

Cross-Epithelial Hydrogen Transfer from the Midgut Compartment Drives Methanogenesis in the Hindgut of Cockroaches

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In the intestinal tracts of animals, methanogenesis from CO₂ and other C₁ compounds strictly depends on the supply of electron donors by fermenting bacteria, but sources and sinks of reducing equivalents may be spatially separated. Microsensor measurements in the intestinal tract of the omnivorous cockroach *Blaberus* sp. showed that molecular hydrogen strongly accumulated in the midgut (H₂ partial pressures of 3 to 26 kPa), whereas it was not detectable (<0.1 kPa) in the posterior hindgut. Moreover, living cockroaches emitted large quantities of CH₄ [105 ± 49 nmol (g of cockroach)⁻¹ h⁻¹] but only traces of H₂. In vitro incubation of isolated gut compartments, however, revealed that the midguts produced considerable amounts of H₂, whereas hindguts emitted only CH₄ [106 ± 58 and 71 ± 50 nmol (g of cockroach)⁻¹ h⁻¹, respectively]. When ligated midgut and hindgut segments were incubated in the same vials, methane emission increased by 28% over that of isolated hindguts, whereas only traces of H₂ accumulated in the headspace. Radial hydrogen profiles obtained under air enriched with H₂ (20 kPa) identified the hindgut as an efficient sink for externally supplied H₂. A cross-epithelial transfer of hydrogen from the midgut to the hindgut compartment was clearly evidenced by the steep H₂ concentration gradients which developed when ligated fragments of midgut and hindgut were placed on top of each other—a configuration that simulates the situation in vivo. These findings emphasize that it is essential to analyze the compartmentalization of the gut and the spatial organization of its microbiota in order to understand the functional interactions among different microbial populations during digestion.

Methanogenesis is an important electron sink in the intestinal tracts of terrestrial arthropods such as *Diplopoda* (millipedes), *Scarabaeidae* (scarab beetles), *Blattidae* (cockroaches), and *Isoptera* (termites) (12). It has been postulated that termites contribute substantially to global methane fluxes (9, 17, 26). According to recent estimates, termites may account for about 4 to 10% of the global production of this greenhouse gas (1, 19). The contribution of all methane-producing arthropods together is likely to be much higher (12, 13).

Methanogenic archaea in arthropods are generally restricted to the hindgut, where they occur free-floating in the gut lumen, attached to chitinous structures of the gut wall, or as intracellular symbionts of gut-dwelling anaerobic protists (12). The most likely electron donors for intestinal methanogens are H₂, formate, and methanol; there is no indication that acetoclastic methanogenesis plays a major role. Hydrogen is the by far most prominent among these potential substrates, particularly in termites. All termites investigated to date are characterized by gut segments with high H₂ partial pressure (10, 20). In (phylogenetically) lower termites, which harbor large numbers of protozoa in their hindguts (3), the carbohydrate fermentation by anaerobic flagellates is likely to be the major source of H₂ (14, 16). In (phylogenetically) higher termites, which possess a largely prokaryotic gut microbiota and typically do not host any

flagellates in their hindgut, and also in other arthropods, the organisms and metabolic pathways that are responsible for the formation of H₂ remain to be identified.

If methane-producing and hydrogen-consuming processes were homogeneously distributed in the gut segments that emit methane, low hydrogen partial pressures would be expected throughout the gut. Notably, microsensor measurements in the methane-emitting hindgut of the lower termite *Reticulitermes flavipes* have shown that hydrogen accumulates to high partial pressures at the gut center, whereas only small fluxes of hydrogen emanate from the hindgut (10). This is due to the presence of significant hydrogen-consuming activities at the periphery of the hindgut and hydrogen-producing activities apparently prevailing in the central region. In other words, the spatial organization of the hydrogen-producing and hydrogen-consuming microbiota controls hydrogen metabolism and methanogenesis in the hindgut of lower termites (5, 10, 24).

In the highly differentiated intestinal tract of higher termites (*Cubitermes* spp.), hydrogen-emitting and methane-producing gut compartments can be discriminated. The latter exhibit a significant hydrogen uptake activity when provided with external hydrogen (20). Based on the proximity of the hydrogen-producing and the hydrogen-consuming, methane-producing gut segments in vivo, a cross-epithelial hydrogen transfer has been postulated (6, 20). Since a striking compartmentalization of the intestinal tract is also present in other methane-producing arthropods (8), it can be assumed that this phenomenon is not restricted to higher termites. Here we describe the cross-

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epithelial transfer of hydrogen in *Blaberus* sp., a large, omnivorous cockroach with a highly differentiated intestinal tract.

MATERIALS AND METHODS

Organisms. Cockroaches (*Blaberus* sp.) were bred at the University of Nijmegen. They were fed a commercial pelleted rabbit diet, supplemented by apples, raw potatoes, and water ad libitum. Only adult cockroaches were used for the experiments.

For measurements with isolated guts or ligated gut segments, cockroaches were anesthetized with N_2 - CO_2 (80:20, vol/vol), decapitated, and dissected in Ringer's solution (7) to prevent dehydration of the gut. When midgut and hindgut were incubated separately, each gut segment was ligated at both ends with thin cotton or nylon thread using a pair of watchmaker's forceps and a stereomicroscope.

CH_4 and H_2 production rates. Living cockroaches were placed in 250-ml glass bottles, which were sealed with butyl-rubber stoppers, and were incubated for several hours under air at room temperature. Ethane (1 ml) was added as an internal standard (11). At regular intervals, gas samples (200 μ l) were taken using hypodermic needles and 1-ml syringes and analyzed for methane and hydrogen by gas chromatography (21, 22). As a rule, gases were measured at 3 h and 17 to 22 h after the start of the incubation.

Gut segments were incubated in 2 ml of HEPES buffer (50 mM HEPES, 50 mM NaCl, 5 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 [pH 7.0]) in 10-ml vials (21). Since pilot experiments had shown that under these conditions hydrogen production was linear for up to 29 h and the production of methane from endogenous substrates was linear for up to 48 h, gas production rates were analyzed using the same protocol as for living cockroaches.

Completely separated midgut and hindgut segments incubated in the same vial did not differ significantly with respect to gas production from intact guts merely ligated between midgut and hindgut; therefore, the data for all incubations were pooled. Since moderate shaking of the vials did not stimulate the production of methane and hydrogen by isolated guts or gut segments, we concluded that gas diffusion from the buffer into the headspace was not limiting under the experimental conditions.

Hydrogen microsensor measurements. Hydrogen concentration profiles were measured with polarographic H_2 microsensors, which had basically the same design as Clark-type O_2 microsensors (18) and were modified according to Witty (25). The microsensors were constructed in our laboratory in Konstanz and were tested and calibrated as described earlier (10). The detection limit for H_2 was about 100 Pa; the stirring sensitivity was always <1% of the signal obtained at a H_2 partial pressure of 20 kPa (calibration gas, N_2 - H_2 , 80:20 [vol/vol]). For profile measurements, the microsensors were mounted on a manual micromanipulator (10). Each set of experiments was reproduced with at least four different animals; the profiles shown in the figures represent typical examples.

For axial hydrogen profiles, cockroaches were dissected and the intact, fully extended gut was placed in an incubation chamber (16 by 16 mm, 100 mm long) and fixed with minutia pins on a thin silicone layer at the bottom of the chamber. The chamber was filled with air-saturated Ringer's solution and irrigated at a continuous flow rate (5 ml min^{-1}). Under these experimental conditions, the dissected guts exhibited a moderate peristalsis that persisted for several hours, indicating that they were still physiologically active.

For radial hydrogen profiles, shorter, ligated sections of midgut or hindgut were embedded in a smaller chamber in Ringer's solution solidified with 0.5% agarose and incubated under a controlled gas headspace. The agarose layer above the gut did not exceed 2 mm to avoid the formation of a diffusion barrier. The chamber was continuously flushed with the desired gas mixture. To demonstrate cross-epithelial transfer of hydrogen, ligated sections of midgut and hindgut that were lying in proximity in vivo (Fig. 1) were positioned in direct contact with each other and embedded in Ringer's solution solidified with 0.5% agarose. The experimental setup was the same as for the other radial profiles; the gut sections were incubated under air. The details of the experimental setup have been described earlier (10).

All hydrogen concentrations are reported as partial pressures. At atmospheric pressure, a partial pressure of 1 kPa equals a mixing ratio of 1% H_2 . At this partial pressure, pure water dissolves about 8.0 μ mol of H_2 per liter (20°C). However, we did not convert H_2 partial pressures to molarities, since this would require knowledge of the exact solubility coefficient(s) for H_2 in different gut contents.

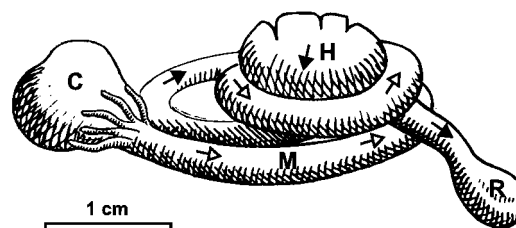


FIG. 1. Semischematic presentation of the intestinal tract of an adult *Blaberus* sp. cockroach, illustrating the intimate intertwining of midgut (M) and hindgut (H) in the abdomen. Open (M) and closed (H) arrows indicate the direction of flow from crop (C) to rectum (R). The coiling of the individual loops in vivo is even tighter, especially in the larvae.

RESULTS

Axial hydrogen profiles and emission rates of H_2 and CH_4 . Axial hydrogen profiles of the intact guts of *Blaberus* sp. revealed large differences among the various gut segments (Fig. 2). While hydrogen partial pressures were always below the detection limit of the microsensor (0.1 kPa) in the posterior hindgut of all animals investigated, the midgut segments accumulated substantial amounts of H_2 . Hydrogen profiles of the midgut varied considerably among individual animals (data not shown), ranging from 3 to 26 kPa H_2 . The average partial pressures of hydrogen in the anterior, median, and posterior regions of the midgut were 17, 6, and 11 kPa ($n = 7$), respectively. In most cases, the accumulation of hydrogen extended into the anterior hindgut. However, hydrogen accumulation was never observed in the posterior part of the hindgut or the crop.

Measurement of hydrogen and methane emissions by intact, living cockroaches (Fig. 3) showed that the animals emitted only traces of H_2 but large quantities of CH_4 [105 ± 49 nmol (g of cockroach) $^{-1} h^{-1}$ ($n = 12$)]. Notably, isolated midguts incubated in vitro emitted significant amounts of H_2 at constant rates [106 ± 58 nmol (g of cockroach) $^{-1} h^{-1}$], whereas isolated hindguts emitted only CH_4 [71 ± 50 nmol (g of cock-

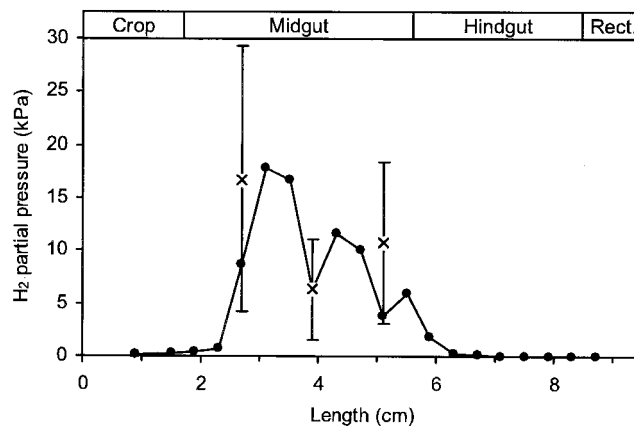


FIG. 2. Typical axial hydrogen profile (●) of an intact, fully extended gut of *Blaberus* sp. and average H_2 partial pressures (×) in the anterior, median, and posterior midguts of different animals (means \pm standard deviations; $n = 7$). Hydrogen was measured with a microsensor; all readings were taken at the gut center.

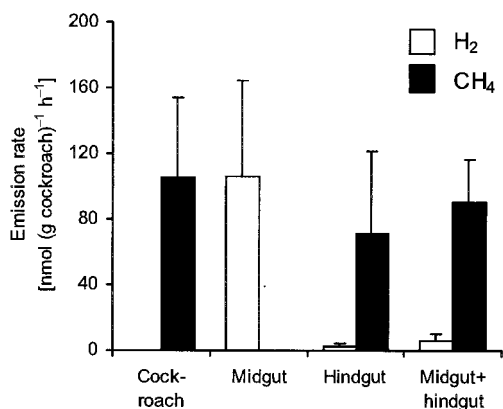


FIG. 3. Hydrogen and methane emission rates of whole insects, intact guts, and ligated midgut and hindgut segments of *Blaberus* sp. Values are means (plus standard deviations) of 12 different assays each. The large variance is due to the individual differences among the animals.

roach)⁻¹ h⁻¹]. When intact intestinal tracts with ligated midgut and hindgut segments were incubated in the same vial, methane emission increased over that of isolated hindguts [91 ± 26 nmol (g of cockroach)⁻¹ h⁻¹], whereas the hydrogen concentrations in the headspace remained low (Fig. 3).

Radial hydrogen profiles. Isolated, agarose-embedded midguts showed the highest hydrogen accumulation at the center of the gut (Fig. 4A). In the gut periphery, H₂ diffused across

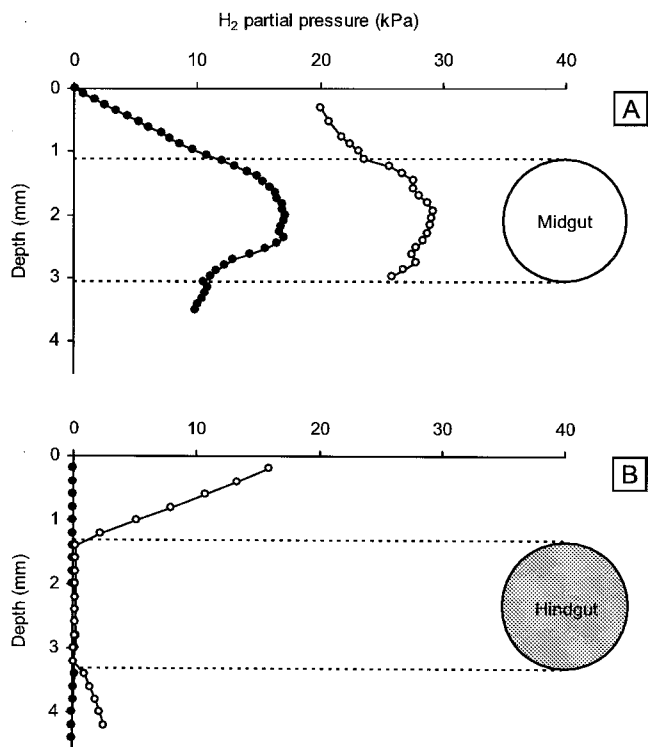


FIG. 4. Typical radial hydrogen profiles of agarose-embedded midgut (A) and hindgut (B) sections of *Blaberus* sp. incubated under a gas headspace of air (●) or air enriched with 20 kPa of H₂ (○). Hydrogen was measured with a microsensor.

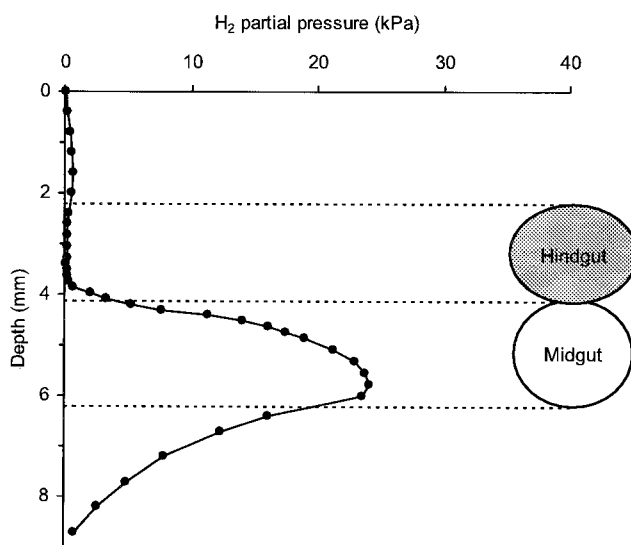


FIG. 5. Typical radial hydrogen profile through ligated midgut and hindgut sections placed in direct contact with each other and embedded in agarose, mimicking the in vivo arrangement of the sections (Fig. 1). Guts were incubated under air, and hydrogen was measured with a microsensor.

the gut epithelium into the surrounding agarose. When midgut sections were incubated under a headspace of air containing 20 kPa of H₂, the hydrogen concentration in the midgut lumen increased, and the shape of hydrogen profiles did not show any evidence of hydrogen uptake activities in the midgut periphery. Isolated hindgut sections, on the other hand, showed no detectable hydrogen accumulation, confirming the results of the axial profiles (Fig. 4B). Notably, all hindgut sections tested (*n* = 12) were efficient hydrogen sinks when incubated under air containing 20 kPa of H₂.

To test whether hydrogen is transferred across the gut epithelium from midgut to hindgut, short, ligated sections of midgut and hindgut that were also in direct contact in vivo (Fig. 1) were placed on top of each other and embedded in agarose. Radial hydrogen profiles revealed an accumulation of hydrogen at the center of the midgut sections and a diffusion of hydrogen into the surrounding agarose. Towards the hindgut sections, however, steep diffusive gradients of hydrogen developed (Fig. 5). Despite its high accumulation in the midgut, hydrogen was completely consumed within the first 100 μm beyond the hindgut epithelium. In the central portion of the hindgut lumen, there was no evidence of any accumulation of hydrogen. Comparable profiles were obtained irrespective of the positioning of the gut sections, i.e., whether the midgut was placed on top of hindgut or vice versa (data not shown). Steep hydrogen gradients developed across the midgut-hindgut interface of all gut sections which were tested in this experimental setup (*n* = 6).

DISCUSSION

The strong accumulation of hydrogen in the midgut of *Blaberus* sp. and its emission from isolated midgut segments contrasts strongly with the nearly complete absence of H₂ in the breath of cockroaches. This implies that almost all H₂ pro-

duced in the midgut is consumed inside the animal. Considering the anatomy of the intestinal tract and the juxtaposition of hydrogen-producing and hydrogen-consuming gut segments in vivo (Fig. 1), it is tempting to assume that the hydrogen formed in the midgut diffuses across the gut epithelia into the hindgut, where it is removed by a hydrogenotrophic microbiota. Such a model has already been proposed to explain a similar phenomenon in soil-feeding termites (20) and is supported by the stimulation of methane emission by externally applied hydrogen observed in living termites (10, 20) and cockroaches (J. H. P. Hackstein and T. van Alen, unpublished results).

This is the first time, however, that the cross-epithelial transfer of endogenously produced hydrogen was measured directly. Coincubation experiments and radial hydrogen concentration profiles of isolated midgut and hindgut segments of *Blaberus* sp. clearly identify the presence of a strong hydrogen source and the absence or insignificance of hydrogen-consuming processes in the midgut and the presence of a highly efficient hydrogen sink in the hindgut. The cross-epithelial transfer of hydrogen is clearly evidenced by the radial hydrogen profiles obtained when ligated fragments of midgut and hindgut were placed on top of each other, a configuration that simulates the in vivo situation (Fig. 1).

Notably, H₂ did not accumulate in the headspace when midgut and hindgut were coincubated in vitro, whereas the methane production increased considerably (Fig. 3). This observation strongly argues for a preferential stimulation of methanogenesis by cross-epithelial hydrogen transfer. The hydrogen emission rates of isolated midguts are about 5.5-fold higher than the corresponding stimulation of methanogenesis during coincubation. Assuming that H₂ consumed by the hindgut is used exclusively for methanogenesis from CO₂, a 4:1 stoichiometry would be expected. Therefore, it is possible that other hydrogen-consuming processes, e.g., reductive acetogenesis, are stimulated as well. The large variation in the emission rates between individual cockroaches, which also depends on developmental stage, sex, and diet (15) (unpublished observations), does not allow a definite conclusion based on this data set. Nevertheless, the stimulation of reductive acetogenesis by exogenous H₂ has been clearly demonstrated in the hindgut of soil-feeding termites with radiotracer techniques (23).

It should be pointed out that there is so far no satisfactory explanation for the fate of the hydrogen which apparently emanates from the side of the midgut which is not in contact with the hindgut in situ (Fig. 5). Most likely, oxygen supply to the gut, which is effected by the tracheal system in the living cockroach (a combination of advective and diffusive transport in the gas phase and only a short-range diffusive transport in the aqueous phase), is restricted by embedding the gut in agarose. The resulting diffusion limitation would create a significant oxygen deficit, which would stimulate the formation of H₂ in the midgut (10). Therefore, it is possible that in vivo fluxes of H₂ from the midgut are lower than those determined under experimental conditions—or that intercompartmental transfer of H₂ is not the only reason for the absence of hydrogen emission in living cockroaches.

The presence of highly differentiated gut segments in a variety of methane-producing arthropods (12) suggests that a cross-epithelial transfer of reducing equivalents between different gut compartments is likely to occur in other animals as

well. The diffusion of hydrogen is facilitated by the inherent permeability of the intestinal epithelia to gases; transepithelial gas exchange between gut and bloodstream (or between gut and tracheal system) is well established in humans (4) and in a number of arthropods (2). Nevertheless, it should be considered that formate and methanol are also potential substrates for methanogenesis in the hindgut. The hemolymph of soil-feeding termites, for example, contains appreciable concentrations of formate (23). Formate seems to be produced by microbial fermentations in the midgut and stimulates methanogenesis in the hindgut (20). A similar situation is present in the larvae of scarab beetles (*Pachnoda* sp.) (T. Lemke and A. Brune, unpublished results). Moreover, methanol is formed as the demethylation product of pectins in the midgut of several species of cockroaches and the larvae of *Pachnoda* (21; J. H. P. Hackstein, J. A. de Gouw, and C. Warneke, unpublished results). It remains to be shown that methanol is transported between the gut compartments, but there is evidence that *Methanomicrococcus blatticola*, an obligately methanol-reducing, methanogenic bacterium, is a major methanogenic organism in the hindgut of the cockroach *Periplaneta americana* (21). While methanol should be able to pass through the epithelial barrier easily, formate and other charged metabolites would require specific transport systems.

The results of the present study emphasize that we are just beginning to understand the interdependence of microbial processes in arthropod guts. Any such gut, no matter how small it is or how simple it seems to be at first glance, is a complex and highly structured environment (6). In order to understand the physiology of the digestive tract and to resolve the functional interactions among the different microbial populations, it is essential to analyze the compartmentalization of the gut and the spatial organization of its microbiota in more detail.

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