Xpf suppresses mutagenic consequences of bacterial phagocytosis in

Dictyostelium

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Summary statement (30 words)

The DNA repair nuclease Xpf helps to maintain the integrity of the genome during bacterial phagocytosis in the amoeba Dictyostelium
Abstract

As time passes, mutations accumulate in the genomes of all living organisms. These changes promote genetic diversity, but also precipitate ageing and the initiation of cancer. Food is a common source of mutagens, but little is known about how nutritional factors cause lasting genetic changes in the consuming organism. Here, we describe an unusual genetic interaction between DNA repair in the unicellular amoeba – *Dictyostelium discoideum* – and its natural bacterial food source. *Dictyostelium* deficient in the DNA repair nuclease Xpf displays a severe and specific growth defect when feeding on bacteria. Despite being proficient in the phagocytosis and digestion of bacteria, over time, *xpf* *Dictyostelium* feeding on bacteria ceases to grow and in many instances die. The Xpf nuclease activity is required for sustained growth using a bacterial food source. Furthermore, the ingestion of this food source leads to a striking accumulation of mutations in the genome of *xpf* *Dictyostelium*. This work therefore establishes *Dictyostelium* as a model genetic system to dissect nutritional genotoxicity, providing insight into how phagocytosis can induce mutagenesis and compromise survival fitness.
Introduction

The DNA damage response is highly conserved and prevents the accumulation of deleterious DNA damage after exposure to environmental mutagens. However, organisms and cell types show enormous variability in their susceptibility to mutagen exposure. For instance, in vertebrates certain lineages such as blood stem cells are highly sensitive to DNA damage, whilst others such as muscle cells are resistant to the same mutagens (Meijne et al., 1991; Rossi et al., 2007). On a quite different scale are organisms such as Deinococcus radiodurans and Dictyostelium discoideum, which are highly resistant to mutagens (Deering, 1968; Zahradka et al., 2006; Zhang et al., 2009). It is clear that these organisms have evolved DNA damage responses capable of rapidly and efficiently repairing extensive DNA damage (Hudson et al., 2005). However, an important question remains: Why have they evolved such effective DNA repair? One possibility is that both D. radiodurans and Dictyostelium have unusual life cycles in that they can survive in dormant desiccated states. Such suspended existence could lead to the accumulation of extensive DNA damage that must be repaired to resume growth. Another possibility is that they are exposed to heavy mutagenesis as a consequence of their life cycle or niche, including food as a source of mutagens. In the wild, Dictyostelium feeds on bacteria by phagocytosis. The ingested microorganism is trapped in a phagolysosome where it is ultimately killed and degraded, resembling professional phagocytes such as macrophages (Cosson and Soldati, 2008). Here we show that Dictyostelium amoebae use the DNA repair nuclease Xpf to protect their genome from mutagens released during consumption of bacteria, revealing an unanticipated role of DNA repair in bacterial phagocytosis.
Results and Discussion

A genetic requirement for the DNA repair gene \( xpf \) to enable \( Dictyostelium \) to feed on \( Klebsiella \)

In the wild, the unicellular amoeba \( Dictyostelium discoideum \) is found in the soil litter feeding predominantly on bacteria and dividing by binary fission (Hohl and Raper, 1963; Weeks and Weijer, 1994). In the laboratory, this organism can be propagated either on agar plates coated with a \( Klebsiella \) \( aerogenes \) \( (K.a.) \) bacterial lawn, or it can be grown in axenic media (Fey et al., 2007). We previously reported that this organism is highly resistant to the mutagen and DNA crosslinking agent Cis-platin (Zhang et al., 2009). This resistance is under genetic control because \( Dictyostelium \) deficient in the excision repair nuclease Xpf \( (xpf) \) is hypersensitive to this DNA crosslinking agent. However, whenever we propagated the \( xpf \) strain we noted a profound growth defect on \( K.a. \) plates (Fig. 1A, B). To address whether this defect was due to a loss of viability, we quantified the colony forming efficiency. As seen in Fig. 1C, the plating efficiency of Xpf-deficient cells was greatly reduced when grown on \( K.a. \). In contrast, this strain grew as well as wild type \( (Ax2) \) \( Dictyostelium \) in axenic media (Fig. 1D, E). Furthermore, the cloning efficiency of \( xpf \) in axenic medium was comparable with \( Ax2 \) (Fig. 1F), indicating that the vegetative growth defect is specifically associated with feeding on \( K.a. \) but not with axenic medium. Thus, these data suggest that the \( xpf \) strain struggles to proliferate when utilizing live \( K.a. \) as a nutritive source.

\( xpf \) \( Dictyostelia \) fail to thrive on a range of bacterial strains

We next tested whether this growth defect could be observed with other known bacterial food sources \( (Escherichia coli, Micrococcus luteus and Bacillus subtilis) \). \( xpf \) amoebae showed a growth defect on all three quite distinct bacteria to varying degrees (Fig. 2A, B, S1A). We then asked if this effect depended on live food or could also be observed when fed dead bacteria. We therefore heat-inactivated \( K.a. \) for 20 min at 121°C. This preparation can still support \( Dictyostelium \) growth but it is not as nutritious as living \( K.a. \) (Fig. 2A, B, S1A). However, heat inactivated \( K.a. \) was also toxic to \( xpf \) amoebae, suggesting that the bacteria do not need to be metabolically active at the time of ingestion to affect \( xpf \) amoebae.
Xpf is not required for efficient uptake and digestion of bacteria

In order to feed on bacteria, *Dictyostelium* must internalise their prey and then digest it to release nutrients (Cosson and Soldati, 2008; Lelong et al., 2011). This whole process contrasts to when *Dictyostelium* grows in axenic media, where nutrient uptake occurs through a process called macropinocytosis (Bloomfield et al., 2015; Cardelli, 2001). We therefore determined whether the *xpf* strain is competent at ingesting and subsequently digesting bacteria. Ax2 and *xpf* strains were pulsed with bacteria for varying times and the number of bacterial colony forming units (CFU) per amoeba was scored for both the total bacteria bound and internalized by *Dictyostelium* (Lelong et al., 2011). The CFU number did not markedly differ between wild type and *xpf* *Dictyostelium*, strongly indicating that *xpf* cells do not present a defect in bacterial killing (Fig. 2C, D). We confirmed the bacterial phagocytic proficiency by quantifying the uptake of tetramethylrhodamine isothiocyanate (TRITC)-labeled yeast (Fig. 2E) (Rivero and Maniak, 2006). Finally, when bacteria are ingested by the amoeba, the nascent phagosome incorporates the membrane protein p80 – a reliable marker of the endocytic pathway (Ravanel et al., 2001). Ax2 and *xpf* strains were therefore pulsed with GFP-expressing *K.a.* and were then visualized by confocal microscopy. Immunofluorescent detection of p80 co-localised with GFP-containing vesicles. These results show that in both Ax2 and *xpf*, GFP-labelled *K.a.* fuse with p80-containing vesicles, causing the decrease of the GFP signal denoting bacterial digestion (Fig. 2F). Supporting this observation, the clearance in the supernatant of a suspension where both *Dictyostelium* and *K.a.* are co-incubated was similar between Ax2 and *xpf* (Fig. 2G). From this set of experiments, we can conclude that whilst the *xpf* strain is sensitive to *K.a.*, this sensitivity was not due to defective uptake or digestion of bacterial food.

The endonuclease activity of Xpf is specifically required to tolerate a bacterial food source

Xpf is an endonuclease that cuts out damaged DNA caused by UV irradiation and interstrand crosslinking agents (Ahmad et al., 2008; Enzlin and Scharer, 2002). We therefore set out to establish if this endonuclease activity is required for *Dictyostelium* to effectively utilize bacteria as a food source. Xpf, and in particular its
nuclease motif carrying a critical metal-binding aspartic acid residue, is highly conserved between humans and Dictyostelium (Fig. 3A) (Enzlin and Scharer, 2002). Consequently, we transfected Ax2 and xpf Dictyostelium cells with plasmids for expression of GFP fusions of either wild type Dictyostelium Xpf (Xpf) or a nuclease-dead mutant form where the key aspartic acid residue in the nuclease motif was mutated to alanine (Xpf-D771A). All transfected strains expressed the recombinant Xpf (Fig. S1B), but only wild type Xpf and not the nuclease-dead mutant rescued the growth defect of xpf cells on K.a. bacterial lawns (Fig. 3B, S1B, S1C). To extend our analysis we then tested if two other DNA repair nuclease-deficient Dictyostelium strains (Mus81 – mus81 and FAN1 – fan1) were also susceptible to growth inhibition on K.a. plates. In fact, none of these mutants exhibited a growth defect on K.a. plates (Fig. 3C). However, Dictyostelium has robust DNA repair systems and it has been described as a gamma-ray resistant organism (Deering, 1968; Hudson et al., 2005). Accordingly, we investigated mutants in Xpf-related DNA repair pathways for their contribution to tolerance of bacterial mutagens and found that Dictyostelium knockouts in the translesion synthesis DNA-repair polymerase Rev3 – rev3 and the global nucleotide excision repair (NER) gene xpc (xpc) showed a mild growth defect, while fncD2, inactivating the Fanconi Anemia DNA Repair pathway, showed comparable growth on K.a. bacterial lawns to the wild type strain Ax2 (Fig. S1D). Taken together, our results show that sustained growth on bacterial plates specifically requires the nuclease activity of Xpf.

Bacterial consumption leads to induced mutagenesis in xpf Dictyostelium

The Xpf endonuclease repairs damaged DNA; we reasoned that in its absence, damaged DNA should accumulate and may eventually lead to mutagenesis. Direct approaches to detect DNA damage were unsuccessful. However, we measured mutagenesis frequency in Dictyostelium by using resistance to methanol (Garcia et al., 2002; Podgorski and Deering, 1980). This mutagenic reporter system relies on the fact that methanol is toxic to Dictyostelium because of its conversion by the enzyme Catalase A (CatA) into toxic formic acid (Garcia et al., 2002). Inactivation of
the catA gene leads to failure to convert methanol into formic acid and hence confers resistance to this alcohol (Fig. 4A). We therefore set up a methanol mutagenesis assay to determine the frequency of resistance to methanol in Ax2 and xpf after exposure to K.a (Fig. 4B). Briefly, we first took Ax2 and xpf cells and expanded them from single clones into six well plates in axenic media, and then plated these out on methanol-containing plates. In parallel, we took the same two strains and plated them out onto K.a. plates, picked single colonies, re-plated on new K.a. agar plates. We then scraped the entire population from a single plate and briefly expanded them in individual wells within a 6 well plate; the expanded population was then plated onto methanol plates. Methanol resistance (as number of resistant colonies per $1 \times 10^6$ viable cells) was then determined on methanol agar plates. A clone that acquired a catA mutation early during growth in any differential condition will show an elevated number of methanol-resistant colonies, but by repeating the assay many times with independent cultures, this fluctuation assay captures the mutation frequency (Luria and Delbruck, 1943). The data in Fig. 4C show that both Ax2 and xpf Dictyostelium developed few methanol-resistant clones when propagated in axenic media. In contrast, when propagated on K.a. plates, the xpf strain shows a striking induction of methanol-resistant colonies compared to Ax2, indicating that Xpf prevents mutagenesis in this growth condition. Next, we sought to determine the mutational pattern underlying these mutagenic events and thus amplified, cloned, and sequenced the catA gene from Ax2 and xpf methanol resistant clones. However, the pattern of mutations did not differ greatly between the two strains (Fig. S2), although it is important to note that the mutational pattern observed here may be biased towards gene-disrupting mutations, which are more likely to cause enzyme inactivation than point mutations.

It is thus very likely that the reason xpf amoebae fail to thrive in the presence of a bacterial food source is due to the accumulation of DNA damage. Xpf participates in several DNA repair pathways, including homologous recombination, NER and the Fanconi pathway (Manandhar et al., 2015), which may explain why its role is so fundamental on phagocytic growth. We can only speculate as to the mechanism responsible for causing DNA damage. The simplest explanation is that genotoxins are produced by the ingested bacteria, although it is unlikely to be an exotoxin, since heat-inactivated bacteria are still toxic. Plausibly, the genotoxin might be an integral
bacterial part that is not inactivated by heat sterilisation, or it could be a substance generated as a consequence of ‘digesting’ this food source. Another intriguing possibility comes from the resemblance of *Dictyostelium* to neutrophils, which kill ingested bacteria by using respiratory burst activity (Chen et al., 2007; Cosson and Soldati, 2008; Zhang et al., 2016). Neutrophil killing generates a battery of reactive molecules such as hypochlorus acid and reactive oxygen species, which are known to be highly mutagenic (Knaapen et al., 2006). It is thus conceivable that the mutagen forms part of an amoebal immune response to bacteria. An intriguing observation is that bacteria differ in their potency to inhibit growth. Exploring which factors determine these differences might give insight into the nature of genotoxicity. Finally, this work highlights the intricate manner by which nutritional sources might stimulate mutagenesis. Consuming food is essential to (heterotrophic) life, but as this work highlights often comes at considerable mutational cost against which the organism must defend itself.
Material and methods

Cell culture and molecular biology procedures

All Dictyostelium strains were routinely grown at 22°C in HL5 (axenic medium) supplemented with streptomycin (200 μg/ml). Bacterial lawn plates were made by spreading 300 μl of an overnight bacterial culture on SM-agar plates and pictures were taken after five days at 22°C. For Micrococcus luteus, 0.5 liter of stationary-phase culture was pelleted and resuspended in 50 ml, and then one ml of this culture was spread on SM-agar plates. Pictures were taken after 9 days. Comparison between two groups was done using t-test in Prism software.

The parental strain was the Kay laboratory version of Ax2, according to the following nomenclature: Ax2 (wild type), HM1403 (xpf), HM1464 (mus81), HM1253 (fncD2), HM1456 (xpc), HM1351 (rev3) (Zhang, et al. 2009), HM1515 (fan1/DDB_G0267916). Colony forming efficiency in HL5 was determined by sorting one cell per well in 96 well plates. After 20 days, the number of confluent wells per plate was scored and represented as percentage. Colony forming efficiency on bacterial plates was scored by plating 25 and 50 Dictyostelium viable cells on K.a. lawns and counting the colonies after 5 days for Ax2, and 6 days for xpf. Growth profiles in axenic media were obtained using a Vi-cell analyzer (Beckman Coulter). Doubling time was determined as previously (Fey et al., 2007). Cloning of Xpf was carried out using primers described in Table S1 in pDM317. The Fan1 knockout was made using a pLPBLP-targeting vector constructed using primers listed in Table S1.

Bacterial killing and phagocytosis assays

Phagocytosis and killing of bacteria were analyzed as described previously (Benghezal et al., 2006). Phagocytosis of fluorescent TRITC (tetramethylrhodamine isothiocyanate)-labeled yeast was based on a published protocol (Rivero and Maniak, 2006). Clearance of bacteria was followed by reading optical density in the supernatant of a phosphate buffer suspension initially containing 1x10^6 Dictyostelium and 1x10^8 K.a.
Microscopy

Endocytosis and intracellular bacteria were visualized by incubating $2.5 \times 10^6$ GFP-expressing _K.a._ from an overnight culture with $5 \times 10^5$ _Dictyostelium_ cells in 500 µl of HL5 medium. p80 H161 monoclonal antibody (2 µg/ml) was used together with an Alexa 647-coupled secondary antibody (Mercanti et al., 2006; Ravanel et al., 2001). Images were acquired on a Zeiss LSM710 confocal microscope and processed in ImageJ.

Methanol sensitivity assay

Ax2 or _xpf_ cells were grown in HL5 to $1 \times 10^6$ cells/ml. From this broth, _Dictyostelium_ was plated to get isolated clones both in HL5-containing 96 well plates and on _K.a._ coated plates. The isolated clones (n=41 from HL5, and n=43 for Ax2 or n=72 for _xpf_ from _K.a._ plates) were expanded in HL5. Then, the clonal population was lifted and up to $5 \times 10^5$ viable amoebae were plated onto 3% methanol-containing SM-agar plates. After 6 days, the number of methanol-resistant colonies per plate was scored and plotted relative to the colony forming efficiency on _K.a._ plates (Garcia et al., 2002; Podgorski and Deering, 1980). Finally, methanol resistant clones were grown in HL5 and the _catA_ gene was cloned into pTOPO for sequencing.
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Competing interests

No competing interests declared.

Author contributions

LP, JL, IVR, XYZ and KJP conceived the study and designed the experimental methods. LP and JL performed the experiments. DT and RRK contributed with \textit{Dictyostelium} strains and critical discussion of the manuscript. LP and KJP wrote the paper.

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References


**Figures**

![Image](image_url)

**Figure 1.** *Dictyostelium* deficient in the DNA repair nuclease Xpf (*xpf*) present a growth defect on *Klebsiella aerogenes* lawns but not in axenic media. (A) Wild type (Ax2) and *xpf* deficient (*xpf*) *Dictyostelium* were plated on agar plates coated with *K. aerogenes* (*K.a.*) – single clones of *Dictyostelium* grow out as punched colonies. (B) Quantification of clonal growth of the two strains on *K.a.* plates, scored as plaque diameter at day 5 after growing at 22°C (n=55 and n=53 for Ax2 and *xpf*, respectively). (C) Colony forming efficiency of Ax2 and *xpf* on *K.a.* plates (n=20). **** $P < 0.0001$. (D) Growth curves for Ax2 and *xpf* in axenic medium. (E) Doubling times calculated from plot D (n=3, mean±s.e.m.). (F) Colony forming efficiency in axenic medium (n=20).
Figure 2. *xpf Dictyostelium* are proficient at bacterial phagocytosis. (A) The *xpf* strain presents a growth defect on *E. coli*, *M. luteus*, *B. subtilis* and heat-inactivated *K.a.* plates. (B) Quantification of A by plaque diameter (n=30). (C) Ax2 and *xpf* strains were incubated with a limiting amount of *K.a.*, and remaining live bacilli were then monitored over time (n=3, mean±s.e.m.). (D) Similar to C, only that the clearance of phagocytized bacteria was monitored (n=3, mean±s.e.m.). (E) Phagocytosis in *Dictyostelium* Ax2 and *xpf* strain scored by incorporation of TRITC-labeled yeast. (F) Uptake of GFP-labeled *K.a.* was monitored by confocal microscopy. White arrowheads indicate *K.a.* co-localization with the endocytic marker p80. The number of fluorescent bacteria was quantified within 120 *Dictyostelium* cells. (G) The clearance of bacteria in the supernatant of a suspension containing only *Dictyostelium* and *Klebsiella* was followed by optical density (O.D.) at 600 nm.
Figure 3: Nuclease activity of Xpf is required for growth on bacteria. (A) Domain organization of the Xpf protein – the C terminal nuclease domain is highlighted to display the high level of conservation – asterisk marks the critical aspartic acid residue (D771) that is known to be essential for the nuclease activity. (B) Expression of wild type Xpf (Xpf) or nuclease inactive point mutant (Xpf(D771A)) (clone 2). Right panel shows the quantification of plaque diameter at day 5 after growing at 22°C. (C) Growth phenotype on K.a. lawns for Dictyostelium mutants deficient in other DNA repair nucleases (Mus81 and Fan1) (n=23).
Figure 4: Consumption of bacteria promotes mutagenesis in *xpf* 
*Dictyostelium*. (A) Schematic outline of the basis of methanol resistance in 
*Dictyostelium*. Mutational inactivation of the catalase A gene (CatA) results in failure 
to break down methanol and hence survival in the presence of this alcohol. (B) 
Experimental outline of the methanol resistance assay to assess if growth in axenic 
media or on *K.a.* plates promotes the accumulation of *catA* mutations. (C) Graph 
depicting the number of methanol-resistant clones per 10⁶ viable cells obtained 
following propagation of Ax2 and *xpf* on either axenic media or *K.a.* plates. *** refers 
to *P* < 0.001 and ** to *P* < 0.01 in one-way ANOVA using Tukey-Kramer test for 
multiple comparison. Each symbol denotes a single clone expanded as shown in B.
Supplementary figure 1: Overexpression of Xpf in Ax2 and growth phenotype of different DNA repair mutants.
(A) Colony forming efficiency on bacteria (quantification for Fig 2A). n = 10 per group for M. luteus and B. subtilis, n = 13 per group for E. coli and dead K.a.
(B) Both Ax2 and xpf cells transfected with plasmids carrying Xpf fused to GFP express Xpf-GFP, as detected by western blot using an anti-GFP antibody (Abcam, ab6673, 1/1000). Lower blot, loading control developed with anti-β-actin antibody. Bottom panel: Growth phenotype of multiple Xpf clones expressing Xpf or Xpf(D771A).
(C) Expression of wild type Xpf (Xpf) and nuclease dead mutant (Xpf(D771A)) does not affect growth of Ax2 strain on K.a. plates.
(D) xpc, fncD2 and rev3 growth phenotype and quantification.
Supplementary figure 2: Mutational landscape of catA gene in methanol resistant clones.
Each line represents one independent methanol resistant clone. On every line the kind of mutation was drawn according to the nomenclature depicted in the figure. Bottom pie charts compare the type of catA mutations found in Ax2 methanol resistant clones with those found in xpf methanol resistant clones. The different colors highlight deletions, insertions, rearrangements and point mutations, as stated in the figure.
Table S1

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