INTERACTIONS OF HUMAN COMMENSAL BACTERIA WITH AMALGAM-DERIVED MERCURY: THE SCIENCE AND ITS IMPLICATIONS FOR INFECTIOUS DISEASE AND NEUROTOXICOLOGY

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SUMMARY FOR NON-SPECIALISTS

Sect. 1. The bacterial mercury transformations. Certain bacteria that colonize the human gastrointestinal (GI) tract methylate inorganic mercury to form methylmercury, $CH_3Hg(I)$. Other bacteria that colonize the gut ecosystem reduce mercuric ion, Hg(II), to monoatomic elemental mercury, Hg(0), a form which readily diffuses across the body's membranes into circulation. The genes for the latter ability are carried on extrachromosomal DNA called plasmids that also carry genes for resistance to all known antibiotics.

Sect. 2. The connection between amalgam mercury and antibiotic resistances. Installing dental amalgam restorations into laboratory animals (monkeys) resulted in a dramatic increase in the proportion of their GI tract (oral and fecal) bacteria able to produce volatile Hg(0). Although these lab animals were never exposed to antibiotics, 80 to 90 percent of their mercury transforming bacteria were also resistant to several antibiotics because selection for the mercury transformation genes results in co-selection for whatever antibiotic resistances happen to be on the same plasmid; i.e. they are genetically linked. Three similar studies done in humans were less clear cut because humans also are exposed to antibiotics and because bacteria regularly migrate among members of the same family. However, many of the findings from the tightly controlled lab animal studies were replicated in human studies, especially the genetic linkage of antibiotic multi-resistance and mercury transformation in individual bacterial strains.

Sect. 3. Human commensal bacteria methylate and reduce mercury. Seven studies dating back to 1973 have shown that commensal bacteria either as individual pure strains or as bulk (fecal or saliva) specimens from humans and experimental animals can methylate inorganic mercury to methyl mercury *in situ* (i.e. inside the human or animal). Indeed, this activity in their intestinal bacteria is one source for methylmercury in fish. The other major source of fish methylmercury is bacteria in the sediments of acidic lakes; these bacteria are also present in the GI tract of about 50% of humans. Other studies have shown that 80% of inhaled monoatomic Hg(0) vapor is absorbed by the lung epithelium. It is reasonable to consider that Hg(0) vapor generated by intestinal bacteria would be similarly permeable to the intestinal epithelium.

Sect. 4. Conclusions and recommendations. Modern medicine now recognizes the profound influence of human commensal bacteria in everything from allergies to cancer. The evidence reviewed here warrants recognition that the activities of human commensal bacteria must be figured into any considerations of the risks of amalgam dental restorations. Because these bacterial communities are very dynamic and idiosyncratic individual humans are likely to have different vulnerabiliites owing to their bacterial differences. Fortunately, all the tools of molecular and chemical analysis are available to do large scale epidemiology to define the bacterial contribution rigorously and devise measures that will minimize their deleterious effects.

1. THE BACTERIAL MERCURY TRANSFORMATIONS

Most people assume that environmental exposure to toxic substances involves only the direct contact between a given agent and the cells of the human subject; microbiologists know otherwise. All plants and animals (including humans) have evolved with and are occupied by complex and dynamic communities of beneficial, and even essential, microorganisms (mostly bacteria) for their

entire lives (Tannock, 1995; Hooper and Gordon, 2001; Yan and Polk, 2004). We host 10-fold more bacterial cells in and on our bodies than we have human cells in our bodies. These bacteria are vastly more diverse metabolically as well as more genetically flexible than we are. They assist us in digestion (Lundberg et al., 2004), protect us from pathogenic bacteria (Conway et al., 2004; Merk et al., 2005), and are required for the development and maintenance of our immune systems (Smits et al., 2004; Kanauchi et al., 2005). Thus, toxic agents to which we expose ourselves impinge not only on our cells, but also on the cells of these bacteria. And, being more metabolically and genetically versatile than our cells, the bacteria can transform toxic substances so that they are no longer toxic to them. This does not always mean that the transformed agent is also less toxic to us as we shall see in the case of mercury (Hg). In this paper I will describe the data supporting three distinct interactions of commensal microbes with Hg that impinge directly on human health: (1) inorganic Hg(II) released from amalgams selects for antibiotic multiresistant bacteria because genes for Hg resistance and antibiotic multiresistance are genetically linked in bacteria, (2) Hg(II) can be converted to methylmercury by common bacteria living in our mouths and gastrointestinal tract and (3) inorganic Hg(II) can also be reduced to volatile, lipid soluble monoatomic Hg(0) which may be absorbed from the GI tract and re-enter circulation.

The subject of bacterial mercury metabolism has recently been comprehensively reviewed (Barkay et al., 2003) and salient points are briefly introduced here. In the early-1960's bacterial resistance to mercury was found to be a distinct genetic trait, inherited both clonally (i.e. by vertical descent from mother to daughter cells) and by plasmid-mediated horizontal transfer in both benign and pathogenic bacteria. In the late 1960's and early 1970's, it was established that the mechanism of bacterial resistance to inorganic ionic mercury, Hg(II), is the reduction of this highly reactive ionic species to relatively inert monoatomic elemental mercury, Hg(0), a volatile gas that readily dissipates from the culture medium consequently detoxifying it. This process was carried out by an enzyme called the mercuric ion reductase (MerA), an evolved variant (homolog) of the common 'housekeeping' enzyme glutathione reductase, which itself is quite sensitive to Hg(II). Bacteria resistant to inorganic mercury sometimes have an additional gene encoding an organomercurial lyase, MerB, which splits the carbon-mercury covalent bond in such compounds as the neurotoxin methylmercury. In this same timeframe it was also found that certain anaerobic bacteria could methylate Hg(II) to form mono- or dimethylmercury, both potent neurotoxins. Considerably later, in the 1990's it was found that aerobic bacteria having the enzyme catalase can oxidize elemental Hg(0) to reactive Hg(II). Bacteria that oxidize sulfides can generate acidic environments (acid mine drainage) and under such conditions even sulfide ores of metals such as cinnabar can be re-solublized. Thus, natural processes found in several different types of bacteria catalyze an environmental "Mercury Cycle", actually a set three key equilibria as depicted in Fig. 1 at the end of this document (the sizes of the arrows indicate the relative rapidity or throughput by these biotic processes in each reaction).

Interestingly, most of the early studies of Hg resistance were done on clinical bacteria such as *E.coli* and *Staphylococcus aureus*. Because mercurial compounds were commonly used as disinfectants in medical settings they selected for Hg resistant bacteria (Porter et al., 1982); in light of such observations, Hg-containing disinfectants were largely replaced by quaternary ammonium disinfectants during the 1980's. By that time, advances in the study of bacterial gene transfer had revealed that plasmids, which are small circles of DNA capable of moving themselves from one cell to another, could carry multiple genes for resistance to several different antibiotics. Plasmids were then revealed to be the major agents mediating the newly recognized spread of antibiotic multi-resistance. Many of the first studied plasmids also carried resistance to mercury and organomercury (e.g. merthiolate) compounds genetically linked to the antibiotic resistance loci on these transferrable bacterial plasmids. Consequently, exposure of bacteria to any agent for which their resident plasmid provided resistance would indirectly select (co-select) for all of the other genetically linked resistance loci on that plasmid (Fig. 2). It was assumed that bacteria carried Hg resistance genes only because they could occasionally encounter Hg in the external environment; indeed, Hg mines and polluted industrial sites were found to have an abundance of Hg resistant bacteria (Olson et al., 1979; Barkay and Olson, 1986; Barkay, 1987). Of course, carriage of antibiotic resistance genes by bacteria was assumed to reflect their occasional exposure to

antibiotics when in a human or animal host being treated for an infection. Since, at the time, so few bacterial plasmids had been studied, no particular significance was placed on the fact that the antibiotic and Hg resistance genes were found genetically linked on the same plasmid.

2. THE CONNECTION BETWEEN AMALGAM MERCURY AND ANTIBIOTIC RESISTANCES

2.1. The Initial Discovery in a Primate Model System

Until the early 1990's it was assumed that the only Hg-containing compounds, apart from methylmercury in fish, to which humans were regularly and directly exposed were disinfectants such as mercurichrome and merthiolate, employed as topical antiseptics and also widely used in feminine douches and contact lens cleaning solutions (Risher et al., 2002). However, in the early 1980's my group observed that people who had no recent exposure to antibiotics often had an abundance of Hg resistant bacteria in their feces. Many such people were significantly (0.001, chi-square) more likely also to have bacteria with a multiple antibiotic resistance genes. So, beginning in 1989, my group in collaboration with investigators at the University of Calgary, specifically tested two hypotheses: that Hg is released from amalgam dental restorations in amounts sufficient to select for Hg resistant bacteria in the commensal microbiota and that the Hg resistance loci thus selected would be linked to antibiotic resistance genes and borne by bacterial plasmids (Summers et al., 1993). Using monkeys housed in a research vivarium and fed an antibiotic-free diet, we showed that installation of 16 occlusal amalgams led to (a) a 10,000-fold rise in the Hg content of their feces, reaching millimolar levels in some animals, (b) a consequent dramatic rise in the proportion of the oral and fecal bacteria that were Hg resistant, and (c) a strong association between Hg resistance and antibiotic resistance in pure bacterial strains of three distinct types of bacteria obtained from feces or from the oral cavity. In two of our three experiments, the amalgam fillings we had installed were replaced after two months by non-metallic restorations. Removal provoked a second spike in fecal Hg concentrations followed by a gradual decline in fecal Hg and slower decline in the multiresistant resistant bacteria, though neither declined to pre-treatment levels during the 2-month follow-up period. The changes observed were statistically significant at 95-99% level, so we could conclude that Hg released from dental amalgam fillings can select for multi-resistant bacteria. This was the first demonstration that a widely used non-antibiotic agent could select for antibiotic multi-resistant bacteria and do so in the primate commensal microbiota. Subsequent work showed that among ca. 500 strains of *Enterobacteriaceae* recovered from these animals the Hg and antibiotic resistance genes are physically associated (i.e. genetically linked) on transferrable plasmids (Wireman et al., 1997b) very much like those in the clinically-derived bacteria studied in the earliest days of plasmid research.

2.2. Studies Extending These Observations to Humans

Although primates are considered the gold standard laboratory animal model for humans, the relevance for human health of Summers' findings was questioned by the American Dental Association. Fortunately, two Scandanavian groups undertook similar work with human study populations and more recently important additions to this area have been made by a British group. The actual observations in these studies largely replicated those of Summers et al. in laboratory animals but some of the human data were sufficiently noisey that it was not possible to decide whether their results agreed with the laboratory primate observations. The following brief critiques of each article show the areas of agreement and the dangers of overlooking the realities of commensal microbiology in designing and interpreting such human experiments.

2.2.1 Studies from the Laboratory of Pentti Houvinen and Colleagues (Turku University, Finland)

A study from this group (Österblad et al., 1995) was the first to address explicitly the influence of amalgam Hg exposure on antibiotic resistance in humans and is significant for that reason. Österblad et al. examined the occurrence of Hg and antibiotic multi-resistance in persons with fillings, persons who had had fillings removed, and persons who had never had fillings. Most of

their data were consistent with those of Summers et al. Despite this, the authors concluded that the earlier observations with monkeys did not apply in humans. Their conclusion arises from the following flaws in their methods and interpretations of their data.:

2.2.1.1. Missing amalgam status data and superficial statistical analysis. Österblad et al. provided no data on the average, standard deviation, and range in the numbers or the types of amalgam fillings in their study cohorts, although such data were at the time well recognized as essential to evaluate Hg exposure from amalgams. They also did not report how many amalgams were removed from their "amalgam removed" subjects and when they had been removed. Consequently, the standard deviations in their three cohorts are larger than the averages for the fecal Hg concentrations by 2.7-fold and 1.5-fold, respectively, for those with amalgams and for those who have had them removed (p. 2500, first paragraph of the Results section). Such large standard deviations strongly suggest clustering in the data; i.e. the existence of a sub-group with unusually high fecal Hg concentrations. Failure to do cluster analysis or even multivariate analysis renders unconvincing Österblad et al.'s conclusion that there is no correlation between fecal Hg concentration and either Hg or antibiotic resistances. It is very surprising that the reviewers missed these points. Certainly no paper concerned with the effect of antibiotic consumption on the commensal microbiota would have been published if the authors failed to distinguish those who had only consumed one tablet of ampicillin from those who took the full course of the drug.

2.2.1.2. The stated conclusions are contradicted by the data. In the abstract (lines 8-9) and also on p. 2500 of the paper (first sentence of the 2nd paragraph of the Results section), the authors state broadly that there were no correlations between antibiotic resistance and Hg resistance in their subjects. However, in Fig. 1, both ampicillin resistance and nalidixic acid resistance are higher in both amalgam-exposed groups than in those who had never had amalgams at a level which is called "marginally significant" (<0.05). This 95% level is typically regarded as solidly significant in most laboratory and epidemiology work.

2.2.1.3. Overlooking the significance of antibiotic multiresistance. In Fig. 2 Houvinen compares the number of resistances per subject in those with Hg resistance and those without Hg resistance and states that there was no difference among the three groups of subjects. However, in every category of multiple resistance (from 1 to 6 additional resistances), the fraction which is also Hg resistant is higher than the fraction which is Hg sensitive by as much as 2-fold or more (e.g. cases with 4-6 additional resistances). In the legend he also notes that 74% of the subjects having Hg resistances have two or more other resistances. So, although Houvinen cannot correlate multiresistances with his different amalgam-exposed groups (not surprising for the reasons noted above), there is a very strong correlation of Hg resistance with antibiotic multiresistance for all of these humans.

2.2.1.4. Accepting an erroneous assumption. The largest error in this work is a conceptual one: that you can learn about the cause of a phenomenon from community prevalence studies. This error has plagued the subject of non-hospital antibiotic resistance since its recognition in the mid-1970's. Hospital-based research on nosocomial antibiotic resistance involves rigorous documentation of individual patients' medical histories, continuous, exacting environmental monitoring (including hospital staff), and often continuous draconian measures to control the spread of an antibiotic resistant infectious agent. In other words, such work is based on longitudinal data on individual humans and their environment. Using such protocols, cause and effect relationships can actually be established.

Houvinen's study is a good example of the limitations of prevalence studies for discerning causal relationships in "free-range" humans. If the eight monkeys which Summers et al. examined were given a random number of fillings (both the number and type unknown to the investigator), had been free to roam among the other animals and humans in the vivarium, sharing food, and interacting ad lib, with uncontrolled exposure to antibiotics over a period of years, and if only a single fecal sample had been taken from each monkey at some random point in its life, it is unlikely that any correlation between fecal Hg concentration, Hg resistance, and single or multiple antibiotic

resistances could have been seen. Prevalence studies can tell us about where we are now; they cannot tell us how we got here, i.e. which of many possible factors caused the present state. Longitudinal studies can make such distinctions, for example, Mark Richmond and colleagues established in their landmark 1973 longitudinal studies (Anderson et al., 1973) that even brief antibiotic therapy spreads antibiotic resistances among multiple bacteria in the human gut flora**.** Thus, well-controlled longitudinal studies on individual humans are necessary to resolve whether there is an effect of acute and/or chronic Hg exposure on antibiotic resistance in their normal flora. And in choosing the control population for such human experiments it will be essential to keep in mind the following realities of commensal microbiology.

2.2.1.5. Exchange of commensal microbiota. We share our microbes with our family members; Caufield and colleagues had documented even before the Österblad et al. study that infants are colonized with their mothers' strain of *Streptococcus mutans* (Caufield et al., 1993; Li and Caufield, 1995). More recently Griffin and colleagues demonstrated the transfer of the bacterial agent of periodontal disease from mother to child (Tuite-McDonnell et al., 1997). We also observed several years ago that children without fillings, or even without teeth, have Hg and antibiotic multiresistant fecal bacteria if their parents have amalgam fillings (Wireman and Summers, unpublished observations). Thus, before deciding that a person is a suitable "amalgam-free" control one would need information on the amalgam status of immediate family members; those with amalgam-bearing kin might well have been colonized by incoming Hg and antibiotic resistant bacteria, perhaps fostering persistance of these acquired microbes each time they took an antibiotic themselves.

In a later paper (Leistevuo et al., 2000) the Finnish group showed that for any given antibiotic the degree of resistance as measured by the minimal inhibitory concentration (MIC) for that antibiotic did not differ for bacteria obtained from persons with amalgams or those without them. Indeed, this is the expected result, since as shown in myriad other studies involving exchangeable genes, a given resistance gene is the same regardless of the bacterial source and the MIC measure only reports on the characteristics of the gene. Unfortunately, the authors did not use any bona fide susceptible and resistant control strains in their survey, so for Hg at least, they had no working definition of Hg resistance and susceptibility against which to compare their measurements. Moreover, it is not the degree of resistance to any one antibiotic that differs in bacteria derived from high Hg environments; it is the number of different kinds of antibiotic resistances that each bacterial strain carries that increases. The authors only surveyed four antibiotics and did not report individual strain phenotypes, so we do not have an answer to the critical question: do the amalgam-exposed bacteria have more antibiotic resistance genes or not?

2.2.2. The Work of Charlotte Edlund and Colleagues, Stockholm University, Sweden

This thorough, well controlled, and well executed longitudinal study (Edlund et al., 1996) was the second to address explicitly the effect of amalgam restorations on antibiotic resistance in the human normal flora. Unlike the earlier study (Österblad et al., 1995) Edlund et al. employed a longitudinal model with 10 subjects with amalgams and 10 subjects without them. Edlund's experimental design involved the removal of pre-existing fillings whereas Summers' work involved the installation of fillings in naive animals. Edlund et al. also examined specific bacterial populations from the feces; their human study agrees with the work on monkeys for the following points:

2.2.2.1. Fecal Hg concentrations People with fillings have high levels of fecal Hg (the average number of fillings was 19) (Fig. 1 in Edlund paper). The pre-removal fecal Hg levels are in the 0.1 to 1 µg/g range which is close to the "steady state" post-installation Hg observed in the monkeys (which had only 16 fillings) (Fig. 5 in (Summers et al., 1993).).

2.2.2.2 Hg dynamics on amalgam removal Removal of the fillings results in a large pulse of Hg going through the feces. One week after the procedure the fecal Hg had increased approximately 3-4 fold (Fig. 1, Edlund paper). The range of concentrations was very much higher,

and even reached the 10 µg/gm level in some persons, just as seen with the monkeys (Fig 5, (Summers et al., 1993).)

2.2.2.3. Bacterial dynamics on amalgam removal. For the group with fillings, the percent of Hg resistant bacteria increased immediately after the fillings were removed and then began to decline (Fig. 2, Edlund paper). This was also seen in the monkeys (Figs. 3,4 (Summers et al., 1993)).

2.2.2.4. Increase in antibiotic resistance In the amalgam group (compared to the controls) minimal inhibitory concentrations (MIC) for several antibiotics increased in *Bacteroides* and *E.coli*. Although Summers' group did not use the MIC method to assess resistance nor did they monitor *Bacteroides*, they did monitor Gram-negative facultative bacteria (including *E. coli*) and similarly found an increase in the numbers of antibiotic resistant isolates during the 4 weeks following amalgam installation.

2.2.2.5 Increase in multiresistance Antibiotic multiresistance (having 2 or more antibiotic resistances) is very strongly associated with being Hg resistant (Tables 2-4) for all three bacterial genera examined (*Bacteroides, E.coli*, and *Enterococci*). These data exactly corroborate Summers original findings in humans which provoked their animal studies ((Summers et al., 1993), Fig. 1) which revealed the same Hg-multiresistance phenomenon ((Wireman et al., 1997a), Fig. 4).

The Edlund group also made one finding which Summers et al. did not make. Specifically, Edlund et al. found more Hg resistant *Bacteroides* in the amalgam subjects than in the controls (Table 1.) Summers et al. did not examine *Bacteroides* whose cultivation involves techniques not available in their lab. Edlund et al. did not find more Hg resistant *E.coli* or *Enterococcus* in the amalgam group compared to the controls, but Summers et al. did find such a difference in the monkeys for these two types of bacteria. It is very interesting that Edlund et al. found increased Hg resistance in *Bacteroides*, a significant component of the intestinal flora. It is of less concern that Edlund et al. did not find Hg resistance in the minor components *Escherichia* and *Enterococcus*. The variability in composition of the gut communities of individual humans and other primates will affect the detectability of the minor components more than in the major ones. Moreover, as noted above, the possible confounding factor of relatives' amalgam fillings might have increased the numbers of Hg resistant bacteria in the controls.

Curiously, despite the extensive concordance with the animal observations, Edlund et al. concluded that amalgams have no effect on the occurrence of Hg and antibiotic resistance in humans because their amalgam-free control group could not be distinguished statistically from the subjects with amalgams. Actually, the reason they could not distinguish the controls from the amalgam-bearers was because of very high, randomly fluctuating levels of Hg resistant bacteria in the control cohort. It would have been correct to state "no conclusion can be drawn" rather than to state as they did that their data show there is no difference between the two groups. This latter conclusion simply cannot be drawn from data with as much variability as theirs. In addition, they used only Wilcoxon, rank (non-parametric) statistics in their data analysis. Such a statistical tool specifically minimizes the ability of very high or very low observations to influence the mean and thus obscures the occurrence of "outliers". At the very least, they should have done both parametric and non-parametric analyses; the latter could have unmasked individual differences in the response to Hg. And, as noted above people share their normal flora with their families. If Edlund's "no amalgam" controls had family members with amalgams, there is some likelihood that they would be colonized with Hg resistant strains. That Edlund et al. did not consider this possible confounder suggests that they were also unaware of the earlier work of Caufield noted above (Caufield et al., 1993; Li and Caufield, 1995).

2.2.3. Work of P. Mullany et al., Eastman Dental Hospital, University College, London.

Beginning in 2002 the group of Peter Mullany has carried out several epidemiological and molecular studies on the relationship of Hg and antibiotic resistances to amalgam exposure on oral bacteria in children.

2.2.3.1. Prevalence and longitudinal studies. Their initial case-control prevalence study found that Hg resistance is common in streptococci of the oral cavity of children regardless of whether they have amalgam restorations, demonstrating again the limitations of prevalence studies on "free range" humans for such studies (Pike et al., 2002). Interestingly, they observed strongly positive skewing in the data, suggestive of large individual variation in carriage of resistant bacteria among children in both groups. A subsequent assessment of the prevalence of Hg and antibiotic resistance in dental placque bacteria in a larger cohort of children lacking amalgams reached the same conclusion (Ready et al., 2003). Mullany and colleagues have also undertaken one longitudinal study (Pike et al., 2003), monitoring changes in Hg and antibiotic resistances in oral bacteria upon placement of amalgam restorations in children. They were not able to find significant differences in the pre- and post-installation oral bacteria, possibly owing to the already high prevalence of both characters in the oral microbiota of children in the general population.

2.2.3.2. Novel Hg resistance genes Moving to molecular analysis, Mullany's group has sequenced the hallmark *merA* gene of their oral bacterial isolates and found that they carry either the classic Gram positive *merA* gene originally defined in *Bacillus cereus RC607* or a completely novel version of *merA* that they have also found in a coagulase-negative *Staphylococcus* strain (Stapleton et al., 2004) located on a novel transposable element. It is clear that Hg resistance genes travel very widely among the Gram positive bacteria, just as they have been found to do among the Gram negative bacteria.

2.2.3.4 Genetic linkage of Hg and antibiotic resistance in Gram positive bacteria. In their two most recent papers, Mullany's group break some especially exciting ground in this area. Firstly, in work following immediately upon their sequencing of the *merA* genes of oral bacteria, they have found in a strain of *Enterococcus* collected from the feces of one of the monkeys used in the Summers' studies that the *mer* operon lies within a new transposon and is genetically linked to streptomycin resistance as both are carried on a conjugative plasmid (Davis et al., 2005b). This is the first clear demonstration that mobile plasmids of Gram positive bacteria have also evolved to carry both Hg- and antibiotic resistances.

2.2.3.5. Genetic linkage of silver and antibiotic resistances in oral bacteria. In a second paper, Mullany's team makes a very important observation concerning silver resistance. Since silver (Ag) has been used as a topical antiseptic in the prevention of neonatal ophthalmic gonorrhea and a genetic locus conferring resistance to silver sulfadiazine has been identified in plasmids recovered from bacteria infecting burn patients, Mullany's group asked whether Ag resistant bacteria might also be recovered from the mouths of persons with amalgam restorations. They found Ag- and antibiotic-multiresistant Gram negative *Enterobacter cloacae* could be isolated from infected teeth with dental restorations (Davis et al., 2005a). They confirmed the presence of the previously defined *sil* operon by PCR amplification of the hallmark *silE* gene and think that the *sil* locus is plasmid-carried in these strains. This finding provides an important clue to the possible origins of the plasmid-encoded Ag resistance (*sil*) locus which has been the bane of burn centers since the mid-1970's (McHugh et al., 1975). All known examples of the *sil* locus are in bacteria isolated from severely burned patients, many of whom died from infection with the silver-sulfadiazine resistant bacteria. Surprisingly, when the original Ag resistance locus was sequenced, it was found to be a very large and complex 9-gene operon for constructing two multi-protein membrane-bound efflux pumps, a two-component regulatory system, and a periplasmic silver binding protein (Silver, 2003), not something that could evolve in a few weeks of growing on a burn patient being treated with silver sulfadiazine, nor for that matter in response to the single dose of ophthalmic silver nitrate given to newborns. However, amalgam fillings can in principle leach silver by abrasion and galvanic corrosion and they remain in the mouth, a rich bacterial ecosystem, for

decades, certainly sufficient time for bacteria to evolve and optimize the silver defense system we have today. It is noteworthy that the first characterized Ag resistant strain carried both the *sil* and *mer* loci along with 5 antibiotic resistant genes genetically linked on the same large, mobile plasmid, pMG101 (McHugh et al., 1975).

In summary, both prevalence and longitudinal studies in "free range" humans are consistent with and extend the findings made with the more controllable laboratory animal longitudinal experiments. Thus, it is reasonable to conclude that Hg and possibly Ag released from amalgam restorations select for antibiotic multiresistant bacteria as effectively as a course of antibiotic does. However, unlike antibiotics which are typically given for only a few days, amalgams are meant to stay for many years and, as demonstrated elsewhere, leak Hg for their entire lifetimes, continuously selecting for multiresistant bacteria, both benign and pathogenic, in the oral and gastrointestinal microbial communities.

3. HUMAN COMMENSAL BACTERIA METHYLATE AND REDUCE MERCURY

The *mer* locus has evolved to defend bacteria against exposure to toxic Hg(II) compounds (Barkay et al., 2003) and, as described above, the abundance of this locus in commensal microbes arises because of frequent or continual exposure to Hg(II) arising from amalgam dental restorations(Summers et al., 1993). The enzymes MerA and MerB, defend the bacteria by transforming inorganic Hg(II) ion to volatile, monoatomic Hg(0) and organic mercurials such as methylmercury or merthiolate to ionic Hg(II), respectively. The Hg(II) produced by MerB can then be reduced by MerA, so, these two enzymes constitute a detoxification pathway. The levels of amalgam-derived inorganic Hg(II) found in the oral cavity and the GI tract are sufficiently high to select for bacteria carrying the *merA* gene in the *mer* operon. But the levels of methylmercury in fish tissue are at least 100-fold lower than what would select for organomercury resistance in bacteria. So, why is the *merB* gene present in commensal bacteria? Is there another source of methylmercury apart from diet?

3.1. Methylation

Although it is well established that inorganic mercury, both the vapor Hg(0) and the soluble ion Hg(II) are released into circulation from amalgam restorations (Lorscheider et al., 1995), it is still widely believed that the only source of the organic form, methyl mercury, is from fish in the diet. Again, microbiologists know otherwise. As noted above, it is well established that the anaerobic sulfate reducing bacteria (SRBs) are the major methylators of Hg in sulfur-rich freshwater and estuarine sediments (King et al., 1999; King et al., 2000). However, it is less well known that human commensal bacteria can methylate Hg(II) (Vonk and Sijpesteijn, 1973; Edwards and McBride, 1975; Rowland et al., 1975; Heintze et al., 1983; Liang and Brooks, 1995; Leistevuo et al., 2001) (Table 1).

3.1.1. Common commensal bacteria methylate Hg These seven studies over the last 30 years, each done in slightly different ways in different laboratories, have observed methylation of Hg by either purified commensal bacteria or directly by saliva or feces. For a meta-analysis of these studies, I took the numbers supplied by the authors and derived estimates for the total possible daily methylmercury output by the corresponding microbial communities assuming that an adult carries about 1 lb (454 g) of feces and swallows about 500 ml of saliva daily. The estimates for methyl mercury production per microbial community vary wildly, certainly owing to the many different approaches used by the authors in their respective experiments as well as to the simplicity of my assumptions. However, all investigators found methylmercury produced by ordinary bacteria or mixed bacterial communities (i.e. feces or saliva). Indeed, there is good evidence that the source of methylmercury in fish is not from the water column alone, but from methylation of by their own intestinal bacteria of ingested inorganic Hg(II) from the water column (Rudd et al., 1980).

3.1.2. Some people are colonized by very active Hg methylating bacteria. Interestingly, in addition to the bacteria considered in these experiments, it is also now known that sulfate reducing

bacteria (SRB's) of the same genus as those which are the most effective methylators in lake sediments (Compeau and Bartha, 1985) also live in the intestines of ca. 50% of healthy humans (Gibson et al., 1993b). These bacteria use sulfates from sloughed intestinal cells and their numbers can also vary with the sulfur content of the diet. Since they produce the corrosive gas H_2S, they have been suspected of a role in one or more chronic bowel disorders. Curiously, in one of the few consistent patterns that has yet been demonstrated in the human bowel microbiota, people who have high numbers of SRB's tend to have low numbers of a completely unrelated "hydrogen-consumer", the methanogens (which do not methylate Hg) and vice versa: those high in methanogens are low in SRB's (Gibson et al., 1993a). The basis for such preferential colonization and whether it is life-long are not known, however, it is a formal possibility that a bowel composition high in SRB's might be risk factor for those with amalgam restorations. It is also a potential confounding factor in assuming that all Hg in hair is methylmercury derived exclusively from fish. Fortunately, molecular techniques are available to quantify the composition of the gut community and identify appropriate case and control cohorts to resolve these questions. It would be good to know the methylation potential of the oral and gut microbiota because ca. 95-99% of ingested or inhaled Hg is eliminated through the GI tract and only 1-5% through urine (Clarkson, 1997). Thus, there will be ample methylatable Hg(II) for the SRB's and other bacteria that cover the huge surface area of our digestive systems.

3.2. Reduction of Hg(II) to Hg(0) and re-absorption into circulation

An additional complexity is that a person may also be colonized with mercury resistant bacteria. Since the organomercurial lyase (MerB) is found in 15-20% of mercury resistant fecal *Enterobacteriaceae* (Liebert et al., 1997), methylmercury formed by SRB's might be demethylated to Hg(II) which although toxic is less lipid-soluble and, thus, less mobile than methylmercury. However, Hg(II) is the substrate for MerA (Fig. 2) and will be rapidly reduced to monoatomic Hg(0) vapor which is relatively inert and, thus, will not be methylated, but it is very lipid soluble and can diffuse through the gut epithelium membrane back into circulation. The precise permeability of the gut epithelium to monoatomic Hg(0) vapor has never been measured but the corresponding value for the lung epithelium has been measured at 80% permeation (Magos and Clarkson, 1978). Thus, mercury volatilizing bacteria in the GI tract might initiate an enterohepatic cycle in which each mercuric ion is reduced to Hg(0) and diffuses through the gut epithelium back into circulation where it can be oxidized to reactive, toxic Hg(II) by catalase in the red blood cells (Hursh et al., 1988) and be delivered to all organs and tissues of the body. Although laboratory data on this point are still lacking, on balance, it would seem that the enzymatic activities provided by the *mer* operon are good for the bacteria and perhaps not so beneficial for their human hosts.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Amalgams and antibiotic resistance. The spectre of a world where antibiotics no longer work is upon us. Thousands of people die each year in the developed world alone from infections that are no longer treatable by any antibiotic (McGowan, 1991; Witte et al., 2002; Office, 2004).The problem is especially acute in the very young and in the elderly as well as for the immunocompromised. Physicians everywhere are working to minimize the use of antibiotics, especially those of "last resort" and the general public has been educated not to demand antibiotics for colds and flu's for which they are not effective. The EU has taken draconian measures to eliminate the use of antibiotics in the food animal industry (http://www.engormix.com/e_news_view.asp?news=8303&AREA=GDL) and equally severe measures are under consideration by the US Congress (http://www.tufts.edu/med/apua/News/AnimalFeed.html).These efforts directed exclusively at the use of antibiotics themselves were begun almost two decades ago and have grown steadily in all sectors of antibiotic use. However, antibiotic resistance continues to spread, so, there must be a missing factor that is overlooked despite our best efforts. A reasonable candidate for this missing factor is amalgam Hg. Amalgam is still the most widely used restorative material in developed countries and the only material available in poorer countries and in the poorest segments of richer nations (Lorscheider et al., 1995). The EU has recently prohibited use of antibiotics as growth

promoters in food animal production because it fosters the increase of antibiotic resistance. Perhaps it is time to recognize that amalgam is fostering the spread of antibiotic resistance directly in humans and should be phased out for that reason as well as for its direct toxic effects.

4. 2. Amalgams as a direct source of methylmercury. The myth that Hg in amalgams is "inert" is still actively promulgated by the dental industry and is generally believed even by educated non-specialists, including physicians. Even those with some knowledge of Hg's remarkable chemical plasticity largely limit themselves to thinking of industrial or abiotic processes for oxidation, reduction and methylation. Those aware of biotic methylation likely are also aware that the agents of this transformation are anaerobic bacteria living in acidified lake sediments. However, fish in many non-acidified rivers, lakes, and the ocean also have actionable levels of Hg, presumed in all cases to be methylmercury (Clarkson, 1997). As noted above, if provided with inorganic Hg(II) in the water, the commensal gut microbes of such fish methylate Hg and this endogenously methylated Hg ends up in their muscle tissue (Rudd et al., 1980).

Notwithstanding the actual chemical form of all Hg in all fish, it is the case that those who eat fish high in methylmercury do excrete more Hg in their urine and in their hair (assumed to be methylated). Urinary and hair Hg excretion both decline when fish consumption ceases (Grandjean et al., 1997). However, what has rarely been measured is how much fish Hg ends up in other tissues of consumers compared to what is excreted. Is it possible that, unlike the methylmercury chloride used in toxicology experiments, the methylmercury in fish, recently shown to have a sulfide, not a chloride, ligand (Harris et al., 2003), is largely excreted? This is a valid question given that the recently reported Faroe Islands (Grandjean et al., 2004) and Seychelles (Axtell et al., 1998; Myers and Davidson, 1998) studies found only a few, very slight or no effects, respectively, in children as a function of fish consumption by their mothers. A very important recent report (Berntssen et al., 2004) has shown that rats excreted more Hg from regular fish tissue (i.e. with its normal form of Hg) than from Hg-free fish tissue doped with methylmercury chloride. The latter preparations also delivered more Hg to blood, liver, kidney, and brain than did the diet of regular fish. As these data begin to raise questions about the long believed dangers of fish methylmercury, studies are warranted to measure methylmercury formation in a model animal not consuming fish but exposed to Hg via amalgams. If commensal bacteria methylate *in situ* the Hg derived from their human host's dental fillings, this would be an entirely new dimension of risk for amalgams. All existing evidence points to this as a real possibility and the question must be asked in an explicit and well-controlled manner.

In summary, there is currently a renaissance of interest in the contributions of the commensal microbiota to many aspects of health and disease from digestion to the development of the immune system and even to effects on the central nervous system (Yan and Polk, 2004; Kanauchi et al., 2005; Macdonald and Monteleone, 2005). One traditional area upon which this new perspective has yet to impinge is toxicology. The vigorous metabolism of all forms of Hg by bacterial members of the commensal microbiota is a rich example of what our small fellow travelers can marshal for their defense against the toxic agents to which we expose ourselves. Hopefully, toxicologists, especially those concerned with the widespread exposure to Hg from amalgam restorations, will begin to collaborate with microbiologists to take into account these bacterial processes (Fig. 1) in understanding the risks of amalgams and devising means to protect those with them and those having them removed.

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The Biotic Hg Cycle

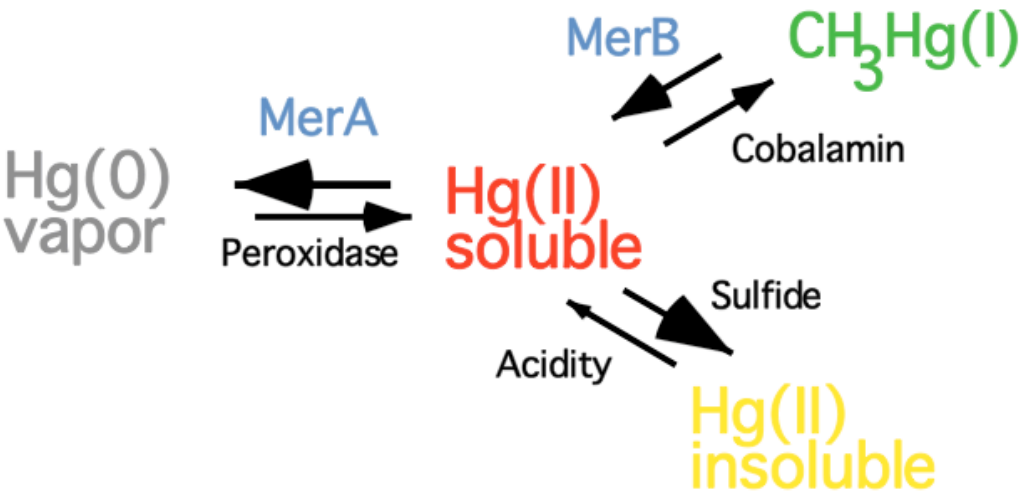

Figure 1. The reactions of the biotic mercury cycle. MerA and MerB are enzymes encoded by the *mer* locus, which is commonly carried by plasmids and transposons in many eubacteria. Most aerobioc bacteria encode peroxidase/catalase activities and cobalamin (vitamin B12) is a widely found metabolic cofactor used in biosynthesis in many bacteria as well as higher organisms. It apparently methylates mercury spontaneously as soon as it is enzymatically methylated to methyl-cobalamin (Wood et al.,). The ability to make sulfide (S-) is characteristic of anaerobes called the sulfate-reducing bacteria (SRB's) that normally grow at neutral pH. As long as the pH remains high, HgS (mercury sulfides) are insoluble. Environmental changes leading to a decrease in pH or an increase in sulfides can re-solublize the Hg(II) by displacement or by the formation of poly-metal sulfides, respectively.

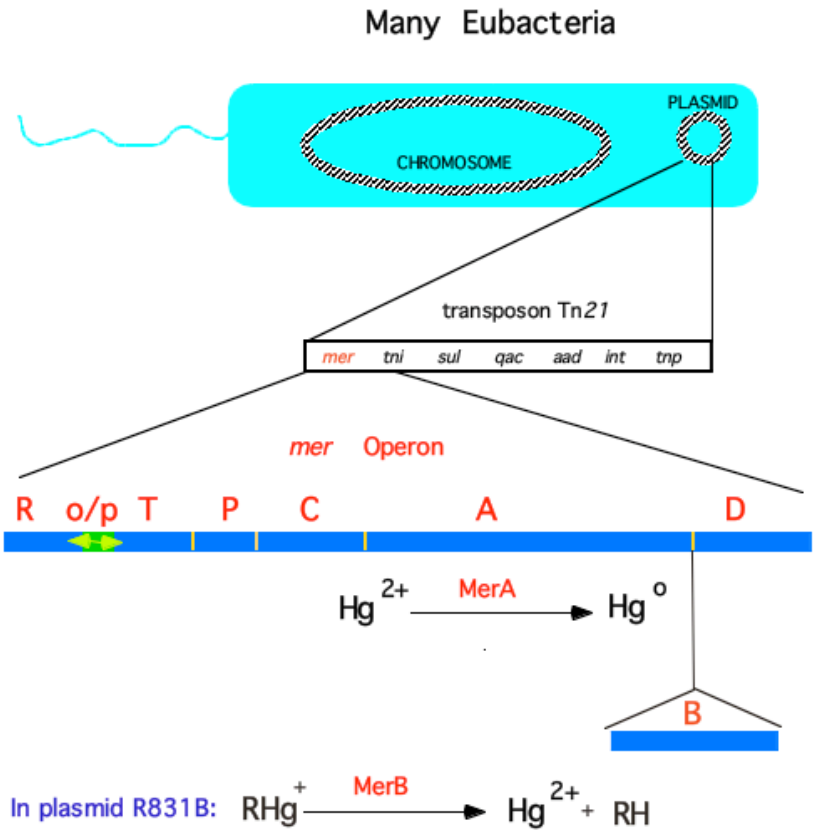

Figure 2. The Genetic Basis for Co-selection of Antibiotic Resistance Genes by Exposure to Mercury. Many common bacteria (eubacteria) have, in addiiton to their circular DNA chromosome, additional small circles of DNA called plasmids. The plasmids themselves can move with relative freedom to other bacteria. In addition, certain regions of plasmids called (transposons) can "jump" (recombine randomly) from the plasmid to the chromosome or to other plasmids in the same cell. One very widely found arrangement is called transposon 21 (Tn*21*). It contains mercury resistance genes (the *mer* operon) and several genes for resistance to antibiotics, sulfadiazine (*sul*) and streptomycin, *aad*; and disinfectants, *qac* (for quaternary ammonium compounds) as well as a novel recombinase called an integrase (*intI)* responsible for establishing and rearranging these tandem arrays of resistance genes. Exposure of any bacterial cell carrying such a plasmid to mercury will select indirectly (co-select) for resistances to the non-metallic genes for resistance to all the agents.

Table 1. Formation of Methyl Mercury by Human Commensal Microbes

Authors	Year	Material		[Hg(II)] Substrate (μM) (Range)	μg MeHg formed/g bacteria•day[a]	Estimated μg MeHg formed/day[b]
Vonk & Sipjesteijn	1973	E.coli		74	0.1	0.45
Rowland et al.	1975	E.coli, Streptococcus		18	3.3	15
		Rat caecal contents		not reported	0.026	12
Edwards & McBride	1975	Human feces		7	0.0025	1
Heintze et al.	1982	Oral streptococci		37	197	894
Liang & Brooks	1995	Saliva		1	(0.12 ng/ml)	(0.06 μg)[c]
Leistevuo et al.	2001	Saliva w/ amalgams	inorganic	0.160 (0.002 - 3.2)		16 [c]
			organic	0.014 (0 - 0.174)		1.4 [c]
		Saliva	inorganic	0.004 (0 - 0.009)		0.4 [c]
			organic	0.004 (0 - 0.011)		0.4 [c]

[a] Columns 5-6 calculated using rate data reported in different formats in each paper.
[b] Assumption: 454 g feces per adult human lower bowel of which *E.coli* and/or *Streptococcus* are 1% at maximum.
[c] Assumption: 500 ml of saliva per day is swallowed.

Table 1 is based on the following reports: Vonk & Sijpesteijn (1973) Antonie van Leeuwenhoek 39:505-513; Rowland et al., (1975) Experientia 31:1064-65; Heintze et al. (1983) Scand. J. Dent. Res. 2:150-152; Liang & Brooks (1995) Wat. Soil Air Poll. 80:103-107; Edwards & McBride (1975) Nature 253:462-464; and Leistevuo et al. (2001) J. Dent. Res. For each paper the methyl-mercury data supplied by the authors were used, with the indicated assumptions, to estimate the total possible methyl-mercury output from the gut contents. In our own experimental work with monkeys (1), Hg(II) content in feces of animals with from 12-16 molar occlusal fillings ranges from 1.5 mM during the week after fillings are installed to 5-10 μM, steady state when fillings have been in for ca. 2 months. Clearly, these estimates for methyl mercury production vary wildly owing to the many different approaches used by the authors in their respective single experiments as well as to the simplicity of the assumptions. However, the estimates indicate the possible production of methyl-mercury equivalent to several servings of tunafish per day using only the Hg(II) released from amalgam fillings.

It is well established that the most important environmental methylators of mercury are the sulfate reducing bacteria (SRB), anaerobic bacteria that reside in freshwater sediments (2). As it happens, these bacteria can frequently be stable members of the oral and intestinal microbiota of humans (3) and, thus, are the most obvious candidates for methyation of Hg(II) arising from amalgam restorations. Lastly, it is important to note that the major source of variation in the real world is the highly idiosyncratic composition and behavior of human commensal communities which can vary as a function of host genetics, diet, age, and general health and can be modulated episodically by exposure to antibiotics and other drugs. These realities require much more sophisticated epidemiological analyses to evaluate the above possibility than have yet been applied to any aspect of the amalgam toxicity question.

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