane, using it as a carbon and energy source. One particularly important enzyme, comprised of three polypeptides, is called particulate methane monooxygenase (pMMO). pMMO catalyzes a reaction between methane and oxygen that produces methanol (Chistoserdova, 2011). This reaction is the first step in a pathway that allows *M. capsulatus* to convert methane into metabolites (***Figure 1***) that become amino acids and other components of new cells.

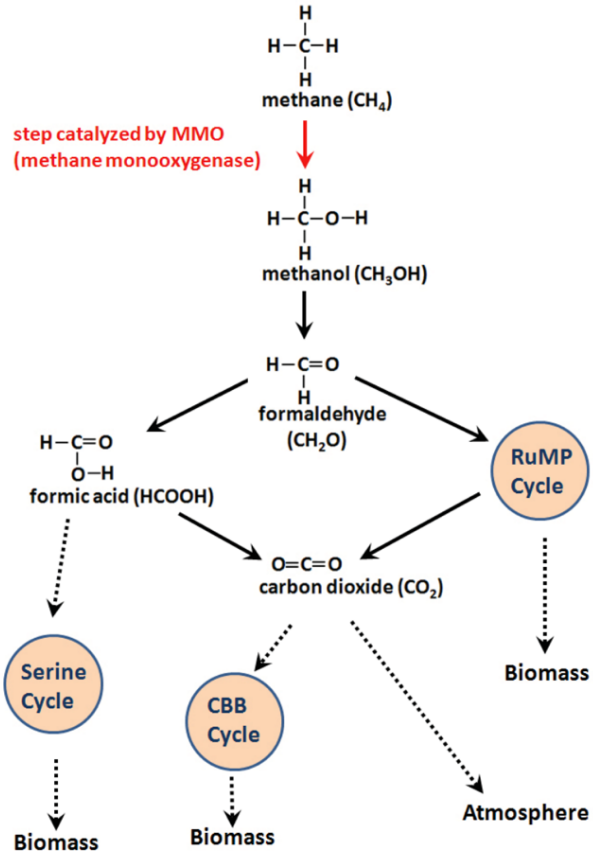

Figure 1 - Metabolic pathways that enable methylotrophic metabolism in Methylococcus capsulatus. Each solid line depicts a chemical reaction catalyzed by a specific enzyme. For example, the first step of this pathway is catalyzed by particulate methane monooxygenase. Dashed lines represent processes involving multiple steps and multiple enzymes, some of which are not yet well understood. CBB denotes the Calvin Benson Bassham cycle; RuMP is the ribulose monophosphate pathway.

As a principal investigator, I have studied *M. capsulatus* for more than twenty years. In a prior funded project, I cloned the *M. capsulatus* genes encoding the enzyme methane monooxygenase (*pmoC, pmoA,* and *pmoB*) and expressed them in *Escherichia coli*.

The digestive system of cattle contains a compartment called a rumen that food passes through before reaching the stomach. The rumen functions similarly to a fermentation vessel, providing a good environment to support the growth of a large, diverse population of microbes: it is large,

has a constant temperature (39°C), constant pH (in the range of 5.5 to 7), and provides a continuous flow of nutrients (in) and waste products (out). The rumen is filled with bacteria and protozoa, some of which degrade cellulose. That's why ruminants, unlike many other animals, can digest cellulose—the microbes do it for them. Food remains in the rumen for about 10 hours, during which time the resident microorganisms hydrolyze ingested cellulose to glucose. Rumenal bacteria then use this glucose as a substrate for fermentation, producing volatile fatty acids and gas including methane. Fermentation in the rumen generates enormous quantities of gas: 30–50 liters per hour in one adult cow (Bowen, 2009), about 25% of which is methane produced by methanogens (Moate et al., 1997). Belching is how ruminants continually get rid of fermentation gases. The term "eructation" describes this release of gas produced by enteric fermentations occurring in the rumen, and is the leading source of bovine methane (Bowen, 2009).

Methods: Naturally occurring methanotrophs cannot survive in the rumen, so we will create one that can survive in this environment by using recombinant DNA strategies. To accomplish this, we will take the genes encoding methane utilization enzymes from the bacterium *Methylcoccus capsulatus* and move them into a well characterized, safe (nonpathogenic) laboratory strain of *E. coli* (*E. coli Nissle*) that has been used previously as a human probiotic (Mutaflor®) and that can colonize the cow rumen. As proof-of-principle that this approach is feasible, we propose a pilot study in which the genes encoding the enzyme particulate methane monooxygenase (*pmoC pmoA pmoB*) will be incorporated into a plasmid and then this plasmid will be introduced into *E. coli* in the laboratory using the process of transformation. This recombinant *E. coli* will be fed to cattle so that it can colonize the rumen. In the rumen, the *E. coli* will grow and produce particulate methane monooxygenase that will metabolize much of the methane produced there by rumenal microorganisms, converting it to methanol. If these initial studies are successful, we will perform more extensive genetic engineering of *E. coli* to introduce the full methane-utilization pathway of *M. capsulatus* into *E. coli Nissle*.

The plasmid directing expression of the methane utilization pathway will be lost from *E. coli* unless we provide selective pressure to force its continued maintenance when our recombinant bacteria are colonizing the cow rumen. Therefore, we have included a selectable marker on the pMMO expression plasmid: the *blaA* gene (encoding β-lactamase) will allow us to use ampicillin resistance as a selectable marker. Thus, the antibiotic ampicillin will be added to cattle feed along with the recombinant *E. coli*. We anticipate that we can use these two simple additions to cattle feed (recombinant methanotrophic *E. coli* and ampicillin) as an effective and inexpensive approach to significantly reduce bovine methane emissions.

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