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# Glycosidase-induced fusion of isoprenoid gentiobiosyl lipid membranes at acidic pH

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**A difficulty in explaining the mechanism whereby archaeal lipid membrane vesicles (archaeosomes) deliver entrapped protein antigens to the MHC class I cytosolic pathway from phagolysosomes of antigen-presenting cells has been the observation that they tend not to fuse. Here, we determine that archaeosomes, composed of archaeal isoprenoid mixtures of glyco and phospholipids, can be highly fusogenic when exposed to the pH and enzymes found in late phagolysosomes. Fusions were strictly dependent on acidic pH and the presence of α- or β-glucosidase. Resonance energy transfer (RET) assays demonstrated that fusion conditions induced lipid mixing of archaeosome lipids with self-unlabeled archaeosomes. Because PC/PG/cholesterol liposomes by themselves did not fuse, it was possible to unequivocally show a fusion of rhodamine-labeled liposomes with archaeosomes by fluorescence microscopy and to demonstrate lipid mixing between labeled liposomes and archaeosomes by the RET assay. Radiotracer and $^1$H NMR studies revealed that glycolipids in fused archaeosomes were not degraded significantly by glucosidase treatment during fusion. Rather, the glucosidases dramatically induced small archaeosomes to rapidly and visually aggregate at pH 4.8, but not 6.8, thus bringing membranes together appropriately as a first step in the fusion process. $^1$H NMR was used to demonstrate that conditions causing aggregation correlated with binding of glucosidase to the archaeosomes. Binding at acidic pH occurred by the electrostatic interaction of positively charged glucosidase with the anionic phospholipids, although the interaction also occurred with the gentiobiosyl lipids. The data indicate a mechanism of membrane–membrane fusion for archaeal glycolipid membranes induced by glycosidase and illustrate the importance for inclusion of glycolipids in compositions of vesicles designed to deliver protein antigens to the cytosol for MHC class I presentation.**

*Keywords:* fusion/glycolipid membranes/glycosidase/glycosyl isoprenoids/phagolysosome

## Introduction

The fusion of archaeal lipid membranes (archaeosomes) is not documented to occur under physiological conditions, presumably because of the unique chemistry of archaeal lipids. Structurally, the lipid cores of archaea are isoprenoid chains, often C-20, that are ether-linked to a glycerol backbone in an archaeal-specific sn-2,3-stereochemistry (archaeol). Thermoacidophilic and some methanogenic archaea have the additional capacity to synthesize caldarchaeol lipids that consist of C-40 isoprenoid chains ether-linked at either end to the two glycerol backbones. The free sn-1 hydroxyls of the glycerols are linked to polar head groups, the structures of which depend on the biosynthetic capabilities of the various archaea. Mixtures of polar lipids extracted from archaea generally hydrate readily to form lipid vesicles, which because of their stability and other unique properties have been called archaeosomes, to distinguish them from strictly bilayer liposomes (Sprott and Krishnan 2007). Bipolar caldarchaeol lipids span the archaeosome membrane as deducted by the absence of intramembrane freeze fracture planes (Beveridge et al. 1993) and contribute to enhanced stability of the vesicle structure dependent on the amount of membrane-spanning lipids present (Elferink et al. 1994; Choquet et al. 1996; Mathai et al. 2001).

Archaeosomes consisting of the total polar lipids extracted from various archaea with different polar lipid profiles all have strong adjuvant activity for an entrapped protein antigen when injected into mice (Krishnan, Dicaire, et al. 2000; Krishnan et al. 2003, 2007; Sprott, Sad, et al. 2003). The *Methanobrevibacter smithii* archaeosome was chosen as a promising adjuvant for a detailed study (Krishnan, Dicaire, et al. 2000; Krishnan et al. 2003; Sprott et al. 1999), consisting of major amounts of β-gentiobiosyl (β-D-glucosyl-(1,6)-β-D-glucosyl-), phosphoserine, phosphoinositol, and phosphate head groups linked to both archaeol and caldarchaeol lipid cores (Sprott et al. 1999). An entrapped protein antigen was tracked following phagocytosis by antigen-presenting cell cultures by monitoring presentation of generated peptides on either MHC class I or class II molecules to $CD8^+$ or $CD4^+$ T cells, respectively (Gurnani et al. 2004). Through the use of inhibitors and Tap knock-out mice, it was established that the antigen was cross-presented to the MHC class I pathway by phagosome-to-cytosol transport at the late phagolysosome stage. This presented a problem mechanistically, as a previous study identified that only a slow fusion of archaeal lipid membranes containing primarily caldarchaeol lipids occurred with self-membranes, and this fusion was restricted to pH near neutrality in the presence of >3.5 mM $Ca^{2+}$ and >5 mM phosphate (Relini et al. 1994, 1996; Elferink et al. 1997), apparently excluding potential to fuse within the parameters of the phagolysosome environment.

Because of the importance of membrane–membrane fusion in biology, there has been considerable interest and recent progress in understanding fusion mechanisms (Martens and McMahon

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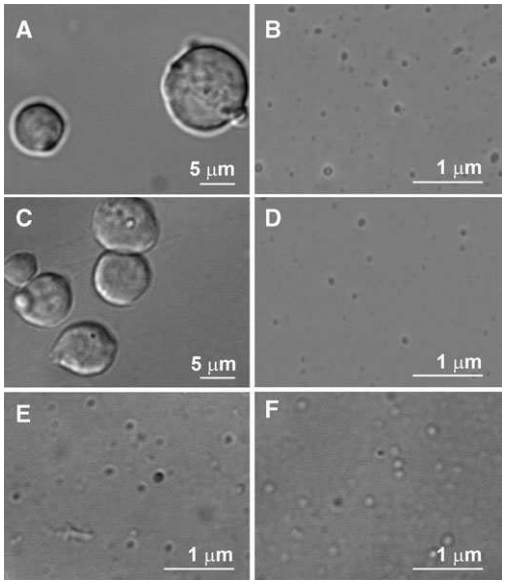


**Fig. 1.** Differential interference contrast microscopy (DIC) images showing fusions of archaeosomes induced by β-glucosidase. Archaeosomes or liposomes (DPPC/DPPG/cholesterol) were incubated at 37°C for 1.5 h in a 60 μL reaction mixture consisting of the 25 mM sodium acetate buffer, pH 4.5, 140 mM NaCl, and 1.0 mM $CaCl_2$. (**A** and **B**) 50 μg archaeosomes; (**C** and **D**) 50 μg archaeosomes plus 50 μg liposomes; or (**E** and **F**) 50 μg liposomes. **A**, **C**, and **E** received 16.5 μg β-glucosidase (almond) to begin the reactions; **B**, **D**, **F** are negative controls lacking β-glucosidase. Average diameters of unfused liposomes were 65 ± 33 nm and archaeosomes were 66 ± 37 nm.

2008). These studies have focused on viral membranes, SNARE proteins, and synaptotagmin membrane protein-mediated fusion mechanisms that would not apply to cytosolic delivery of antigen by pure-lipid archaeosomes. However, the process of exocytosis during which a vesicle fuses with another, in this case an archaeosome with a phagolysosome membrane, would result in the passage of the vesicle content across the phagolysosome membrane and explain cytosolic antigen delivery. Although pH sensitive liposomes containing oleic acid or palmitoylhomocysteine fuse in response to lowering pH from 7 to 5 (Connor et al. 1984; Straubinger et al. 1985) and may have application in cytosolic delivery (Connor and Huang 1985; Straubinger et al. 1985), archaeosomes in contrast are quite stable to acidic pH (Choquet et al. 1994). Here, we test whether glycosidases and phospholipases, acidic pH, and ions found in late phagolysosomes may separately or in combination promote the fusion of glycolipid/phospholipid archaeosomes.

## Results

*Glucosidase, calcium, and NaCl-dependent fusion balls*

Small archaeosomes of about 65 nm diameter when incubated at pH 4.5 in the presence of 1.0 mM $CaCl_2$ did not fuse or change in appearance, as monitored by differential interference contrast (DIC) microscopy (Figure 1B), until β-glucosidase was added. β-Glucosidase had the dramatic effect of inducing the formation of large fusion balls within 1.5 h that often exceeded 5 μm, with the corresponding disappearance of small archaeosomes (Figure 1A). This same effect was observed for mixtures of archaeosomes and liposomes (Figure 1C and D), but not for liposomes incubated without archaeosomes (Figure 1E and F).

The parameters required for fusion ball formation from archaeosomes were explored further. As illustrated in Figure 2A–C, the formation of large fusion balls depended on the amount of β-glucosidase added, with essentially all small archaeosomes disappearing at 0.3 μg/μL. $CaCl_2$ was also essential for the conversion of lipid aggregates or amorphous lipid products to form fusion balls, although microscopic observation (Figure 2D) was suggestive that lipid mixing, in contrast to the formation of fusion balls, might occur without $CaCl_2$. As $CaCl_2$ was increased to 0.5 mM, irregular aggregates formed more rounded structures and at 1.0 mM rounded large fusion balls appeared. These effects were not reproduced when the same concentrations of $MgCl_2$ were substituted for $CaCl_2$. In addition to a $Ca^{2+}$ requirement for induction of fusion ball formation by β-glucosidase, there was a requirement for physiological concentrations of NaCl (substituted by KCl, not shown) to convert apparently fused, irregular lipid aggregates to fusion balls (Figure 2G–I). Strikingly, the fusion of small archaeosomes exposed to β-glucosidase, $Ca^{2+}$, and $Na^+$ was strictly pH-dependent, with essentially no activity at pH > 5.6 and best activity at pH < 4.8 (Figure 2J–L). No requirement for added phosphate was demonstrated even after the extensive dialysis of β-glucosidase against water, and the presence of up to 0.5 mM phosphate was not detrimental (data not shown).

Albumin at 0.1 μg/μL has been reported to induce the fusion of phosphatidylserine liposomes in the presence of >1 mM $CaCl_2$ and pH 7.4 (Papahadjopoulos et al. 1974). To explore the specificity for β-glucosidase, various other proteins were substituted using the standard fusion conditions at pH 4.8 and pH 6.8, with proteins at 0.15 μg/μL. The conditions used and results for the β-glucosidase control were as shown in Figure 2B. The following proteins had no effect microscopically on small archaeosomes: β-glucuronidase, phospholipase C, inositol-specific phospholipase C, lysozyme, or ovalbumin. However, α-glucosidase had the same dramatic effect as the β-isomer to induce fusion balls from small archaeosomes. Fusions induced by α-glucosidase showed the same requirements as β-glucosidase for $Ca^{2+}$, $Na^+$, and acidic pH (data not shown).

*Glucosidase and archaeosomes*

α-Glucosidase and β-glucosidase when added to an archaeosome suspension containing $Ca^{2+}$ and $Na^+$ had an immediate visual effect to increase the turbidity, and by microscopic observation to form aggregates consisting of small archaeosomes. This turbidity increase monitored at 660 nm approached steady state after 30 min, achieving half the change in turbidity within the first 1–2 min. Turbidity increases correlated well under fusion conditions with respect to being strictly-dependent on acidic pH and to the concentration of glucosidase required (Figure 3).

Lipid mixing between NBD/rhodamine-labeled archaeosomes and unlabeled archaeosomes was monitored following the addition of α- and β-glucosidase to start the fusion reaction (Figure 4). Lipid mixing occurred rapidly with archaeosome self-fusions and was independent of the addition of $Ca^{2+}$, indicating that archaeosome–archaeosome lipid mixing can occur without the formation of discrete fusion ball structures (B).

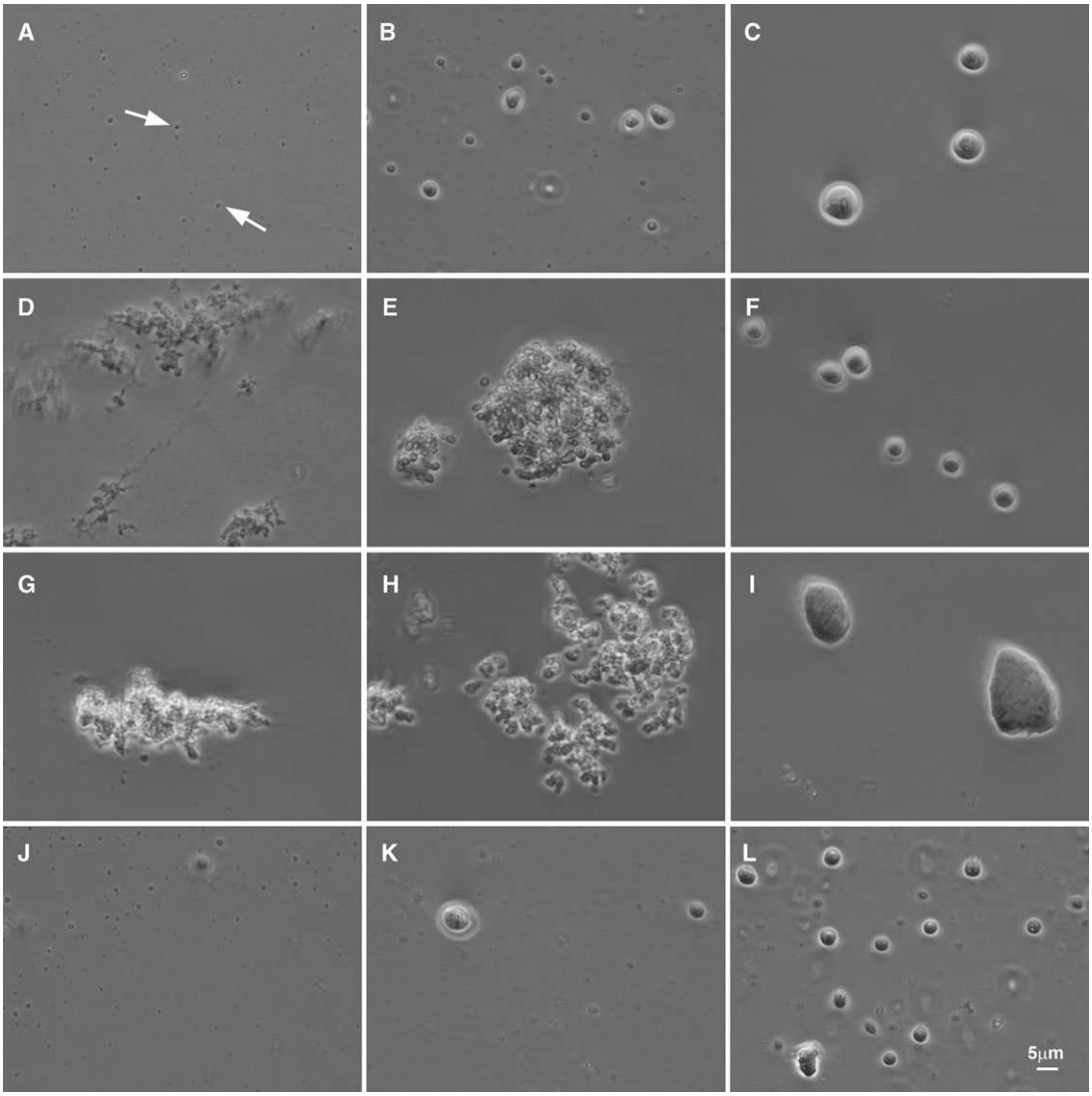


**Fig. 2.** Phase contrast microscopy images defining the fusion parameters of β-glucosidase concentration, $CaCl_2$ molarity, NaCl molarity, and pH. 50 μg archaeosomes were incubated at 37°C for 1.5 h in 60 μL reaction mixtures consisting of the 25 mM sodium acetate buffer, pH 4.5, 140 mM NaCl, 1.0 mM $CaCl_2$, and 18 μg (0.3 μg/μL) β-glucosidase (almond) with the following variations. (**A–C**) contained β-glucosidase at zero (**A**), 0.16 (**B**), and 0.3 (**C**) μg/μL. (**D–F**) contained $CaCl_2$ at no addition (**D**), 0.5 mM (**E**), and 1.0 mM (**F**). (**G–I**) contained NaCl at 25 mM (**G**), 92 mM (**H**), and 165 mM (**I**). (**J–L**) contained sodium acetate adjusted to pH 5.6 (**J**), 5.3 (**K**), or 4.8 (**L**) with β-glucosidase in this case at 0.16 μg/μL. Arrows point to small archaeosomes (sized as 66 ± 37 nm) in a complete reaction mixture lacking protein (**A**). The magnification bar shown in **L** was the same as for **A–L**.

Further, lipid mixing was not induced by either α- or β-glucosidase at pH 6.8 (C). Fluorescence changes were minimal in the absence of unlabeled archaeosomes (D) and were dependent on the presence of NBD/rhodamine (E).

*Archaeosome/liposome fusion*

To evaluate whether archaeosomes can fuse with other nonfusogenic membranes under phagolysosome conditions, PC/PG/cholesterol liposomes were used. These liposomes were incapable of fusion in the absence of archaeosomes as shown previously (Figure 1E and F). Fluorescence microscopy evidence for archaeosome–liposome fusion is presented in Figure 5. Rhodamine–liposomes mixed with unlabeled archaeosomes resulted in large fusion balls upon incubation with β-glucosidase in the fusion assay. All large fusion balls observed by DIC microscopy (Figure 5A) were labeled (Figure 5C) indicative of liposome–archaeosome fusion. As expected, since archaeosomes self-fuse, rhodamine-labeled archaeosomes incubated in the fusion assay with unlabeled liposomes also resulted in labeling of all large fusion balls (Figure 5B and D). In keeping with archaeosome–liposome fusions, very few small liposomes remained after fusions were allowed to proceed for 1.5 h.

Resonance energy transfer (RET) assays confirmed that archaeosome–liposome fusions occurred and that fusions were dependent on the presence of β-glucosidase. Lipid mixing occurred between rhodamine/NBD-labeled liposomes and archaeosomes (Figure 6A) albeit with slower kinetics than archaeosome self-fusions (Figure 4). Lipid mixing did not occur in the absence of archaeosomes (Figure 6B), or if unlabeled archaeosome addition was replaced by unlabeled liposomes (Figure 6C).

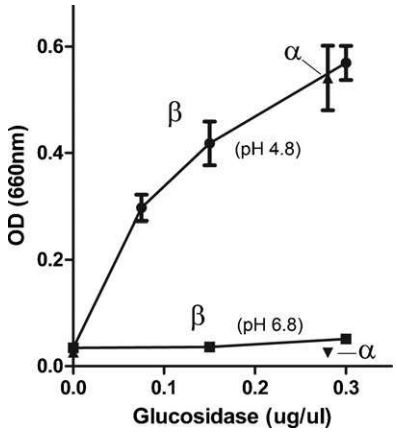


**Fig. 3.** Light scattering measurements conducted under fusion conditions (α-glucosidase or β-glucosidase at pH 4.8) and nonfusion conditions (α-glucosidase or β-glucosidase at pH 6.8). Steady-state conditions occurred after 30 min incubations at 37°C. Reaction mixtures consisted of the 25 mM sodium acetate buffer at pH 4.8 or 6.8, 140 mM NaCl, 1.0 mM $CaCl_2$, and 170 μg archaeosomes in a volume of 0.5 mL. The reaction was started with either α- or β-glucosidase at the concentrations indicated. Results are the average of triplicate reaction mixtures ± SEM.

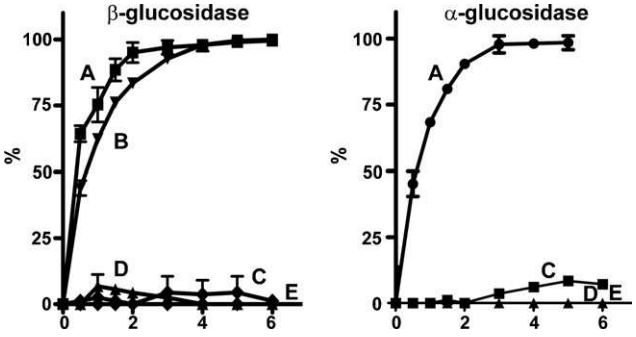


**Fig. 4.** RET lipid mixing assay demonstrating membrane self-fusions of archaeosomes. Hundred micrograms archaeosomes containing rhodamine-PE and NBD-PE fluorescent probes were incubated in a 1.0 mL reaction mixture containing (unless stated otherwise) the 25 mM acetate buffer, pH 4.8, with 140 mM NaCl, 1.0 mM $CaCl_2$, and 170 μg of unlabeled archaeosomes. 0.5 μg/μL of either β-glucosidase or α-glucosidase started the reaction. The complete reaction at pH 4.8 is shown by curves A. Other reaction mixtures were as for A except that $CaCl_2$ was omitted and 400 μM EGTA added in B, and the pH was 6.8 in C; unlabeled archaeosomes were absent in D, and fluorescent archaeosomes were absent in E. Data are shown as average of two assays ± SEM. Fluorescent archaeosomes were sized at 124 ± 61 nm and unlabeled archaeosomes were 66 ± 37 nm. 100% fluorescence was determined as the fluorescence reading following treatment with detergent.

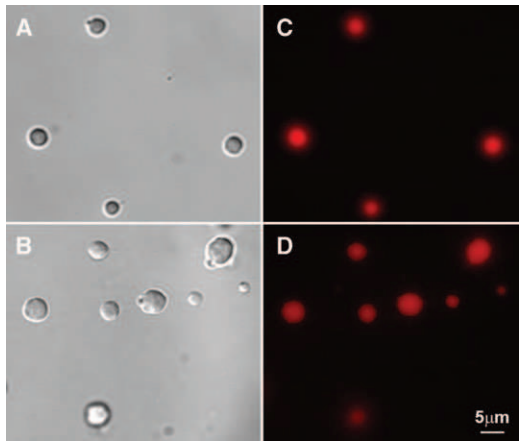


**Fig. 5.** Fluorescence microscopy evidence for the fusion of archaeosomes with DPPC/DPPG/cholesterol liposomes. Large fusion vesicles were induced by β-glucosidase over 1.5 h at 37°C in mixtures of archaeosomes and liposomes in complete reaction mix (Figure 2 legend). (**A** and **C**) Show rhodamine-PE liposomes mixed with unlabeled archaeosomes; (**B** and **D**) show rhodamine-PE archaeosomes mixed with unlabeled liposomes. Results of DIC microscopy are shown in **A** and **B**. Fluorescence microscopy results to detect the rhodamine label are shown in **C** and **D**. Average diameters of unfused rhodamine-PE DPPC/DPPG/cholesterol liposomes, rhodamine-PE archaeosomes, unlabeled archaeosomes, and unlabeled liposomes (no β-glucosidase) were in the 50–150 nm range. The magnification bar shown in **D** was the same as for **A**–**D**.

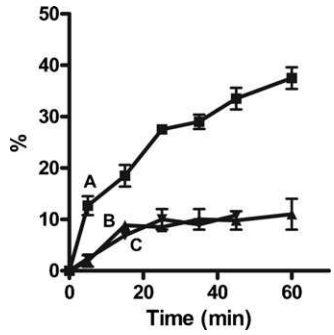


**Fig. 6.** RET assay demonstrating β-glucosidase-induced lipid mixing of unlabeled archaeosomes with PC/PG/cholesterol liposomes labeled with rhodamine-PE and NBD-PE fluorescent probes. Lipid mixing is shown (**A**) in a 1.0 mL complete reaction mix consisting of acetate buffer, pH 4.8, 140 mM NaCl, 1.0 mM $CaCl_2$, 100 μg rhodamine-PE liposomes, and 200 μg unlabeled archaeosomes. Fusions were induced by adding 0.48 μg/μL of β-glucosidase at time zero. (**B**) The uncorrected background effect of omitting unlabeled archaeosomes and (**C**) of replacing unlabeled archaeosomes by 200 μg of unlabeled liposomes. Particle sizes were unlabeled archaeosomes 66 ± 37 nm, labeled liposomes 48 ± 31 nm, and unlabeled liposomes 65 ± 33. Data are the average of duplicate fusion assays ± SD.

### β-*Glucosidase and lipid metabolism*

To evaluate quantitatively the effect of β-glucosidase on lipid structural integrity in archaeosomes, [$^{14}$C]mevalonate was used to label the isoprenoid chains of *M. smithii* lipids. [$^{14}$C]archaeosomes were prepared and subjected to fusion conditions. The archaeosome lipids were then separated by thin-layer chromatography and quantified. β-Gentiobiosyl-caldarchaeol and β-gentiobiosyl-archaeol lipids were compared to archaetidylserine and archaetidic acid as phospholipid controls, as these lipids would not be expected to change (Figure 7). Following fusions, any degradation of the polar gentiobiosyl lipids that might have occurred as a result of β-glucosidase activity was found not to be statistically significant. To confirm that fusions had occurred, microscopic observation showed that large fusion balls formed at pH 4.8 with β-glucosidase, not at pH 4.8 in the absence of β-glucosidase, and not at pH 6.9 with β-glucosidase, as observed in other assays.

That β-glucosidase does not degrade the β-gentiobiosyl-caldarchaeol and β-gentiobiosyl-archaeol lipids in *M. smithii* archaeosomes was supported further by $^1$H NMR. The addition of β-glucosidase to the free β-gentiobiose disaccharide under conditions used for vesicle fusion led to the complete conversion of $^1$H NMR resonances associated with the disaccharide to those of free glucose after several hours, shown by the loss of those $H'_1$ signals corresponding to the nonreducing glucose

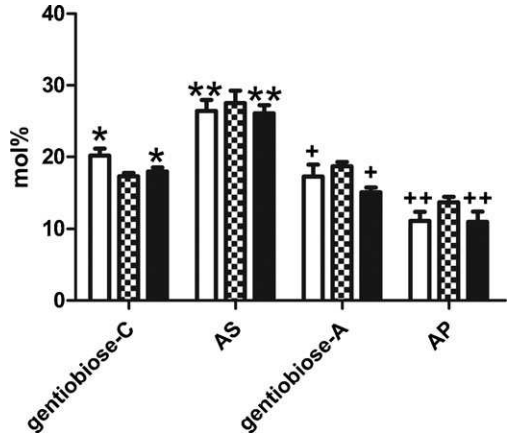


**Fig. 7.** The absence of statistically significant lipid degradation by β-glucosidase during the self-fusion of archaeosomes. Archaeal polar lipids labeled by [$^{14}$C]mevalonate were used to prepare [$^{14}$C]archaeosomes (97 ± 49 nm). [$^{14}$C]archaeosomes were incubated for 1.5 h at 37°C under conditions where fusion does not occur (open bars, no β-glucosidase; hatched bars, pH 6.9) or under fusion conditions in complete reaction mix, pH 4.8 (black bars). The lipids were then recovered and subjected to separation by thin-layer chromatography. Spots corresponding to gentiobiosyl-caldarchaeol lipids (β-gentiobiosyl-caldarchaetidylserine and β-gentiobiosyl-caldarchaetidylinositol), gentiobiosyl-archaeol, archaetidylserine (AS), and archaetidic acid (AP) were removed for quantification by liquid scintillation counting. Counts were converted to mole% for each lipid based on the total label spotted. Means of marked pairs were not significant based on unpaired *t* tests, $n = 8$, where *P* values were 0.0722 (*), 0.8577 (**), 0.2367 ($^{+}$), and 0.9482 ($^{++}$).

of gentiobiose (supplementary Figure 1B). The metabolism of β-gentiobiose, as anticipated, was not detected following α-glucosidase treatment (supplementary Figure 1A). A $^{1}$H NMR spectrum of *M. smithii* archaeosomes under fusion conditions showed no evidence of the $H_1$-β signals, indicative of no released glucose (supplementary Figure 1C).

*pH gradient and fusion*

We considered it possible that a pH gradient maintained across the archaeosome membrane might induce acid pH-dependent fusion through changes in phospholipid asymmetry. This was tested in two ways. First, the protonophore CCCP was added and found to have no effect on archaeosome self-fusion at concentrations as high as 50 μM. Second, lipids hydrated in the acetate buffer, pH 4.8, and assayed for fusion at pH 4.8 to ensure that ΔpH was zero, exhibited normal fusion demonstrating no requirement for a transmembrane pH gradient in the fusion process.

*Fusion of other archaeosome types*

To explore the specificity of β-glucosidase in the fusion of archaeal lipid membranes, several different lipid compositions of archaeosomes known to induce cytotoxic CD8$^{+}$ T cell (CTL) responses in mice (Krishnan, Sad, et al. 2000; Sprott, Sad, et al. 2003) were tested in the fusion assay at pH 4.8. *Methanococcus voltae* archaeosomes containing β-gentiobiosyl-archaeol (Ferrante et al. 1986) dramatically aggregated upon the addition of β-glucosidase, followed by large fusion ball formation. Other compositions lacking this lipid head group (*Natronobacterium magadii* (Kates 1978), *Halobacterium salinarum* (Kates 1978), and *Methanospirillun hungatei* (Sprott, Ferrante, et al. 1994) did not. In addition, *M. mazei* archaeosomes showed the same β-glucosidase-induced fusion ball formation as *M. smithii* archaeosomes despite having only a small proportion of glycolipid identified as a glucosyl archaetidylinositol (Nishihara et al. 1992).

*Binding of glucosidase to archaeosome/liposome surfaces*

Both of the glucosidases that are associated with archaeosome fusion are positively charged at pH values below 5 because their pI values are 5–5.5 (Takii et al. 1992; Gomez et al. 2005). Therefore, one may expect that association with negatively charged membranes will involve electrostatic interactions. As a result, the interaction will lead to charge neutralization of the membrane surface. In addition, because the proteins have a multiply positively charged surface, cross-linking and aggregation of vesicles would be anticipated. $^{1}$H NMR was used to examine the electrostatic nature of glucosidase-membrane association, including the binding of both glucosidases to *M. smithii* archaeosomes as well as to model liposomes.

In comparison to glucosidases, archaeosomes of 65 nm size and larger, as well as their aggregates, are expected to tumble much more slowly in the aqueous buffer. As a result, protein bound to archaeosomes, their aggregates and fusion products will exhibit $^{1}$H NMR spectra with line shapes that are much broader, mostly beyond detection, than the free protein. The interaction of α- and β-glucosidase with *M. smithii* archaeosomes under conditions giving rise to fusion produced $^{1}$H NMR spectra in which proton resonances for the aromatic/amide region (where there are no interfering lipid resonances) were significantly reduced, indicating binding (Figure 8A and B). Interestingly, it appears that α-glucosidase bound to the archaeosomes at pH 7.2, a result that would not be expected if binding were determined solely by the electrostatic interaction. The β-glucosidase protein preparation was not as pure as the α-glucosidase, which may account in part for the apparent lesser binding implied by the results in Figure 8. As a result, subsequent experiments focused on the purified α-glucosidase.

Since glucosidase-mediated archaeosome fusion only occurs when glycolipid is present, it is tempting to speculate that binding of α-glucosidase to the archaeosomes at pH 7.2 (Figure 8C) may occur through the interaction with the gentiobiosyl head group. Interestingly, at pH 7.2, archaeosomes in the presence of either glucosidase do not aggregate or fuse. To delineate further the role of liposome and protein surface charge as well as glycolipid on protein binding and on vesicle aggregation, model phospholipid vesicles of defined composition were examined. To investigate the role of charge on binding, the binding of α-glucosidase to anionic phospholipid vesicles containing high and low concentrations of anionic phosphatidylglycerol was compared (Figure 9). At pH 4.5, the presence of the glucosidase gave rise to extensive aggregation (supplementary Table 1) with concomitant binding of the enzyme to vesicles containing 80 mole% phosphatidylglycerol (Figure 9A). Corresponding microscopic observations clearly showed only aggregates composed of small liposomes (data not shown). At pH 7.2, little or no vesicle aggregation was detectable for the same highly charged vesicles. Vesicles containing a 20-fold reduction in phosphatidylglycerol, and thus lower surface charge, exhibited much reduced aggregation (supplementary Table 1) and much reduced enzyme binding (Figure 9B) at pH 4.5.

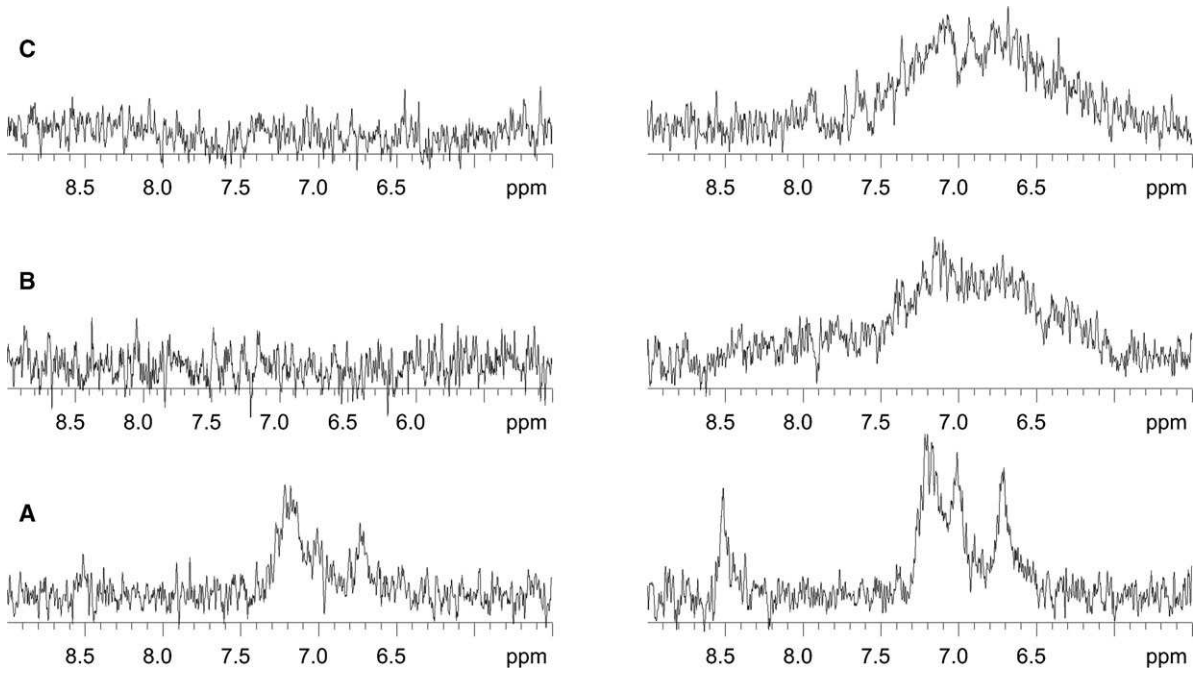


**Fig. 8.** $^1$H NMR spectra of glucosidase admixed with *M. smithii* archaeosomes: (**A**) β-glucosidase–archaeosomes at pH 4.5 (left) and free β-glucosidase (right); (**B**) α-glucosidase–archaeosomes at pH 4.5 (left) and free α-glucosidase (right); (**C**) α-glucosidase–archaeosomes at pH 7.2 (left) and free α-glucosidase (right). The concentration of glucosidases was identical for all samples.

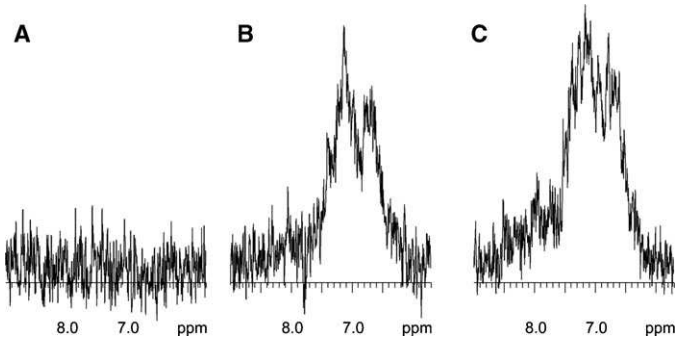


**Fig. 9.** $^1$H NMR spectra of α-glucosidase admixed with liposomes at pH 4.5. (**A**) DPPG/cholesterol (80:20 mole%); (**B**) DPPC/DPPG/cholesterol (37/4/59 mole%); and (**C**) free α-glucosidase. The concentration of α-glucosidase was identical for all samples.

To elucidate the role of β-gentiobiosyl–glycerolipids in glucosidase-promoted vesicle aggregation, vesicles of anionic phospholipids containing β-gentiobiosyl-archaeol (25 mole%) and having low surface charge were treated with α-glucosidase. It should be noted that phospholipid vesicles containing β-gentiobiosyl-archaeol at 25 mole% required some charged lipid (DPPG, 4 mole%) to form stable vesicles and hydrate well. At pH 4.5, α-glucosidase induced lipid aggregation of β-gentiobiosyl-archaeol vesicles, compared to no aggregation of DPPC/DPPG/cholesterol (4 mole% DPPG) liposomes, although aggregation was to a lesser degree than for DPPG/cholesterol (80 mole% DPPG) liposomes (supplementary Table 1). A comparison of the $^1$H NMR spectra of vesicles, with and without glycolipid and having the same charge density, suggests that there is some enzyme bound in the presence of β-gentiobiosyl-archaeol at pH 4.5 (supplementary Figure 2A). However, at pH 7.2, in the presence of glycolipid, enzyme binding declined compared to the degree of binding at acidic pH (supplementary Figure 2B).

## Discussion

Archaeosome lipid vesicles carrying an entrapped antigen are capable of antigen presentation to both MHC classes I and II. Archaeosomes prepared from the polar lipids of *M. smithii* have major amounts of β-gentiobiosyl and phosphoserine lipids (Sprott et al. 1999) and are especially good adjuvants to promote the MHC class I (CTL) response in mice (Krishnan, Sad, et al. 2000; Krishnan et al. 2003). Because these archaeosomes are taken up by antigen-presenting cells by receptor-mediated phagocytosis and deliver protein antigen to the cytosol from acidic phagolysosomes, a mechanism involving archaeosome fusion with the phagolysosome membrane and exocytosis of antigen to the cytosol is likely. In apparent contradiction to this mechanism, archaeosomes remain fusion-free for long periods during storage at acidic pH (Choquet et al. 1994). Although little is known regarding the fusion of archaeosome membranes generally, one archaeosome type prepared from a high proportion of membrane-spanning caldarchaeol lipids from *Sulfolobus acidocaldarius* did slowly fuse, but only at neutral pH values, and was dependent on phosphate $> 5$ mM and $Ca^{2+} > 3.5$ mM (Elferink et al. 1997). This finding appeared to exclude fusion within the phagolysosome. It was reasoned herein that fusion in the context of antigen delivery should be tested using conditions reported for the phagolysosome environment. Free $Ca^{2+}$ has been measured in macrophage lysosomes at 0.4–0.6 mM resulting in an inwardly directed gradient (inside high) that was dependent

on the proton gradient (inside acidic) (Christensen et al. 2002). Further, phagosomes maintain a pH of 4.0–5.0 (Mellman et al. 1986) and contain various carbohydrate and many other processing enzymes (Garin et al. 2001). The primary finding from this present study is that *M. smithii* archaeosomes dramatically fuse, strictly dependent on acidic pH and the presence of glucosidase. Also, $Ca^{2+}$ and $Na^{+}$ exert a major influence on the shape of the fusion product obtained in vitro.

Lipid mixing during fusion could be demonstrated for both *M. smithii* archaeosome self-fusion and for the fusion of archaeosomes with nonfusogenic PC/PG/cholesterol liposomes. Induction of fusion was specific to β-glucosidase (or α-glucosidase), not occurring with various other enzymes and proteins tested, suggesting specificity for the β-gentiobiosyl lipids of these archaeosomes. Further, the fusion of *M. voltae* archaeosomes containing β-gentiobiosyl head groups was also induced by glucosidase, whereas archaeosomes lacking glycolipids (*N. magadii*) were unaffected.

Enzymatic removal of glucose units from the archaeosome surface imparting vesicle instability and fusion was unlikely to be the mechanism for the following reasons. First, α-glucosidase was equally active to β-glucosidase, both demonstrating the same requirements for acidic pH despite the fact that the pH optimum for activity of these enzymes is approximately 7 and 5, respectively. Further, no significant degradation of β-gentiobiosyl lipids during fusion could be demonstrated by either radiotracer or $^{1}H$ NMR methods. Although it is not possible to exclude the possibility that a very few head group cleavages could be involved, taken together the results indicate that glucosidase-induced fusion is not the result of lipid degradation.

The first step in membrane–membrane fusion processes is promotion of membrane proximity (Martens and McMahon 2008). That negatively charged surfaces of archaeosomes containing acidic phospholipid and neutral glycolipid may be brought together through the interaction with glucosidase at acidic pH, is demonstrated by the fact that both α- and β-glucosidases induced rapid, acidic pH-dependent aggregation. At pH values below 5.0, the α- and β-glucosidases used in this study are positively charged and bind to the anionic liposomes by electrostatic interactions. The degree of liposome aggregation and hence cross-linking of liposomes by glucosidase is dependent on the charge density in the liposomes. Polylysine has been shown to aggregate liposomes prepared from cell membranes and phospholipids in a similar manner (Rosier et al. 1979). In addition, polylysine has been shown to induce the pH-dependent fusion of phosphatidylserine-containing phospholipid vesicles (Walter et al. 1986). The latter study concluded that optimal conditions for fusion are when charge neutralization occurs without completely covering the vesicles with peptide. *M. smithii* archaeosomes have a high content of the anionic lipids archaetidylserine (30 mol%) and gentiobiosyl-caldarchaetidylserine (>10 mole%). At pH 4.5, near the pKa of the serine carboxyl group, lipid head group protonation may be expected to complement charge neutralization by the enzyme. Thus, the glucosidases may promote archaeosome–lysosome membrane fusion at low pH by mediating close membrane apposition through aggregation and charge neutralization.

Because a transmembrane pH gradient may induce a redistribution of phospholipids in liposomes (Hope et al. 1989) and archaeal lipid membranes maintain a pH gradient through their reduced permeability to protons (Elferink et al. 1994; Komatsu and Chong 1998; Mathai et al. 2001), the possibility was tested that fusion might depend on induction of a proton gradient upon adding archaeosomes to a low pH buffer. This was excluded by showing fusion in the presence of a protonophore and in the absence of ΔpH where inside and outside pH was 4.8. The role of acidic pH, then, was attributed to membrane–membrane contact via glucosidase. A role for $Ca^{2+}$ may be anticipated in the fusion process, as $Ca^{2+}$ has an affinity for phosphatidylserine liposomes and induces the fusion of these without protein involvement (Papahadjopoulos et al. 1974), and *M. smithii* archaeosomes contain major amounts of phosphoserine isoprenoids. Although lipid mixing still occurred in the absence of added $Ca^{2+}$ in the case of archaeosome self-fusions (Figure 4), $Ca^{2+}$ (and $Na^{+}$) ions dramatically affected the shape of the fusion product obtained (Figure 2). It is clear that the content of $Ca^{2+}$, $Na^{+}$, (or $K^{+}$) required for the formation of fusion balls in this in vitro study are likely within physiological amounts expected in phagolysosomes, but the importance of $Ca^{2+}$ or $Na^{+}$ in exocytosis of antigen in vivo has yet to be determined. The ability of archaeosomes to effectively adjuvant the CTL response in animals, with long lasting memory in the case of *M. smithii* archaeosomes (Gurnani et al. 2004; Krishnan et al. 2007), correlates to fusions which would efficiently deliver antigen by exocytosis specifically from the phagolysosome of phagocytic antigen-presenting cells.

Archaeosomes composed primarily of bipolar caldarchaeol lipids of *S. acidocaldarius* have been shown to aggregate in very high concentrations of $Ca^{2+} > 10$ mM, with the concentration of $Ca^{2+}$ depending on pH and temperature (Kanichay et al. 2003). *M. smithii* archaeosomes composed of 60 wt% archaeol monopolar and 40 wt% caldarchaeol bipolar lipids also aggregate in approximately 15 mM $Ca^{2+}$ to form very large aggregated structures (Patel et al. 2008). Aggregation of these archaeosomes observed in this study and measured in the presence of 1 mM $Ca^{2+}$ differs, as it occurred only at acidic pH and in the presence of glucosidase. Further, archaeosomes prepared from *M. voltae* and *M. mazei* polar lipids that lack caldarchaeols completely fused to form the same dramatic large fusion balls by a process that was also dependent on the presence of glucosidase, acidic pH, $Ca^{2+}$, and $Na^{+}$. It can be concluded that the presence of caldarchaeol lipids is neither a requirement for glucosidase-induced fusion to occur nor does the presence of up to 40 wt% caldarchaeols prevent glucosidase-induced fusion. This is consistent with the finding that archaeosomes could be induced to fuse, albeit under different conditions, in the presence of up to 95% membrane-spanning caldarchaeols (Relini et al. 1996).

We have demonstrated that, at acidic pH, glucosidase can dramatically induce the fusion of archaeosomes containing glucosyl isoprenoid lipids. It may be anticipated that different enzymes present in phagolysosomes may account for the fusion of lipid vesicles having other glyco head groups with differing in vivo fusion efficiencies. Recent results have shown that the potency of synthetic archaeal lipid archaeosomes as CTL adjuvants depends on the presence and structure of a glycolipid (Sprott et al. 2008), thus further supporting the notion to include glycolipids in the design of lipid-based adjuvant delivery systems. The phagolysosome environment appears to promote fusion and exocytosis of antigen from glycolipid/phospholipid archaeosomes, resulting in cytosolic delivery for MHC class I processing.

## Material and methods

*Preparation of liposomes and archaeosomes*

Liposomes were prepared from DPPG/cholesterol (80/20 mole%) and DPPC/DPPG/cholesterol (37/4/59 mole%) (Avanti Polar Lipids, Inc, Alabaster, AL, USA). Archaeosomes consisted of total polar lipids prepared from *M. smithii* AL1 (DSM 2375) as described (Sprott, Patel, et al. 2003). To prepare rhodamine-phosphatidylethanolamine-labeled (rhodamine-PE, Molecular Probes, Eugene, OR) archaeosomes and liposomes, the label dissolved in chloroform was added to lipids at 0.5% (w/w). In some cases, the total polar lipids of other archaea were prepared as for *M. smithii*. Biomass was generated for the archaea *N. magadii* (Kamekura and Kates 1999), *H. salinarum* (Kamekura and Kates 1999), *M. voltae* (Ferrante et al. 1986), *M. mazei* (Sprott, Dicaire, et al. 1994), and *M. hungatei* (Sprott, Ferrante, et al. 1994). Lipids (30 mg) were dried from $CHCl_3/CH_3OH$ and the dried lipid film was hydrated in 2.5 mL deionized water at 37°C. Average vesicle diameters were decreased to approximately 100 nm by sonicating in a heated water bath sonicator. Liposomes or archaeosomes were then filter sterilized using PVDF 0.45 μm membranes (Millipore) and stored at refrigeration temperature. Vesicles initially characterized by particle size (Nicomp 370) and salt-free dry weight measurements remained stable indefinitely. Archaeosomes and liposomes for $^1$H NMR experiments were prepared as above, except hydration was in $D_2O$ rather than water.

*Fusion assay*

The assay for membrane fusion consisted of the 25 mM sodium acetate buffer, pH 4.8, 140 mM NaCl, 1.0 mM $CaCl_2$, 0.8 μg archaeosomes and/or liposomes/μL, and to start the fusion process 0.3 μg β-glucosidase/μL. Variations and further details are given in figure legends. Fusions were at 37°C for as long as 1.5 h. β-Glucosidase (EC 3.2.1.21 from almond, essentially salt-free) was replaced in some trials by α-glucosidase (EC 3.2.1.20 from *Bacillus stearothermophilus*), β-glucuronidase (EC 3.2.1.31 from bovine liver), phospholipase C (EC 3.1.4.3 from *Bacillus cereus*), phosphatidylinositol-specific phospholipase C (EC 4.6.1.13 from *Bacillus cereus*), lysozyme, and ovalbumin. All proteins were purchased from Sigma.

*Microscopy*

DIC and fluorescence microscopy were performed using a Zeiss Axiovert 200M microscope. For detection of rhodamine, label excitation was at 550 nm and emission 580 nm. Phase contrast images were photographed using an Olympus BX 51 microscope.

*Light scattering*

Turbidity changes that occur upon the addition of α- or β-glucosidase to archaeosomes were monitored over time using a Coleman 575 spectrophotometer at 660 nm, 1 cm light path. Steady-state turbidity changes were recorded for 30 min following the addition of glucosidase. Reaction mixtures (0.5 mL) were 25 mM sodium acetate, pH 4.8 or 6.8, 140 mM NaCl, 1.0 mM $CaCl_2$, and 170 μg archaeosomes. Glucosidase at various concentrations started the reactions.

*Lipid mixing RET assay*

RET assays to assess lipid mixing between lipid membranes (Struck et al. 1981) were essentially as described by Ahmad et al. (2001). Lipid mixing was monitored between NBD/rhodamine-labeled archaeosomes and unlabeled archaeosomes, and between NBD/rhodamine-labeled liposomes and unlabeled archaeosomes. Fluorescent probes (Molecular Probes), L-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (rhodamine-PE), were incorporated into lipid vesicles by adding to lipids at 1.0 and 0.6 mol%, respectively, prior to drying and hydration. Reaction mixture details are given in figure legends. RET was followed between NBD and rhodamine using a Turner fluorometer with excitation at 470 nm and fluorescence monitored at 515 nm. Maximal dequenching was determined by disrupting vesicles with 0.5% octyl-β-D-glucopyranoside detergent and taken as a measure to estimate 100% fusion.

*Metabolism of lipids*

*M. smithii* was grown anaerobically (Sprott, Patel, et al. 2003) in 100 mL medium contained in 1 L sealed bottles. To label the isoprenoid chains of polar lipids, 10 μCi of [2-$^{14}$C]mevalonic acid (Amersham Biosciences, Piscataway, NJ) was included during growth. Labeled lipids were extracted from the biomass and the total polar lipids freed of any neutral lipids by several precipitations from cold acetone (Sprott et al. 1999). $^{14}$C-polar lipids were mixed 10-fold with unlabeled archaeal total polar lipids from *M. smithii* prior to forming archaeosomes. Fusion assays in 8.0 mL total volume contained the 25 mM sodium acetate buffer, pH 4.8 or 6.9, 140 mM NaCl, 1.0 mM $CaCl_2$, 0.76 μg $^{14}$C-archaeosomes/μL, and 0.3 μg/μL of β-glucosidase to start the reaction. Incubation was at 37°C for 1.5 h. In addition, a control included the same reaction mix at pH 4.8, but without β-glucosidase. Lipids were then extracted into $CHCl_3$ by the Bligh/Dyer method (Kates 1986) and counted by liquid scintillation using Ecolite (MP Biomedicals, Solon, OH). Lipids (2,500 DPM/spot) were applied to 20 × 20 cm, 0.25 mm, silica gel 60 thin-layer plates (Merck) and developed using $CHCl_3/CH_3OH$/acetic acid/water (85/22.5/10/4, v/v). Lipids were located using $I_2$ vapor and identified based on inclusion of a β-gentiobiosyl-archaeol standard (Sprott et al. 2008) along with unlabeled total polar lipids (Sprott et al. 1999) that were separated and stained for sugar and phospho groups (Kates 1986). Spots corresponding to major lipids β-gentiobiosyl-caldarchaetidylserine and β-gentiobiosyl-caldarchaetidylinositol (of similar Rf) were combined, and β-gentiobiosyl-archaeol, archaetidylserine, and archaetidic acid were removed and counted.

*Glucosidase/lipid membrane binding assays using $^1$H NMR*

Binding was conducted in the sodium acetate buffer, at pH 4.5 or 7.2, containing 140 mM NaCl, 1.0 mM $CaCl_2$, and 0.34 μg/μL of α- or β-glucosidase. Archaeosomes and/or liposomes at 2.15 μg/μL initiated binding which was allowed to proceed at 37°C for 30 min prior to $^1$H NMR data acquisitions. Solutions were prepared in $D_2O$ and lipids were hydrated in $D_2O$. β-Gentiobiosyl-archaeol was synthesized previously by D.M. Whitfield (Sprott et al. 2008). Final sample size was typically 160 μL in a 3 mm (O.D.) NMR tube.

$^1$H NMR spectra were acquired at 25°C on a Varian Inova spectrometer operating at 500 MHz. Spectra were acquired with a spectral width of 8 kHz, 8 K complex data points, and a recycle time of 2 s. The data were processed with zero filling to 32 K complex data points and 3 Hz apodization prior to Fourier transformation.

## Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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## Conflict of interest statement

None declared.

## Abbreviations

Archaeol, 2,3-di-*O*-phytanyl-sn-glycerol; archaeosomes, liposomes prepared from archaeal polar lipids; β-gentiobiosyl, β-D-glucosyl-(1,6)-β-D-glucosyl; caldarchaeol, 2,2′,3,3′-tetra-*O*-dibiphytanyl-sn-diglycerol; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CTL, cytotoxic $CD8^+$ T cell response; DIC, differential interference contrast microscopy; EGTA, ethylene glycol tetraacetic acid; RET, resonance energy transfer.

## References

Ahmad N, Masood AK, Owais M. 2001. Fusogenic potential of prokaryotic membrane lipids. Implication in vaccine development. *Eur J Biochem.* 268:5667–5675.

Beveridge TJ, Choquet CG, Patel GB, Sprott GD. 1993. Freeze-fracture planes of methanogen membranes correlate with the content of tetraether lipids. *J Bacteriol.* 175:1191–1197.

Choquet CG, Patel GB, Beveridge TJ, Sprott GD. 1994. Stability of pressure-extruded liposomes made from archaeobacterial ether lipids. *Appl Microbiol Biotechnol.* 42:375–384.

Choquet CG, Patel GB, Sprott GD. 1996. Heat sterilization of archaeal liposomes. *Can J Microbiol.* 42:183–186.

Christensen KA, Myers JT, Swanson JA. 2002. pH-dependent regulation of lysosomal calcium in macrophages. *J Cell Sci.* 115:599–607.

Connor J, Huang L. 1985. Efficient cytoplasmic delivery of a fluorescent dye by pH-sensitive immunoliposomes. *J Cell Biol.* 101:582–589.

Connor J, Yatvin MB, Huang L. 1984. pH-sensitive liposomes: Acid-induced liposome fusion. *Proc Natl Acad Sci USA.* 81:1715–1718.

Elferink MG, van Breemen J, Konings WN, Driessen AJ, Wilschut J. 1997. Slow fusion of liposomes composed of membrane-spanning lipids. *Chem Phys Lipids.* 88:37–43.

Elferink MGL, deWit JG, Driessen AJM, Konings WN. 1994. Stability and proton-permeability of liposomes composed of archaeal tetraether lipids. *Biochim Biophys Acta.* 1193:247–254.

Ferrante G, Ekiel I, Sprott GD. 1986. Structural characterization of the lipids of *Methanococcus voltae*, including a novel *N*-acetylglucosamine 1-phosphate diether. *J Biol Chem.* 261:17062–17066.

Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, Sadoul R, Rondeau C, Desjardins M. 2001. The phagosome proteome: Insight into phagosome functions. *J Cell Biol.* 152:165–180.

Gomez JM, Romero MD, Fernandez TM. 2005. Immobilization of β-glucosidase on carbon nanotubes. *Catalysis Lett.* 101:275–278.

Gurnani K, Kennedy J, Sad S, Sprott GD, Krishnan L. 2004. Phosphatidylserine receptor-mediated recognition of archaeosome adjuvant promotes endocytosis and MHC class I cross-presentation of the entrapped antigen by phagosome-to-cytosol transport and classical processing. *J Immunol.* 173:566–578.

Hope MJ, Redelmeier TE, Wong KF, Rodrigueza W, Cullis PR. 1989. Phospholipid asymmetry in large unilamellar vesicles induced by transmembrane pH gradients. *Biochemistry.* 28:4181–4187.

Kamekura M, Kates m. 1999. Structural diversity of membrane lipids in members of *Halobacteriaceae*. *Biosci Biotechnol Biochem.* 63:969–972.

Kanichay R, Boni LT, Cooke PH, Khan TK, Chong PL. 2003. Calcium-induced aggregation of archaeal bipolar tetraether liposomes derived from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. *Archaea.* 1:175–183.

Kates M. 1978. The phytanyl ether-linked polar lipids and isoprenoid neutral lipids of extremely halophilic bacteria. *Prog Chem Fats Other Lipids.* 15: 301–342.

Kates M. 1986. *Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids*. New York: Elsevier.

Komatsu H, Chong PL. 1998. Low permeability of liposomal membranes composed of bipolar tetraether lipids from thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. *Biochemistry.* 37:107–115.

Krishnan L, Dicaire CJ, Patel GB, Sprott GD. 2000. Archaeosome vaccine adjuvants induce strong humoral, cell-mediated, and memory responses: Comparison to conventional liposomes and alum. *Infect Immun.* 68:54–63.

Krishnan L, Gurnani K, Dicaire CJ, van Faassen H, Zafer A, Kirschning CJ, Sad S, Sprott GD. 2007. Rapid clonal expansion and prolonged maintenance of memory $CD8^+$ T cells of the effector (CD44highCD62Llow) and central (CD44highCD62Lhigh) phenotype by an archaeosome adjuvant independent of TLR2. *J Immunol.* 178:2396–2406.

Krishnan L, Sad S, Patel GB, Sprott GD. 2000. Archaeosomes induce long-term $CD8^+$ cytotoxic T cell response to entrapped soluble protein by the exogenous cytosolic pathway, in the absence of $CD4^+$ T cell help. *J Immunol.* 165:5177–5185.

Krishnan L, Sad S, Patel GB, Sprott GD. 2003. Archaeosomes induce enhanced cytotoxic T lymphocyte responses to entrapped soluble protein in the absence of interleukin 12 and protect against tumor challenge. *Cancer Res.* 63:2526–2534.

Martens S, McMahon HT. 2008. Mechanisms of membrane fusion: Disparate players and common principles. *Nat Rev Mol Cell Biol.* 9:543–556.

Mathai JC, Sprott GD, Zeidel ML. 2001. Molecular mechanisms of water and solute transport across archaebacterial lipid membranes. *J Biol Chem.* 276:27266–27271.

Mellman I, Fuchs R, Helenius A. 1986. Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem.* 55:663–700.

Nishihara M, Utagawa M, Akutsu H, Koga Y. 1992. Archaea contain a novel diether phosphoglycolipid with a polar head group identical to the conserved core of eucaryal glycosyl phosphatidylinositol. *J Biol Chem.* 267:12432–12435.

Papahadjopoulos D, Poste G, Schaeffer BE, Vail WJ. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. *Biochim Biophys Acta.* 352:10–28.

Patel GB, Ponce A, Zhou H, Chen W. 2008. Structural characterization of archaeal lipid mucosal vaccine adjuvant and delivery (AMVAD) formulations prepared by different protocols and their efficacy upon intranasal immunization of mice. *J Liposome Res.* 18:127–143.

Relini A, Cassinadri D, Fan Q, Gulik A, Mirghani Z, De RM, Gliozzi A. 1996. Effect of physical constraints on the mechanisms of membrane fusion: Bolaform lipid vesicles as model systems. *Biophys J.* 71:1789–1795.

Relini A, Cassinadri D, Mirghani Z, Brandt O, Gambacorta A, Trincone A, De RM, Gliozzi A. 1994. Calcium-induced interaction and fusion of archaeobacterial lipid vesicles: A fluorescence study. *Biochim Biophys Acta.* 1194:17–24.

Rosier RN, Gunter TE, Tucker DA, Gunter KK. 1979. A rapid method for separating small vesicles from suspension. *Anal Biochem.* 96:384–390.

Sprott GD, Brisson J, Dicaire CJ, Pelletier AK, Deschatelets LA, Krishnan L, Patel GB. 1999. A structural comparison of the total polar lipids from the human archaea *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* and its relevance to the adjuvant activities of their liposomes. *Biochim Biophys Acta.* 1440:275–288.

Sprott GD, Dicaire CJ, Cote JP, Whitfield DM. 2008. Adjuvant potential of archaeal synthetic glycolipid mimetics critically depends on the glyco head group structure. *Glycobiology.* 18:559–565.

Sprott GD, Dicaire CJ, Patel GB. 1994. The ether lipids of *Methanosarcina mazei* and other *Methanosarcina* species, compared by fast atom bombardment mass spectrometry. *Can J Microbiol.* 40:837–843.

Sprott GD, Ferrante G, Ekiel I. 1994. Tetraether lipids of *Methanospirillum hungatei* with head groups consisting of phospho-*N*,*N*-dimethylaminopentanetetrol, phospho-*N*,*N*,*N*-trimethylaminopentanetetrol, and carbohydrates. *Biochim Biophys Acta.* 1214:234–242.

Sprott GD, Krishnan L. 2007. Archaeosome vaccines. In: *Archaea*. Washington (DC): ASM Press. p. 496–510.

Sprott GD, Patel GB, Krishnan L. 2003. Archaeobacterial ether lipid liposomes as vaccine adjuvants. *Methods Enzymol.* 373:155–172.

Sprott GD, Sad S, Fleming LP, Dicaire CJ, Patel GB, Krishnan L. 2003. Archaeosomes varying in lipid composition differ in receptor-mediated endocytosis and differentially adjuvant immune responses to entrapped protein. *Archaea.* 1:151–164.

Straubinger RM, Duzgunes N, Papahadjopoulos D. 1985. pH-sensitive liposomes mediate cytoplasmic delivery of encapsulated macromolecules. *FEBS Lett.* 179:148–154.

Struck DK, Hoekstra D, Pagano RE. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry.* 20:4093–4099.

Takii Y, Daimon K, Suzuki Y. 1992. Cloning and expression of a thermostable exo-α-1,4-glucosidase gene from *Bacillus stearothermophilus* ATCC12016 in *Escherichia coli*. *Appl Microbiol Biotechnol.* 38:243–247.

Walter A, Steer CJ, Blumenthal R. 1986. Polylysine induces pH-dependent fusion of acidic phospholipid vesicles: A model for polycation-induced fusion. *Biochim Biophys Acta* 861:319–330.