

Medical bioremediation: prospects for the application of microbial catabolic diversity to aging and several major age-related diseases

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Abstract

Several major diseases of old age, including atherosclerosis, macular degeneration and neurodegenerative diseases, are associated with the intracellular accumulation of substances that impair cellular function and viability. Moreover, the accumulation of lipofuscin, a substance that may have similarly deleterious effects, is one of the most universal markers of aging in postmitotic cells. Reversing this accumulation may thus be valuable, but has proven challenging, doubtless because substances resistant to cellular catabolism are inherently hard to degrade. We suggest a radically new approach: augmenting humans' natural catabolic machinery with microbial enzymes. Many recalcitrant organic molecules are naturally degraded in the soil. Since the soil in certain environments – graveyards, for example – is enriched in human remains but does not accumulate these substances, it presumably harbours microbes that degrade them. The enzymes responsible could be identified and engineered to metabolise these substances *in vivo*. Here we survey a range of such substances, their putative roles in age-related diseases and the possible benefits of their removal. We discuss how microbes capable of degrading them can be isolated, characterised and their relevant enzymes engineered for this purpose, and ways to avoid potential side-effects.

1. Introduction

Mammalian cells possess numerous components that degrade damaged or otherwise unwanted material. These range from single-polypeptide proteolytic enzymes present throughout the cell and in the extracellular milieu, more complex assemblies such as the proteasome, up to the most versatile degradation machinery of all, the lysosome. The term “lysosomal storage disease” (LSD) describes a range of inherited defects in the ability to break down material that would normally be degraded in the lysosome. Such material consequently accumulates, resulting in diminishing cell and tissue function and premature death (Schiffmann and Brady, 2002).

The fully-endowed lysosome is not omnipotent either, however, and material accumulates in various cells of all individuals (though more slowly than in LSDs). The most ubiquitous example of this is a highly heterogeneous protein- and lipid-rich amalgam termed lipofuscin, which is seen in virtually all postmitotic cells throughout the animal kingdom. It is thought to derive largely from oxidatively damaged components of mitochondria; it accumulates especially rapidly in highly oxidative cells such as

cardiomyocytes, in which it reaches roughly 10% of total cell volume by old age (Nakano et al., 1993). Compelling *in vitro* studies support the view that it impairs lysosomal function and thence general cellular performance (Brunk and Terman, 2002). However, it remains to be demonstrated conclusively that the levels of lipofuscin observed *in vivo* are significantly deleterious. In this article we will therefore focus on three cases in which both the chemical composition and the possible causal role of the accumulating material are clearer, while noting that the same concepts discussed here are equally applicable in principle to lipofuscin. The three cases are:

- cholesterol and related molecules in arterial macrophages;
- certain specific proteins in neurons;
- A2E (a derivative of vitamin A aldehyde) and related molecules, in the retinal pigmented epithelium (RPE) of the eye.

It should be noted that the aggregate which forms in the RPE consists of a family of related compounds, of which A2E may be one of the most abundant. It is fluorescent and resistant to degradation, leading to its often being termed “lipofuscin”, but its spectral properties are distinct from the standard lipofuscin mentioned above. Thus, for clarity in the present discussion of several different substances in different tissues, we hereafter refer to the retinal aggregate as “bisretinoid-based material”.

In all these cases there is abundant evidence of the progressive impairment of cellular function, leading eventually to cell dysfunction and death, and there is strong evidence that the substances just mentioned (or some subset of them) play a primary causal role in bringing this dysfunction about. The pathology it causes is clear: it leads, respectively, to advanced atherosclerosis, neurodegeneration and macular degeneration. (These diseases will hereafter be described collectively as “age-related storage diseases”.) Atherosclerosis is the cause of the large majority of heart attacks and strokes, which together are the most common cause of death in the industrialised world. Neurodegeneration, especially Alzheimer’s disease, is the most common cause of debility in the industrialised world. Macular degeneration is the most common cause of blindness in the elderly. Hence, any intervention that removed these substances would be a highly promising candidate for alleviating ailments that account for a large proportion of global health expenditure, and indeed a proportion that is set to increase as life expectancy continues to climb. The social and economic consequences of such an advance need not be belaboured.

One of the more unfortunate side-effects of humanity’s technological ingenuity is the increasing abundance of waste products in the environment. Increasingly this is being recognised and addressed by the preferential use of material that is rapidly biodegradable, but this cannot of itself diminish the legacy of more durable substances that we have been discarding for so long. Fortunately, a wide range of substances that would not normally be considered biodegradable appear not to accumulate in the environment at the rate that might be expected from their rate of release. This observation has led to the realisation that essentially any organic, energy-rich compound introduced into the environment is a potential nutrient for microorganisms that encounter it. Compounds that are initially resistant to biodegradation therefore constitute a strong selective pressure on microbial life in their vicinity to evolve the ability to degrade them. There will be a selective advantage for microorganisms that out-compete others by extracting energy from difficult-to-degrade compounds, instead of growing very rapidly by utilizing easily degradable compounds: such microorganisms must occupy an ecological niche by being more “tortoises” than “hares,” so to speak. This concept was voiced over 50 years ago by Gayle, who termed it the “microbial infallibility hypothesis” (Gayle, 1952). Since that time, interest in microbial catabolic diversity has flourished, inspiring the commercially highly successful area of environmental microbiology and biochemistry known as bioremediation. Bioaugmentation is a bioremediation strategy for environmental decontamination by isolation and expansion of the bacteria or fungi responsible for this process and their reintroduction into the contaminated area in quantities sufficient to deplete the contaminant to levels acceptable for the area’s intended use.

The relevance of bioremediation to age-related storage diseases may not be immediately apparent – and indeed was first noted, by one of us (A. de G.) only recently (de Grey, 2002). It arises from the fact that

certain environmental sites are enriched in human remains, and thus in the substances that accumulate pathogenically with age in human tissues. These substances fit the criteria of being organic and energy-rich – and, sure enough, there is no evidence of them in locations where human material that does not fit those criteria (such as bone) indeed accumulates. This circumstantial evidence for the existence of microbes able to degrade such substances was provisionally reinforced by a pilot study (de Grey and Archer, 2001) which, suggested that microbes capable of digesting at least some constituents of lipofuscin could be isolated from the soil with ease. de Grey (de Grey, 2002) proposed a therapeutic approach to the age-related storage diseases, involving the identification of enzymes responsible for this activity, their engineering for appropriate localisation and function in mammalian cells, and their introduction by either gene therapy or enzyme replacement therapy as a means of removing the offending material.

In this article we explore this proposal in depth, surveying the present state of knowledge of the etiology of age-related storage diseases, the available mechanistic hypotheses implicating accumulation of degradation-resistant substances in the progression of these conditions, the prospects for applying the tools of bioremediation to eliminate those substances, and the options for addressing potential side-effects of such a therapy. We conclude that this strategy holds exceptional promise as a technique for preventing and treating several of the most fearsome medical challenges facing the industrialised world today. The increasing incidence of these diseases that will result from the rising life expectancy forecast to occur throughout the developed world in coming decades makes the exploration of radical but potentially very powerful approaches to these diseases all the more urgent.

2. The major age-related storage diseases

2.1. Age-related macular degeneration (AMD)

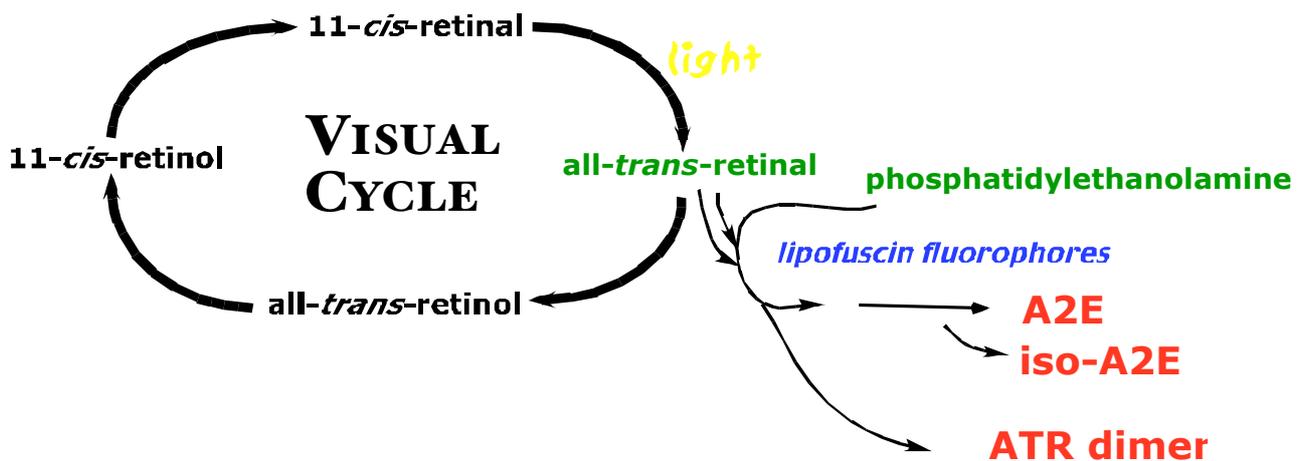


Figure 1. The visual cycle of light absorption and the formation of A2E.

The biochemical basis of vision is the absorbance of photons by molecules that convert their energy into electrical impulses transmitted to the optic nerve and thence to the visual cortex. The first step in this process is the light-driven stereoisomerisation of 11-*cis*-retinal to all-*trans*-retinal. Normally, all-*trans*-retinal is then enzymatically reduced to all-*trans*-retinol, stereoisomerised to 11-*cis*-retinal and oxidised back to 11-*cis*-retinal, which can then initiate another cycle. Rarely, however, all-*trans*-retinal spontaneously reacts in a 2:1 ratio with the major membrane phospholipid phosphatidylethanolamine to form a phosphatidyl-pyridinium bisretinoid termed A2PE. A2PE then undergoes phosphate hydrolysis, probably mainly via the action of phospholipase D, to form A2E (Figure 1) (Sparrow et al., 2003c). [It should be noted that while A2E is often said to be *N*-retinylidene-*N*-retinylethanolamine, that name describes a structure inconsistent with A2E.] A2E spontaneously and reversibly stereoisomerises, under the influence of light, to a form known as iso-A2E. This isomer constitutes roughly 20% of the total A2E

at equilibrium; other stereoisomers of A2E are also seen at lower abundance (Parish et al., 1998; Ben-Shabat et al., 2002a). Intriguingly, it may be that A2E is more often a product of rods than cones, since A2E levels are particularly low in the fovea, the central area of the retina in which all photoreceptor cells are cones (Delori et al., 2001).

The visual cycle described in Figure 1 happens partly in the photoreceptor cells and partly in the adjacent cells of the retinal pigmented epithelium (RPE), which exchange retinol and retinal with the photoreceptor cells. The RPE has a second, equally crucial role in supporting the photoreceptor cells: it is responsible for the degradation of photoreceptor cell membrane that has been discarded for the purpose of membrane renewal. It achieves this by daily phagocytosis of a small portion of the least-recently synthesised sections of the photoreceptor outer segment (Boulton and Dayhaw-Barker, 2001). That material is thereby enclosed in an endosome within the RPE cell, which subsequently fuses with acidified organelles (lysosomes) to initiate the degradation of the material. Most of the material is rapidly disposed of, but A2E is evidently not a substrate for any lysosomal enzyme and therefore remains, reaching up to 20% of the RPE cell by mass in old age (Feeney-Burns et al., 1984). RPE cells with high loads of such aggregates eventually begin to atrophy; consequently, the photoreceptor cells that require RPE cell support subsequently die, leading to progressive loss of sight (Zarbin, 2004).

Multiple mechanisms have been identified whereby A2E may be toxic to RPE cells. Firstly, its amphiphilic structure causes it to accumulate in membranes to the detriment of their integrity (Sparrow et al., 1999). Second, it can itself absorb light, and blue light absorption by A2E promotes the creation of singlet oxygen ($^1\text{O}_2$), a highly reactive high-energy state of molecular oxygen (Sparrow et al., 2000, 2002; Ben-Shabat et al., 2002b). A2E then reacts with $^1\text{O}_2$, resulting in multiple epoxide moieties on both carbon chains and rings; unfortunately, these A2E epoxides are themselves highly prone to react with various macromolecules including DNA and proteins (Sparrow et al., 2003a, 2003b). Finally, it was recently reported that A2E binds to and inhibits the vacuolar ATPase, the enzyme responsible for maintaining a low intralysosomal pH (Bergmann et al., 2004). Even a slight alkalisation of the lysosome is potentially highly detrimental to the activity of many of its constituent enzymes, as will be noted below.

2.2. Atherosclerosis

The major cause of heart attacks and strokes is the rupture of an atherosclerotic plaque and the resultant formation of an emboli or occlusive thrombi which inhibits blood flow to the heart or brain. Thus, treatments aimed at preventing heart attacks and strokes typically target the atherosclerotic plaque, and especially those with a relatively thin, easily ruptured surface encasing the lipid-rich core (Shastry, 2003). This approach has proven highly challenging, because such lesions tend to grow slowly and asymptotically becoming progressively but silently more rupture-prone. The early stages of this process actually begin during childhood (Strong et al., 1958). Once begun, lesion growth is in part driven by the continuous arrival of monocytes from the blood, attracted by the inflammatory signals that the cells of the plaque secrete (Libby, 1992; Stary et al., 1994; Babiak and Rudel, 1987; Johnson et al., 1997). Within the artery wall the monocytes differentiate into macrophages and engulf lipid that is present in the extracellular space of the arterial intima in the form of lipoproteins (primarily native and modified LDL) and lipid particles (Hoff and Morton, 1985; Guyton and Klemp, 1994). The lipid-engorged macrophages are called foam cells because of the foamy appearance that excess lipid imparts (Figure 2). The major stored lipid in the artery wall and foam cells is cholesterol and cholesteryl esters (Brown and Goldstein, 1983). Some of the initial cholesterol accumulation is the result of lysosomal degradation of cholesteryl esters to free cholesterol in lysosomes, export of free cholesterol out of the lysosome, and the subsequent reesterification to a fatty acid in the cytoplasm to form a cholesteryl ester droplet (Brown and Goldstein, 1983). However, as the disease progresses, much of the internalized cholesterol becomes trapped in lysosomes (Peters et al., 1972; Jerome and Lewis, 1990). Thus, as in the RPE, this later, more clinically important stage, is a classic example of homeostasis gone awry, in which the cells attempt to engulf and digest the material they encounter but are unable to degrade it. This can accentuate cell death and further exacerbate the problem (Johnson et al., 1996; Lucas and Greaves, 2001).

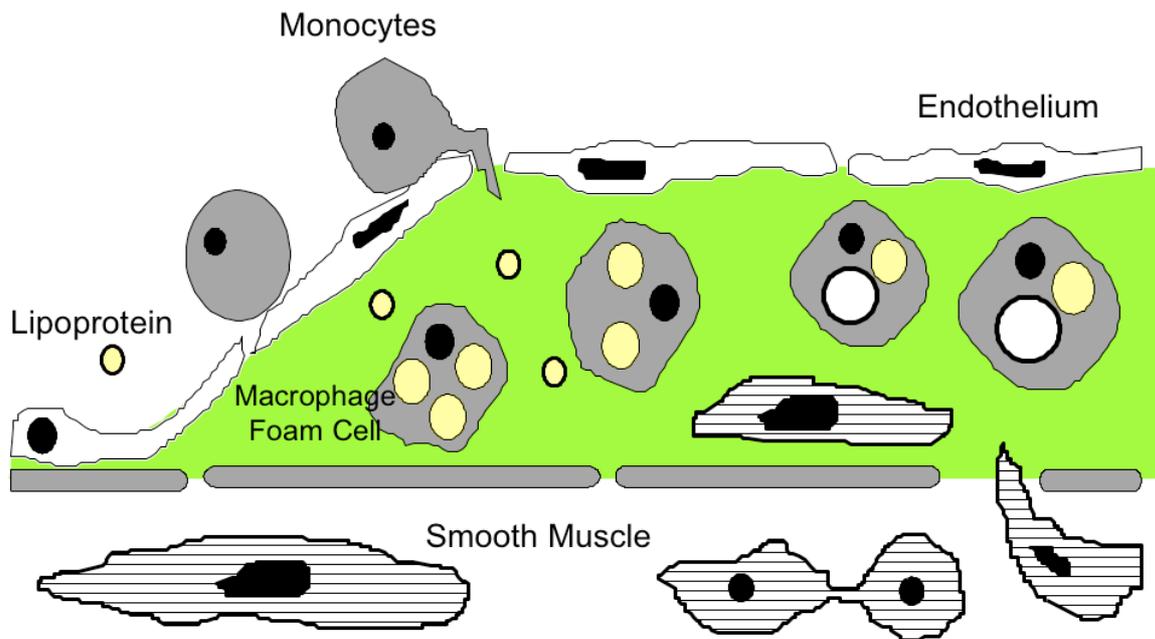


Figure 2. The early etiology of atherosclerosis.

The reasons for the lysosomal accumulation are not clear. However, it does indicate that the digestive processing of plaque lipids is error-prone. Moreover, various modifications such as oxidation and aggregation can promote the retention of such particles in the artery and accelerate their uptake by macrophages (Jerome and Yancey, 2003). These “foam cells” are still alive but can perform only limited further lysosomal function (Yancey and Jerome, 2001). The macrophages in the artery wall continuously engulf new modified lipoproteins and other acellular lipid material that increasingly dominates this lipid-rich core as a result of the death and disintegration of foam cells. A mature plaque becomes increasingly chaotic at the histological level and more susceptible to rupture. Thus, alleviating lysosomal accumulation could significantly decrease plaque size and vulnerability.

LDL containing oxidized lipids have been identified within atherosclerotic lesions. (101, 76). This observation gives rise to one of two hypotheses for the molecular mechanism of lysosomal lipid accumulation, that oxidized lipids or other toxic materials inhibit lysosomal hydrolysis of cholesteryl ester (Yla-Herttuala et al., 1989; Rosenfeld et al., 1991; Maor et al., 1995; O'Neil et al., 2003). Although only a minor component of the lipids, oxidized sterols can be very potent. Indeed, the major oxidized form of cholesterol present in oxidized LDL, 7-ketocholesterol (7KC), causes lysosomal sequestration of esterified native cholesterol as well as esterified 7KC (Jessup et al., 2004). The bulk of cholesterol in an LDL particle is in the esterified form, and this is hydrolysed by lysosomal acid lipase to release free (unesterified) cholesterol (Johnson et al., 1997). Thus, inhibition of hydrolysis by a minor contaminant of LDL would prevent cholesterol export from the lysosome by preventing processing of the ester. Foam cell lysosomes have an abundance of free as well as esterified cholesterol, however, so failure of de-esterification cannot be a complete explanation for lysosomal sequestration (Dhaliwal and Steinbrecher, 2000). The export of free cholesterol from the lysosome is a poorly understood process that depends on specific cholesterol transporters and perhaps also on the intralysosomal pH (Liscum and Munn, 1999; Lange et al., 2002). Modified lipids could inhibit export of cholesterol by poisoning the vacuolar ATPase (V-ATPase) that maintains the acidic lysosome environment or by inhibiting transporters. This could be instead of or in addition to inhibiting cholesteryl ester hydrolysis.

The second hypothesis is inspired by two major challenge to the first. One is that only a minority of the lipid in lesions consists of plausibly toxic substances, principally various oxidation products of cholesterol (Jerome and Yancey, 2003). The other is that unoxidised LDL when vortexed so as to

aggregate before exposure to macrophages, produces lysosomal cholesterol accumulation at a rate comparable to that with oxidised LDL (Khoo et al., 1988). The ability to induce engorgement without any chemical modification of the LDL appears irreconcilable with the “toxic contaminant” hypothesis outlined in the previous paragraph. Thus, the second hypothesis states that macrophage engorgement is caused not by small amounts of a toxic compound but by large amounts of a normally non-toxic one, such as free or esterified cholesterol. The arrival in the lysosome of a bolus of lipid considerably larger than an LDL particle may overwhelm the cholesterol processing machinery and cause intralysosomal pH – or perhaps other aspects of the lysosomal milieu that are necessary for appropriate enzymatic function – to become altered so severely that it cannot recover. Moreover, increased in the concentration of free cholesterol in membranes is known to effect membrane function and has been suggested to inhibit proton pumps (D'Souza et al., 1987).

In reality the inhibition of lysosomal lipoprotein hydrolysis is probably complex, multifactorial, and may involve aspects of both hypotheses. Nonetheless, for both hypotheses it is easy to envision that screening of microbial products could uncover either single molecules or, more likely, a group of separate products capable of remediating specific or multiple defects. Such molecules might remove the toxic contaminants, bypass the metabolic block, replace non-functional enzymes, and/or alter the lysosomal membrane or luminal contents in ways that would re-establish hydrolysis and clearance of cholesterol. If found, these bioremediation agents potentially could alter the rupture-vulnerable late-stage plaque, creating one less likely to produce thrombosis or emboli.

2.3. Neurodegeneration

Alzheimer's disease (AD) is by far the commonest neurodegenerative disease. The hallmark of AD is the presence of two distinct aggregates. One, the β -amyloid plaque, is extracellular and its main constituent is a peptide 40-43 amino acids long, called amyloid beta ($A\beta$) peptide. The other, the neurofibrillary tangle (NFT), is found within neurons and is mainly composed of a different protein, tau, that is present in tangles in an abnormally hyperphosphorylated state. Other neurodegenerative diseases are also characterised by aggregates of distinctive morphology and composition, which, in these cases, are only intracellular. For example, sufferers from Parkinson's disease accumulate Lewy bodies, which predominantly consist of the protein α -synuclein (Bharath and Andersen, 2004); Huntington's disease shows aggregates of huntingtin (Shimohata et al., 2002; Bulteau and Friguet, 2004); and spinocerebellar disorders and motor neuron diseases accumulate specific proteins encoded by additional causative gene mutations (Schools et al., 2003; Wood et al., 2003).

We focus here mainly on the problem of intracellular aggregates. Regarding the exceptional example of the extracellular amyloid plaque, recent studies have shown that vaccinating mice against $A\beta$ causes its uptake and apparent rapid degradation by microglia (the brain's equivalent of the macrophage), indicating that it may be easily handled by a normally functioning lysosome (Schenk et al., 1999). This approach has already entered clinical trials; first-generation vaccines proved to have unacceptable vascular side-effects (Schenk, 2002), but improved versions are being developed. It should be noted, however, that a promising approach to slowing the increase of plaque burden is to stimulate the degradation of $A\beta$ while it is still intracellular. Indeed, there is growing suspicion that accumulating $A\beta$ monomers or oligomers inside cells are more toxic than the fibrillar aggregates outside (Ghosh et al., 2003; Kakkis et al., 2004). Much of this $A\beta$ is formed in, or accumulates in, the endosomal-lysosomal system of neurons (Takahashi et al., 2002; Cataldo et al., 2004) and astrocytes, the most abundant type of glial cell (Nagele et al., 2004), in the latter case possibly via endocytosis of plaque material and secreted $A\beta$ peptide. The havoc that $A\beta$ in these cellular compartments may wreak on proteolytic function and on the integrity of hydrolase-laden lysosomes is only now beginning to be explored (Glabe, 2001; Nixon, 2005).

An intriguing feature of the intracellular aggregates in the various neurodegenerative diseases is that they are not always intralysosomal. Perhaps the clearest situation is in certain polyglutamine repeat diseases, where the largest aggregates (aggresomes) tend to be found close to the nuclear membrane in the vicinity of the microtubule-organising centre (Shimohata et al., 2002). This finding suggests that cytosolic

aggregation is a directed process, with target proteins being trafficked along microtubules; indeed, mutation of a microtubule attachment protein impairs aggresome formation (Kawaguchi et al., 2003). The purpose of this process is postulated to be a reduction in the target proteins' toxicity by simple surface-to-volume manipulation – proteins embedded in an aggregate cannot interact with bioactive cellular components. The question remains, however, of why these proteins are not simply targeted to cytosolic proteases or, if that is ineffective, to the lysosome.

This paradox, together with the related one of why microglia do not spontaneously engulf senile plaques rather than having to be encouraged by vaccination, is in a way the main source of the debate currently raging within neurodegeneration research concerning whether aggregates are causal, innocuous or actively protective in the progression of disease (Mudher and Lovestone, 2002). An interpretation that we feel merits more attention than it has yet received is that extralysosomal (perhaps including extracellular, perhaps not) aggregation is a protective strategy that emerges as a fallback when the default lysosomal pathway is (by whatever means) disabled. For example, mutations in α -synuclein identified in Parkinson's disease patients inhibit its degradation by chaperone-mediated autophagy (CMA, discussed in detail below), a mechanism that efficiently degrades the wild-type protein (Cuervo, 2004). Aggregation is thus the cell's response to the increased cytosolic levels of the mutant protein. In this model the aggregates would not themselves be protective, but they would initially be innocuous and would become pathogenic only when their abundance began to impair the cell's ability to continue aggregating and "neutralising" newly-synthesised toxic material. Conversely, restoration or enhancement of lysosomal function may stimulate autophagy and lysosomal degradation of previously-formed aggresomes; indeed, stimulation of macroautophagy (a mechanism that delivers cytosolic components to lysosomes) in one type of glial cell, the Schwann cell, has been shown to clear aggresomes (Fortun et al., 2003).

Unlike macrophages, neurons can accumulate histologically conspicuous extralysosomal material during normal aging. In the example given above, the causative mutant protein fails to gain access to the lysosomal degradative compartment, thereby promoting its accumulation in the cytosol. A variant of this mechanism seems to occur in a mouse model of Huntington's disease (Ravikumar et al., 2004) where mutant forms of huntingtin reach lysosomes by autophagy but are inefficiently degraded and possibly may even interfere with endosomal-lysosomal function (Ravikumar et al., 2002) leading to altered proteolysis of other proteins. Pharmacologically stimulating lysosomal turnover (macroautophagy) not only reduces aggregate accumulation but also partially ameliorates neurologic deficits in these mice (Ravikumar et al., 2004). Primary failures of the lysosomal system are another well-known pathologic scenario leading to intralysosomal protein building, lysosomal dysfunction, and often prominent neurodegeneration, which are best illustrated by the inherited lysosomal storage diseases (Tyynela et al., 2000; Cooper, 2003).

Is there any relevance of these findings to AD? In AD, neurons also do not seem to accumulate histologically conspicuous material within their lysosomes, but something similar happens: autophagosomes and other autophagy-related compartments accumulate in striking numbers within dendrites of the affected neurons, often resulting in enormously distended expanses along the dendrite's length (Nixon et al., 2005). Autophagosomes are double-membrane-bounded structures created by the intracellular process of macroautophagy, whereby a free piece of membrane (probably derived from the endoplasmic reticulum) grows, with a curved morphology, until it eventually surrounds a portion of cytoplasm (Yoshimori, 2004). The trapped cytoplasm may contain proteinaceous or other aggregates, organelles such as mitochondria and peroxisomes destined for destruction, or other soluble cytosolic components. Destruction is achieved in very much the same way that cells liberate cholesterol from LDL: the initially neutral vesicle fuses with an acidified lysosome. This first causes the degradation of the inner autophagosomal membrane and then of its contents. AD neurons exhibit a proliferation of abnormal autophagosomes, including multilamellar structures that presumably result from the necessary autophagy of damaged/degenerating neuronal membranes, organelles and proteins (Nixon et al., 2005). The most straightforward interpretation of this phenotype is that the process of fusion of autophagosomes with lysosomes is faulty in these neurons. This process has also been shown to be impaired during normal

aging (Cuervo, 2003).

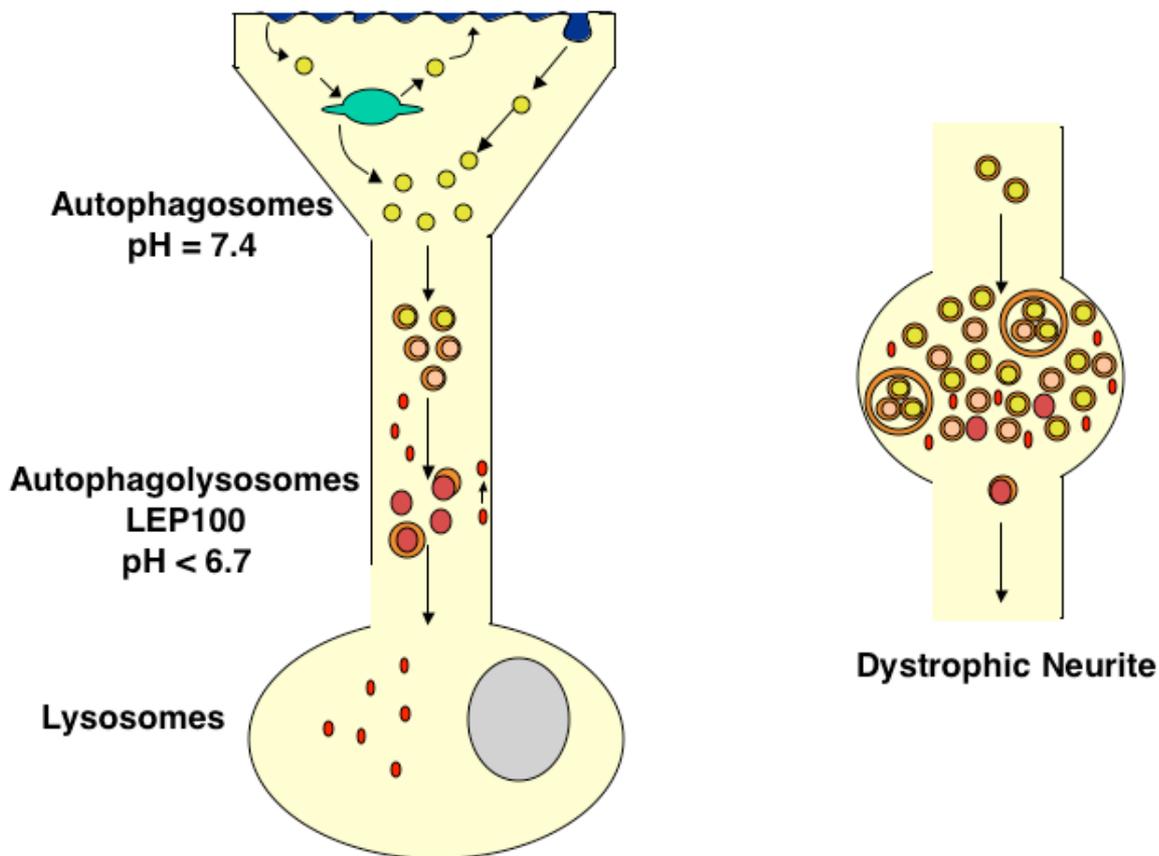


Figure 3. A model for lysosomal impairment in AD.

What might cause this defect? A strong hint comes from the remarkable overlap of risk factors for AD and atherosclerosis. For example, apolipoprotein E genotype is the most overt genetic risk factor for both diseases, with the e4 allele conferring a many-times greater risk of developing them at a given age than the e2 or e3 alleles (Mahley and Huang, 1999). This suggests that, as in atherosclerosis, the causative agent in AD may be cholesterol or a derivative. Similarly, a convergence of mechanisms is represented by Niemann-Pick type C disease (NPC), a lysosomal storage disorder arising from defects in the cellular trafficking of cholesterol (Pentchev et al., 1994; Carstea et al., 1997; Vincent et al., 2003) and caused by mutations of a resident endosomal-lysosomal protein. NPC has attracted particular attention as one of a very few disorders in which NFT robustly form in the brain in the absence of tau mutations or β amyloid deposition (Love et al., 1995; Ohm et al., 2003). In addition to sharing the tau-related neurofibrillary pathology with AD, NPC also shows abnormal amyloidogenic APP processing, another highly characteristic endosome dysfunction seen in AD (Nixon, 2004). Intralysosomal pH is again a prime suspect, as inadequately acidified lysosomes do not efficiently degrade autophagosomes (Nakamura et al., 1997). Neurons are highly oxidative, so the autophagic pathway (which delivers mitochondria – perhaps preferentially damaged ones (de Grey, 1997) – to lysosomes) may be the main source of material for the neuronal lysosome. In the context of the “toxic contaminant” hypothesis for foam cell formation discussed earlier, a model for dysfunction of the lysosomal apparatus in neurons can thus be proposed (Figure 3), in which lysosomes are not unduly burdened with material that they have become unable to degrade but instead have become unable to access material that the cell needs to eliminate. A corresponding model analogous to the “bolus” hypothesis may also be possible. Accordingly, enzymes effective at preventing and treating atherosclerosis may be effective against neurodegeneration too: rather than seeking enzymes capable of degrading proteinaceous aggregates, we may merely need ones capable of reactivating the lysosomal apparatus, following which the accumulated proteinaceous material (whether it be A β , tau, or autophagosomes) may be summarily disposed of by existing proteolytic

functions. Straightforward experiments might shed light on this: for example, exposure of macrophages to aggregates of hyperphosphorylated tau would determine whether lysosomes can usually degrade it.

3. Delivery of exogenously synthesised lysosomal enzymes

In previous sections we have made the case that it is at least reasonable, if not proven, that all three diseases being considered here are ultimately LSDs – caused, via more or less direct routes, by the presence in lysosomes of material that impairs cell function. On this assumption, microbial enzymes capable of degrading this material could have enormous therapeutic potential. That potential can be realised, however, only if the enzymes can be effectively delivered to their target. We are fortunate that a precedent for the necessary technology exists in the form of treatments for early-onset, heritable lysosomal storage diseases.

Enzyme replacement therapy (ERT) is the treatment of a genetic deficiency by the intravenous injection of the missing enzyme, typically synthesised recombinantly or produced by gene activation. It is presently an FDA-approved treatment for two major LSDs, non-neuronopathic Gaucher disease and Fabry disease, and clinical trials are underway for a number of other LSDs. In each case, the cell type in which enzyme deficiency is most deleterious to tissue function is one easily accessible by intravenous injection: in Gaucher it is the macrophage and in Fabry it is the vascular endothelium (Brady, 2003). Enzyme injected into the circulation is taken up by cells if its glycoform is matched to a lectin on the cell surface; conveniently, different cell types have lectins that recognise different sugars (Das et al., 1985). It has thus been possible to introduce, via endocytosis, therapeutic quantities of the enzymes for which Gaucher and Fabry patients are genetically deficient into the lysosomes of the appropriate cells. One consequence is that more than 4,000 Gaucher sufferers are now receiving enzyme replacement therapy; many patients are now able to lead normal productive lives with minimal symptoms from long-term ERT (Brady, 2003). The ability to target particular cell types by modification of surface sugars is of considerable importance; moreover, glycosylation-independent targeting is now also being developed (LeBowitz et al., 2004).

The major downside of ERT is the amount of enzyme needed. Even with efficient targeting of the enzyme to the correct cells, injections must be frequent, simply because lysosomal enzymes themselves are only modestly protected from being degraded by lysosomal proteases. Accordingly, ERT is extremely expensive. A second shortcoming, addressed in more detail below, is that it cannot easily address neurological defects, because these enzymes do not naturally cross the blood-brain barrier when injected intravenously.

4. Engineering *in situ* synthesis of new lysosomal enzymes

An approach that would, in principle, circumvent these difficulties is to introduce not the enzyme but the gene encoding it. As with any genetic therapy, this may be achieved either by somatic transduction (typically using viruses to deliver the therapeutic gene) or by *ex vivo* manipulation of precursor cells such as bone marrow stem cells, which are introduced after a successful transfection has been verified. The latter approach has the advantage of greater safety, whereas the former is capable of reaching cells not readily repopulated from stem cell pools, including (for some delivery systems) postmitotic cells such as neurons (Lai and Brady, 2002). Gene therapy has not yet emerged as a therapy for LSDs, doubtless because safety concerns are still a major issue in all gene therapy applications; however, encouraging progress is being made (Hofling et al., 2004). Even in an optimistic scenario, it will be several years before any potentially therapeutic microbial enzymes have been isolated, re-engineered and tested in rodents and perhaps non-human primates; gene therapy may be considerably closer to the clinic by then.

4.1. The mannose 6-phosphate pathway

In addition to the “in bulk” delivery of cytosolic components to lysosomes through macroautophagy, selective delivery of soluble proteins to the mammalian lysosome is also possible through two intracellular pathways, both of which are triggered by signals on the target protein (Figure 4). Lysosomal enzymes are generally delivered by a vesicular pathway triggered by the presence of mannose-6-phosphate (M6P) adducts. M6P is attached to N-linked oligosaccharides of proteins with an appropriate

tertiary structure: there is apparently no specific primary sequence motif required. This occurs in the cis-Golgi network and causes appropriate sorting in the trans-Golgi network (Ghosh et al., 2003). Most lysosomal membrane proteins also follow vesicle-mediated pathways, but in contrast to the M6P-mediated pathway they are targeted via motifs present in their amino acid sequence (Eskelinen et al., 2003).

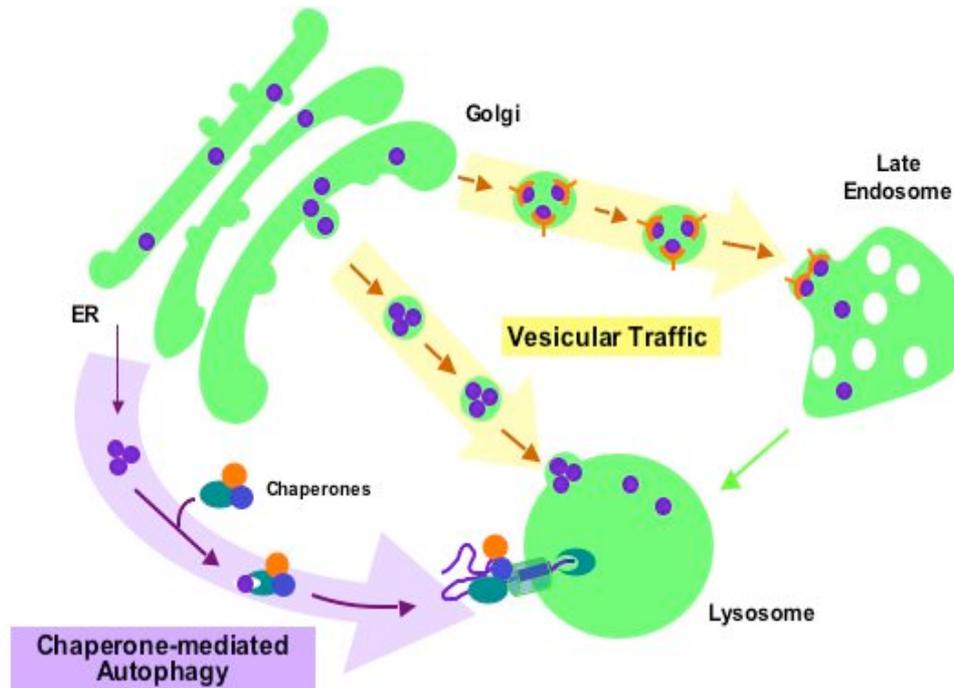


Figure 4. Pathways for the delivery of proteins to the lysosome. In mammalian cells selective delivery of proteins to lysosomes can take place via both vesicular and nonvesicular pathways. Most constituent lysosomal proteins that initially follow the secretory pathway (ER to Golgi), are sorted in the Golgi on account of particular motifs (M6P or amino acid sequence signals) and then packed into vesicles that fuse with late endosomes and lysosomes to deliver their contents. Selective cytosolic proteins containing a KFERQ-related motif are recognized by cytosolic chaperones and targeted to lysosomes. In this pathway, known as chaperone-mediated autophagy, transport into lysosomes takes place directly through the membrane and it is mediated by a membrane receptor and a luminal chaperone.

4.2. Chaperone-mediated autophagy

The second pathway for selective lysosomal targeting is chaperone-mediated autophagy (CMA) (Massey et al., 2004). Unlike the vesicular pathways, CMA operates on cytosolic proteins with a specific pentapeptide motif, which is named “KFERQ” after an early example but is in fact quite relaxed, consisting of a tetrapeptide sequence, located anywhere in the protein, satisfying the pattern “positive; negative; hydrophobic; hydrophobic or positive” (in either direction) and either directly preceded or directly followed by a glutamine residue (Dice, 1990). CMA is not naturally used for delivery of lysosomal enzymes, but of substrate proteins (proteins that should be removed from the cytosol). In fact, few lysosomal enzymes possess a KFERQ motif (cathepsin D being an exception); however, several proteins involved in neurodegenerative diseases possess it, including the amyloid precursor protein, β -synuclein and huntingtin. Although the removal of the aggregated forms of these proteins seems ultimately to take place through macroautophagy (Nixon, 2004; Lee et al., 2004; Ravikumar et al., 2004), at least for synuclein, degradation via CMA of the soluble wild-type protein has recently been demonstrated (Cuervo et al., 2004). The major components of the CMA system are: a cytosolic chaperone, hsc70, that recognizes the targeting signal; the lysosomal membrane protein type 2a, lamp-2a, that acts as a receptor; and a lysosomal luminal variant of hsc70, required for substrate translocation

across the lysosomal membrane (Massey et al., 2004). This luminal hsc70 is extremely pH-sensitive, being degraded when intralysosomal pH rises even to half a unit above normal, and consequently stopping protein translocation through CMA (Massey et al., 2004). This clearly points to the possibility that mild impairment of lysosomal acidification in neurons may cause cytosolic accumulation of CMA substrates such as α -synuclein merely by slowing down their translocation into lysosomes for degradation.

It is, naturally, premature to anticipate which of these pathways would be likely to prove more useful for delivery of non-human enzymes to the lysosome. Thus, we merely note that the existence of multiple well-characterised alternatives gives cause for optimism.

5. Finding candidate microbial strains and genes

5.1. Background

Bioremediation has progressed considerably since Gayle's microbial infallibility hypothesis. As so often for an idea that shows so much promise, but is not trivial to implement, bioremediation has had its ups and downs. In the 1970s, relatively simplistic approaches of adding specialized bacteria to contaminated sites often did not achieve the anticipated results, and bioremediation was sometimes categorized with "snake oil salesmen." (National Research Council, 1993) However, momentum was regained by the 1990s as a better understanding of the fundamental processes involved was obtained (National Research Council, 1993, 1994, 2000). This was especially reflected in the emergence of microbial ecology as a key discipline within bioremediation (Table 1; Rittmann and McCarty, 2001).

Decade	Event
1900's:	Development of biological processes to treat municipal wastewater and sludge.
1950's:	Development of industrial wastewater treatment processes that rely on biodegradation. Microbial infallibility hypothesis proposed by Gayle (29), borne out of aerobic lab studies.
1960's:	Research on the biodegradation of synthetic organic chemicals present as "micropollutants" in wastewaters.
1970's:	Environmental statutes of unprecedented scope pass, and regulatory pressure (1976 RCRA & TSCA, 1980 CERCLA-Superfund) stimulates development of remediation technologies. Bioremediation successes are reported for the cleanup of aquifers contaminated by gasoline releases, and the first bioremediation patent is granted to Dick Raymond (74). Nevertheless, adding acclimated microorganisms to contaminated sites becomes common practice. Earlier proponents of bioaugmentation often fail to recognize that indigenous bacteria already present might be better suited genetically and physiologically to degrade the pollutants, but biodegradation might be limited by contaminant bioavailability or unfavorable redox conditions rather than by a lack of catabolic potential. Failure to meet expectations creates a bandwagon effect and prompts a major downturn in bioremediation.
1980's:	It becomes clear that fundamental hydrogeologic and biogeochemical processes inherent to bioremediation need to be understood before a successful technology can be designed. This realization, along with the fear of liability and Federal funding (e.g., Superfund), stimulates the blending of science and engineering to tackle environmental problems and improve bioremediation practice.
1990's:	Many successful bioremediation technologies (mainly <i>ex situ</i>) and hybrid (microbial/chemical) approaches are developed (often reflecting that adaptation is

part of innovation). However, many decision makers continue to regard bioremediation as a risky technology and continue to select (relatively ineffective) pump and treat (P&T) technologies for remediation purposes. A 1994 NRC study reports that conventional P&T technologies restored contaminated groundwater to regulatory standards at only 8 of 77 sites. Superfund is depleted. Poor cleanup record and resource allocation problems stimulate paradigm shift towards natural attenuation and risk-based corrective action (RBCA).

2000's: *In situ* bioremediation and monitored natural attenuation are widely accepted as cost-effective cleanup alternatives for sites contaminated with a wide variety of organic pollutants, and the interest in bioaugmentation grows following successful trials for enhancing the removal of recalcitrant compounds.

Table 1. Historical perspective of bioremediation

Virtually any natural environment contains a wide variety of microorganisms with hugely varying biodegradation capabilities. One reason for the high diversity is that the microorganisms live in communities whose members usually cooperate to ensure complete biodegradation of organic compounds. Completely biodegrading complex organic materials demands many steps of hydrolysis, oxidation and reduction. Often, several different microorganisms are responsible for a set of reactions within the entire biodegradation pathway and communicate and cooperate syntrophically by providing each other with degradation products useful to the others. In addition, microbial communities exhibit diversity *because* they usually have metabolic redundancy: several microorganisms are able to carry out the same steps, sometimes using two or more versions of the same kind of enzyme. It must be stressed that this does not imply a need to add numerous enzymes to the human lysosome to replicate complete microbial metabolic pathways for the mineralisation of indigestible material because for therapeutic purposes all that is required is to convert the target compound into one that mammalian metabolism can already handle.

Although the principles underlying bioremediation are relatively well understood today, the strains responsible for degradation of a substance of interest will typically not have been previously characterised. Microbial ecologists estimate that we have isolated less than 1% of the diversity among microorganisms. To harvest a critical enzyme for use in therapy, we must identify and ultimately isolate the microorganisms responsible for producing it. Fortunately, identification and isolation are becoming more tractable today through the development of molecular techniques that directly target the cells' DNA and RNA. Many new methods are being developed each year, and two approaches are especially noteworthy in this context: molecular fingerprinting and the DNA microarray.

5.2. Molecular fingerprinting

Molecular fingerprinting relies on the fact that the sequences of genes that code for like protein products are usually similar. In that case, a primer can be generated for a class of genes. When such a primer is used to amplify the DNA in the polymerase chain reaction (PCR), the amplified products are from similar genes from all microorganisms in the community. We can separate the different amplified products by electrophoresis to create a DNA fingerprint, and we can track the emergence of important community members or important genes even when we do not know the identity of the microorganism.

One target for amplification is the small subunit of the ribosomal RNA, or the SSU rRNA. All living organisms contain ribosomal RNA, and it is a measure of phylogenetic identity of the organism. Therefore, a primer that amplifies the gene for the SSU rRNA allows us to track the identity of the microorganism itself. Specific types of metabolic genes can also be amplified with specific primers to search for the source of important new enzymes. For example, hydrolysis is often the first step in the biodegradation of complex organic molecules; amplifying hydrolases that relate to structures of the aggregates of age-related diseases may be a good way to identify and track a gene for a critical enzyme.

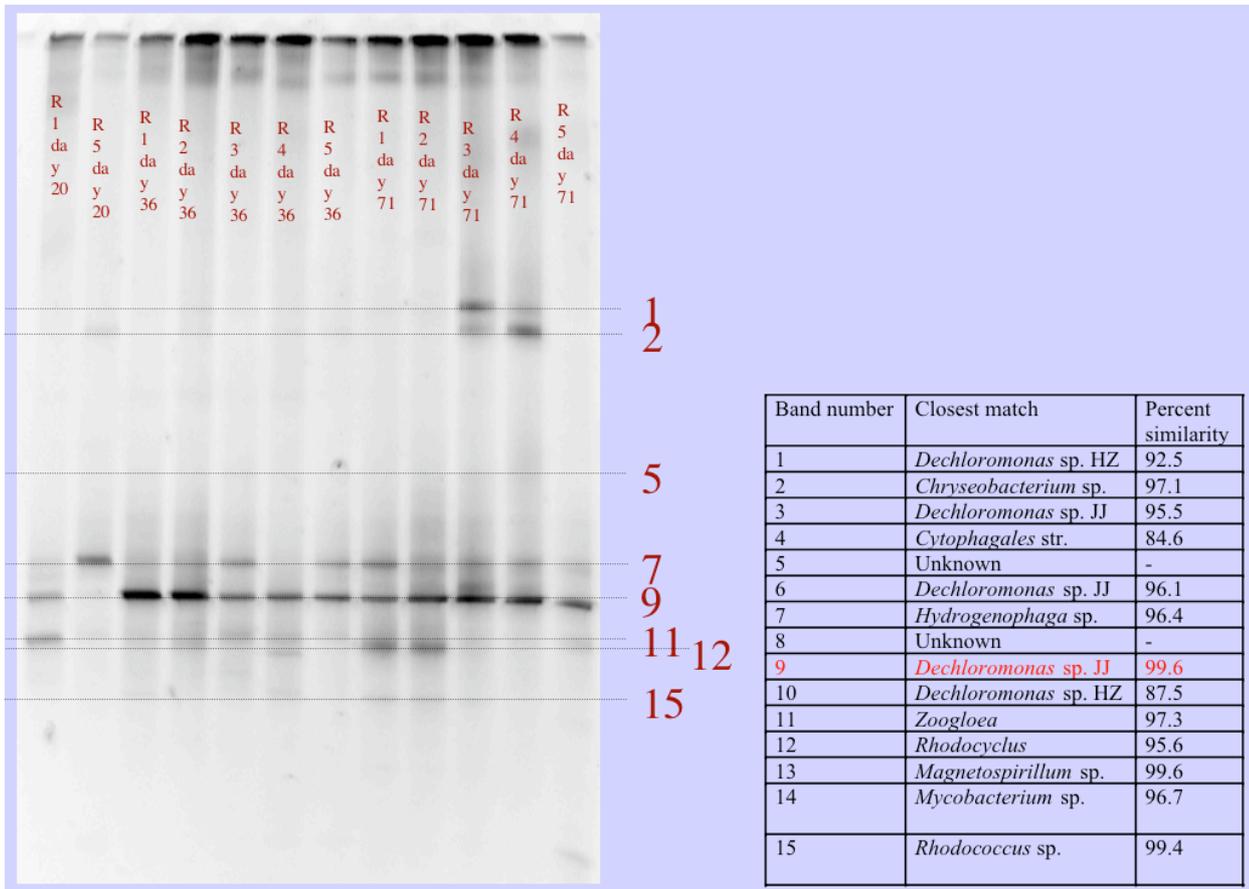


Figure 5. A DGGE gel showing results from perchlorate-reducing, membrane biofilm reactors.

Several fingerprinting techniques are available today, and the number is growing. Perhaps the best one for the goals outlined here is denaturing gradient gel electrophoresis, or DGGE. Here, the amplified DNA is separated on an electrophoresis gel that has a gradient of denaturing chemical. Based on the size and G+C content of the DNA, its double strand form denatures at different positions on the gel (Figure 5). We can follow that position to track what appears to be an important community member. One reason why DGGE is so effective here is that the amplified DNA can be excised from the gel and sequenced. The sequence can give us a phylogenetic identity (based on SSU rDNA) or knowledge of the function of the metabolic gene. In addition, the sequence allows us to design oligonucleotide probes that can be used in the second new technology, the DNA microarray.

5.3. DNA microarrays

DNA-microarray technology is emerging as a viable new addition to the bioremediator's arsenal. Chips can be synthesised with arrays of hundreds to thousands of oligonucleotides from well-characterised or partially characterized strains. Figure 6 illustrates the array format of a gel-pad microarray. At least in principle, we can track the presence of many interesting microorganisms (by using oligonucleotide probes that targeting amplified SSU rDNA), genes (by targeting amplified DNA of the genes), and gene expression (by tracking messenger RNA) with one assay. Microarray technology greatly expands the power of DNA-based tracking, particularly when we must deal with microbial communities that have many members and even more potentially useful enzymes.

Having identified strains with the desired catabolic ability and the promising enzymes, we should be able study the enzymes by standard genetic and genomic techniques. Some strains are amenable to transposon-mediated mutagenesis (Fernandes et al., 2001), which allows the random disruption of genes and the molecular identification of the disrupted gene after identification of the appropriate phenotype (in this case, loss of the ability to degrade the target material). It may be possible to manipulate the genome to constitutively over-express the catabolic enzyme of interest, precluding the need for specific inducers to

be present above specific thresholds and avoiding repression of gene expression by alternative (labile) substrates.

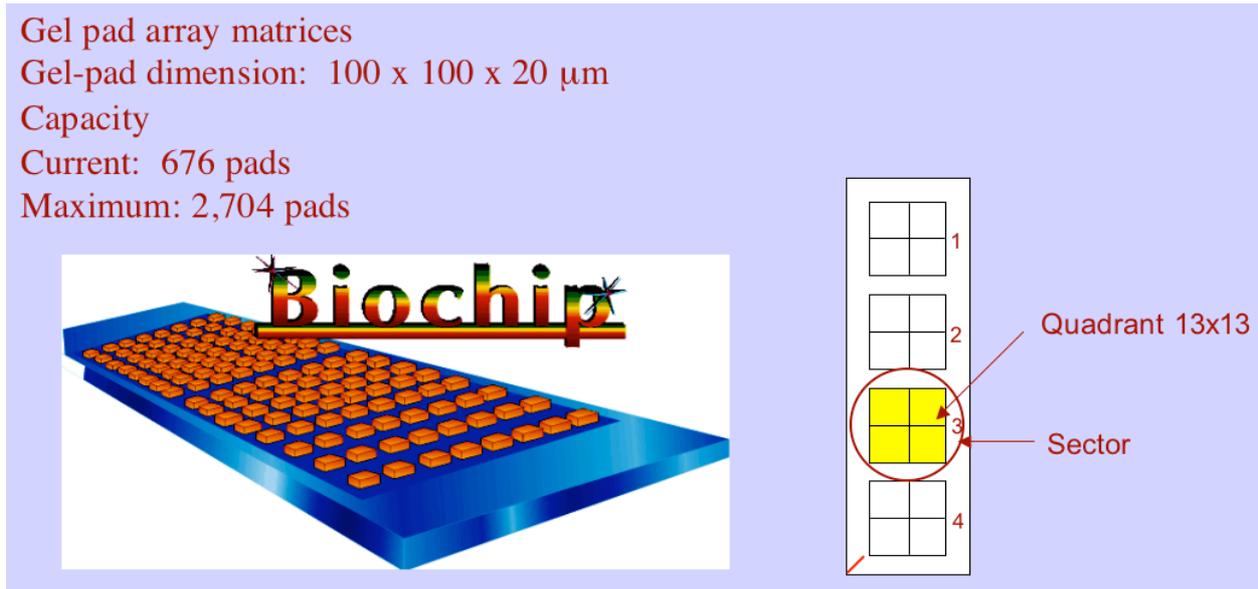


Figure 6. The array format of a gel-pad microarray.

5.4. Presence of promising enzyme families in soil microbes

Despite the arguments and analogies just presented, it would be comforting to those searching for candidate microbial strains if there were enzymes already known with activities similar to those sought. Information in this regard is encouraging. Concerning cholesterol and its derivatives, many bacteria have monooxygenase and dioxygenase enzymes that use molecular oxygen as a co-reactant to insert hydroxyl groups onto highly reduced aromatics and aliphatics with double bonds. Once one or a few hydroxyl groups are inserted, the molecule then becomes accessible to dehydrogenase and hydroxylase attacks (Gottschalk, 1986). Bacterial and fungal proteases are also highly diverse and are widely used commercially for baking, cleaning, tenderizing, and medical applications (Madigan et al., 2003). While aggregated proteins have the problem of inaccessibility of most peptide bonds, it is plausible that lysosomal aggregation of highly proteinaceous material (such as lipofuscin) results from the formation of a “Gordian knot” in which some bonds are exposed, just not ones amenable to cleavage by human lysosomal enzymes, and thus that such aggregates can be albeit maybe only slowly) dismantled by the initial action of microbial enzymes followed by the action of human enzymes on previously inaccessible peptide bonds. Further, as noted earlier, non-lysosomal aggregates characteristic of neurodegenerative diseases may spontaneously disaggregate or undergo autophagy on rejuvenation of the lysosomal compartment.

6. Side-effects, obstacles and options for avoiding them

6.1. Inactivity and toxicity

The possibility clearly exists that microbial genes introduced into mammalian cells will be either inactive or toxic. The blanket answer to this difficulty is just to carry on looking until we find ones that are both effective and non-toxic, but clearly a more directed approach would help to get results sooner. Lack of function can result from a disparity of pH optimum between the microbe and the lysosome; this may be a reason to explore fungal strains more assiduously than is normal in bioremediation, where their relatively slow growth is an obstacle to large-scale expansion. A second reason to explore fungi is the strong similarity of vacuolar targeting mechanisms in fungi and mammals (Levine and Kliensky, 2004), which may facilitate manipulation of the chosen enzymes for appropriate localisation. Use of fungi may also motivate the *in vitro* evolution of enzymes which work in their native environment; this is a technique that has reproducibly optimised characteristics such as pH optimum in other contexts and may allow the

enzyme to be expressed at a lower level than otherwise. Toxicity, most simply the degradation of vital extralysosomal material, may be more straightforward to address: firstly it may be minimised by precisely the pH sensitivity just mentioned, and where this does not suffice the option exists to synthesise the enzyme as a proenzyme requiring intralysosomal proteolytic cleavage for activation, as most mammalian lysosomal enzymes already are (Wittlin et al., 1999).

6.2. Immune rejection

A more profound difficulty that this therapy may face is the response of the immune system, since clearly any microbial enzyme is likely, by default, to be detected as “non-self” and cells expressing or containing it thus attacked by lymphocytes. (Note: for brevity we refer throughout this section only to enzymes, but our comments apply equally to other proteins, e.g. a transporter that restores lysosomal acidity.) Here, however, we can again derive confidence from the experience of ERT, in which large quantities of enzyme are injected without, in many cases, inducing a serious immune response; moreover, in cases where a response is initially substantial it often declines with continued treatment (Brooks et al., 2003). Since the patients receiving these enzymes are congenitally deficient for them, in many cases due to deletion or nonsense mutations (80), those enzymes are just as “non-self” to the patients as a microbial enzyme would be. The tolerance of these enzymes may largely be because they are taken up directly into the vesicular apparatus and are thus never exposed to the immunoproteasome. Hence, peptide fragments from them are never loaded onto HLA complexes for cell surface presentation. The humoral arm of the immune system is thus the only source of any response.

This would seem to motivate ERT, rather than gene therapy, as a delivery modality for these enzymes, but that conclusion may be premature. Progress is currently rapid in the field of tolerisation, the education of the immune system to recognise a new protein as “self” by expressing it within the bone marrow, and some such work has been done with lysosomal enzymes (Kakkis et al., 2004). Moreover, the declining immune response often seen in long-term ERT recipients indicates a spontaneous tolerisation process. This may suffice to allow expression of such proteins at will by the time we have them in hand. We should also bear in mind that the target substances which these enzymes will degrade accumulate extremely slowly – which is why they do not become pathogenic until middle age – and thus that very low, or occasional, enzyme expression should suffice to reverse this accumulation. Enzymes normally expressed at very low levels, such as telomerase, have been found to be good antigens: telomerase is being energetically pursued in this regard as an anti-cancer therapy (Vonderheide, 2002). Brief, periodic (typically drug-induced) expression, on the other hand, could be combined with robust immunosuppression without severely compromising the patient’s immune system long-term.

6.3. The blood-brain barrier

An additional argument in favour of a gene therapeutic approach concerns the delivery of enzymes to the brain. ERT as currently practised is of very limited benefit to neuronopathic LSD patients, on account of the failure of the therapeutic enzyme to cross the blood-brain barrier. While it may in principle be possible to engineer transcytosis of selected proteins, perhaps by manipulation of the transferrin receptor (Bickel et al., 2001), the versatility of this technique remains uncertain. Hence, viral or other methods that deliver transgenes to the brain may prove to be essential components of a comprehensive lysosomal enhancement strategy for the treatment of age-related storage diseases. Progress in this area is encouraging, though important hurdles also remain (Sanftner et al., 2004).

7. Conclusion

We argue here that the application of bioremediation methodologies to the degradation of the various recalcitrant aggregates that form in human tissues throughout life has the potential to give rise to unprecedented therapeutic options for the treatment of several of the most debilitating and common diseases in the modern industrialised world. The wide differences between researchers in the age-related diseases and bioremediation make this goal scientifically challenging. However, the potential benefit is so great that new collaborations should be pursued expeditiously and energetically.

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